Dipartimento di Scienze Agrarie e Forestali

Corso di Dottorato di Ricerca in

Scienze delle Produzioni Vegetali e Animali - XXXI Ciclo

NOVEL APPLICATIONS OF PLANT-DERIVED POLYPHENOLIC COMPOUNDS

BIO/15 - Pharmaceutical Botany
CHIM/06 - Organic Chemistry

PhD student

Elisa Pannucci

PhD Coordinator

Prof. Stefania Masci

Supervisors

Prof. Luca Santi

Prof. Roberta Bernini

A.A. 2018/19

Abstract

Polyphenol compounds represent one of the most numerous and ubiquitous groups of plant metabolites. Ranging from rather simple to highly polymerized compounds, this complex group of molecules is extremely variable. They possess interesting biological properties as anti-inflammatory, antioxidant and antimicrobial effects. Interest in plant-derived polyphenolic compounds has recently increased, due to their possible beneficial implications in human health such as the treatment and prevention of cancer and cardiovascular diseases.
Considering their diffusion into the plant kingdom, phenolic derivatives naturally can be found also in agro-industrial by-products. These could be managed and utilized more efficiently, becoming new and low-cost sources of valuable compounds. In this way, plant-derived polyphenols can be recycled to produce pharmaceuticals, cosmetics and food preservatives, generating both economic and environmental benefits as required by the principles of "circular economy". A multidisciplinary research effort, combining chemistry and biology, allowed to reach the main goal of this study: the valorization of kiwifruit (*Actinidia deliciosa*, cv. Hayward) peel and olive (*Olea europaea*) oil by-products to obtain extracts rich in procyanidins and hydroxytyrosol, respectively. Polyphenol-rich extracts were prepared and investigated for their anti-inflammatory, antioxidant and antimicrobial activities. In particular, procyanidins-rich extract was tested for the anti-inflammatory activity, demonstrating to be able to inhibit multiple key molecular targets involved in inflammatory diseases; hydroxytyrosol-rich extract was tested for the antioxidant and antimicrobial activities, demonstrating to be a strong antioxidant and to exert an antimicrobial activity against *Pseudomonas savastanoi* and *Agrobacterium tumefaciens*, two olive pathogens. Finally, with the aim of applying the hydroxytyrosol-rich extract as antioxidant in active food packaging, hydroxytyrosol synthetized in our laboratory was preliminary used as active ingredient. The corresponding formulations developed, based on poly(vinyl alcohol) (PVA), nanostructured starch and hydroxytyrosol, showed a gradual release of the antioxidant suggesting novel and eco-friendly solutions for active food packaging.

Index

1. Introduction

1.1 Plant natural products

Plants synthesise a wide range of bioactive compounds, which have important ecological functions. Plant-derived natural compounds represent a central theme in research between biology and chemistry.[1] Historically natural products from plants and their derivatives have been the main therapeutic resources. For centuries they have been used relying on indications provided by traditional medicine which, however, have often proved to lack scientific foundations. Recently, due to the continuous scientific research on new, safer and more effective drugs, there has been a rediscovery of natural substances as a potential source of high-added-value compounds and molecules, which can be used not only in the pharmaceutical field but also for food and cosmetic applications, and for crop protection strategies.[2-4]

Despite the huge number of organic compounds produced by plants, the great majority of these do not appear to participate directly in plant growth and development. These substances, named secondary metabolites, are often distributed differentially among taxonomic groups within the plant kingdom. The primary metabolites, in contrast, are found in all plants and play a fundamental role in metabolism and reproduction.[5]

Secondary metabolites are characterized by an enormous structural variability and are known to play a major role in the adaptation of plants to their environment, but also to represent an important source of active pharmaceuticals. These metabolites are produced by pathways derived from primary metabolic routes (Figure 1). Substances produced in the fundamental processes such as photosynthesis, glycolysis and Krebs cycle can generate biosynthetic intermediates called “biosynthetic bricks”, by means of which a large variety of natural substances can be built.[6] Among plant secondary metabolites, polyphenols are derivatives of the shikimate/phenylpropanoid and/or the polyketide pathways[7] that represent one of the most widely distributed groups of phytochemicals in the plant kingdom. These compounds play important roles in plants growth and reproduction, providing protection against pathogens, and, also, contribute for colours and sensory characteristics of fruits and vegetables.[8, 9] Moreover, phenolic compounds have numerous physiological properties, and have been associated with health benefits.[9, 10] In this work, the focus was particularly on polyphenols.

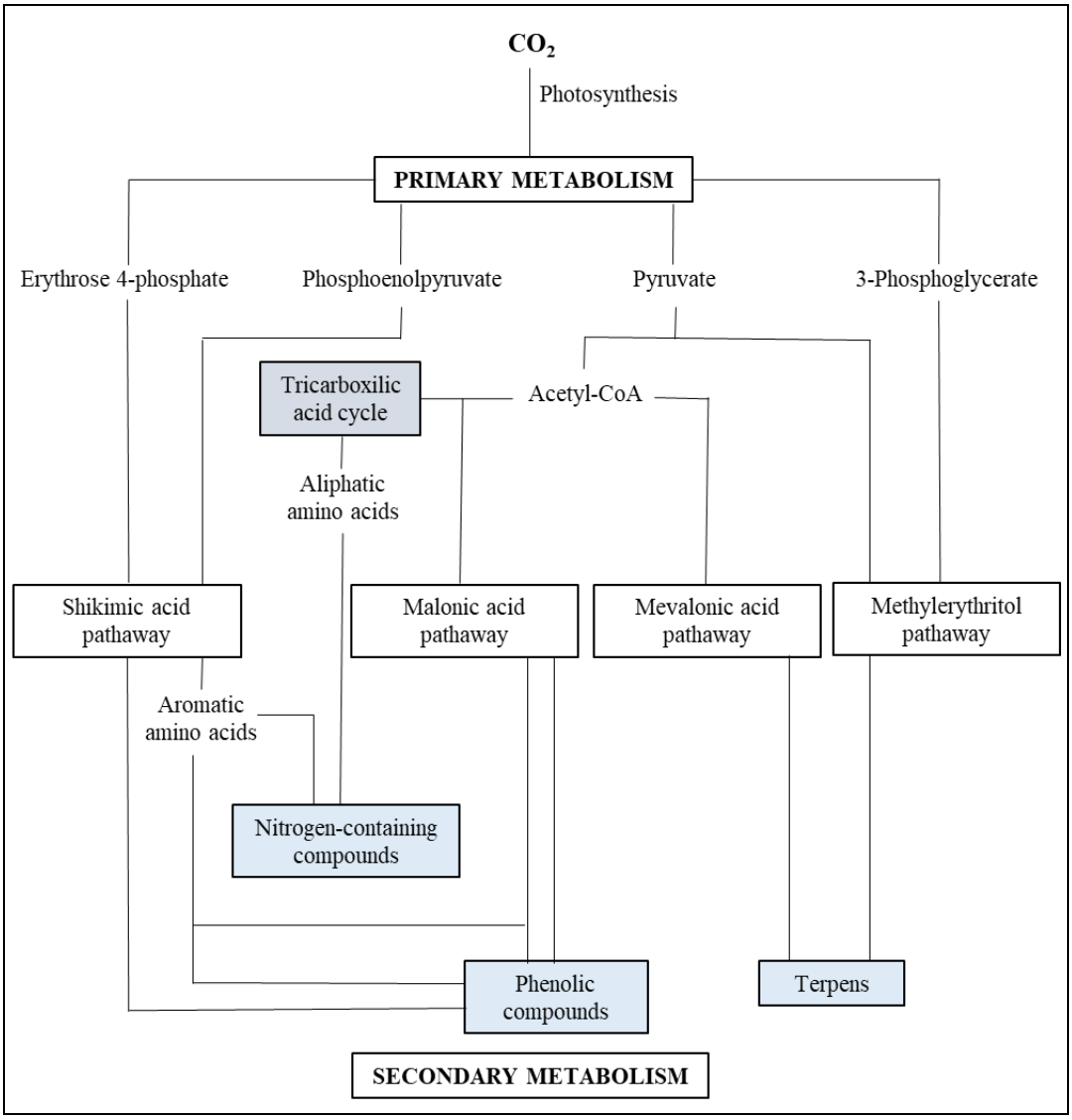

Figure 1: General overview of the biosynthetic pathways involved in the biosynthesis of secondary metabolites.[11]

1.1.1 Plant-polyphenols

Polyphenol compounds structurally range from simple phenolic molecules to complex high-molecular-weight polymers. The basic monomer in polyphenols is the phenolic ring, characterized by an aromatic ring bearing at least one hydroxyl group.[12] Thousands of polyphenolic structures have been identified, and they can be divided into several classes according to the number of phenol rings that they contain and based on structural elements that bind these rings to one another.[13] Polyphenols include (Figure 2):

- phenolic acids
- phenethyl alcohols
- stilbenes
- coumarins
- flavonoids

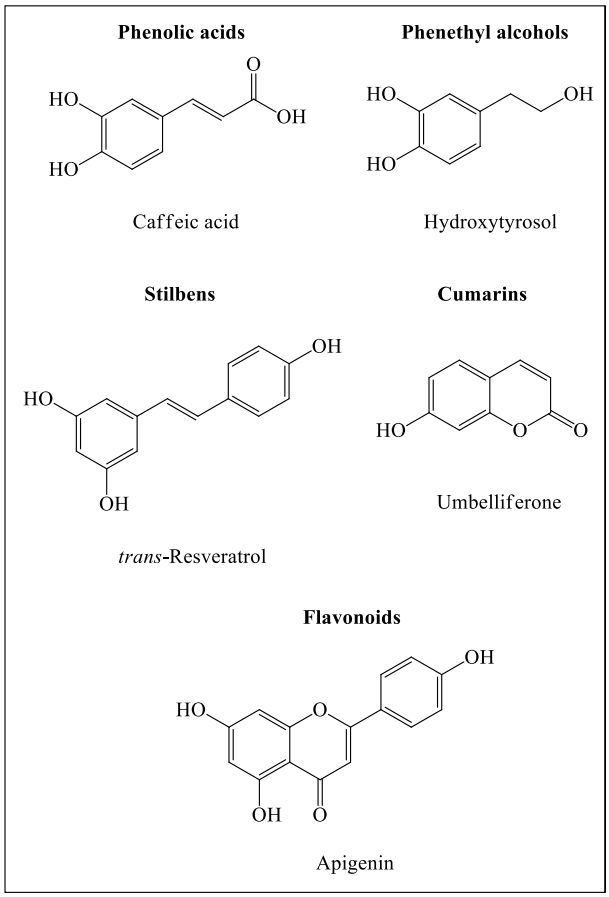

Figure 2: Chemical structures of major classes of polyphenols.

Phenolic acids are found abundantly in almost all the plant-derived foods, representing an important part of the human diet,[14] and include benzoic acids and cinnamic acids, having backbones of C_1-C_6 and C_3-C_6, respectively.[15]

Phenethyl alcohols structurally are characterized by a phenethyl-carbon skeleton containing a primary oxygenated functional group. The main substances belonging to this group are tyrosol (4-hydroxyphenylethanol) and hydroxytyrosol (3,4-dihydroxyphenylethanol) found mainly in extra-virgin olive oil. Recently, research on tyrosol and hydroxytyrosol has increased due to the role that these compounds may play in the prevention of serious diseases (cardiovascular, metabolic, neurodegenerative diseases and cancer).[16]

Stilbenes show a C_6-C_2-C_6 structure and are characterized by two phenolic moieties connected by a double bond mainly in *trans* configuration. Occurrence of stilbenes in the human diet is quite low. The most representative naturally occurring stilbene is resveratrol (3,4',5-trihydroxystilbene) found largely in red grapes and red wine. Most stilbenes in plants are synthesized in response to infection or injury and then act as antifungal phytoalexins.[17]

Coumarins are classified as members of the benzopyrone family, sharing a benzene ring joined to a pyranic ring. There are four main coumarin sub-types: simple coumarins, furanocoumarins, pyranocoumarins and pyrone-substituted coumarins. Coumarins are of great interest due to their biological properties. Indeed, their physiological, bacteriostatic and anti-cancer activities make these class of compounds attractive as novel therapeutic agents.[18]

Flavonoids represent the most abundant group of polyphenols. Their general backbone structure is C_6-C_3-C_6. The common basic structure consisting of two phenyl rings (ring A and B) joined by benzopyranic ring (ring C). Based on the oxidation state of the ring C, flavonoids are divided into several subclasses as flavones, flavanones, flavanols (catechins and proanthocyanidins), anthocyanidins and isoflavones.[12]

In general, polyphenols are an essential part of the human diet, which can be found in large quantities mainly in fruits, vegetables, whole grains, and beverages such as red wine, tea, and coffee.[19] They have been reported to have multiple biological effects. In fact, many epidemiological studies have associated the consumption of fruits, vegetables, and whole grains with a decreased risk of chronic diseases such as cardiovascular disease (CVD), cancer, and diabetes, specifically attributed to their polyphenolic content.[20-22]

1.1.2 Biological activities

As mentioned, polyphenols exhibit a wide range of physiological activities, such as anti-allergenic, anti-atherogenic, anti-inflammatory, antimicrobial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects (Figure 3).[23-25]

Among the various potential health benefits, shown in Figure 3, polyphenols have drawn increasing attention due to their antioxidant and anti-inflammatory activity.[26] Both oxidative stress and inflammation can cause the pathogenesis of chronic diseases.[27] As well as other biological functions, the mechanisms of the antioxidant and anti-inflammatory effects of polyphenols have been largely attributed to their particular chemical structures and arise from their ability in scavenging free radicals and regulating cytokine-induced inflammation.

At the same time, polyphenols have drawn attention due to their antimicrobial activity. The increasing demand for new antimicrobials led the research to concentrate on new antimicrobial agents obtained from natural sources. As evidenced in many studies, some plant extracts have shown antimicrobial properties directly related to their polyphenolic content.[28] This suggested a potential use of phenolic compounds as antimicrobials; thus, these could be applied not only in the pharmaceutical field,[29] but also in the food-industry and in agriculture for the management of food spoilage and plant diseases, respectively.[30]

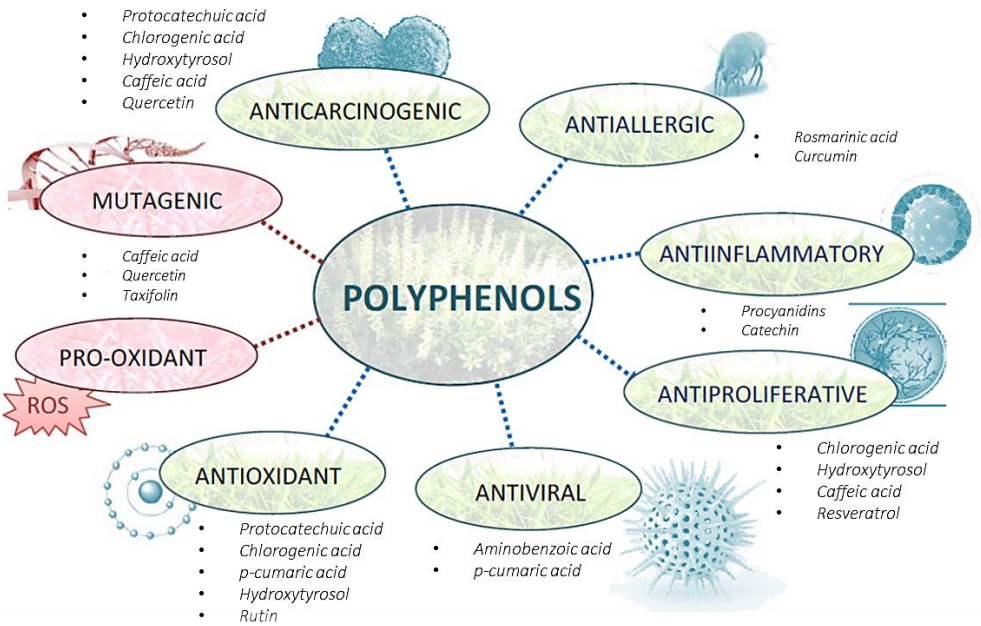

Figure 3: Biological properties of polyphenols.[26]

1.1.2.1 Inflammation and anti-inflammatory activity

Inflammation is a physiological response of our body to infections or tissue injury. Many factors like physical damage, ultra-violet irradiation, microbial infections, and immune reactions are responsible for inflammation, which is characterized by redness, heat, swelling, and pain. There are two major categories of inflammation: acute and chronic. Acute inflammation occurs within minutes, hours, and days, while chronic inflammation is characterized by long-term inflammation that occurs for several months and even years. The sources of inflammation are widespread and include microbial and viral infections, exposure to allergens, autoimmune and chronic diseases, obesity, consumption of alcohol and tobacco, and diet. Normally inflammation is a self-limiting process, because the production of anti-inflammatory mediators follows closely the production of pro-inflammatory mediators. However, in some cases, this physiological process gets out of control. It can be detrimental when the acute inflammatory response switches to chronic inflammation, leading to inflammatory diseases and cancers. Chronic inflammations seem to be due to the persistence of the triggering factors or to the failure in the mechanisms necessary for the resolution of the inflammatory response.[31]

During inflammation mast cells and leukocytes are recruited to the site of damage, leading to a "respiratory burst" due to an increased uptake of oxygen, and thus, an increased accumulation of Reactive Oxygen Species (ROS) at the site of damage. Also, inflammatory cells can produce soluble mediators, such as metabolites of arachidonic acid, cytokines and chemokines, which recruit other inflammatory cells to the site of damage. These mediators can activate signal transduction cascades and induce changes in transcription factors, which mediate cellular stress responses. This sustained inflammatory/oxidative environment leads to a vicious circle, which can damage healthy neighbouring cells and over a long period of time may lead to carcinogenesis.[32, 33]

Important players in the orchestration of the inflammatory response are represented by monocytes and monocyte-derived cells. The monocyte-mediated inflammatory response, although necessary for eliminating invading pathogens, when hyper-activated or persistent may contribute to the pathophysiology of many diseases, as chronic liver disease, rheumatoid arthritis, cardiovascular diseases and obesity.[34-36] A well-known activity is the overproduction by monocytes of pro-inflammatory cytokines such as interleukin-6/IL-6, IL-1β and tumor necrosis factor α/TNF-α, which activate immune cells and the inflammatory microenvironment. Another activity is the release by stimulated monocytes of high mobility

group box 1 (HMGB1), which acts as a “damage-associated molecular pattern” (DAMP) molecule in the extracellular milieu, inducing cytokine secretion and leucocyte recruitment (Figure 4).[37] Monocytes can also produce and release the serine protease granzyme B. The latter, when released in the extracellular milieu, might function on the one hand as DAMP and on the other as enzyme, cleaving several substrates like extracellular matrix (ECM) proteins and cytokines, leading to inflammatory cell recruitment and activation of inflammatory cytokines, as IL-18 and IL-1α.[38] Furthermore, the aberrant hyper-activation of signal transducer and activator of transcription 3 (STAT3), an upstream signalling pathway of multiple pro-inflammatory mediators upon stimuli, may underlie the overproduction of various inflammatory molecules by monocytes (Figure 4). [39] Finally, the dysregulation of autophagy, a widely recognized homeostatic cellular process, can affect transcription, processing and secretion of several cytokines in monocytes, leading to inflammatory diseases.[40]

Many studies have shown a potential effect of polyphenols in attenuating inflammations. The ability of polyphenols to reduce inflammation depends mainly on their ability to act as antioxidants, interfering with oxidative stress signalling, and suppressing the pro-inflammatory signalling transductions. It is known that inflammation and oxidative stress are interrelated processes: the Reactive Oxygen Species (ROS) produced during the inflammatory response promote the synthesis of pro-inflammatory cytokines through the activation of inflammasome.[33]

Current evidences strongly support that polyphenols can act as regulatory molecules of inflammatory biomarkers through different signalling pathways; several studies have shown that polyphenols are able to inhibit inflammasome activation and IL-1β secretion. In addition, phenolic compounds can also attenuate the nuclear factor kappa B (NFκB) induced pro-inflammatory signalling transduction, as well as the expression of inflammatory mediators induced during inflammations.[41-44] Effects on these mediators are targets of therapeutic drugs, therefore the ability of polyphenols on pro-inflammatory cytokines and other signalling molecules can have a significantly positive impact on the prevention of inflammatory diseases.

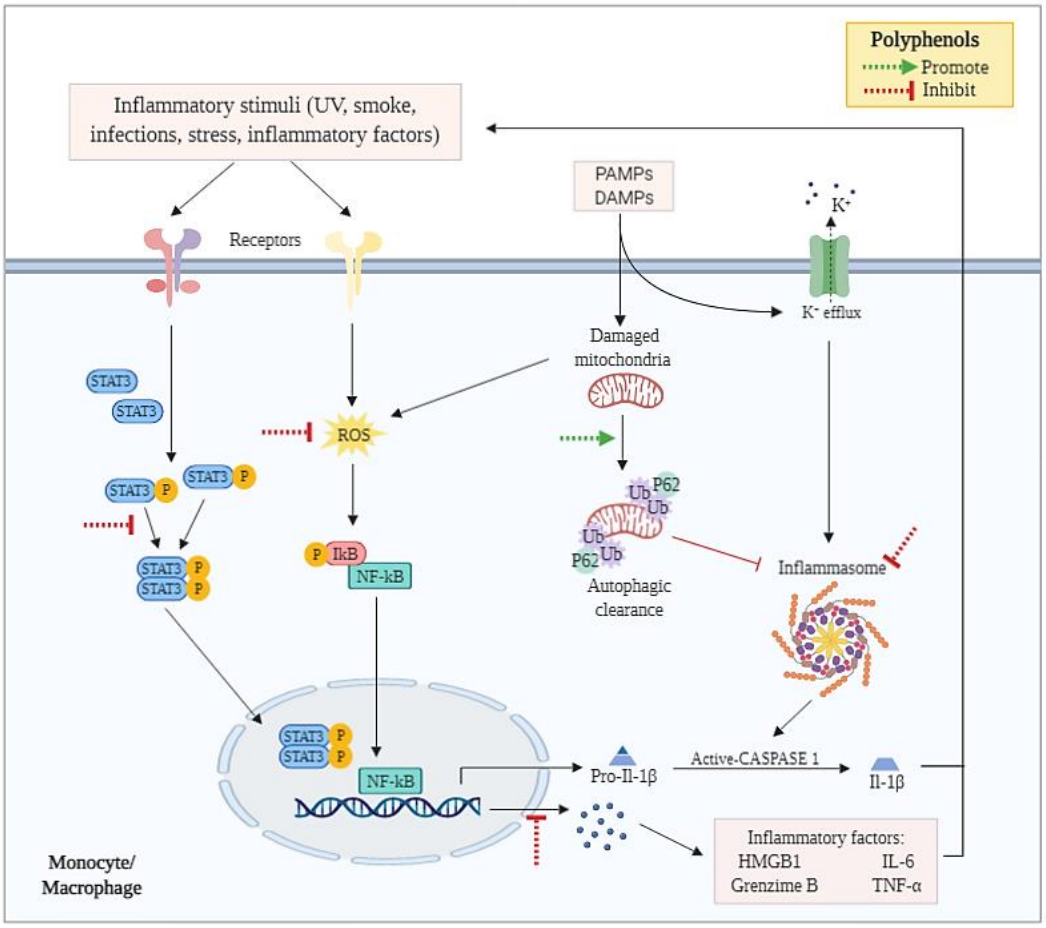

Figure 4: Schematic representation of monocyte/macrophage inflammatory response and the potential role of polyphenols.

1.1.2.2 Antioxidant activity

In every cell of the human body, as a result of the normal essential metabolic processes, free radicals are produced. Free radicals are species containing one or more unpaired electrons in the most external atomic or molecular orbital. The energy corresponding to this configuration gives these species a high instability and reactivity. The most known free radicals are those that contain oxygen, called Reactive Oxygen Species (ROS). Among the most common ROS there are the superoxide anion ($O_2^{\bullet-}$) and the hydroxyl radical ($^{\bullet}OH$); also, other non-radical species like the hydrogen peroxide (H_2O_2), can easily lead to free radical reactions in living organisms.

