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DOTTORATO DI RICERCA IN SCIENZE DELLA VITA

XXXII CICLO

AUTOCHTHONOUS TUSCAN OLIVE LEAVES
(*olea europaea* var. *Olivastra seggianesse*) AS ANTIOXIDANT,
ANTIMICROBIAL AND IMMUNOMODULATORY SOURCE FOR
BIOMEDICINE AND TISSUE ENGINEERING

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ABSTRACT

Background. *Olea europaea* is one of the most ancient trees of the Mediterranean region. Olive leaf extracts (OLE) have aroused interest in researchers from different scientific disciplines mainly due to the distinctive phenolic composition allegedly related to potent biological activities. ***Objectives***. The aim of the present s study was to evaluate: A) the properties of OLE extracted from the Tuscan *Olea europaea* to protect endothelial cells against oxidative stress generated by reactive oxygen species (ROS) in 2D culture and innovative 3D scaffold (P(VDF-TrFE)); B) to investigate the antimicrobial effect of OLE versus Cold Atmospheric Plasma (CAP) technology or their combination against pathogens, i.e., *Escherichia coli*, *Staphylococcus aureus* and *Listeria innocua*, grown to exponential (24h) or stationary (6h) phase; C) to characterize electrospun OLE-loaded PHBHV based composite fibers for wound healing applications; D) to evaluate the ability of OLE incorporated in Polyhydroxyalkanoates (PHAs) fibers to modulate the release of cytokines from healthy Human Keratinocytes (HaCaT). ***Methods***: OLE total polyphenols (TPs) were characterized by the Folin–Ciocalteu method. Endothelial cells were grown in conventional cultures (two-dimensional, 2D) and on three-dimensional scaffold fabricated via electrospinning. Cell viability and ROS production after H_2O_2 insults were evaluated by WST-1, AlamarBlue and Probe CM-H_2DCFDA assays. The *E.coli, S.aureus* and *L.innocua* growth were assessed by CFU/mL and CAP methods; Real-time polymer chain reaction (PCR) was carried out to evaluate the immunomodulatory properties; Fourier Transform Infrared Spectroscopy (FT-IR) Analysis was performed to discriminate the chemical composition in both electrospun fibers. Moreover, Gel Permeation Chromatography (GPC) to allow biodegradation analysis, SEM microscopy to study fiber morphology and HPLC to carry out the release study, were performed. ***Results***: OLE TP content was 23.29 mg of gallic acid equivalent (GAE)/g, and oleuropein was the principal compound. The dose-dependent viability curve highlighted the absence of significant cytotoxic effects at OLE concentrations below 250 µg GAE/ml TPs. OLE preconditioning at 100 µg GAE/ml was protective against ROS in both models. The combination of CAP and OLE resulted in substantial microbial inactivation against all strains at exponential phase showing a complete inactivation. OLE possess a significant anti-inflammatory activity, downregulating the expression of all proinflammatory cytokines, upregulating IL-8, IL- 1α and TNF-α in HaCaT model. ***Conclusion***. OLE possess a significant antioxidant and anti-inflammatory activities; PHBHV+OLE retains OLE beneficial effects and represents a high-value 3D scaffold with great potential in tissue regeneration. CAP and OLE have synergistic antibacterial activity; therefore, CAP technology in combination with OLE can be utilized for effective decontamination when required for example in wound healing and biomedical devices.

Keywords: *Olea europaea*, Olive Leaves Extract (OLE), Endothelial cells, ROS, Scaffold, Polyhydroxyalkanoates (PHA), PHBHV, Gene expression, Biodegradation, electrospinning, Cold Atmospheric Plasma (CAP), anti-inflammatory, wound healing, antioxidant, tissue regeneration.

RIASSUNTO

Background. L'*Olea europaea è* uno degli alberi più antichi della regione mediterranea. Gli estratti di foglie di olivo (Olive Leaf Extracts, OLE) hanno suscitato interesse nei ricercatori di diverse discipline scientifiche principalmente a causa della particolare composizione fenolica, presumibilmente correlata alla sua potente attività biologica. ***Obiettivi***. Lo scopo di questo studio è stato valutare: *a)* le proprietà degli OLE di *Olea europaea* Toscana per proteggere le cellule endoteliali dallo stress ossidativo generato dalle specie reattive dell'ossigeno (ROS), sia in coltura 2D sia in innovativi scaffold 3D (P (VDF-TrFE)); *b)* studiare l'effetto antimicrobico di OLE rispetto alla tecnologia Cold Atmospheric Plasma (CAP), o la loro combinazione, contro agenti patogeni, ad esempio *Escherichia coli*, *Staphylococcus aureus* e *Listeria innocua*, cresciuti a livello esponenziale o in fase stazionaria; *c)* caratterizzare fibre composite elettrofilate a base di PHBHV caricate con OLE per applicazioni nella guarigione delle ferite; *d)* valutare la capacità di OLE incorporato nelle fibre di poliidrossialcanoati (PHA) di modulare il rilascio di citochine da cheratinociti umani sani (HaCaT). ***Metodi***: I polifenoli totali (total polyphenols, TP) di OLE sono stati caratterizzati con il metodo di Folin-Ciocalteu. Le cellule endoteliali sono state coltivate in colture convenzionali (cioè bidimensionali, 2D) e su impalcature tridimensionali fabbricate tramite elettrofilatura. La vitalità cellulare e la misurazione dei ROS dopo lo stress indotto da H_2O_2 sono state eseguite tramite WST-1, alamar Blue e la sonda CM-H_2DCFDA per ROS. La crescita di *E. coli, S. aureus* e *L. innocua* è stata valutata mediante il test di unità (CFU) formanti colonie (espressa in CFU/ml) e CAP. È stata eseguita la reazione a catena della polimerasi in tempo reale (real-time PCR) per valutare le proprietà immunomodulatorie; analisi di spettroscopia infrarossa in trasformata di Fourier (FT-IR), per discriminare la composizione chimica in entrambe le fibre elettrofilate; cromatografia a permeazione su gel (GPC) per consentire l'analisi della biodegradazione; microscopia SEM per lo studio della morfologia delle fibre ed in fine, HPLC per eseguire lo studio di rilascio. ***Risultati***: il contenuto in TP di OLE era 23,29 mg di acido gallico equivalente (GAE)/g e l'oleuropeina era il composto principale. La curva di vitalità dose-dipendente ha evidenziato l'assenza di effetti citotossici significativi a concentrazioni di OLE inferiori a 250 μg GAE/ml di TP. Il pretrattamento di 100 μg GAE/ml di TP di OLE ha avuto effetti protettivi sullo stress ossidativo indotto da H_2O_2 in entrambi i modelli (2D e 3D). La combinazione di CAP e OLE ha determinato una sostanziale inattivazione microbica contro tutti i ceppi in fase esponenziale mostrando una completa inattivazione. OLE ha dimostrato una significativa attività antinfiammatoria, sottoregolando l'espressione di tutte le citochine proinfiammatorie e sovraregolando IL-8, IL-1α e TNF-α nel modello HaCaT. ***Conclusione***: OLE possiede una significativa attività antiossidante e antinfiammatoria; la fibra a base di PHBHV + OLE preserva gli effetti benefici di OLE e rappresenta uno scaffold 3D di alto valore con un grande potenziale nella rigenerazione dei tessuti. CAP e OLE possiedono un'attività antibatterica sinergica; pertanto, la tecnologia CAP in combinazione con OLE può essere utilizzata per una decontaminazione efficace, ad esempio nella guarigione delle ferite ed in dispositivi biomedicali.

Parole chiavi: *Olea europaea*, estratto di foglie di olivo, cellule endoteliali, ROS, scaffold, poliidrossialcanoati, PHBHV, espressione genica, biodegradazione, elettrofilatura, plasma atmosferico freddo, antinfiammatorio, cicatrizzante, antiossidante, rigenerazione dei tessuti.

