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Diospyros digyna Jacq. Fruit: Exploring Bioactive Compounds, Functional Properties, and Involved Mechanisms

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Table of contents

Introduction

Functional Nutrition: an approach to wellness and disease prevention

Functional nutrition is an emergent paradigm in the field of healthcare that highlights the key role of nutrition in health promotion and disease prevention.

Although Hippocrates proclaimed over 2500 years ago, "Let food be the medicine and medicine be the food," it is only in recent years that this concept has garnered substantial interest within the field of food science. Traditionally, nutrition was primarily concerned with meeting basic survival needs, maintaining normal physiological functions, and preventing severe nutrient deficiencies or excesses by adhering to essential dietary guidelines encompassing a balanced intake of macronutrients and micronutrients. However, the modern understanding of nutrition has evolved over the past few decades, driven by advances in scientific research and evolving healthcare needs. Indeed, due to the increasing prevalence of "lifestyle diseases", like chronic inflammation and cardiovascular diseases, the intricate relationship between nutrition and health has been explored more extensively, with a specific focus on the functional role of nutrition. Going beyond the conventional approach of only tallying calories and nutrient intake, functional nutrition introduces an innovative perspective that underscores the vital role of food as both a source of nourishment and a means of healing. Recent studies have convincingly demonstrated that a well-balanced diet not only supplies energy but also exerts a fundamental influence on maintaining health and preventing diseases (Downer et al., 2020; Hanekamp et al., 2015; Luvián-Morales et al., 2022; G. Wu, 2023). This functional role of specific dietary regimes, notably those rich in plant-based foods, can be attributed to the presence of components in these foods that, while not fitting the classical definition of "nutrients", possess unique biological activities that contribute to improve human health (El Sohaimy, 2012; Eussen et al., 2011; Peluso et al., 2017).

Today, health is no longer defined merely as the absence of disease but, in accordance with the World Health Organization's definition, as "a state of complete physical, mental, and social well-being", which is increasingly desired by consumers (World HealthOrganization, 2020). Consequently, this growing emphasis on understanding the health benefits of specific foods and the mechanisms behind their protective effects has become a top priority in both nutrition and medical research.

Bioactive compounds: the healing touch of natural chemistry

A bioactive compound refers to a substance exhibiting biological reactivity, impacting the function of living organisms, tissue, or individual cells.

Plants serve as the primary reservoir of biologically active compounds. Bioactive molecules derived from plant are known as phytochemicals They constitute a heterogeneous group of substances, predominantly devoid of direct nutritional roles, but exhibiting a significant health-promoting potential. These plant compounds are organic molecules with low molecular weight generated as secondary metabolites. They play accessory roles in living plants, including protection, attraction, signalling, and indirectly support processes like reproduction, growth, and regulation of host plant's metabolism (Liu, 2013).

Numerous studies have provided substantial evidence that phytochemicals exhibit a wide range of biological activities, including antioxidant, anti-inflammatory, antiproliferative, and antimicrobial actions (Peluso et al., 2017). These findings strongly suggest a multitude of protective effects and potential benefits for human health. Consistent with these observations, the intake of phytochemical-rich foods and supplements can be associated with the enhancement of the overall health and well-being. This has been already proven by clinical, epidemiological, and experimental evidence that have consistently demonstrated inverse correlations between the consumption of plant-based foods and the development of various degenerative and chronic diseases, such as cardiovascular diseases, gastrointestinal disorders, diabetes, and cancer (Asgary et al., 2018; Cossarizza et al., 2011; Ekström et al., 2011; George &

Abrahamse, 2021; González-Quilen et al., 2020; Guasch-Ferré et al., 2017; Olfert & Wattick, 2018; Romier-Crouzet et al., 2009; Widmer et al., 2015).

To date, over 5000 individual phytochemicals have been isolated and identified in fruits, and vegetables (Liu, 2013). However, a substantial percentage of these compounds still eludes identification and remains unknown.

The dietary phytochemicals, known up to now, can be classified into various groups, including polyphenols, alkaloids, nitrogen-containing compounds, organosulfur compounds, phytosterols, and carotenoids (Patra et al., 2021). Among these groups, polyphenols have received the most extensive research attention.

They are a complex category characterized by one or more aromatic ring structures with single or multiple hydroxyl (OH) groups bound to them. Based on their chemical structure, these compounds can be systematically categorized into distinct subgroups, including flavonoids, phenolic acids, stilbenes and lignans (Patra et al., 2021). Among them, flavonoids and phenolic acids are the most extensively distributed. Flavonoids are typically water-soluble and exhibit a generic structure characterized by the presence of two benzene rings linked to a three-carbon chain forming an oxygenated heterocyclic ring. Variations in the structure of this heterocycle ring further classify them into distinct subgroups, including flavonols, flavones, flavanols, flavanones, flavanonols, anthocyanidins, and isoflavones (Dias et al., 2021). On the other hand, phenolic acids are typically characterized by the presence of a single carboxylic acid group and are primarily categorized into two subgroups: hydroxybenzoic and hydroxycinnamic acids (Santos-Buelga et al., 2023). (Figure 1)

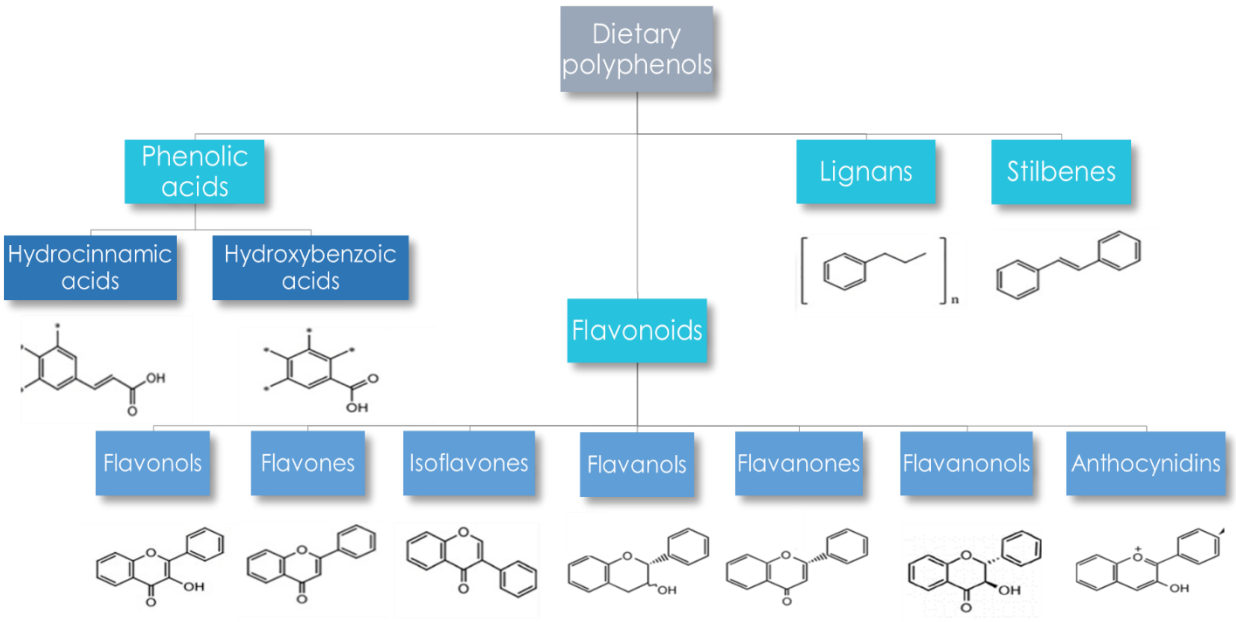

Figure 1: Classification and chemical structures of dietary polyphenols

The significance of structural arrangement cannot be overstated, as it plays a central role in influencing activity, but also bioavailability of dietary polyphenols. Indeed, multiple factors, including chemical structure and complexity, degree of polymerization, conjugation with other phenolic compounds, molecular size, glycosylation, acylation, and hydroxylation, collectively determine how these compounds interact with the body and impact their overall effectiveness in promoting health (Quideau et al., 2011).

The chemical structure of phytochemicals is intricately intertwined with their redox-active properties, granting them an extraordinary proficiency in transferring electrons to potential oxidizing agents. As a result, they undergo self-oxidation, forming a relatively stable radical through the extensive delocalization of electrons across both aromatic and aliphatic structures (Patra et al., 2021). Empirical evidence highlights the essential contribution of phytochemicals in preserving cellular redox equilibrium, serving as a potent defence mechanism against oxidative damage (Guan et al., 2021). This capability may, in part, explain the well-documented inverse correlation between

the consumption of fruits and vegetables and the occurrence of various chronic diseases, particularly inflammation, often triggered by prolonged oxidative stress (Gentile, 2021; Zhang et al., 2015)

Antioxidants and inflammation

Oxidative Stress and Inflammation: A Dual Challenge to Health

Oxidative stress, initially defined by Sies (Sies, 1997), refers to a disruption in the oxidant to antioxidant balance, favouring oxidant species and potentially leading to cellular damage. Oxidant species are generated due to the inefficient utilization of molecular oxygen during the oxidative breakdown of nutrient molecules, within mitochondria and peroxisomes. This inefficiency results in the partial reduction of oxygen, giving rise to both radical and non-radical species collectively termed Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). Additionally, specific enzymatic activities, such as myeloperoxidase, xanthine oxidase, and monoamine oxidase, contribute to the production of ROS and RNS by catalysing the oxidation of particular substrates in the presence of molecular oxygen. On the other hand, the production of reactive species can be increased by exposure to various chemical and physical agents, including those present in environmental pollutants such as cigarette smoke, alcohol, ionizing and UV radiation, pesticides, and ozone (Aseervatham et al., 2013).

ROS and RNS, as highly oxidising species, pose a significant threat to the integrity and proper functioning of cellular structures. On the other hand, they also can act as signalling molecules, playing a key role in the regulation of different process including proliferation, differentiation, apoptosis, and inflammation (Sies & Jones, 2020).

The main ROS and RNS include the superoxide radical anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), singlet oxygen, nitric oxide (NO) and peroxynitrite (NOO^-) (Pizzino et al., 2017). Most of these oxidant species are free radicals, resulting in high reactivity and chemical instability.

The superoxide radical anion is produced enzymatically in inflammatory cells and non-enzymatically in mitochondria during the oxidative phosphorylation process. Although

it can mediate direct cytotoxic actions, the cellular damage associated with increased production of the superoxide radical anion is to be correlated with secondary oxidants (Pervaiz & Clement, 2007). Under normal conditions, the generation of superoxide is immediately followed by its reduction to molecular oxygen and hydrogen peroxide in a rapid dismutation process catalysed by superoxide dismutase (SOD). The effectiveness of this enzyme is remarkable, as it efficiently eliminates superoxide before it can interact with biological targets. Its crucial role in maintaining cellular redox balance and preventing O_2 cytotoxicity becomes apparent when one considers that at physiological concentrations of superoxide (approximately 10μM), the spontaneous dismutation of this potent free radical would otherwise extend its life to seconds (Yasui & Baba, 2006).

Currently, three distinct isoforms of superoxide dismutase (SOD) have been identified, each playing a crucial role in cellular defence mechanisms. Both SOD1 and SOD3 incorporate Cu and Zn into their catalytic centres. SOD1 is ubiquitously distributed across intracellular cytoplasmic compartments and is responsible for scavenging superoxide radicals in the cytoplasm and nucleus. In contrast, SOD3 has extracellular localization, and due to its recent discovery, its function remains not well-characterized. On the other hand, SOD2 employs Mn as a cofactor and predominantly localizes to the mitochondria of aerobic cells. It specifically targets superoxide radicals generated within mitochondria during aerobic respiration preserving normal cellular processes (Zelko et al., 2002). Scientific data indicate that dysfunction of SOD enzymes has been associated with various health conditions, including neurodegenerative diseases, cancer, and cardiovascular disorders (Johnson & Giulivi, 2005).

Hydrogen peroxide (H_2O_2) is produced enzymatically *in vivo* by a variety of systems including SOD-dependent dismutation of superoxide radical anion. *In vivo*, H_2O_2 exhibits modest oxidant power and is a neutrally charged reactive species. This property gives it the ability to cross biological membranes and react away from its point of origin (Sies, 2014). The removal of H_2O_2 is facilitated by glutathione peroxidase (GPx) and catalase (CAT), a heme-containing enzyme that converts hydrogen peroxide to water. In the presence of transition metals, usually iron ions, the relatively inert hydrogen peroxide is converted to the highly reactive hydroxyl radical ($OH^{\cdot}$) by the Fenton or Haber-Weiss

reaction. This radical, which has an exceptionally high reduction potential (E°' = 2.3) (Halliwell, 2006), is the oxyradical with the highest reactivity and the lowest selectivity.

The enzymatic oxidation of the guanidine group of L-arginine results in the production of nitric oxide (NO), a relatively stable free radical with implications for the regulation of various biological processes. Nitric oxide exerts its effects primarily through the activation of guanylate cyclase, although some cGMP-independent effects have been documented (Pan et al., 2017).

Under inflammatory stimuli, an inducible form of NO synthase (iNOS or NOS2) leads to increased and sustained production of NO. While the production of NO during inflammation and the up-regulation of iNOS have been linked to the cellular damage observed in various inflammatory pathologies, a substantial proportion of the cytotoxic effects attributed to NO are more likely to be due to peroxynitrite.

Peroxynitrite is the product of the reaction between nitric oxide and the superoxide radical anion (Squadrito & Pryor, 1998) (Figure 2). The kinetic constant of this reaction is remarkably high, competing with the SOD-catalysed dismutation of superoxide (Padmaja & Huie, 1993). The protonated form of peroxynitrite (ONOOH) is a potent oxidant too. Direct cytotoxic effects of peroxynitrite include protein nitration, lipid peroxidation, enzymatic inactivation, oxidation of biologically important thiols and direct effects on DNA

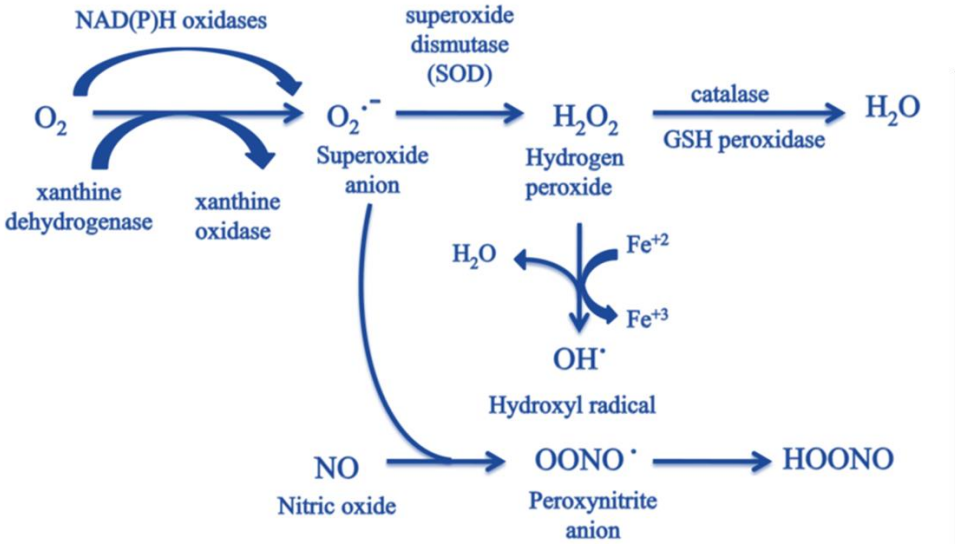

Figure 2: ROS and RNS production.

Guardians of Balance: The crucial role of antioxidants in inflammation regulation

Under physiological conditions, the production of ROS and RNS is controlled by endogenous antioxidant defence mechanisms. These mechanisms include enzymes and soluble antioxidants, which have a role in the prevention of oxidative damage (Halliwell, 2006). As defined by Halliwell and Gutteridge, an antioxidant is described as a substance capable of competing with oxidisable substrates to retard or inhibit their oxidation (Halliwell & Gutteridge, 1995). Among the soluble antioxidants, glutathione (GSH), a tripeptide derivative with a redox-active sulfhydryl group, serves as the most important endogenous antioxidant. It can transfer electrons to oxidising species and undergo oxidation itself to form the stable product GSSG. Through an enzymatically catalysed process involving glutathione reductase, an NADPH-dependent enzyme, GSSG is reduced back to GSH, restoring the active form of glutathione (Deponte, 2013). Other endogenous molecules contribute to antioxidant defence, each with a different primary role. Melatonin, synthesised by the pineal gland and involved in the regulation of circadian rhythms, and uric acid and bilirubin, by-products of the catabolism of endogenous molecules, partly evade elimination mechanisms due to their antioxidant defence potential (Mannino et al., 2021).

Enzymes involved in antioxidant defence mechanisms include GPx, SOD, CAT, which functions are intricately interconnected, ensuring a coordinated and effective response to oxidative stress. Superoxide dismutase (SOD), catalysing the dismutation reaction of $O_2^{\cdot -}$, produces H_2O_2 which serves as crucial substrate for both CAT and GPx. They then come into play to handle the generated hydrogen peroxide. CAT, located in cellular peroxisomes, efficiently decomposes H_2O_2 into H_2O and O_2. Simultaneously, GPx, relying on GSH, facilitates the reduction of H_2O_2, protecting the cell from potential oxidative damage. This interconnected enzymatic network forms a sophisticated defence mechanism against the harmful effects of reactive oxygen species.

Many phytochemicals possess redox-active properties and incorporating them into the daily diet through plant foods contributes to enhancing endogenous antioxidant defences, preserving redox homeostasis and promoting human health. Furthermore,

experimental evidence indicates that dietary phytochemicals go beyond direct scavenging of reactive species. They also influence the expression and activity of antioxidant enzymes, further contributing to the maintenance of redox balance (Mannino et al., 2020, 2022).

On the other hand, dietary phytochemicals go beyond merely shielding cells from oxidative stress. Indeed, subtle alterations in the cellular redox state, triggering structural modifications and subsequent functional changes in numerous proteins with redox-sensitive sites, have the potential to affect a multitude of signal transduction pathways. Extensive documentation supports the notion that pathways orchestrating the inflammatory response feature numerous proteins highly responsive to the cellular redox state (Janssen-Heininger et al., 2008; Mannino et al., 2019), Consequently, numerous experimental studies have investigated and demonstrated anti-inflammatory activity of antioxidant phytochemicals, both individually and within extracts (Gentile et al., 2015; Hollebeeck et al., 2012; Xu et al., 2022).

Moreover, the bioactivity of phytochemicals is not solely derived from their redox properties. Experimental data has demonstrated that many phytochemicals possess the ability to interact with a diverse range of biological targets, such as proteins and lipid membranes, thereby influencing their structure and function (Caradonna et al., 2020) (Figure 3).

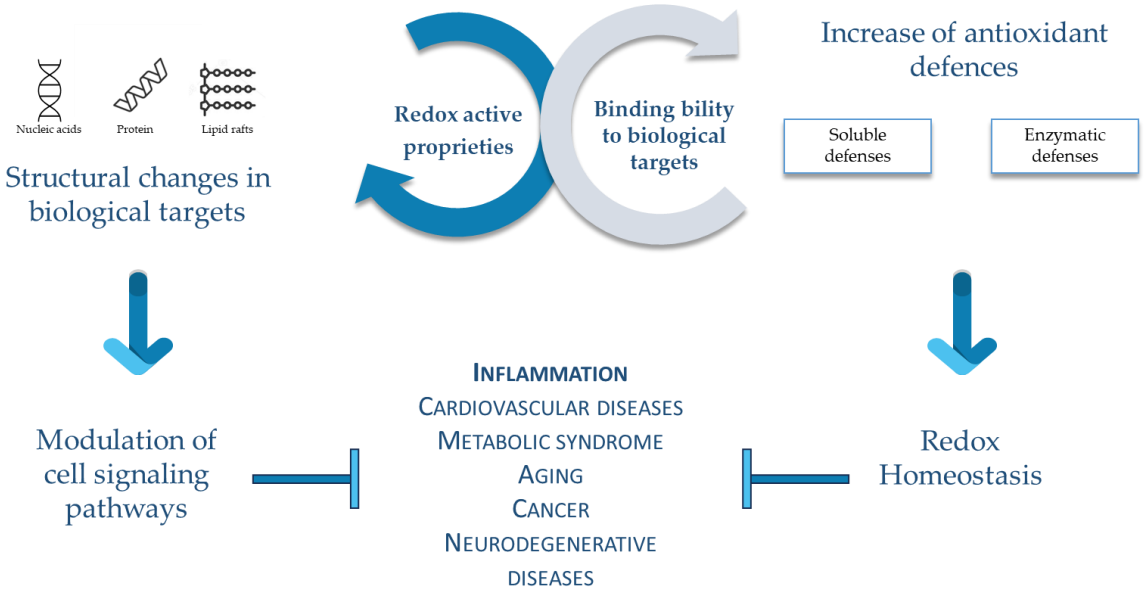

Figure 3: Biochemical mechanisms of phytochemical bioactivity.

Intestinal inflammation

Intestinal inflammation is a natural and protective response essential for preserving the integrity and function of the gut. Deregulation of this physiological process is associated with disruptions in the normal balance of the immune system within the digestive tract.

This imbalance can result in chronic inflammation, which is linked to various gastrointestinal disorders, including inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis (Mayer, 2010). IBD comprises a spectrum of inflammatory, chronic, and progressive conditions affecting the intestinal tract, resulting in ulcerations in the intestinal mucosa and chronic dysfunction of the microvascular system and endothelium (Ko & Auyeung, 2014). The consequences of IBD vary based on triggering factors giving rise to a spectrum of symptoms ranging from abdominal cramps, vomiting, loss of appetite, and diarrhea to poor digestion, flatulence, fever, blood and mucus in stools, and weight loss (Oz et al., 2016).

The incidence and prevalence of IBD are on the rise globally. Initially recognized as distinct entities in Western Europe and North America in the late eighteenth century, the incidence of IBD escalated rapidly in industrialized areas during the latter half of the twentieth century. Presently, over 1 million residents in the USA and 2.5 million in

Europe are estimated suffered of IBD, with the condition manifesting at any age and approximately 25% of patients presenting before the age of 20 (Kaplan, 2015; Richardson et al., 2018). The correlation between the incidence and changes in lifestyle and environment is increasingly apparent, with diet playing a significant role. It is well-established that a diet rich in red meat, high-fat foods, and refined sugars is associated with IBD (Borowitz, 2023; Ng et al., 2013). On the other hand, it has been demonstrated that a substantial intake of fruits and vegetables, especially those rich in polyphenols, is linked to a decreased risk of developing chronic intestinal inflammation and colorectal cancer (Jedrychowski et al., 2009; H. Wu et al., 2009).In addition, numerous *in vivo* and *in vitro* studies suggest the promising potential of various polyphenolic compounds and plant extracts abundant in polyphenols to proficiently regulate intestinal inflammation (Jarmakiewicz-Czaja et al., 2022; Romier-Crouzet et al., 2009; Sergent et al., 2010).

While various cell types are involved in intestinal inflammation, intestinal epithelial cells play a pivotal role. Positioned strategically at the interface between the luminal antigenic environment and the internal milieu, these cells serve a dual purpose. On one hand, they function as a physical barrier, regulating the direct exposure of immune cells to luminal antigens. On the other hand, they actively contribute to the physiological inflammatory response in the intestine by synthesizing and secreting soluble inflammatory mediators (Okumura & Takeda, 2017)initiating a self-amplification process (Cashman & Shanahan, 2003; Kiesler et al., 2001) (Figure 4).

The physical integrity of the intestinal barrier, upheld by intestinal epithelial cells, depends on tight junctions (TJs) – specialized intercellular connections essential for regulating paracellular transport and controlling permeability. Their assembly involves multiple integral transmembrane proteins, such as occludin, claudins, and junctional adhesion molecule (JAM), forming a network between adjacent cell membranes. Meanwhile, peripheral membrane adaptor proteins like zonula occludens-1 (ZO-1), ZO-2, and ZO-3 act connecting the integral transmembrane proteins to the actin cytoskeleton and other signaling proteins (Otani & Furuse, 2020). The activation of intestinal epithelial cells during inflammation leads to deficiencies in the barrier function of the epithelium. This results in the disruption of tight junctions, along with the

redistribution and loss of associated proteins. As a consequence, there is an elevation in paracellular permeability, allowing increased exposure of immune cells to luminal antigens (Farré et al., 2020). This exposure, in turn, further activates intestinal epithelial cells, creating a feedback loop that exacerbates the compromise to the epithelium's barrier functions (R. M. Al-Sadi & Ma, 2007).

Fascinatingly, matrix metalloproteases (MMPs) play a pivotal role in compromising the integrity of the intestinal epithelial barrier. These zinc-dependent enzymes are crucial contributors to the dynamic remodeling of the extracellular matrix (ECM). Beyond their primary function in ECM turnover, MMPs are implicated in both the proteolytic activation and degradation of various non-extracellular matrix substrates. These substrates include chemokines, cytokines, growth factors, and junctional proteins, some of which play key roles in the inflammatory response (Ravi et al., 2007).

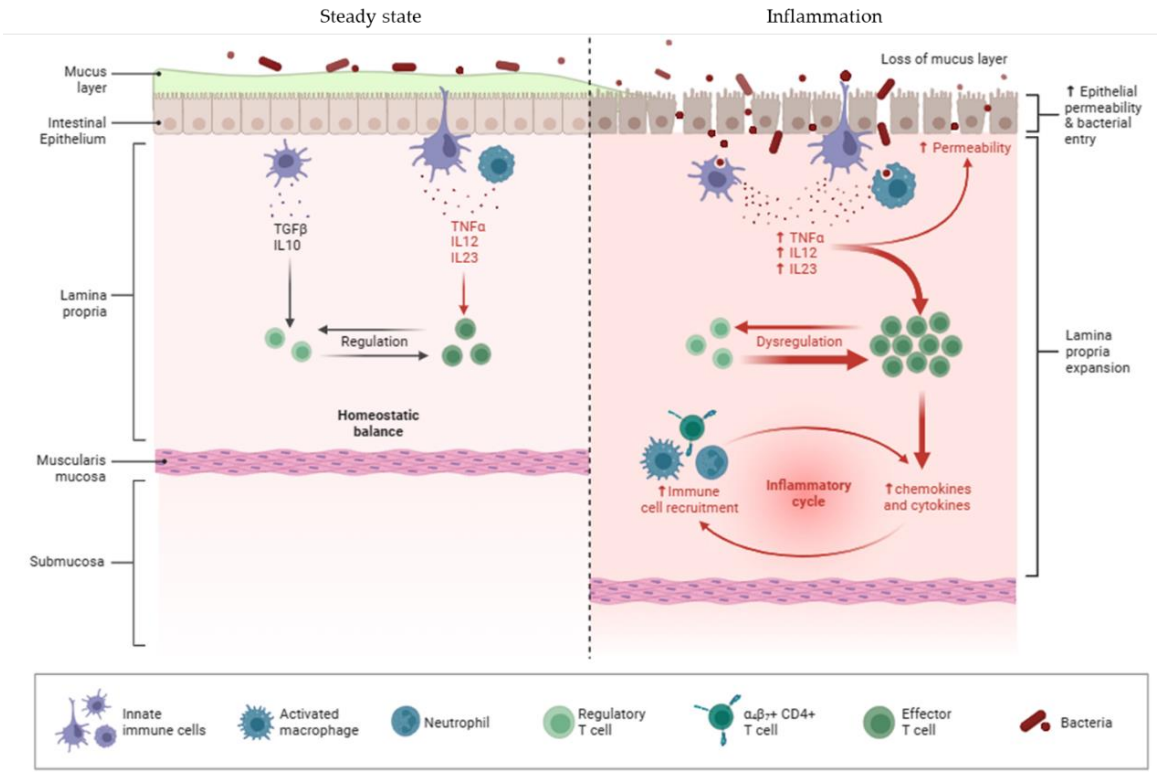

Figure 4: Homeostasis and inflammation in the intestine (Adapted from www.biorender.com)

In the context of intestinal inflammatory response, Interleukin-1β (IL-1β) emerges as a crucial pro-inflammatory mediator. Upon activation of specific pathways, this cytokine stimulates the transcription of inflammatory genes, including those encoding IL-8 (Schuerer-Maly et al., 1994), IL-6 (Parikh et al., 1997), and the inducible forms of nitric oxide synthase and cyclooxygenase (iNOS and COX-2) (Chavez et al., 1999; Duque et al., 2006). In contrast, Interleukin-10 (IL-10), a renowned anti-inflammatory cytokine, exerts a regulatory role in intestinal physiology by limiting and ultimately terminating the inflammatory process (Li & He, 2004).

Turning attention to the intracellular pathways crucial in the inflammatory response, including those of intestinal epithelial cells, particular interest lies in those involving nuclear factor-κB (NF-κB). In the absence of stimulation, the predominant form of NF-κB within the cell is the inactive cytosolic heterodimer p65/p50, bound via the p65 subunit to the inhibitory protein IκB-α (Lawrence, 2009). Various pro-inflammatory stimuli, such as IL-1β, bacterial LPS, or TNF-α, trigger the phosphorylation of IκB-α at serine residues S32 and S36, orchestrated by the IκB kinase complex (IKK α,β,γ trimer). Phosphorylated IκB-α is subsequently degraded, allowing translocation of the p65/p50 dimer to the nucleus. In the nucleus, this dimer can regulate the transcription of specific genes by interacting with response elements in their promoter regions (Barnes & Karin, 1997; Jobin & Balfour Sartor, 2000; Schmid & Adler, 2000).

The regulation of NF-kB is intricately linked to the cellular redox state, and numerous in vitro and *in vivo* studies highlight a clear association between inflammation, NF-kB activation, and oxidative stress (Winyard & Blake, 1996). The redox modulation of NF-kB is notably cell-specific, manifesting at various levels, including phosphorylation and proteasomal degradation of the inhibitor, nuclear translocation of the free transcription factor, and gene transcription (Meyer et al., 1994). For instance, it has been proposed that the redox-dependent phosphorylation of the cytosolic inhibitor IkB, necessary for detachment from NF-kB, may be influenced by polyphenols. These compounds have the potential to inhibit kinase phosphorylation, thereby preventing NF-kB translocation and subsequently impeding the transcription of pro-inflammatory mediators (Khan et al., 2020; Romier et al., 2008)

Nuclear factor erythroid 2p45-related factor 2 (Nrf2), a well-characterised transcription factor known for its role as an oxidant/electrophile sensor, is another key player in the inflammatory response. Under physiological cellular ROS levels, Nrf2 expression is regulated by the Kelch-like ECH-associated protein 1 (Keap 1), which targets Nrf2 for ubiquitylation and subsequent proteasomal degradation. Keap1 includes 27 cysteine residues, some acting as sensors for oxidative stress. The oxidation state of these sites is indeed influenced by the cellular redox state and, consequently, by the balance between levels of oxidizing species and the availability of antioxidant molecules. Redox-sensitive modification of Keap1 induces a conformational change, preventing its binding to newly synthesised Nrf2 and immediate accumulation of Nrf2 in the cell nucleus. Once in the nucleus, Nrf2 binds to the antioxidant response element (ARE) and orchestrates the regulation of genes encoding proteins involved in maintaining cellular redox homeostasis, facilitating detoxification, repairing macromolecular damage, and regulating metabolism (Nguyen et al., 2009).

Nrf2 and NF-κB represent key pathways that intricately regulate the delicate balance of cellular redox status and responses to stress and inflammation. The crosstalk between these pathways involves a complex network of molecular interactions and can vary based on cell type and tissue context (Nguyen et al., 2009). Notably, the NF-κB activation has a negative impact on the Nrf2 signaling pathway. In this context, Nrf2 plays a role in reducing ROS levels, preventing the proteasomal degradation of IκB-α, and inhibiting the nuclear translocation of NF-κB. Moreover, the upregulation of Nrf2 results in elevated levels of heme oxygenase (HO-1), and the subsequent increase in expression of phase II enzymes hinders the degradation of IκB-α (Krajka-Kuźniak & Baer-Dubowska, 2021) (Figure 5).