ROS can derive from both endogenous (mitochondria, peroxisomes, endoplasmic reticulum, phagocytic cells etc.) and exogenous sources (pollution, alcohol, tobacco smoke, heavy metals, industrial solvents, pesticides, certain drugs, radiation etc.).[45]

ROS formation occurs continuously in the cells and is controlled by endogenous enzymes (superoxide dismutase, glutathione by oxidase, catalase, etc.). ROS are recognised for playing a dual role, because they can be either beneficial or harmful to living systems.[46] Beneficial effects occur at low concentrations and involve physiological roles, for example in defence against infections and in the functioning of several cellular signalling systems. The condition of oxidative damage, associated with a damage to a wide range of biomolecules including lipids, proteins, and nucleic acids, arises as a result of an imbalance between ROS production and antioxidant defences, known as oxidative stress. Short-term oxidative stress may occur in tissues injured by trauma, infection, and excessive exercise. ROS accumulation is reported to be involved in several diseases such as diabetes mellitus, neurodegenerative disorders (Parkinson's disease and Alzheimer's disease), cardiovascular diseases (atherosclerosis and hypertension), respiratory diseases and in various cancers (colorectal, prostate, breast, lung, bladder cancers).[47]

Many compounds can protect cells against degenerative effects of ROS, playing an important antioxidant role. An antioxidant is defined as a molecule able to donate an electron to a free radical and neutralize it, thus reducing its damage capacity and replacing the chemical equilibrium of the system in which it operates. The prerequisite to act as antioxidants is that, once oxidized, their radical form is non-reactive towards other molecules. Antioxidants perform their action at low concentrations and can interact with free radicals before vital molecules as lipids, proteins, and DNA are damaged. Some antioxidants are produced during normal metabolism, while other are found in the diet.[48]

Two principal mechanisms of action of antioxidants have been proposed. The first is a "chain-breaking" mechanism in which the antioxidant acts as free radical inactivator by two fundamental techniques: through the transfer of a hydrogen atom (Hydrogen Atom Transfer, HAT) or through the transfer of a single electron (Single Electron Transfer, SET) to the radical species (Figure 5).[49, 50]

The second is a "scavenger" mechanism in which the antioxidant prevents the formation of free radicals coupling with the radical (Figure 5). The scavenging activity also includes the chelation of metals.[49]

However, in nature, the distinction between these classes of antioxidants is not so clear, both because some of the mechanisms of action with which certain antioxidant substances act have not been well defined, and because there are substances, such as phenolic compounds, which can act simultaneously as antioxidants of first and second type.

Synthetic and natural antioxidants are used routinely in foods and medicines. There are several synthetic phenolic antioxidants, like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), which have been widely used as antioxidants in food, cosmetics, and therapeutic industry. However, some physical properties of BHT and BHA, strict legislation on the use of synthetic additives and consumer preferences have shifted the attention from synthetic to natural antioxidants. The use of natural antioxidants in food, cosmetic, and therapeutic industry would be a promising alternative considering the higher compatibility with dietary intake, lower harmful effects and potentially lower cost. There are a number of studies on the biological activities of phenolics, which act as strong antioxidants and free radical scavengers.[51-53]

Polyphenols are characterized by an optimal chemical structure for radical scavenger activity. The structural characteristic responsible for their antioxidant activity is the presence of the phenolic group, which is able to donate the hydrogen atom to the free radicals, interrupting the propagation of the chain reactions typical of oxidative processes. Moreover, the effectiveness of these compounds is mainly due to their ability to delocalize the unpaired electron of the phenolic radical in the aromatic ring and to the possible chelation of the transition metals.[54]

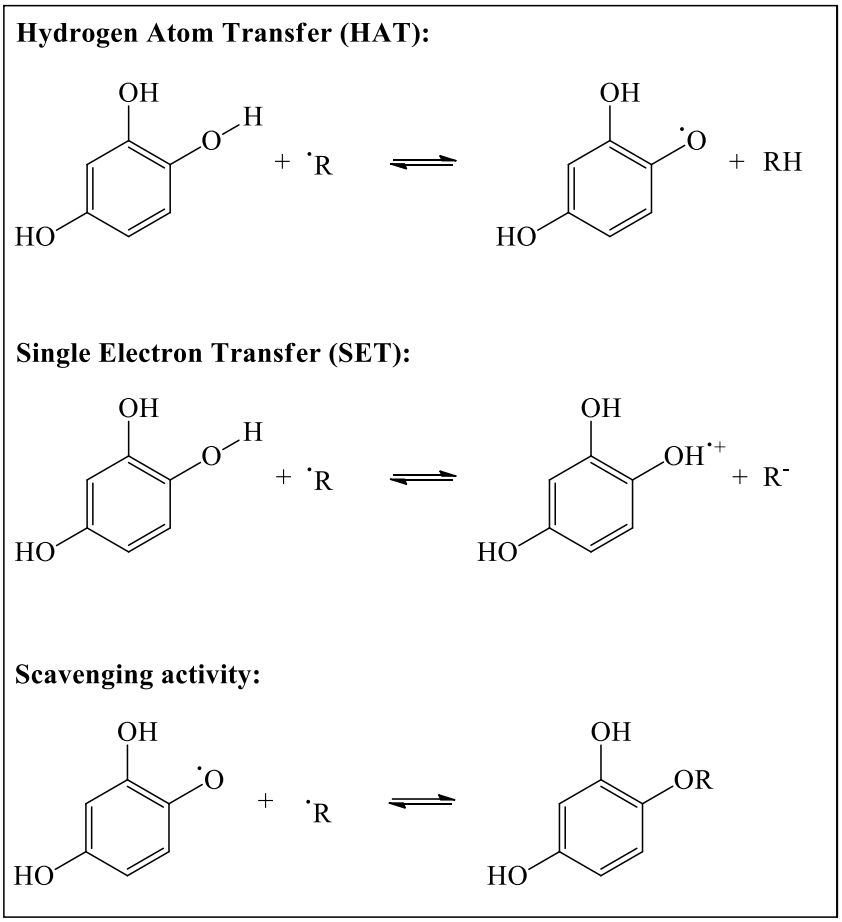

Figure 5: Principal mechanisms by which antioxidants play their protective role.

1.1.2.3 Antimicrobial activity

Phenolic compounds, essential for a wide range of plant functions, are important players in plant-microbe interactions. As evidenced in numerous studies, some classes of phenolic compounds are involved in plants defence mechanisms against bacterial, fungal or viral infections.[55] This activity is manifested both by pre-existing compounds and metabolites formed following the establishment of the infectious process, as phytoalexins.[56]

Providing new and effective antimicrobial drugs has become crucial due to the growing microbial resistance. In this context, plants and their by-products[57] represent promising potential sources of new, efficient and cheap antimicrobial compounds. Literature provides many reports on the antimicrobial activity of extracts rich in polyphenols:[58, 59] in particular, flavonoids have shown antiviral, antifungal and a strong antibacterial activity.[60] The latter property is associated to different mechanisms of action, such as the inhibition of nucleic acid synthesis, the alteration of the membrane permeability, the inhibition of biofilm formation and the attenuation of pathogenicity. Also, polyphenols showed an *in vitro* synergistic effect when tested in combination with conventional antibiotics.[29]

The antimicrobial effect of plant derived polyphenolic compounds could be exploited not only in the biomedical field but also for food and agronomic applications. The use of natural antimicrobial compounds in food applications achieved much interest by the food industry due to the rise of antibiotic resistant foodborne pathogens. As highlighted in many studies, compounds derived from natural sources could be used in the food industry as preservatives capable of improving the safety and quality of food.[57, 61]

Natural products with antibacterial or antifungal activities could be also exploited in agronomic applications.[56, 62] Recently, in response to public concern, there has been a pressure on agriculture to reduce chemical pesticides and look for better alternatives. Pesticides in general are persistent in nature, they can cause ecological imbalances and their indiscriminate use can lead to the development of resistant populations of plant pathogens. Furthermore, their negative effects on human health have encouraged researchers to look for alternative solutions to the currently used pesticides. Therefore, plants and plant by-products can be exploited as sources of bioactive compounds that could be used for the development of new pesticides.[63] For example, olive by-products have interesting antimicrobial activities that could be exploited for the management of plant diseases; as evidenced by Yangui and co-workers[64] hydroxytyrosol-rich extract obtained from olive mill wastewater exerts a broad

fungicidal capacity against *Botrytis cinerea*, a plant-pathogen that causes grey mould on several economically important crops.[64]

1.1.3 Polyphenol extraction methods

As described above, polyphenols are a large family of natural compounds and exhibit a wide range of beneficial properties. Polyphenols can be extracted from plant materials. Due to the huge structural diversity of polyphenolic compounds and their sources, the development of an efficient procedure for the extraction is a challenging task. The structural diversity results in large variability of the physical-chemical properties influencing the extraction procedures, hence no global common strategy has been developed.[65]

The phenolic compounds extraction is a crucial step in the sample preparation process. For the extraction step from plant materials several methods have been developed.[66] Many factors such as chemical nature, solvent composition, extraction time and temperature, and solvent to solid ratio may significantly influence the extraction efficacy and yield.[66] Phenolics can be extracted from fresh, frozen or dried plant samples. The solvents commonly employed are methanol, ethanol, acetone, and their water solutions. In particular, mixtures of alcohols and water have revealed to be more effective in extracting phenolics than the corresponding mono-component solvent systems. Also, the extraction efficiency depends on the extraction temperature and time. It increases at higher working temperatures, but the latter affect the stability of the phenolic compounds, which also depends on their chemical structure. High processing temperatures (>70°C) should be avoided to prevent degradation of the molecules. In the same way, longer extractions increase the chance of oxidation of phenolics, decreasing the phenolic yield in the extracts.[66]

Below, the most common extraction methods will be described, starting from the conventional, up to the most eco-sustainable and innovative ones (Figure 6), with reference to an extensive review by Dai and Mumper.[66] These authors provide an overview on phenolic extraction, purification, analysis and quantification, as well as polyphenols antioxidant and anticancer properties.

The most commonly used and easiest methods of extraction for phenolics is the Solid-Liquid Extraction (SLE).[67, 68] This method is among the most used due to its ease of use, good efficiency and wide applicability. The solvent diffuses into the matrix and dissolves the soluble compounds separating them from the less soluble ones. Despite efforts to optimize the

processes, this conventional method is considered to have a high environmental impact; therefore, new and more eco-sustainable techniques were developed, such as solvent extraction assisted by ultrasound, microwaves and enzymes, and techniques based on compressed fluids as extraction agents (Supercritical Fluid Extraction, SFE or Accelerated Solvent Extraction, ASE).[66]

The Ultrasound-Assisted Extraction (UAE) does not require complex instruments and high costs. The process exploits the phenomenon of cavitation, generated by ultrasounds irradiation, which causes the swelling of cells or the breakdown of cell walls, thus facilitating the release of compounds.[69]

The Microwave-Assisted Extraction (MAE) requires the use of the electromagnetic waves that interact with the polar molecules generating heat. The overheating of the water inside the cells causes a breakdown of the cell walls allowing a rapid release of the chemical substances into the surrounding solvent. The use of this method reduces the extraction time and the quantity of the solvent used, but it is not the best method for the extraction of substances sensitive to high temperatures.[69]

The Enzyme-Assisted Extraction (EAE) involves the use of enzymes such as cellulase, pectinase and hemicellulase. They hydrolyze the structural components of the cell wall and to increase the permeability of the cell wall facilitating the extraction of polyphenols. This method is excellent from the point of view of yield, time and process sustainability. Nevertheless, it shows limitations of application on an industrial scale due to the high cost of the enzymes.[66] Accelerated solvent extraction (ASE) is performed at high temperatures and pressures. The action of the pressure ensures that the solvent is kept in liquid state at high temperatures and pushes the liquid into the matrix; therefore, it accelerates the diffusivity of the solvent and the extraction efficiency. The ASE is mainly used for the rapid extraction of thermally stable organic compounds.[66, 70]

Supercritical Fluid Extraction (SFE) uses temperatures and pressures above the critical point of the solvent. It is conducted in the absence of light and air, thus significantly reducing oxidation phenomena compared to other extraction methods. Many supercritical fluids can be used (ethylene, methane, nitrogen, xenon or fluorocarbons), however CO_2 is the most common. This technique involves high pressures; thus, the required instrumentation entails high costs, which exceed the technical benefits obtained from its use on an industrial scale.[65, 66]

A valid alternative to these conventional processes is represented by membrane processes. The latter offer interesting advantages due to their intrinsic properties in terms of lower

operating and maintenance costs, milder operating conditions of temperature and pressure, greater ease of control and scale-up, and more selective separations. The general principle of this technology is based on the selective permeability of the membranes to allow the target substances to penetrate through the membrane, while unwanted substances are rejected. Various methods, like high pressures, concentration gradients and chemical or electrical potential differences, may be adopted as driving force for the membrane separations. Membrane techniques are generally classified into microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) based on the size of the substances to be separated. In addition, the combination of membrane units in integrated systems offers several benefits in terms of energy consumption, product quality, process capacity and selectivity.[71] Several studies are focused on optimizing the extraction techniques of polyphenols from agro-industrial by-products,[9, 72, 73] since these matrices are valid sources of high-added-value compounds.

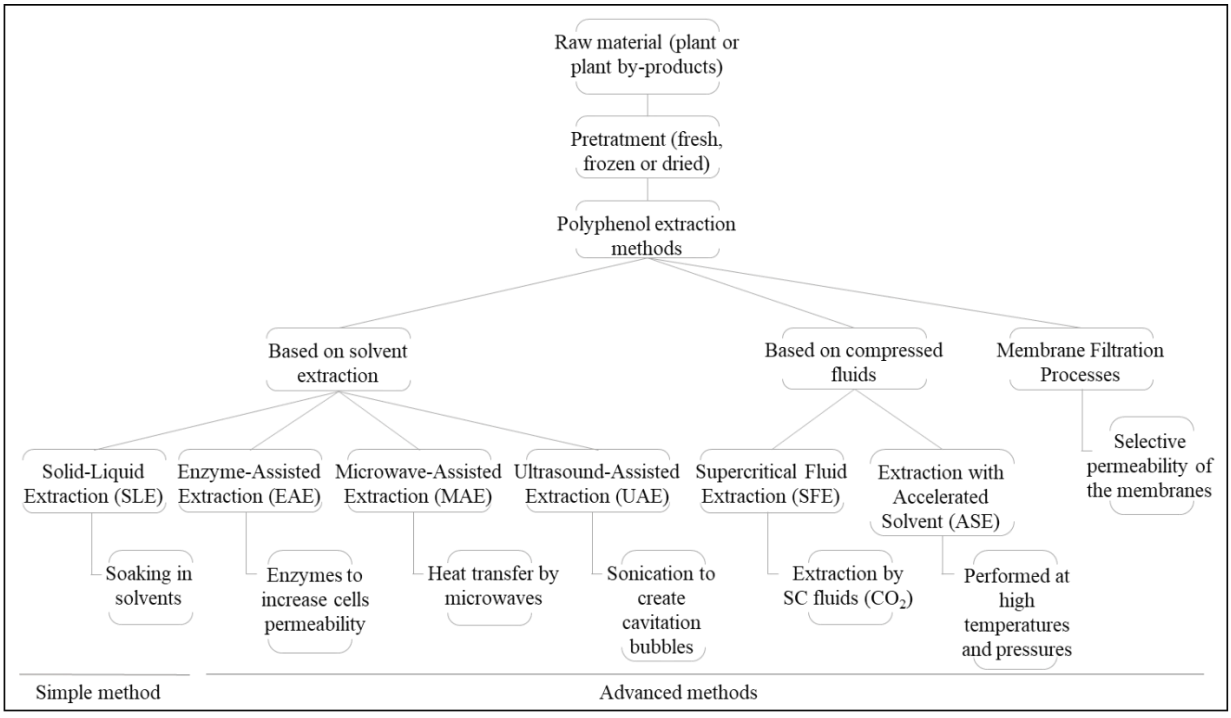

Figure 6: Conventional and advanced methods for plant polyphenols extraction.

1.1.4 Polyphenols and agro-industrial waste

Phenolic compounds are present in almost all fruits and vegetables. Therefore, the processing of agro-food results in the production of by-products that are rich sources of bioactive compounds, including phenolic compounds.[9] The valorisation of food processing by-products is an opportunity for the sustainable and competitive development of several industrial sectors.[74-76] In addition, the reuse of by-products as a source of natural biomolecules may reduce environmental risks caused by waste disposal. For example, the peels of several fruits as also the by-products of the olive-oil industry have attracted considerable interest because have been found to contain high amounts of phenolic compounds.[9, 77] Therefore, agro-industrial by-products, in the light of their huge amount and very low costs, associated to an interesting chemical composition, could be considered highly attractive materials to be recovered and reused, as suggested by the principles of the circular economy.[78, 79]

1.1.5 The circular economy

Among the efforts towards sustainable development, circular economy has emerged as a strategy to re-target production processes in contrast with the linear economy. The latter could be summarised as a "take, make and dispose" model, with raw materials at one end and waste at the other. The negative effects caused by the linear economic model are threatening the stability of the economies and the integrity of the natural ecosystems. On the contrary, the circular economy aims to "close the loop" of product lifecycles: waste products become resources to be recovered and revalorised, through recycling, creating benefits both for the environment and for the economy.[80] In the last years these implications and advantages have been extensively demonstrated,[81, 82] as a mean for achieving a better balance among economy, environment and human health, thus promoting a more environmentally friendly use of resources. The underlying idea is to imitate the circularity of biological processes, ensuring that raw materials used in production processes can easily be recycled at the end of their life through their reintroduction into the environment or into a new production process, as secondary raw material. The circular economy is, in fact, a closed-circuit development model that avoids, where possible, waste generation.[83]

The circular economy approach has been adopted also by the European Community[84] to promote economic growth, increase global competitiveness and create new jobs by saving resources and energy.[85] In this sense, as part of the "Thematic Strategy" on the prevention and recycling of waste, the European Commission distinguished between waste and by-products. While the European law defines waste as "any substance or object which the holder discards or intends or is required to discard", by-product is defined as "a substance or object, resulting from a production process, the primary aim of which is not the production of that item". By-products could have very different environmental impacts, and an incorrect classification could cause environmental damages or unnecessary costs.[86]

The concept of circular economy can be applied to almost all manufacturing fields. Looking more specifically at the agro-industry, by-products offer a good opportunity, as their production in this sector covers the entire life cycle of products, from agricultural to industrial production and processing. If treated as waste, their huge amount, as well as being a great loss of precious materials, would also raise serious management problems, both from an economic and from an environmental point of view. In this context, the application of the circular economy principles can lead to achieve an increased recycling and valorisation of agro-industrial by-products, by maximising their reuse. Indeed, by-products constitute an attractive sustainable resource for industry because they still contain a wide range of bioactive compounds that could be recycled and could be used as active ingredients for agronomic, cosmetic, food, and pharmaceutical formulations.[77, 79]

Below a focus will be provided on two valuable sources of phenolic compounds, represented by the by-products generated during kiwifruit and olive processing.

1.1.5.1 Kiwifruit extracts

The kiwifruit is the edible berry of the *Actinidia* plant (Actinidiaceae). Also known as the "Chinese gooseberry", it was renamed by New Zealand exporters to kiwifruit. This name comes from "the kiwi", a brown flightless bird and New Zealand's national symbol. The genus *Actinidia* is native to South China and is broadly distributed on the Asian continent. Out of all species, *Actinidia deliciosa* is intensely cultivated all over the world, in particular the most common variety is the green-fleshed berry of the *Actinidia deliciosa* (C. F. Liang, A. E. Ferguson) "Hayward" (A. Chev) cultivar. It was developed by Hayward Wright in New Zealand around 1924 and this is the most widely grown cultivar in the world.[87] According to data provided by the Food and Agriculture Organization of the United Nations (FAO), kiwifruits cultivation is relevant in China, Italy, New Zealand and Chile, which represent the four largest producers in the world.[88] Kiwifruit contains several health-beneficial phytoconstituents and it is recognized as an interesting source of bioactive compounds. In particular, strongly acidic compounds (phenolic and hydroxycinnamic acids such as *p*-coumaric acid, protocatechuic acid, and syringic acid) and weakly acidic compounds (flavonoids as catechin, epicatechin, naringenin, quercetin) have been identified in fruit pulp crude and juice extracts.[89] Several research studies have been focused on the biological activities of the extracts obtained from the edible part of the fruits. For example, Iwasawa and Co-Authors[90] reported stronger *in vitro* antioxidant activities of kiwifruit compared with other fruits like orange and grapefruit: particularly, inhibitory effects on lipid oxidation, H_2O_2-elimination properties and superoxide dismutase (SOD)-like activities were reported.[90] Also, as shown by Jung *et al*.,[91] kiwifruit extract has *in vitro* protective effects against cardiovascular disease.[91] On the contrary, little has been reported on the biological activity of kiwifruit peel extracts.[92, 93] Residues from kiwifruit can represent up to 30% of the total kiwifruit crop.[94] These by-products derived from kiwifruit processing and still contain appreciable amounts of valuable bioactive compounds. As evidenced by Pinelli *et al*.[95] kiwifruit peels could be valuable sources of functional compounds, since they contain different classes of phenolic compounds such as phenolic acid and hydroxycinnamic acid derivatives, flavonols and procyanidins.[95] In particular, procyanidins, a subclass of flavonoids composed of flavan-3-ol (epi)catechin monomers and their respective oligomers (Figure 7), have attracted increasing attention. Procyanidins, found in commonly consumed foods, are of great interest in nutrition and medicine because of their antioxidant capacity and possible protective effects on human health in reducing the risk of chronic diseases such as

cardiovascular diseases and cancers.[96, 97] Therefore, kiwifruit by-products could be used as sources of high value-added ingredients useful in other sectors, such as the pharmaceutical and food industry, generating environmental and economic benefits.

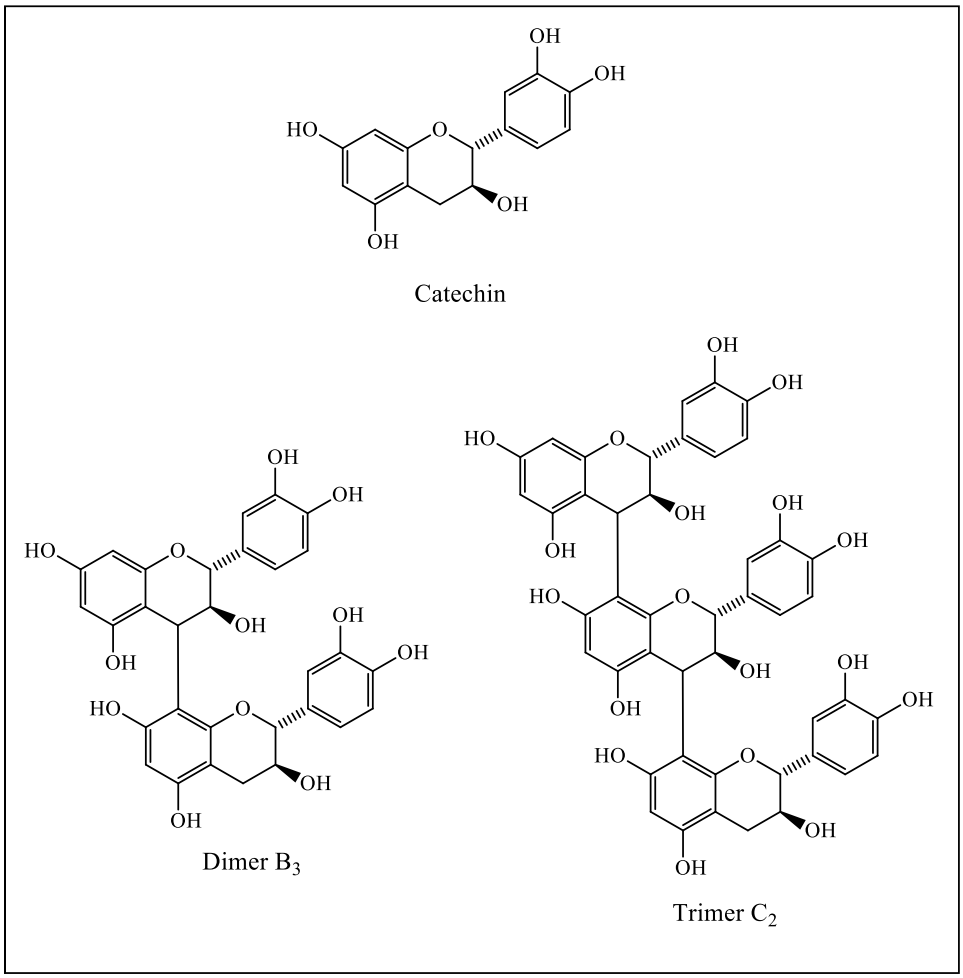

Figure 7: Chemical structures of catechin, procyanidin dimer B_3, and trimer C_2.