To my dear
Mother, Father and Siblings

To my team of the Cardiovascular Research Laboratory and Oto-Lab Group
To the Real Amazing Spiderman, a.k.a. Gianfranco Serecchia
To my dear closest friends

ACKNOWLEDGEMENTS

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ACRONYMS

AA	Acrylic Acid
ACN	Acetonitrile
ADP	Adenosin diphosphate
AMPK	5' adenosine monophosphate-activated protein kinase
CAP	Cold Atmospheric Plasma
CM-H$_2$DCFDA	5-(and-6)-chloromethyl-2',7'-dichloro-di-hydro-fluorescein diacetate, acetyl
COX	Cyclooxygenase
CRP	C Reactive Protein
CVD	Cardiovascular Diseases
CRP	C-Reactive Protein
DAPI	4',6-Diamidino-2-phenylindole dihydrochloride
DBD	Dielectric Barrier Discharge
DCM	Dichloromethane
ECM	Extracellular Matrix
EGM-2	Endothelial Cell Growth Medium
EVOO	Extra Virgin Olive Oil
FBS	Fetal Bovine Serum
GAE	Gallic Acid Equivalent
H$_2$O$_2$	Hydrogen Peroxide
HaCaT	Human keratinocytes
Hcy	Homocysteine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High-Performance Liquid Chromatography
HT	Hydroxytyrosol
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM-1	Intercellular Adhesion Molecule 1
IL-1β	Interleukin-6
iNOS	Inducible form of nitric oxide synthase
M199	Medium 199
MD	Mediterranean Diet
MeOH	Methanol
MEK	Methyl Ethyl Ketone
MMP-9	Metalloproteinases-9
mTOR	Mammalian target of rapamycin
Na$_2$CO$_3$	Sodium Carbonate
NCD	Non-communicable diseases
NF-kB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NO	Nitric Oxide
LC3	Autophagy-specific marker
LDPE	Low Density Poly-Ethylene
OLE	Olive Leave Extract
oxLDL	Oxidized low-density lipoprotein
PARP1	Poly (ADP-ribose) polymerase
PBMC	Peripheral Blood Mononuclear Cells
PET	Polyethylene Terephthalate
P(3HO –co-3HD)	Poly(3-hydroxyoctanoate-co-3-hydroxydecanoate)
PHBHV	Poly(hydroxybutyrate-co-hydroxyvalerate)
(P(VDF–TrFE)	Poly(vinylidene fluoride–trifluoroethylene)

ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
SAR	Structure Activity Relationship
SEM	Scanning Electronic Microscopy
SIRT-1	NAD-dependent deacetylase sirtuin-1
SHR	Spontaneously Hypertensive Rats
TE	Tissue Engineering
TFEB	Transcription factor EB
TP	Total Polyphenol
UV	Ultraviolet
WST-1	4-[3-(4-iodophenyl)-2-(4nitrophenyl)-2H-5-tetrazolium]-1,3-benzenedisulfonate ester
VCAM	Vascular Cell Adhesion Molecule 1.

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STATE-OF-THE-ART

1. INTRODUCTION

1.1 Uses of Plant: Medicinal Approach

In many countries in the world, people still rely on traditional plant-based medicines for their primary healthcare. This is especially true for many rural communities in Africa, parts of Asia, and Central and South America, where plants and knowledge of their traditional use are accessible and affordable. In other countries, many of these traditional plant-based medicines are being integrated through regulations into mainstream health systems (Allkin, B., 2017). A variety of plant extracts have been used in traditional medicine over the centuries and some plants and herbs have been identified for provide evidence in the prevention or treatment in many diseases including cardiovascular diseases (CVD), diabetes, weight loss, inflammation, etc. (Poswal, F. et al., 2019), due to the presence of different polyphenols.

Polyphenols, both flavonoids and phenolic acids, are ubiquitous in plants and are mainly present as glycosides. Upon their chemical structure, polyphenols produce different colours from yellow-green to blue-red, especially in flowers. Therefore, they can act as attractants for honeybees or attraction for other pollinators. Moreover, polyphenols can implement structural functions, such as the defence against ultraviolet (UV) radiation or the protection of plants against microbial invasion and herbivores (Harborne and Williams 2000; Manach, C et al. 2004; Taiz and Zeiger, 2009). For human nutrition, polyphenols are well-known for their health properties. Dependent on their chemical structure, polyphenols are antioxidant (or pro-oxidant), anti-inflammatory, or anti-carcinogenic. However, their bioavailability is generally low (Sahin, S et al. 2018), due to the sugar moiety of the flavonoid glycoside can affect the bioavailability, even though only aglycones can be absorbed.

In Western-style diets, chlorogenic acid due to coffee consumption is the major source of polyphenols. Moreover, the quercetin and kaempferol are the most consumed flavonoids and are present in high concentrations in, for example, onion or *Brassica* vegetables.

Many plant species, such as *Crataegus* species, comprehends to be valuable in treating CVDs particularly angina heart failure and hyperlipidemia (Chan, Q. et al., 2002). The leaves, flowers and fruits of *Crataegus* species contain varying quantities of a number of biologically active substances such as oligomeric, procyanidins, flavonoids and catechins. The extract is suggested to have antioxidant properties and inhibits the formation of thromboxane (Bahorun, T. et al.,1994; Vibes, J. et al.,1994). In traditional Chinese medicine the fruit of the hawthorn (usually *Crataegus*) is ideally used for many indications including digestive disorders and for lowering cholesterol and blood pressure (Chang, W.T. et al., 2005). *C. oxyacantha* berry extract antagonized dietary induced increases in cholesterol triglycerides and phospholipid levels in D fractions and very low density

lipoprotein fractions in rats (Shanthi, S. et al., 2004). Thus, it could inhibit the progression of atherosclerosis (Rajendran, S. et al., 1996). Other species like *Rubus* spp. (*Rosaceae*) leaves have been used as antimicrobial, anticonvulsant, and muscle-relaxing agents. *Morus alba* (*Moraceae*) leaves have been applied in these examples clearly demonstrate that leaves can be at least equally interesting as fruits or other parts of plants. Importantly, they are also a much more accessible source of polyphenols than fruits. *Abelmoschus esculentus* also has a beneficial impact on the cardiovascular system. Sabitha *et al*. demonstrated that *A. esculentus* peel and seed powder reduced the blood glucose level and improved the lipid profile level in diabetic male Wistar rats (Sabitha, V et al., 2011). Tea (*Camellia sinensis*, Theaceae), the most important non-alcoholic beverage in the world, has been extensively studied for its putative disease preventive effects. Tea leaves are well known as an abundant source of polyphenols with strong antioxidant properties (Gramza, A. et al., 2005). Regular tea intake prevents cancer and vascular disorders and regulates the digestive system (Khan, N. and Mukhtar, H., 2013). Animal, as well as human studies, point to the cardioprotective effect of black tea by lowering cholesterol level. Also, the ability of black tea to decrease some inflammatory markers and mediators expressed by endothelium clearly points to the beneficial prosperities of this plant towards the vascular system (Dudzińska, D. et al., 2015). Also, *Urtica dioica* (*Urticaceae*) roots and leaves are a remedy for hypertension, diabetes, prostate hyperplasia, and cancer (Ziyyat, A. et al., 1997; Konrad, L. et al., 2000). *Urtica dioica* possesses anti-inflammatory, anti-hyperglycaemic, antimicrobial, antioxidant, anti-ulcer, and analgesic activity (Gülçin, I. et al., 2004). *Urtica dioica* leaf extract, when administered before glucose loading, has demonstrated strong ability to decrease glucose level in alloxan-induced diabetic rats (Bhouham, M. et al., 2003). Extract from *U. dioica* also appears to be an effective scavenger of free radicals, including superoxide anion radicals and hydrogen peroxide. *Allium sativum* (*Amaryllidaceae*), known since ancient times for its healing properties, may be a beneficial agent for the treatment of CVD (Agarwal, K.C. et al., 1996; Neil, H.A., 1996). *Allium sativum* can normalize plasma lipids, enhance fibrinolytic activity, inhibit platelet aggregation, and reduce blood pressure and blood glucose level. In experiments on platelet aggregation evoked by Adenosin diphosphate (ADP) , collagen, or arachidonic acid, Hiyasat *et al*. compared the anti-platelet activity of methanolic and aqueous extracts isolated from the leaves of *A. ursinum* and *sativum*. Alcoholic extracts of both species and an aqueous extract of *A. sativum* most efficiently inhibited ADP-induced platelet aggregation, while an aqueous extract of *A. ursinum* inhibited platelet aggregation, but the effect did not depend on the type of platelet agonist (Hiyasat, B. et al., 2009). Other plants like *Ginkgo* extract also demonstrates cardioprotective activity, which has been demonstrated in an experiment with HgCl2-induced oxidative damage in Wistar albino male and female rats. While HgCl2 has been shown to significantly increase thromboplastic activity and malondialdehyde levels or decrease glutathione levels in serum and tissue samples, this effect has