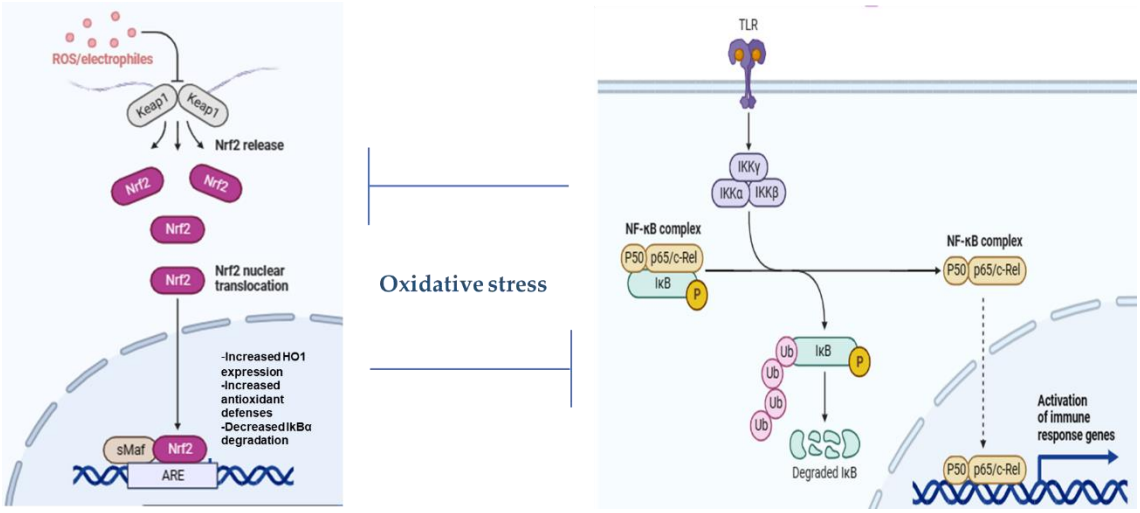

Figure 5: Crosstalk between NRF-2/NF-κB pathways. (Adapted from www.biorender.com)

Potential Preventive and Therapeutic Role of Antioxidant Phytochemicals in Intestinal Inflammation

Currently, the available therapeutic options for IBD include aminosalicylates, glucocorticosteroids, immunosuppressants, and biologic agents. However, these treatments offer only temporary remission and lack a curative solution. Their primary goal is to manage the inflammatory process within the intestines, aiming to prevent irreversible structural damage. Unfortunately, the therapeutic outcomes achieved so far have been less satisfactory than anticipated (Triantafillidis et al., 2011). The introduction of specific anti-TNF alpha has been a significant advancement. However, up to 40% of patients in clinical trials with Crohn's disease (CD) do not respond to therapy, and one-third of responders lose the response after one year of treatment (Cai et al., 2021). Therefore, there is an imperative need to delve into the pathophysiology of IBD and urgently explore novel therapeutic possibilities, including cellular therapies.

Recent attention has focused on naturally occurring phytochemicals as alternative candidates for IBD therapy (Somani et al., 2015). This underscores the pivotal role of diet in preventive measures, as dietary components reaching the intestinal lumen may

create favourable conditions for influencing essential physiological processes crucial for digestive health. This is particularly relevant to compounds with poor absorption and stability under gastrointestinal digestion conditions, such as proanthocyanidins.

Notably, proanthocyanidins have shown protective effects on recurrent colitis by reducing the expression of TNF-α and IKKα/β, translocation of NF-κB, promoting damaged tissue repair, and improving colonic oxidative stress (Wang et al., 2011). Several studies highlight the efficacy of phytochemicals in different animal models. For example, phenolic acids like vanillic acid and caffeic acid exhibit beneficial effects on DSS-induced ulcerative colitis by inhibiting COX-2 expression, NF-κB activation, and reducing TNF-α and IL-6 levels (S. J. Kim et al., 2010; Zielińska et al., 2021)

Resveratrol demonstrates anti-ulcerative effects in TNBS-induced colitis by reducing neutrophil infiltration, inhibiting adhesive molecules, restoring redox status, and diminishing cytokines and enzymes (Abdallah & Ismael, 2011). Quercetin prevents intestinal barrier disruption by enhancing the expression of tight junction proteins (Amasheh et al., 2008). Naringenin abrogates experimental colitis by down-regulating pro-inflammatory mediators (Picos-Salas et al., 2023). Extracts from *Boswellia serrata*, *Panax notoginseng*, and *Cistanche tubulosa* show activity in animal models of intestinal inflammation by suppressing inflammatory mediators and restoring intestinal epithelium function (Jia et al., 2014; Kiela et al., 2005; Wen et al., 2014).

Despite abundant preclinical studies, mainly in rodent models, investigations regarding the safety and beneficial effects of phytochemicals in humans are scarce. Curcumin, known for its anti-inflammatory properties, has demonstrated positive outcomes in IBD patients, with a higher clinical response, remission, and improvement in endoscopy compared to the placebo group (Singla et al., 2014). *B. serrata* extract has shown promising effects on patients with collagenous colitis but lacks significant efficacy in maintaining remission in CD patients, despite good tolerability (Abdel-Tawab et al., 2011).

Endothelial Progenitor Cells' Influence on Intestinal Health

Beyond localized inflammation, IBDs are distinguished by abnormal angiogenesis and vasculogenesis processes, with presence of immature vessels, and intensified responsiveness of the dysfunctional microvasculature to growth factors. This condition leads to persistent tissue hypoperfusion and ischemia in the intestinal mucosa, further contributing to the maintenance of chronic inflammation (Chidlow et al., 2007)

The process of intestinal mucosal repair involves two major components: epithelial regeneration and mesenchymal reconstruction. In this context, angiogenesis, a multistep process in the formation of new blood vessels from preexisting vessels, plays a central role in tissue repair, ensuring the supply of oxygen and nutrients to the regenerating tissue (Koutroubakis et al., 2006).

Recent insights support the exploration of therapeutic approaches aimed at enhancing neovascularization processes to restore a healthy vascular condition in the intestinal mucosa. While angiogenesis remains a key aspect, growing evidence suggests that neovascularization may also involve bone marrow-derived endothelial progenitor cells (EPCs) (Deng et al., 2011; Flores, 2015; Khoo et al., 2008). Notably, promising results have been achieved using EPCs for the treatment of hindlimb ischemia and myocardial ischemia. The transplantation of EPCs into fetal sheep demonstrated efficient migration and homing within the mucosal layer, contributing significantly to the vasculogenesis of the intestine (Wood et al., 2012). These findings underscore the potential of EPCs to serve as an additional cellular resource for cellular therapy in IBD, either independently or in conjunction with other stem cells such as mesenchymal stem cells (MSCs).

EPCs are a heterogeneous single-nucleated cell population originating in the bone marrow (BM) and represent a circulating reservoir of endothelial progeny. They express the main endothelial markers (CD31, CD144 and VEGFR-2) and possess stem/progenitor cell characteristics, including high clonogenicity and proliferation rate. EPCs, through differentiation into mature endothelial cells (ECs), play a crucial role in endothelial regeneration, angiogenesis and the formation of new blood vessels (Murasawa & Asahara, 2005; Naserian et al., 2020). Consequently, a reduction in the number and

function of EPCs can increase the risk of vascular disease and inhibit the restoration of a healthy vascular condition.

Due to their regenerative potential, EPCs are widely studied as possible cell sources for revascularisation strategies in cell therapy for ischaemia and various degenerative diseases. Although EPCs can be isolated from both umbilical cord blood (CB) and adult peripheral blood (APB), cord blood-derived EPCs (CB-EPCs) demonstrate stability in culture through multiple passages and, unlike APB endothelial cells, do not specialise in tissue-specific endothelial phenotypes (Au et al., 2008). Consequently, CB-EPCs endothelial cells prove extremely useful as a generic endothelial cell model to evaluate the effects of different molecules on the regenerative potential of the vascular endothelium. Moreover, due to their greater stability in culture, they can be considered excellent candidates for initial *ex vivo* expansion in anticipation of future autologous transplants aimed at treating ischaemia and related pathologies.

However, prolonged *ex vivo* expansion prior to transplantation has been associated with an impaired ability of CB-EPCs for vascular repair (Banno & Yoder, 2018). Furthermore, the survival of transplanted cells in a highly inflamed and oxidised environment of injured ischaemic tissues represents a challenge for successful clinical treatments (Barzegar et al., 2019). In this context, optimising the *ex vivo* expansion of EPCs and enhancing EPCs activity by pharmacological modulation could represent a novel strategy to increase cell survival and endothelial function, thus contributing to the effectiveness of using endothelial progenitor cells in regenerative medicine. Although some molecules, such as statins and VEGF, have been identified as being capable of enhancing the number and functional capacity of EPCs, it is important to note that these compounds have also been shown to carry side effects that could limit their use (Ma et al., 2012; Sirtori, 2014; Stancu & Sima, 2001). This raises the urgency to explore new agents that, on the one hand, are characterised by a lower incidence of adverse effects and, on the other hand, preserve or even enhance the positive effects on EPCs function. By preventing oxidative stress, antioxidant molecules have been shown to protect the vascular system and help restore normal vascular function after exposure to different

types of stress (Gentile et al., 2004). Based on these findings, the clinical applications of antioxidant treatments on EPCs should not be neglected.

Diospyros dygina Jacq.

The awareness of the benefits that food can bring to health has certainly changed the concept of food quality. In response to new consumer needs, research in the agri-food field has aimed at obtaining products that are not only of good organoleptic and nutritional value but also nutraceutical (Cocetta & Ferrante, 2020; Giovannetti et al., 2013).

Within this framework, there has been an increase in the consumption of tropical fruits both in the local and international markets. In Italy, particularly, the market for tropical and subtropical fruits has grown exponentially (Migliore et al., 2017). Although tropical fruits are mainly imported in Italy, there has been consideration for cultivating exotic fruit species domestically for several years. The cultivation of such species on national territory, particularly in Sicily, has been explored due to its unique climatic conditions (Gentile et al., 2019, 2021). Sicily, with its specific climate, has been deemed especially suitable for the cultivation of tropical and subtropical species according to scientific agricultural research (Calabrese et al., 1984).

Currently, in Sicily, interest in cultivating exotic fruit species continues to grow continuously. However, while the cultivation of species such as mango, lychee, and avocado has achieved significant commercial success, the cultivation of other exotic fruit species remains very limited and practically in the experimental phase.

Diospyros digyna Jacq. is an evergreen tropical tree belonging to the Ebenaceae family. Diospyros genus includes over 700 species of evergreen trees and shrubs, that are pantropically distributed (Ramírez-Briones et al., 2019). However, not all the species belonging to Diospyros genus produce edible fruits, and the most studied are kaki persimmon (D. kaki L.), date-plum (D. lotus L.) and mabolo (D. blancoi A. DC) (Jiménez-González & Guerrero-Beltrán, 2021).

Originally indigenous to South and Central America, notably Mexico and Colombia (Lim & Lim, 2012), *Diospyros digyna* fruit was initially consumed by the native populations of Mexico. It was introduced to Eastern Asia by the Spaniards towards the late 17th century, first arriving on the island of Amboina, present-day Indonesia, and later in the Philippines. From there, the plant spread to regions spanning Malaysia up to Australia (Morton, 1987). Presently, the cultivation of this plant holds significant commercial importance in the Philippines, Australia, and Florida, USA.

The plant reaches a height of about 25 meters, with a trunk diameter of 75 centimetres and black bark. The flowers, white in colour, tubular, and with a large persistent green calyx, can be either hermaphroditic or male, isolated or in clusters of 3 to 7. The leaves are alternate, oblong-lanceolate in shape, tapering at the ends or rounded at the base, and sharply pointed at the apex. They are leathery, glossy, and range from 10 to 30 cm in length (Morton, 1987). The fruit, oblate or almost round, get an external diameter of about 5–15 cm, having olive epicarp, dark coloured pulp, and a considerable number of smooth and brown seeds (Jiménez-González & Guerrero-Beltrán, 2021) (Figure 6). The pulp has a soft texture, and it is commonly consumed fresh or, thanks to its creamy consistence and sweat taste, for the preparation of ice cream, jams, and mousse (Jiménez-González & Guerrero-Beltrán, 2021). Due to this peculiar soft and dark chocolate pulp, the fruit is called black persimmon or chocolate pudding fruit (Yahia et al., 2011).

Figure 6: Diospyros digyna and fruits at two ripening states

Roots, wood, leaves, fruits, and seeds of *D. digyna* have been successfully used in folk medicine for the treatment of asthma, dermatitis, hypertension, diabetes, atherosclerosis, and insomnia (Jiménez-González & Guerrero-Beltrán, 2021). On the other hand, despite studies evaluating the nutritional and nutraceutical profile of black persimmon have reported how this fruit has an interesting proximate, mineral, vitamin, and phytochemical composition (Ramírez-Briones et al., 2019; Yahia et al., 2011), it may be mistakenly considered unattractive due to the particular aspect (Jiménez-González & Guerrero-Beltrán, 2021). Nevertheless, the use of black persimmon pulp as food is almost exclusively limited to countries of origin. In particular, black persimmon is a climacteric fruit that becomes rapidly perishable after the harvesting, as demonstrated by the early peaks in CO_2 and ethylene production (Arellano-Gómez et al., 2005). In addition, the optimal transport and storage conditions are not actually well-known (Yahia et al., 2011). Consequently, the export of black persimmon is currently a hard challenge and the local production in European area may be the only alternative. Numerous scientific evidence highlighting how byproducts derived from food processing could be considered rich sources of phytochemicals with interesting beneficial properties (Sagar et al., 2018). In particular, several data have been demonstrated interesting phytochemical profile and antioxidant properties in different experimental models of extracts from *Diospyros kaki* peel (Jang et al., 2010; S. Y. Kim et al., 2006; Lee et al., 2008; Yokozawa et al., 2007). On the contrary, the phytochemical profile and functional properties of *Diospyros digyna* peel and seeds have not still been evaluated and currently they are discarded as waste (Peláez-Cid et al., 2020).

Aim of the work

The increasing interest in elucidating the health benefits of specific plant foods has sparked extensive research endeavours. As the demand for exotic fruits continues to grow, tropical crop plants are now being cultivated beyond their native regions, including along the Tyrrhenian and Ionian coasts of Sicily. *Diospyros digyna* Jacq., among these, has shown promising results in cultivation trials in the Mediterranean region.

This study aims to comprehensively evaluate the nutraceutical value of both the edible and residual parts—peel and seeds—of *D. digyna* fruits obtained from Sicilian cultivation. Building upon preliminary data, our primary objective is to conduct an in-depth exploration of the phytochemical composition of peel, pulp, and seed extracts from *D. digyna* fruits using a combination of colorimetric and HPLC methods. With the goal of investigating the functional value of Diospyros digyna Jacq fruit, we assess the impact of the peel, pulp, and seed extracts in three different *in vitro* cell models:

Our first aim is to evaluate the potential of *D. digyna* extracts to protect hepatic epithelial cells from oxidative damage induced by prooxidant stimuli, assessing their ability to enhance soluble and enzymatic endogenous antioxidant defenses.

Secondly, we aim to assess the effect of *D. digyna* extracts in a model of intestinal inflammation, involving intestinal epithelial cells subjected to the proinflammatory activity of IL-1β.

Finally, we aim to evaluate the effect of the extracts on a vascular endothelium model, using *ex-vivo* expanded human endothelial progenitor cells.

Through this integrated approach, our study aims to uncover the nutraceutical potential of *D. digyna* fruits, shedding light on the possible benefits associated with consumption. Additionally, by examining the phytochemical composition and associated bioactivities of not only the edible flesh but also the peel and seeds, our study could have implications in various fields including nutraceuticals, pharmacology, and cosmetics. These findings could pave the way for innovative strategies to more effectively utilize agro-industrial wastes.

Section I

"Phytochemical profile and antioxidant properties of the edible and non-edible portions of *Diospyros digyna* Jacq. fruit"

The content of this section incorporates the findings and insights published in the study by Mannino, G.*, Serio, G.*, Bertea, C.M., Chiarelli, R., Lauria, A., Gentile, C. *Phytochemical profile and antioxidant properties of the edible and non-edible portions of black persimmon (Diospyros digyna Jacq.) (2022)*. Food Chemistry, 380, art. no. 132137 https://doi.org/10.1016/j.foodchem.2022.132137 (*Co-first)

Abstract

This study evaluated the phytochemical profile and antioxidative properties of the edible and non-edible portions of black persimmon. The phytochemical analysis highlighted the presence of several bioactive compounds, differently distributed among peel, pulp and seeds. In particular, the peel resulted rich of flavan-3-ols and proanthocyanidins, whereas seeds contained high amount of organic acids, including ferulic, citric and sinapic acids. Concerning functional properties, both edible and non-edible portions showed a significant prevention of lipid peroxidation in a cell-based model. Moreover, the results suggested that the antioxidant protection involved both redox active properties and gene expression modulation. Concerning redox active properties, peel extracts showed an antioxidant activity 7/12-fold higher than the edible portion, while seed extracts were more active in increasing catalase gene expression. The obtained results confirmed that black persimmon is a good source of antioxidant phytochemicals, and its non-edible portions have a great potential in the production of functional foods and supplements.

Materials and methods

Plant material

Black persimmons were harvested from trees grown in Vivai Torre (Milazzo, Sicily, Italy; 38◦190 N, 15◦240 E; 20 m a.s.l.). The fruits were collected when still unripe and stored at room temperature for three days until they were ready for consumption. The fruits were peeled, and the pulp was manually separated from seeds and peel. The pulp, peel and seeds were then distinctly used for extraction process.

Extract preparation

For the preparation of the extracts, the pulp, peel, and seeds were thawed. The pulp was homogenized by using a homogenizer (Shaoxing Worner Lab Equipment Co., Ltd. Shaoxing, Zhejiang, China), meanwhile the seeds and peel were manually crashed using mortar and pestle. Then, samples were weighted and extracted with a mixture composed by 70:30 (v/v) ethanol:water using 1:10 (w/v) ratio. The samples were mixed by vortexing for 5 min, sonicated at room temperature (RT) for 30 min, and stirred on a plate shaker overnight at 4 ◦C in the dark. After the extraction time, each sample was centrifuged (10 min at 8000g, 4 ◦C). After isolation of the supernatants, the obtained residues were again extracted twice using 1:5 (w/v) ratio of the same solvent mixture. After, vortexing, sonication, stirring, and centrifugation under the same experimental condition previously described, the supernatants derived from the three extraction cycles were collected, filtered (Millex HV 0.45 µm, Millipore, Billerica, MA), and stored at − 80 ◦C until further chemical and biological analyses.

Total polyphenolic content – Folin-Ciocalteu assay

The total polyphenolic content (TPC) was measured via colorimetric assay using the reaction that leads to the reduction of Folin-Ciocalteu Reagent in a blue pigment under alkaline environment, as previously described (Mannino, Perrone, et al., 2020). Briefly,

the peel, pulp, and seed hydroalcoholic extracts of black persimmon were diluted 1:10 (v/ v) in 70% (v/v) ethanol. Then, 10 µL of diluted-extract was incubated for 1 min at 80 ◦C and for 30 min at RT with 190 µL of reaction mixture composed by 10 µL of Folin-Ciocalteu reagent, 40 µL of 20% (w/v) Na2CO3, and 140 µL of deionized water. After the incubation time, the sample absorbances were measured at 740 nm using a microplate reader (iMark™ Microplate Absorbance Reader, Biorad®, Milan, Italy) against a blank simply containing 190 µL of the same reaction mixture and 10 µL of pure 70% (v/v) Ethanol. Gallic acid (GA) was used to quantify the total content of polyphenols via external calibration curve (y = 0.3649x + 0.0277; R2 = 0.9987). TPC was then expressed as mg GA equivalents (GAE) per 100 g fresh weight (FW). All analyses were run in triplicate.

Total proanthocyanidin content – the Brunswick Laboratories 4-dimethylaminocinnamaldehyde (BL-DMAC) assay

The total proanthocyanidin content (TPAC) was measured via spectrophotometric assay using the reaction that leads to transformation of DMAC reagent in a green pigment under acidic conditions, as previously described (Mannino, Di Stefano, et al., 2020). Briefly, the extracts of peel, pulp, and seeds of black persimmon were diluted 1:10 (v/v) ratio in 75% (v/v) acetone acidified with 0.5 % (v/v) acetic acid. Then, 50 µL of each diluted extract was incubated with 150 µL of 0.1% (w/v) DMAC reagent dissolved in 75% (v/v) ethanol acidified with 12.5% (v/v) HCl. The sample absorbances were after 20 min of incubation in the dark at 640 nm, against a blank containing the same reaction mixture and pure 75% (v/v) acetone acidified with 0.5 % (v/v) acetic acid. A2-type PAC (PAC-A) was used to quantify the content of proanthocyanidins via external calibration curve (Y = 0.085x + 0.001; R2 = 0.9979). TPAC was then expressed as mg PAC-A equivalent (PAC-AE) per 100 g of FW. All measurements were repeated three times.

Identification and quantification of phenolic compounds via HPLC/DAD-MS/MS

The identification and quantification of polyphenols was performed using High Performance Liquid Chromatography (HPLC) coupled to a Diode Array Detector (DAD) and a 6330 Series Ion Trap Mass Spectrometer (MS) System equipped with an electrospray ionization (ESI) source (Agilent Technologies, USA). The chromatographic separation was carried out as previously described (Mannino, Perrone, et al., 2020). Briefly, it was used a constant flow rate (0.2 mL min− 1) and a reverse phase C18 Luna column (3.00 µm, 150 × 3.0 mm i.d., Phenomenex, USA) thermo-maintained at 25 ◦C. The mobile phase was prepared by using MilliQ H2O acidified with 0.1% (v/v) formic acid (Solvent A) (Millipore, Billerica, MA, USA) and acetonitrile acidified with 0.1% (v/v) formic acid (Solvent B) as previously reported (Mannino, Perrone, et al., 2020). The samples were separated using 97% A and 3% B as initial conditions, 70% A and 30% B for 35 min, and then 2% A and 98% B for 5 min. The concentration of A was maintained at 2% for 5 min and raised again to the initial condition before the subsequent injection. The compounds were identified comparing the obtained retention time and UV–Vis or MS/MS spectra with those of reference compounds, whereas their quantification was performed using calibration curves of the respective pure standard of each compound (range: 0.001 – 0.250 mg/ mL), except for proanthocyanidins that were quantified using PAC-A standard due to the unavailability on the market of other pure compounds. The results were expressed as mg per 100 g FW.

Cellular antioxidant activity (CAA)

Cell culture

Cell line HepG2 (hepatocarcinoma cells, American Type Culture Collection ATCC, Rockville, MD, USA) was cultured in RPMI supplemented with 5% (v/v) FBS, 2 mM L-glutamine, 50 IU/mL penicillin, and 50 µg/mL streptomycin and maintained in a humidified atmosphere with 5% CO2 at 37 ◦C (Mannino, Perrone, et al., 2020).Cells were

cultured in 75 cm2 culture flasks and were trypsinized using trypsinEDTA before the confluence was reached.

CAA assay

CAA assay was performed as previously described (Wolfe and Liu, 2007) with minor changes (Mannino, Perrone, et al., 2020). Briefly, the cells were seeded in 96-well plates in complete culture medium at the density of 6.0×10^4 cells/well and incubated for 24 h. Subsequently, the medium was removed, and the cells were treated with 25 µM DCFH-DA and the ethanolic extracts at different concentrations for 2 h. Ethanol concentration never exceeded 0.25% (v/v). Culture medium with 0.25% ethanol (v/v) was used as control. After the incubation time, the cells were washed with PBS, then 600 µM ABAP in HBSS was added and the fluorescence was evaluated by using a plate-reader at 37 ◦C (λ emission 538 nm, λ excitation 485 nm) every 5 min for 1 h. Control wells were preincubated with DCFH-DA and then incubated with ABAP in HBSS; blank wells contained cells treated with DCFH-DA and HBSS without oxidant. Each plate included triplicate control and blank wells. The CAA value was calculated as follows:

CAA = [100- (∫ SA/ ∫ CA)] * 100

where: ∫ SA is the integrated area of the sample curve and ∫ CA is the integrated area of the control curve. The concentration necessary for 50% of DCF formation inhibition (CAA50) for each extract was calculated from concentration-response (CAA) curves using linear regression analysis. Each result is the average of three separate experiments.

Radical scavenging and metal reducing activity

The determination of antioxidant activity was performed via spectrophotometric assays. In particular, ABTS and DPPH assays were used to evaluate the radical scavenging activity (Mannino, Gentile, et al., 2020), and FRAP assay to measure the metal reducing antioxidant power(Mannino, Perrone, et al., 2020). Briefly, for ABTS assay, the radical

$ABTS^{\bullet+}$ was previously prepared by incubating 7 mM ABTS with 2.45 mM K2S2O8 at room temperature overnight. The formed solution containing the radical $ABTS^{\bullet+}$ was diluted in methanol until reaching a final absorbance equal to 0.70 at 734 nm. The decay of the radical $ABTS^{\bullet+}$ resulted by the incubation with different extract dilutions was monitored reading the colour decrease at 734 nm. Regarding DPPH assay, the radical $DPPH^{\bullet}$ dissolved in 95% (v/v) ethanol was added at different concentrations of the samples. After vigorous shaking, the mixture was incubated for 30 min at 25◦ C in the dark. The reduction of the radical DPPH was monitored at 517 nm. The inhibition percentage for both ABTS and DPPH assay was measured using the following equation:

$AA\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100$

Where: AA% is the percentage of colour reduction of the reagent; A_{blank} is the absorbance measured at the specific wavelength of each assay after the incubation of the reaction mixture with 70% (v/v) ethanol only; A_{sample} is the absorbance of the sample read at the specific wavelength of each assay after the incubation time. The concentration necessary for 50% of colour reduction (IC_{50}) for each extract was calculated from concentration-response curves using linear regression analysis. Each result is the average of three separate experiments. Trolox was employed as reference compound and the antioxidant activity of each assay was expressed as mmol of Trolox Equivalent (TE) per 100 g of FW. Finally, for FRAP assay, the reaction mixture composed by 300 mM CH3COONa (pH = 3.6, HCl), 10 mM TPTZ, and 20 mM FeCl3 in 8:1:1 (v/ v/v) ratio were added to the sample previously diluted. Then, samples were incubated at 37 ◦C for 10 min and the absorbance was monitored at 595 nm. The quantification of the reducing activity was measured using a calibration curve of Trolox as external standard. All measurements were repeated three times. The results were expressed as mmol TE per 100 g of FW.

Expression of antioxidant enzyme genes

HepG2 cells were seeded at a density of 5×10^5 cells/well in 24-multiwell plates. The media were discarded after 24 h and the cells were treated for 2 h with the extracts in

fresh FBS-free DMEM. Then, cells were exposed to 200 µM H2O2 for 24 h, the total RNA was isolated (RNAXPress™ Reagent, HiMedia, China) and reverse-transcribed (OneScript® Reverse Transcriptase, HiMedia, China) as previously reported (Mannino, Perrone, et al., 2020). The resulting cDNA was employed as a template for quantitative real-time polymerase chain reaction (qRTPCR) using the BrightGreen 2XqPCR MasterMix-Low ROX (Abm, Canada) on a Stratagene® Mx3000P Real-Time PCR system. Primers for human CuZnSOD, MnSOD, GPx and CAT genes and for β-Actin (Table 1.1) were designed and purchased by Integrated DNA Technologies (Leuven, Belgium). Quantitative real-time PCR was performed according to a previously described method (Mannino, Perrone, et al., 2020) and the relative expression levels of each gene were estimated using the method of Pfaffl (Pfaffl, 2001).

Table1.1: Primer sequences used for quantitative real-time PCR analysis

Genes		Primer sequences	Accession
CuZnSOD	**F**	5′-ACGGTGGGCCAAAGGATGAA-3′	AC026776.4
	R	5′-TCATGGACCACCAGTGTGCG-3′	
MnSOD	**F**	5′-AGAAGCACAGCCTCCCCGAC-3′	NM_000636.4
	R	5′-GGCCAACGCCTCCTGGTACT-3′	
GPx	**F**	5′-TCGGTGTATGCCTTCTCGGC-3′	NM_000581.4
	R	5′-CCGCTGCAGCTCGTTCATCT-3′	
CAT	**F**	5′-CCAACAGCTTTGGTGCTCCG-3′	NM_001752.4
	R	5′-GGCCGGCAATGTTCTCACAC-3′	
β-Actin	**F**	5′-CGGGAAATCGTGCGTGACAT-3′	NM_001101.5
	R	5′-GGACTCCATGCCCAGGAAGG-3′	

Statistical analysis

All results were expressed as mean ± standard deviation (SD). Student t-test or ANOVA followed by Tukey's test were used to evaluate significant ($p \leq 0.05$) differences among the experimental conditions. All the statistical analyses were carried out using SPSS ver. 24 software (IBM, USA).

Results and discussion

Phytochemical profile of black persimmon

Phytochemicals are bioactive compounds produced by plants which may exert potential effect on human health, since these molecules show antioxidant, anti-inflammatory, antimicrobial, anti-lipidemic, and antidiabetic properties. Eating plant foods represent an easy way to intake phytochemicals. In recent years, it has been shown that different tropical fruits are a very rich source of these bioactive compounds, some of which limitedly distributed in the plant kingdom. In this work, a phytochemical characterization of black persimmon was carried out by spectrophotometric assays, and TPC and TPAC in peel, pulp and seeds were evaluated. Moreover, HPLC-DAD-MS/MS analysis was aimed to profile and quantify each individual compound present in analysed extracts.

Total phenolic content

TPC evaluated via Folin-Ciocalteu method is a parameter largely used for the estimation of the nutraceutical potential of plant materials and their derived-extracts. The analysis showed that black persimmon fruit contains high amounts of polyphenols, especially in the peel. On the contrary, Jang and co-authors, evaluating TPC of different parts on *Diospyros Kaki* fruit, found a higher TPC in the seeds than in other fruit portion (Jang et al., 2010). In this study, the values recorded were 374.34 ± 10.12, 134.58 ± 10.32 and 34.96 ± 8.93 mg GAE per 100 g of FW respectively in the peel, pulp, and seed (Figure 1.1). Despite TPC of the peels was close to that measured by Can-Cauich in methanolic extracts from black persimmon peel (Can-Cauich et al., 2017), the content obtained for pulp was lower than those determined in other studies (Corral-Aguayo et al., 2008; Yahia & Gutierrez-Orozco, 2011). However, the TPC measured for the pulp was in the range of that previously reported for the 50 fruits with the highest polyphenolic content (Pérez-Jiménez et al., 2010), indicating a real possibility to obtain high quality tropical fruits also in Mediterranean climate.

Total proanthocyanidin content

The edible part of the fruit showed a very low TPAC (2.19 ± 0.99 mg PAC-A equivalent per 100 g of FW) with respect to other common fruits, such as grape, apple, cranberry, bilberry, or strawberry (Grace et al., 2019; Ma et al., 2019; Mannino, Di Stefano, et al., 2020; Payne et al., 2010) and other tropical fruits like, cherimoya, jaboticaba, and mango. On the other hand, the analysis recorded a significant PAC amount in the extracts from black persimmon peel 50-fold higher than the pulp, with an estimated value of 101.08 ± 5.48 mg of PAC-A equivalents per 100 g of FW. Significant concentration of PAC was recorded also in peel extracts of other Diospyros species, in particular *Diospyros kaki* (Lee et al., 2008). Finally, PAC content in seed extracts was 10-fold lower than the value measured in the peel (Figure 1.1). The highest content of polyphenolic compounds measured in the peels is not surprising, since polyphenols are involved in the antioxidant defence of plants against abiotic and biotic menaces and these phytochemicals tend to be mainly stored in the outermost part of the fruits (Patiño-Rodríguez et al., 2020). However, the peels and the seeds of black persimmon are not eaten or utilized for commercial purposes, and they represent both the major byproducts discarded as a waste and a potential source of environmental pollution. Consequently, peel and seeds might represent an eco-suitable opportunity to provide low-cost raw material for the preparation of dietary supplements.