1.1.5.2 Olive extracts

Olive tree (*Olea europaea* L., Oleaceae) is among the most extensively cultivated crop in the world, especially in Mediterranean countries. Olive fruits are used for olive oil extraction and consumed as table olives. During olive fruits harvesting and processing many by-products are generated. The main by-products include olive pomace, wastewater, and a mixture of leaves, small twigs, and branches.[98] These matrices are rich in phenolic compounds with biological and pharmaceutical interest. Several biological effects have been proved including antiradical, antimicrobial, anti-inflammatory, anticancer and neuroprotective activities.[99-102] Qualitative and quantitative characterization of solid and aqueous by-products suggested that the latter can be considered as an important natural source of phenolic compounds, which, after suitable purification, could be used as food antioxidants or as ingredients in nutraceutical or pharmaceutical products.[103] Polyphenols contained into these by-products belong to different classes, such as phenolic acids and alcohols, secoiridoids, lignans, and flavones.[99] In order to recover high-added value compounds and to reduce pollution of wastewaters generated during the olive oil production, many researches are focused on the recovery of the phenolic compounds in olive mill wastewaters, testing different extraction systems.[104, 105]

Among olive polyphenols, hydroxytyrosol (HTyr) represent the most exhaustively studied due to its interesting biological activities like antioxidant and anticancer activities, with many promising applications in foods, cosmetics, and medicine.[100, 106] HTyr is one of the main phenethyl alcohols in olives; in plant materials, it is esterified by elenolic acid to get oleuropein. During the milling process for olive oil production the released endogenous β-glycosidase hydrolyze the ester bond producing elenolic acid and HTyr (Figure 8), which is partitioned between the oil and water phases.[107] As a consequence of its polar character, olive oil by-products represent the major source of HTyr, although it can be found also in the olive oil and in the olive leaves. HTyr plays an important role in pharmaceutical and food industry for its beneficial health properties and for its strong antioxidant capacity. This latter property makes HTyr a good candidate as a preservative in the food sector, which constantly requires new, powerful and safe antioxidants to increase products shelf life. In this context, HTyr could potentially replace synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). HTyr can be both synthetized[100, 108] and recovered from waste[74, 77].

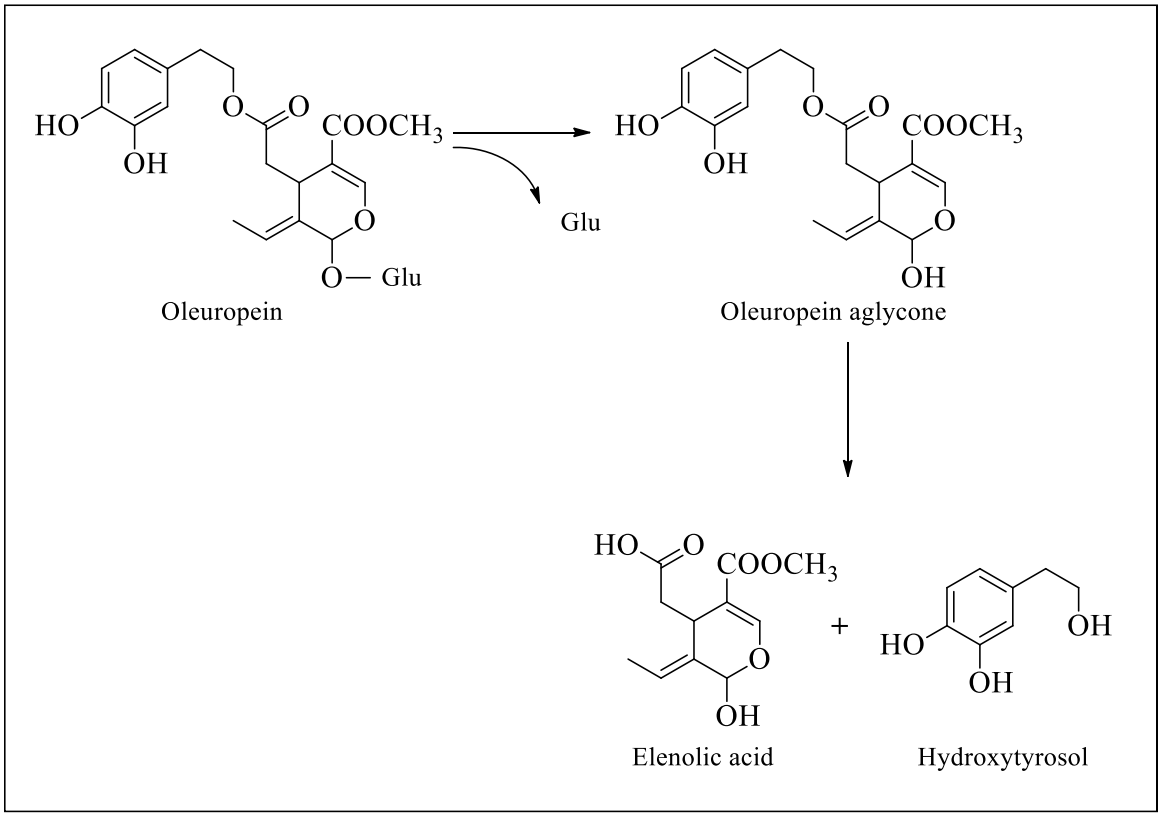

Figure 8: Hydrolysis of Oleuropein.

1.2 Natural antioxidants for active food packaging

In a recent study carried out by FAO is reported that every year about one-third of food produced for human consumption, consisting in about 1.3 billion tons, is globally wasted or lost.[109] These alarming data have significant adverse implications from ethical, economic and environmental points of view. In this scenario, all strategies should aim to maximise the amount of food available for human consumption, reducing the environmental and the economic impacts of its production and improving food safety. Among them, the measures focused on food preservation play an important role. Food preservation consists in controlling all the aspects involved in food deterioration, which cause loss in nutritional value and food safety. The most relevant are the oxidative processes, which are responsible, for example, for degradation of vitamins, alteration of flavours and visual deterioration of food such as the browning of fruits, vegetables and meat. For these reasons, active food packaging solutions represent strategies spanning through the entire food chain from producers and distributors to consumers.[110] In fact, polymeric films used to prepare food packaging, can incorporate ingredients active in preventing degradations by slowing down such processes. Recently, several phenolic compounds[111, 112] and natural extracts[113, 114] have been investigated for this purpose. Among naturally-occurring polyphenols, hydroxytyrosol[77, 100, 107] and derivatives[115, 116] were successfully used in antioxidant food packaging strategies. Recently, both hydroxytyrosol and hydroxytyrosol methyl carbonate (HTyr-MC), a synthetic HTyr-derived compound, were successfully used for antioxidant food packaging solutions, demonstrating to exert a strong antioxidant activity, but, in both cases the release from the films reached the maximum level during the first day of contact with a food simulant, thus representing a limitation for food packaging applications.[117, 118]

In the food packaging sector, over the last decades, nanostructured materials have been intensely investigated for improving films performances for industrial applications.[119-122] In fact, the nanometric size grants to these materials a greater surface area per unit of mass and this feature may permit a slower and thus more controlled release of an active ingredient from a film matrix.[123, 124] Among the possible polymers suitable for the production of nanomaterials, great attention has been paid to starch, a natural, renewable and biodegradable polymer. Structurally, it consists of two macromolecules: amylose and amylopectin. Amylose is a slightly branched polymer[125] of α-D-glucose units linked by α-1,4-glycosidic bonds; amylopectin is a highly branched polymer consisting of α-D-glucose units linked by α-1,4-glycosidic bonds and α-D-glucose units by α-1,6-glycosidic bonds (Figure 9). The ratio of

these two polymers is generally related to the botanic origin of starch[126] and is responsible for the shape, size and crystalline organization of starch granules.

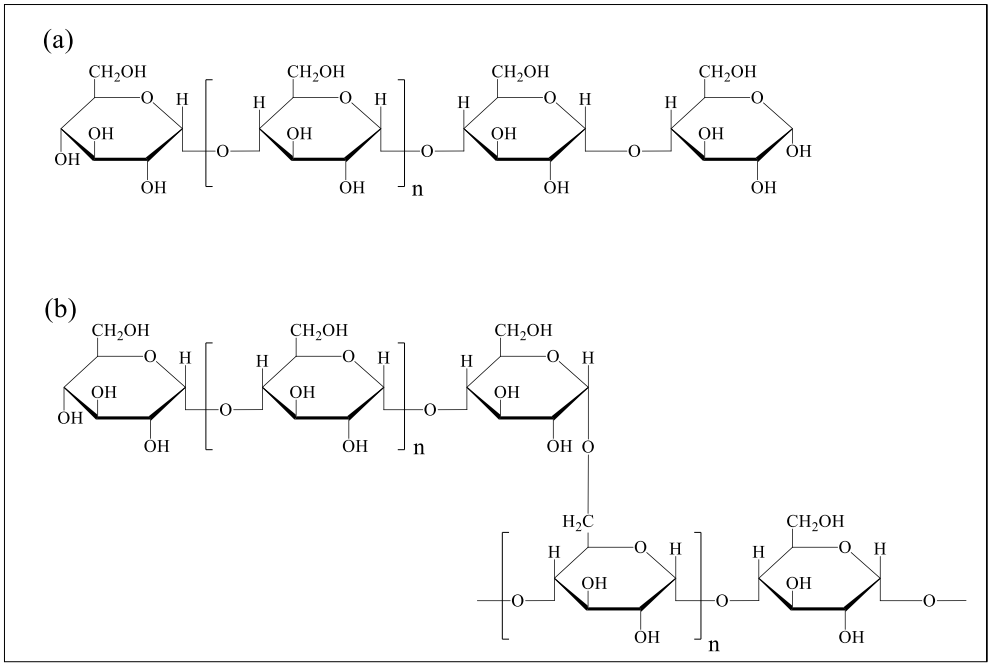

Figure 9: Structure of (a) amylose α-(1→4)-glucan and (b) amylopectin α-(1→6) branching points.

2. Aim of the work

Polyphenols are one the most important group of secondary metabolites, they have received considerable attention thanks to their physiological functions, including the anti-inflammatory, antioxidant and antimicrobial properties. Polyphenolic compounds are widely distributed in plants, fruits and vegetables, and therefore in their by-products. The latter cannot be considered waste, but starting materials to obtain new products of industrial interest. This is required by international waste management strategies, aiming at more adequate, environmentally and economically sustainable disposal solutions. In this light, the experimental activity carried out during the three-year PhD, as a part of the research in the field of natural substances, was involved in a multidisciplinary research topic: the valorisation of agro-food by-products to obtain biologically active materials.

The activity carried out can be divided into three parts:

1. Valorisation of kiwifruit (*Actinidia deliciosa*, cv. Hayward) by-products through the preparation and the characterization of a procyanidin-rich extract from *Actinidia deliciosa* peel, the most relevant by-product deriving from fruits processing. The *in vitro* anti-inflammatory activity of the whole extract on human THP-1 monocytes was evaluated. In particular, its capability to target multiple processes involved in monocyte-mediated inflammation, such as the production of inflammatory molecules (including IL-6, IL-1β and TNF-α pro-inflammatory cytokines, HMGB1 danger signal and granzyme B serine protease), the activation of STAT3 signalling and autophagy, was investigated.
2. Valorisation of olive (*Olea europaea*) oil by-products testing a standardized hydroxytyrosol-rich extract, obtained from olive mill waste waters (OMWW) with an eco-friendly procedure, for its antioxidant and antimicrobial activities. All the experiments were carried out using the synthetic hydroxytyrosol as reference compound. The antioxidant properties were assessed by the ABTS (2,2'-azinobis (3-ethylbenzothiazoline 6-sulfonate) radical scavenging capacity assay. Also, antimicrobial activity, against two olive pathogens (*Pseudomonas savastanoi* pv. *savastanoi* and *Agrobacterium tumefaciens*), was assessed with both qualitative and quantitative assays. The qualitative drop diffusion method was used to determine the effective dose, while the quantitative broth dilution method was used to determine the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) of the hydroxytyrosol-rich extract.

3. Finally, with the aim of applying the hydroxytyrosol-rich extract as antioxidant in active food packaging, hydroxytyrosol standard was used as active ingredient. To develop novel ternary multifunctional films the antioxidant (HTyr), starch nanocrystals (NC) or starch nanoparticles (NP) were included, by solvent casting technique, into a poly(vinyl alcohol) (PVA) film matrix. Two type of nanostructured starches were used, from the bread wheat variety Cadenza (WT, amylose content 33%) and from a derived-high amylose line (HA, amylose content 75%). All formulations, named PVA/NCWT/HTyr, PVA/NCHA/HTyr, PVA/NPWT/HTyr and PVA/NPHA/HTyr, were investigated for their optical, morphological, thermal and mechanical properties. Indeed, in order to evaluate the kinetic release of HTyr from each film, overall and specific migration tests were performed using a food simulant according to the current European legislation. Furthermore, antioxidant properties were tested considering the final application as antioxidant food packaging systems.

This work was made in collaboration with the research groups of Prof. Annalisa Romani, Prof. Francesca Romana Velotti, Prof. Leonardo Varvaro, Prof. Domenico Lafiandra, Prof. Josè Maria Kenny and Prof. Laura Dugo. Thanks to these collaborations it was possible to obtain the results that will be shown.

The results obtained led to the following publications:

Luzi, F., Fortunati, E., Di Michele, A., **Pannucci**, E., Botticella, E., Santi, L., Kenny, J.M., Torre, L., Bernini, R. (2018). *Nanostructured starch combined with hydroxytyrosol in poly (vinyl alcohol) based ternary films as active packaging system*. Carbohydrate polymers, 193, 239-248.

D'Eliseo, D., **Pannucci**, E., Bernini, R., Campo, M., Romani, A., Santi, L., Velotti, F. (2019). *In vitro studies on anti-inflammatory activities of kiwifruit peel extract in human THP-1 monocytes*. Journal of ethnopharmacology, 233, 41-46.

Pannucci, E., Caracciolo, R., Romani, A., Cacciola, F., Dugo, P., Bernini, R., Varvaro, L., Santi, L. (2019). *An hydroxytyrosol enriched extract from olive mill wastewaters exerts antioxidant activity and antimicrobial activity on Pseudomonas savastanoi pv. savastanoi and Agrobacterium tumefaciens*. Natural Product Research. Accepted for publication.

3. Material and methods

3.1 Plant material

Low-weight fruits of *Actinidia deliciosa* (A.Chev.) C.F.Liang & A.R.Ferguson, cv Hayward were collected in Cisterna di Latina (Latina, Italy; Latitude: 41°31'42.8''N; Longitude: 12°47'31.3''E) from Stefano Della Bianca orchard in July 2016. A representative of the cultivar has been transplanted in the "*Orto dei semplici*" section of the "*Angelo Rambelli*" Botanical Garden of the University of Tuscia, under the accession GS159.
Olive Mill Waste Waters (OMWW) were collected in a farm of Molfetta (Puglia, Italy; Latitude: 41°09'56.97''N; Longitude: 16°33'34.17''E).

3.2 Standards, solvents and antibodies

Standards and reagents were of analytical grade and were purchased by Sigma-Aldrich Company (Milan, Italy). All solvents were of HPLC grade (Anal R Normapur, VWR chemicals). 2-Iodoxybenzoicacid (IBX) was freshly prepared as described in literature.[127]
The following primary antibodies were used: rabbit polyclonal anti-IL-6 (Gene Tex), rabbit polyclonal anti-IL-1β (Gene Tex), mouse monoclonal anti-TNFα (Santa Cruz), rabbit polyclonal anti-HMGB1 (Abcam), mouse monoclonal anti-granzyme B (Calbiochem), mouse monoclonal anti-phospho-STAT3 (pY705) (BD Transduction Laboratories), mouse monoclonal anti-STAT3 (BD Transduction Laboratories), rabbit polyclonal anti-LC3 (Novus Biologicals), mouse monoclonal anti-p62 (BD Transduction Laboratories) and mouse monoclonal anti-β-actin Ac-40 (Sigma-Aldrich).

3.3 Hydroxytyrosol synthesis and characterization

Hydroxytyrosol [2-(3',4'-dihydroxyphenyl)ethanol] was synthesized from tyrosol according to a procedure optimized and patented in our laboratory consisting of three steps (Figure 10).[108]

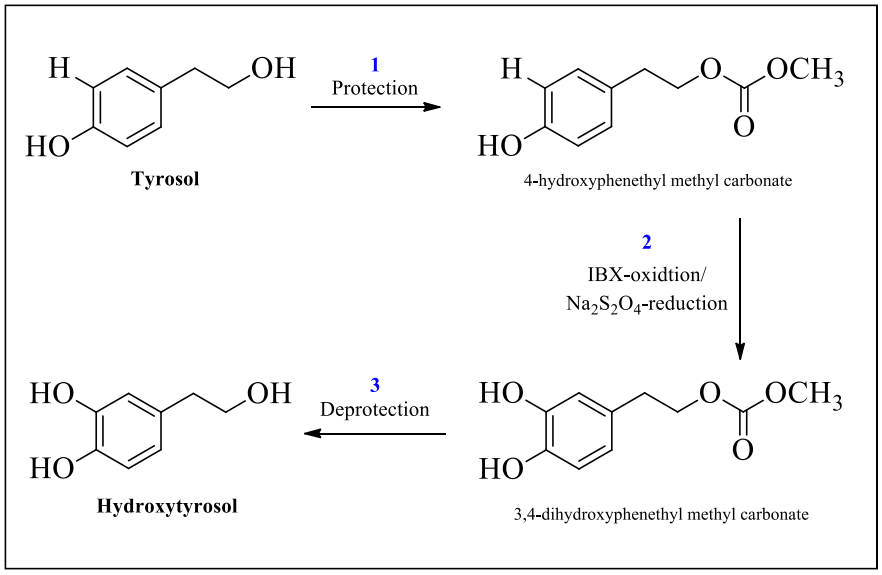

Figure 10: Synthetic procedure to obtain hydroxytyrosol from tyrosol (IT Patent 0001381959, filing date 09/27/2010).

(1) Tyrosol (138 mg, 1.0 mmol) was dissolved in DMC (3mL) and then H_2SO_4 96% (10.7 µL, 0.2 mmol) was added. The solution was kept at 90 °C for 7 h under stirring. At the end, it was cooled to room temperature (RT), and DMC was evaporated under vacuum. The residue was extracted with ethyl acetate, washed with a saturated solution of NaCl, and dried over Na_2SO_4. After filtration and evaporation under vacuum, tyrosol methyl carbonate was obtained.

(2) Tyrosol methyl carbonate (1.0 mmol) was dissolved in CH_3OH (4 mL), and then IBX (336 mg, 1.2 mmol) was added. The solution obtained was kept at 0 °C for 30 minutes under stirring. Then, water (4 mL) and $Na_2S_2O_4$ (348 mg, 2.0 mmol) were added, and the solution was stirred for 5 minutes at RT. At the end, the solvent was evaporated under vacuum and the residue was solubilized with ethyl acetate and a saturated solution of $NaHCO_3$ was added. The aqueous phase was extracted with ethyl acetate. The collected organic phases were washed with a saturated solution of NaCl and dried over Na_2SO_4. Once the solvent was evaporated, hydroxytyrosol methyl carbonate was obtained.

(3) Hydroxytyrosol methyl carbonate (226 mg, 1.0 mmol) in THF was added to KOH 1 M (3 mL, 3.0 mmol). The mixture was kept at RT for 30 minutes under stirring. At the end, the solvent was evaporated, and the residue was solubilized with ethyl acetate. The solution was

treated with HCl 1 M. The aqueous phase was extracted with ethyl acetate. The collected organic phases were washed with a saturated solution of NaCl and dried over Na_2SO_4. After the solvent evaporation, the product was obtained (85% yield) and characterized by ^{1}H-NMR and ^{13}C-NMR.[108] NMR spectra were recorded with the 400 MHz Nuclear Magnetic Resonance spectrometer Avance III (Bruker, Germany) using methanol-d_4, 99.8% in deuterium (VWR chemicals), as solvent. Chemical shifts were expressed in parts per million (δ scale) and referred to the residual protons of the solvent.

3.4 Extracts preparation

The extracts preparation was carried in collaboration with the research group of Prof. Annalisa Romani at University of Florence, Department of Statistics, Computing, Applications "G. Parenti".

Kiwifruit peel extract

The kiwifruit peel extract was prepared according to a procedure already optimized.[95] Kiwifruit peels were isolated from fruits, frozen in liquid nitrogen and crushed in a mortar. 1 g of the resulting powder was extracted with 50 mL of ethanol/acidified water (70/30 v/v; pH=3.2, obtained by adding formic acid) under magnetic stirring for 24 h at RT. The mixture was centrifugated (4500 rpm, 15 minutes) and the supernatant was recovered and analyzed through high-performance liquid chromatography coupled to photodiode array detector and mass spectrometer (HPLC/DAD/ESI-MS).

For the biological activities a stock solution of the extract was prepared removing the solvent from the extract solution using a rotary evaporator (Heidolph). The powder obtained was weighted and solubilized in a known volume of ethanol/water=70/30 to reach a concentration of 12 mg/mL (stock solution).

Hydroxytyrosol-enriched extract

The hydroxytyrosol-enriched extract (HTyr-enriched extract) was provided by PhytoLab (University of Florence, Department of Statistics, Computing, Applications "G. Parenti"); it was obtained from Olive (*Olea europaea* L.) Mill Waste Waters (OMWW) using an eco-friendly solid-liquid extraction technology followed by an innovative separation process, according to a patented procedure.[128] The extraction process starts from a solid–liquid

extraction in a pneumatic pressure extractor using water as solvent at 60 °C. Then, the separation was performed with physical technologies, consisting of an integrated system of membrane filtration steps: Microfiltration (MF), Ultrafiltration (UF) and Nanofiltration (NF), followed by Reverse Osmosis (RO). The filtration steps are characterized by different filtration degrees. The output product is further concentrated with an heat pump evaporator to obtain a powdered standardized extract.[129]

For the biological activities a stock solution, corresponding to a concentration of 100 mg/mL, was prepared solubilizing the powder in sterile pure water.

3.5 Extracts characterization

HPLC/DAD/ESI-MS analysis

A sample of the extract obtained (10 mg) was solubilized in methanol (2 mL) and then filtered (0.45 mm). HPLC/DAD/ESI-MS analysis of kiwifruit peel extract was made in collaboration with the research group of Prof. Annalisa Romani. The quali-quantitative characterization of polyphenols was carried out using a HP-1100 liquid chromatograph equipped with a DAD detector and a HP 1100 MSD API-electrospray (Agilent Technologies) operating both in negative and positive ionization mode and a column Zorbax SB-Aq 4.6mm ID × 150mm (5 μm, Agilent).[95] Polyphenols were identified by retention times, spectroscopic and MS data. Procyanidins and hydroxycinnamic acid derivatives were quantified at 280 and 330 nm using catechin and caffeic acid as reference standard, respectively. The quantitative determination was carried out using five-point regression curves (with $r^2 > 0.9998$). All determinations were carried out in triplicate. Results are given as averages and expressed as mg per gram of peel, considering that each mL of extract is referred to 0.02 g of kiwifruit peel (standard error was always< 5%).[130]

HPLC/DAD/ESI-MS analysis of HTyr-enriched extract was provided by the research group of Prof. Paola Dugo, University of Messina, Dipartimento di Scienze Chimiche, Biologiche, Farmaceutiche ed Ambientali. HPLC/DAD/ESI-MS analyses were carried out on a Shimadzu instrument, consisting of two binary solvent pumps LC-20AD, a SPD-M20A photodiode array detector (DAD) and a LCMS-2020 mass spectrometer detector (MS). MS detector was equipped with an electrospray ionization (ESI) source operating in negative ionization (NI) mode and single quadrupole MS. Separations were attained on an Ascentis Express C18 (150 × 4.6 mm, 2.7 μm) analytical column (Merck Life Science).

NMR analysis

The ^{1}H NMR spectrum of HTyr-enriched extract was recorded using the 400 MHz Nuclear Magnetic Resonance spectrometer Avance III (Bruker, Germany). A sample of the extract (15 mg) was solubilized in methanol-d_4, 99.8% in deuterium. Chemical shifts were expressed in parts per million (δ scale) and referred to the residual protons of the solvent.

3.6 Anti-inflammatory activity of kiwifruit peel extract

The anti-inflammatory properties of kiwifruit peel extract and its effects on the expression and production of several inflammatory mediators were investigated. This part of the work was obtained through a collaboration with the research group of Prof. Francesca Romana Velotti, University of Tuscia, Department of Ecological and Biological Sciences.

3.6.1 Cell culture

THP-1 human monocytic cells, derived from a patient with acute monocytic leukemia (AML), from American Type Culture Collection (ATCC® TIB-202™), were cultured in RPMI 1640 medium containing 10% FCS (HyClone), 100 μg/mL of streptomycin and 100 IU/mL penicillin, and maintained in a 5% CO_2 incubator at 37 °C. Cells were mycoplasma free (EZ-PCR Mycoplasma test kit, Biological Industries).