been effectively reversed by *Ginkgo* leaf extract (Tunali-Akbay, T. et al., 2006). *Dicksonia sellowiana* (*Dicksoniaceae*) is a common tree in Central and South America; its leaves are used in a folk medicine to treat scabies, pruritus, parasitic diseases, and asthma. Hydroalcoholic extract of *D. sellowiana* (HEDS) decreases hypertension and induces endothelium-dependent relaxation in spontaneously hypertensive rat (SHR) aortic rings. Hydroalcoholic extract of *D. sellowiana* induces aortic relaxation by activation of muscarinic receptors and stimulation of the NO pathway in SHR rat aortic endothelium. Others like sweet cherry (*Prunus avium*) and tart or sour cherry (*Prunus cerasus*) have global trading importance and are now growing widely around the world. Although the commercial cultivation of sweet cherries is more difficult and expensive than that of tart cherry, the first ones are prized for their excellent taste and nutritious nature, thanks to the high presence of sugars and the low level of acidity. Depending on pre- and post-harvest factors, sweet cherry also contains high levels of nutrients, including glucose, fructose, vitamin C, and bioactive compounds, which present various health benefits (Chockchaisawasdee, S. et al., 2016). Several clinical studies have shown that cherry fruit or juice consumption plays an important role in the struggle against inflammatory diseases (Kelley, D.S., 2018). Moreover, a recent study confirmed the protective effects of cherry extracts to reduce ROS accumulation *in vitro* and the main role of its polyphenols in inflammation reduction and endothelial dysfunction improvement (Beconcini, D. et al, 2019). One of the most common plant known for its properties in the human health is the olive tree, mainly the oil olive, fruit, and leaves (Romani, A. et al., 2019). Specifically, the use of the olive leaves (extract) has been explored due to its phenolic composition. Such bioactive ingredients are used in medicines, pharmaceuticals or cosmetics, to improve the human health as well as to develop functional foods (Abaza, L. et al., 2015). The main uses of plant components in the biomedical field are summarized in table 1.

Plant species	Bioactive component	Biomedical application/Uses	References
Crataegus spp. **Extract**	Oligomeric, procyanidins, flavonoids and catechins	-Angina heart failure and hyperlipidemia	Chan, Q. et al., 2002
Rubus **spp Leaves Extract**	Polyphenols	-Antimicrobial -Anticonvulsant, -Muscle-relaxing agents.	Rajendran, S., et al., 1996
Abelmoschus esculentus **Peel and seed extract**	Polyphenols	- Peel and seed powder reduced the blood glucose level and improved the lipid profile level in diabetic male Wistar rats.	Sabitha, V., et al., 2011
Camellia sinensis (Tea) **Leaves Extract**	Antioxidants	-Studied for its putative disease preventive effects. - Studies for vascular disorders and regulates the digestive system	Gramza, A., et al., 2005 Khan, N., and Mukhtar, H., 2013
Urtica dioica **Extract.**	Polyphenols	-Anti-inflammatory. -Anti-hyperglycaemic, -Antimicrobial -Antioxidant -Anti-ulcer	Gülçin, I., et al., 2004 Bhouham, M., et al., 2003

		-Analgesic activity effective scavenger of free radicals.	
Allium sativum	Allicin	-Normalize plasma lipids. -Enhance fibrinolytic activity. -Inhibit platelet aggregation. - Reduce blood pressure and blood glucose level.	Hiyasat, B., et al., 2009
Ginko biloba	Polyphenols	-Cardioprotective activity, demonstrated in an experiment with HgCl2-induced oxidative damage in Wistar albino male and female rats.	Tunali-Akbay, T., et al., 2006
Dicksonia sellowiana	Polyphenols	- Studies showed decreases hypertension and induces endothelium-dependent relaxation in spontaneously hypertensive rat (SHR) aortic rings.	Rattmann, Y. et al., 2012
Prunus avium **Fruit Extract**	Polyphenols	- Reduce ROS accumulation *in vitro*	Beconcini, D. et al., 2019
Oregano oil (from Origanum vulgare)	Polyphenols. Nanoemulsion	- Controlled and reduced the growth of food-borne bacteria (L. monocytogenes, S. Typhimurium, and E. coli) on fresh lettuce.	Bhargava, K., et al., 2015
Silybum marianum- **loaded PLGA. Nanosphere**	Silymarin	- Improved encapsulation efficiency, sustained release, high internalization by cells and preferential toxicity to prostate cancer cells.	Snima, K.S., et al. 2014
Curcuma longa. **Nanocrystal**	Curcumin	-Improved solubility, stability, bioavailability and biodistribution. Enhanced skin penetration and uptake, and targeting hair follicles are also seen in vitro using the porcine skin	Vidlarova, L., et al., 2016
Aloe vera **Nanofibers**	Aloin, Aloesin	-Increased hydrophilicity of fabricated nanofiber. Enhanced in vitro biocompatibility of gel-coated scaffold on fibroblast cells using MTT assay.	Jouybar, A., et al., 2017
Rosmarinus officinalis **Extract.**	Antioxidants	-Antimicrobial effects against pathogenic microorganism.	Amaral, G.P. et al., 2018
Theobroma cacao **Powder Extract**	Antioxidants.	-Recent In vitro studies showed effects against ROS-induced oxidative stress in Human Umbilical Vein Endothelial Cells (HUVECs)	Felice, F. et al., 2020

Table 1. Uses/applications of plant components in biomedical field.

1.2 Olive Leaves Extract: Uses, Characteristics and Properties

Olea europaea is one of the most ancient trees of the Mediterranean region. Olive leaf extracts (OLE) have been an interest in researchers from different scientific disciplines mainly due to the distinctive phenolic composition allegedly related to potent biological activities (Goulas V., et al., 2009). Olive leaves contain considerable bio-phenols as the other parts of the olive tree (Sahin, S., and Bilgin, M., 2017). OLE is used principally as a food supplement and an over-the-counter drug for a variety of beneficial effects, including anti-inflammatory and anti-atherosclerotic ones. Phenolic compounds in OLE are secondary metabolites of plant, which play important roles in disease resistance (Servili and Montedoro, 2002; Ryan, D., et al., 1999), protection against pests and species dissemination. The interest in these compounds is related with their antioxidant activity and promotion of health benefits (Ryan, D et al., 2002). The Mediterranean diet's healthy effects can in particular be attributed not only to the high relationship between unsaturated and saturated fatty acids in olive oil but also to the antioxidant property of its phenolic compounds. The main phenolic compounds, hydroxytyrosol (HT) and oleuropein, which give extra-virgin olive oil its bitter, pungent taste, have powerful antioxidant activity both in vivo and in vitro. (Manna et al., 1999; Servili et al., 2004; De la Torre-Carbot, et al., 2005). Besides, literature reports several compounds of the class of flavonoids that may occur in appreciable amounts (Savournin, et al., 2001) and become the major leaf constituents in certain phenological stages (Papoti & Tsimidou, 2009). Consequently, phenolic compounds have antioxidant activity, being attributed to the capacity of scavenging free radicals, donating hydrogen atoms, electrons, or chelate metal cations, characteristics studies in molecules as Quercetin and Catechin (Afanas, I.V., et al,1989). The number and positions of the hydroxyl groups, and the nature of substitutions on the aromatic rings, gives to phenolic compounds the capacity of inactivating free radicals, which is referred to as structure–activity relationships (SARs) (Minatel, I.V., et al, 2017). In Figure 1 is schematized the principal biomolecules in OLE.
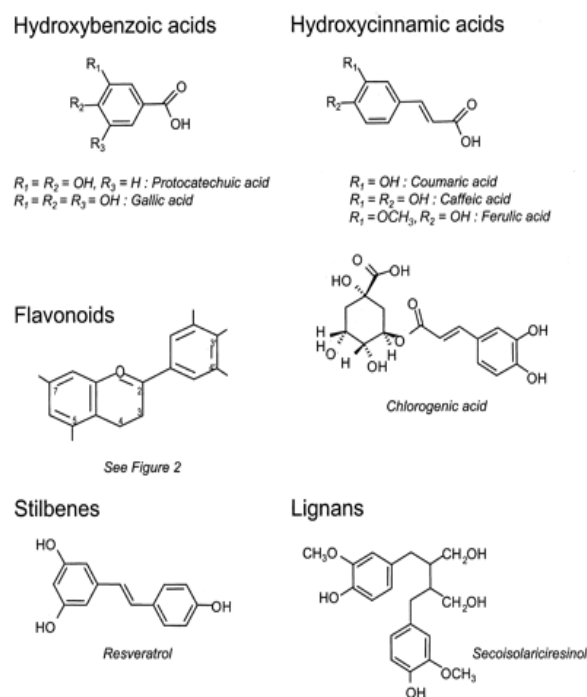
Figure 1. *Chemical structure of polyphenols. Source: © 2004 American Society for Clinical Nutrition*

The OLE are characterized by the presence of polyphenols responsible for some health properties of olive oil, including anti-atherogenic, anti-inflammatory, anti-aging, anti-tumour, anti-viral, and immune modulatory activities (Sahin, S., et al, 2018; Susalit, E., et al., 2011; Boss, A., et al.,2016; Magrone, T., et al., 2018).