Qualitative and quantitative phenolic compound profile

To separate and determine individual phytochemicals in peel, pulp, and seeds of black persimmon, HPLC-DAD-ESI-MS/MS was employed. This analysis allowed the identification of 29 compounds (Figure 2.1). Among them, four were organic acids [Cinnamic Acid (#2), Fumaric Acid (#5), Malic Acid (#6) and Citric Acid (#22)], six were phenolic acids [pHydroxybenzoic Acid (#3), Protocatechuic Acid (#9), Coumaric Acid (#10), Caffeic Acid (#21), Ferulic Acid (#23), and Sinapic Acid (#26)], two were ester derivatives of hydroxycinnamates [Chlorogenic Acid (#12), and Isochlorogenic Acid (#15)], three were flavan-3-ols [Catechin (#1), Gallocatechol (#4), Epigallocatechin

(#28)], five were condensed-tannins [PAC A-type dimer (#11), PAC A-type trimer (#19), PAC A-type tetramer (#25), PAC B-type dimer (#24), PAC B-type trimer (#29), and PAC B-type tetramer (#20)], six were flavon-3-ols [Myricetin (#7), Rutin (#13), Myricetrin (#14), Isohermetin (#16), Kaempherolglicoside (#18), and Quercetin (#27)], a flavanonol [Dihydromyricetin (#8)], and a flavone [Cynaroside (#17)]. These compounds were differently distributed between peel, pulp, and seeds (Table 2.2). All the detected phenolic acids were found in the edible part of the fruit. Also, the non-edible part of the black persimmon showed an interesting phytochemical composition. In particular, about 70% of the identified compounds in seed extract were organic acids, and fumaric acid and citric acid were the most representative, whereas the peel contained all the detected flavan-3-ols that reached 30% of the total quantified compounds. Myricitrin was the most abundant flavon-3-ol (194.17 mg/ 100 g FW). Moreover, in the peel extract also two phenolic acids, ferulic acid and sinapic acid were present. Compared to other detected compounds, their content was the highest representing 25% of the quantified compounds in the peel. The large number of organic acids, including phenolic acids, in peel and seeds should not be underestimated. Indeed, recent scientific research have shown how feed enriched with plant by-products may be useful as sustainable and low-cost alternative for animal dietary supplementation (Viana et al., 2020). In particular, Shaojun He and co-authors reported that the dietary supplementation of broilers with fumaric acid positively affected their growth performance, immune response, relative weight and antioxidant status (He et al., 2020). The most representative compound in the black persimmon extracts was sinapic acid, reaching 17%, 22%, and 11% of the total identified compounds in peel, pulp, and seeds, respectively. This compound displayed several biological properties, including antioxidant, anti-inflammatory, anticarcinogenic and antibacterial activity (Nićiforović & Abramovič, 2014). It is found in high quantities mainly in by-products derived from food processing, such as seeds and peel of several fruits of little economic value. Various studies suggested that sinapic acid may be not only a natural preservative for different food and cosmetic products, but also represents an attractive ingredient for the development of new functional foods and dietary supplements (Nićiforović & Abramovič, 2014; Yang et al., 2019) . Finally, HPLC-DAD-MS/MS analysis revealed that PAC were present in both edible and non-edible parts of

black persimmon. In particular, while all the extracts contained the A-type and B-type dimers and the A-type trimer, the edible portion was the only one containing the A-type trimer and the B-type tetramers. Nevertheless, A-type PACs were always more abundant than B-type. This data is an interesting result due to both the limited occurrence of A-type PACs in food sources and to their higher bioactivity with respect to B-type PACs (Gentile et al., 2021; Mannino, Di Stefano, et al., 2020). Moreover, concerning the polymerization degree, even though we were unable to detect PACs with high molecular weight, dimers and trimers of PACs were present in the extracts. In particular, a significant amount of A-type trimers was measured in the seeds extract Although PAC bioavailability is quite limited at intestinal level, previously reported scientific data indicated that dimers and trimers are resistant to gastric digestion and also easily absorbed (Deprez et al., 2001).

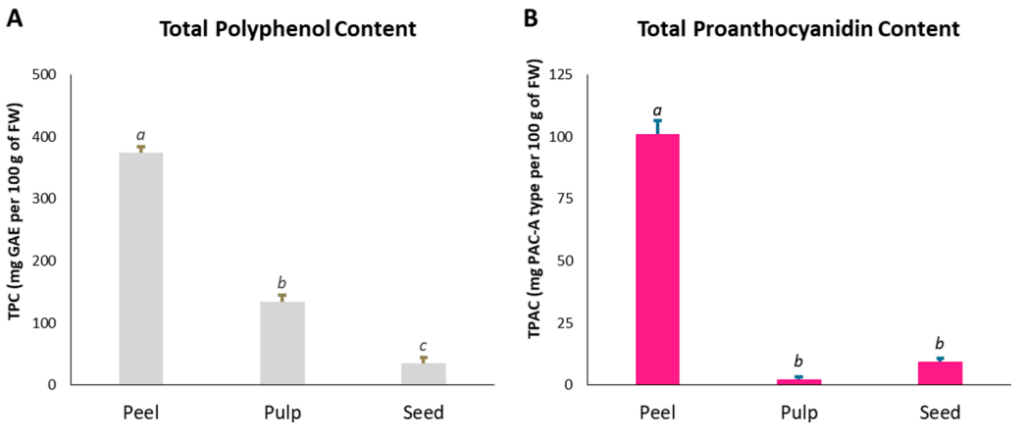

Figure 1.1: Total polyphenol content (TPC) and total proanthocyanidin content (TPAC) in extracts from seeds, pulp, and peel of *Diospyros digyna* fruits. Results are expressed as mean ± SD of three experiments carried out in triplicate. For each panel, different letters indicate significant differences at $p \leq 0.05$, as measured by one-way ANOVA followed by Tukey's test.

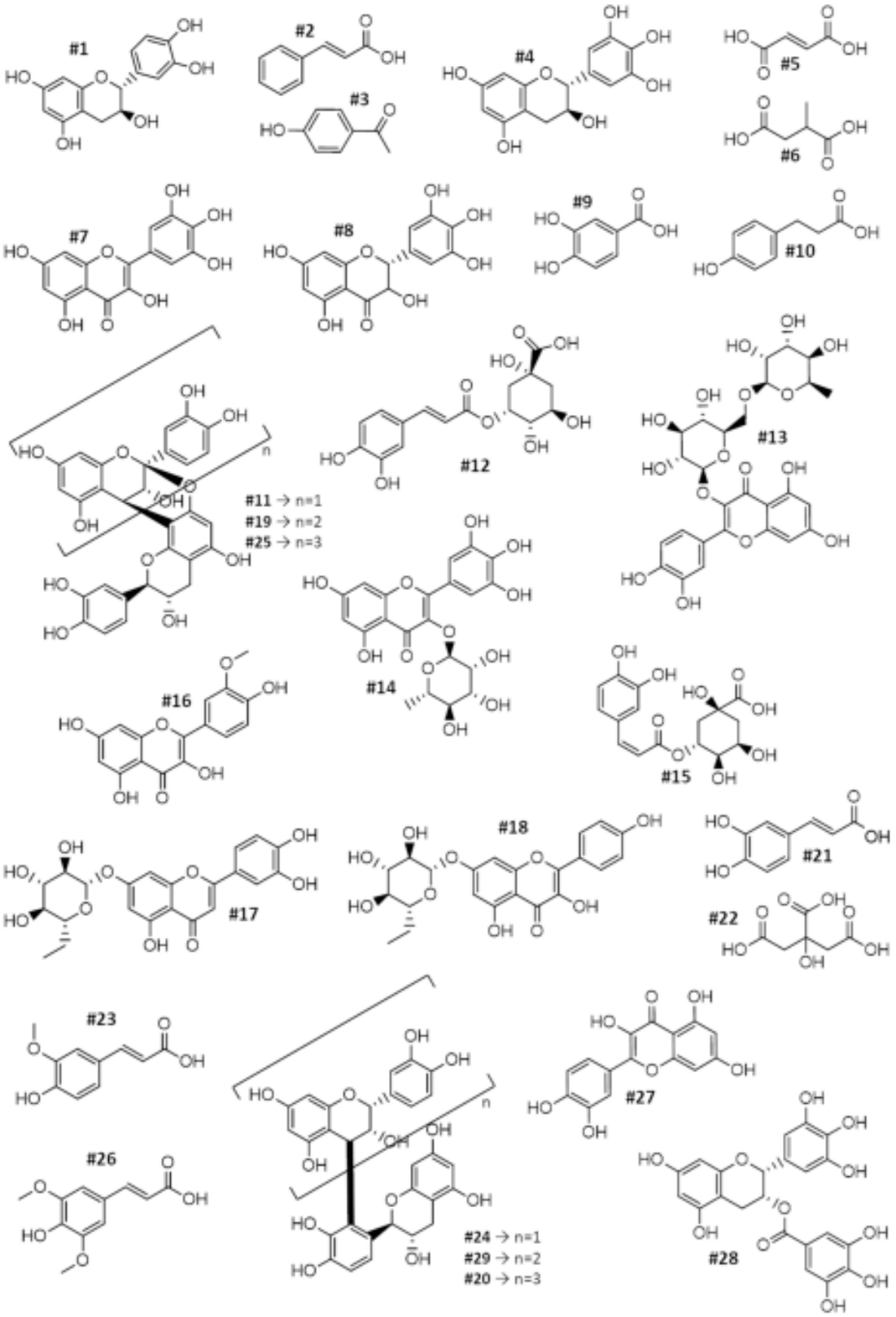

Figure 2.1. Phenolic compounds characterized and quantified via HPLC-DAD-ESI-MS/MS in extracts from seeds, pulp, and peel of *Diospyros digyna* fruits.

#	RT	Compound(s)	MW	*m/z*	MS/MS	λ	Peel	Pulp	Seed
Organic Acids									
2	4.1	Cinnamic Acid	148.15	147.15	103	280	n.d.	n.d.	74.03 ± 5.41
5	5.3	Fumaric Acid	194.18	193.18	91	280	n.d.	109.02 ± 3.27^{b}	794.59 ± 42.41^{c}
6	5.5	Malic Acid	134.18	133.18	71; 43	280	191.19 ± 9.43^{a}	49.35 ± 4.56^{c}	87.25 ± 5.35^{b}
22	32.6	Citric Acid	192.12	191.12	111; 87	280	n.d.	43.09 ± 1.77^{b}	272.97 ± 10.05^{c}
Phenolic Acids									
3	4.4	p-Hydroxybenzoic Acid	138.12	137.12	93; 59	280	n.d.	75.25 ± 2.06	n.d.
9	15.1	Protocatechuic Acid	154.12	153.12	109	280	n.d.	37.51 ± 3.16	n.d.
10	17.1	Coumaric Acid	164.15	163.15	119; 91	280	n.d.	91.27 ± 2.37	n.d.
21	32.3	Caffeic Acid	180.16	179.16	135	280	n.d.	44.18 ± 3.88	n.d.
23	32.6	Ferullic Acid	194.18	193.18	133	280	219.03 ± 8.51^{a}	65.27 ± 3.96^{b}	32.20 ± 1.25^{c}
26	38.5	Sinapic Acid	224.21	223.21	121; 93	280	426.74 ± 35.98^{a}	251.83 ± 7.83^{b}	201.43 ± 4.14^{c}
Ester derivates of hydroxycinnamates									
12	22.1	Chlorogenic Acid	354.31	353.31	191; 135	325	176.96 ± 8.42^{a}	77.89 ± 2.95^{b}	63.3 ± 1.33^{c}
15	23.7	Isochlorogenic Acid	354.31	353.31	191; 135	325	183.07 ± 5.77	n.d.	n.d.
Flavan-3-ols									
1	3.1	Catechin	290.26	289.26	245	280	n.d.	n.d.	0.13 ± 0.01
4	5.2	Gallocatechol	306.27	305.27	455; 289	350	178.12 ± 13.88^{a}	40.39 ± 3.81^{c}	65.36 ± 5.97^{b}
28	41.9	Epigallocatechin	458.37	457.37	295	350	n.d.	13.01 ± 1.19	n.d.
Condensed tannins									
11	21.4	dymer-PAC A type	576.51	575.51	449; 423; 289	350	6.71 ± 0.46^{a}	2.01 ± 0.08^{b}	1.10 ± 0.11^{c}
19	31.2	tetramer PAC A type	1152.14	1151.14	999; 575; 449; 423; 289	350	n.d.	0.63 ± 0.01	n.d.
20	31.4	tetramer PAC B type	1154.25	1153.25	576; 522; 448	350	n.d.	0.02 ± 0.01	n.d.
24	32.9	dymer-PAC B type	578.14	577.14	451; 407; 289	350	2.04 ± 0.11^{a}	0.26 ± 0.01^{b}	2.05 ± 0.17^{a}
25	38.4	trimer-PAC A type	862.37	861.37	575; 449; 423; 289	350	2.03 ± 0.16^{a}	0.12 ± 0.01^{b}	n.d.
29	45.8	trimer PAC B type	866.24	865.24	577; 449; 423; 289	350	n.d.	0.02 ± 0.01	n.d.
Flavon-3-ols									
7	5.5	Myricetin	318.16	317.16	178; 151	360	179.78 ± 12.06^{a}	84.87 ± 1.36^{b}	71.22 ± 2.74^{b}
13	22.2	Rutin	610.52	609.52	463; 301	260; 360	60.78 ± 4.51^{a}	n.d.	17.39 ± 1.66^{b}
14	23.2	Myricetrin	464.37	463.37	301	360	194.17 ± 10.19^{a}	n.d.	32.75 ± 1.42^{b}
16	24.2	Isohermetin	478.36	477.36	316	260; 360	184.22 ± 7.07	n.d.	n.d.
18	29.7	Kaempherol-4′-glucoside	448.36	447.36	285	280; 380	52.71 ± 3.91	n.d.	n.d.
27	39.7	Quercetin	302.23	301.23	178; 151	260; 360	67.68 ± 4.94^{a}	28.54 ± 1.02^{b}	23.74 ± 1.96^{b}
Flavanonol									
8	5.6	Dihydromyricetin	320.18	319.18	175; 149	360	192.14 ± 15.01^{a}	107.14 ± 1.79^{b}	87.98 ± 3.07^{b}
Flavone									
17	24.5	Cynaroside	448.37	447.37	285	465	174.97 ± 5.47^{a}	n.d.	18.57 ± 1.02^{b}
Total Identified Compounds							2492.34 ± 145.88^{a}	1121.67 ± 45.11^{c}	1846.06 ± 88.07^{b}

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Table 2.1: Qualitative and quantitative analysis of the phytochemicals determined in extracts from seeds, pulp, and peel of *Diospyros digyna* fruits. Results are expressed as mean ± SD of three different experiments carried out in triplicate and the values are expressed as mg per 100 g of fresh weight (FW). For each row, different letters indicate significant differences at $p \leq 0.05$, as measured by one-way ANOVA followed by Tukey's test. MW: Molecular weight; m/z: mass-to-charge ratio; MS/MS: detected fragmentations; λ: maximum absorption wavelength.

Antioxidant properties of black persimmon

Cellular antioxidant activity (CAA)

The health effects related to the intake of plant foods have been frequently ascribed to the phytochemical antioxidant properties. We have evaluated the antioxidant properties of the ethanolic extracts from peel, pulp, and seeds of *D. digyna* fruits in a

cell-based oxidation model. This model allows evaluating the ability of extract components to render cells more resistant to oxidation induced by a pro-oxidant stimulus (Mannino, Perrone, et al., 2020). In our experimental conditions, the CAA50 value measured for the fruit peel extract (0.32 ± 0.06 mg FW per mL of cell medium) indicates antioxidant activity 3 to 6 times higher than that estimated for the pulp extract (0.94 ± 0.03 mg FW per mL of cell medium) and seed extract (1.80 ± 0.06 mg FW per mL of cell medium), respectively (Figure 3.1, Panel A). On the other hand, comparing our results with literature data demonstrates that the antioxidant activity of black sapote extracts is 10 to 100 times that determined for many more familiar fruits. The high correlation between CAA50 values and TPC values of the three extracts is indicative of a substantial contribution of phenolic components in the extracts to the estimated antioxidant activity (Figure 1.1). Although the HPLC analysis did not demonstrate the presence of proanthocyanidin tetramers or pentamers in the extracts, it cannot be ruled out that higher polymerization degree proanthocyanidin polymers may be present. It is hypothesized, on the other hand, that PACs with a polymerization degree > 3, having little tendency to cross membranes (Rauf et al., 2019), may contribute little to the observed antioxidant action. The CAA assay evaluates the antioxidant ability of species capable of entering cells. Furthermore, although the tendency of proanthocyanidin polymers to interact with membranes is known, since in our experimental conditions the cells were washed with PBS after treatment with the extracts and before exposure to the pro-oxidant agent, it is to be excluded that high molecular weight polyphenols incapable of crossing the cell membrane exerted protective actions through interaction with components of the cell surface (Wolfe et al., 2008).

In order to limit the consequences of the overproduction of reactive species, endogenous antioxidant defence systems are aimed to maintain redox homeostasis in living systems. In particular, this effect is obtained through both the direct scavenging of reactive species by the soluble antioxidant defences and/or preventing their production via the action of antioxidant enzymes (Mannino, Perrone, et al., 2020). In this context, bioactive compounds may not only act as antioxidants due to their redox-active properties, but they can also regulate both the activity and expression of

enzymatic defences (Mannino, Perrone, et al., 2020). Here, in order to clarify the mechanism underlying the antioxidant action observed in HepG2 cells, we evaluated both the radical scavenging and metal reducing activity of black persimmon extracts through in solution assays, together with the potential regulation of the antioxidant enzyme gene expression via qRT-PCR analysis.

Radical scavenging and reducing activity

Considering the different mechanisms involved in the antioxidant properties of plant extract components and the potential synergies among them, various assays have been introduced to measure their antioxidant capacity. In particular, different experimental protocols are often necessary to better understand the dynamics involved in the observed effects (Gentile et al., 2021). Despite the criticisms due to obvious limitations of in solution assays, spectrophotometric methodologies evaluating the antioxidant properties are very popular and almost necessary to provide valuable information about the intrinsic redox-active properties of complex mixtures such as plant extracts (Gentile et al., 2021; Mannino, Perrone, et al., 2020). In recent years, a wide range of colorimetric assays has been adopted to easily measure the antioxidant capacity of foods and plant extracts, and among them ABTS, DPPH, and FRAP assays are very common. ABTS and DPPH assays are based on the decolouration of a synthetic, nonphysiological and coloured radical that may be scavenged by the redox-active compounds via the transfer of one or two electrons, respectively. On the other hand, in FRAP assay there are no free radicals involved, but the antioxidant properties are measured as metal-reducing activity through the monitoring of the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}) ion. In particular, it has been reported that polyphenol compounds having meta- or ortho-hydroxyl substitutions are able to chelate the ferric iron, following in a stronger reducing activity measured through FRAP assay (Gentile et al., 2021; Mannino, Perrone, et al., 2020). Here, to evaluate the overall intrinsic reducing capability of extracts from peel, pulp, and seeds of black persimmon, ABTS (Figure 3.1, Panel B), DPPH (Figure 3.1, Panel C), and FRAP (Fig. 3.1, Panel D) assays were performed. The analysis revealed that black persimmon possesses high antioxidant capacity. DPPH values were higher than those

measured by ABTS and FRAP assays. In particular, the mean values of the antioxidant activity for all analysed extracts were 3.64 ± 0.48, 7.88 ± 0.86 mmol, and 4.26 ± 0.60 mmol TE per 100 g FW respectively for ABTS, DPPH and FRAP assay. FRAP values were 10–20 fold higher compared to those recorded for other common fruits (Thaipong et al., 2006). The higher metal reducing activity may be linked to the presence of high content of flavonoid compounds having meta- or ortho- hydroxyl substitutions on A-ring or B-ring, as those determined in the analysed extracts. Moreover, the variability observed between the different assays could be linked to peculiar characteristics of the reaction mixtures or to specific differences in the electronic transfer mechanism (Mannino, Gentile, et al., 2020). Despite the variability evaluated in the antioxidant activity, the trend among peel, pulp and seed extracts were not influenced by the assays. Precisely, peel extracts always showed the highest antioxidant properties in term of both radical scavenging and metal reducing activity, whereas seed extracts the lowest one.

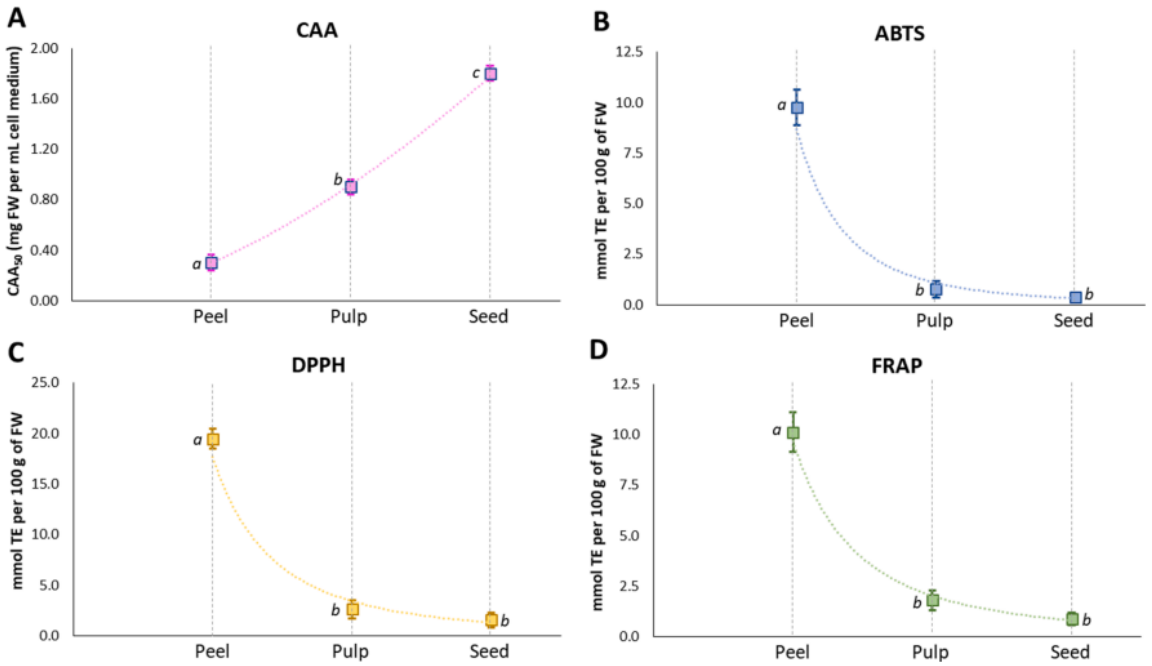


Figure 3.1: Cellular Antioxidant Activity (Panel A: CAA assay), radical scavenging activity (Panel B: ABTS assay; Panel C: DPPH assay), and ferric reducing antioxidant power (Panel D: FRAP assay) of extracts from seed, pulp, and peel of *Diospyros digyna* fruits. Results are expressed as mean

± SD of three experiments carried out in triplicate. For each panel, different letters indicate significant differences at $p \leq 0.05$, as measured by one-way ANOVA followed by Tukey's test.

Gene expression of antioxidant enzymes in HepG2 cells after treatment with black persimmon extracts

In order to limit the consequences of the overproduction of reactive species, endogenous defences are activated to maintain redox homeostasis. They can act by the directly scavenging of reactive species or by preventing their production through the modulation of antioxidant enzymes. Experimental data show that the antioxidant action of phytochemicals can also involve their ability to influence the enzymatic antioxidant defences by interacting with them or influencing their expression (Kang et al., 2020; Mannino, Perrone, et al., 2020). The main antioxidant enzymes include SOD, GPx, and CAT. SOD reacts with superoxide anion radical (O^{2-}) to produce oxygen (O_2) and the less-reactive H_2O_2, while CAT and GPx neutralize H_2O_2 by forming H_2O and O_2. In this study, we evaluated the effects of the peel, pulp, and seed extracts from black persimmon on the expression of CuZnSOD, MnSOD, GPx and CAT in HepG2 cells, under both non-oxidative condition and after cell incubation with 200 µM H_2O_2 (Figure 4.1). In these experimental conditions, HepG2 cells expressed all target genes. Following cell incubation with 200 µM H_2O_2, the expression of MnSOD (47% inhibition), CuZnSOD (27% inhibition), GPx (49% inhibition), and CAT (62% inhibition) was significantly reduced when compared to cells that were not exposed to oxidative stress (Figure 4.1, Panel A). These results are in accordance with those reported in our previous work (Mannino, Perrone, et al., 2020) and to those reported by Hong and co-authors (Hong et al., 2015) in which the antioxidant enzymes were downregulated in a similar manner. Cell incubation with black persimmon peel, pulp, and seed extracts for 2 h did not change the basal expression of target genes (Figure 4.1, Panel B). On the other hand, when HepG2 cells were pre-treated for 2 h with ethanolic extracts of black persimmon and then incubated with 200 µM H_2O_2 for 24 h, changes in the level of expression of the target genes were observed (Figure 4.1, Panel C). In particular, while the treatment with seed extracts did not change the expression of both SOD isoforms, MnSOD expression

was up-regulated about 2-fold after cell exposure to pulp and peel extracts. Moreover, a stronger effect was observed on the CuZnSOD expression after the treatment with pulp extract. Concerning GPx, a similar gene regulation was observed when HepG2 cells were treated with pulp (+92%) and peel (+91%) extracts. The most interesting effect was observed on the gene expression of the enzymes catalysing the H_2O_2 dismutation in H_2O and O_2. In particular, the seed extract was able to induce 10-fold CAT expression. The different modulation of the antioxidant enzymes observed under oxidative stress conditions and after treatment with the ethanolic extracts of peel, pulp, and seeds from black persimmon may be linked to the phytochemical profile of each extract. HPLCDAD-MS/MS analysis revealed that the seed extract was rich in fumaric acid. It is well-known that this molecule and its ester derivatives show hepatoprotective action by involving antioxidant activity (Šilhavý et al., 2014). Specifically, it has been proven that fumaric acid induces the activation of nuclear factor-erythroid-2 (Nrf-2) that is considered the "master regulator" of the antioxidant response, able to modulate the expression of hundreds of genes, including those coding for the antioxidant enzymes (Hybertson et al., 2011). Finally, these findings strongly suggest that phytochemicals from black persimmon may participate to antioxidant cellular protection both by scavenging ROS and stimulating the activation of adaptive response in which antioxidant enzymes are involved.

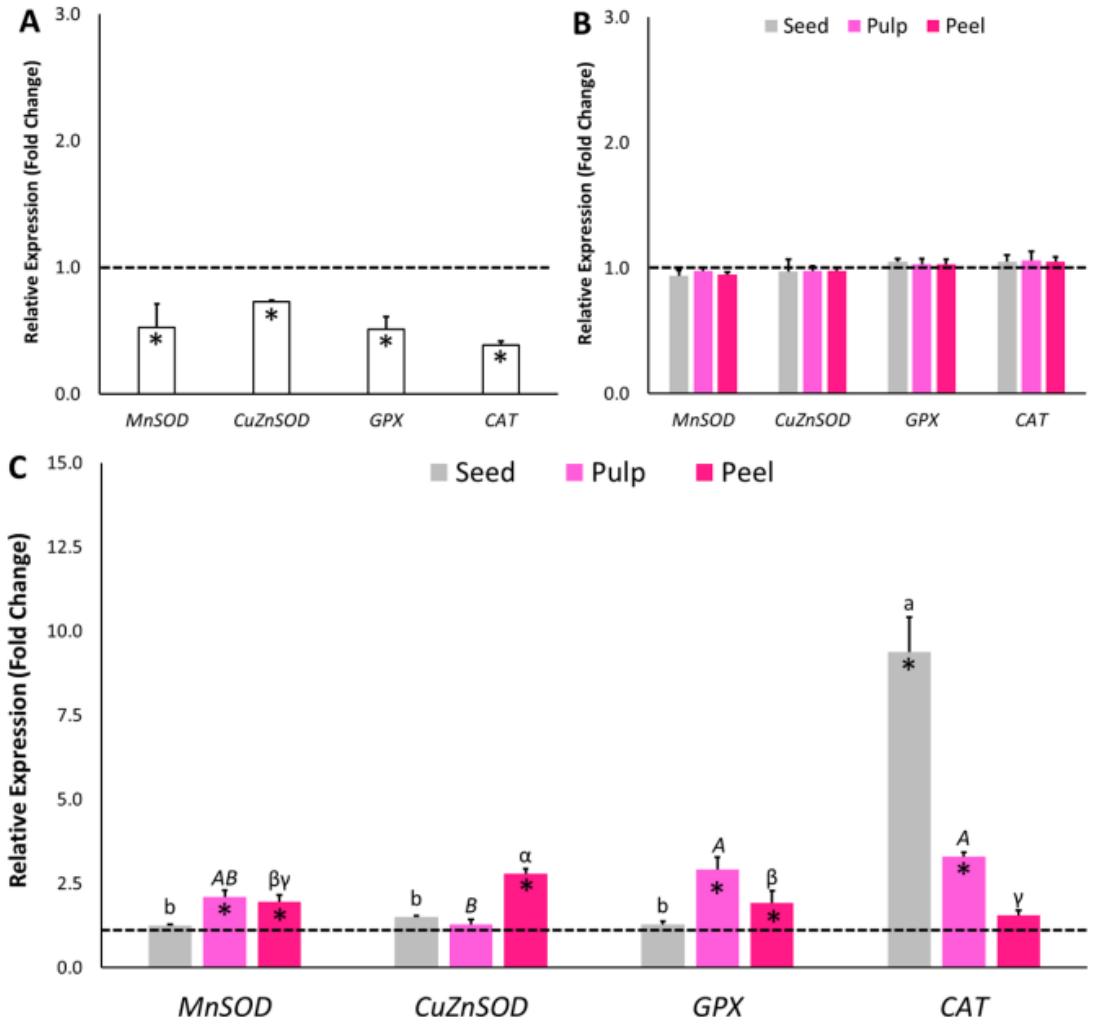


Figure 4.1. Effect of H_2O_2 or extracts from seed, pulp, and peel of *Diospyros digyna* fruits on the gene expression of SOD, CAT and GPx on HepG2 cells. After the seeding, cells were incubated in the absence of additional treatments (untreated cells) and treated for 24 h with 200 µM H_2O_2 (Panel A) or with the different extracts (Panel B). For both Panel A and B, cells untreated and not exposed to H_2O_2 were used as control. Panel C displays the effect of the extracts on the gene expression of SOD, CAT and GPx on HepG2 cells treated for 2 h with the extracts and then exposed to 200 µM H_2O_2 for 24 h. For this experimentation, cells untreated but exposed to H2O2 were used as positive control. After the different treatments, the cells were collected, and total cellular RNA was isolated and retro-transcribed to cDNA. The cDNA was used as template for

quantitative real-time PCR using β-actin as a reference gene. Bars represent the mean ± SD of three qRT-PCR analyses carried out in triplicate. Data were calculated as fold-change, and the bars represent the mean ± SD. Different lowercase letters on the top of each bar indicate significantly different values at $p \leq 0.05$ as measured by Tukey's test. The asterisk (*), when present, indicates statistical ($p \leq 0.05$) differences between control (dashed line) and treated condition.

Conclusions

The results obtained in this work provided information on the phytochemical composition and functional properties of the edible and non-edible parts of black persimmon. Specifically, we showed that the pulp of this fruit is a rich source of polyphenols, in particular sinapic acid and proanthocyanidins. On the other hand, peel, and seeds, considered a waste product, also showed an interesting phytochemical composition thanks to the presence of fumaric and sinapic acids. Moreover, although the phytochemical profile of seeds, pulp, and peel of black persimmon was different, remarkable antioxidant properties both in in solution and in a cellular model system were determined for all the extracts. In addition, bioactive compounds of seeds and peel are also able to enhance the expression of genes encoding for antioxidant enzymes. Finally, the results suggest that black persimmon by-products may find a potential use as alternative raw materials for the preparation of food supplements or functional foods.

Section II

"Effects of *Diospyros digyna* Jacq. fruits in an *in vitro* model of intestinal inflammation"

The contents covered in this chapter have been incorporated into the following manuscript currently under preparation: "*Modulation of inflammatory responses in an in vitro model of intestinal inflammation by extracts from edible and non-edible portions of Diospyros digyna Jacq. fruit"*

Serio G., Chiarelli R., Mannino G., Geraci F, Gentile C.