3.6.2 Cell treatments

For THP-1 activation, cells were cultured with 100 ng/mL lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 for 24 h. THP-1 cells were incubated with the ethanolic solution alone (Control) or with different concentrations of the extract (25, 50, 100 and 200 μg/mL) by diluting the stock solution in the cell culture medium. The final concentration of ethanol in the medium was always< 1.6%, compatible with cell viability (assessed by the trypan blue dye exclusion assay) and function (assessed by the analysis of cell proliferation).

For the autophagic investigation, THP-1 cells were cultured with the extract (50 μg/mL) for 24 h and then treated with Bafilomycin A1 (Baf) (20 nM) (Santa Cruz Biotechnology Inc.), an

inhibitor of vacuolar-H^+-ATPase, for the last 2 h. In some experiments, 3-methyladenine (3-MA) (0.2 mM) (Santa Cruz Biotechnology Inc.) was added 2 h before the extract treatment.

3.6.3 Cell viability and proliferation assay

After each treatment, cell viability was assessed by the trypan blue dye exclusion assay, as previously reported.[131] The analysis of cell proliferation was performed using 0.05% trypan blue solution to count the number of live cells within a Neubauer chamber. At least three replicate counts were conducted by the same operator at each time.

3.6.4 Cytokine measurement, western blot and densitometric analysis

Cytokines were measured in conditioned media using the MILLIPLEX® MAP Human Cytokine/Chemokine Magnetic Bead panel (Millipore) according to the manufacturer's instructions and detected by Bio-Plex® MAGPIX™ Multiplex Reader (Bio-Rad).

For western blot assays cells were washed twice in PBS and cell lysates were prepared by a solution containing 50 mM TRIS-HCl pH=7.6, 150 mM NaCl, 0.5% TRITON X-100, 0.5% Sodium deoxycolate, 0.1% SDS and the protease inhibitor mixture ''Complete'' (Roche Diagnostic GmbH). For testing the extracellular release of inflammatory mediators, the conditioned media was also collected. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes. The membranes were blocked with 5% Non-Fat Dry Milk, probed with specific primary antibodies overnight, at +4°C, washed and incubated with appropriated secondary antibodies. The reaction was revealed by horseradish peroxidase (HRP)-coupled secondary reagents (Bio-Rad) and developed by enhanced chemiluminescence (Amersham ECL Western Blotting Detection Reagent, GE Healthcare).

The quantification of protein bands was performed by densitometric analysis using Quantity One 1-D analysis software (Bio-Rad) and band intensities (b.i., band volume/area x mean pixel intensity), normalized for β-actin, as previously reported. [131]

3.7 Antimicrobial activity of hydroxytyrosol enriched extract

The HTyr-enriched extract was tested against the olive pathogens *Pseudomonas savastanoi* pv. *savastanoi* and *Agrobacterium tumefaciens* to evaluate its antibacterial potential. Pure hydroxytyrosol was used as reference compound. This part of the work was made in collaboration with the research group of Prof. Leonardo Varvaro, University of Tuscia, Department of Agriculture and Forest Sciences.

3.7.1 Microbial strains

The microorganisms used were *Pseudomonas savastanoi* pv. *savastanoi* strain 326 (from olive) and *Agrobacterium tumefaciens* strain 129 (=NCPPB 2437, from apple). Both strains from the collection of Plant Pathology laboratory of University of Tuscia. Frozen stocks of the strains were recovered and grown for 24 h at 28 °C in Luria-Bertani (LB) broth (Sigma-Aldrich).

3.7.2 Drop diffusion test

Petri dishes (90 mm in diameter), containing 15 mL of LB-agar, were overlaid with melted ‘‘soft’’ agar (5 mL), prepared with LB-broth and 0.7% agar. Fresh bacterial cultures were prepared in LB broth and added at the melted soft agar, cooled to about +40°C, to reach a final bacterial concentration of 1×10^7 Colony Forming Units per mL (CFU/mL) in each petri dish. Plates were dried for 15 minutes and used for the test. HTyr-enriched extract or pure HTyr (0.5, 0.25, 0.1, 0.05,0.01 and 0.0 mg/drop) solutions were dropped (10 µL) on the surface of the inoculated plates and incubated for 24 h at +28°C. Experiments were performed in triplicate.[132, 133]

3.7.3 Broth-dilution test

Overnight broth cultures were prepared and diluted in 5 ml of LB-broth to reach a final concentration of 1×10^8 (CFU/mL) in each test tube. Different concentrations (1, 0.5, 0.3, 0.1 mg/mL) of HTyr-enriched extract or pure HTyr were added to the tubes and were incubated at 28 °C and 250 rpm for 24 h. Control tubes containing sterile water were included.

Experiments were performed in triplicate. After incubation, Minimum Inhibitory Concentration (MIC) was determined as the lowest concentrations that ended with no visible growth. [134, 135]

From each of the experimental culture tube described, at the end of the incubation period, 100 µL-aliquots were collected to prepare serial dilutions (1:10) in sterile water. From each dilution 100 µL were taken, plated on LB-agar and incubated for 24 h at +28 °C. After incubation, the number of Colony Forming Units per mL (CFU/mL) was determined and the Minimum Bactericidal Concentration (MBC) was established as the first concentration in which the microorganisms were completely killed. [134, 135]

3.8 Antioxidant activity of hydroxytyrosol-enriched extract

Antioxidant activity of both HTyr-enriched extract and pure HTyr was evaluated by the ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] radical-scavenging assay, according to a procedure previously described with some modifications.[136] Stock solutions of HTyr-enriched extract or pure HTyr were prepared in phosphate buffer (PBS, pH=7.4) to reach final concentrations of 2 mg/mL and 1 mg/mL, respectively. The ABTS radical was generated by mixing ABTS (7 mM) and potassium persulfate (2.5 mM) in PBS, pH=7.4. The mixture was kept in the dark for 16 h at RT. The ABTS radical solution prepared was diluted with PBS, pH=7.4, to an absorbance of 0.70 ± 0.02 at λ=734 nm at the time of use. Each sample (100 µL) at different concentrations (from 10 to 50 µg/ml for the extract and from 25 to 150 µg/ml for the HTyr) was added to 1 mL of the diluted ABTS radical solution and mixed vigorously. After reaction at RT for 10 minutes, the absorbance at 734 nm was measured with a spectrophotometer (Shimadzu UV-2600). All tests were carried out in triplicate and a blank with PBS alone was included. The decrease of ABTS absorbance in percent (I%) was calculated according to the following Equation:

$$I\% = \frac{(A_0 - A_t)}{A_0} \times 100$$

where A_0 is the absorbance of the blank and A_t is the absorbance of the sample. Dose-response curves were extrapolated plotting I% against the sample concentrations. The antioxidant activities were determined using Trolox as standard and were reported as Trolox Equivalent Antioxidant Capacity (TEAC), defined as the concentration (mg/L) of Trolox

having the antioxidant capacity equivalent to that of a 1.0 mg/L solution of the sample investigated. Results are expressed as averages ± S.D..

3.9 Active food packaging

Novel ternary films for active food packaging were developed incorporating nanostructured starch and HTyr into a poly(vinyl alcohol) (PVA) matrix. This part of the work was carried out in collaboration with the research groups of Prof. Domenico Lafiandra (University of Tuscia, Department of Agriculture and Forest Sciences), Prof. José Maria Kenny (University of Perugia, Civil and Environmental Engineering Department) and Prof. Laura Dugo (University Campus Bio-Medico of Rome, Centre of Integrated Research).

3.9.1 Starch extraction

Starch was obtained from the bread wheat variety Cadenza (WT, amylose content 33%) and from a derived high amylose line (HA, amylose content 75%) as described by Botticella *et al*. (2018).[137] Starch was isolated from whole flour of the WT and HA wheats by the dough ball method.[138] In particular, 50 g of whole flour were mixed with 60% of water and hand kneaded until optimal dough development. Starch was washed from the dough with water (300 mL) and, after centrifugation (1500 rpm) the starch pellet was washed twice with 20 mL of water. The thin yellow/grey layer deposited on the surface of the starch pellet was scraped off by a spatula. Starch pellet was subsequently dried for two days at +25 °C.

3.9.2 Starch nanocrystals and starch nanoparticles preparation and characterization

Starch nanocrystals (NC) were prepared by acid hydrolysis of starch granules. The latter (25 g of both WT or HA), were mixed with 200 mL of sulfuric acid (3.16 M) for 5 days at +40 °C, with a stirring speed of about 100 rpm as previously reported.[139] After hydrolysis, the obtained suspension was diluted with distilled water, centrifuged (4000 rpm for 20 minutes) and filtered (filter mesh 2 μm). The resultant NC suspensions were ultrasonicated by means of a tip sonicator (Vibracell, 750) for 10 minutes, and the final yield was calculated as % of

initial weight of raw material. The obtained NC were characterized by Field Emission Scanning Electron Microscopy (FESEM) using a Field Emission Gun Electron Scanning Microscopy LEO 1525 (Zeiss); the morphology was observed after metallization with Cr.

Furthermore, the change in NC crystallinity structures was evaluated by X-ray powder diffraction (XRD: PANalytical X'Pert Pro, Cu Ka radiation) operating at 40 kV and 40 mA, step size 0.0170 2 h and step scan 20 s.[140]

Starch nanoparticles (NP) were prepared by ultrasonication. A high-power ultrasound generator titanium horn type 750W (VCX 750 Sonic and Materials), 20 kHz, with a tip diameter of 13 mm, was used. Starches (1 g of both WT or HA), were placed in a bottle with 100 mL of pure water. Bottles were hermetically sealed to the horn and then subjected, for 50 minutes at +8 °C, under argon flow (100 mL/min), to high power ultrasound irradiation (50% amplitude). The ultrasonic apparatus used in this research activity was described in literature.[141] After ultrasonication, each colloid suspension was centrifuged at 4000 rpm for 20 minutes. The resulting concentration of the aqueous WT suspension was 0.13 mg/mL, while HA suspension was 0.35 mg/mL. The obtained NP were characterized by Field Emission Scanning Electron Microscopy (FESEM) and by XRD, using the same procedure as described above for NC characterization.

3.9.3 Preparation and characterization of binary and ternary formulations

Preparation of binary and ternary formulations

Poly(vinyl alcohol) (PVA) binary and ternary formulations were prepared (Table 1). Binary films were made using PVA as polymeric matrix and HTyr as antioxidant; ternary films were made using PVA, HTyr and nanostructured starch as reinforcement phase.

Based on previous results, the concentration of HTyr was fixed at 5 wt%.[118] For the preparation of binary formulations two different concentrations, 1 and 3 wt%, of nanostructured starch (NC and NP) were used; these binary formulations were employed to select the better starch nanostructures concentration to apply in ternary formulations. After that, ternary films combining 5 wt% HTyr and 1 wt% NC or NP were produced.

PVA based formulations were prepared by the solvent casting technique using a procedure described in literature.[118] Firstly, 0.5 g of high purity grade PVA (average Mw 124–146 kg mol−1,99% hydrolyzed) was dissolved in 10 mL of distilled water under magnetic stirring for 2 h at +80 °C. The polymeric solution was kept under magnetic stirring to reach RT.

PVA/HTyr was obtained mixing, under magnetic stirring, the polymeric solution with HTyr previously dispersed in distilled water (0.1 g of HTyr in 10 mL of distilled water) for 1 h at RT. Uniform dispersion of HTyr in aqueous solution was obtained applying a magnetic stirring and a sonication bath, both at RT for 1 h.

PVA/NC or PVA/NP were obtained mixing the nanostructures in aqueous solution with polymeric aqueous suspensions, to achieve a uniform dispersion of nanofillers and active ingredient, the mixture was kept under magnetic stirring for 1 h at RT.

Ternary formulations were first obtained mixing the NC or NP with HTyr in aqueous suspension, then the obtained solution was added to PVA. PVA/HTyr/NC or PVA/HTyr/ NP in aqueous solution were mixed under magnetic stirring for 1 h at RT.

The polymeric solutions of neat, binary and ternary films were casted in a *Teflon*® mold and evaporated at RT. Films (0.10 mm thick) obtained were equilibrated for 7 days at 53% RH in desiccators, using a magnesium nitrate-6-hydrate oversaturated solution, as described in literature.[118, 142]

Formulations	***PVA** (wt%)*	***Starch NC/NP** (wt%)*	***HTyr** (wt%)*
PVA	100	-	-
Binary formulations			
PVA/HTyr	95	-	5
$PVA/1NC_{WT}$	99	1	-
$PVA/3NC_{WT}$	97	3	-
$PVA/1NC_{HA}$	99	1	-
$PVA/3NC_{HA}$	97	3	-
$PVA/1NP_{WT}$	99	1	-
$PVA/3NP_{WT}$	97	3	-
$PVA/1NP_{HA}$	99	1	-
$PVA/3NP_{HA}$	97	3	-
Ternary formulations			
PVA/NC_{WT}/HTyr	94	1	5
PVA/NC_{HA}/HTyr	94	1	5
PVA/NP_{WT}/HTyr	94	1	5
PVA/NP_{HA}/HTyr	94	1	5

Table 1: Material formulations for binary and ternary films.[140]

Characterization of binary formulations

To select the appropriate content of starch nanofillers (NC and NP) for ternary formulations, different characterization methods were used.

Thermal properties were investigated by a Differential Scanning Calorimeter (DSC-TA Instrument, Q200) to verify the effect of nanostructured starches on PVA crystallization phenomena and crystallinity degree. Measurements were performed in the temperature range −25 - +240 °C, at a heating rate of 10 °C min^{-1}. Three samples were used to characterize each material. Moreover, tensile tests of neat PVA and PVA binary systems were performed to evaluate the effect of the nanostructured starch-based fillers on the mechanical response of the PVA matrix. The test was performed on rectangular samples (50×10 mm^2) based on UNI ISO 527 with a crosshead speed of 5 $mmmin^{-1}$, a load cell of 500 N and an initial gauge length of 25 mm. The measurements were done at RT and five samples for each formulation were tested.[140]

Characterization of ternary formulations

The morphological and optical, thermal, mechanical, and water absorption properties of ternary films were investigated.

The microstructure of PVA/NC_{WT}/HTyr, PVA/NC_{HA}/HTyr, PVA/NP_{WT}/HTyr, PVA/NP_{HA}/HTyr fractured surfaces was investigated by scanning electron microscope (FESEM, Supra 25-Zeiss). Surfaces were gold sputtered to provide electric conductivity and samples were observed using an accelerating voltage of 2.5 kV. The optical properties of neat PVA and ternary films were investigated by UV–vis spectroscopy using a Perkin Elmer Lambda 35 in the range 250–900 nm.

Thermal characterization of each ternary formulation was performed by both DSC and Thermogravimetric Analysis (TGA) analysis.

DSC measurements as well as mechanical properties of ternary formulations were carried out according to the procedure reported above for the binary systems.

TGA (Seiko Exstar 6300) experiments were performed for each sample from +30 to +600 °C at 10 °C min^{-1} under a nitrogen atmosphere (250 mL min^{-1}) to evaluate the effect of both starch fillers and HTyr addition on the thermal stability of PVA matrix. The first degradation temperature (Td_I), the second/maximum degradation rate temperature (Td_{IImax}) and its onset ($T_{onsetmainpeak}$), the third (Td_{III}) and the residual mass at +600 °C were obtained from the weight loss and its derivative curves.

Water Absorption Capacity (WAC) of neat PVA and ternary films was measured on rectangular plastic probes of 10mm x 20 mm. Specimens were first dried in a vacuum oven at +40 °C for 72 h, then cooled down in a desiccator and immediately weighted. Conditioned systems were fully immersed in a container filled with distilled water. In order to simulate the

practical condition of a packaging material, tests were conducted in triplicate at +4, +20 and +37 °C. Samples were removed from the water bath at different times (1, 3, 6, and 24 h) and the surface were blotted using filter paper, and then weighed.[140]

3.9.4 Overall and specific migration analysis in food simulant

To simulate the use of package formulations with food, the overall and specific migration tests of ternary films were performed. Tests were run in triplicate in simulant A [10% (v/v) ethanol water solution] according to current legislation.[143] For overall migration test rectangular strips of 10 cm^2 in 10 mL of food simulant were used.[143] Samples were kept in a controlled chamber at +40 °C, removed after 10 days according to EN-1186 standard, and the simulant was evaporated. Residues were weighed and migration values in mg/kg were determined. Specific migration tests were carried out with double-sided (12 cm^2) films in total immersion (20 mL of simulant A), area-to-volume ratio around 6 dm^2/L, in triplicate at +40 °C in an oven. Samples were taken at 1, 3, 7, 10, 21 days; a blank test for the simulant was also included. After the migration tests, each solution was recovered and stored at −4 °C before analysis.

3.9.5 HTyr release studies in food simulant

HTyr released by binary film PVA/HTyr and each ternary formulation in food simulant for every contact time (1, 3, 7, 10 and 21 days) was analyzed by UV-2600 spectrophotometer (Shimadzu). Absorbance values were measured at λ=280 nm and the concentration of the antioxidant released into the simulant was determined by a calibration curve for HTyr (range: 50–400 μM) as described in literature.[117, 118]

3.9.6 Antioxidant activity of binary and ternary formulations

Antioxidant activity of HTyr released from binary and ternary formulations was evaluated as reported above (section 3.8, Antioxidant activity of hydroxytyrosol-enriched extract). After the preparation of the radical ABTS solution, a volume of 10 μL of each sample was added to a 96-multiwell plate. The reaction was initiated by the addition of 190 μL of the diluted ABTS

radical solution. Absorbance readings were taken at λ=734 nm after 90 s in a microplate reader (Infinite®, 200 Pro multimode reader, TECAN). The activity of each sample was determined using Trolox as a standard, a calibration curve (range: 50–700 μM, r^2=0.997) was made, and the antioxidant activity was expressed as Trolox Equivalent Antioxidant Capacity (TEAC) as reported in literature.[117, 118] All determinations were carried out in triplicate.

3.10 Statistical analysis

All experiments were replicated three times. For anti-inflammatory experiments, data were analysed using Student's *t* test. Data from antimicrobial activities and antioxidant tests were analysed using analysis of variance (ANOVA) and Tukey's (HSD) multiple range test.
Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 20.0. A level of P≤0.05 was chosen as the criteria for statistical significance.

4. Results and discussions

The results that will be shown were obtained thanks to the collaboration with the research groups of Prof. Annalisa Romani, Prof. Francesca Romana Velotti, Prof. Leonardo Varvaro, Prof. Domenico Lafiandra, Prof. Josè Maria Kenny and Prof. Laura Dugo.

4.1 Kiwifruit peel extract

Inflammation is a physiological response of our body to infections or tissue injury. However, this physiological process can be detrimental when the acute inflammatory response switches to chronic inflammation, leading to inflammatory diseases and cancers.[31] Growing interest concerns the use of natural extracts as anti-inflammatory agents and in this context the anti-inflammatory effect of kiwifruit peel extract, obtained from waste materials, was studied. The extract was characterized by HPLC-DAD-ESI-MS analysis and its effect on key targets of inflammation was studied on THP-1 cell line.

4.1.1 Preparation and characterization

Kiwifruit peel extract rich in polyphenols, derived from low weight kiwifruit peels of *Actinidia deliciosa* (A.Chev.) C.F.Liang & A.R.Ferguson, cv Hayward, was obtained by solvent extraction with ethanol/acidified water (pH 3.2) = 70/30 v/v according to a procedure already optimized.[95] The extraction process is an important step to isolate and identify polyphenols. It should allow the complete extraction of the compounds of interest, avoiding degradations and modifications. The polarity and the type of extraction solvent could affect the polyphenol extraction efficiency. For this reason, ethanol/water = 70/30 v/v was chosen as extraction solvent and the pH was decreased.[95, 144, 145]

The quali-quantitative analysis of the extracted polyphenols was performed by HPLC-DAD-ESI-MS; components were identified based on their spectroscopic and spectrometric data including retention time (tR), molecular weight (MW), negative ions (m/z), and quantified with specific reference standards. As shown in Table 2, procyanidins in the form of dimers, trimers and tetramers resulted the main class of polyphenols present in the extract. In fact, with 5.422 ± 0.209 mg per gram of peel, they represent the 92% w/w of the total polyphenols. Procyanidins, commonly found in fruits, vegetables, nuts, legumes, and grains, are a class of

flavonoids built from flavan-3-ols (+)-catechin and (−)-epicatechin, monomers can form linkages leading to oligomers, further forming polymers. Oligomeric procyanidins have received increasing interest due to their potential health benefits.[146]

The remaining 8% w/w of the total polyphenols is constituted by hydroxycinnamic acid derivatives (Table 2).

Compound	***mg/g*** [a]
6-Hydroxy-7- (β-D-glucopiranolyloxy coumarin)	Traces
Caffeic acid derivative	0.243 ± 0.007
Ferulic acid glucoside	0.174 ± 0.006
Ferulic acid dehydrodimer	0.042 ± 0.002
Dimethyl caffeic acid hexoside	Traces
Procyanidin trimer	1.193 ± 0.042
Procyanidin trimer	0.659 ± 0.025
Procyanidin trimer	0.665 ± 0.019
Procyanidin tetramer	0.142 ± 0.003
Procyanidin trimer	0.880 ± 0.035
Procyanidin trimer	1.187 ± 0.047
Procyanidin dimer	0.696 ± 0.038
Total hydroxycinnamic derivatives	0.459 ± 0.015
Total procyanidins	5.422 ± 0.209
Total polyphenols	5.881 ± 0.224

Table 2: Analysis of phenolic compounds found in the kiwifruit peel extract carried out by HPLC-DAD-ESI-MS analysis.[130]

[a] mg of phenolic compound per gram of peel.

4.1.2 Effects on THP-1 cell viability and proliferation

THP-1 is a human acute monocytic leukaemia (AML) cell line, but also resemble primary monocytes and macrophages in morphology and differentiation properties. The trypan-blue dye exclusion assay was used to test the kiwifruit peel extract cytotoxicity on THP-1 cells. The latter were cultured in presence of increasing amounts of extract (from 12.5 to 200 μg/mL) for 24 h. Results showed that the extract did not affect the viability of THP-1 cells at none of the concentrations tested (Figure 11a). Moreover, the evaluation of cell growth showed that cell number was not affected up to an extract concentration of 100 μg/mL, and

then decreased in a dose-dependent manner (Figure 11b), suggesting that a proliferative arrest might occur at high concentrations. Thus, all subsequent experiments were performed treating THP-1 cells with a dose of 50 µg/mL for 24 h.

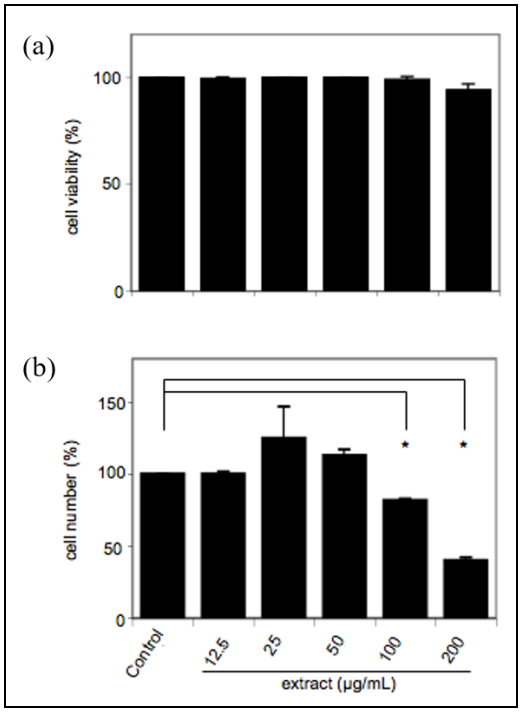

Figure 11: Effects of kiwifruit peel extract on THP-1 cell viability (a) and proliferation (b) at 24 h of treatment. Data show averages of the percentage (%) plus S.D. of three independent experiments; *$p < 0.005$.[130]

4.1.3 Effects on LPS-inducible and constitutive IL-6, IL-1β and TNF-α production

Cytokines are important mediator proteins, essential in networking communication for a proper immune response, they represent key modulators of inflammation participating in acute and chronic inflammation in a complex network of interactions. Pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β), are involved in the up-regulation of inflammatory reactions. The inhibition of these inflammatory mediators is important in the prevention and treatment of inflammatory diseases. Therefore, the capability of the extract to influence the production of these cytokines, in activated monocytes, was investigated. The lipopolysaccharide (LPS) is a component of Gram-negative bacteria cell walls and represents their major virulence factor.