The polyphenols in OLE that are the most common in the human diet are not necessarily the most active within the body, either because they have a lower intrinsic activity or because they are poorly absorbed from the intestine, highly metabolized, or rapidly eliminated. In addition, the metabolites that are found in blood and target organs and that result from digestive or hepatic activity may differ from the native substances in terms of biological activity. Extensive knowledge of the bioavailability of polyphenols is thus essential if their health effects are to be understood. The aglycones can be absorbed from the small intestine. However, most polyphenols are present in food in the form of esters, glycosides, or polymers that cannot be absorbed in their native form. These substances must be hydrolyzed by intestinal enzymes or by the colonic microflora before they can be absorbed. When the flora is involved, the efficiency of absorption is often reduced because the flora also degrades the aglycones that it releases and produces various simple aromatic acids in the process. During absorption, polyphenols are conjugated in the small intestine and later in the liver. This process mainly includes methylation, sulfation, and glucuronidation (Figure 2).
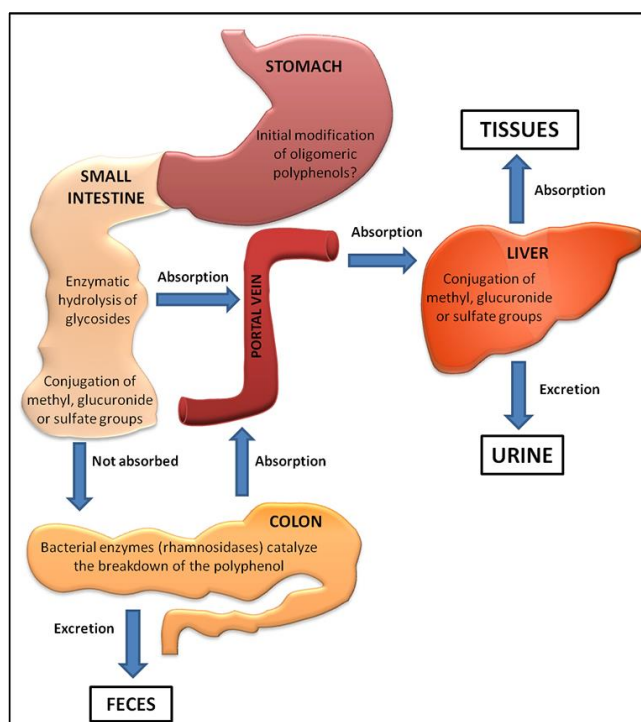
Figure 2. Predicted routes for absorption of dietary phenolics. Source: Igor Otavio Minatel, Cristine Vanz Borges, Maria Izabela Ferreira, Hector Alonzo Gomez Gomez, Chung-Yen Oliver Chen and Giuseppina Pace Pereira Lima (March 8th, 2017). Phenolic Compounds: Functional Properties, Impact of Processing and Bioavailability, Phenolic Compounds - Biological Activity, Marcos Soto-Hernandez, Mariana Palma-Tenango and Maria del Rosario Garcia-Mateos, IntechOpen, DOI: 10.5772/66368. Available from: https://www.intechopen.com/books/phenolic-compounds-biological-activity/phenolic-compounds functional-properties-impact-of-processing-and-bioavailability

Oleuropein (Figure 3), related secoiridoids and other derivatives are the principal compounds of olive leaves, but other phenolic compounds are present in both olive (*Olea europaea* L.) fruit and leaves. These phenolic compounds include, among others, HT, tyrosol, rutin, verbascoside, luteolin-7-glucoside, and oleuropein.

Oleuropein, is the major and most abundant phenolic compound in olive leaves and fruits and is responsible for the characteristic bitterness of the olive fruit (Tayoub, G. et al., 2012). The concentration of oleuropein can reach up to 140 mg/g (14%) on a dry matter basis in young olives and 60-90 mg/g of dry matter in the leaves.
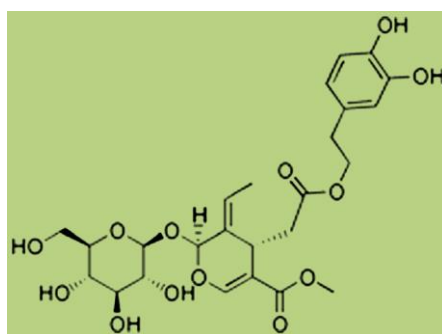
Figure 3. Structure of oleuropein, the major phenolic compound in olive leaves.

1.2.1 Endothelial Dysfunction and OLE

Oxidative stress is characterized by an imbalance between reactive oxygen species (ROS) and reactive nitrogen species (RNS) levels, and the enzymatic antioxidant protection system and plays a key role in the pathophysiological mechanisms leading to CVD. ROS refers to a group of small reactive molecules and are produced under both the normal life process and the various pathological conditions. ROS can function as a signaling molecule or a risk factor for the occurrence of diseases (Xu, T et al 2019). The levels of intracellular ROS are precisely regulated to limit it to a certain level. The central role in the pathogenesis belongs to the vascular endothelium is correlated to the oxidative stress. Vascular biology assumes a pivotal role in the initiation and perpetuation of cardiovascular tissue and organ damages. In various pathophysiological states, an imbalance due to a reduced nitric oxide (NO) production and an increased ROS production, so-called "oxidative stress", may promote endothelial dysfunction and lead to cardiovascular complications, inflammation, increased expression of redox-sensitive proinflammatory genes and cell adhesion molecules (Kouka, P., et al., 2017; Woywodt, A. et al., 2002). All these are closely interrelated and establish a deadly combination that leads to endothelial dysfunction, vascular smooth muscle and cardiac dysfunction, hypertension, vascular disease, atherosclerosis, and CVD (Micucci, M., et al., 2014). Understanding the mechanism of ROS production, ROS-related signaling pathways, and their different roles played under different pathological conditions, is essential for increasing the chance of success during CVD treatment (Xu, T. et al., 2019). Figure 4 reports the pathways leading to the production of ROS. Due to high oxygen consumption by increased mitochondrial activity, the transfer of a single electron to molecular oxygen gives rise to a monovalent reduction of oxygen, which leads to the formation of superoxide ions. The enzymatic process can also promote superoxide production through NADPH oxidase enzymes or the xanthine/xanthine oxidase system. Moreover, in the presence of transition metal ions (e.g., Fe^{2+n3+}, Cu^{+n2+}), hydrogen peroxide (H_2O_2) produces the highly reactive hydroxyl radical ($OH^{\cdot}$) and hydroxyl ion ($OH-$), according to the Fenton reaction.
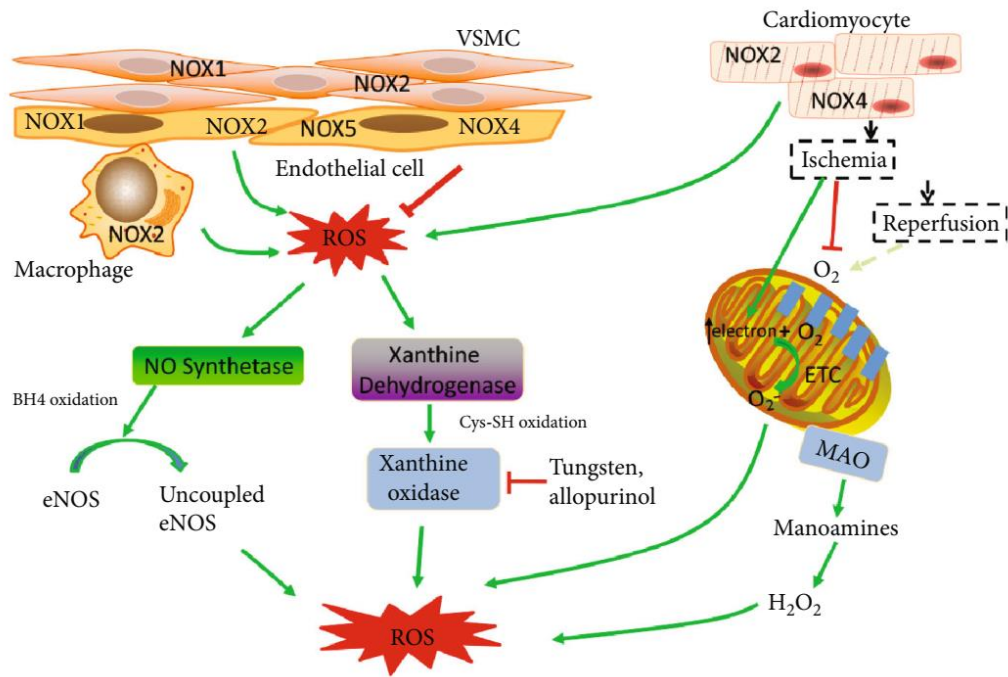
Figure 4: ROS resources during cardiovascular diseases. The NOX-derived ROS are the primary ROS resources. NOX1, NOX2, NOX4, and NOX5 are expressed in the endothelial cell. NOX1 and NOX2 are expressed in the VSMC. NOX2 and NOX4 are abundant in cardiomyocyte. The activity of NOX2 in the immune cells also contributes to the ROS production under pathological condition. NOX-derived ROS can uncouple the NO synthase and promote O2-generation. The xanthine dehydrogenase is transformed into xanthine oxidase by oxidation which uses oxygen as an electron acceptor and produces ROS. Ischemia disrupts the oxygen supply and promotes the electron accumulation of electron transport chain. Reperfusion recovers the oxygen and promotes O2-production. Monoamine oxidase (MAO) anchored on the mitochondrial outer membrane degrades the monoamines and produces H2O2. Source: Xu, T., et al., 2019.