Abstract

Oxidative stress is a pivotal factor in the etiology and progression of chronic diseases, including cancer and inflammation. Notably, reactive species actively participate in cellular activation during inflammation, influencing redox-sensitive proteins crucial for inflammatory signaling pathways. The demonstrated anti-inflammatory potential of various antioxidant phytochemicals emphasizes the prospect of dietary antioxidants in safeguarding against oxidative stress-related diseases.

Expanding on this understanding, our prior research spotlighted black persimmon as an abundant source of antioxidant compounds distributed across its diverse components.

This study sought to evaluate the impact of black persimmon extracts on IL-1β-induced intestinal inflammation in Caco-2 cell monolayers. Extracts from pulp, seeds, and peel effectively mitigated the up-regulation of proinflammatory genes induced by IL-1β while concurrently preserving the integrity of the epithelial barrier. Noteworthy is the significant anti-inflammatory impact of non-edible components, particularly seed extracts, highlighting the potential value of typically discarded fruit parts.

The observed anti-inflammatory effects correlated with the modulation of the NF-κB signaling pathway, evident in reduced IκB-α phosphorylation and p65/p50 translocation. Furthermore, the upregulation of antioxidant enzyme genes (*CuZnSOD, MnSOD*, and *GPx*) and increased NrF2 expression elucidate a clear correlation between the anti-inflammatory and antioxidant actions of the bioactive compounds in the extracts. This suggests that black persimmon extracts may alleviate epithelial inflammatory responses by modulating redox-sensitive targets.

In conclusion, these findings underscore the promising anti-inflammatory effects of black persimmon extracts in the context of intestinal inflammation, providing insights into their potential to modulate cellular signaling pathways and supporting for a sustainable approach to waste management.

Material and Methods

Plant material

D. digyna fruits were harvested from trees cultivated in Vivai Torre, located in Milazzo, Sicily, Italy (38.190° N, 15.240° E; 20 m a.s.l.). Harvesting was performed at the immature stage, and the fruits were stored at room temperature for three days until reaching ripeness. Following this, the fruits underwent peeling, and the pulp was manually separated from the seeds and peel. Each distinct portion, including pulp, peel, and seeds, was then processed separately for the extraction procedure.

Extraction process

The extraction procedure closely adhered to a previously outlined method (Mannino et al., 2022). The pulp, peel, and seeds were thawed, weighed, and subjected to extraction using a 70:30 (v/v) ethanol:water mixture with a 1:10 (w/v) ratio. The samples underwent vortexing for 5 minutes, followed by sonication for 30 minutes at room temperature (RT), and stirring on a plate shaker overnight at 4°C in darkness. Post-extraction, each sample underwent centrifugation (10 minutes at 8000g, 4°C). The resulting supernatants were collected, and the residues underwent two additional extraction cycles using a 1:5 (w/v) ratio of the same solvent mixture. The supernatants from all three extraction cycles were combined, filtered (Millex HV 0.45 µm, Millipore, Billerica, MA), and subsequently stored at -80°C until further analysis.

Total polyphenolic content

The total polyphenolic content (TPC) was measured via colorimetric assay using the reaction that leads to the reduction of Folin-Ciocalteu Reagent in a blue pigment under alkaline environment, as previously described (Mannino, Perrone, et al., 2020). Briefly, the peel, pulp, and seed hydroalcoholic extracts of black persimmon were diluted 1:10 (v/ v) in 70% (v/v) ethanol. Then, 10 µL of diluted-extract was incubated for 1 min at 80

◦C and for 30 min at RT with 190 µL of reaction mixture composed by 10 µL of Folin-Ciocalteu reagent, 40 µL of 20% (w/v) Na2CO3, and 140 µL of deionized water. After the incubation time, the sample absorbances were measured at 740 nm using a microplate reader (iMark™ Microplate Absorbance Reader, Biorad®, Milan, Italy) against a blank simply containing 190 µL of the same reaction mixture and 10 µL of pure 70% (v/v) Ethanol. Gallic acid (GA) was used to quantify the total content of polyphenols via external calibration curve. TPC was then expressed as mg GA equivalents (GAE) per 100 g fresh weight (FW). All analyses were run in triplicate.

Cell culture

The Caco-2 cell line (human colon adenocarcinoma cells, American Type Culture Collection ATCC) was cultured in Dulbecco's Modified Eagle Medium (DMEM) with Glutamax-I supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 50 IU/mL penicillin, and 50 µg/mL streptomycin, 1% (v/v) non-essential amino acids (NEAA) (Invitrogen), and maintained in a humidified atmosphere with 5% CO2 at 37 ◦C.

For experimental purposes, cells were cultivated at the density of 60×10^3 cells/cm^2 for 21 days in 6-well plates, until the complete differentiation, established by measuring the Trans-Epithelial Electrical Resistance (TEER). The media was replaced every 3 days. In alternative experiments, treatments were applied to differentiated cells cultured in Transwell inserts (polycarbonate membrane, 0.4 µm pore size, Corning Costar Corp.) positioned in 12-well plates.

Cell treatment

For the treatments, cells underwent washing with phosphate-buffered saline, pH 7.4 (PBS). Only freshly prepared and filtered (0.2 µm) dilution of black persimmon extracts and medium were utilized. Differentiated cells were preincubated with or without peel, pulp and seed extracts at concentrations between 5 and 10 µM GAE for 2 hours. Subsequently, they were subjected to the proinflammatory action of IL-1β (10 ng/ml) for an appropriate incubation time. Cells not preincubated with the extracts and

exposed to the proinflammatory stimulus were used as positive controls. Ethanol concentration never exceeded 0.25% (v/v). Culture medium with 0.25% ethanol (v/v) was used as control. After incubation, supernatants were collected, and underwent centrifugation at 16,000 g for 10 minutes and then stored at -20°C until the analysis. In addition, in some experiments, cells were used to extract either proteins or RNA.

Gene Expression Analysis of Antioxidant Enzymes and Inflammatory Proteins

Differentiated Caco-2 cell monolayers underwent a 2-hour preincubation in DMEM, either with or without the addition of black persimmon extracts. Subsequently, cells were incubated with or without IL-1β for 24 hours. The total RNA was isolated (RNAXPress™ Reagent, HiMedia, China) and reverse transcribed (OneScript® Reverse Transcriptase, HiMedia, China) as previously reported (Mannino, Perrone, et al., 2020). The resulting cDNA was employed as a template for quantitative real-time polymerase chain reaction (qRTPCR) using the BrightGreen 2XqPCR MasterMix-Low ROX (Abm, Canada) on a Stratagene® Mx3000P Real-Time PCR system. Primers for human *CuMnSOD, ZnSOD, GPx*, *IL-6, IL-8, IL-10, IL-12, iNOS e COX2* genes and for *β-Actin* (Table 1) were designed and purchased by Integrated DNA Technologies (Leuven, Belgium). Quantitative real-time PCR was performed according to a previously described method (Mannino, Perrone, et al., 2020) and the relative expression levels of each gene were estimated using the method of Pfaffl (Pfaffl, 2001)

Table 1. Primer sequences used for quantitative real-time PCR analysis.

Genes		Primer sequences	Accesion
IL6	F	5'-AGACAGCCACTCACCTCTTCAG-3'	BC015511
	R	5'-TTCTGCCAGTGCCTCTTTGCTG-3'	
IL8	F	5'-GAGAGTGATTGAGAGTGGACCAC-3'	NM_000584
	R	5'-CACAACCCTCTGCACCCAGTTT-3'	
IL10	F	5'-TCTCCGAGATGCCTTCAGCAGA-3'	BC034024
	R	5'-TCAGACAAGGCTTGGCAACCCA-3'	
IL12	F	5'-TGCCTTCACCACTCCCAAAACC-3'	NM_000882
	R	5'-CAATCTCTTCAGAAGTGCAAGGG-3'	
TNFα	F	5'-CTCTTCTGCCTGCTGCACTTTG-3'	NM_000594
	R	5'-ATGGGCTACAGGCTTGTCACTC-3'	
INOs	F	5'-GCTCTACACCTCCAATGTGACC-3'	NM_000625
	R	5'-CTGCCGAGATTTGAGCCTCATG-3'	
COX2	F	5'-CGGTGAAACTCTGGCTAGACAG-3'	NM_000963
	R	5'-GCAAACCGTAGATGCTCAGGGA-3'	
β-Actin	F	5'-CACCATTGGCAATGAGCGGTTC-3'	NM_001101.5
	R	5'-AGGTCTTTGCGGATGTCCACGT-3'	
CuZnSOD	F	5'-ACGGTGGGCCAAACCATGAA-3'	AC026776.4
	R	5'-TCATGGACCACCAGTGTGCG-3'	
MnSOD	F	5'-AGAAGCACAGCCTCCCGAC-3'	NM_000636.4
	R	5'-GGCCAACGCCTCCTGGTACT-3'	
GPx	F	5'-TCGGTCTATGCCTTCTCGGC-3'	NM_000581.4
	R	5'-CCGCTGCAGCTCGTTCATCT-3'	

IL-6, IL-8, IL-10, IL-12, and INF-γ release

The quantification of IL-6, IL-8, IL-10, IL-12, INF-γ proteins in the supernatant was performed utilizing sandwich ELISA kits (Thermo Fisher-Invitrogen) following the manufacturer's protocol.

iNOS, COX-2, and NrF2 protein levels

The levels of inducible isoform of Nitric Oxide Synthase (iNOS), Cyclooxygenase-2 (COX-2), and Nuclear factor erythroid 2-related factor 2 (NrF2) were assessed through Western blot analysis, conducted following established procedures (Chiarelli et al., 2021). In brief, total protein samples were extracted from both control and treated

differentiated Caco-2 cells using a lysis buffer (7 M Urea, 2% CHAPS, and 10 mM DTT) containing a protease inhibitor cocktail (Sigma P8340). The protein samples (25 µg) were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and were subsequently transferred to 0.45 µm nitrocellulose blotting membranes. These membranes were incubated with primary antibodies, anti-iNOS (Invitrogen, 14592082), anti-COX-2 (Invitrogen, 358500), and anti-NrF2 (Invitrogen PA5-27882) at 4◦C overnight. Following thorough washing, the membranes were exposed to peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Promega W4021 and W4011,) at room temperature for 1 hour. Visualization of protein expression was achieved using a Molecular Imager Versadoc MP imaging System (Bio-Rad, Hercules, CA, USA) and the Immun Star™ WesternC™ chemiluminescent kit (Bio-Rad). Quantitative analysis of the bands was conducted using Quantity One v.4.6.6 software (Bio-Rad), with normalization to actin band intensity, serving as a loading control.

NF-κB Activation: IκB-α Phosphorylation and p65/p50 Translocation

The phopho-IκB-α/IκB-α ratio was evaluated by Western blot. Preparation of cell extracts, along with protein separation and immunoblots using anti-IκB-α (LS Bio 148310) and anti-phospho-IκB-α (SANTA CRUZ BIOTECHNOLOGY, INC., SC1017113), were carried out as described above.

NF-κB p65/p50 translocation was performed using immunofluorescence *in situ* assay. Briefly, after differentiation and treatment, the cells were trypsinised and collected in medium and centrifuged twice for 7 minutes at 800 rpm. Subsequently, they were fixed in 3.7% (v/v) paraformaldehyde for 1 hour. The cells underwent an additional centrifugation for 7 minutes at 2000 rpm, the supernatant was removed, and PBS was added. The cytospin method was employed to mount the cells on polylysine-coated glass slides, initiating *in situ* immunocytochemistry experiments. The cells underwent a PBS wash and were permeabilized for 10 minutes at 4°C in a solution of 0.1% Triton X-100 and 0.1% sodium citrate in PBS. After three additional PBS washes, the cells were

incubated overnight at 4°C with the primary antibody, monoclonal Anti-NF-κB (SIGMA N8523) in a solution containing 3% BSA in PBS. For negative controls, the primary antibody was omitted. Following three PBS rinses, cells were incubated with a secondary antibodies Goat anti-Mouse IgG (H+L), Alexa Fluor™ 488, A28175), for 1 hour.

The cells were stained for 10 minutes with Hoechst 33342 (Invitrogen) and then mounted in a 10 µl DABCO solution (de-ionized H_2O, 1 M Tris–HCl pH 8, 2 mM DABCO, glycerol). The cells were examined using a fluorescence microscope (Olympus BX50). Multiple optical sections of CaCo2 cells were acquired using a digital camera (Nikon Sight DS-U1). The intensity of autofluorescence emitted by the negative control served as a threshold for the remaining samples. The immunofluorescence analysis of NF-κB activation was conducted by counting the percentage of cells positive for nuclear NF-κB in three separate experiments.

Epithelial Intestinal Barrier Function

Evaluation of paracellular permeability - transport of FSA

The evaluation of black persimmon extracts on the barrier function of Caco-2 cell monolayers involved assessing the apparent permeability coefficient (P_{app}) in the apical-to-basolateral direction of the fluorescent marker, fluorescein-5-(and-6)-sulfonic acid trisodium salt (FSA) (Invitrogen)—a well-established paracellular marker (Banan et al., 2000) Caco-2 cells were cultured on 12-well tissue culture plate inserts with a 0.4 µm pore diameter (Transwell insert, Corning Costar Corp) for 21 days, as previously described, enabling access to both the basolateral and apical sides of the cells, representing the serosal and luminal poles of the intestinal epithelium.

Cell monolayers underwent a 2-hour preincubation in DMEM, either with or without the addition of black persimmon extracts to the apical compartment. Subsequently, the cells were incubated with or without IL-1β in the basolateral compartment for 24 hours. Following this, culture media in both compartments were replaced with Hank's balanced solution, and FSA (0.42 mM final concentration) was added to the apical compartment. Cultures were incubated, and the acceptor medium was collected at 30-minute intervals

between 0 and 150 minutes, with fresh HBSS replacing it. Concentrations of FSA in basolateral fluids were fluorometrically estimated using a fluorescence multiplate reader (FSA excitation, 485 nm; FSA emission, 530 nm). Unknown FSA concentrations in the samples were determined using standard curves generated with appropriate solutions of known FSA concentrations. The P_{app} ($cm*s^{-1}$) was calculated using the formula:

$P_{app} = (V/A * C_0) * dC/dt$

where *V* is the volume of the solution in the receiving compartment, *A* is the membrane surface area (1.12 cm^2), C_0 is the concentration of FSA in the apical compartment at time zero, and *dC/dt* is the steady-state flux across the monolayer, calculated as the slope of the curve depicting FSA concentration in the basolateral compartment over time.

Localization of the tight junction protein, ZO-1

Caco-2 cells were seeded and cultured for an extensive 21-day period on glass inserts within 6-multiwell plates to achieve optimal differentiation. These fully differentiated Caco-2 cell monolayers were subjected to the previously described treatments. Subsequent to these treatments, the Caco-2 cell monolayers underwent fixation in 3.7% paraformaldehyde for 1 hour. Following a PBS wash, cells were permeabilized for 10 minutes at 4°C using a solution comprising 0.1% Triton X-100 and 0.1% sodium citrate in PBS. After three additional PBS washes, the cells were incubated overnight at 4°C with the primary antibody, anti-ZO-1 (Invitrogen 33-9100) in a solution containing 3% BSA in PBS. Negative controls were established by omitting the primary antibody. Following three PBS rinses, cells were incubated with a secondary antibodies (Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 633) 1 hour. Subsequently, the samples were incubated with Hoechst 33342 and FITC-phalloidin and mounted in a 10 µl DABCO solution (de-ionized H_2O, 1 M Tris–HCl pH 8, 2 mM DABCO, glycerol). Several optical sections were acquired by confocal laser scanning (Olympus FV10i).

Assessment of the membrane type matrix metalloproteinases expression

The expression levels of matrix metalloproteinases (MMPs) MMP-2, MMP-9, and MMP-14 were evaluated using Western blot analysis. This assessment was carried out following established procedures, as previously described.

Statistical analysis

All results were expressed as mean ± standard deviation (SD). Student t-test or ANOVA followed by Tukey's test were used to evaluate significant ($p \leq 0.05$) differences among the experimental conditions. All the statistical analyses were carried out using Prism (GraphPad) software.

%

Results and Discussion

Multiple scientific findings underscore the profound impact of diet on the physiology of the gastrointestinal tract. Epidemiological investigations have notably illuminated associations between specific dietary patterns and the susceptibility to gastrointestinal diseases. Particularly, diets characterized by high levels of saturated fats, refined sugars, and red meat are implicated in an elevated risk of chronic inflammation and intestinal cancer (Chan et al., 2011; Rajendran & Kumar, 2010; Willett et al., 1990). Conversely, a substantial intake of fruits and vegetables in general, and polyphenols in particular, exhibits an inverse correlation with the risk of developing colorectal cancer (M. Xu et al., 2016; Yammine et al., 2021). In particular, an abundance of evidence from *in vivo* studies involving diverse animal models and *in vitro* studies, utilizing intestinal epithelial cell lines activated by various inflammatory stimuli, indicates that diverse polyphenolic phytochemicals and plant extracts possess the capacity to modulate intestinal inflammation (Cicio et al., 2023; Gentile et al., 2015; Kim et al., 2018; Romier-Crouzet et al., 2009; Ruiz & Haller, 2006; Sergent et al., 2010). Green tea polyphenols (epigallocatechin-3-gallate-rich polyphenon E) (Dryden et al., 2013),curcumin (Sreedhar et al., 2016), resveratrol (Samsami-kor et al., 2015),bilberry anthocyanins(Joseph et al., 2014), and fish oils (LORENZ et al., 1989) have demonstrated anti-inflammatory activity in models of intestinal inflammation. The demonstrated anti-inflammatory activity of dietary phytochemicals in models of intestinal inflammation suggests their genuine potential in the prevention and treatment of Inflammatory Bowel Diseases (IBD). Indeed, dietary components reach the intestinal lumen in higher quantities compared to other regions, encountering optimal conditions to exert their biological activity. This holds especially true when dietary components remain stable during gastrointestinal digestion and exhibit limited absorption. Notably, proanthocyanidins, polymeric flavonoids predominantly found in hydroalcoholic extracts of black persimmon, show resistance to gastrointestinal digestive conditions and low bioavailability. This allows them to attain significant concentrations in the intestinal lumen (Rios et al., 2002; Spencer et al., 2000).

In our experimental investigation, we assessed the anti-inflammatory potential of black persimmon extracts across specified concentration ranges: 0.25-0.5, 1-2 mg/ml, and 3-6 mg fresh weight (FW)/ml cell medium for peel, pulp, and seed, respectively. Folin-Ciocalteau analysis unveiled TPC values for peel, pulp, and seed extracts as 340, 87, and 29 mg gallic acid equivalent/100 g of FW. Each selected concentration range for cell treatments corresponds to a TPC between 5 and 10 µM gallic acid equivalent, aligning with phenolic-rich extract concentrations utilized in analogous experimental models.

Notably, the chosen pulp extract concentration, given the volume of intestinal fluid of 600 ml (Mahé et al., 1992), is an order of magnitude lower than the ideally achieved in the gut after ingestion of 25 g of fruit pulp.

Preliminary studies did not reveal cytotoxic effects of peel, pulp, and seed extracts on Caco-2 cells under all experimental conditions (data not shown).

Black persimmon extracts influence the expression of gene codifying inflammatory mediators

During the inflammatory process, intracellular signaling pathways leading to the transcriptional activation of proinflammatory genes, a pivotal mechanism in coordinating the immune response (Medzhitov & Horng, 2009). The heightened expression of these genes contributes to the synthesis of inflammatory mediators, intensifying the immune response, recruiting immune cells to the inflammation site and fostering the progression of inflammation.

Among inflammatory stimuli IL-1β is a key player in the orchestration of intestinal inflammation. In the intestinal inflammatory environment, various cells, including epithelial cells, produce IL-1β. This cytokine is pivotal in initiating and amplifying the cascade of events that characterize inflammation in the intestine.

IL-1β doesn't act in isolation; it collaborates with other proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and IL-6 (Eskan et al., 2008; Li et al., 2022; Shahini & Shahini, 2023; Zelová & Hošek, 2013). At the molecular level, IL-1β activates

intracellular signaling pathways, notably NF-κB pathway. This activation results in the transcriptional upregulation of a myriad of proinflammatory genes, perpetuating the inflammatory response (Lawrence, 2009).

The influence of black persimmon extracts on the expression of genes encoding proteins involved in the epithelial inflammatory response (IL-6, IL-8, IL-10, IL-12, iNOS, and COX-2) was evaluated through qRT-PCR. To induce inflammation in our experimental setup, we utilized an IL-1β concentration of 10 ng/ml. At this working concentration, IL-1β demonstrated the capacity to induce in Caco-2 cells an inflammatory condition comparable to that triggered by IL-1β set at 25 ng/ml (Van De Walle et al., 2010).

Cellular exposure to IL-1β at a concentration of 10 ng/ml resulted in a notable upregulation of all examined pro-inflammatory cytokine-encoding genes compared to basal levels (non-stimulated cells). Pre-treatment of cells with black persimmon extracts demonstrated a substantial inhibition of the expression of the targeted genes in the majority of cases.

Specifically, concerning the *IL6* and *IL12* genes, cellular exposure to the extracts resulted in gene expression levels returning to baseline, with more pronounced effects observed in cells treated with the seed extract, where the expression levels of both genes were reduced even compared to those of untreated cells (CTR^-).

Regarding the expression of the *IL8* gene, while no inhibitory effects were detected after treatment with the low concentrations (5 μM GAE) of peel and pulp extracts, both extracts, as well as the seed extract even at the low concentration, significantly inhibited gene expression, displaying down-regulations of 70%, 73.6%, and 98%, for peel, pulp, and seed extracts, respectively, assayed at 10 μM GAE.

Regarding the expression of genes encoding antioxidant enzymes, while all extracts induced a comparable down-regulation of *iNOS* gene expression, returning expression levels to those of the control cells, differences among the extracts were observed concerning the expression levels of *COX-2*. Specifically, while the peel extract had no significant effects on gene expression, the high concentration of the pulp extract, as well as the seed extract at both tested concentrations, restored expression levels to those of

untreated cells (Figure 1.2).Significantly, in our experimental setting, the seed extract exhibited the most pronounced inhibitory effect, restoring the expression levels of all proinflammatory genes considered to those of non-stimulated cells. The distinctive phytochemical profile of this extract, characterized by a higher concentration of organic acids or specific proanthocyanin polymerization levels—currently undetermined—could potentially account for the observed variations in activity.

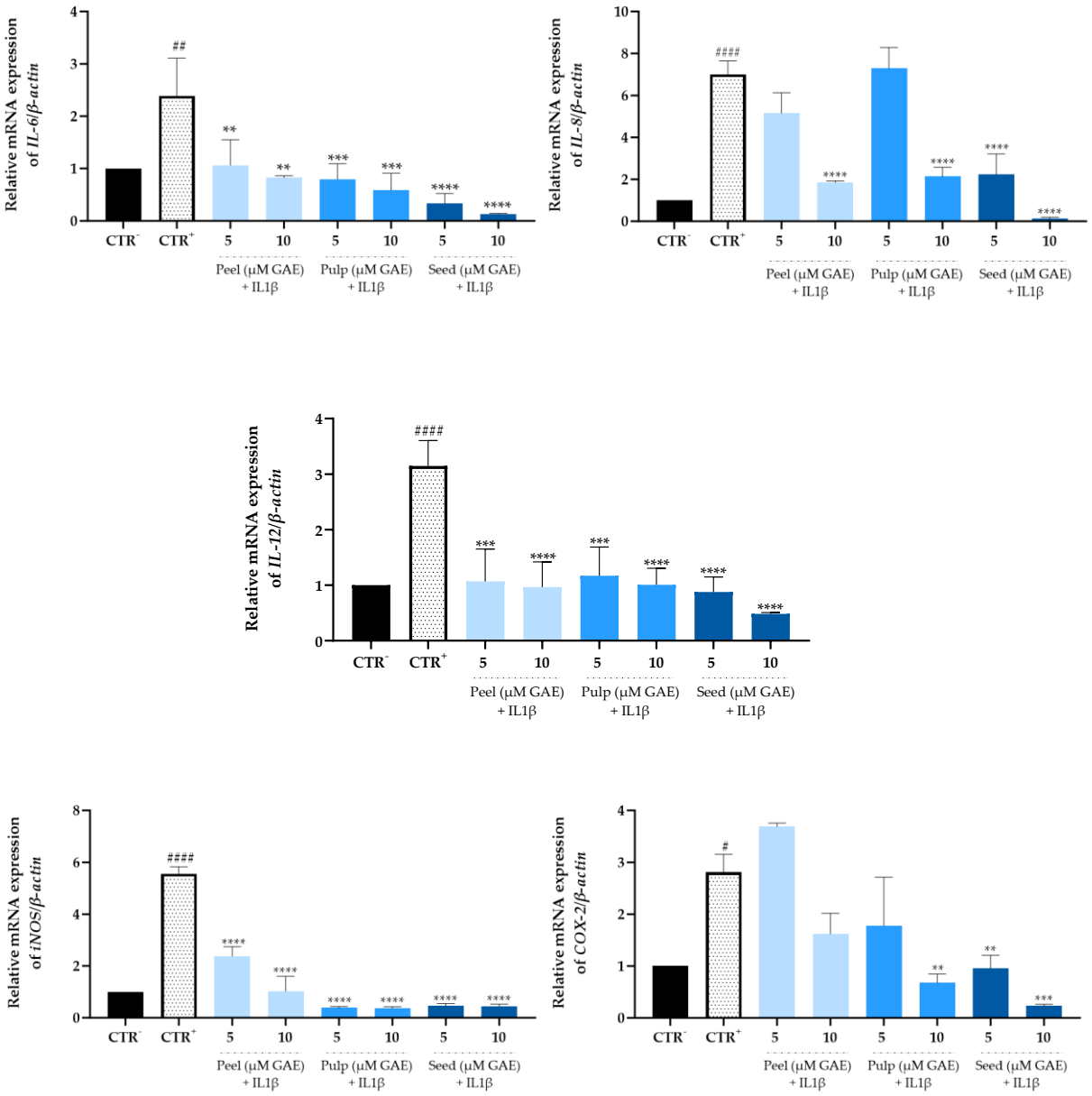


Figura 1.2: Expression of inflammatory genes (*IL-6*, *IL-8*, *IL-12*, *iNOS,* and *COX2*) from Caco-2 cells exposed to the proinflammatory action of IL-1β. Gene expression was determined by qRT-PCR. β-actin was used as the reference gene. Cells were incubated in the absence of additional treatments (CTR^-), preincubated in the absence of additional treatments and subsequently incubated for 24 h with 10 ng/mL IL-1β (CTR^+), or preincubated for 2 h in the presence of different concentration of peel, pulp, and seed extracts and subsequently co-incubated for 24 h with 10 ng/mL IL-1β. After the different treatments, cells were collected, and total cellular RNA was isolated and retro-transcribed to cDNA. The cDNA was used as template for quantitative real-time PCR using β-actin as a reference gene. Bars represent the mean ± SD of three qRT-PCR analyses carried out in triplicate. Data were calculated as fold-change. One-way ANOVA followed by Tukey's test was used to generate p values. "#" indicates significative differences compared to CTR^- ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ $^{\#\#\#\#}p < 0.0001$); "*" indicates significative differences compared to CTR^+ (*p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).

In contrast with the observed effects on proinflammatory gene expression, our findings demonstrated a counteractive impact of the black persimmon extracts on the *IL-10* gene expression. IL-10 plays a pivotal role in inhibiting the inflammatory response of both innate and adaptive immune cells. Additionally, the spontaneous development of intestinal inflammation in *IL-10* gene - or IL-10 receptor- knockout rats suggests a crucial role for this interleukin in maintaining mucosal intestinal homeostasis (Shouval et al., 2014). Under our experimental conditions, differentiated Caco-2 cells express the *IL-10* gene and release the corresponding cytokine into the medium. The treatment of cells with IL-1β, in the absence or presence of pulp and peel extracts, does not influence the expression of the gene encoding IL-10. However, a significant upregulation (2.8-fold) of the gene is observed when stimulated cells are exposed to the highest concentration (10 μM GAE) of the seed extract (Figure 2.2).

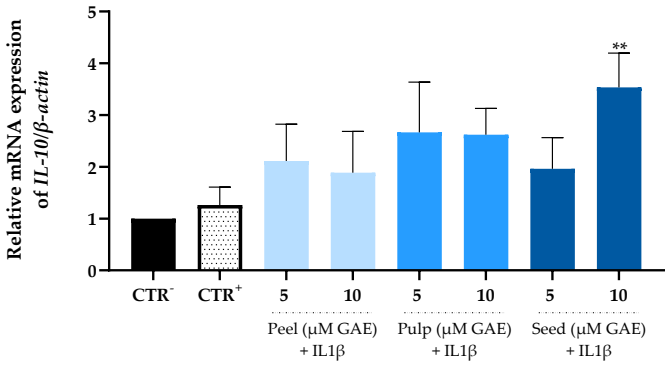


Figure 2.2: *IL-10* gene expression in Caco-2 cells exposed to IL-1β. Gene expression was determined by qRT-PCR. β-actin was used as the reference gene. Cells were incubated in the absence of additional treatments (CTR^-), preincubated in the absence of additional treatments and subsequently incubated for 24 h with 10 ng/mL IL-1β (CTR^+), or preincubated for 2 h in the presence of different concentration of peel, pulp, and seed extracts and subsequently co-incubated for 24 h with 10 ng/mL IL-1β. After the different treatments, cells were collected, and total cellular RNA was isolated and retro-transcribed to cDNA. The cDNA was used as template for quantitative real-time PCR using β-actin as a reference gene. Bars represent the mean ± SD of three qRT-PCR analyses carried out in triplicate. Data were calculated as fold-change. One-way ANOVA followed by Tukey's test was used to generate p values. "#" indicates significative differences compared to CTR^- ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ $^{\#\#\#\#}p < 0.0001$); "*" indicates significative differences compared to CTR^+ ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

Effects of black persimmon on IL-6, IL.8, and IL-10 release

The amplification of pro-inflammatory cytokine release from epithelial cells plays a crucial role in advancing intestinal inflammation, concurrently activating cells within the gut-associated lymphoid tissue (Gentile et al., 2015). Limited data currently exist regarding the inhibitory potential of plant extracts on the expression and release of pro-inflammatory cytokines in intestinal inflammation models. Previous studies have demonstrated inhibitory effects on the expression and secretion of IL-8 and IL-6 in differentiated Caco-2 monolayers subjected to pro-inflammatory stimuli when treated with hydrophilic extracts from green tea, pistachio, and pomegranate fruits (Gentile et

al., 2015; Romier-Crouzet et al., 2009; H. Wu et al., 2009). In our experimental setup, exposing differentiated epithelial monolayers to IL-1β for 24 hours results in a significant increase in the release of two pro-inflammatory cytokines, IL-8 and IL-6, quantified by ELISA, compared to unstimulated cells.

Consistent with the effects on the expression of corresponding genes, both pulp and seed extracts dose-dependently reduce the release of IL-6 and IL-8 induced by IL-1β stimulation. Notably, the seed extract exerts the most quantitatively significant inhibitory effect, restoring the release values of the two cytokines to the levels observed in unstimulated cells.

Concerning the extract derived from black sapote peel, no significant effects on the secretion of IL-6 and IL-8 are observed in cells activated by IL-1β, despite the demonstrated dose-dependent downregulation of the two corresponding genes. A lesser intensity of the inhibitory effects of the peel extract compared to those of pulp and seed on the expression of IL-6 and IL-8, along with a delay between downregulation and the actual decrease in the concentration of these cytokines in the cellular supernatant, might justify the lack of consistency for the peel extract between gene expression data and secretion data. On the other hand, it cannot be ruled out that other genes or signaling pathways activated by components in the peel extract may compensate for the downregulation of the considered genes, masking the expected effect on the levels of the corresponding proteins.