Since LPS can induce oxidative stress in macrophages,[147] LPS-stimulated and -unstimulated (Control) THP-1 cells were treated with the extract. Subsequently expression levels of IL-6, IL-1β and TNF-α proteins were measured by Western blot analysis and normalized on β-actin or Ponceau stain. Since it has been previously reported that the maximum of TNF-α release occurs at earlier times than IL-6 and IL-1β, the release of TNF-α was analysed at 4 h post treatment, whereas IL-6 and IL-1β releases were measured at 24 h post treatment.[148] Basal levels of both intracellular and extracellular cytokines were detected in stimulated and unstimulated THP-1 cells. As expected, cytokines increased when THP-1 cells were stimulated with LPS (100 ng/mL), as shown in Figure 12. However, when the cell cultures were treated with the extract, a nearly complete reduction of the intracellular content (Figure 12a), associated to a significant decrease of the release (Figure 12b) of all the three cytokines, was observed in both LPS-stimulated and -unstimulated THP-1 cells. Moreover, the inhibition of monocyte-mediated release of cytokines by the extract was confirmed by Luminex multiplex assay (Figure 12c).

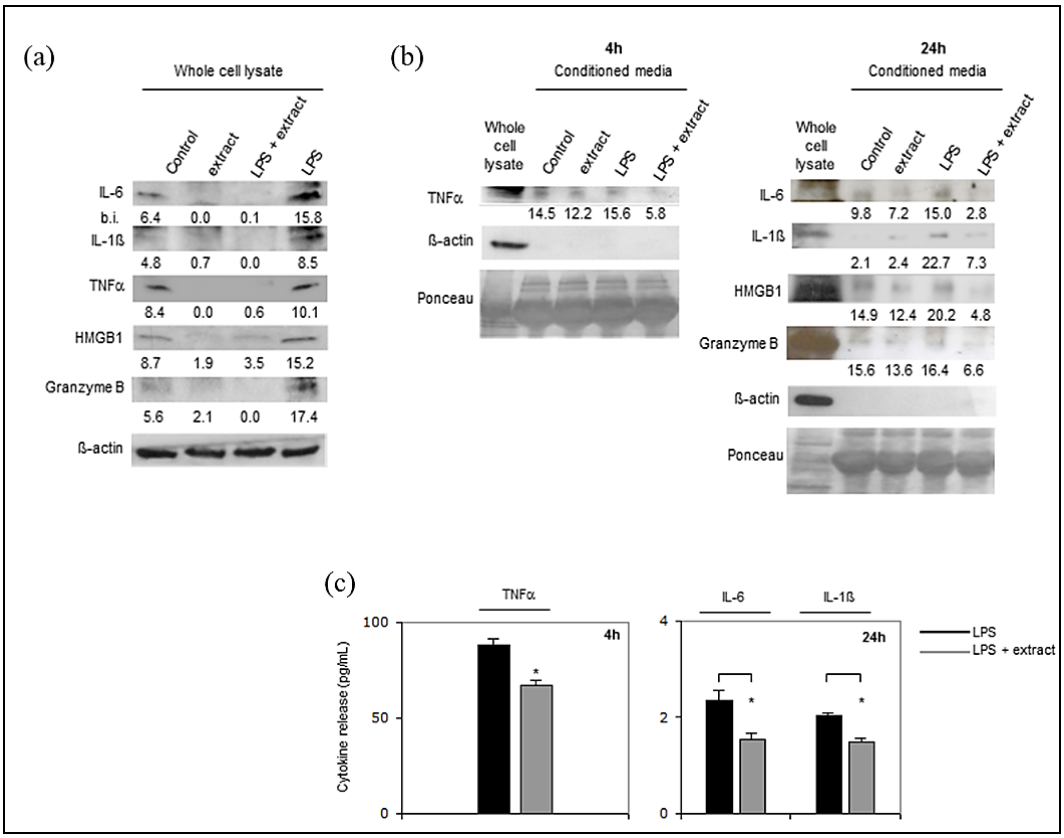

Figure 12: Effects of kiwifruit peel extract on the intracellular content (a) and extracellular release (b, c) of inflammatory mediators on THP-1 cells. Data show Western blot on cell lysates (a) and conditioned media (b); β-Actin and Ponceau staining served as loading controls; band intensities (b.i.) were quantified using Quantity One 1-D analysis software. Histograms represent the averages plus S.D. of cytokine concentrations evaluated in conditioned media by Luminex multiplex assay, (c); experiments were carried out in triplicate, *p< 0.005.[130]

4.1.4 Effects on LPS-inducible and constitutive HMGB1 production

High-Mobility Group Box 1 (HMGB1) is a non-histonic nuclear protein regulating gene expression, but it acts also as a Damage-Associated Molecular Pattern (DAMP), triggering inflammatory responses when released into extracellular milieu.[37] HMGB1 is passively released or actively secreted in several human inflammatory diseases (trauma, ischemia, chronic inflammatory disorders, autoimmune diseases and cancer).[149] Extracellular HMGB1 represents a key anti-inflammatory target which triggers and sustains the inflammatory response by inducing cytokine release and recruiting leucocytes.[150, 151]

Thus, the capability of the extract to modulate HMGB1 production in activated monocytes was investigated. Results of Western blot analysis using an anti HMGB1 antibody showed that both the intracellular content (Figure 12a) and the extracellular release (Figure 12b, right panel) of HMGB1 were significantly inhibited by the extract in LPS-stimulated and -unstimulated THP-1 cells.

4.1.5 Effects on LPS-inducible and constitutive granzyme B production

The serine protease granzyme B is involved in inflammation, cytokine activation and autoimmunity; furthermore high levels of soluble granzyme B have been reported in plasma of patients with inflammatory diseases.[38, 152, 153] It has been demonstrated that monocytes/macrophages express granzyme B in the lesion areas of inflammatory diseases, including atherosclerosis and rheumatoid arthritis.[38] Given that targeting granzyme B could provide new pharmaceutical agents for inflammatory disorders,[154] it was analysed whether the extract could affects the production of granzyme B in THP-1 cells. The extract significantly inhibited both intracellular expression (Figure 12a) and extracellular release (Figure 12b, right panel) of granzyme B in LPS-stimulated and -unstimulated THP-1 cells as reveled by Western blot analysis using an anti-granzyme B antibody.

4.1.6 Effect on STAT3 activation

Signal Transducer and Activator of Transcription 3 (STAT3) is a member of the STAT protein family. STATs are cytoplasmic transcription factors that convey signals from the cell surface to the nucleus through activation by cytokines and growth factors. Many cytokines and growth factors, involved in the pathogenesis of autoimmune, inflammatory and cancer diseases, use STATs to transduce intracellular signals. In particular, STAT3 regulates nuclear genes controlling cell proliferation, survival, and inflammation and plays a key role in inducing and maintaining a pro-carcinogenic inflammatory microenvironment. Aberrant STAT signalling has been demonstrated to contribute to cancer progression and metastatic development. Its activation by pathogens, growth factors or cytokines, such as IL-6, induces the production of pro-inflammatory cytokines and regulates several inflammatory processes.[39, 155, 156] Agents blocking constitutive or inducible activation of STAT3 have a potential for the treatment of inflammation and inflammatory-associated cancers.[39]

Thus, the capability of the extract to modulate STAT3 activation was examined analysing its phosphorylation at Tyrosine705. Tyr-705-phospho-STAT3 is taken as an active form of STAT3, the phosphorylation of the Tyr705 residue is required for STAT3 dimerization, nuclear translocation, and DNA binding.[157, 158] Phospho-STAT3 (Tyr705) was measured by Western blot analysis using an anti-phospho-Stat3 (Tyr705)-specific antibody.

As expected, STAT3 phosphorylation (p-STAT3) levels significantly increased when THP-1 cells were exposed to LPS (Figure 13). However, treatment with the extract inhibited p-STAT3 in both LPS-stimulated and -unstimulated THP-1 cells (Figure 13). Moreover, considering that STAT3 signalling is triggered by IL-6 upon interaction with IL-6 receptor, it can be hypothesised a correlation between the inhibition of IL-6 production (Figure 12) and the inhibition of STAT3 activation (Figure 13). Inhibition of IL-6 production and STAT3 activation by the extract are both relevant findings, since numerous studies demonstrated the association of down-regulation of IL-6 and/or IL-6 signalling with therapeutic results in inflammatory diseases.[159, 160] In addition, an important role of STAT3 has been identified in the development of different cancers, including AML.[161] Moreover the inhibition of the constitutive level of STAT3 in unstimulated THP-1 monocytic leukemia cells suggests that the extract may also exerts certain anti-cancer activity in AML.

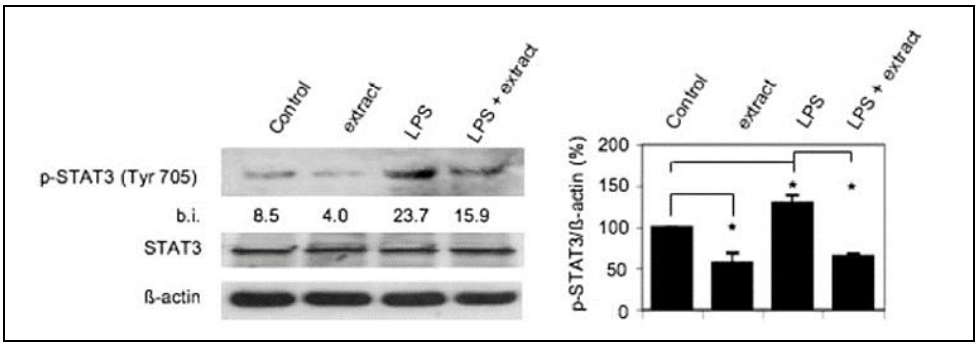

Figure 13: Effect of kiwifruit peel extract on STAT3 activation in THP-1 cells. Data show STAT3 Tyr705 phosphorylation (p-STAT3), evaluated by Western blot (left panel); total STAT3 and β-actin served as controls; numbers indicate band intensities (b.i.), quantified using Quantity One 1-D analysis software; experiments were carried out in triplicate. Histograms represent the percentage (%), respect to the control, of the mean plus S.D. of the densitometric analysis of p-STAT3/β-actin ratio of three different experiments (right panel); *$p < 0.005$.[130]

4.1.7 Effect on autophagy

Autophagy is a cellular waste disposal process that normally maintains cellular homeostasis. Autophagy mediates the clearance of cytoplasmic molecules, organelles and pathogens, and, orchestrates inflammation, immunity and cancer. [40] In particular, activation of autophagy in monocytes exerts anti-inflammatory activity, affecting inflammatory cytokine production through different mechanisms. Autophagy can inhibit inflammasomes in monocytes/macrophages, thus suppressing the maturation of interleukin 1β (IL-1β).[162] Defects in autophagy have been linked to a wide range of diseases (cardiomyopathy, neurodegenerative disorders, cancers).[40] An altered or disturbed autophagy is believed to play an important role in inflammatory diseases and cancer.[163]

Therefore, whether the kiwifruit peel extract affects autophagy in THP-1 cells was examined. To investigate the autophagic flux, two main autophagic markers, the microtubule-associated protein 1 light chain 3 (LC3) and the sequestosome-1 (SQSTM1)/ubiquitin-binding protein (p62), were evaluated by Western blot analysis.[164] During autophagy, LC3 is post-translationally processed into soluble LC3-I and is, in turn, converted to membrane-bound LC3-II, that correlates with the extent of autophagosomes. So, LC3, in the LC3-II form, was examined. As shown in Figure 14, LC3-II formation significantly increased in THP-1 cells after treatment with the extract, suggesting an induction of autophagy. Moreover, bafilomycin A1 (Baf), a vacuolar H+-ATPase inhibitor, was added to the culture blocking LC3-II degradation. This allows the evaluation of LC3 formation and consequently the completeness of the autophagic flux.[164] As shown in Figure 14, a further accumulation of LC3-II was observed when the extract treated cells were cultured in presence of Baf, confirming the induction of a complete autophagic flux by the extract. Moreover, as a control, the autophagic inhibitor 3-MA was added to extract-treated THP-1 cells.[165] As shown in Figure 14, LC3-II formation decreased in presence of 3-MA, confirming that LC3-II protein levels were the result of the autophagic process. Subsequently SQSTM1/p62 was analysed. This autophagic marker, being part of the assembled autophagosome, which is then degraded in autolysosomes, serves as an index of autophagic degradation. As shown in Figure 14, SQSTM1/p62 levels, evaluated by Western blot analysis using an anti-p62 antibody, decreased in THP-1 cells treated with the extract, indicating that the extract was able to promote a complete autophagic process in THP-1 cells. This is indeed a relevant finding, since compounds capable of modulating autophagy have recently received attention for applications on several inflammatory diseases.[40]

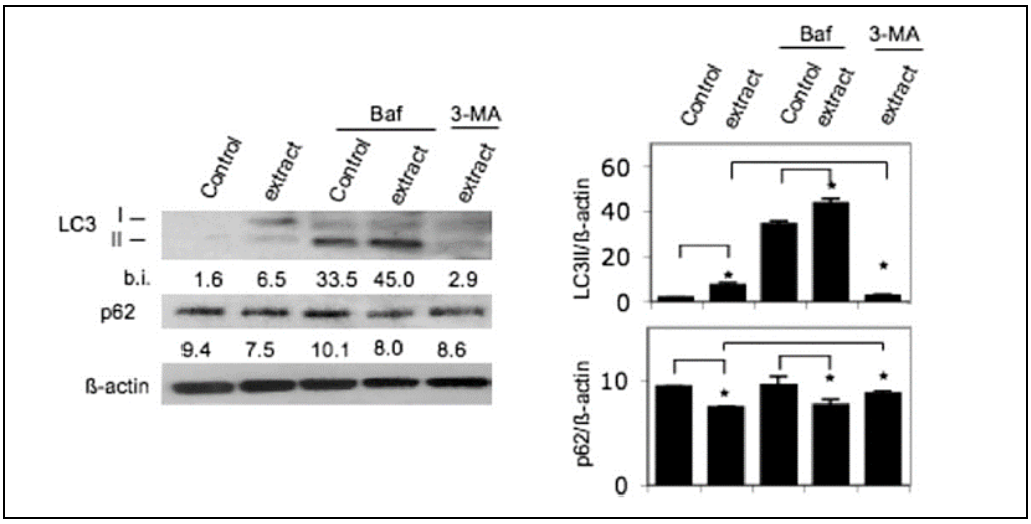

Figure 14: Effect of kiwifruit peel extract on autophagy in THP-1 cells. Data show the expression of LC3I/II and SQSTM1/p62, analysed by Western blot (left panel); β-actin was included as loading control; numbers indicate band intensities (b.i.) normalized for β-actin and quantified using Quantity One 1-D analysis software; experiments were carried out in triplicate. Histograms represent the mean plus S.D. of the densitometric analysis of LC3II/β-actin and p62/β-actin ratio of three independent experiments (right panel); *$p < 0.005$.[130]

4.2 Hydroxytyrosol enriched extract

Considering the biological properties of polyphenols, many efforts have been made to obtain these molecules from natural sources. In particular, the recovery of HTyr from the Olive Mill Waste Waters (OMWW) has attracted much interest for both the demonstrated biological activities of HTyr and the environmental benefits that could be achieved by a more ecological disposal of OMWW. Therefore, the HTyr enriched extract from OMWW, product of an eco-friendly extraction procedure, was investigated for its chemical composition, antioxidant and *in vitro* antimicrobial activities and correlated with those of the HTyr synthetized.

4.2.1 Hydroxytyrosol synthesis and characterization

HTyr is principally found in olive fruit and in olive by-products and is considered one of the most powerful antioxidants among olive phenolic compounds. It has a wide spectrum of beneficial effects on human health and has therefore generated much research interest.[166] In the last years, several procedures have been proposed to synthetize pure HTyr.[100, 166]

In this work, HTyr was synthesized from tyrosol, a natural and low-cost compound, using cheap and green reagents.[108, 167]

According to this method it was possible to obtain the HTyr (98% purity) with satisfactory yield (85%) and short time, at much lower costs compared to the commercially available products.[168] Chemical structure and purity of synthetic HTyr were confirmed by NMR analysis (Figure 15).

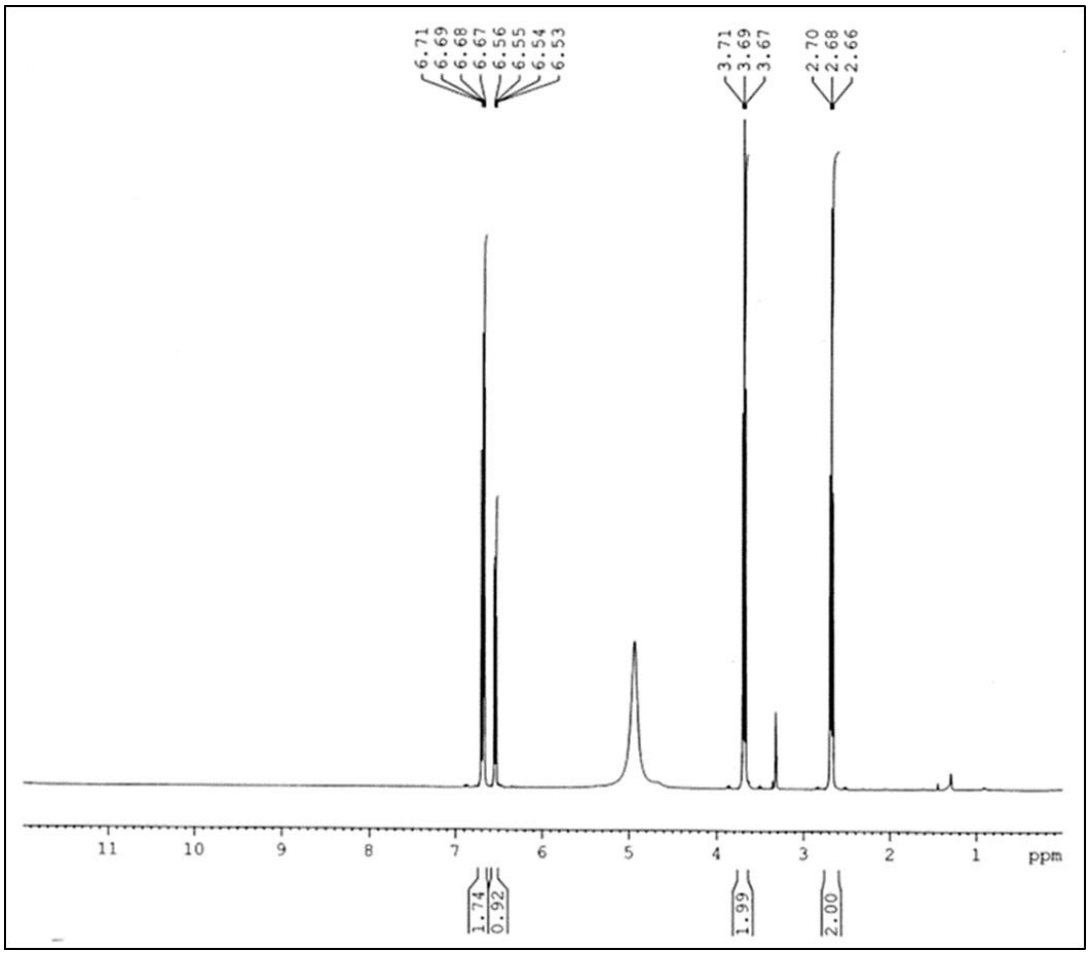

Figure 15: Hydroxytyrosol ^{1}H NMR (CD_3OD, δ ppm). 6.71-6.67 (m, $2H_{aromatic}$), 6.56-6.53 (m, $1H_{aromatic}$), 3.69 (t, 2H, OCH_2, J=8Hz), 2.68 (t, 2H, CH_2CH_2, J=8Hz).[108]

Synthetic HTyr served both as reference compound, for antioxidant and antimicrobial experiments, and as antioxidant additive for active food packaging materials.

4.2.2 Hydroxytyrosol-enriched extract preparation and characterization

Membrane-based technologies are physical separation processes allowing the separation of different high-added-value compounds from aqueous systems.[169] These are promising technologies for the treatment of agro-food by-products aimed at the recovery of valuable compounds. For example, fractions enriched in HTyr can be obtained from OMWW by an innovative process based on membrane technologies.[128] Thus, the HTyr-enriched extract from OMWW was obtained according to a patented procedure.[77, 115, 128] The process started

from an aqueous extraction, obtained using a pneumatic pressure extractor; then the extract was purified by successive filtration steps; finally the output was reduced in powder by a spray drying process. The latter procedure allowed to obtain a powder standardized for HTyr content.[128] The extract was characterized by HPLC/DAD/ESI-MS (Figure 16 and Table 3) and ^{1}H-NMR analysis (Figure 17).

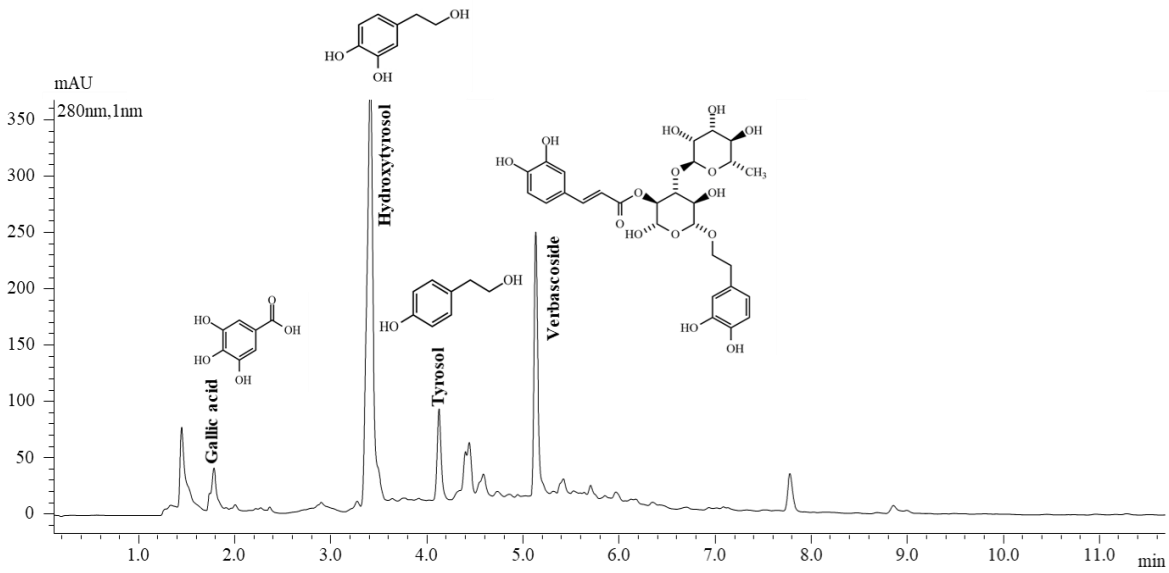

Figure 16: Chromatographic profile at 280 nm of HTyr-enriched extract.

The quali-quantitative analysis showed a total polyphenol content of 50.7 mg/g and the extract resulted enriched in HTyr (32.83 mg/g), which represents 64.7% w/w of total polyphenols. Besides HTyr, verbascoside (12.88 mg/g), being the 25.4%, w/w represents another important component, along with minor components represented by tyrosol, gallic acid and verbascoside derivatives, which in total constitute the 9.6% w/w of total polyphenols, as shown in Table 3.

Compound		***mg/g*** [a]
Hydroxytyrosol		32.83 ± 0.001
Gallic acid		0.89 ± 0.006
Tyrosol		3.58 ± 0.003
Verbascoside der. [M-H-caffeoyl]		0.52 ± 0.002
Verbascoside		12.88 ± 0.006
	Total polyphenols	50.7 ± 0.018

Table 3: Quantitative analysis of phenolic compounds found in the HTyr-enriched extract. [a] mg of phenolic compound per gram of powder.