Despite the fact that small quantities of intracellular ROS are constantly produced in the cells, the excessive generation of ROS, caused by pathological stimuli or by the failure of ROS clearance system, is the major cause of various vascular dysfunctions. Several evidences suggest that excessive ROS production contributes to the altered vascular functions including endothelial dysfunction, vascular smooth muscle cell overgrowth, and structural remodelling. Furthermore, oxidative stress could induce vascular inflammation and injury through activation of the transcription factors, upregulation of adhesion molecules, stimulation of chemokine production, and recruitment of inflammatory cells (Xu, T., et al., 2019; Förstermann, U., et al., 2008; Thomas, S.R., et al., 2008).

The leaves of the olive tree (*Olea europaea* L.) have been used since ancient times to combat issues related to high blood pressure, atherosclerosis, diabetes and also in endothelial dysfunction, hypertension and for other medicinal purposes (Romero, M., et al., 2016). Nowadays, endothelial dysfunction is a trademark underlying vascular disease caused by some risk factor like aging, diabetes, or arterial hypertension (Figure 5). In the aorta, NO is the leading factor accounting for endothelium-dependent relaxation (Vanhoutte, P.M., and Miller, V.M., 1985). Thus, the diminished acetylcholine-induced relaxation indicates an impaired agonist-induced NO bioactivity. In an animal model using hypertensive rats, OLE treatment can eliminate the altered responses to acetylcholine

observed in aortae, indicating a protective role in agonist-induced NO bioactivity (Romero, M., et al., 2016). Additionally, HT, the main metabolite of oleuropein (Del Boccio, P., et al., 2003), also reversed the reduced intracellular NO levels stimulated by acetylcholine in endothelial cells incubated *in vitro* with high glucose and free fatty acids (Storniolo, C.E., et al., 2014). eNOS is a constitutive enzyme, controlled at the transcriptional and post-transcriptional levels. The post-transcriptional eNOS regulation is dependent on the phosphorylation state, mainly on a serine residue (Ser-1177) on the reductase domain and on a threonine residue (Thr-495) within the calcium/calmodulin residue. Thus, the functional changes observed in endothelium-dependent relaxation should be attributed to an alteration in NO synthesis and/or its bioavailability. A key mechanism of endothelial dysfunction in hypertension involves the vascular production of ROS, particularly superoxide ($O_2^{\cdot-}$), which reacts rapidly with NO and inactivates it (Tschudi, M.R., et al., 1996; Kobayasi, R., et al., 2010). The enhancement of ROS production, in particular $O_2^{\cdot-}$, affects the endothelial function not only by reducing NO bioavailability, but also by promoting inflammation (Vila, E . and Salaices, M., 2005) via MAPK signaling pathways, that is also modulated by ROS (Griendling, K.K., et al., 2000). OLE administration, which reduces the ROS level, also reduces p38 activation and the inducible form of nitric oxide synthase (iNOS) enzyme found in the aorta from Spontaneously Hypertensive Rats (SHR) (Romero, M., et al., 2016)
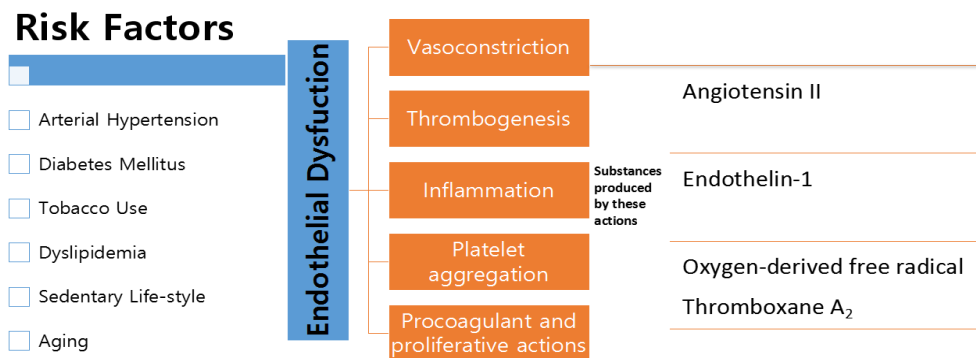
Figure 5. Risk factors and endothelial dysfunction.

However, the mechanisms by which the biomolecules of OLE acts are unclear but possible path execution have been proposed for oleuropein and oleuropein metabolites absorption. As reported by Manna et al. (2003), oleuropein-glycoside may diffuse through the lipid bilayer of the epithelial cell membrane and be absorbed via a glucose transporter or, as additional mechanisms, via the paracellular or transcellular passive diffusion (Manna, C., et al., 2003).

Oleuropein presents two isoforms, glycosidic form and aglycone, that have recently attracted scientific attention by virtue of their health benefits not only in cardiovascular system, but also as antioxidants, anti-inflammatory, neuro-protective, and anti-cancer compounds. These

pharmacological activities are mainly due to their putative radical scavenging features, due to the ortho-diphenolic group. It is important to say that the mechanistic studies indicate that these compounds are also able to act at different sites, interfering with protein function and gene expression, or modifying cellular pathways relevant to the non-communicable diseases (NCDs) i.e. a group of long-lasting and slowly progressive chronic disorders pathological processes (Nediani, C., et al., 2019), suggesting that the actions of oleuropein in various disorders may result from shared molecular mechanisms. For example, dysregulated autophagy is a common feature of NCDs. This dysregulation seems to be due to increased oxidative stress, so, although these mechanisms are generally viewed as cell autonomous, recent evidence suggests an occurrence of an interplay between autophagy and oxidative stress that influences the inflammatory state of tissues, linked with NCD development (Pena-Oyarzun, D., et al., 2018) (Figure 6).
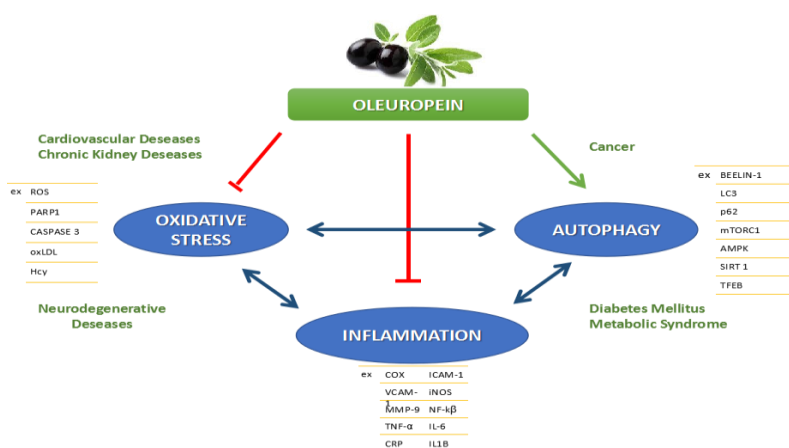
Figure 6. Effect of oleuropein on interplay between oxidative stress, autophaghy and inflammation in non-communicable diseases. AMPK, 5' adenosine monophosphate-activated protein kinase; Beclin-1 autophagy-specific marker; COX, Cyclooxygenase; CRP, C Reactive Protein; Hcy, homocysteine; ICAM-1, Intercellular Adhesion Molecule 1; IL-1β, interleukin-1β; IL-6, interleukin-6; iNOS, inducible form of nitric oxide synthase; LC3 autophagy-specifific marker; MMP-9, metalloproteinases-9; mTOR, mammalian target of rapamycin; NF-kB, Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells; oxLDL, oxidized low-density lipoprotein; p62 autophagy-specifific marker; PARP1, Poly (ADP-ribose) polymerase; ROS, Reactive Oxygen Species; SIRT-1, NAD-dependent deacetylase sirtuin-1; TFEB, Transcription factor EB; TNF-α, tumour necrosis factor-α; VCAM-1, Vascular Cell Adhesion Molecule 1. Source: Illustration Modified from Nediani et al., 2019.