Conversely, in accordance with the gene expression data, exposure of differentiated epithelial monolayers to IL-1β for 24 hours results in a significant decrease in IL-10 release. No changes in IL-10 release were found in cells pre-treated with peel extract, compared to IL-1β stimulated cells. On the other hand, pre-treatment of Caco-2 cells with black persimmon seed extract results in a substantial 11-fold increase in the release of the anti-inflammatory cytokine. Unexpectedly, even treatment of IL-1β-stimulated cells with pulp extract demonstrates activity in enhancing the release of IL-10 (Figura 3.2).

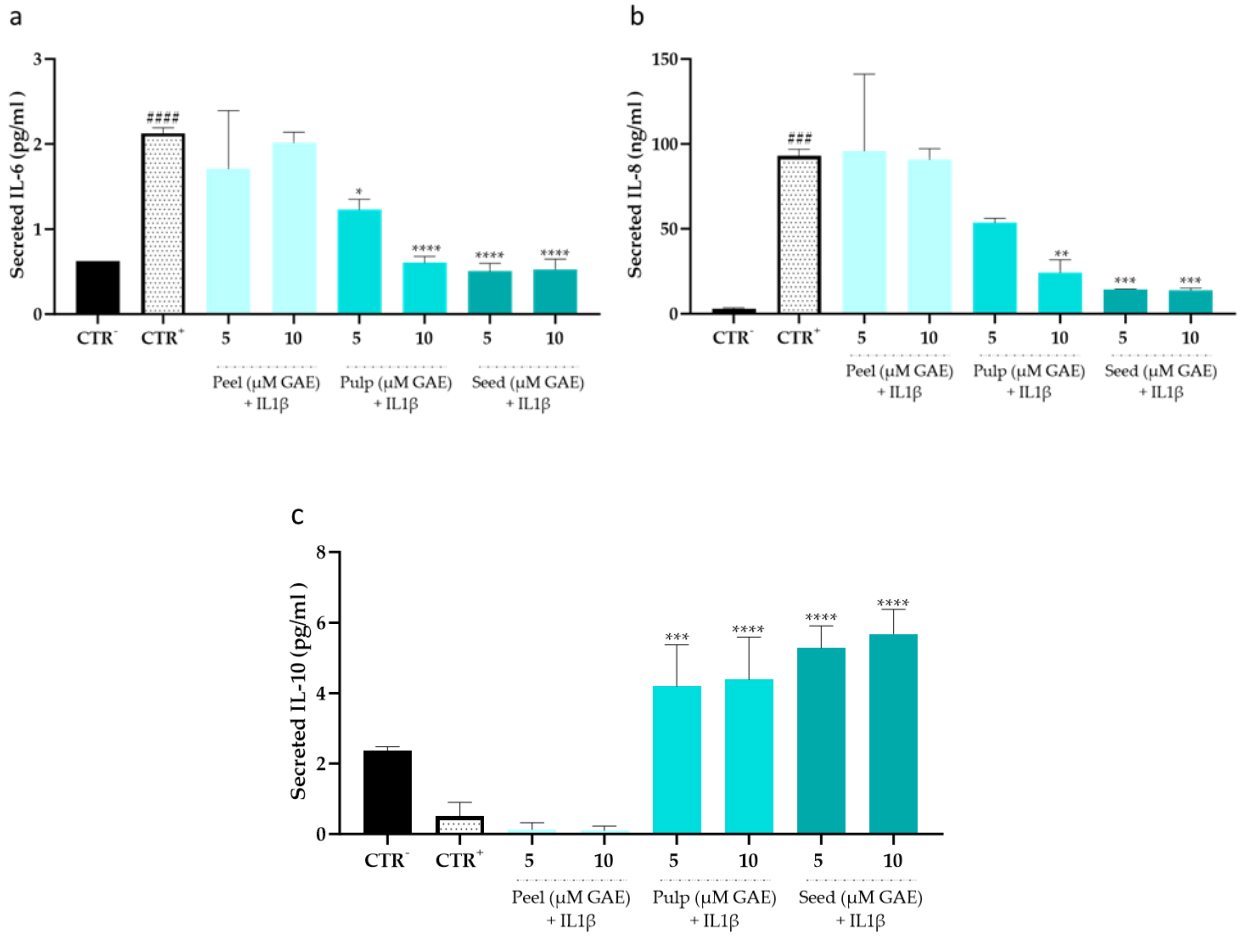


Figure 3.2: IL-6 (a), IL-8 (b), and IL-10 (c) secretion from Caco-2 cells and effect of black persimmon extracts. Cells were incubated in the absence of additional treatments (CTR^-), preincubated in the absence of additional treatments and subsequently incubated for 24 h with 10 ng/mL IL-1β (CTR^+), or preincubated for 2 h in the presence of different concentration of peel, pulp, and seed extracts and subsequently co-incubated for 24 h with 10 ng/mL IL-1β. Supernatants were collected, and IL-6, IL-8, and IL-10 concentrations were measured by ELISA. Each value is the mean ± SD of three experiments carried out in triplicate. One-way ANOVA followed by Tukey's test was used to generate p values. "#" indicates significative differences compared to CTR^- ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ $^{\#\#\#\#}p < 0.0001$); "*" indicates significative differences compared to CTR^+ ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

Effects of black persimmon on the release of INF-γ

IFN-γ acts as a potent activator of the inflammatory response in phagocytic cells and plays a crucial role in both cell-mediated and humoral immune responses. While T and B lymphocytes are traditionally associated with the production and release of this cytokine, existing literature also highlights the involvement of intestinal epithelial cells in IFN-γ production and release (Romier-Crouzet et al., 2009).In our experimental setup, exposure of differentiated epithelial monolayers to IL-1β leads to an increased release of IFN-γ in the cell supernatants. Remarkably, preincubation of cells with black persimmon extracts significantly hinders the release of IFN-γ. While pulp and seed extracts result in inhibitions of 52% and 43%, respectively, the peel extract at the highest concentration demonstrates the most pronounced inhibitory effect (80% compared to the positive control) (Figure 4.2). The demonstrated activity may be ascribed to the elevated concentrations of chlorogenic acid present in the peel extract, potentially elucidating the observed robust activity. *In vivo* investigations conducted in murine models have substantiated the decline in IFN-γ levels in both the jejunum and colon following supplementation with chlorogenic acid (Ruan et al., 2014).

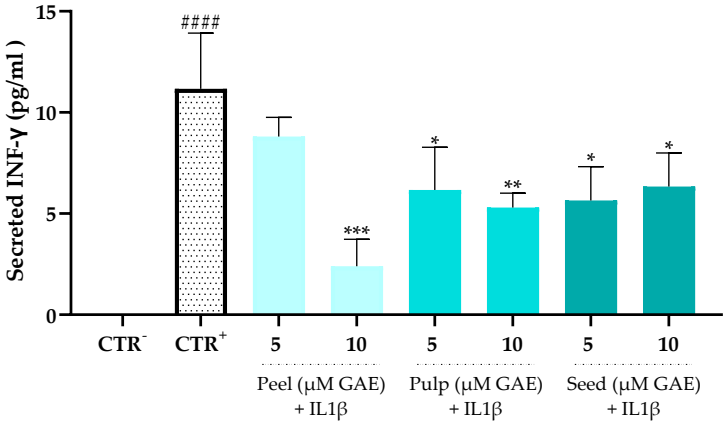


Figure 4.2: INF-γ secretion from Caco-2 cells and effect of black persimmon extracts. Cells were incubated in the absence of additional treatments (CTR$^-$), preincubated in the absence of additional treatments and subsequently incubated for 24 h with 10 ng/mL IL-1β (CTR$^+$), or preincubated for 2 h in the presence of

different concentration of peel, pulp, and seed extracts and subsequently co-incubated for 24 h with 10 ng/mL IL-1β. Supernatants were collected, and INF-γ concentrations were measured by ELISA. Each value is the mean ± SD of three experiments carried out in triplicate. Oneway ANOVA followed by Tukey's test was used to generate p values. "#" indicates significative differences compared to CTR^- ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ $^{\#\#\#\#}p < 0.0001$); "*" indicates significative differences compared to CTR^+ (*p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).

Black persimmon effect on IL-1β-induced COX-2 protein expression

Among proinflammatory mediators, COX-2 assumes a pivotal role. In stark contrast to its constitutive counterpart, COX-1, COX-2 maintains minimal detection levels under normal physiological conditions but quickly responds to proinflammatory stimuli. The prolonged overexpression of COX-2 precipitates the release of prostaglandins, notably PGE2, recognized for their implication in the pathogenesis of IBDs and colorectal cancer (Wang et al., 2010). Within various epithelial tissues, including the intestinal epithelium, IL-1β triggers the upregulation of COX-2 expression, culminating in the release of PGE2 (Duque et al., 2006). Notably, polyphenol-rich extracts have been reported to effectively inhibit this release (Romier-Crouzet et al., 2009).

The expression of COX-2 in Caco-2 cells was evaluated through Western blot analysis. Under our experimental conditions, untreated Caco-2 cells exhibited detectable levels of COX-2 protein. Despite 24 hours of treatment with 10 ng/ml IL-1β being sufficient to demonstrate the upregulation of the COX-2 gene, it did not induce significant variations in enzyme expression (data not shown). However, prolonged exposure to IL-1β for 48 hours resulted in a tenfold increase in protein expression compared to untreated cells. In these experimental conditions, cell exposure to black persimmon extracts from both peel and seed (5-10 μM GAE) maintained protein levels comparable to those of untreated cells. Unexpectedly, coincubation with IL-1β and pulp extract further increased enzyme expression compared to cells treated only with IL-1β (Figure 5.2). Our overall results indicate that components of black sapote extracts have specific and selective effects on COX-2 expression induced by proinflammatory stimuli, without influencing basal expression (data not shown).

The consequences of the observed variations, whether in the same or opposite direction regarding enzyme levels, remain to be determined. Although excessive COX-2 expression is commonly associated with pathological conditions, scientific data demonstrate that in specific contexts, increased COX-2 expression can have protective effects or be associated with beneficial physiological responses. This is noteworthy considering that some products of COX-2 play a significant role in protecting gastric mucosa and regulating renal blood flow. In this context, concerning the pathogenesis of NSAID-enteropathy, although inhibition of COX-1 is of pivotal importance, COX-2 inhibition may have a synergistic detrimental action (Hotz-Behofsits et al., 2003). In addition, some studies suggest that increased COX-2 expression may be important in stimulating the immune response.

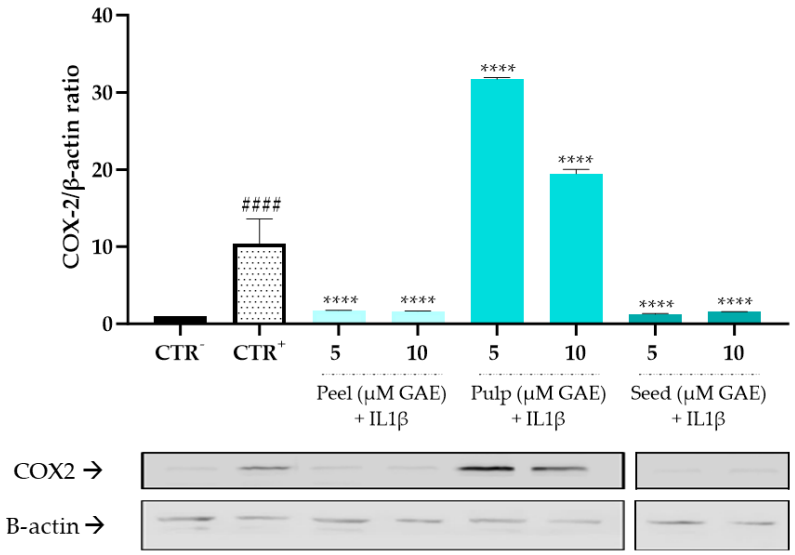


Figura 5.2: COX-2 protein expression in Caco-2 cells and effect of black persimmon extracts. Cells were incubated in the absence of additional treatments (CTR^-), preincubated in the absence of additional treatments and subsequently incubated for 48 h with 10 ng/mL IL-1β (CTR^+), or preincubated for 2 h in the presence of different concentration of peel, pulp, and seed extracts and subsequently co-incubated for 48 h with 10 ng/mL IL-1β. Cells were collected, and the proteins were isolated for Western blot analysis

as described in ‘‘Materials and methods’’. Panel shows representative Western blot and densitometric analysis. The values represent the relative expression levels normalized to β-actin and are the mean ± SD of three separate experiments with similar results. One-way ANOVA followed by Tukey's test was used to generate p values. "#" indicates significative differences compared to CTR^- ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ $^{\#\#\#\#}p < 0.0001$); "*" indicates significative differences compared to CTR^+ (*p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).

Black persimmon effect on IL-1β-induced iNOS protein expression

Nitric Oxide Synthase (NOS) enzymes play a critical role in catalyzing the conversion of arginine to nitric oxide (NO), a pivotal signaling molecule involved in diverse physiological processes, including immune responses and inflammation. The overproduction of NO by inducibile NOS (iNOS) has been implicated in various inflammatory conditions and autoimmune diseases. Its categorization as "inducible" stems from its heightened production triggered by external factors such as infection, inflammatory cytokines, and immune-related signals (Cinelli et al., 2020).

Cell exposure to IL-1β for 24 hours leads to the upregulation of iNOS production, according with the expression data of its associated gene. Remarkably, pre-treatment of the cells with black persimmon extracts, particularly at the highest concentration (10 µM GAE), aligns with the observed downregulation of the gene. This pre-treatment demonstrates a consequential suppression of the induced enzyme expression levels mediated by IL-1β, ultimately restoring the basal levels observed in untreated cells (Figure 6.2).

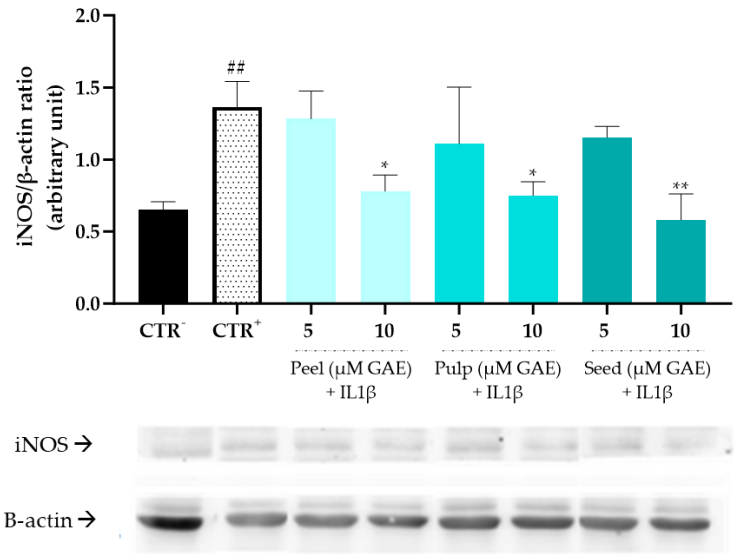


Figure 6.2: iNOS protein expression in Caco-2 cells and effect of black persimmon extracts. Cells were incubated in the absence of additional treatments (CTR^-), preincubated in the absence of additional treatments and subsequently incubated for 24h with 10 ng/mL IL-1β (CTR^+), or preincubated for 2 h in the presence of different concentration of peel, pulp, and seed extracts and subsequently co-incubated for 24 h with 10 ng/mL IL-1β. Cells were collected, and the proteins were isolated for Western blot analysis as described in ''Materials and methods''. Panel shows representative Western blot and densitometric analysis. The values represent the relative expression levels normalized to β-actin and are the mean ± SD of three separate experiments with similar results. One-way ANOVA followed by Tukey's test was used to generate p values. "#" indicates significative differences compared to CTR^- ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ $^{\#\#\#\#}p < 0.0001$); "*" indicates significative differences compared to CTR^+ (*p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).

Black persimmon effects on NF-κB activation

Within the intricate web of inflammatory cascades, the pathways orchestrated by NF-κB emerge as a focal point of considerable interest (Lawrence, 2009). Indeed, the intricate control over the expression of genes encoding numerous proinflammatory mediators is, in part, mediated by the transcriptional activation of NF-κB (Barnes & Karin, 1997; Duque et al., 2006). In the context of Caco-2 cells, IL-1β assumes a pivotal role as the major driver behind the release of IL-6, IL-8, and PGE2 (Van De Walle et al., 2010), concurrently acting as the most potent activator of NF-κB within this cellular milieu.

A shared intracellular mechanism, responding to proinflammatory stimuli such as IL-1β and LPS, unfolds through the swift phosphorylation of the N-terminal region of two serine residues of the cytosolic inhibitor of NF-κB, IκB-α. This intricate process, orchestrated by an IκB kinase complex, is promptly succeeded by ubiquitination and subsequent degradation of IκB-α. This series of events culminates in the activation of NF-κB, facilitating the translocation of the p65/p50 dimer from the cytosol to the nucleus (Barnes & Karin, 1997).

The impact of black persimmon extracts on IL-1β-induced NF-κB activation was evaluated through the assessment of both the IκB-α phosphorylation and the p65/p50 heterodimer translocation into the nucleus. Interestingly, while the treatment of Caco-2 cells with black persimmon pulp extracts appears to enhance NF-κB activation, cell exposure to the seed extract presents noteworthy dose-dependent reduction in the phosphorylation of IκB-α induced by IL-1β. This reduction is clearly evident through the presence of elevated quantities of the non-phosphorylated protein, as observed in comparison to cell lysates treated solely with IL-1β (Figure 7.2). These findings underscore a nuanced and potentially divergent regulatory role of different components within black persimmon extracts, shedding light on the complex interplay between cellular responses and specific extract constituents.

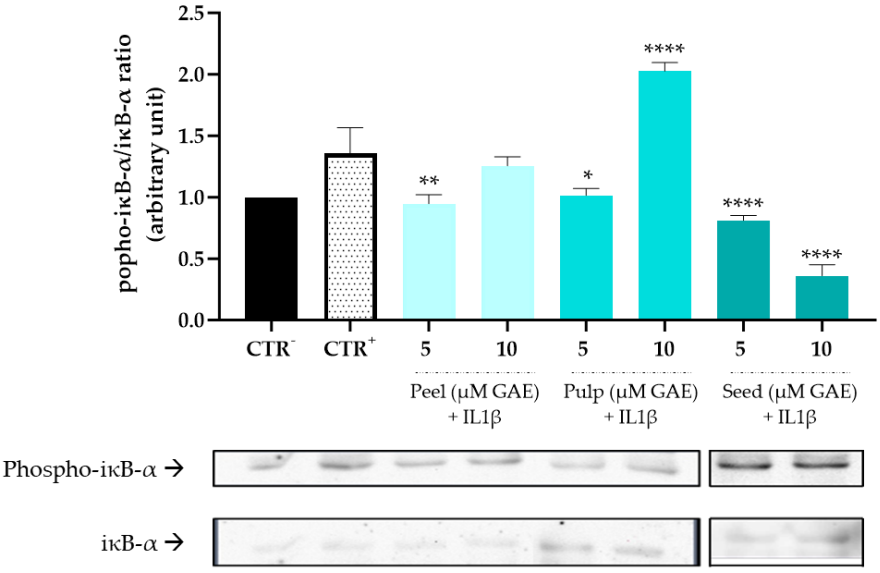
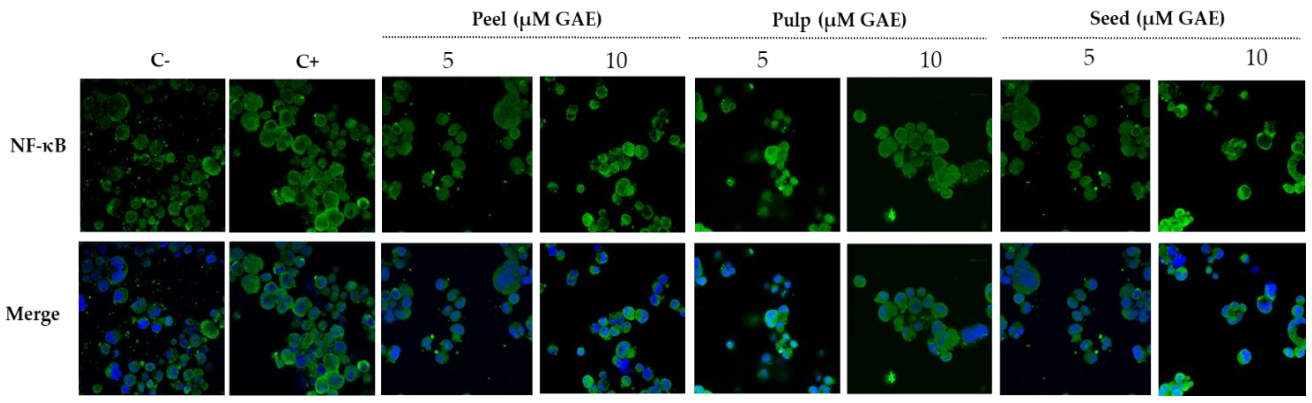


Figure 7.2: Effects of black persimmon extracts treatment on IκB-α phosphorylation in Caco-2 cells. Cells were incubated in the absence of additional treatments (CTR^-), preincubated in the absence of additional treatments and subsequently incubated for 24h with 10 ng/mL IL-1β (CTR^+), or preincubated for 2 h in the presence of different concentration of peel, pulp, and seed extracts and subsequently co-incubated for 24 h with 10 ng/mL IL-1β. Cells were collected, and the proteins were isolated for Western blot analysis as described in ''Materials and methods''. Panel shows representative Western blot and densitometric analysis. The values represent the relative expression levels normalized to β-actin and are the mean ± SD of three separate experiments with similar results. One-way ANOVA followed by Tukey's test was used to generate p values. "#" indicates significative differences compared to CTR^- ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ $^{\#\#\#\#}p < 0.0001$); "*" indicates significative differences compared to CTR^+ (*p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).

The inhibitory impact of the seed extracts on NF-κB activation is further substantiated through the analysis of the p65/p50 heterodimer translocation using an immunofluorescence *in situ* assay. The data obtained indicate that, following IL-1β stimulation, more than 80% of the observed cells exhibit a positive nuclear staining for p65. A comparable NF-κB activation is noted in cells exposed to the pulp extract. In stark contrast, treatment with peel and seed extracts reveals a dose-dependent inhibition of the p65/p50 translocation into the nuclei. This effect is particularly noteworthy when cells were treated with the high concentration of the extracts as the treatment restores the percentage of p65/p50 NF-κB nuclei-positive cells to that of negative control (Figure 8.2).

A



B

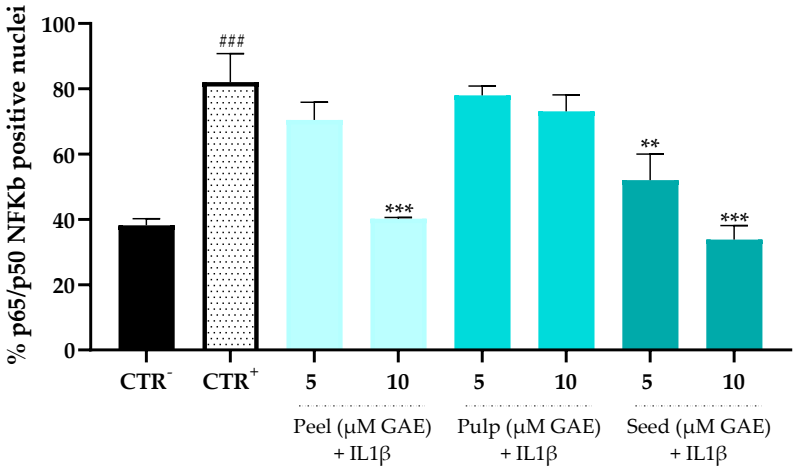


Figure 8.2: Immunofluorescence analysis of NF-kB activation. **A:** Immunofluorescence analysis of NF-kB activation. **A:** Differentiated CaCo-2 cells. Cells were incubated in the absence of additional treatments (CTR^-), preincubated in the absence of additional treatments and subsequently incubated for 24h with 10 ng/mL IL-1β (CTR^+), or preincubated for 2 h in the presence of different concentration of peel, pulp, and seed extracts and subsequently co-incubated for 24 h with 10 ng/mL IL-1β.The nuclear translocation were determined by NF-kB p65/p50 immunolocalization (green fluorescence). Nuclei were counterstained with Hoechst 33342 (blue fluorescence). **B:** Percentage of nucleic positive cells to p65/p50 NFκB. The results are representative of three independent experiments. One-way ANOVA followed by Tukey's test was used to generate p values. "#" indicates significative differences compared to CTR^- ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ $^{\#\#\#\#}p < 0.0001$); "*" indicates significative differences compared to CTR^+ (*p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).

The robust findings from our study strongly indicate that the protective effects demonstrated by black persimmon extracts, with a notable emphasis on the seed extract, in our experimental model of intestinal inflammation are closely tied to a noticeable reduction in NF-κB activation. These compelling results underscore the potential therapeutic significance of black persimmon seed extract in effectively modulating inflammatory responses within the intricate environment of the intestinal system. This insight opens up promising avenues for further exploration and development of black persimmon-derived interventions as potential agents in mitigating inflammation-related challenges in the gastrointestinal context.

Black persimmon effects on NrF2 expression

While NF-κB plays a central role in orchestrating the inflammatory response and generating pro-inflammatory molecules, Nrf2 functions as a protective mechanism against reactive species and inflammatory insults by activating the cytoprotective pathway. This activation induces the expression of a myriad of cytoprotective enzymes and proteins crucial for cellular defence against ROS, and electrophilic species (Nguyen et al., 2009). Today, the interaction between the NF-κB and Nrf2 signaling pathways is widely acknowledged and carries substantial importance (Wardyn et al., 2015).

In particular, the extant crosstalk between Nrf2 and NF-κB identifies them as pivotal targets. Bioactive compounds with the ability to activate Nrf2 and concurrently diminish NF-κB activity emerge as promising candidates for an effective strategy in the prevention of inflammation. Accumulating evidence underscores the ability of various phytochemicals to target a wide array of signaling pathways, including both Nrf2 and NF-κB pathways (Krajka-Kuźniak & Baer-Dubowska, 2021; Wu et al., 2022; Xu et al., 2022). Consequently, the impact of plant food matrices on Nrf2 and NF-κB signaling pathways has been a subject of numerous studies, including human intervention trials (Chen et al., 2015; Ding et al., 2020; Na et al., 2008).

Therapeutic interventions utilizing extracts derived from coffee, bilberry pomace, virgin olive oil, sunflower seed oils, cherry juice, grape, and broccoli sprouts have shown a notable augmentation in the overall expression of the Nrf2 gene. The duration of the clinical trial and the specific time points assessed exert discernible influences on the observed outcomes (Krajka-Kuźniak & Baer-Dubowska, 2021).

In our experimental design, we subjected differentiated Caco-2 monolayers to IL-1β exposure for 24 hours, observing minimal impact on the basal expression level of the transcription factor Nrf2. Exposure of cells to black persimmon extracts did not yield significant effects on the basal quantity of Nrf2 (data not shown). Conversely, while pre-treating cells with pulp extract reduced Nrf2 levels in IL-1β-exposed cells, pre-treatment with seed extract increased these levels in a dose-dependent manner. No significant

variation was observed when IL-1β-stimulated cells were pre-treated with peel extract (Figure 9.2).

The cumulative impact of black persimmon pulp extract constituents on IL-1β-stimulated cells, particularly the reduction in Nrf2 levels concomitant with an augmented NF-kB activation, would suggest a potential pro-inflammatory influence. This hypothesis finds support in the observed elevation of IL-8 release and COX-2 levels subsequent to pre-treatment with pulp extract before IL-1β stimulation. Further investigation is warranted to elucidate this hypothesis, especially considering that the extract with potential pro-inflammatory properties is derived from the edible portion of the fruit.

On the other hand, the demonstrated effect of seed extract on Nrf2 levels, aligned with its proven inhibitory effects on NF-kB activation and the expression and release of pro-inflammatory mediators, further indicates a genuine protective potential against intestinal inflammation attributed to the phytochemical characteristics of this fruit portion. It is noteworthy that the phytochemical profile of black persimmon is characterized, among other compounds, by a significant presence of fumaric acid, whose positive action on Nrf2 activation is extensively documented (Hybertson et al., 2011).

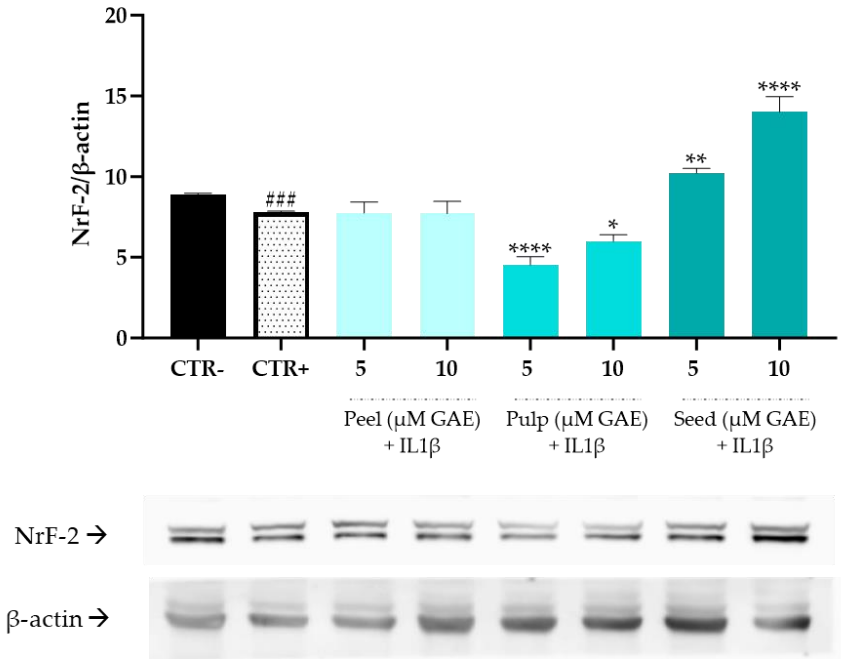


Figure 9.2: Nrf2 protein expression in Caco-2 cells and effect of black persimmon extracts. Cells were incubated in the absence of additional treatments (CTR^-), preincubated in the absence of additional treatments and subsequently incubated for 24h with 10 ng/mL IL-1β (CTR^+), or preincubated for 2 h in the presence of different concentration of peel, pulp, and seed extracts and subsequently co-incubated for 24 h with 10 ng/mL IL-1β. Cells were collected, and the proteins were isolated for Western blot analysis as described in ''Materials and methods''. Panel shows representative Western blot and densitometric analysis. The values represent the relative expression levels normalized to β-actin and are the mean ± SD of three separate experiments with similar results. One-way ANOVA followed by Tukey's test was used to generate p values. "#" indicates significative differences compared to CTR^- ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ $^{\#\#\#\#}p < 0.0001$); "*" indicates significative differences compared to CTR^+ (*p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001

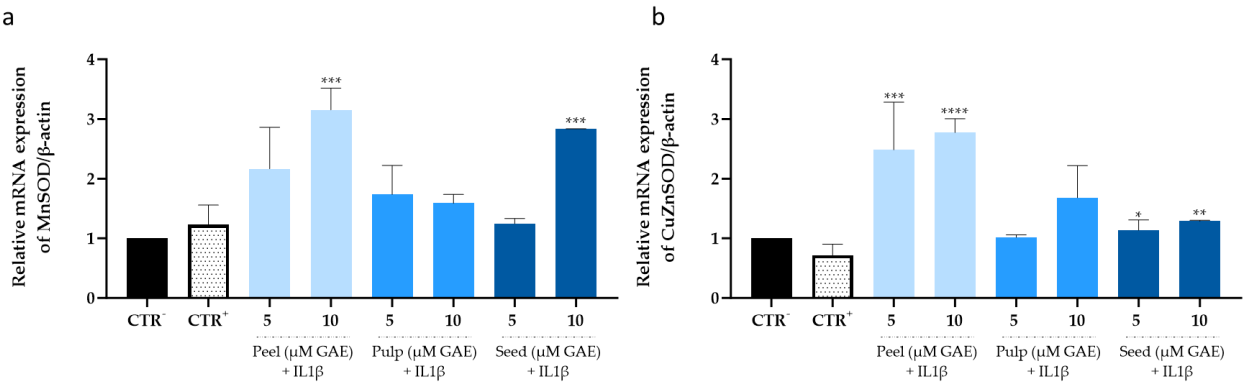
Black persimmon effects on expression of genes encoding antioxidant enzymes

Experimental evidence underscores that the influence of diverse phytochemicals on cellular redox balance extends beyond their redox-active properties, encompassing their regulatory impact on antioxidant enzymes' activity or expression (Mannino, Perrone, et al., 2020). This influence appears to be intricately linked with the modulation of redox-sensitive sites in biological targets, notably Nrf2, which govern the expression of these enzymes.