Subsequently, the ^{1}H NMR spectrum of the extract was compared with HTyr standard spectrum (Figure 15). The extract spectrum evidenced a very complex behaviour, although it was possible to identify the typical signals of the HTyr moiety, in particular in the aromatic region (Figure 17). The presence of HTyr is clearly revealed, in fact it was possible to identify the signals of the three aromatic protons of HTyr (δ, ppm: dd, 6.53-6.56, *J*=8.0 and 4.0 Hz, 1H; d, 6.67, *J*=4.0 Hz, 1H; d, 6.70, *J*=8.0 Hz, 1H) confirming its presence as the main compound.[108]

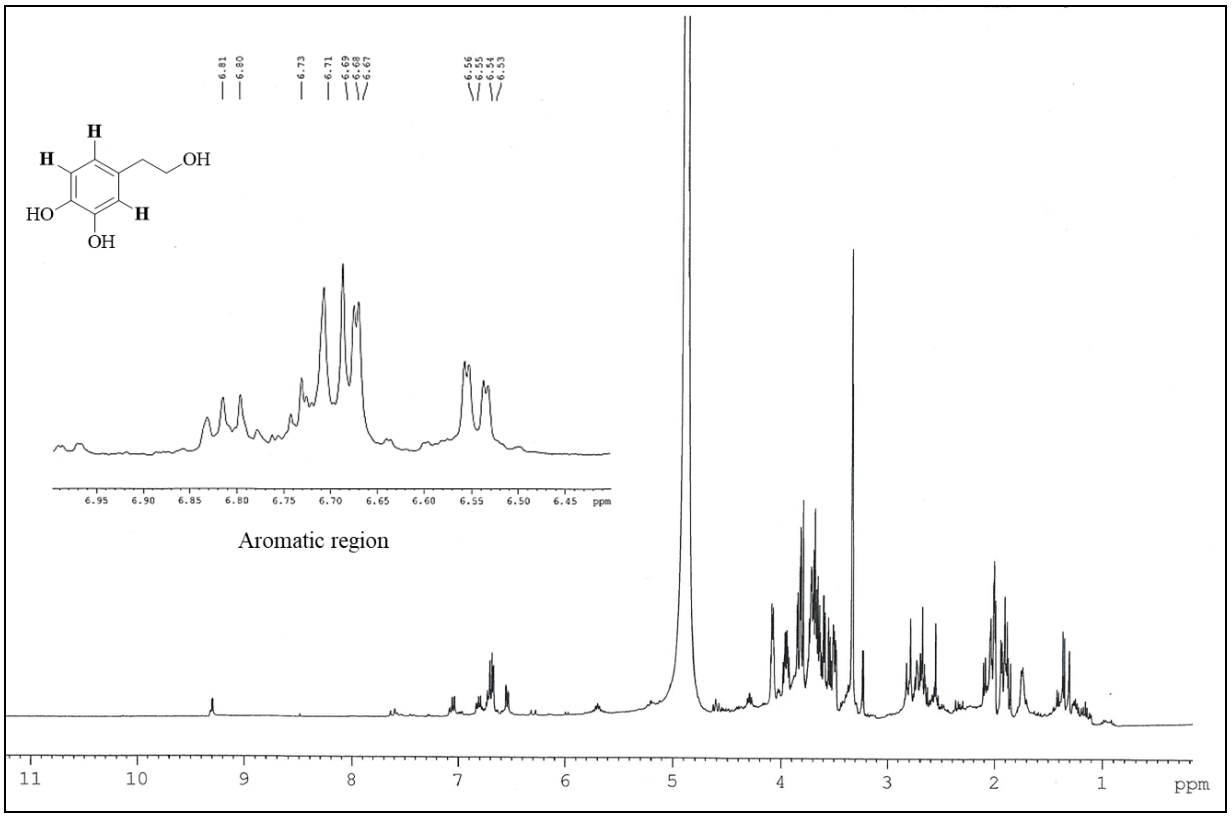

Figure 17: ^{1}H NMR spectra of HTyr-enriched extract (CD_3OD).

4.2.3 Antioxidant activity

The antioxidant activity of plant extracts containing polyphenols is well known.[170, 171] In particular, OMWW contains large quantities of precious polyphenols to be recovered: among these, HTyr is one of the major ones.[172] Therefore, the antioxidant activity of HTyr-enriched extract against ABTS radical was determined. HTyr standard was also analysed. As a result, HTyr-enriched extract (1.88 ± 0.012 TEAC mg/L) exhibited a stronger antioxidant effect than the standard (0.71 ± 0.002 TEAC mg/L). This suggests that possible interactions between HTyr and other antioxidant components of the extract were also influent in determining overall antioxidant activity.

4.2.4 Antimicrobial activity

Plant extracts rich in polyphenols have generated much interest also for their proven capability to exert a significant antimicrobial activity against many different bacteria. This property has a great potential to be exploited, for instance, in pharmaceutical, food and agricultural fields. In this context, OMWW could be used as a low-cost source of natural antimicrobials. In particular, antibacterial properties could be exploited to develop natural products for the management of plant microbial disease in agriculture. Plants are constantly exposed to a large number of pathogenic microorganisms in their environment and bacterial diseases lead to considerable losses in productivity.[173, 174] Therefore, the HTyr-enriched fraction from OMWW was investigated for its *in vitro* antimicrobial activities against two plant pathogens: *Pseudomonas savastanoi* pv. *savastanoi* (Pss) and *Agrobacterium tumefaciens* (At). Pss is the causal agent of one of the most important olive diseases: olive knot disease. The pathogen is usually introduced in new areas through infected vegetal material. It penetrates plant through wounds, like leaf scars or those caused by pruning, harvesting, frost or hail. The presence of knots, even only in a single tree, normally leads to the rapid infection of the entire orchard.[175] At causes crown gall disease on various plant species, including *Olea europaea*. The crown gall is considered as an economically relevant bacterial disease that causes significant economic losses in nurseries and orchards. Infections are facilitated by wounds. The crown gall disease affects mature plants by reducing the size of the roots and/or interrupting the vascular flow in the stems. Also, infected plants become more susceptible to other pathogens.[176]

Thus, the inhibitory effects of HTyr-enriched extract and pure HTyr against Pss and At were investigated. The pathogens susceptibility against pure HTyr was firstly determined. Given that pure HTyr was found to be active in a concentration range between 1 and 0.1 mg/ml the HTyr-enriched extract was tested at 30.4, 15.2, 9.1 and 3 mg/ml, which correspond to 1, 0.5, 0.3, 0.1 mg/ml referred to the HTyr content in the extract, and compared to pure HTyr activity, at the same concentrations. Furthermore, the minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the HTyr-enriched extract were determined (Figure 18). Results showed that the extract was able to inhibit completely Pss at 15.2 mg/ml (that correspond to 0.5 mg/ml of HTyr content). Differently, At resulted completely inhibited at a dose of 30.4 mg/ml (1 mg/ml of HTyr content), as shown in Figure 18. Results (Figure 18) suggesting a broad antimicrobial activity of HTyr-enriched extract. MIC and MBC values for HTyr-enriched extract coincided, corresponding to 15.2 and 30.4

mg/ml for Pss and At respectively. Moreover, at the tested conditions the HTyr-enriched extract has shown a stronger antibacterial activity than pure HTyr for both pathogens. This suggests a possible synergistic effect among the constituents of the extract that reinforce the response. The presence of other components with antimicrobial activity, like verbscoside and gallic acid,[177, 178] albeit in lower concentration, can contribute to the antimicrobial effect of the HTyr-enriched extract.[20]

Results obtained indicate that HTyr-enriched extract could be a good antimicrobial agent. However, further analysis will be needed to clarify the mechanisms underlying this activity.

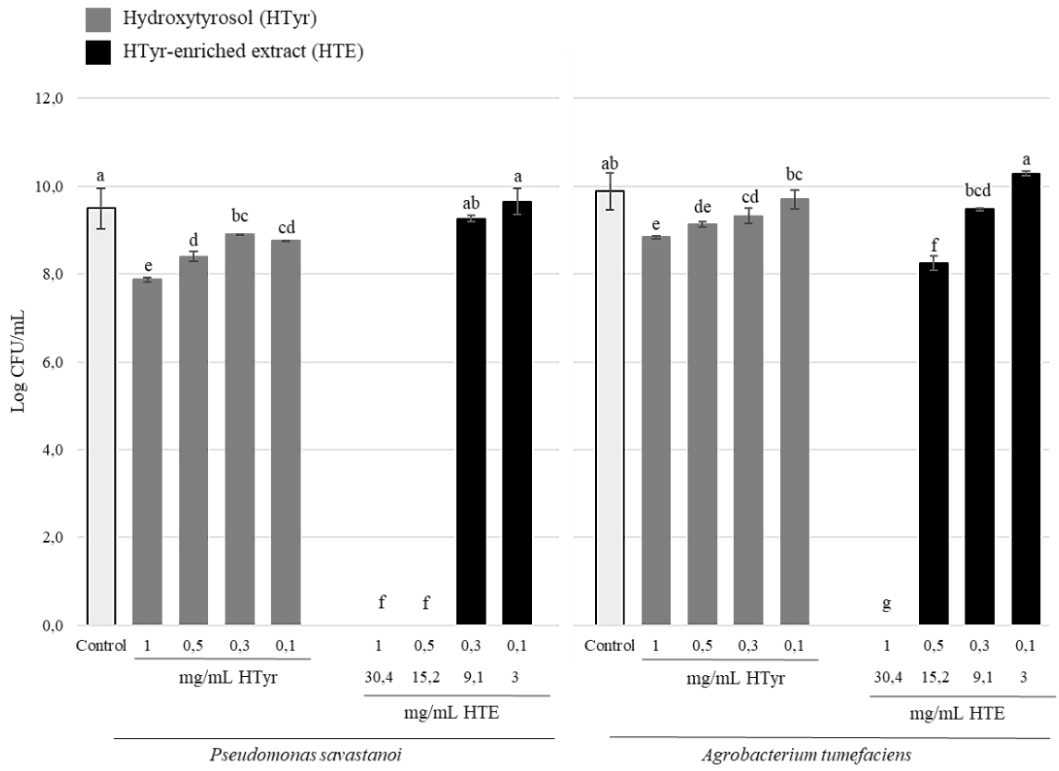

Figure 18: Antimicrobial activity of HTyr and HTE against Pss and At at different doses of HTyr and HTyr-enriched extract. Average values and S.D. among three independent experiments are shown. Labels indicate significant differences ($p<0.05$).

4.3 Food packaging and active food packaging

Packaging solutions aim to protect packaged food from physical damages and chemical and biological contaminations until the time of consumption. The increased use of traditional plastics, such as polyethylene (PE), polypropylene (PP) and polystyrene (PS), has led to serious ecological problems due to their non-biodegradability.[118] Thus, one of the main objectives in the development of new functional materials is to obtain ecological and biodegradable solutions able to extend product shelf life. Some packaging materials obtained from renewable resources have been proposed as alternatives to conventional polymers. Among these poly(vinyl alcohol) (PVA), a polymer produced by the hydrolysis of poly(vinyl acetate), possess properties that are suitable for packaging applications like good thermomechanical properties, thermal stability, strength and flexibility. Moreover, PVA is totally biodegradable, biocompatible, water soluble, non-toxic, cheap and is approved by the Food and Drug Administration in products which are in contact with food.[117]

In polymer industry synthetic antioxidants are added to polymers to improve flexibility and stretchability and to prevent the oxidative degradation of polymeric films.[179] However the potential toxicity derived from their migration into the food make their application controversial. An alternative approach consisting in the use of natural antioxidants, particularly plant extracts or antioxidant agents from food industrial waste, is of high interest. Natural antioxidants could act as processing stabilizers for polymers. Moreover, their release during product storage may reduce food oxidation processes and can even increase food nutritional value.[179] The use of packaging materials that incorporate natural antioxidants may represent an attractive option for active food packaging solutions. The latter is a strategy based on the use of packaging material to enhance the primary role of traditional food packaging, namely conserving and protecting food. It aims to extend the shelf life of packaged products guaranteeing quality and safety, the addition of natural antioxidant allows their continued release during food storage, extending the product shelf-life.[180] Among natural antioxidants, HTyr, which is categorized as Generally Recognised as Safe (GRAS) by the US Food and Drug Administration, represent a valid alternative. The use of HTyr in packaging formulations can bring advantages both in improving the characteristics of the polymer and in extending the shelf life of the food.[118]

PVA/HTyr films could be very promising active food packaging systems as sustainable alternatives to petroleum-based materials.[118] However, as reported by Fortunati *et al*.,[118] the functional release of the antioxidant was not carried out gradually representing a considerable

limitation for active food packaging applications. For improving drug delivery and films performances in industrial applications nanostructured materials have been used.[120] The latter, may permit a slower and thus more controlled release of an active ingredient from a film matrix.[123] Among the polymers suitable for the production of nanomaterials, starch, a natural, renewable and biodegradable polymer, represent a valid alternative.

In this context, novel ternary films were developed. Films were obtained incorporating the antioxidant HTyr and nanostructured starch into a poly(vinyl alcohol) (PVA) matrix. Taking into account the final application, films were characterized for their morphological, optical and thermal properties, water absorption capacity, overall and specific migration into a food simulant and, finally, antioxidant properties.

4.3.1 Nanostructured starch synthesis and characterization

Starch represent an attractive raw material for packaging industry because of its low cost, renewability, and biodegradability. Moreover, it has good mechanical and barrier properties which can allow wide applications in the packaging sector.[181] In particular, nano-sized starch, used as a filler in composites, can improve not only the mechanical properties but also the biodegradability of the matrix.[181] Indeed, several reports show that films made of starches with different amylose/amylopectin ratios possess different rheological properties, these differences have been attributed mainly to the amylose content of the starch.[182]

Films based on high-amylose starch exhibited enhanced mechanical performances than films containing normal levels of amylose, the high amylose content contributes to improve the tensile strength and gas barrier properties of starch-based films.[183] Thus, nano-sized starch from both wild-type (WT) and high-amylose (HA) wheat were produced.

Starch nanocrystals (NC_{WT}, NC_{HA}) and starch nanoparticles (NP_{WT}, NP_{HA}) were obtained, respectively, by acid hydrolysis and ultrasound irradiation of native starch extracted from WT and HA wheats. With the aim of understanding the effects of the different treatments (acid hydrolysis and ultrasound irradiation) on the morphologies of WT and HA starch, FESEM analysis was performed. The results, shown in Figure 19, revealed that after both acid hydrolysis (Figure 19, c, d) and ultrasonic treatment (Figure 19, e, f) the starch granules were fragmented to reach nanometric size (20-50 nm).

Furthermore, X-ray diffraction (XRD) was used to study the change in starch crystallinity caused by acid hydrolysis and ultrasonic treatment of WT and HA native starch. As expected,

the WT native starch exhibited the typical diffraction pattern of cereal starch.[140, 184] On the contrary, HA starch exhibited a different XRD spectrum due to the higher content of amylose, which reduced crystallinity.[140, 185]

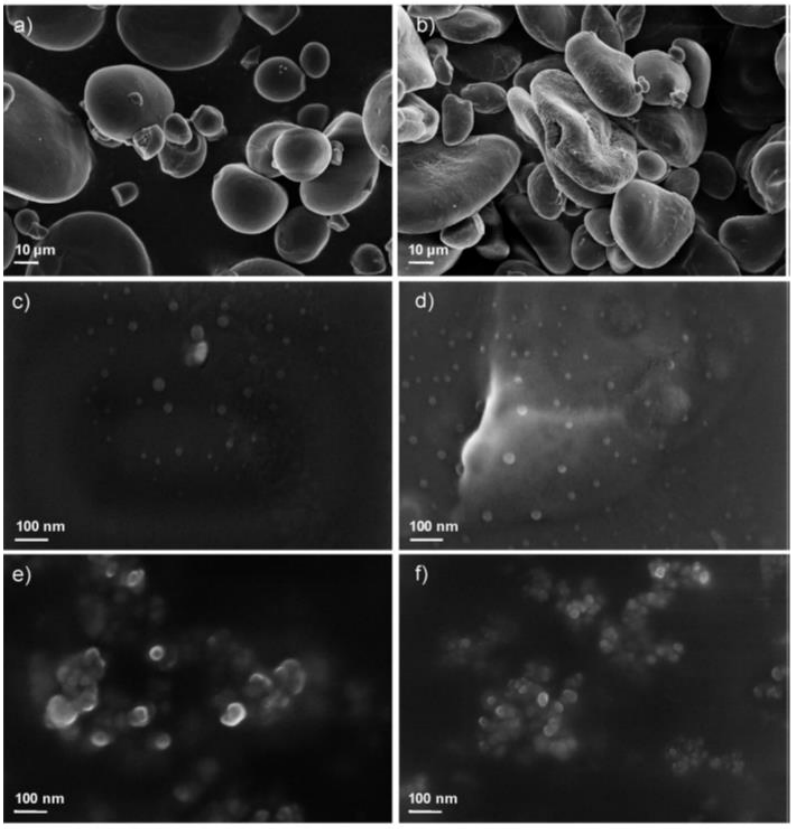

Figure 19: FESEM images of starch particles. (a) Native WT starch; (b) native HA starch; (c) WT starch after acid hydrolysis treatment; (d) HA starch after acid hydrolysis treatment; (e) WT starch after ultrasound treatment; (f) HA starch after ultrasound treatment.[140]

4.3.2 Preparation of ternary films: optimization procedure from binary systems

In order to select the best content of starch nanocrystals or nanoparticles to use in the ternary formulations, binary PVA films, reinforced with 1 or 3 wt% NC_{HA}, NC_{WT}, NP_{HA} or NP_{WT} (namely PVA/1NC_{WT}, PVA/3NC_{WT}, PVA/1NC_{HA}, PVA/3NC_{HA}), were produced and characterized. Thermal properties were investigated by DSC analysis to verify the effect of nanostructured starches on PVA crystallization phenomena and crystallinity degree. Moreover, tensile tests of neat PVA and PVA binary systems were performed to evaluate the effect of the nanostructured starch-based fillers on the mechanical response of the PVA

matrix. The incorporation of starch nanocrystals induced an increase in crystallinity. However, no significant enhancements were induced by the addition of 3 wt% of starch nanocrystals respect to the lower content (1 wt%).[140] Tensile tests for NC-based formulations showed a decrease of the elongation at break of the films, that was more evident in PVA/3NC_{WT} systems.[140]

Similar results were also detected for NP-based formulations. A general increase in crystallinity degree values, although less evident respect to NC based systems, was detected for NP-based films, except for PVA/3NP_{WT} film.[140]

Based on the results obtained, ternary films were prepared by adding 1 wt% of nanostructured starches (NC_{HA}, NC_{WT}, NP_{HA} or NP_{WT}) combined with 5 wt% of HTyr[118] in PVA matrix, by solvent casting technique.[140]

4.3.3 Characterization of ternary formulations

Morphological and optical properties

The cross-section surfaces of PVA-based systems were analysed by FESEM (Figure 20, a), to evaluate the influence of both nanostructured starch (NC and NP) and HTyr on PVA microstructure. A smooth and homogeneous fractured surface was observed in neat PVA film. As previously reported,[118] no alterations were induced by the addition of 5 wt% of HTyr into the PVA matrix. No alterations were observed either in PVA/NC/HTyr and in PVA/NP/HTyr films. The FESEM images underlined, in fact, no separation phases or holes, highlighting that homogeneous dispersion of the antioxidant agent and nanostructured starch in the polymeric compound was obtained.[140]

Visual aspect and transparency of PVA and PVA ternary films were also analysed. As shown in Figure 20 (b), the visual observation (PVA/NP_{WT}/HTyr) underling the transparent nature of PVA ternary films with a uniform colour distribution.[140]

The results from UV-vis spectra of the different films produced (Figure 20, c) confirmed that the transparent nature of PVA (transmittance of 94% at a wavelength of 600 nm) was slightly affected by the addition of both active agent and nanostructured starches. Transmittances between 92% and 90% were, in fact, detected for PVA/HTyr binary films and for all the ternary systems produced. This result highlights the possibility of using these formulations to make transparent packaging in the wavelength range of 600-900 nm. Furthermore, the transparency rapidly decreased from 600 nm to lower wavelength and all the studied systems

showed a visible absorption band at 280 nm, due to the presence of HTyr, as previously reported.[118, 140]

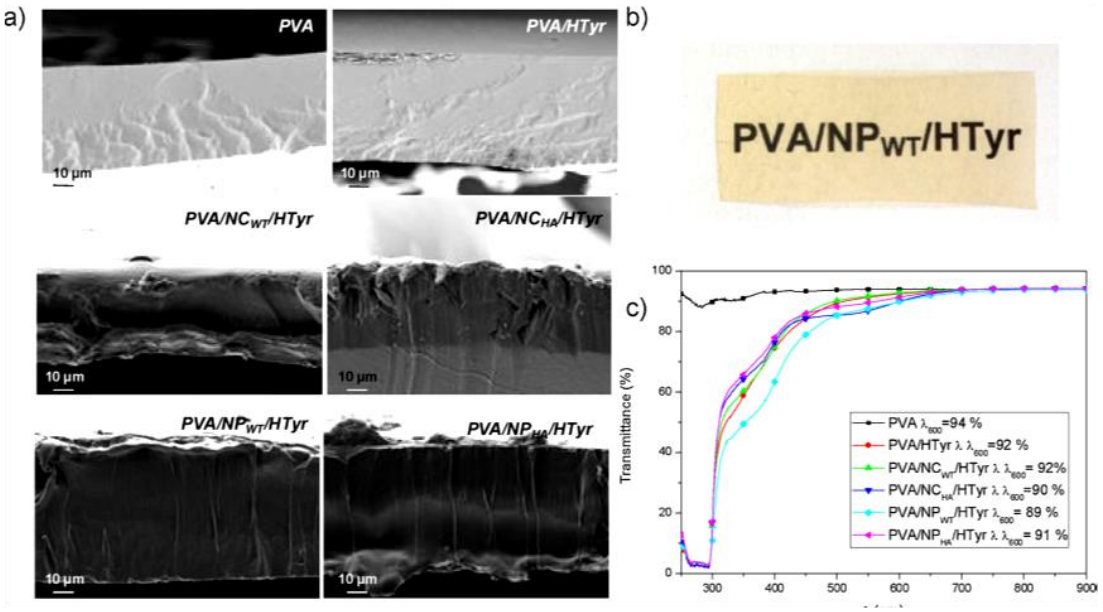

Figure 20: FESEM investigation of fractured surface (a), visual image of PVA/NP$_{WT}$/HTyr (b) and UV–vis analysis (c) of PVA, PVA/HTyr, PVA/NC$_{WT}$/HTyr, PVA/NC$_{HA}$/HTyr, PVA/NP$_{WT}$/HTyr and PVA/NP$_{HA}$/HTyr.[140]

Thermal analyses, mechanical response and water absorption capacity

With the aim of evaluating the effect of both HTyr and nanostructured starch on the thermal stability of PVA matrix, thermal properties of PVA and PVA-based films were evaluated by Differential Scanning Calorimetry (DSC) and Thermogravimetric Analysis (TGA).

To evaluate the effect of NC and NP combined with HTyr on the crystallization and melting phenomena of PVA, DSC analyses were performed. As previously reported in literature, the presence of HTyr promotes the crystallization phenomena of PVA/HTyr.[117] However, the combination of HTyr and starch nanofiller in PVA matrix did not induce a proportional increase in terms of crystallinity degree. Data obtained[140] highlighted, as demonstrated in literature,[118, 186] that HTyr favours the crystallization phenomena of PVA/HTyr-based films positively affecting the final thermal response of PVA matrix.

The effect of HTyr and nanostructured starch on the thermal degradative properties of PVA matrix was also investigated by TGA. All the studied formulations were characterized by the

presence of a multi-step degradation behaviour. The first weight loss corresponds to the evaporation of the water or a weight loss of low molecular weight compounds.[187] The second and the third degradation temperatures correspond to the degradation of neat PVA.[187-189] As previously reported, the presence of HTyr in the PVA matrix shifted the main degradation temperature to higher values, whereas no alterations in the main degradation temperatures were induced by the presence of different nanostructured starch fillers. Finally, the third degradation step was poorly affected by the presence of both HTyr and starch nanofillers.[118]

The mechanical responses of ternary formulations were also evaluated. Considering the final application of the films in the industrial sector, in which the mechanical properties are a crucial issue, the performed tensile test permitted the evaluation of the influence of both HTyr and nanostructured starches on PVA. The addition of 5 wt% of HTyr to PVA induced an increase of about 100% respect to PVA matrix, underlining and confirming the plasticization effect of the selected active ingredient. This effect was, in part, maintained also in the case of ternary systems. The presence of NC or NP combined with HTyr induce a reduction of elongation at break. These results suggested the possibility to modulate the final mechanical response incorporating the NC and NPs in PVA/HTyr film.[140]

Experimental data showed that pure HTyr induced a positive effect on the PVA matrix enhancing mechanical and thermal properties. Ternary films, compromising between elastic and plastic response of the film, which are useful characteristics for packaging sector.