1.2.1.1 Endothelial dysfunction: Human Umbilical Vein Endothelial Cells (HUVEC) as model system.

Endothelial cells (ECs) are important cell that line the blood vessels regulate both vascular tone and blood vessel permeability and are very sensitive to injury caused by oxidative stress (Dejana, E., et al., 2001). The results from the injury leads to compensatory responses that alter the normal

homeostatic properties of the ECs. Thus, the injury increases the adhesiveness of the endothelium to leukocytes and platelets, as well as its permeability. The injury also induces a procoagulant state and the release of vasoactive molecules, cytokines, and growth factors (Xu, T., et al., 2019).

The Human Umbilical Vein Endothelial Cells (HUVECs) are cells derived from the endothelium of veins from the umbilical cord, these cells offer a classic model system to study the different aspects associated to endothelial function and disease, such as normal, abnormal and tumor-associated angiogenesis, oxidative stress, hypoxia and inflammation related pathways in endothelial under normal and pathological conditions, cardiovascular-related complications associated with various diseases, mode of action and cardiovascular protection effects of various compounds, and so on (Park, et al., 2006). In previously works, the HUVECs have been selected and tested to demonstrate stimulation dependent angiogenesis and key endothelial cell signaling pathways (e.g., phosphorylation of VEGFR, Akt, MAPK, and expression of Tie2, eNOS, Axl and Etk/Bmx). The obtained evidence indicated that HUVECs are an important *in vitro* model useful in molecular medicine including pathophysiology of atherosclerosis and plaque formation, and mechanisms for the control of angiogenesis or neovascularization in response to hypoxia and inflammation in tumors, ischemic tissue, and in embryogenesis (De Paola and Burn 2005; Park et.al, 2006). A nutraceutical intervention with OLE using HUVEC as model could be a new perspective for the study of dysfunction of the endothelium and damages in the vascular system.

Other studies have been developed resulting in promising outcomes in cardiovascular diseases and problems related as is show in Table 2.

Aim of study	Relevant outcomes	References
Hyperlipidemia	- OLE extract (1.200 mg/day for 28 days) decreased total cholesterol, LDL cholesterol, total cholesterol/HDL cholesterol ratio, oxidized LDL, and GGT in Granada, Spain 39 hypercholesterolemic subjects (aged 45.0 ± 8.8 years)	Fonolla, Diaz-Ropero, de la Fuente, & Quintela, 2010
Inflammation	- In vitro study with Andalusian extract inhibited pro-inflammatory mediator NO in LPS stimulated RAW264.7 cells	Talhaoui, et al. 2016
	- Double-blind randomized crossover using OLE polyphenols (6 mg hydroxytyrosol, 136 mg oleuropein) for 6 weeks reduced the interleukin−8 in 60 experimental participants.	Lockyer, et al. 2017
Antihypertensive	- Double-blind randomized crossover. OLE polyphenols (6 mg hydroxytyrosol, 136 mg oleuropein) for 6 weeks reduced BP without the alternation in inflammation, glucose metabolism and vascular function biomarkers in 60 experimental participants.	Lockyer, et al. 2017
	- In vivo study demostraded that Oleuropein reduced SBP in male SHR Sprague Dawley rats	Ghibu, et al. 2015
	- Study in human. OLE extract EFLA$^{®}$943 (500−1,000 mg) tablets to hypertensive monozygotic twins (40n, age: 16–60), significant reduction in BP was observed after 8 weeks.	Perrinjaquet-Moccetti, et al. 2008
	- In vivo study using OLE (30 mg/kg/day bw 5 weeks) to SHR rat reduced SBP by modulating the pro-oxidative and pro-inflammatory status and improved vascular function.	Romero, et al., 2019
Oxidative Stress	- Animal model. OLE extract 50–100 g/kg bw for 8 weeks to the pigs significantly protected RBCs hemolysis from AAPH or H_2O_2 initiators in dose-dependent manner.	Paiva-Martins, et al. 2014

Obesity	- Randomized, double-blind, placebo-controlled study, 77 healthy adult overweight/obese subjects (aged 56 ± 10 years and BMI 29.0 ± 2.7 kg/m^2) with total cholesterol levels of 5.0–8.0 mmol/L (5.9 ± 0.7 mmol/L) were randomly assigned to receive 500 mg of OLE ($n = 39$) or placebo ($n = 38$) for 8 weeks. OLE supplementation did not significantly affect blood lipid levels after 4 weeks or after 8 weeks compared to placebo (all $p > 0.05$).	Stevens, Y., et al., 2020
Cardioprotective. hepatic, and metabolic signs of a high-carbohydrate	- Animal model. Results strongly suggest that an OLE containing polyphenols such as oleuropein and hydroxytyrosol reverses the chronic inflammation and oxidative stress that induces the cardiovascular, hepatic, and metabolic symptoms in this rat model of diet-induced obesity and diabetes without changing blood pressure.	Poudyal, H., et al. 2010

Table 2. State-of-the-art of application of OLE in cardiovascular diseases and related.

1.2.2 Wound Healing and OLE

Wound is still one of the main health concerns due to the complications from co-morbidities such as diabetes or infection (Sahana, T.G. and Rekha, P.D., 2018). Wound healing is a complex and dynamic process connecting a cascade of biological reactions initiated in response to an injury. The process mainly involves the interaction of immune and non-immune cells (i.e., endothelial cells, fibroblasts, keratinocytes) with soluble mediators (i.e., cytokines and growth factors), and extracellular matrix (ECM) components (Sahana, T.G., et al., 2018; Singer, A.J., 1999; Gurtner, G.C.,et al., 2008) (Figures 7 and 8). The rate of healing in acute wounds differs from that in chronic wounds and is also dependent on the immunological status of the patient (Demidova-Rice, T.N., et al., 2012). Skin ulcers represents a healthcare challenge for the medical sectors affecting several million people worldwide (WHO).
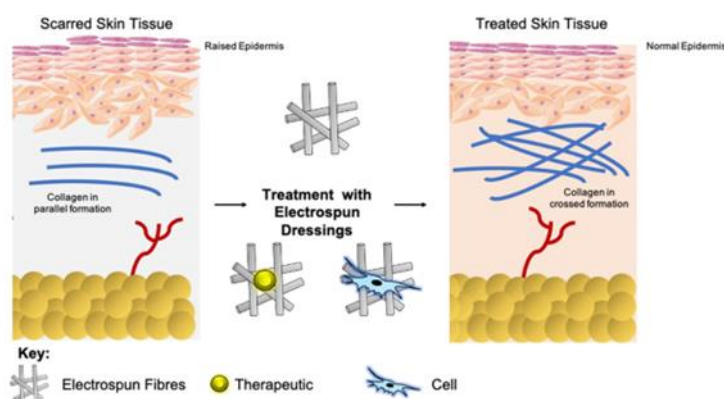
Figure 7. *General schematic of wound healing scar. Source: Mulholland EJ (2020) Electrospun Biomaterials in the Treatment and Prevention of Scars in Skin Wound Healing. Front. Bioeng. Biotechnol. 8:481. doi: 10.3389/fbioe.2020.0048*