Previously our data delineate that preincubation with black persimmon extracts on human hepatocytes exposed to hydrogen peroxide results in a heightened expression of genes encoding pivotal antioxidant enzymes (CuZnSOD, MnSOD, GPx, and CAT). In addition, our investigations reveal that preincubation of intestinal epithelial cells with black persimmon seed extract prior to IL-1β stimulation augments Nrf2 protein levels.

To elucidate the impact of antioxidant phytochemicals within black persimmon extracts on the observed anti-inflammatory activity, we evaluated the influence of the three extracts on the expression of *CuZnSOD, MnSOD,* and *GPx* genes in differentiated Caco-2 cells subjected to the proinflammatory effects of IL-1β.

Intestinal epithelial monolayers express detectable levels of the three considered genes, with their expression remaining unaltered following a 24-hour exposure to 10 ng/ml IL-1β or a two-hour exposure to black persimmon extracts. Despite the absence of a notable effect on *GPx* gene expression in cells stimulated with IL-1β, pretreatment with the extracts from black persimmon peel and seed induces a significant upregulation of genes encoding the two isoforms of SOD (Figure 10.2). Notably, the prominent effect of the peel extract on the expression of antioxidant enzymes is in line with the observed protective effect in HepG2 cells subjected to a pro-oxidant stimulus (Mannino et al., 2022).On the other hand, the effects observed with the seed extract align with its influence on Nrf2 protein levels, underscoring the pivotal role of this extract's specific phytochemical profile in modulating the antioxidant response of cells subjected to a proinflammatory stimulus. HPLCDAD-MS/MS analysis uncovered a notable presence of fumaric acid in the seed extract. Fumaric acid, a well-recognized compound along with its ester derivatives, is renowned for its hepatoprotective properties attributed to its engagement in antioxidant mechanisms (Šilhavý et al., 2014). Significantly, prior research has validated the ability of fumaric acid to activate Nrf2, the "master regulator" of the antioxidant response. This activation, as documented by Hybertson et al. (2011), plays a pivotal role in modulating the expression of numerous genes, including those responsible for encoding antioxidant enzymes (Hybertson et al., 2011).



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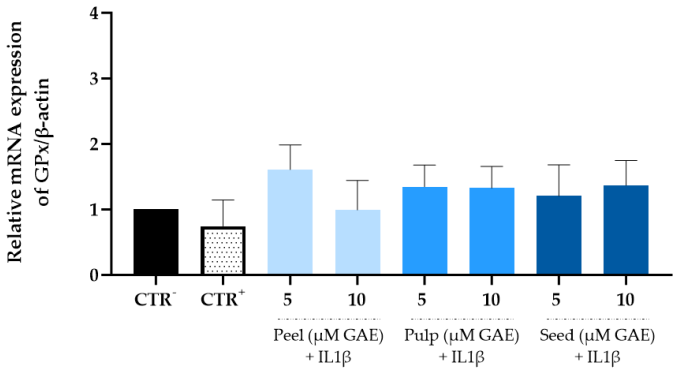

Figure 10.2: Expression of (a) MnSOD, (b) CuZnSOD and (c) GPx in IL-1β-stimulated Caco-2 cells. Gene expression was determined by qRT-PCR. β-actin was used as the reference gene. Cells were incubated in the absence of additional treatments (CTR^-), preincubated in the absence of additional treatments and subsequently incubated for 24 h with 10 ng/mL IL-1β (CTR^+), or preincubated for 2 h in the presence of different concentration of peel, pulp, and seed extracts and subsequently co-incubated for 24 h with 10 ng/mL IL-1β. After the different treatments, cells were collected, and total cellular RNA was isolated and retro-transcribed to cDNA. The cDNA was used as template for quantitative real-time PCR using β-actin as a reference gene. Bars represent the mean ± SD of three qRT-PCR analyses carried out in triplicate. Data were calculated as fold-change. One-way ANOVA followed by Tukey's test was used to generate p values. "#" indicates significative differences compared to CTR^- ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ $^{\#\#\#\#}p < 0.0001$); "*" indicates significative differences compared to CTR^+ (*p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).

Epithelial intestinal barrier function

Evaluation of paracellular permeability - transport of FSA

The barrier function of intestinal epithelial cells plays a crucial role in maintaining the integrity of the gastrointestinal tract and regulating the passage of substances between the gut lumen and the underlying tissues. The barrier functions depend critically on the organization of tight junctions, which are specialized structures formed by adhesive proteins that connect adjacent epithelial cells, establishing complex interactions with cytoskeletal and cytoplasmic proteins. Tight junctions contribute to the selective

permeability of the epithelium by reducing the likelihood of paracellular leakage and maintaining a tight seal between adjacent cells. Indeed, they create a selectively permeable barrier, preventing the uncontrolled entry of unwanted substances, including pathogens and toxins. The proper functioning of this barrier is essential for overall gut health.

On the other hand, increased permeability is a pivotal event in the context of intestinal inflammation (Bruewer et al., 2003). It stems from compromised integrity of the intestinal barrier, characterized by the formation of intercellular gaps in the mucosa, leading to heightened paracellular permeability (DeMeo et al., 2002). This, in turn, amplifies the exposure of immune cells to potential proinflammatory agents that are typically confined to the intestinal lumen. The increase in epithelial permeability, contributing to the inflammatory process, establishes a self-sustaining cycle.

The genesis of gaps within the intestinal mucosa during inflammation can be attributed to cell death or the reorganization of epithelial adhesion complexes, resulting in alterations in cell shape. Convincing studies have demonstrated that the perturbation of epithelial Tight Junctions, arising from the altered expression and distribution of junctional proteins, represents a significant mechanism through which proinflammatory cytokines induce heightened intestinal permeability (R. M. Al-Sadi & Ma, 2007).

To assess the impact of black persimmon extracts on the induced increase in epithelial permeability following exposure to IL-1β, we evaluated the effects of pretreatment with extracts from the peel, pulp, and seed of black persimmons on the permeability across differentiated Caco-2 cells. FSA, a well-established marker transported paracellularly, was employed for this assessment as previously reported (Gentile et al., 2015).

In addition, we evaluate the expression level and distribution of the Tigh junction protein ZO-1.

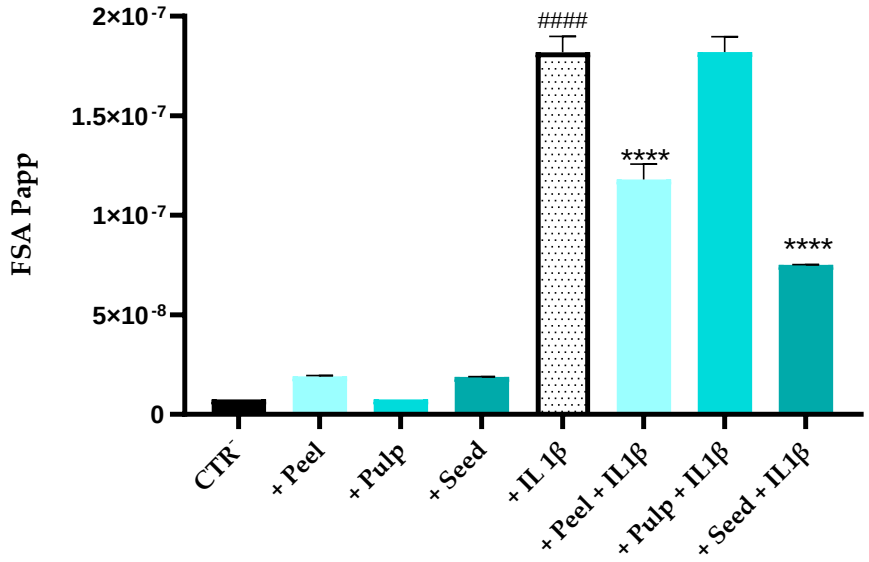

Figure 11.2: Effects of black persimmon extracts treatment on the transport of fluorescein sulfonic acid (FSA) across Caco-2 cell monolayer. Cells were incubated in the absence of additional treatment (CTR^-), preincubated in the absence of additional treatment and subsequently incubated for 24 h with 10 ng/mL IL-1β (CTR^+), or preincubated for 2h in the presence of peel, pulp, and seed extracts (10 µM GAE) and subsequently co-incubated for 24 h with or without 10 ng/ mL IL-1β. Results are a mean ± SE of three independent experiments. Oneway ANOVA followed by Tukey's test was used to generate p values. "*" indicates significative differences compared to CTR^+ (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

IL-1β initiates a notable increase in paracellular permeability within differentiated Caco-2 cell monolayers cultured on transwells, evidenced by an elevated flow of fluorescein sodium salt (FSA). In our experimental setup, the apparent permeability coefficient (Papp) of FSA surges by 25-fold after 24 hours of exposure of differentiated monolayers to 10 ng/ml IL-1β, which is added to the culture medium in the basolateral compartment. Intriguingly, when black persimmon peel and seed extracts are introduced into the apical compartment, a restrained increase in the Papp of FSA induced by IL-1β is observed (Figure 11.2). These findings compellingly illustrate that, under our experimental conditions, both peel and seed extracts significantly reinforce the resistance of epithelial monolayers against the integrity loss induced by IL-1β. The

demonstrated presence of specific components, such as proanthocyanidins, within these extracts could contribute to this observed effect (Mannino et al., 2022).

Proanthocyanidins, especially those with a high degree of polymerization, are indeed recognized for their stability in gastrointestinal conditions and poor absorption in the intestinal lumen (Rios et al., 2002). They actively engage with cell membranes, significantly contributing to the protection of the gastrointestinal tract. Previous studies have demonstrated that pure proanthocyanidins or those within the context of plant extracts interact with the membranes of intestinal epithelial monolayers (Gentile et al., 2015).In particular, Erlejman et al. (2006) demonstrated that hexameric proanthocyanidins from cocoa prevent the increase in paracellular permeability induced by deoxycholic acid in Caco-2 cells. This prevention occurs by hindering the stimulus-induced redistribution of junctional proteins associated with pro-inflammatory processes (Erlejman et al., 2006).

Localization of tight junction protein ZO-1

The organization of tight junctions involves several proteins, including adhesion proteins (claudins and occludins), cytoskeletal actin, and several adaptor proteins linking the adhesion proteins to the cytoskeleton.

Among the adaptor proteins, the most important in tight junctions are Zonula Occludens (ZO) Proteins, including ZO-1, ZO-2, and ZO-3.

ZO-1, a pivotal scaffolding protein, plays a central role in linking transmembrane proteins of tight junctions to the actin cytoskeleton within the cell. This linkage contributes significantly to the formation and stabilization of tight junctions, serving as a selectively permeable barrier anchored by ZO-1. Beyond its structural role, ZO-1 actively engages in cell signaling by interacting with various signaling proteins and kinases, influencing cellular responses to environmental stimuli. In the context of inflammation, ZO-1 may undergo modulation through signaling pathways, thereby impacting the dynamics of tight junctions and overall barrier function.

In particular, inflammatory signals, such as cytokines and other immune mediators, can lead to the downregulation of ZO-1 expression. Research indicates that during episodes of intestinal inflammation, ZO-1 mRNA and protein levels may decrease (Kuo et al., 2022). This downregulation is closely associated with tight junction disruption, resulting in compromised barrier function and increased permeability. Numerous studies on patients affected by conditions like Crohn's disease and ulcerative colitis, as well as animal models, have highlighted alterations in ZO-1 expression and distribution and tight junction function, underscoring the potential role of ZO-1 in the pathogenesis of these diseases (Poritz et al., 2007; Tan et al., 2019). Previous studies have also indicated a connection between NF-κB activation and the downregulation and redistribution of ZO-1. For instance, it was demonstrated that TNF-α-induced increases in Caco-2 tight junction permeability were accompanied by downregulation of ZO-1 proteins and alterations in their junctional localization. Importantly, inhibiting TNF-α-induced NF-κB activation prevented the increase in Caco-2 tight junction permeability, as well as the alterations in junctional localization of ZO-1 proteins and their expression (Lee et al., 2018; Ma et al., 2004).

Various studies have explored the beneficial role of dietary phytochemicals in maintaining tight junction integrity. For instance, treatment with curcumin in IL-1β-stimulated Caco-2 cells resulted in the upregulation of ZO-1 expression, positively influencing tight junction function and barrier permeability (Sergent et al., 2010).

In our experimental conditions, differentiated CaCo-2 cells exposed to 10 ng/ml of IL1β for 24 hours exhibit a noticeable redistribution of the ZO-1 protein, with an increased localization in the cytoplasm compared to what is observed in cells not exposed to the inflammatory stimulus. Pre-treatment with black sapote peel and seed extract, but not with pulp extract, prevents the alterations observed in the distribution of the junctional protein induced by the proinflammatory stimulus, with an increased localization of ZO-1 in the cell membrane (Figure 12.2).

The gathered results, in accordance with paracellular permeability data, contribute to suggesting that components present in black sapote peel and seed extracts may prove beneficial in attenuating the inflammatory response of intestinal epithelial cells, not only

by preventing the release of proinflammatory mediators but also by preserving the integrity of the epithelial barrier that the proinflammatory stimulus tends to compromise.

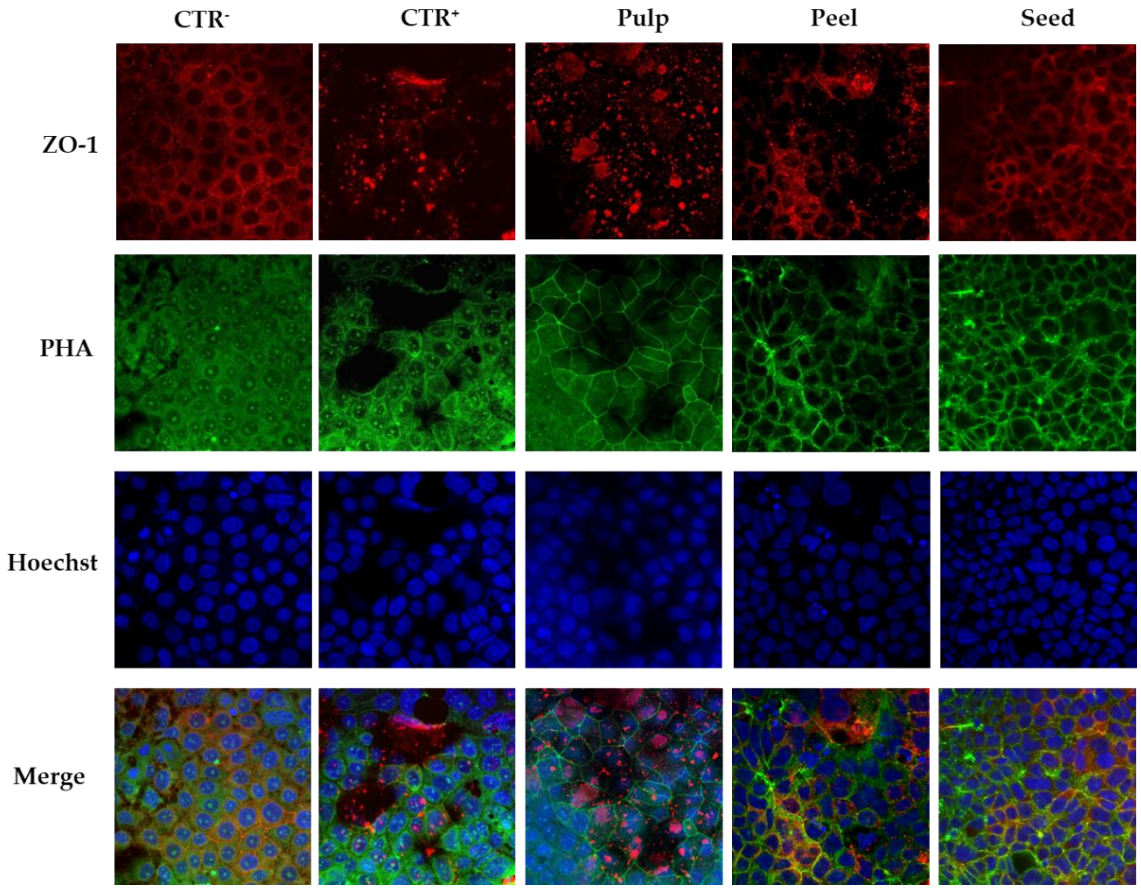

Figure 12.2: Immunofluorescence analysis for ZO-1 localization. Cells were incubated in the absence of additional treatment (CTR^-), preincubated in the absence of additional treatment and subsequently incubated for 24 h with 10 ng/mL IL-1β (CTR^+), or preincubated for 2h in the presence of peel, pulp, and seed extracts (10 µM GAE) and subsequently co-incubated for 24 h with or without 10 ng/ mL IL-1β. ZO-1 (red fluorescence). F-Actin cytoskeleton was marked with FITC-phalloidin (green fluorescence). Nuclei were stained with Hoechst 33342 (blue fluorescence. The images were acquired using a confocal laser scanning microscope (CLSM) (Olympus FV10i).

Assessment of membrane type matrix metalloproteinases expression

Matrix metalloproteinases (MMPs) constitute a vital family of zinc-dependent enzymes crucially involved in the dynamic remodelling of the extracellular matrix (ECM). Their influence extends across a spectrum of physiological and pathological processes, prominently in the realm of inflammation. Apart from their central role in ECM turnover, MMPs exhibit proteolytic ability in activating or degrading diverse non-matrix substrates, such as chemokines, cytokines, growth factors, and junctional proteins. Consequently, MMPs are increasingly acknowledged as pivotal contributors to inflammatory responses (Parks et al., 2004).

Accumulating evidence from multiple studies underscores the involvement of MMPs in disrupting the integrity of the intestinal epithelial barrier, thereby contributing to the pathogenesis of IBD (Marônek et al., 2021; Naito & Yoshikawa, 2005). While certain MMPs are constitutively expressed and contribute to the protective aspect of IBD by maintaining cellular homeostasis, others are induced during inflammation-induced tissue damage. For example, MMP-2 and MMP-9, despite sharing structural and substrate similarities, exert opposing effects on the development of intestinal inflammation.

In particular, during IBD, MMP-2 undergoes significant upregulation dependent on MMP-14. a membrane-type protease activating MMP-2 in the extracellular matrix. Research indicates that MMP-2 may play a role in promoting epithelial barrier function, as demonstrated by MMP-2 knockout mice exhibiting barrier dysfunction compared to their wild-type counterparts (Garg et al., 2006). MMP-2 might affect barrier function by modulating tight junction organization directly by associating with the involved adhesion proteins (Miyamori et al., 2001)

Conversely, MMP-9 emerges as the most abundantly expressed protease in IBD, with its production correlating with disease activity (Nighot et al., 2015). Inflammation triggers MMP-9 expression in both immune and epithelial cells. In contrast to MMP-2, MMP-9 mediates tissue damage during intestinal inflammation. Recent investigations have identified defective re-epithelialization, reduced adhesion complex integrity leading to

impaired wound healing (Castaneda et al., 2005), increased endothelial permeability (R. Al-Sadi et al., 2021; Nighot et al., 2015) as potential mechanisms through which MMP-9 orchestrates the inflammatory response.

In this study, we investigated the expression of MMP-2, MMP-9, and MMP-14 using Western blot analysis. Under our experimental conditions, IL-1β significantly increased the expression levels of MMP-14 and MMP-9, while MMP-2 levels remained unchanged. Notably, preincubation of cells with black sapote peel and pulp extracts resulted in an enhanced expression of MMP-2 and MMP-14 compared to cells treated only with IL-1β. Conversely, preincubation with pulp extract led to a downregulation of these two metalloproteases, suggesting a potential proinflammatory effect of components in the black sapote pulp extract within our experimental model. Interestingly, no significant variation in the expression of MMP-9 was observed, regardless of the treatment (Figure 13.2).

The acquired data, consistent with the effects of black sapote extracts on paracellular permeability and the distribution of the junctional protein ZO-1, contribute to demonstrating that the specific phytochemical profile of extracts from the non-edible parts of black sapote can, through various mechanisms, effectively preserve the integrity of the intestinal barrier compromised by proinflammatory stimuli.

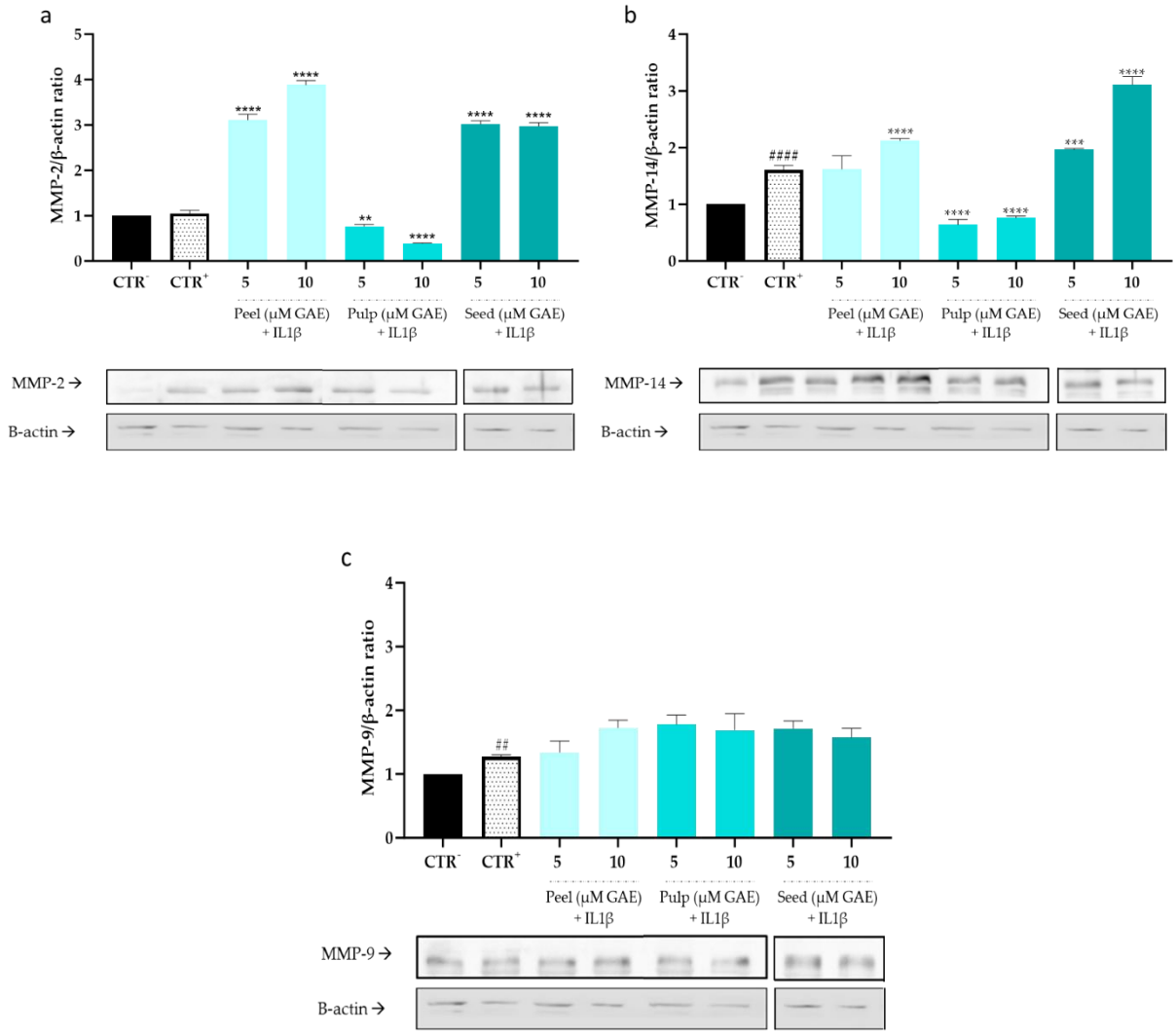

Figure 13.2: : (a) MMP-2, (b) MMP-14, and (c) MMP-9 protein expression in Caco-2 cells and effect of black persimmon extracts. Cells were collected, and the proteins were isolated for Western blot analysis as described in ''Materials and methods''. Panel shows representative Western blot and densitometric analysis. The values represent the relative expression levels normalized to β-actin and are the mean ± SD of three separate experiments with similar results. Cells were incubated in the absence of additional treatments (CTR^-), preincubated in the absence of additional treatments and subsequently incubated for 24 h with 10 ng/mL IL-1β (CTR^+), or preincubated for 2 h in the presence of different concentration of peel, pulp, and seed extracts and subsequently co-incubated for 24 h with 10 ng/mL IL-1β. Oneway ANOVA followed by Tukey's test was used to generate p values. "#" indicates significative differences compared to CTR^- ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ $^{\#\#\#\#}p < 0.0001$); "*" indicates significative differences compared to CTR^+ (*p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).

Conclusions

In this study, we evaluated the anti-inflammatory potential of hydroalcoholic extracts obtained from different parts of black persimmon in an *in vitro* model of intestinal inflammation, involving intestinal epithelial cells stimulated by the proinflammatory cytokine IL-1β.

Employing a comprehensive array of analytical methodologies, such as qRT-PCR, western blot, ELISA, and immunohistochemistry, our findings demonstrate that extracts from the non-edible parts of black persimmon, especially the seed extract, exhibit dose-dependent inhibition of the IL-1β-activated proinflammatory response in intestinal epithelium. The anti-inflammatory activity is manifested not only through the downregulation of proinflammatory mediator-encoding genes and subsequent attenuation of their corresponding protein levels but also in preserving epithelial barrier function and limiting the increase in paracellular permeability induced by the inflammatory stimulus. The collected data suggest that the pivotal anti-inflammatory activity observed is intricately linked to the inhibition of the NF-kB activation.

Furthermore, our findings highlight a significant correlation between the antioxidant activity of phytochemicals present in the extracts and the observed anti-inflammatory effects. Indeed, both seed and peel extracts of black persimmon not only decrease the expression of inflammatory markers but also concurrently activate the expression of genes encoding antioxidant enzymes and enhance the expression of Nrf2, the most important regulator of cellular redox response.

In summary, our data, for the first time, convincingly demonstrates that components of *Diospyros digyna* fruit by-products exhibit robust anti-inflammatory activity even at very low concentrations. These findings highlight their potential as effective agents in attenuating the inflammatory response of intestinal epithelial cells, suggesting a positive impact for the physiology of the gastrointestinal tract.

Finally, the collected results also suggest that some components of the fruit pulp extract may exacerbate the inflammatory response of intestinal epithelial cells, as demonstrated by increased NF-kB activation, elevated expression of IL-8 and COX-2, and reduced expression of NrF2. This possibility warrants further investigation, considering

that the extract in question is obtained from the edible portion of the fruit, and notably, the tested concentrations in the experimental model are very low, likely lower than those achievable after ingesting small amounts of the fruit.

Section III

"*In vitro* demonstration of regenerative and anti-senescence effect of black persimmon (*Diospyros digyna* Jacq.)"

The contents covered in this chapter have been incorporated into the following manuscript currently under preparation: "*Effects of peel, pulp and seed extracts from Diospyros digyna Jacq. fruit on EPCs' function and senescence*"

Serio G., Naserian S., Uzan G., Gentile C.

Abstract

Endothelial progenitor cells (EPCs) play a crucial role in vascular system regeneration. Investigated for their regenerative potential, EPCs are promising cell sources for revascularization in ischemia and degenerative diseases. Cord blood derived EPCs (CB-EPCs), characterized by remarkable stability and a lack of specialization in tissue-specific phenotypes, serve as a valuable model for assessing the impact of different molecules on vascular endothelial regeneration. With enhanced stability, these cells are excellent candidates for initial *ex vivo* expansion, anticipating autologous transplants for ischemia and related pathologies.

However, prolonged *ex vivo* expansion poses challenges, impairing vascular repair capacity, and the survival of transplanted cells in inflamed and oxidized environments complicates clinical success. Addressing these issues, optimizing EPC *ex vivo* expansion and enhancing activity through pharmacological modulation emerges as a novel strategy. Beyond conventional clinical drugs, various antioxidative agents with anti-inflammatory properties hold the potential to boost EPC function by averting oxidative stress, suppressing pro-inflammatory pathways, and enhancing anti-inflammatory responses.

Our prior investigation emphasized the rich polyphenol content in black persimmon, distinctively distributed among its edible and non-edible parts, showcasing notable antioxidant and anti-inflammatory properties. Based on these findings, the use of black persimmon extracts holds promise for protecting vascular health and deserves consideration in clinical applications involving EPCs.

The present study, for the first time, demonstrates the efficacy of black persimmon extracts in enhancing the functions of CB-EPCs. Peel, pulp, and seed extracts not only enhance mobility and migration without inducing toxicity or negative impacts on proliferation and endothelial phenotype but also demonstrate a promising potential in preventing and treating cellular senescence induced by a pro-oxidative stimulus. This highlights their potential in optimizing the *ex vivo* expansion process of endothelial progenitor cells for use in regenerative medicine applications. Notably, among the

extracts, the seed extract emerges as the most influential, showcasing robust positive effects on the regenerative activity of *ex vivo* expanded CB-EPCs.

Material and methods

Isolation and Culture of CB-EPCs

Samples of CB were obtained from the St Louis Hospital CB bank (Paris, France; authorization no. AC-2022-5325) from healthy term neonates. The use of human samples was in accordance with the Helsinki Declaration. Before being overlaid on Pancoll (Pan biotech, Aidenbach, Germany), CB was diluted in phosphate-buffered saline (PBS) containing 2 mM ethylenediaminetetraacetic acid (EDTA). After density gradient centrifugation, the obtained cells were seeded into 12-multiwell plates pre-coated with type-I rat-tail collagen (Corning, Glendale, AZ, USA), and cultured in endothelial cell basal medium-2 (EBM2) supplemented with endothelial single quotes kit containing 5% FBS (Lonza, Basel, Switzerland) (EGM2). 24 h later, a wash with PBS was done to remove non-adherent cells. The medium was replaced daily for the first 7 days and bi-daily thereafter until colony appearance (7-20 days). Only early passages (3 to 8) were used in subsequent experiments.

Characterization of Human CB-EPCs

The phenotypic characterisation of the isolated CB-EPCs was confirmed by flowcytometric analysis. Cells were trypsinised, washed with PBS containing 3% FBS and incubated for 20 min at 4°C with (APC) conjugated anti-endothelial growth factor receptor 2/kinase insert region receptor (VEGFR-2/KDR), (PE) conjugated anti-CD144 (vascular endothelial [VE] cadherin) and (FITC) conjugated anti-CD31 (Miltenyi, Germany). Non-stained cells were used as a negative control.

Plant material

D. digyna fruits were picked from trees grown in Vivai Torre (Milazzo, Sicily, Italy; 38◦190 N, 15◦240 E; 20 m a.s.l.). They were collected when still immature and stored at room temperature for three days until their ripeness. Peeling was carried out, and the pulp was manually separated from seeds and peel. The obtained portion of pulp, peel and seeds were then distinctly used for extraction process.

Extract preparation

The extracts were prepared as previously described (Mannino et al., 2022). Briefly, pulp, peel, and seeds were thawed. After homogenization, the samples were weighted and extracted with a 70:30 (v/v) ethanol:water mixture using 1:10 (w/v) ratio. Samples were mixed by vortexing for 5 min, sonicated for 30 min at room temperature (RT) and stirred on a plate shaker overnight at 4◦C in the dark. Each sample was centrifuged (10 min at 8000g, 4 ◦C) at the end of the extraction period. After collection of the supernatants, the resulting residues were extracted again twice using 1:5 (w/v) of the same solvent mixture. Finally, the supernatants from the three extraction cycles were collected, filtered (Millex HV 0.45 µm, Millipore, Billerica, MA), and stored at − 80 ◦C until further analysis.