The water absorption capacity (WAC) of ternary films at different temperatures has been studied. Since it is directly linked to food quality and safety, WAC is one of the most important properties to study for biodegradable packages. The amount of water absorbed on the package material can, in fact, influence the microorganism growth and lead to an increased deterioration of the contained food products, thus decreasing their shelf life.[118]

In order to evaluate the effect of different components and their combinations in the PVA matrix, WAC of PVA-based formulations was investigated. Three different temperatures (4, 20, and 37 °C) were selected to reproduce the different conditions in which food products are exposed during transport and storage. All the formulations showed a fast absorption mechanism with a saturation limit reached within the first 24 h,[118] due to the hydrophilic nature of the polymer matrix that justifies this behaviour.[187, 190, 191] In PVA ternary based films, the presence of starch nanofillers influence the WAC%, specifically their presence determined an increase of swelling phenomena, due to the hydrophilic nature of PVA and starch nanostructures.[187, 190, 192] Considering data obtained from ternary based formulations, no particular differences were experimentally obtained by using NC or NP in the polymer

matrix in terms of WAC% at the temperatures tested. A slight increase in terms of WAC% were registered only in the case of PVA ternary films combined with NC_{WT} or NP_{WT}. It is widely known that the increase in amylose limits the water absorption capacity of starch due to the tight interaction among its linear glycosidic chains.[140, 193, 194]

4.3.5 Overall migration and release tests in food simulant

Overall migration tests of each formulation were performed in food simulant according to the European Union Regulation no. 10/2011, which defines the limits at which additives can migrate into food during the storage, transport and commercialization period of foodstuff.[143]
To perform migration tests EU legislators have agreed on food simulants which reflect the properties of the food that will come in contact with the packaging materials. The simulant allowed are: ethanol 10% v/v (Simulant A) for water-based foods, acetic acid 3% (Simulant B) for hydrophilic food (pH<4.5), ethanol 20% (Simulant C) for alcoholic more lipophilic food, ethanol 50% (Simulant D1) for lipophilic/alcoholic food and oil in water emulsion and vegetable oil (Simulant D2) for lipophilic food.[143]
Given the hydrophilic properties of HTyr the food simulant representing the characteristics of fresh food with high water content, like fruit and vegetables, was chosen. The migration values, obtained in the food simulant A (ethanol 10% v/v), for all the studied formulations were lower than the migration limits for food contact materials, 60 mg kg^{-1} simulant, established by the current legislation,[143] demonstrating the possible practical application of the produced films in contact with foods.
The final aim of an active packaging system is to play an active role in preserving food, hence to define a packaging film as active a functional activity is required. Regarding the antioxidant release from packaging materials, one of the main advantages is that the active materials can act as a source of antioxidants that are released to food at controlled rates, compensating for the continuous consumption of the antioxidant during storage.[195]
Thus, specific migration tests were carried to define the HTyr release from the PVA matrix, with the further aim of verifying the effect of starch-based nanomaterials on the release profile of the antioxidant.
Release tests were performed according to European Standard EN 13130-200522 and European Commission Regulation 10/2011.[118, 143] Binary film PVA/HTyr and each ternary formulation (PVA/NC_{WT}/HTyr, PVA/NC_{HA}/HTyr, PVA/NP_{WT}/HTyr, PVA/NP_{HA}/HTyr) was

placed into the simulant A for 21 days. All samples were analysed at 1, 3, 7, 10 and 21 days measuring HTyr released by UV–vis spectrophotometry. As shown in Figure 21 (a), all the ternary formulations tested exhibited a gradual release of HTyr during the 21 days of the test, while with the binary film PVA/HTyr the concentration of HTyr released did not sensibly grow over time, in accordance with a previous work.[118] In fact, HTyr concentrations in the simulant solutions ranged from 1118 (at day 1) to 962 μM (at day 21) for PVA/HTyr; from 1337 to 2109 μM for PVA/NC_{WT}/HTyr; from 1269 to 1747 μM for PVA/NC_{HA}/HTyr; from 1004 to 2438 μM for PVA/NP_{WT}/HTyr and from 663 to 1467 μM for PVA/NP_{HA}/HTyr. As described, ternary systems overall were able to promote a greater release of HTyr compared to the binary film, demonstrating the active role of nanostructured starch in the release process. Among all formulations, PVA/NP_{WT}/HTyr allowed the most gradual HTyr release during the 21 days of the test.[140]

4.3.6 Antioxidant activity

The antioxidant activity of binary and ternary formulations was evaluated through the ABTS assay performed on the food simulant solutions at different times (1, 3, 7, 10 and 21 days). As shown in Figure 21 (b), a remarkable antioxidant activity was observed for all ternary formulations tested over the time compared to binary film PVA/HTyr, as a consequence of the different amount of HTyr released. In addition, the effect of the different formulations agreed with the experimental data of the specific migration. In particular, the antioxidant activity increased from 458 (at day 1) to 255 TE/μM (at day 21) for PVA/HTyr; from 950 to 1201 TE/μM for PVA/NC_{WT}/HTyr; from 854 to 987 TE/μM for PVA/NC_{HA}/HTyr; from 824 to 1286 TE/μM for PVA/NP_{WT}/HTyr and from 479 to 762 TE/μM for PVA/NP_{HA}/HTyr. As already stated in a previous paper, the diffusion was the only mechanism of HTyr release from PVA film over the time.[118, 140]

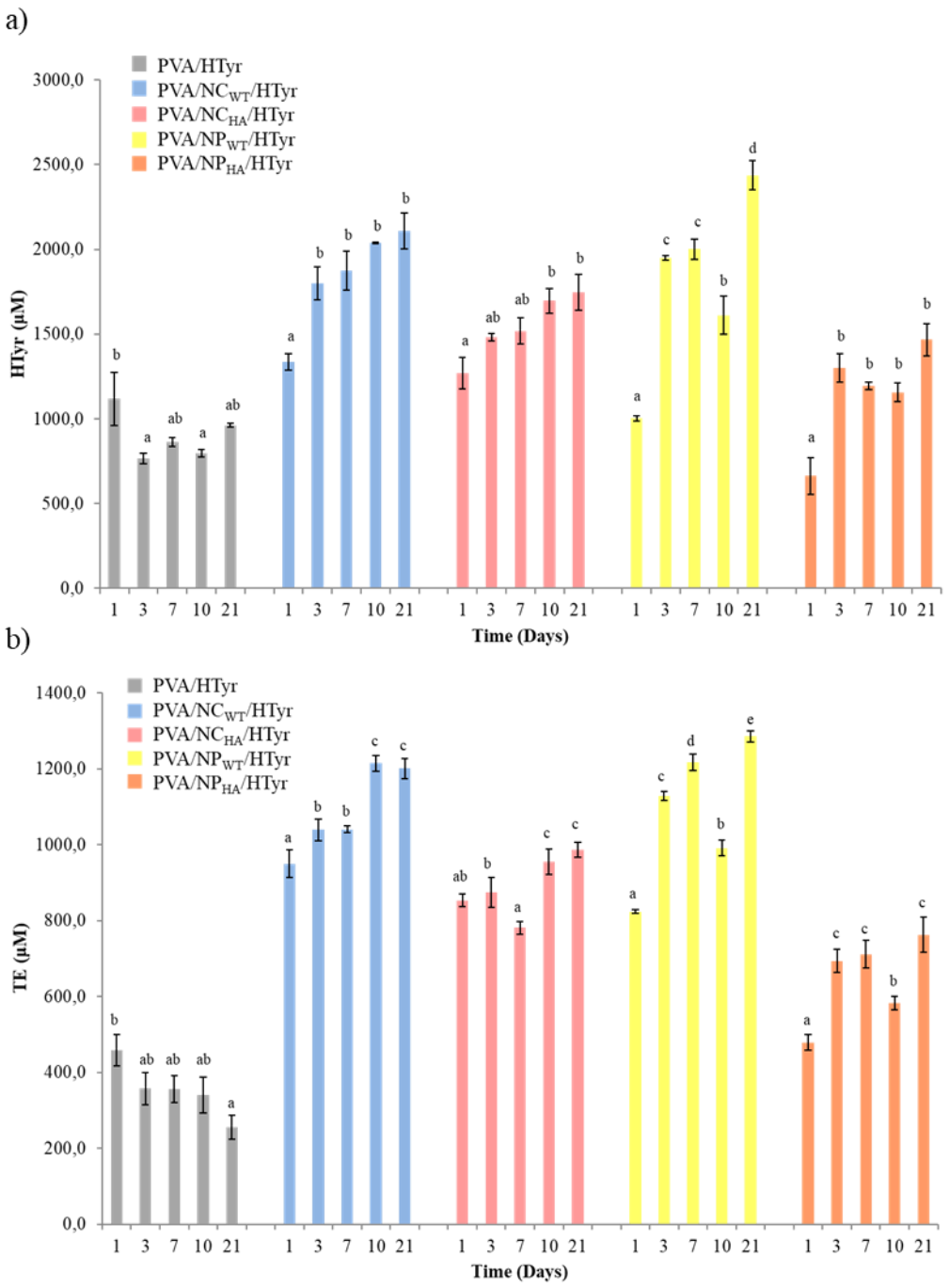

Figure 21: (a) HTyr released (µM) from PVA/HTyr binary film and from each ternary film in food simulant (ethanol 10% v/v); (b) antioxidant activity (TE, µM) of food simulant solutions after specific migration tests from PVA/HTyr binary film and from each ternary film evaluated by ABTS assay. Labels indicate significant statistical differences between different time tests for each formulation ($P < 0.05$).[140]

5. Conclusions

The research carried out in the three-year PhD period was aimed at the valorisation of agro-food by-products to obtain biologically active materials of industrial interest in the light of circular economy principles. In particular, the first part of the work was focused on the valorisation of kiwifruit by-products exploring the anti-inflammatory activity of a kiwifruit peel extract rich in procyanidins. The results obtained showed that the kiwifruit peel extract suppressed the production of several inflammatory mediators, such as pro-inflammatory cytokines, HMGB1, granzyme B and STAT3, and, also, was able to promote the autophagic process in human activated THP-1 monocytes. Considering all these findings, the extract could represent a promising anti-inflammatory natural ingredient for pharmaceutical and nutraceutical formulations, as topical anti-inflammatory agent for the treatment of cutaneous inflammatory diseases or as dietary supplementation in complementary therapies for the treatment of inflammatory disorders. However, further research is needed on *in vivo* models.

The second part of the work focused on the valorisation of olive oil by-products, analysing the antioxidant and antimicrobial activities of the HTyr-enriched extract obtained from OMWW. The latter showed good antioxidant and antimicrobic activities against two olive three pathogens, *Pseudomonas savastanoi* and *Agrobacterium tumefaciens*, when compared with HTyr standard. This approach would lead to close the production circle by using compounds obtained from OMWW directly on olive trees, however, further research is needed to better understand the mechanisms underlying these activities.

Finally, with the future goal of applying the HTyr-enriched extract as antioxidant in active food packaging, HTyr standard was used as active ingredient. Experimental data showed that produced films, based on PVA, nanostructured starch and HTyr, were able to release the antioxidant in the food simulant in a gradual fashion and the resulting solution maintained the antioxidant activity during the 21 days of the test. These results suggest that ternary films may represent a very promising strategy for active food packaging applications, exploitable for preserving fresh food with high water content, such as fruit and vegetables.

Acknowledgements

I would like to thank who helped me in writing the thesis with suggestions and observations.
In particular I would like to thank Prof. Luca Santi and Prof. Roberta Bernini for their valuable scientific supervision, provided with professional competence and kindness. Their commitment made this experience a beautiful opportunity of human and scientific growth.
My sincere thanks also goes to Prof. Annalisa Romani, Prof. Francesca Romana Velotti, Prof. Leonardo Varvaro, Prof. Domenico Lafiandra, Prof. Josè Maria Kenny and Prof. Laura Dugo. They provided me an opportunity to join their research teams and laboratories. Without their precious support it would not have been possible to conduct this research.
Moreover, I would like to thank Prof. Heribert Warzecha, Plant Biotechnology and Metabolic Engineering, Technische Universität Darmstadt, for receiving me in his laboratory as guest student and for supporting me during my experience in Darmstadt.
Finally, I must express my gratitude to my parents and to my boyfriend for supporting me with continuous encouragements.
The research was carried out in the frame of the MIUR initiative “Department of excellence” (Law 232/2016).
A part of the project was funded by Cariplo Foundation within the “Agroalimentare e Ricerca” (AGER) program. Project AGER2-Rif.2016-0169, “Valorizzazione dei prodotti italiani derivanti dall’oliva attraverso Tecniche Analitiche Innovative”-“VIOLIN”.

References:

1. Croteau, R., Kutchan, T.M. and Lewis, N.G. *Natural products (secondary metabolites).* Biochemistry and Molecular Biology of Plants, 2000. **24**: p. 1250-1319.
2. Dias, D.A., Urban, S. and Roessner, U. *A historical overview of natural products in drug discovery.* Metabolites, 2012. **2**(2): p. 303-336.
3. Harvey, A.L., *Natural products in drug discovery.* Drug Discovery Today, 2008. **13**(19-20): p. 894-901.
4. Atanasov, A.G., *et al*., *Discovery and resupply of pharmacologically active plant-derived natural products: a review.* Biotechnology Advances, 2015. **33**(8): p. 1582-1614.
5. Crozier, A., Clifford, M.N. and Ashihara, H. *Plant secondary metabolites: occurrence, structure and role in the human diet*. 2008: John Wiley & Sons.
6. Dewick, P.M., *Medicinal natural products: a biosynthetic approach*. 2002: John Wiley & Sons.
7. GP. *Groupe Polyphénols (GP) society*. 2019;

   Available from: https://www.groupepolyphenols.com/. (Last visit: 19/04/2019)
8. Alasalvar, C., *et al., Comparison of volatiles, phenolics, sugars, antioxidant vitamins, and sensory quality of different colored carrot varieties.* Journal of Agricultural and Food Chemistry, 2001. **49**(3): p. 1410-1416.
9. Balasundram, N., Sundram, K. and Samman, S. *Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses.* Food Chemistry, 2006. **99**(1): p. 191-203.
10. Crozier, A., Jaganath, I.B. and Clifford, M.N. *Dietary phenolics: chemistry, bioavailability and effects on health.* Natural Product Reports, 2009. **26**(8): p. 1001-1043.
11. Ncube, B. and Van Staden, J. *Tilting plant metabolism for improved metabolite biosynthesis and enhanced human benefit.* Molecules, 2015. **20**(7): p. 12698-12731.
12. Manach, C., *et al*., *Polyphenols: food sources and bioavailability.* The American Journal of Clinical Nutrition, 2004. **79**(5): p. 727-747.
13. Tsao, R., *Chemistry and biochemistry of dietary polyphenols.* Nutrients, 2010. **2**(12): p. 1231-1246.
14. Heleno, S.A., *et al., Bioactivity of phenolic acids: Metabolites versus parent compounds: A review.* Food Chemistry, 2015. **173**: p. 501-513.
15. Goleniowski, M., *et al*., *Phenolic acids*, in *Natural Products*. 2013, Springer. p. 1951-1973.
16. Rodríguez-Morató, J., *et al*., *Metabolic disposition and biological significance of simple phenols of dietary origin: Hydroxytyrosol and tyrosol.* Drug Metabolism Reviews, 2016. **48**(2): p. 218-236.
17. Chong, J., Poutaraud, A. and Hugueney, P. *Metabolism and roles of stilbenes in plants.* Plant Science, 2009. **177**(3): p. 143-155.

18. Jain, P. and Joshi, H. *Coumarin: chemical and pharmacological profile.* Journal of Applied Pharmaceutical Science, 2012. **2**(6): p. 236-240.

19. D Archivio, M., *et al*., *Polyphenols, dietary sources and bioavailability.* Annali-Istituto Superiore di Sanita, 2007. **43**(4): p. 348.

20. Liu, R.H., *Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals.* The American Journal of Clinical Nutrition, 2003. **78**(3): p. 517S-520S.

21. Arts, I.C. and Hollman, P.C. *Polyphenols and disease risk in epidemiologic studies–.* The American Journal of Clinical Nutrition, 2005. **81**(1): p. 317S-325S.

22. Vauzour, D., *et al*., *Polyphenols and human health: prevention of disease and mechanisms of action.* Nutrients, 2010. **2**(11): p. 1106-1131.

23. Manach, C., Mazur, A. and Scalbert, A. *Polyphenols and prevention of cardiovascular diseases.* Current Opinion in Lipidology, 2005. **16**(1): p. 77-84.

24. Puupponen-Pimiä, R., *et al*., *Antimicrobial properties of phenolic compounds from berries.* Journal of Applied Microbiology, 2001. **90**(4): p. 494-507.

25. Watson, R.R., Preedy, V.R. and Zibadi, S. *Polyphenols in human health and disease*. 2013: Academic press.

26. Zitka, O., *et al.*, *Comparison of various easy-to-use procedures for extraction of phenols from apricot fruits.* Molecules, 2011. **16**(4): p. 2914-2936.

27. Han, X., Shen, T. and Lou, *Dietary polyphenols and their biological significance.* International Journal of Molecular Sciences, 2007. **8**(9): p. 950-988.

28. Cowan, M.M., *Plant products as antimicrobial agents.* Clinical Microbiology Reviews, 1999. **12**(4): p. 564-582.

29. Sanhueza, L., *et al*., *Synergistic interactions between phenolic compounds identified in grape pomace extract with antibiotics of different classes against Staphylococcus aureus and Escherichia coli.* PLoS One, 2017. **12**(2): p. e0172273.

30. Scavo, A., *et al*., *Antimicrobial activity of cultivated cardoon (Cynara cardunculus L. var. altilis DC.) leaf extracts against bacterial species of agricultural and food interest.* Industrial Crops and Products, 2019. **129**: p. 206-211.

31. Coussens, L.M. and Werb, Z. *Inflammation and cancer.* Nature, 2002. **420**(6917): p. 860.

32. Kaplan, M., *et al*., *Use of herbal preparations in the treatment of oxidant-mediated inflammatory disorders.* Complementary Therapies in Medicine, 2007. **15**(3): p. 207-216.

33. Reuter, S., *et al*., *Oxidative stress, inflammation, and cancer: how are they linked?* Free Radical Biology and Medicine, 2010. **49**(11): p. 1603-1616.

34. Shi, C. and Pamer, E.G. *Monocyte recruitment during infection and inflammation.* Nature Reviews Immunology, 2011. **11**(11): p. 762.

35. Yang, J., *et al*., *Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases.* Biomarker Research, 2014. **2**(1): p. 1.

36. Murray, P.J. and Wynn, T.A. *Protective and pathogenic functions of macrophage subsets.* Nature Reviews Immunology, 2011. **11**(11): p. 723.

37. Bianchi, M.E., *DAMPs, PAMPs and alarmins: all we need to know about danger.* Journal of Leukocyte Biology, 2007. **81**(1): p. 1-5.

38. Wensink, A.C., Hack, C.E. and Bovenschen, N. *Granzymes regulate proinflammatory cytokine responses.* The Journal of Immunology, 2015. **194**(2): p. 491-497.

39. Yu, H., Pardoll, D. and Jove, R. *STATs in cancer inflammation and immunity: a leading role for STAT3.* Nature Reviews Cancer, 2009. **9**(11): p. 798.

40. Levine, B., Mizushima, N. and Virgin, H.W. *Autophagy in immunity and inflammation.* Nature, 2011. **469**(7330): p. 323.

41. Romier, B., *et al*., *Dietary polyphenols can modulate the intestinal inflammatory response.* Nutrition Reviews, 2009. **67**(7): p. 363-378.

42. Zhang, X., *et al*., *Flavonoid apigenin inhibits lipopolysaccharide-induced inflammatory response through multiple mechanisms in macrophages.* PLoS One, 2014. **9**(9): p. e107072.

43. Martinez-Micaelo, N., *et al*., *Procyanidin B2 inhibits inflammasome-mediated IL-1β production in lipopolysaccharide-stimulated macrophages.* Molecular Nutrition & Food Research, 2015. **59**(2): p. 262-269.

44. Bisht, K., Wagner, K.-H. and Bulmer, A.C. *Curcumin, resveratrol and flavonoids as anti-inflammatory, cyto-and DNA-protective dietary compounds.* Toxicology, 2010. **278**(1): p. 88-100.

45. Genestra, M., *Oxyl radicals, redox-sensitive signalling cascades and antioxidants.* Cellular Signalling, 2007. **19**(9): p. 1807-1819.

46. Valko, M., *et al*., *Free radicals, metals and antioxidants in oxidative stress-induced cancer*. Chemico-Biological Interactions, 2006. **160**(1): p. 1-40.

47. Pham-Huy, L.A., He, H. and Pham-Huy, C. *Free radicals, antioxidants in disease and health.* International Journal of Biomedical Science: IJBS, 2008. **4**(2): p. 89.

48. Lobo, V., *et al*., *Free radicals, antioxidants and functional foods: Impact on human health.* Pharmacognosy Reviews, 2010. **4**(8): p. 118.

49. Leopoldini, M., Russo, N. and Toscano, M. *The molecular basis of working mechanism of natural polyphenolic antioxidants.* Food Chemistry, 2011. **125**(2): p. 288-306.

50. Di Meo, F., *et al*., *Free radical scavenging by natural polyphenols: atom versus electron transfer*. The Journal of Physical Chemistry A, 2013. **117**(10): p. 2082-2092.

51. Naczk, M. and Shahidi, F. *Extraction and analysis of phenolics in food.* Journal of Chromatography A, 2004. **1054**(1-2): p. 95-111.

52. Naczk, M. and Shahidi, F. *Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis.* Journal of Pharmaceutical and Biomedical Analysis, 2006. **41**(5): p. 1523-1542.

53. Tung, Y.-T., *et al*., *Antioxidant activities of natural phenolic compounds from Acacia confusa bark.* Bioresource Technology, 2007. **98**(5): p. 1120-1123.

54. Rice-Evans, C., Miller, N. and Paganga, G. *Antioxidant properties of phenolic compounds.* Trends in Plant Science, 1997. **2**(4): p. 152-159.

55. Lattanzio, V., Lattanzio, V.M. and Cardinali, A. *Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects.* Phytochemistry: Advances in Research, 2006. **661**(2): p. 23-67.

56. Dayan, F.E., Cantrell, C.L. and Duke, S.O. *Natural products in crop protection.* Bioorganic & Medicinal Chemistry, 2009. **17**(12): p. 4022-4034.

57. Gyawali, R. and Ibrahim, S.A. *Natural products as antimicrobial agents.* Food Control, 2014. **46**: p. 412-429.

58. Bernal, P., Zloh, M. and Taylor, P.W. *Disruption of d-alanyl esterification of Staphylococcus aureus cell wall teichoic acid by the β-lactam resistance modifier (−)-epicatechin gallate.* Journal of Antimicrobial Chemotherapy, 2009. **63**(6): p. 1156-1162.

59. Hemaiswarya, S., Kruthiventi, A.K. and Doble, M. *Synergism between natural products and antibiotics against infectious diseases.* Phytomedicine, 2008. **15**(8): p. 639-652.

60. Savoia, D., *Plant-derived antimicrobial compounds: alternatives to antibiotics.* Future Microbiology, 2012. **7**(8): p. 979-990.

61. Mild, R.M., *et al*., *Antimicrobial edible apple films inactivate antibiotic resistant and susceptible Campylobacter jejuni strains on chicken breast.* Journal of Food Science, 2011. **76**(3): p. M163-M168.

62. Maddox, C.E., Laur, L.M. and Tian, L. *Antibacterial activity of phenolic compounds against the phytopathogen Xylella fastidiosa.* Current Microbiology, 2010. **60**(1): p. 53.

63. Dubey, N., *et al*., *Global scenario on the application of natural products in integrated pest management programmes.* Natural Products in Plant Pest Management, 2011. **1**: p. 1-20.

64. Yangui, T., *et al*., *Potential use of hydroxytyrosol-rich extract from olive mill wastewater as a biological fungicide against Botrytis cinerea in tomato.* Journal of Pest Science, 2010. **83**(4): p. 437-445.

65. Ajila, C., *et al*., *Extraction and analysis of polyphenols: recent trends.* Critical Reviews in Biotechnology, 2011. **31**(3): p. 227-249.

66. Dai, J. and Mumper, R.J. *Plant phenolics: extraction, analysis and their antioxidant and anticancer properties.* Molecules, 2010. **15**(10): p. 7313-7352.

67. Lapornik, B., Prošek, M. and Wondra, A.G. *Comparison of extracts prepared from plant by-products using different solvents and extraction time.* Journal of Food Engineering, 2005. **71**(2): p. 214-222.

68. Ignat, I., Volf, I. and Popa, V.I. *A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables.* Food Chemistry, 2011. **126**(4): p. 1821-1835.

69. Garcia-Salas, P., *et al*., *Phenolic-compound-extraction systems for fruit and vegetable samples.* Molecules, 2010. **15**(12): p. 8813-8826.

70. Azmir, J., *et al*., *Techniques for extraction of bioactive compounds from plant materials: a review*. Journal of Food Engineering, 2013. **117**(4): p. 426-436.