Moreover, some processes skin regeneration involving the expression of defensin in the epidermal cells of tissue-engineered skin can be activated, which may play an important role in the effectiveness ulcer replacements (Rezaie, F., et al., 2019; Baltazar, T., et al., 2020). However, several factors affecting the specific tissue microenvironment, sustained by the perseverance of inflammation, still make ulcer healing an unmet clinical need, which deserves additional research (Sen, C.K., et al., 2009). On the other hand, other critical process in skin regeneration is the re-epithelialization of wounded skin due to requires the rapid and coordinated migration of keratinocytes (KCs) into the wound bed. Almost immediately after wounding, cells present at or attracted to the wound site begin to secrete a complex milieu of growth factors. Growth factors are molecules that stimulate cellular growth, proliferation, differentiation, and/or migration. Growth factors can be small molecules such as hormones or macromolecules such as proteins and can be secreted as fully functional molecules or as molecules that require further posttranslational processing to be activated. These growth factors exert mitogenic and motogenic effects on KCs, inducing the rapid proliferation and migration of KCs at the wound edge (Seeger, M.A., and Paller, A.M., 2014). In fact, these growth factors in KCs have a role in the motility of the cells. The activities of these growth factors are regulated both temporally and spatially in the wound site to establish the well-choreographed process of wound healing. On wounding, KCs along the wound edge begin a dramatic rearrangement of their cytoplasmic and membrane structures. These changes include the disassembly of most hemidesmosomes (e.g., cell-ECM contacts) and many desmosomes (e.g., cell–cell contacts), retraction of cytoplasmic keratin intermediate filaments from the periphery of the cell, and rearrangements of the actin cytoskeleton that facilitate the formation and retraction of lamellipodia and focal adhesions (Seeger, M.A and Paller, A.M., 2014).
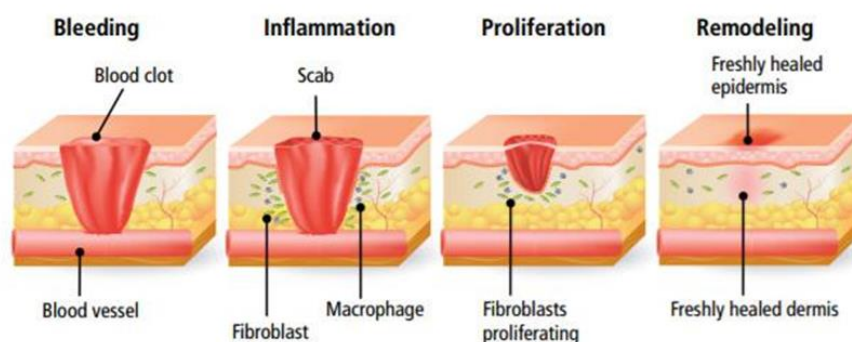
Figure 8. The Wound Healing Phases. Source:https://www.skincol.com/en/info/mechanic-of-action.html

Leaves and fruits of *Olea europaea* L. have been used externally as an emollient for skin ulcers and for healing of inflammatory wounds. Moreover, in animal study the aqueous extract of *O. europaea* leaves displayed wound healing activity (Koca, U., et al., 2011). It is believed that OLE acts as a free radical scavenger by a synergy of its many polyphenols. It is thus important a means for local delivery of OLE inside ulcers, which at the same time, induce connective tissue regeneration. In Table 3 is summarized some relevant studies of the utilization of OLE in wound healing.

Plant origin	Outcomes	References
Razi Herbal Medicine Institute, Lorestan, Iran.	- Results suggested that Oleuropein accelerates skin wound healing in aged male Balb/c mice. These findings can be useful for clinical application of Oleuropein in expediting wound healing after surgery.	Mehraein, F., et al., 2013
Olea europaea var. europaea were collected from Bahcelievler region, Turkey.	- In vivo model resulted in animals treated with the aqueous extract demonstrated increased contraction (87.1%) on excision and a significant increase in wound tensile strength (34.8%) on incision models as compared to other groups	Koca, U., et al., 2011
Olea europaea from Turkey	In vitro study. NIH-3T3 cells were treated with crude OLE and H2O2 as controls in wound scratch assay. In stress condition results indicated that lower concentrations (between 1-50 µM) of H2O2 promoted cell migration.	Erdogan, et al., 2018
Olea europaea leaves from Iran.	The results showed that control group significantly showed higher wound area, total bacterial count, and higher expressions of IL-1β and TNF-α (*P*<0.05) and lower expressions for IL-10 and TGF-β (*P*<0.05). The treatment with OLE could significantly decrease wound area, total bacterial count, expressions of IL-	Jazideh, F et al., 2020

16

1β and TNF-α, and increase the expression of IL-10 and TGF-β (*P*<0.05).

Olea europaea from Turkey.	- OLE incorporated in dressing showed in a Spraque Dawley rats diabetic group; wounds closure time of 24.80 days ± 1.48 in OLE wound dressing and 28.00 ± 2.31 days in classical wound dressing.	Samancioğlu, S. et al., 2015

Table 3. OLE and wound healing studies.

1.2.3 Immunomodulation and OLE

The complexity of immune reactions occurs via the production of both pro- and anti-inflammatory cytokines and antibacterial molecules with different time scale, which ultimately ensure an efficient tissue response to foreign agents. The biological mediators of innate immunity and inflammation are cytokines, biological molecules that act as soluble mediators of natural immunity and the immune response. They are described as multi-functional molecules, together with chemokines and adhesion molecules, which play important biological activities (Mantovani, A., et al., 2004).

In recent years numerous studies have attributed to the polyphenols a broad range of biological activities including anti-inflammatory and immune-modulatory actions (Santangelo, C., et al., 2007; Yahfouti, N. et al., 2018). Inflammation is known to be a significant reason connected to various human issues including cancer, diabetes type II, arthritis, neurodegenerative diseases, and cardiovascular issues i.e. a multifactorial response (Bengmark, S., 2004). Polyphenols have demonstrated positive effects *in vitro* and *in vivo* studies and beneficial role as therapeutic tools in multiple acute and chronic disorders (Gonzalez, R., et al., 2011). Numerous epidemiological and experimental researches have evidenced the anti-inflammatory and immune modulation activities of dietary polyphenols, including OLE, showing the ability of these natural compounds to modify the expression of several pro-inflammatory genes, like multiple cytokines, lipoxygenase, iNOS, cyclooxygenase (COX), in addition to their antioxidant characteristics (Santangelo, C., et al., 2007). The first evidences about the anti-inflammatory and immunomodulatory effects of natural compounds was in 2006 in the clinical trial called PREDIMED. The research showed the anti-inflammatory effect of the Mediterranean Diet supplemented with extra virgin olive oil (EVOO) for a period of three months in comparison with a low fat diet. The authors found a significant decrease of serum C-Reactive Protein (CRP), interleukine (IL) IL-6, adhesion molecules (ICAM-1 and VCAM-1), and chemokines, in a group of 722 participants (Estruch, R., 2010). Also, in another

human trial, 18 healthy volunteers, who had consumed on a single occasion OLEs (51 mg oleuropein and 10 mg HT), were evaluated for vascular function via digital volume pulse (DVP) and cytokine production from endotoxin-stimulated peripheral blood mononuclear cells (PBMCs) (Lockyer, S., et al., 2015). DVP stiffness index and IL-8 production were significantly reduced in comparison to matched untreated controls. Oleuropein and HT urinary metabolites peaked after 8-24 h from administration. Considering the evidences based in MD together with administration of OLE, data suggest that oleuropein and HT exert various protective effects in the host, such as enhanced NO generation by murine macrophages (Manna, C., et al., 2004), induction of the anti-inflammatory pathway in human whole blood cultures (Magrone, T., et al., 2017), prevention of oxidative myocardial injury due to ischemia and reperfusion (Vinson, J.A., et al., 1995; Miao, J., et al., 2019), decrease in blood pressure, and platelet aggregation, inhibition of 5- and 12-lipooxygenases and increased free radical scavenging (Miles, E., et al., 2005), as well as to reduce chronic inflammation and oxidative stress responsible for cardiac and hepatic damage, also preventing the outcome of metabolic syndrome, as reported in an animal model (Pirozzi, C., et al., 2016). Significant advances have been elucidated with respect to the anti-inflammatory mechanisms exerted by OLEs. Macrophages represent one of the principal factors in the inflammatory response, because can produce ROS, but also pro-inflammatory cytokines and chemokines, including IL-1, IL-6, TNF-a, and IFN-γ (Nediani, C., et al., 2019) (Figure 9).
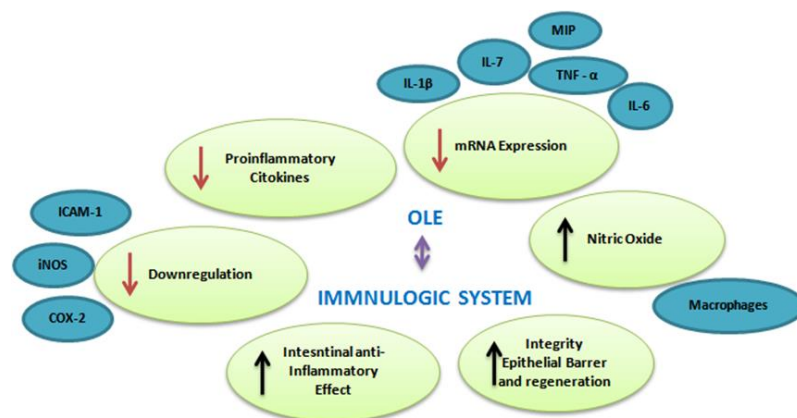
Figure 9. Characteristics of OLE in the immune system studied. MIP (Macrophage inflammatory protein-2), ICAM (intercellular adhesion molecule 1), iNOS (inducible nitric oxide synthase), TNF (tumor necrosis factor), IL (Interleukine).