Cell proliferation

MTS and dye dilution assays were used to assess CB-EPCs proliferation. For MTS assay cells were seeded in four 96 well plates at a density of $9x10^3$ cells/cm^2 in EGM2 medium supplemented with different doses of peel, pulp and seed extracts (0.5, 5, and 50 µg fresh weight (FW)/ ml cell medium) and incubated at 37 °C in a humidified, 5 % CO2 atmosphere. Cells treated with medium only were used as control. MTS (Promega, Madison, WI, USA) was opportunely diluted with EGM2 in the dark and added to the plate that was successively incubated for 2 h at 37 ◦C. After the incubation time, the absorbance was measured at 490 nm and 630 nm, as suggested by the manufacturer. The first plate was analysed 8h after seeding. The other plates were analysed 24, 48, and 72 h after seeding. Using control wells (without cells) containing MTS solution only, readings were corrected for background absorbance at 490 nm. For the dye dilution assay, cells were labelled using a CellTraceTM Cell Proliferation Kit (CFSE, Invitrogen) according to the manufacturer's instructions. Subsequent, cells were treated with different doses of peel, pulp and seed extracts (0.5, 5, and 50 µg fresh weight (FW)/ ml cell medium). Labelled and untreated cells were used as control. After 72h, labeled cells were harvested and analysed by flow cytometric analysis. Due to the dilution after each division, the decreasing in CFSE expression was indicative of divided cells. Events were

acquired on a LSRFORTESSA flow cytometer (BD-Biosciences) and analyzed using FlowJo software v10 (FlowJo-LLC).

Expression of endothelial surface protein markers

To assess the effect of black persimmon extracts on the expression of the main endothelial surface markers, CB-EPCs were seeded in 12-well plates in EGM2 medium and incubated at 37 °C in a humidified, 5% CO_2 atmosphere. At 80 % confluence, the cells were washed two times with PBS, and then treated with different doses of peel, pulp and seed extracts (0.5, 5, and 50 µg FW/ ml cell medium). After 24 h, the cells were trypsinized, and the expression of the surface markers was estimated with flowcytometric analysis, as described above.

Nitric Oxide Production

CB-EPCs were cultured in 75 cm^2 culture flask in EGM2 to reach 90 % confluence. Cells were successively washed with PBS and starved with basal medium supplemented with 0.2% foetal bovine serum for 4 h at 37 °C in a humidified, 5% CO_2 atmosphere. After the incubation time, cells were washed with PBS and treated with different concentration of peel, pulp and seed extracts (0.5, 5, and 50 µg FW/ ml cell medium). Untreated cells cultured in EGM2 were used as controls. After 24 h, the cells were washed with PBS and incubated for 1 h at 37 °C in a humidified, 5% CO_2 atmosphere with a 1 µM 4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate (DAF) probe (Thermofisher, Waltham, MA, USA). The cells were then trypsinized and resuspended in PBS containing 3% FBS. Flow cytometry (Fortessa, BD bioscience, Waltham, MA, USA) was used to assess the labelling. Data were analysed in the FITC channel using FlowJo V10 software (Salem, OR, USA).

Tube Formation

Geltrex (Thermofisher, Waltham, MA, USA) was used to investigate the effects of black persimmon extracts on the ability of CB-EPCs to form tubes. Cells were seeded on wells coated with geltrex and cultured in the presence of different concentrations of the extracts. Cells cultured in EGM2 were used as control. The images of tube formation

were taken each 2h for 24h and analysed with ImageJ software. Total tube length, number of closed structures, and branching points were measured.

Wound Healing

CB-EPCs were seeded in 12-well plates in EGM2 medium at high density, and incubated at 37 °C in a humidified, 5% CO_2 atmosphere. At 90% confluence, cell monolayers were scratched using 200 µL pipette tips. Cells were washed two times with PBS and treated with different concentration of peel, pulp and seed extracts (0.5, 5, and 50 µg FW/ ml cell medium). Un treated cells cultured in EGM2 were used as controls. Images of the scratches were taken at time 0h (T0) and 24h by a Nikon D5300 (Nikon corporation, Tokyo, Japan). ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to quantify the wound area.

Transwell Migration

The effects on the regenerative function of CB-EPCs was further validated by Transwell migration assay. Cells were starved overnight using basal medium supplemented with the 0.2% of FBS. After detachment, cells were plated at a density of 16×10^4 cells per cm^2 on the top layer of Falcon 8-micron pore cell culture inserts. (Corning, Glendale, AZ, USA), placed in 24-well plates. 1 ml of starvation medium containing 1ng/ml of vascular endothelial growth factor (VEGF) or various concentrations of peel, pulp and seed extracts (0.5, 5, and 50 µg FW/ ml cell medium) was added below the cell permeable membrane. After an incubation period of 5h, cells adhering to the top of the filter membrane were removed with a Q-tip. The migrated cells through the membrane were fixed with paraformaldehyde (PFA) 4% and stained with blue RAL 555 (RAL Diagnostics, Martillac, France). ImageJ software was used to count the number of migrated cells.

Inflammation Markers

Reached 90% confluence, CB-EPCs cultured in EGM2 were treated for 24h with various concentrations of peel, pulp and seed extracts (0.5, 5, and 50 µg FW/ ml cell medium) or with TNFα (1 ng/mL and 10 ng/mL). Cells treated only with medium were used as negative control. After the incubation time, cells were trypsinised, washed with PBS

containing 3% FBS and stained with a mix of antibodies: Biotin-anti-ICAM (CD54), PE-anti-VCAM (CD106), PECY7-anti-TNFR1, APC-anti-TIE2 (Miltenyi, Gladbach, Germany), and streptavidin-PE-cys5 (Thermofisher, Waltham, MA, USA). The labeling was evaluated by flow cytometry Fortessa (BD bioscience, Franklin Lakes, NJ, USA) and data were analysed by FlowJo V10 software (FlowJo, LLC, Ashland, OR, USA).

Senescence

CB-EPCs were seeded into 12-well plates at a density of $12.5x10^4$ cells/cm^2 in EGM2. At 80% of confluence, cells were washed with PBS and starved with basal medium supplemented with 2% of FBS overnight at 37 °C in a humidified, 5% CO_2 atmosphere. After the starvation time, cells were treated for 24h with different concentrations of peel, pulp and seed extracts (0.5, 5, and 50 µg FW/ ml cell medium), before and after the induction of senescence with H2O2 400 µM for 4 h. Cells only treated with EGM2 and H_2O_2-induced cells were used as negative and positive controls, respectively. Senescent cells were identified using un histochemical stain assay for β-galactosidase activity at pH 6 (Senescence Cells Histochemical Staining Kit, Sigma-Aldrich, St-Louis, USA). The assay was performed according to the manufacturer's instructions. Images of stained and unstained cells were taken by a Nikon D5300 (Nikon corporation, Tokyo, Japan) using an inverted microscope (X10). The cell numbers were assessed by Image J software (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

Statistical analyses were performed with Prism (GraphPad) software. Data were expressed as means ± SD. For cytometry, the MFI was normalized with cells cultured in EGM2. One-way ANOVA followed by Tukey's test was used to generate p values The p value < 0.05 was considered statistically significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Characterization of Human CB-EPCs

The initial step involved the identity of CB-EPCs that was confirmed by assessing the expression of key endothelial cell surface markers, including CD31, KDR, and CD144.

EPCs were isolated from human CB samples and characterized via flow cytometry. It is known that EPCs can be identified based on the expression of some endothelial cell surface markers, such as CD31, vascular endothelial [VE]-cadherin (CD144), and endothelial growth factor receptor-2/kinase insert domain receptor (VEGFR-2/KDR). Cells were cultured in the EGM2 medium. After colonies appearance, between 7 and 20 days, flow cytometry analysis showed that CB-EPCs were positive for the previously mentioned endothelial markers (Figure 1.3).

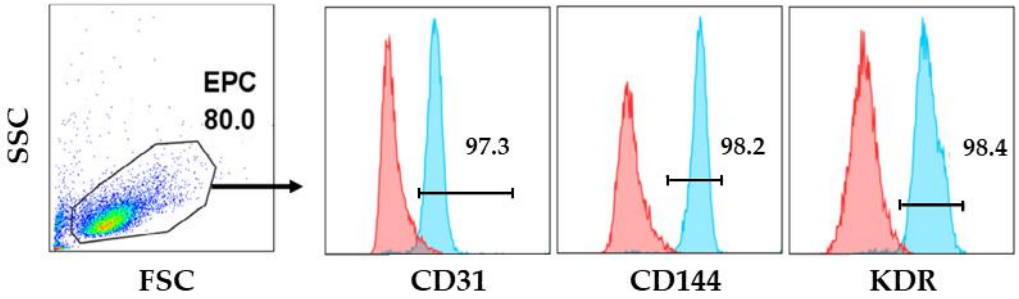

Figure 1.3: EPCs characterization. Flow cytometry was used to assess the expression of endothelial surface markers by cells obtained from samples of human cord blood. Cells were positive for CD31, CD144, and KDR expression. SSC: side scatter channel; FSC: Forward scatter channel.

Expression of endothelial surface protein markers

The peculiar cell phenotype of CB-EPCs is closely correlated to their action on endothelial regeneration, angiogenesis, and formation of new blood vessels. It is therefore essential that CB-EPCs treatments does not affect it. In order to evaluate the effect of black persimmon extracts on the expression of endothelial characteristic markers. Cells were treated for 24h with different concentrations of peel, pulp and seed

extracts (0.5, 5, and 50 µg FW/ ml cell medium). Flow cytometry analysis showed that the exposure of CB-EPCs to black persimmon extracts did not induce alterations in the expression of endothelial markers (CD31, CD144, and VGFR-2), suggesting no negative effect on endothelial cell phenotype (Figure 2.3).

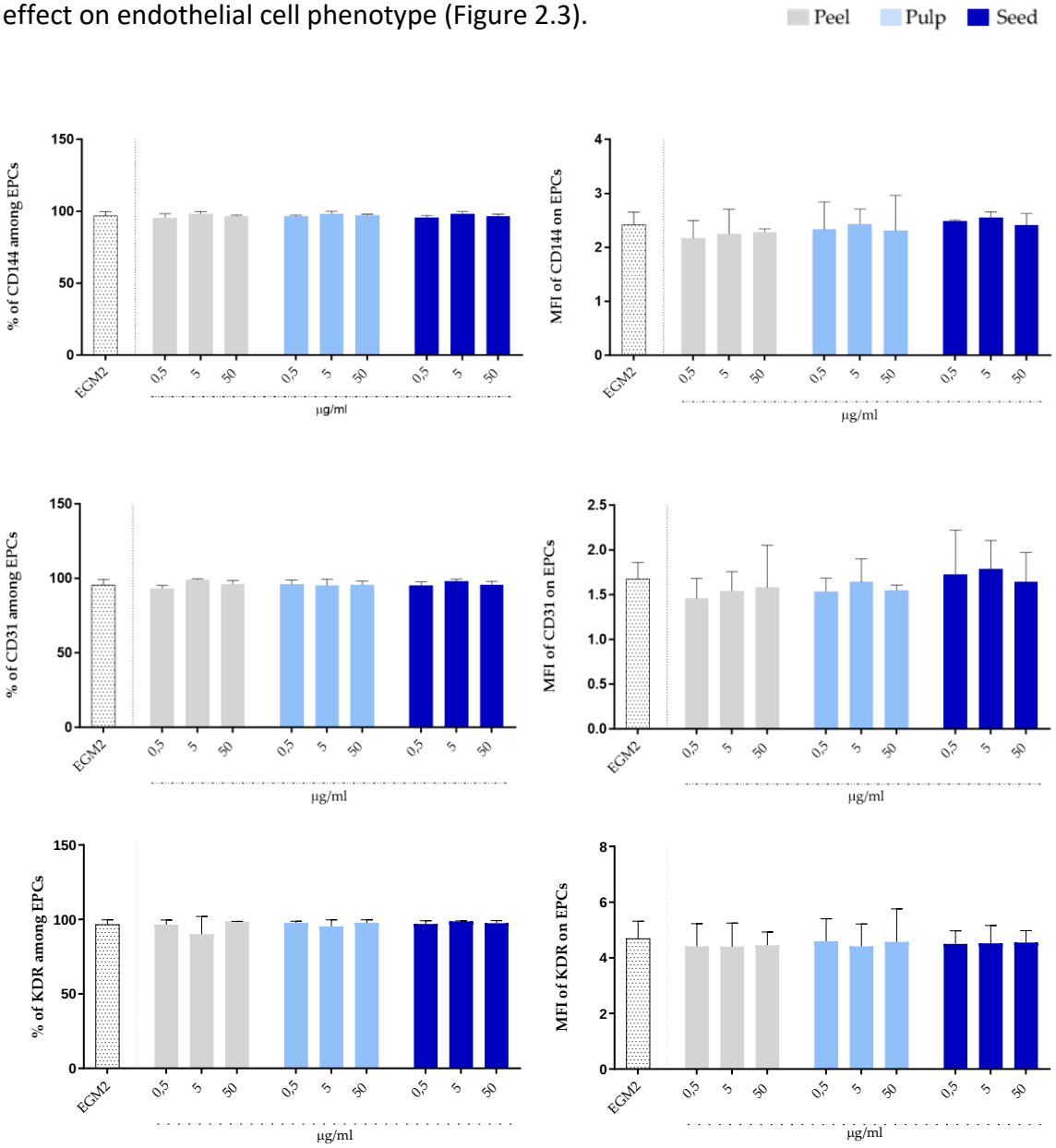

Figure 2.3: Effects of black persimmon extracts on the expression of CB-EPCs' principal markers. EPCs were cultured for 24 h in EGM2 with or wothout different concentration of peel, pulp, and seed extracts. Their expression of endothelial markers CD31, CD144, and KDR was studied by flow cytometry. Cells cultured in EGM2 were considered as the control group. Results are

expressed as mean ± SD of three experiments carried out in triplicate. One-way ANOVA followed by Tukey's test was used to generate p values. "*" indicates significative differences compared to the control group (* $p < 0.05$). EGM2: endothelial cell basal medium-2 (EBM2) + EGM-2 endothelial single quotes kit (Lonza) + 5% FBS (Lonza).

Cell proliferation and NO production

The influence of black persimmon on CB-EPCs proliferation was evaluated using the MTS assay. Notably, the administration of different doses (0.5, 5, and 50 µg FW/mL of cell medium) of the three extracts had no observable impact on cell proliferation, even over an extended 72-hour treatment period. This consistent level of proliferation in treated cells, as compared to control cells, was evident in a non-dose-dependent manner. These results underscore the absence of toxic effects on CB-EPCs' viability. These results were also confirmed by flow cytometry using tracing dye carboxyfluorescein diacetate succinimidyl ester (CellTrace™ CFSE). After the suitable incubation time, no significant differences in the % of CFSE were recovered between treated and untreated cells. In addition, the influence of black persimmon extracts treatments was also investigated on NO production, which reduction is closely correlated to endothelial dysfunction (Cyr et al., 2020). The findings revealed that treated cells exhibited no significant alterations in NO production when compared to control cells, suggesting not adversely impact on NO production and absence of any detrimental effects on CB-EPCs (Figure 3.3).

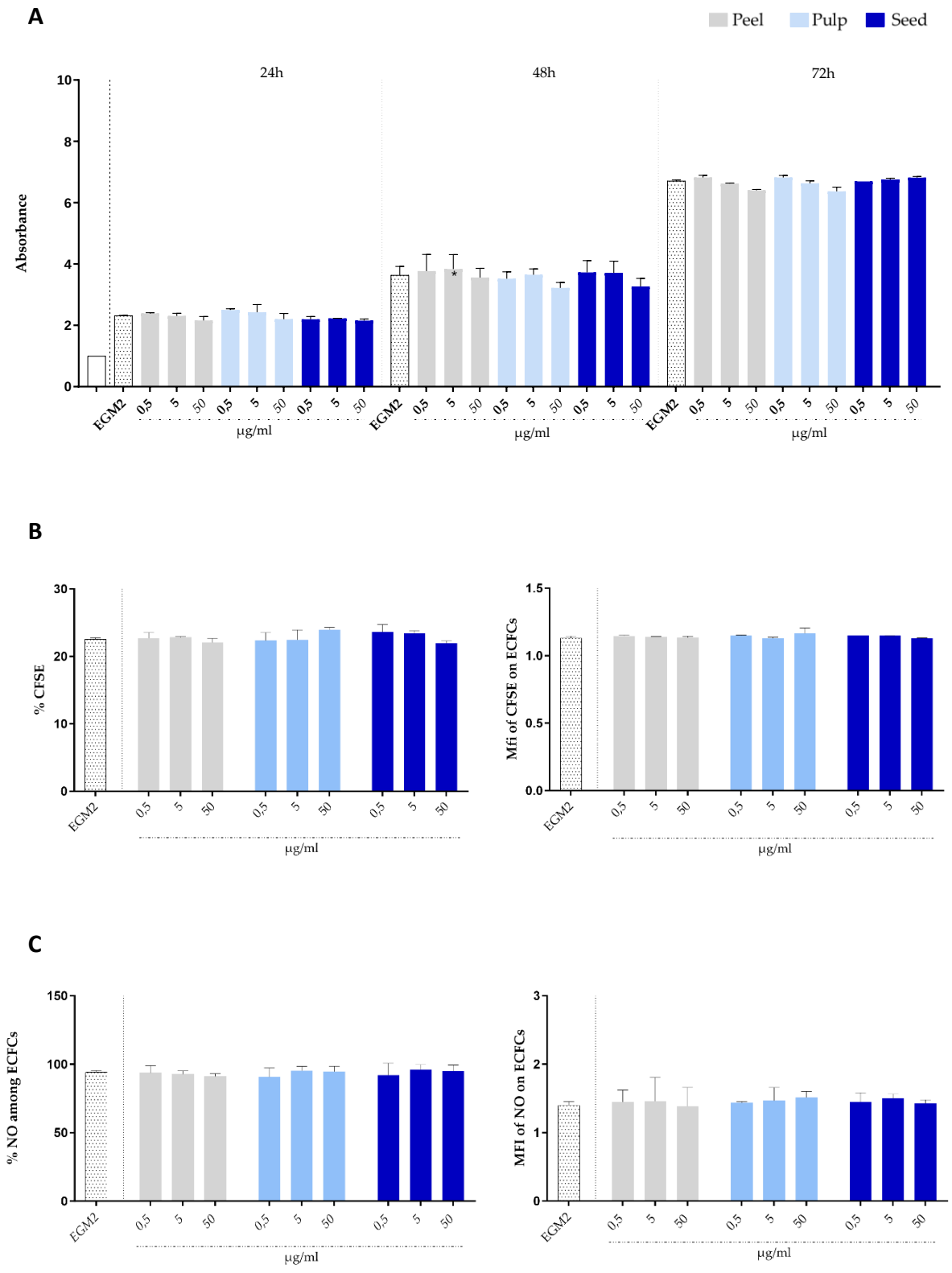

Figure 3.3: Effect of black persimmon extracts on CB-EPCs proliferation and NO production. (A) Cells were seeded in 96- well plates with or without peel, pulp and seed extracts. The absorbance was then measured after 2 h of incubation with MTS reagent and then analysed with a plate

reader (Multiskan EX, ThermoLabsystems). Cells cultured in only EGM2 were considered as control. (B) Dye dilution assay, cells were labelled using a CellTraceTM Cell Proliferation Kit (CFSE, Invitrogen), and treated with different doses of peel, pulp and seed extracts. Labelled and untreated cells were used as control. After 72h, labelled cells were harvested and analysed by flow cytometric analysis. (C) To assess the impact of the extracts on the NO production, cells were cultured for 24 h in EGM2 with or without extracts; then, the NO production was assessed by flow cytometry and the percentage of NO-producing cells determined. Cells cultured in only EGM2 were considered as the control group; MFI values have been normalized with cells cultured in EGM2 medium. Results are expressed as mean ± SD of three experiments carried out in triplicate. One-way ANOVA followed by Tukey's test was used to generate p values. "*" indicates significative differences compared to the control gropup (* $p < 0.05$). EGM2: endothelial cell basal medium-2 (EBM2) + EGM-2 endothelial single quotes kit (Lonza) + 5% FBS (Lonza).

Migration and mobility

Given the importance of specific EPCs functions, such as mobility, migration, and network formation, as parameters in endothelial pro-angiogenic effects (Fraj et al., 2023), It was conducted an assessment to determine the impact of peel, pulp and seed on these functions. Prior studies have reported positive effects of antioxidants, such as resveratrol and ginkgo biloba extracts (Chen et al., 2004; Gu et al., 2006; Wang et al., 2007), on the re-endothelialisation ability of EPCs, enhancing cell migratory and mobility activities or preventing negative effects on migration induced by stress conditions (Felice et al., 2012).

Tube formation

EPCs have the remarkable ability to self-organize into tube-like structures when placed in the presence of an ECM. To evaluate the impact of black persimmon extracts on the ability of CB-EPCs to form tubes, cells were seeded on Geltrex extracellular matrix with either EGM2 alone or with the addition of the extracts (5 µg FW/ ml medium). Tube length, the number of enclosed structures, and branching points were monitored by capturing photos every 2 hours following seeding. The treatment with black persimmon

extracts did not result in a significative alteration in the capacity of CB-EPCs to form tubes when in the presence of the extracellular matrix (Figure 4.3).

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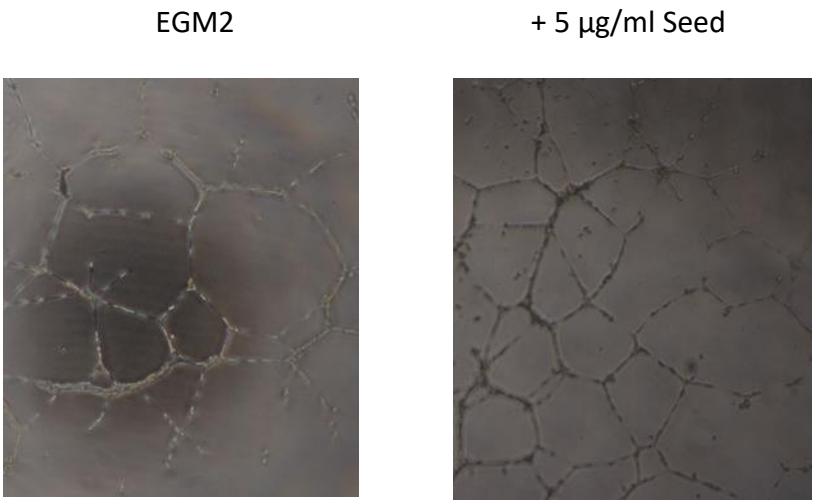

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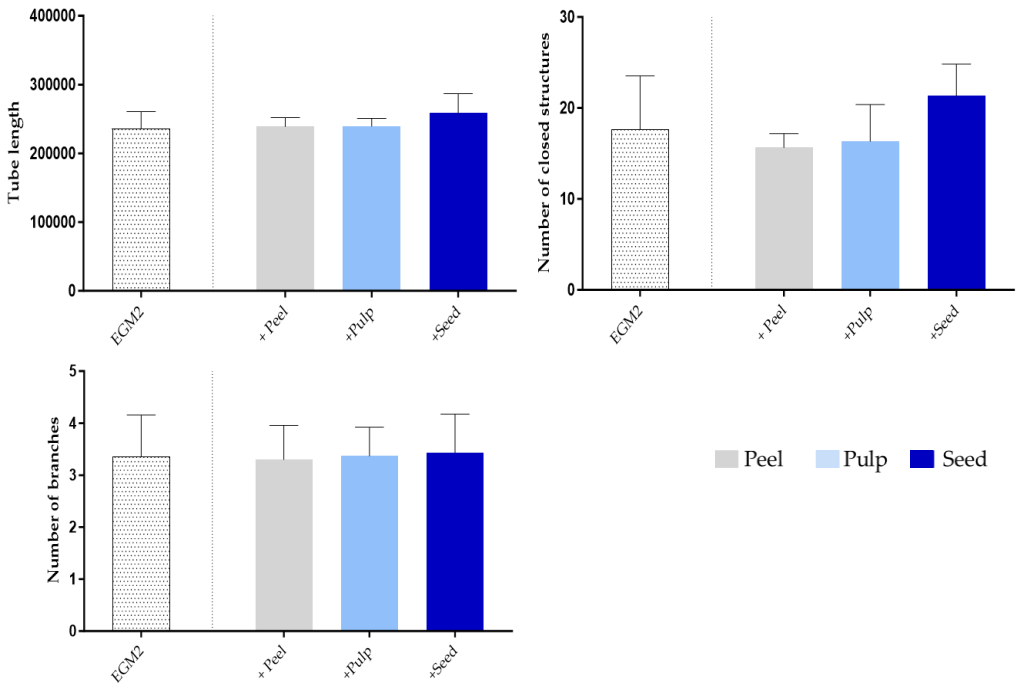
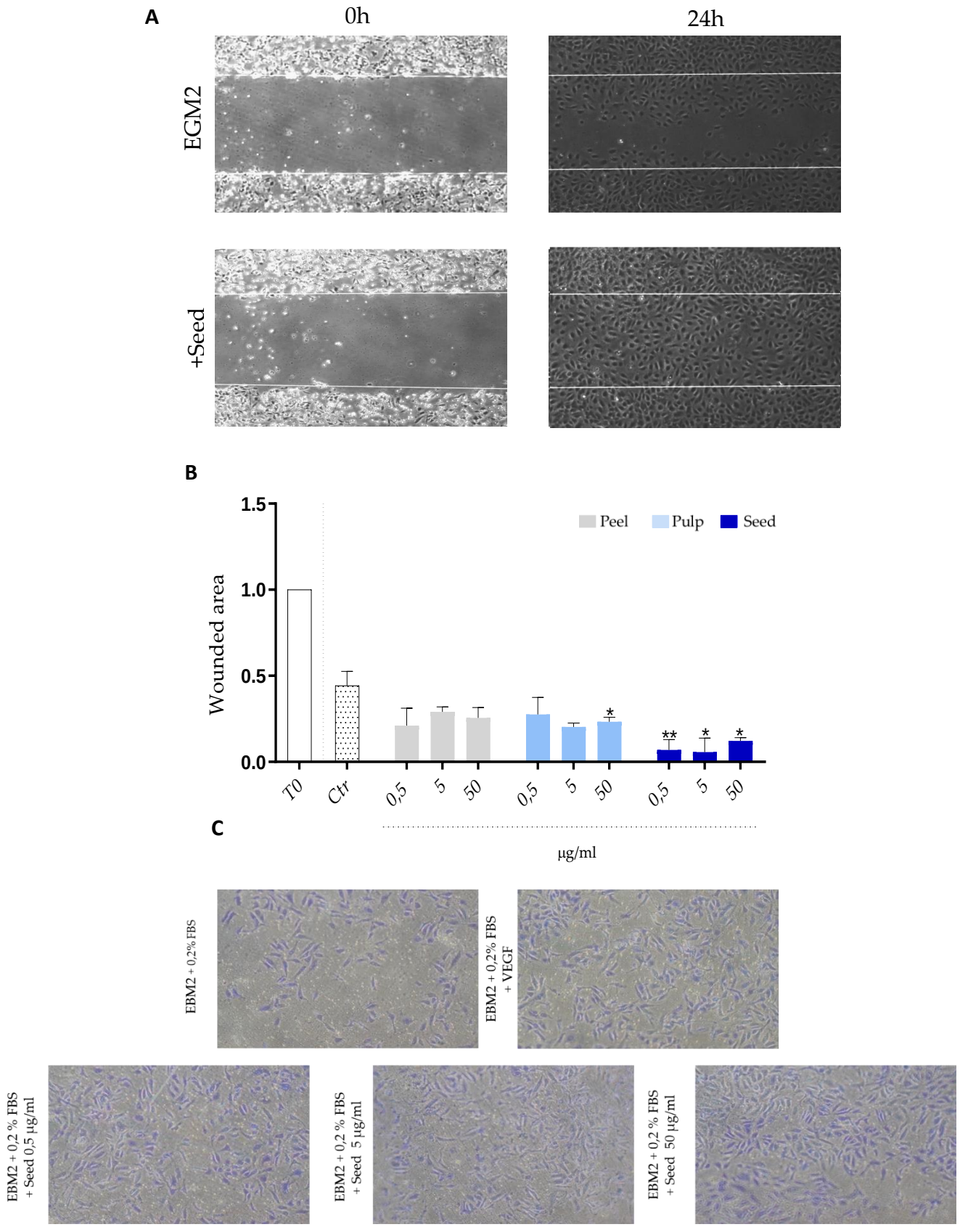


Figure 4.3: CB-EPs' tube formation was assessed on extracellular matrix components (Geltrex). Cells were seeded with or without black persimmon extracts on Geltrex. (A) Images of tube formation (X10). (B) Quantitative analysis of tube length and the number of closed structures and branches Results are depicted as the mean ±SD of 3 independent experiments. Cells

cultured in only EGM2 medium are used as control. One-way ANOVA followed by Tukey's test was used to generate p values (* $p < 0.05$). EGM2: endothelial cell basal medium-2 (EBM2) + EGM-2 endothelial single quotes kit (Lonza) + 5% FBS (Lonza).

Wound healing and migration assay

Although no changes were found in the ability to form capillary-like tubes, treated cells showed positive influence on the regenerative activity of *ex vivo* expanded CB-EPCs. To assess this potential, evaluations of regenerative functions, including mobility and migration, were conducted, using wound healing and Transwell migration assays. In the wound healing assay, where a confluent layer of EPCs underwent a scratch, treated cells exhibited improved mobility in a no-dose-dependent manner compared to the control cells. Specifically, cells treatment with the seed extracts showed the fastest wound healing, evident from a significant reduction in the wound area after 20 hours in comparison to cells treated with EGM2 alone (Figure 5.3). These findings were further validated by Transwell migration assay. Notably, cells treatment with black persimmon extracts enhanced the migration ability of CB-EPCs compared to untreated cells, with a better effect determined by treatment with the seed extract. Furthermore, as vascular endothelial growth factor (VEGF) plays a role in promoting angiogenesis and endothelial cell migration, we compared its effect with that of black persimmon peel, pulp and seed on CB-EPCs' migration. Interestingly, seed extracts treatment produced stronger effects than those obtained by exposing cells to VEGF.


A
0h
24h
EGM2
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1.5
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µg/ml
C
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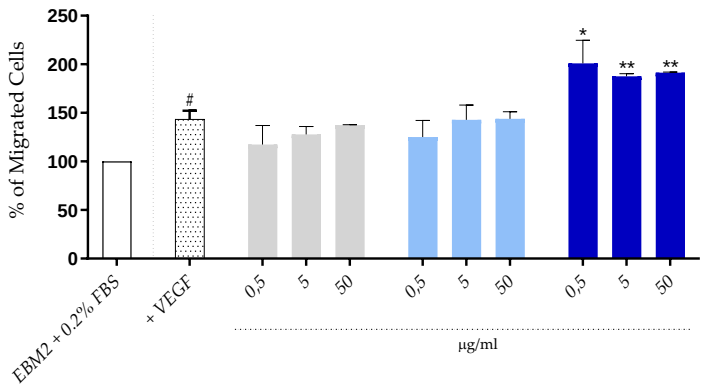


Figure 5.3: CB-EPCs migration was assessed using wound healing and the transwell method. (A) Pictures were taken with an inverted microscope (X4) showing the level of migration area. (B) Quantitative analysis of results from wound healing assay: promotion of EPCs migration is confirmed by the decrease in the scratched area when they are treated with black persimmon extracts. Cells cultured in EGM2 are considered as control (CTR). (C) Pictures were taken with an inverted microscope (X10) showing migrated cells. (D) The number of migrated cells was counted using Image J software. Cells cultured in a starving medium (EBM2 (Lonza) + 0.2% FBS) are considered the negative control group. Each dot presents a measured value (n = 3) collected from 3 independent experiments. All values per group are expressed as a mean ± SD. One-way ANOVA followed by Tukey's test was used to generate p values. "#" indicates significative differences compared to the negative control ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ $^{\#\#\#\#}p < 0.0001$); "*" indicates significative differences compared to +the VEGF treatment (*p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001) (0 h:0 h, 24 h:24 h, EBM2: basal medium.).