71. Conidi, C., Drioli, E. and Cassano, A. *Membrane-based agro-food production processes for polyphenol separation, purification and concentration.* Current Opinion in Food Science, 2018. **23**: p. 149-164.

72. Federici, F., *et al*., *Valorisation of agro-industrial by-products, effluents and waste: concept, opportunities and the case of olive mill wastewaters.* Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology, 2009. **84**(6): p. 895-900.

73. Agourram, A., *et al*., *Phenolic content, antioxidant potential, and antimicrobial activities of fruit and vegetable by-product extracts.* International Journal of Food Properties, 2013. **16**(5): p. 1092-1104.

74. Mincione, E. and Bernini, R. *Produzione di molecole bioattive da residui agroindustriali.*

75. Saladino, R., Bernini, R. and Mincione, E. *Valorizzazione di reflui agroindustriali.* La Chimica e L'industria, 2002. **84**(2).

76. MincionE, E., *et al*., *Phenolic Natural Compounds with Chromanic Structure: Their Valorization by Green Chemistry Oxidative Methodologies.* ChemInform, 2003. **34**(38): p. no-no.

77. Romani, A., *et al*., *Sustainability, Innovation, and Green Chemistry in the Production and Valorization of Phenolic Extracts from Olea europaea L.* Sustainability, 2016. **8**(10): p. 1002.

78. Pfaltzgraff, L.A., *et al*., *Food waste biomass: a resource for high-value chemicals.* Green Chemistry, 2013. **15**(2): p. 307-314.

79. Mirabella, N., Castellani, V. and Sala, S. *Current options for the valorization of food manufacturing waste: a review.* Journal of Cleaner Production, 2014. **65**: p. 28-41.

80. Schulte, U.G., *New business models for a radical change in resource efficiency.* Environmental Innovation and Societal Transitions, 2013. **9**: p. 43-47.

81. Lieder, M. and Rashid, A. *Towards circular economy implementation: a comprehensive review in context of manufacturing industry.* Journal of Cleaner Production, 2016. **115**: p. 36-51.

82. Scheel, C., *Beyond sustainability. Transforming industrial zero-valued residues into increasing economic returns.* Journal of Cleaner Production, 2016. **131**: p. 376-386.

83. Ruggieri, A., *et al*., *A meta-model of inter-organisational cooperation for the transition to a circular economy.* Sustainability, 2016. **8**(11): p. 1153.

84. EU Commission. *Circular Economy. Implementation of the Circular Economy Action Plan*. 2018;

Available from: http://ec.europa.eu/environment/circular-economy/index_en.htm.

85. Stahel, W.R., *The circular economy.* Nature News, 2016. **531**(7595): p. 435.

86. European Commission, *Guidance on the Interpretation of Key Provisions of Directive 2008/98/EC on Waste*. 2012, Directorate Environment Brussels.

87. Shastri, K.V., *et al*., *Actinidia deliciosa: a Review.* International Journal of Pharmaceutical Sciences and Research, 2012. **3**(10): p. 3543.

88. FAOSTAT. *Food and agriculture data*. 2018;

Available from: http://www.fao.org/faostat/en/#home. (Last visit: 10/02/2019)

89. Fiorentino, A., *et al*., *Identification and assessment of antioxidant capacity of phytochemicals from kiwi fruits.* Journal of Agricultural and Food Chemistry, 2009. **57**(10): p. 4148-4155.

90. Iwasawa, H., *et al*., *Anti-oxidant effects of kiwi fruit in vitro and in vivo.* Biological and Pharmaceutical Bulletin, 2011. **34**(1): p. 128-134.

91. Jung, K.-A., *et al*., *Cardiovascular protective properties of kiwifruit extracts in vitro.* Biological and Pharmaceutical Bulletin, 2005. **28**(9): p. 1782-1785.

92. Lee, C.-C., Lee, B.-H. and Wu, S.-C. *Actinidia callosa peel (kiwi fruit) ethanol extracts protected neural cells apoptosis induced by methylglyoxal through Nrf2 activation.* Pharmaceutical Biology, 2014. **52**(5): p. 628-636.

93. Yang, H., *et al*., *Green and gold kiwifruit peel ethanol extracts potentiate pentobarbital-induced sleep in mice via a GABAergic mechanism.* Food Chemistry, 2013. **136**(1): p. 160-163.

94. Schieber, A., Stintzing, F. and Carle, R. *By-products of plant food processing as a source of functional compounds—recent developments.* Trends in Food Science & Technology, 2001. **12**(11): p. 401-413.

95. Pinelli, P., *et al*., *Characterisation of the Polyphenol Content in the Kiwifruit (Actinidia deliciosa) Exocarp for the Calibration of a Fruit-sorting Optical Sensor.* Phytochemical Analysis, 2013. **24**(5): p. 460-466.

96. Hammerstone, J.F., Lazarus, S.A. and Schmitz, H.H. *Procyanidin content and variation in some commonly consumed foods.* The Journal of Nutrition, 2000. **130**(8): p. 2086S-2092S.

97. Corder, R., *et al*., *Oenology: red wine procyanidins and vascular health.* Nature, 2006. **444**(7119): p. 566.

98. Lozano-Sánchez, J., *et al*., *Wastes generated during the storage of extra virgin olive oil as a natural source of phenolic compounds.* Journal of Agricultural and Food Chemistry, 2011. **59**(21): p. 11491-11500.

99. Rodriguez-Morato, J., *et al.*, *Potential role of olive oil phenolic compounds in the prevention of neurodegenerative diseases.* Molecules, 2015. **20**(3): p. 4655-4680.

100. Bernini, R., *et al*., *Naturally occurring hydroxytyrosol: synthesis and anticancer potential.* Current Medicinal Chemistry, 2013. **20**(5): p. 655-670.

101. Thielmann, J., Kohnen, S. and Hauser, C. *Antimicrobial activity of Olea europaea Linné extracts and their applicability as natural food preservative agents.* International Journal of Food Microbiology, 2017. **251**: p. 48-66.

102. Fernandez-Pastor, I., *et al*., *Synthesis and antioxidant activity of hydroxytyrosol alkyl-carbonate derivatives.* Journal of Natural Products, 2016. **79**(7): p. 1737-1745.

103. Visioli, F., *et al*., *Hydroxytyrosol, as a component of olive mill waste water, is dose-dependently absorbed and increases the antioxidant capacity of rat plasma.* Free Radical Research, 2001. **34**(3): p. 301-305.

104. Cardinali, A., *et al.*, *Verbascoside, isoverbascoside, and their derivatives recovered from olive mill wastewater as possible food antioxidants.* Journal of Agricultural and Food Chemistry, 2012. **60**(7): p. 1822-1829.

105. Bertin, L., *et al.*, *Recovery of high added value natural polyphenols from actual olive mill wastewater through solid phase extraction.* Chemical Engineering Journal, 2011. **171**(3): p. 1287-1293.

106. Granados-Principal, S., *et al.*, *Hydroxytyrosol: from laboratory investigations to future clinical trials.* Nutrition Reviews, 2010. **68**(4): p. 191-206.

107. Gambacorta, A., *et al*., *High-yielding preparation of a stable precursor of hydroxytyrosol by total synthesis and from the natural glycoside oleuropein.* Journal of Agricultural and Food Chemistry, 2007. **55**(9): p. 3386-3391.

108. Bernini, R., *et al*., *Convenient synthesis of hydroxytyrosol and its lipophilic derivatives from tyrosol or homovanillyl alcohol.* Journal of Agricultural and Food Chemistry, 2008. **56**(19): p. 8897-8904.

109. FAO. *Food wastage footprint. Impacts on Natural Resources. Summary Report.* 2013;

Available from: http://www.fao.org/3/i3347e/i3347e.pdf. (Last visit: 10/02/2019)

110. Lee, S.Y., *et al*., *Current topics in active and intelligent food packaging for preservation of fresh foods.* Journal of the Science of Food and Agriculture, 2015. **95**(14): p. 2799-2810.

111. Agustin-Salazar, S., *et al*., *From nutraceutics to materials: effect of resveratrol on the stability of polylactide.* ACS Sustainable Chemistry & Engineering, 2014. **2**(6): p. 1534-1542.

112. Castro López, M.a.d.M., *et al*., *Improving the capacity of polypropylene to be used in antioxidant active films: incorporation of plasticizer and natural antioxidants.* Journal of Agricultural and Food Chemistry, 2013. **61**(35): p. 8462-8470.

113. Ambrogi, V., *et al*., *Natural antioxidants for polypropylene stabilization.* Polymer Degradation and Stability, 2011. **96**(12): p. 2152-2158.

114. Cerruti, P., *et al*., *Effect of natural antioxidants on the stability of polypropylene films.* Polymer Degradation and Stability, 2009. **94**(11): p. 2095-2100.

115. Bernini, R., *et al*., *Lipophilization of hydroxytyrosol-enriched fractions from Olea europaea L. byproducts and evaluation of the in vitro effects on a model of colorectal cancer cells.* Journal of Agricultural and Food Chemistry, 2017. **65**(31): p. 6506-6512.

116. Bernini, R., *et al*., *Hydroxytyrosol-derived compounds: A basis for the creation of new pharmacological agents for cancer prevention and therapy.* Journal of Medicinal Chemistry, 2015. **58**(23): p. 9089-9107.

117. Fortunati, E., *et al*., *Effect of hydroxytyrosol methyl carbonate on the thermal, migration and antioxidant properties of PVA-based films for active food packaging.* Polymer International, 2016. **65**(8): p. 872-882.

118. Fortunati, E., *et al*., *Hydroxytyrosol as active ingredient in poly (vinyl alcohol) films for food packaging applications.* Journal of Renewable Materials, 2017. **5**(2): p. 81-95.

119. Domingues, R.M., *et al*., *Development of injectable hyaluronic acid/cellulose nanocrystals bionanocomposite hydrogels for tissue engineering applications.* Bioconjugate Chemistry, 2015. **26**(8): p. 1571-1581.

120. Luzi, F., *et al.*, *Production and characterization of PLA_PBS biodegradable blends reinforced with cellulose nanocrystals extracted from hemp fibres.* Industrial Crops and Products, 2016. **93**: p. 276-289.

121. Stark, W.J., *et al*., *Industrial applications of nanoparticles.* Chemical Society Reviews, 2015. **44**(16): p. 5793-5805.

122. Wang, Y., *et al.*, *Mesoporous silica nanoparticles in drug delivery and biomedical applications.* Nanomedicine: Nanotechnology, Biology and Medicine, 2015. **11**(2): p. 313-327.

123. Acevedo-Fani, A., Soliva-Fortuny, R. and Martin-Belloso, O. *Nanostructured emulsions and nanolaminates for delivery of active ingredients: Improving food safety and functionality.* Trends in Food Science & Technology, 2017. **60**: p. 12-22.

124. Siró, I., *et al*., *Release of alpha-tocopherol from antioxidative low-density polyethylene film into fatty food simulant: influence of complexation in beta-cyclodextrin.* Food Additives and Contaminants, 2006. **23**(8): p. 845-853.

125. Takeda, Y., Shitaozono, T. and Hizukuri, S. *Structures of sub-fractions of corn amylose.* Carbohydrate Research, 1990. **199**(2): p. 207-214.

126. LeCorre, D., Bras, J. and Dufresne, A. *Influence of botanic origin and amylose content on the morphology of starch nanocrystals.* Journal of Nanoparticle Research, 2011. **13**(12): p. 7193-7208.

127. Frigerio, M., Santagostino, M. and Sputore, S. *A user-friendly entry to 2-iodoxybenzoic acid (IBX).* The Journal of Organic Chemistry, 1999. **64**(12): p. 4537-4538.

128. Pizzichini, D., *et al*., *Process for producing concentrated and refined actives from tissues and byproducts of Olea europaea with membrane technologies.* Patent No. EP2338500A1, 2011.

129. Romani, A., Scardigli, A. and Pinelli, P. *An environmentally friendly process for the production of extracts rich in phenolic antioxidants from Olea europaea L. and Cynara scolymus L. matrices.* European Food Research and Technology, 2017. **243**(7): p. 1229-1238.

130. D'Eliseo, D., *et al*., *In vitro studies on anti-inflammatory activities of kiwifruit peel extract in human THP-1 monocytes.* Journal of Ethnopharmacology, 2019. **233**: p. 41-46.

131. D'Eliseo, D., *et al*., *Docosahexaenoic acid (DHA) promotes immunogenic apoptosis in human multiple myeloma cells, induces autophagy and inhibits STAT3 in both tumor and dendritic cells.* Genes & Cancer, 2017. **8**(1-2): p. 426.

132. Berić, T., *et al*., *Antimicrobial activity of Bacillus sp. natural isolates and their potential use in the biocontrol of phytopathogenic bacteria.* Food Technology and Biotechnology, 2012. **50**(1): p. 25-31.

133. Pingitore, E.V., *et al*., *Different strategies for purification of antimicrobial peptides from lactic acid bacteria (LAB).* Communicating Current Research and Educational Topics and Trends in Applied Microbiology, 2007. **1**: p. 557-568.

134. Andrews, J.M., *Determination of minimum inhibitory concentrations.* Journal of Antimicrobial Chemotherapy, 2001. **48**(suppl_1): p. 5-16.

135. Díaz-Gómez, R., *et al*., *Combined effect of gallic acid and catechin against Escherichia coli.* LWT-Food Science and Technology, 2014. **59**(2): p. 896-900.

136. Re, R., *et al*., *Antioxidant activity applying an improved ABTS radical cation decolorization assay.* Free Radical Biology and Medicine, 1999. **26**(9-10): p. 1231-1237.

137. Botticella, E., *et al., Combining mutations at genes encoding key enzymes involved in starch synthesis affects the amylose content, carbohydrate allocation and hardness in the wheat grain.* Plant Biotechnology Journal, 2018. **16**(10): p. 1723-1734.

138. Wolf, M., *Wheat starch isolation.* Methods Carbohydr. Chem, 1964. **4**(6).

139. Angellier, H., *et al*., *Optimization of the preparation of aqueous suspensions of waxy maize starch nanocrystals using a response surface methodology.* Biomacromolecules, 2004. **5**(4): p. 1545-1551.

140. Luzi, F., *et al*., *Nanostructured starch combined with hydroxytyrosol in poly (vinyl alcohol) based ternary films as active packaging system.* Carbohydrate Polymers, 2018. **193**: p. 239-248.

141. Di Michele, A., *et al*., *Mercury acetate produced by metallic mercury subjected to acoustic cavitation in a solution of acetic acid in water.* Ultrasonics Sonochemistry, 2009. **16**(1): p. 141-144.

142. Cano, A.I., *et al., Physical and microstructural properties of biodegradable films based on pea starch and PVA.* Journal of Food Engineering, 2015. **167**: p. 59-64.

143. European Commission, *Commission Regulation (EU) No 10/2011 of 14 January 2011 on plastic materials and articles intended to come into contact with food.* Off J Eur Union, 2011. **12**: p. 1-89.

144. Pérez-Jiménez, J. and Saura-Calixto, F. *Effect of solvent and certain food constituents on different antioxidant capacity assays.* Food Research International, 2006. **39**(7): p. 791-800.

145. Sun-Waterhouse, D., *et al*., *Evaluation of the extraction efficiency for polyphenol extracts from by-products of green kiwifruit juicing.* International Journal of Food Science & Technology, 2009. **44**(12): p. 2644-2652.

146. Rue, E.A., Rush, M.D. and van Breemen, R.B. *Procyanidins: a comprehensive review encompassing structure elucidation via mass spectrometry.* Phytochemistry Reviews, 2018. **17**(1): p. 1-16.

147. Gou, Z., *et al*., *Equol inhibits LPS-induced oxidative stress and enhances the immune response in chicken HD11 macrophages.* Cellular Physiology and Biochemistry, 2015. **36**(2): p. 611-621.

148. Singh, U., *et al*., *Development of an in vitro screening assay to test the antiinflammatory properties of dietary supplements and pharmacologic agents.* Clinical Chemistry, 2005. **51**(12): p. 2252-2256.

149. Bae, J.-S., *Role of high mobility group box 1 in inflammatory disease: focus on sepsis.* Archives of Pharmacal Research, 2012. **35**(9): p. 1511-1523.

150. Yang, H. and Tracey, K.J. *Targeting HMGB1 in inflammation.* Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms, 2010. **1799**(1-2): p. 149-156.

151. Venereau, E., *et al., HMGB1 as biomarker and drug target.* Pharmacological Research, 2016. **111**: p. 534-544.

152. D'eliseo, D., *et al*., *Granzyme B is expressed in urothelial carcinoma and promotes cancer cell invasion.* International Journal of Cancer, 2010. **127**(6): p. 1283-1294.

153. Hiebert, P.R. and Granville, D.J. *Granzyme B in injury, inflammation, and repair.* Trends in Molecular Medicine, 2012. **18**(12): p. 732-741.

154. Marcet-Palacios, M., *et al*., *Design and characterization of a novel human Granzyme B inhibitor.* Protein Engineering, Design & Selection, 2014. **28**(1): p. 9-17.

155. Chen, M., *et al*., *STAT3 activation in circulating monocytes contributes to neovascular age-related macular degeneration.* Current Molecular Medicine, 2016. **16**(4): p. 412-423.

156. Wu, W.-Y., *et al*., *STAT3 activation in monocytes accelerates liver cancer progression.* BMC Cancer, 2011. **11**(1): p. 506.

157. Johnston, P.A. and Grandis, J.R. *STAT3 signaling: anticancer strategies and challenges.* Molecular Interventions, 2011. **11**(1): p. 18.

158. Levy, D.E. and Lee, C.-k. *What does Stat3 do?* The Journal of Clinical Investigation, 2002. **109**(9): p. 1143-1148.

159. Waldner, M.J. and Neurath. M.F. *Master regulator of intestinal disease: IL-6 in chronic inflammation and cancer development*. in *Seminars in immunology*. 2014: Elsevier.

160. Hodge, D.R., Hurt, E.M. and Farrar, W.L. *The role of IL-6 and STAT3 in inflammation and cancer*. European Journal of Cancer, 2005. **41**(16): p. 2502-2512.

161. Redell, M.S., *et al*., *Stat3 signaling in acute myeloid leukemia: ligand-dependent and-independent activation and induction of apoptosis by a novel small-molecule Stat3 inhibitor.* Blood, 2011. **117**(21): p. 5701-5709.

162. Saitoh, T., *et al*., *Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1β production.* Nature, 2008. **456**(7219): p. 264.

163. Zhong, Z., Sanchez-Lopez, E. and Karin, M. *Autophagy, inflammation, and immunity: a troika governing cancer and its treatment.* Cell, 2016. **166**(2): p. 288-298.

164. Klionsky, D.J., *et al*., *Guidelines for the use and interpretation of assays for monitoring autophagy.* Autophagy, 2016. **12**(1): p. 1-222.

165. D'Eliseo, D. and Velotti, F. *Omega-3 fatty acids and cancer cell cytotoxicity: implications for multi-targeted cancer therapy.* Journal of Clinical Medicine, 2016. **5**(2): p. 15.

166. Fernandez-Bolanos, J.G., *et al., Hydroxytyrosol and derivatives: isolation, synthesis, and biological properties.* Current Organic Chemistry, 2008. **12**(6): p. 442-463.

167. Bernini, R., *et al., Dimethyl carbonate: an environmentally friendly solvent for hydrogen peroxide (H2O2)/methyltrioxorhenium (CH3ReO3, MTO) catalytic oxidations.* Tetrahedron, 2007. **63**(29): p. 6895-6900.

168. Bernini, R., *et al., Procedimento per la preparazione di derivati dell'idrossitirosolo e di idrossitirosolo.* IT Patent, 2010. **1381959**.

169. Castro-Muñoz, R. and Fíla, V. *Membrane-based technologies as an emerging tool for separating high-added-value compounds from natural products.* Trends in Food Science & Technology, 2018.

170. Zheng, W. and Wang, S.Y. *Antioxidant activity and phenolic compounds in selected herbs.* Journal of Agricultural and Food Chemistry, 2001. **49**(11): p. 5165-5170.

171. El Gharras, H., *Polyphenols: food sources, properties and applications–a review.* International Journal of Food Science & Technology, 2009. **44**(12): p. 2512-2518.

172. Azaizeh, H., *et al., Antioxidant activity of phenolic fractions in olive mill wastewater.* Food Chemistry, 2012. **134**(4): p. 2226-2234.

173. Escobar, M.A. and Dandekar, A.M. *Agrobacterium tumefaciens as an agent of disease.* Trends in Plant Science, 2003. **8**(8): p. 380-386.

174. Hammami, I., *et al., Optimization and biochemical characterization of a bacteriocin from a newly isolated Bacillus subtilis strain 14B for biocontrol of Agrobacterium spp. strains.* Letters in Applied Microbiology, 2009. **48**(2): p. 253-260.

175. Ramos, C., *et al., P seudomonas savastanoi pv. savastanoi: some like it knot.* Molecular Plant Pathology, 2012. **13**(9): p. 998-1009.

176. Yangui, T., *et al., Efficacy of olive mill waste water and its derivatives in the suppression of crown gall disease of bitter almond.* European Journal of Plant Pathology, 2008. **122**(4): p. 495-504.

177. Borges, A., *et al., Antibacterial activity and mode of action of ferulic and gallic acids against pathogenic bacteria.* Microbial Drug Resistance, 2013. **19**(4): p. 256-265.

178. Avila, J.G., *et al., Mode of action of Buddleja cordata verbascoside against Staphylococcus aureus.* Journal of Ethnopharmacology, 1999. **66**(1): p. 75-78.

179. Bhunia, K., *et al., Migration of chemical compounds from packaging polymers during microwave, conventional heat treatment, and storage.* Comprehensive Reviews in Food Science and Food Safety, 2013. **12**(5): p. 523-545.

180. Gómez-Estaca, J., *et al., Advances in antioxidant active food packaging.* Trends in Food Science & Technology, 2014. **35**(1): p. 42-51.

181. Abreu, A.S., *et al., Antimicrobial nanostructured starch based films for packaging.* Carbohydrate Polymers, 2015. **129**: p. 127-134.

182. Lourdin, D., Della Valle, G. and Colonna, P. *Influence of amylose content on starch films and foams.* Carbohydrate Polymers, 1995. **27**(4): p. 261-270.

183. Cano, A., *et al., Properties and ageing behaviour of pea starch films as affected by blend with poly (vinyl alcohol).* Food Hydrocolloids, 2015. **48**: p. 84-93.

184. Whistler, R.L., *Methods in carbohydrate chemistry. Volume 4.* Methods in Carbohydrate Chemistry. Volume 4., 1964.

185. Cheetham, N.W. and Tao, L. *Variation in crystalline type with amylose content in maize starch granules: an X-ray powder diffraction study.* Carbohydrate Polymers, 1998. **36**(4): p. 277-284.

186. Beltrán, A., *et al., Characterization of poly (ε-caprolactone)-based nanocomposites containing hydroxytyrosol for active food packaging.* Journal of Agricultural and Food Chemistry, 2014. **62**(10): p. 2244-2252.

187. Fortunati, E., *et al., Binary PVA bio-nanocomposites containing cellulose nanocrystals extracted from different natural sources: Part I.* Carbohydrate Polymers, 2013. **97**(2): p. 825-836.

188. Frone, A.N., *et al., Preparation and characterization of PVA composites with cellulose nanofibers obtained by ultrasonication.* BioResources, 2011. **6**(1): p. 487-512.

189. Thomas, P., *et al., FTIR study of the thermal degradation of poly (vinyl alcohol).* Journal of Thermal Analysis and Calorimetry, 2001. **64**(2): p. 501-508.

190. Cano, A., *et al., Development and characterization of active films based on starch-PVA, containing silver nanoparticles.* Food Packaging and Shelf Life, 2016. **10**: p. 16-24.

191. Kavoosi, G., *et al., Antioxidant, antifungal, water binding, and mechanical properties of poly (vinyl alcohol) film incorporated with essential oil as a potential wound dressing material.* Journal of Applied Polymer Science, 2014. **131**(20).

192. Luzi, F., *et al., Cellulose nanocrystals from Actinidia deliciosa pruning residues combined with carvacrol in PVA_CH films with antioxidant/antimicrobial properties for packaging applications.* International Journal of Biological Macromolecules, 2017. **104**: p. 43-55.

193. Frison, E., Graner, A. and Tuberosa, R. *Genomics of plant genetic resources*. 2014: Springer.

194. Sestili, F., Botticella, E. and Lafiandra, D. *TILLING for improved starch composition in wheat*, in *Genomics of plant genetic resources*. 2014, Springer. p. 467-487.

195. LaCoste, A., *et al., Advancing controlled release packaging through smart blending.* Packaging Technology and Science: An International Journal, 2005. **18**(2): p. 77-87.