Collectively, while the tube formation assay indicated that treatment with the extracts did not significantly alter CB-EPCs ability to form tubes in the presence of extracellular matrix, it was demonstrated that the same treatment improved both, mobility and migration. Notably, seed extract treatment demonstrated a remarkable influence in these functions. Indeed, cell treatment with the seed extract determined the fastest healing activity compared to untreated cells, and a more pronounced effect on migration than that achieved with VEGF addition, suggesting a real possibility to enhance the regenerative function of CB-EPCs.

Inflammatory condition

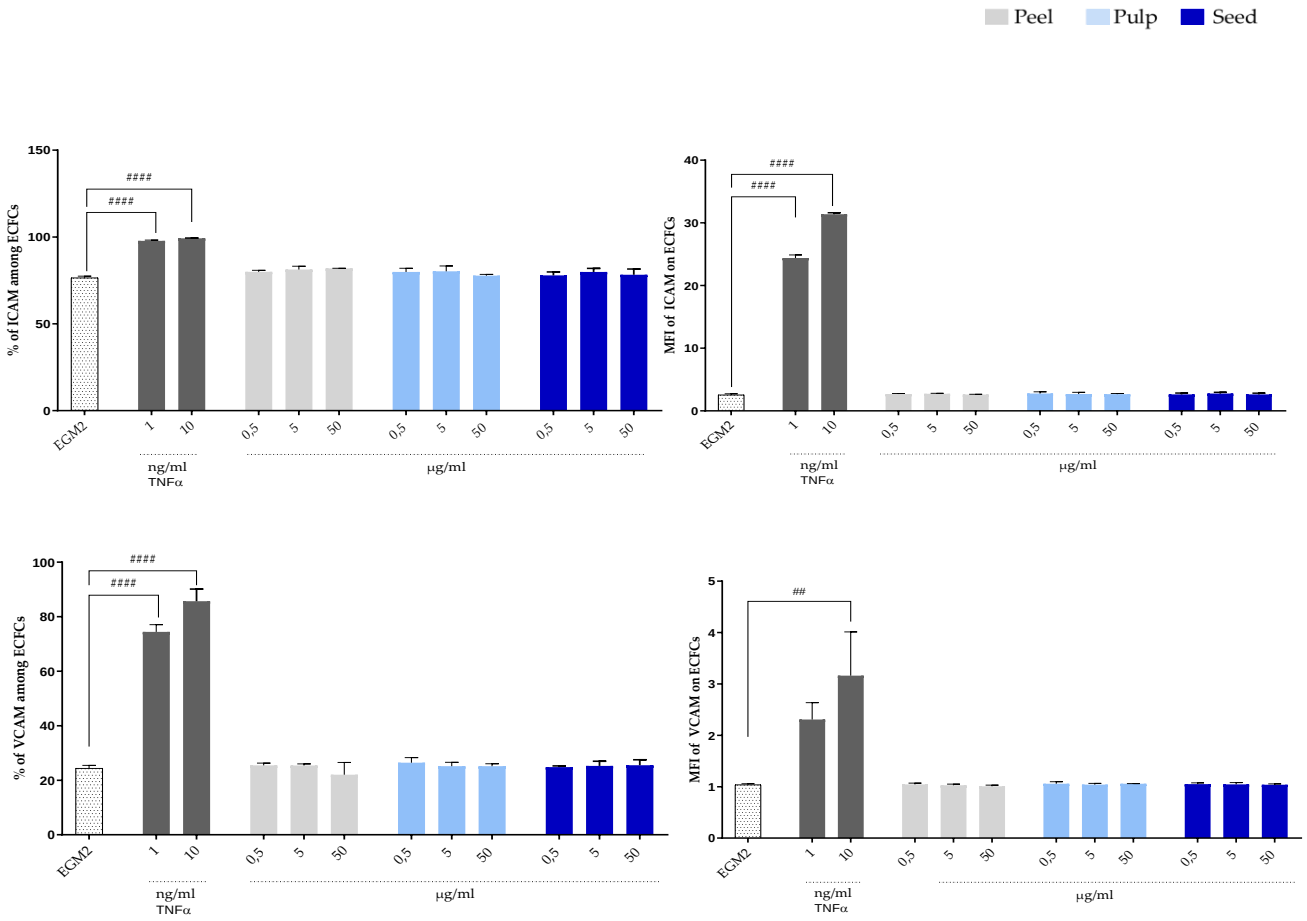
Recent studies have indicated that inflammation and oxidative stress play crucial roles in modulating the functions of EPCs (Gabriele & Pucci, 2017; Incalza et al., 2018; Lin et al., 2013). Clinical medications possessing both anti-inflammatory and antioxidant properties, such as statins, have demonstrated positive effects on EPCs (António et al., 2010; Umemura & Higashi, 2008). However, in addition to clinical drugs, various antioxidative agents with anti-inflammatory properties could have the potential to enhance EPC function by suppressing pro-inflammatory pathways and improving anti-inflammatory responses.

Several studies have highlighted the anti-inflammatory activities of antioxidant agents on endothelial cells. For instance, Huang et al. showed that pretreatment with resveratrol significantly inhibited TNF-α-induced mRNA expression of ICAM-1, iNOS, and IL-1β in human coronary arterial endothelial cells (HCAECs). Similarly, Liu et al. demonstrated that leonurine, an active alkaloid from Traditional Chinese Medicine Herba leonuri, exhibited cardioprotective effects through its anti-oxidative activity in human umbilical vein endothelial cells (HUVEC), attenuating LPS-mediated expression/release of ICAM-1, VCAM-1, COX-2, and TNF-α (Huang et al., 2010; Liu et al., 2012).

On the other hand, EPCs are also highly sensitive to pro-inflammatory environments (Garolla et al., 2009). Pre-treatment of EPCs with increasing doses of TNFα has been found to directly correlate with the upregulation of endothelial activation and inflammatory markers such as ICAM, VCAM, TIE2, and TNFR1 (Naserian et al., 2020; Nouri Barkestani et al., 2021).

To investigate the influence on the expression of inflammatory markers, CB-EPCs were exposed to varying doses of the extracts for 24 hours. The expression of several pro-inflammatory markers, including Intercellular Adhesion Molecule (ICAM), Vascular Cell Adhesion Molecule (VCAM), TIE2, and Tumor necrosis factor receptor 1 (TNFR1) was assessed. Notably, treatment with peel, pulp and seed did not result in an increase in the expression of the examined inflammatory markers. Our previous study

demonstrated that a concentration of 1 ng/mL of the pro-inflammatory cytokine TNFα serves as the threshold distinguishing between is considered the threshold between a low and an extensive inflammatory response, while a concentration of 10 ng/mL is associated with impaired inflammation. In light of these findings, the effects of different doses of black persimmon extracts were compared with those of TNFα. Both concentrations of TNFα (1 ng/mL and 10 ng/mL) produced a more pronounce increase in the expression of all the tested markers. Our results showed an expression of inflammatory markers always lower than that with 1 ng/mL of TNFα, suggesting that peel, pulp and seed do not induce uncontrolled inflammation (Figure 6.3).



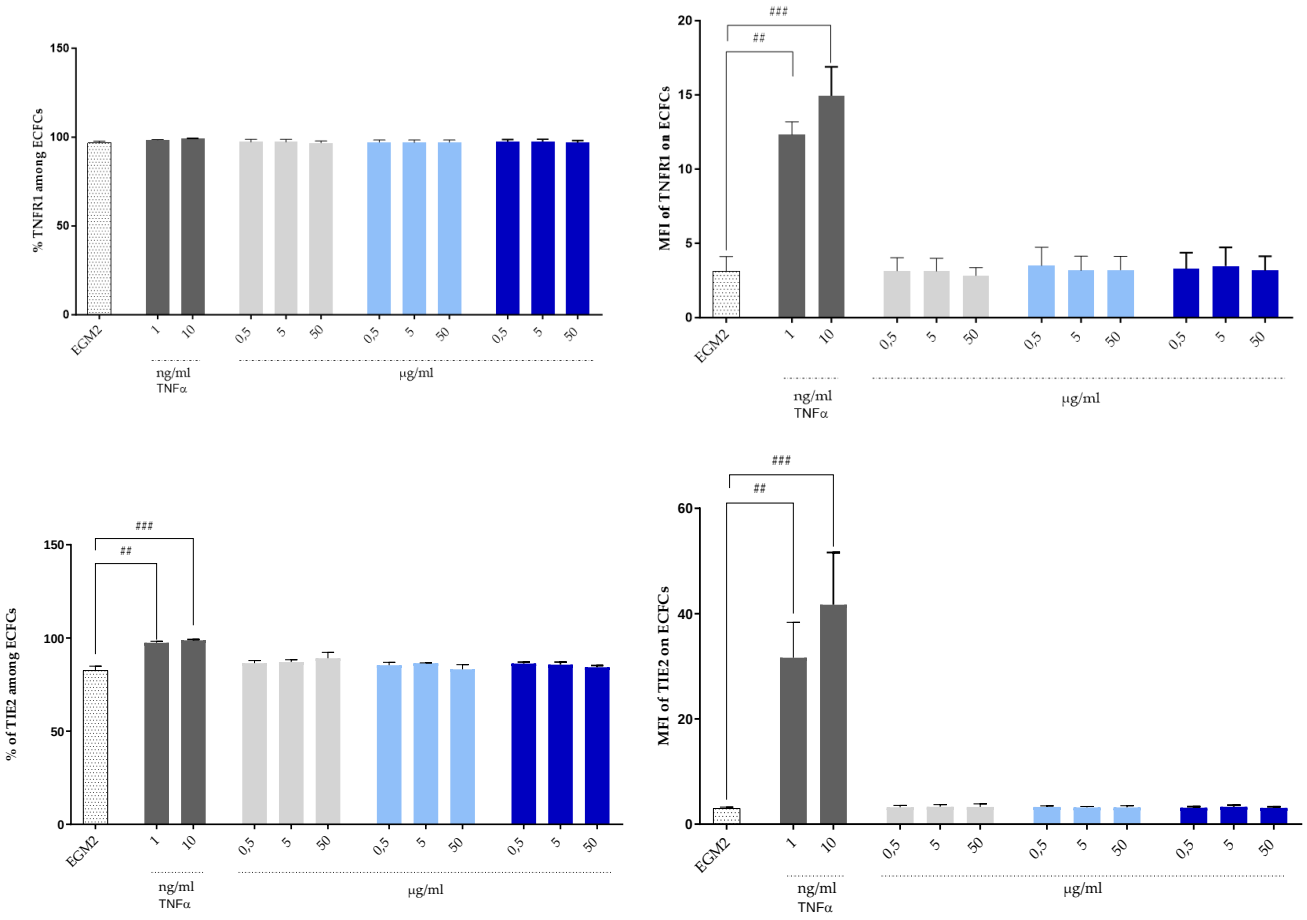


Figure 6.3: The impact of black persimmon extracts on EPCs inflammatory markers expression. CB-EPCs were cultured for 24 h in EGM2 with different concentrations of peel, pulp and seed extracts or with 2 doses of TNFα (1 ng/mL and 10 ng/mL), then assessed for the percentage of expression and the mean fluorescent intensity of the inflammatory surface markers ICAM, VCAM, TIE2, TNFR1. Results are depicted as mean ± SD of 3 independent experiments. Cells cultured in EGM2 without TNFα are considered the control group. MFI values have been normalized with cells cultured in EGM2. One-way ANOVA followed by Tukey's test was used to generate p values "#" indicates significative differences compared to control (# $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$). (TNFα: Tumor Necrosis Factor-alpha).

Senescence

Cellular senescence is a biological process characterized by the cessation of cell division. The isolation and *in vitro* expansion of endothelial cells often lead to the gradual acquisition of a senescent phenotype, resulting in reduced proliferation rates and

functional capabilities. Nevertheless, this process could slow down through the treatment of cells with anti-aging agents. *Tinospora cordifolia* leaf and *Withania somnifera* root extracts, along with resveratrol, have demonstrated the ability to delay senescence, as confirmed by β-galactosidase senescence assays in mesenchymal stem cells (MSCs), without compromising cell viability (Hu & Li, 2019; Sanap et al., 2017). To investigate the impact of black persimmon extracts on the prevention and treatment of cellular senescence, β-galactosidase measurement assay was conducted. The results demonstrated that all the three extracts reduce the senescence levels either before (prevention) or after (curing) the induction of senescence by 400 µM H_2O_2 compared to the positive control (cells treated with cultured medium and H_2O_2). However, treated cells with the seed extract led to the restoration of senescence levels comparable to those observed in the negative control, consisting of CB-EPCs treated with the culture medium EGM2 but without H_2O_2 (Figure 7.3). These findings strongly suggest that the phytochemicals found in the seed extract not only have the capability to prevent senescence during the *ex vivo* expansion of CB-EPCs but also possess the ability to counteract the aging phenotype in cells that have already entered a senescent state.

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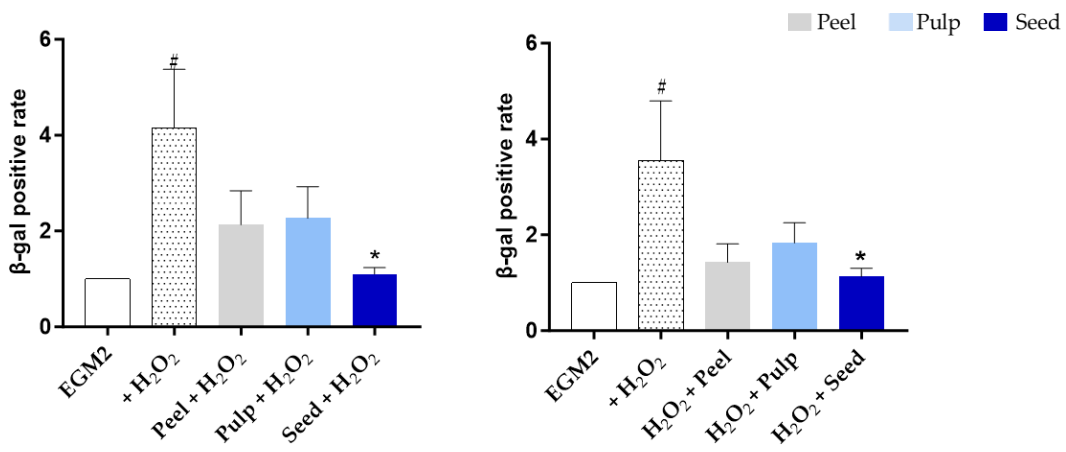

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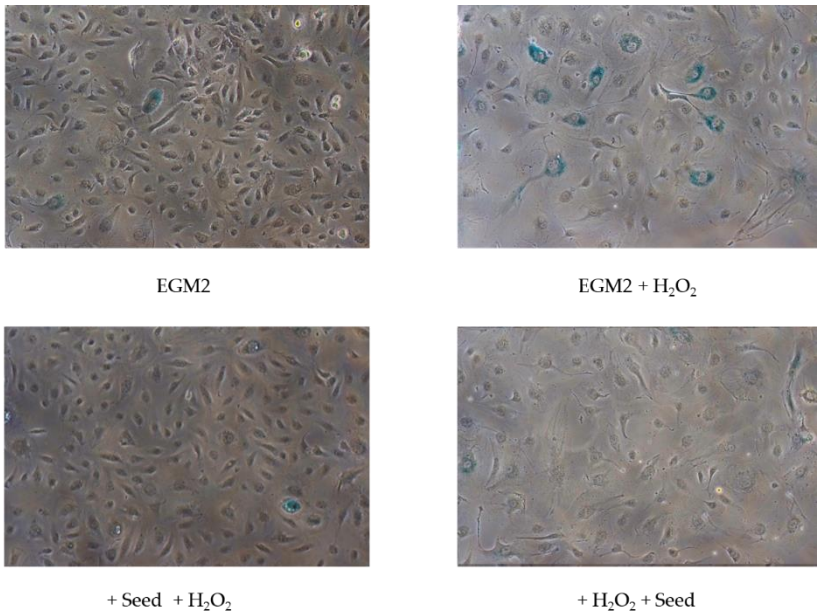

Figure 7.3: CB-EPCs were seeded into 12-well plates and treated with different concentrations of peel, pulp and seed extracts before and after the induction of senescence with H_2O_2 400 µM for 4 h. Cells only treated with EGM2 and H_2O_2-induced cells were used as negative and positive controls, respectively. Senescent cells were identified using un histochemical stain assay for β-galactosidase (Senescence Cells Histochemical Staining Kit, Sigma-Aldrich, St-Louis, USA). (A) Quantitative analysis of β-galactosidase positive cells. Results are depicted as mean ± SD of 3 independent experiments). One-way ANOVA followed by Tukey's test was used to generate p values. "#" indicates significative differences compared to the negative control ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ $^{\#\#\#\#}p < 0.0001$); "*" indicates significative differences compared to the positive control (*p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001) (B) Images of stained and unstained cells were taken by a Nikon D5300 (Nikon corporation, Tokyo, Japan) using an inverted microscope (X10). The cell numbers were assessed by Image J software (National Institutes of Health, Bethesda, MD, USA).

Conclusions

In this study, black persimmon peels, pulp and seed extracts were shown to be positively and strongly involved in the biological functions of CB-EPCs. In particular, the data obtained showed that treatment with the seed extract resulted in a significant increase in CB-EPCs mobility, migration abilities without affecting their viability and identity. Furthermore, a very interesting anti-aging activity was detected. Collectively, further investigations are warranted to elucidate the precise mechanisms by which the phytochemicals found in black persimmon extracts, especially the seed one, enhance the function and activity of CB-EPCs

Conclusion of the work

In summary, our comprehensive analysis of black persimmon (*Diospyros digyna*) underscores its significance as a potent repository of bioactive compounds endowed with exceptional functional properties. The phytochemical exploration of hydroalcoholic extracts from various parts of the fruit, including the pulp and non-edible components such as peel and seeds, has unveiled substantial concentrations of redox-active polyphenols. These extracts demonstrate robust radical-scavenging and metal-reducing activities across diverse solution models.

Furthermore, our investigation reveals that even at minimal concentrations, the hydroalcoholic extracts play a pivotal role in mitigating oxidative stress in liver epithelial cells subjected to pro-oxidant stimuli, concurrently enhancing the gene expression of crucial antioxidant enzymes. The demonstrated functional prowess of phytochemical compounds from *D. digyna* extends to intestinal epithelial cells exposed to the inflammatory impact of IL-1β. Here, our extracts exhibit anti-inflammatory effects by preserving the integrity of the epithelial barrier and modulating the gene expression and protein levels of key inflammatory mediators. Notably, the protective actions are associated with the downregulation of NF-κB activation and the upregulation of the transcription factor Nrf2.

In a vascular endothelium model constructed from endothelial cells derived through ex-vivo expansion of human endothelial progenitor cells, our extracts, particularly the seed-derived variant, showcase positive influences on endothelial function. These include the promotion of cell migration and reparative properties, all achieved without adversely impacting cell proliferation or the endothelial phenotype. Significantly, our results demonstrate that the extracts not only prevent but also reverse cell senescence induced by pro-oxidant stimuli, hinting at their potential in optimizing the ex-vivo expansion of endothelial progenitor cells for application in regenerative medicine.

In conclusion, the multifaceted functional activities of *D. digyna* extracts, as elucidated in this study, not only contribute to our understanding of the therapeutic potential of

this unique fruit but also open avenues for further exploration in the realms of nutrition, medicine, and regenerative therapies.

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List of Abbreviations

ABAP - 2,2’-Azobis(2-methylpropionamidine) dihydrochloride

ABTS - 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

APB – Adult Peripheral Blood

BM – Bone Marrow

BSA - Bovine Serum Albumin

CAA – Cellular Antioxidant Activity

CAT - Catalase

CB – Cord blood

CB-EPC - Cord Blood Endothelial Progenitor Cell

CD – Crohn's disease

COX - Cyclooxygenase

CuZnSOD - Copper-Zinc Superoxide Dismutase

DABCO - 1,4-Diazabicyclo[2.2.2]octane

DAF - 4',6-Diamidino-2-phenylindole

DCFH-DA - 2',7'-Dichlorodihydrofluorescin diacetate

DMAC - N,N-Dimethylacetamide

DNA - Deoxyribonucleic Acid

DPPH - 2,2-Diphenyl-1-picrylhydrazyl

DTT - Dithiothreitol

EBM2 - Endothelial Basal Medium 2

EC - Endothelial Cell

ECFC - Endothelial Colony-Forming Cell

ECM - Extracellular Matrix

EGM2 - Endothelial Growth Medium 2

EPC - Endothelial Progenitor Cell

FRAP – Ferric Reducing Antioxidant Power

FSA - Fluorescein-5-(and-6)-sulfonic acid trisodium salt

FW – Fresh weight

GAE - Gibberellic Acid Equivalent

GPx - Glutathione Peroxidase

GSH - Glutathione

HBSS - Hanks' Balanced Salt Solution

HCAEC - Human Coronary Artery Endothelial Cell

HO-1 - Heme Oxygenase-1

HPLC-DAD-ESI-MS/MS - High-Performance Liquid Chromatography with Diode Array Detector and Electrospray Ionization Tandem Mass Spectrometry

HUVEC - Human Umbilical Vein Endothelial Cell

IBD - Inflammatory Bowel Disease

ICAM - Intercellular Adhesion Molecule

IL - Interleukin

INF-γ - Interferon-gamma

iNOS - Inducible Nitric Oxide Synthase

iκB - Inhibitor of Nuclear Factor Kappa B

JAM - Junctional Adhesion Molecule

Keap 1 - Kelch-like ECH-Associated Protein 1

MMP - Matrix Metalloproteinase

MnSOD - Manganese Superoxide Dismutase

MSC - Mesenchymal Stem Cell

MTS - 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

MTT - 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NFκB - Nuclear Factor Kappa B

NO - Nitric Oxide

Nrf2 - Nuclear Factor Erythroid 2-Related Factor 2

Papp - Apparent Permeability

PBS - Phosphate-Buffered Saline

PFA - Paraformaldehyde

qRTPCR - Quantitative Reverse Transcription Polymerase Chain Reaction

RNA - Ribonucleic Acid

RNS - Reactive Nitrogen Species

ROS - Reactive Oxygen Species

SDS - Sodium Dodecyl Sulfate

SOD - Superoxide Dismutase

TE – Trolox Euivalent

TJ - Tight Junction

TNBS - Trinitrobenzenesulfonic Acid

TNFR-1 – Tumor necrosis farctor receptor 1

TNF-α - Tumor Necrosis Factor-alpha

TPAC - Total Polyphenol Content

TPC - Total Phenolic Content

TPTZ - 2,4,6-Tri(2-pyridyl)-s-triazine

UC - Ulcerative Colitis

VCAM - Vascular Cell Adhesion Molecule

VEGF - Vascular Endothelial Growth Factor

ZO - Zonula Occludens

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Biography and CV of Graziella Serio

- July 2019: Graduated in Chemistry and Pharmaceutical Technology with honors at the University of Palermo with a thesis entitled “Composti bioattivi, attività radical scavenging e antiossidante di frutti di *Diospyros digyna* Jacq.” (supervisor Prof. Carla Gentile)

- November 2020: Enrolled in the Ph.D. program in Molecular and Biomolecular Sciences at the University of Palermo under the supervision of Prof. Carla Gentile. Research focuses on evaluating the functional properties and nutraceutical value of hydro-alcoholic extracts from D. digyna fruits grown in Sicily.

- February – March 2021: Visiting student at the Department of Life Sciences and Systems Biology, Innovation Centre, University of Turin, Turin, Italy

- February – May 2022: Visiting student at the Institut national de la santé et de la recherche médicale (INSERM) – U 1197, Hôpital Paul Brousse, Villejuif, France

List of peer-reviewed pubblications

1. Gaglio, R., La Rosa, L., Serio, G., Mannino, G., Alfonzo, A., Franciosi, E., Settanni, L., Gentile, C. *Rubus idaeus by-products: Sustainable improvement of the antioxidant value of sourdough bread by a new end-use of exhausted seeds still containing bioactive compounds (2023)* Innovative Food Science and Emerging Technologies, 90, art. no. 103517 https://doi.org/10.1016/j.ifset.2023.103517

2. Mannino, G., **Serio, G**., Gaglio, R., Maffei, M.E., Settanni, L., Di Stefano, V., Gentile, C. *Biological Activity and Metabolomics of Griffonia simplicifolia Seeds Extracted with Different Methodologies (2023).*Antioxidants, 12 (9), art. no. 1709. https://doi.org/10.3390/antiox12091709

3. Ragusa, M.A., Naselli, F., Cruciata, I., Volpes, S., Schimmenti, C., **Serio, G**., Mauro, M., Librizzi, M., Luparello, C., Chiarelli, R., La Rosa, C., Lauria, A., Gentile, C., Caradonna, F. *Indicaxanthin Induces Autophagy in Intestinal Epithelial Cancer Cells by Epigenetic Mechanisms Involving DNA Methylation (2023).*Nutrients, 15 (15), art. no. 3495 https://doi.org/10.3390/nu15153495

4. Mannino, G., Ricciardi, M., Gatti, N., **Serio, G**., Vigliante, I., Contartese, V., Gentile, C., Bertea, C.M. *Changes in the Phytochemical Profile and Antioxidant Properties of Prunus persica Fruits after the Application of a Commercial Biostimulant Based on Seaweed and Yeast Extract (2022).* International Journal of Molecular Sciences, 23 (24), art. no. 15911. https://doi.org/10.3390/ijms232415911

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6. Mannino, G., **Serio, G.***, Asteggiano, A., Gatti, N., Bertea, C.M., Medana, C., Gentile, C. *Bioactive Compounds and Antioxidant Properties with Involved Mechanisms of Eugenia involucrata DC Fruits (2022).* Antioxidants, 11 (9), art. no. 1769. https://doi.org/10.3390/antiox11091769

7. Mannino, G., **Serio, G.***, Gaglio, R., Busetta, G., La Rosa, L., Lauria, A., Settanni, L., Gentile, C. *Phytochemical Profile and Antioxidant, Antiproliferative, and Antimicrobial Properties of Rubus idaeus Seed Powder (2022).* Foods, 11 (17), art. no. 2605. https://doi.org/10.3390/foods11172605

8. Mannino, G., **Serio, G.*,** Bertea, C.M., Chiarelli, R., Lauria, A., Gentile, C.*Phytochemical profile and antioxidant properties of the edible and non-edible portions of black sapote (Diospyros digyna Jacq.) (2022).* Food Chemistry, 380, art. no. 13213 https://doi.org/10.1016/j.foodchem.2022.132137

9. Mannino, G., Iovino, P., Lauria, A., Genova, T., Asteggiano, A., Notarbartolo, M., Porcu, A., **Serio, G**., Chinigò, G., Occhipinti, A., Capuzzo, A., Medana, C., Munaron, L., Gentile, C. *Bioactive triterpenes of protium heptaphyllum gum resin extract display cholesterol- lowering potential (2021).* International Journal of Molecular Sciences, 22 (5), art. no. 2664, pp. 1-22. https://doi.org/10.3390/ijms22052664

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13. Mannino, G., Gentile, C., Ertani, A., **Serio, G**., Bertea, C.M. *Anthocyanins: Biosynthesis, distribution, ecological role, and use of biostimulants to increase their content in plant foods—a review (2021).* Agriculture (Switzerland), 11 (3), art. no. 212, pp. 1-25. https://doi.org/10.3390/agriculture11030212

List of conference abstracts

1. **Serio, G**., Mannino, G., Bertea, C.M., Gentile, C.*Chemical Characterization, radical-scavenging and antioxidant activity of Diospyros digyna fruits***.** In: 93rd National Congress of the Italian Society for Experimental Biology. 22-25 Aprile 2021, Palermo.

2. Mannino G., Iovino P., Lauria A., Tullio G, Asteggiano A., Notarbartolo M., Porcu A., **SerioG**., Chinicò G., Occhipinti A., Capuzzo A., Medana C., Munaron L., Gentile C.. *Bioactive triterpenes of Protium heptaphyllum gum resin extract displayed cholesterol-lowering potential.* In*:* 93rd National Congress of the Italian Society for Experimental Biology. 22-25 Aprile 2021, Palermo

3. Mannino G., **Serio G.**, Bertea C.M., Lauria A., Chiarelli R., Gentile C. *Black sapote (Diospyros digyna Jacq): phytochemical characterization and antioxidant properties of seed, pulp and peel extracts.* In: Plant Biology Europe 2021 conference. 28-30 Giugno 2021, Torino

4. **Serio, G.,** Mannino, G., Bertea, C.M., Chiarelli, R., Lauria, A., Gentile, C. *Functional properties of the edible and non-edible portions of black sapote (Diospyros digyna Jacq): valorisation of the fruit waste as a new source of phytochemicals*. In: 61° SIB (Società italiana di Biochimica e Biologia molecolare) meeting. 23-24 Settembre 2021, Virtual edition

5. **Serio G.,** Mannino G., Asteggiano A., Gatti N., Bertea C.M., Medana C., Gentile C. *Phytochemical profile and antioxidant proprieties of fruits of Eugenia involucrata DC.* In: 94th National Congress of the Italian Society for Experimental Biology. 6-9 Aprile 2022,Torino.

6. **Serio G.,** Mannino G., Gaglio, R., Settanni, L., Gentile C. *Valorization of waste raspberry seed powder: phytochemical profile, antioxidant proprieties and nutraceutical applications.* In: 94th National Congress of the Italian Society for Experimental Biology. 6-9 Aprile 2022, Torino.

7. Mannino G., Iovino P., Lauria A., Tullio G., Asteggiano A., Notarbartolo M., Porcus A.,**Serio G**., Chinicò G., Occhipinti A., Capuzzo A., Medana C., Munaron L., Gentile C. *Bioactive triterpenes of protium heptaphyllum gum resin extract display cholesterol-lowering potential.* In: 94th National Congress of the Italian Society for ExperimentalBiology. 6-9 Aprile 2022, Torino.

8. Asteggiano, A., Occhipinti, A,, Mecarelli, E., Aigotti, R., Medana, C., Munaron, L., Mannino, G., Iovino, P., Lauria, A., Porcu, A., Notabartolo, M., **Serio, G.,** Chinigo, G., Gentile, C. *Protium Heptaphyllum resin extracts: chemical and biological characterization of a promising anti-hyperlipidemic and anti-hypercholesterolemic natural product.* In: 3rd international Congress on Food Bioactives and Health. 21-24 Giugno 2022, Parma, Italia

9. **Serio, G.,** La Rosa, L., Mannino, G., Gaglio, R., Lauria, A., Settanni, L., Gentile, C., *Phytochemical Profile and Functional Properties of Rubus idaeus Seed Powder*. In: FirstSTeBICeF young researcher workshop. 12 Gennaio 2023, Palermo

10. . **Serio, G.,** Mannino, G., La Rosa, L., Chiarelli,R., Bertea, C.M., Gentile, C., *Anti-inflammatory activity of Diospyros Digyna Jacq fruit extracts in an in vitro model of intestinal inflammation.* In: 95th National Congress of the Italian Society for Experimental Biology. 12-15 Aprile 2023, Trieste

11. La Rosa, L., **Serio, G.,** Mannino, G., Gaglio, R., Settanni, L., Gentile, C. *Valorization of food waste: Raspberry seed powder (Rubus Idaeus L.) as a functionalizing agent of a bakery product*. In: 95th National Congress of the Italian Society for Experimental Biology. 12-15 Aprile 2023, Trieste

12. Gentile, C., Ragusa, M.A., Schimmenti, C., **Serio, G.,** Naselli, F., Cruciata, I., Chiarelli, R., Volpes, S., La Rosa, L., Cardinale, P.S., Lauria, A., Caradonna, F. *Pro-autophagic activity of dietary indicaxanthin in intestinal epithelial cancer cells.* In: 95th National Congress of the Italian Society for Experimental Biology. 12-15 Aprile 2023, Trieste

13. Gaglio, R., La Rosa, L., **Serio, G.,** Alfonso, A., Mannino, G., Franciosi, E., Gentile, C., Settanni L. *Recycling wastes from Rubus idaeus by-products: sourdough breadproduction as a new end- use of exhausted seeds still containing active compounds.* In: 7th international conference on microbial diversity (Agrifood microbiota as a tool for a sustainable future). 26-29 Settembre 2023, Parma