Therefore, besides the release of several inflammatory cytokines or mediators, damaged tissues also release monocyte chemoattractant proteins (MCP-1), COX, iNOS, metalloproteinases (MMP), and adhesion molecules. In addition, nuclear factor Kappa β (NF-kβ) occupies a key upstream position in a complex signal transduction pathway, controlling the production of countless pro-inflammatory mediators (Hassen, I. et al., 2015). Notwithstanding, Toll-like receptor (TLR) and TNF receptor activation leads to inflammatory gene expression and production of COX-2, IL-1 and IL-8 (Cohen,

P., 2014). In this context, *in vitro* oleuropein-mediated down-regulation of TLR activation by lipopolysaccharide (LPS) has been documented as a result of iNOS, COX-2, extracellular signal-regulated kinases (ERK)1/2, c-JunN- Terminal kinase (JNK) and NF-k light poly-peptide gene enhancer in B-cell inhibitor (IKB) phosphorylation decrease, respectively (Matsuguchi, T., et al., 2003; Ryu, S.J. et al., 2015).

1.2.4 OLE as Antimicrobial Resource

There is an increased societal interest for sustainable industrial routes, with special emphasis in the food and biomedical sector to manufacture safe yet sustainable packaging for edible products and surgical devices equivalently (Guillard, V., et al., 2018; Isbary, G., et al., 2013). Consequently, there is a growing interest towards minimal processing technologies able to replace conventional decontamination benchmarks. To this end, natural antimicrobial compounds, including vegetable bioactive molecules, offer an emerging strategy to control microbial contamination by virtue of their specific bioactive molecules. Indeed, some molecules and plant-derivatives demonstrate good antimicrobial activity: against pathogenic bacteria present in (i) either packaging or processing steps in the food industry (Guillard, V., et al., 2018) or in (ii) biomedical devices, such as surgical tools and supporting parts, the latter being very important in healthcare since the inaccurate sterilization is responsible for at least 1.5%–7.2% of post-operational complications (Isbary, G., et al., 2019; Ben-Othman, S. et al., 2020). Antibacterial properties of OLE have been widely explored exhibiting antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria, as reported in Table 4.

Table 4. *OLE containing antimicrobial compounds against Gram-positive and Gram-negative bacteria*

Specie	Olive variety and origin	TP Extraction Method	Concentration	Reference
Klebsiella pneumoniae	*Olea europaea (*Turkey, west Anatolian*)*	Aqueous	30 µl OLE; 15% (w/v)	Tranter, H.S., et al., 1993
Staphylococcus aureus	*Olea europaea* (Portugal)	Aqueous	5 mg/mL	Pereira, A.P., et al., 2007 Tranter, H.S., et al., 1993
Bacillus cereus	*Olea europaea* (Portugal)	Aqueous	5 mg/mL	Pereira, A.P., et al., 2007
Bacillus subtilis	*Olea europaea* (Portugal)	Ethanol	27.2 mg/g	Pereira, A.P., et al., 2007
Pseudomonas aeruginosa	*Olea europaea* (Portugal)	Aqueous	5 mg/mL	Pereira, A.P., et al., 2007
Campylobacter jejuni	*Olea europaea* (Australia)	No described	No described	Friedman, M., 2015 Sudjana, A.N., et al., 2009
Helicobacter pylori	*Olea europaea* (Australia)	No described	No described	Sudjana, A.N., et al., 2009
Escherichia coli	*Olea europaea* (Several countries)	Water, ethanol.	Variable	Pereira, A.P., et al., 2007 Albertos, I., et al., 2017
Salmonella enterica	*Olea europaea var. Sylvestris* (Algeria)	Methanol/water	198.7 mg GA/g (1 g)	Friedman, M., 2015 Albertos, I., et al., 2017 Djanane, D., et al., 2019
Listeria monocytogenes	Commercial Extract (USA)	Water/ethanol	62.5 mg/ml	Albertos, I., et al., 2017 Bourke, P., et al., 2017

According to some studies, OLE exerts antimicrobial effects due to its high phenolic compound content (Poudyal, H., et al., 2010; Lee, O.H. and Lee B.Y., 2010). Some studies with OLE reported the growth inhibition in some bacteria species but these findings are contradictory and may depend on the concentration and/or the bacteria used. For example, Sudjana et al., demonstrated that OLE does not show broad-spectrum activity having appreciable activity only against *C. jejuni, H. pylori*

and *Staphylococcus* spp. (Sudjana, A.N., et al., 2009). Djanane D. et al., showed that 5% OLE was more efficient as compared to 1% OLE extract against the pathogens *Salmonella* and *E.coli* O157:H7 in Halal meat. Albertos I., et al., showed that OLE had antimicrobial activity against *L. monocytogenes* in edible films intended for cold-smoked fish preservation. Furthermore, in a study by Pereira A.P., et al., (2007) OLE was screened for its antimicrobial activity against *B. subtilis*, *S. aureus*, *E. coli*, *P. aeruginosa, K. pneumoniae* bacteria and against the fungi *C. albicans* and *C. Neoformans.* Therefore, OLE used as an antimicrobial treatment is considered still unreliable.

1.2.4.1 Cold Atmospheric Plasma

Another emerging sustainable technology is Cold Atmospheric Plasma (CAP). Plasma is commonly referred to as the fourth state of matter, namely, an ionized state of the gas, 'plasma', which exhibits unique properties. The plasma state is pervasive, being found in diverse entities (Bourke, P., et al., 2017). Cold plasma is an ionized gas composed of ions, electrons, free radicals as well as positively and negatively charged particles. CAP treatment generates several reactive short and long-lived species including ROS and RNS, which have been shown to play an important role in antibacterial activity (Bermudez-Aguirre, D., 2019). CAP is a technology demonstrated as a potential alternative to conventional methods attributable to its non-thermal nature, like the use of chlorine and other such as thermal processing as drying, chilling, freezing and pasteurization contributed to improve the microbiological procedure safety in food packaging (Guo, J., et al., 2015). In the biomedical field this technique can be applied surface disinfection, sterilization of surgical instruments or decontamination of devices (Isbary G., et al., 2013). In fact, new studies has had published related to application of CAP for biomedical application developing promising perspectives in this field, for example Cheng, Z., et al. developed a single-electrode tiny plasma jet and evaluated its use for clinical biomedical applications investigating the effect of voltage input and flow rate on the jet length and studied the physical parameters of the plasma jet, including discharge voltage, average gas and subject temperature, and optical emissions via spectroscopy (OES) with the objective of evaluate the interactions between the tiny plasma jet and five subjects [de-ionized (DI) water, metal, cardboard, pork belly, and pork muscle], they evidenced in high-resolution images of belly and pork after 2 min tiny plasma treatment no damages or burning on tissues' surfaces (Cheng, Z., et al., 2021); applying OLE at the system might a strategy to improve this procedure. Other recent studies chosen Poly(2-oxazolines) (POx) an attractive material in a new strategy for the preparation of bioactive based coatings at the surface of chemically inert PTFE by using effective "cold" plasma generated by diffuse coplanar surface barrier discharge (DCSBD) at atmospheric pressure, using 3T3 mice fibroblast cell line observed that after the air plasma treatment of PTFE surface, the number of adherent cells

significantly increased to 434 cells/mm^2, all this results suggest that the good biocompatibility play a crucial role in the interaction of foreign material with the body, being the first cells to attach to the healing wound or implanted biomaterial (Šrámková, P., et al., 2020). Moreover, plasma devices – indirect argon plasma devices (MicroPlaSter alpha and beta) –have been applied in a clinical trial in patients with chronic infected wounds. Some studies reported that CAP exhibited excellent antibacterial efficacy against target food pathogens, their spores, and biofilms (Guo, J. et al., 2015). In addition, new information has been elucidated, explaining the effective application of cold plasma in package functionalization and for toxin elimination, pesticide degradation and food (Cullen, P.J., 2018).

Plasma has also been investigated as a pre-treatment step to activate or modify material surfaces (Chang, Y., et al., 2008), because it can improve the efficiency of post-grafting or incorporating antimicrobial components onto the surface. Currently, there is no information describing the use of plasma to bind OLE components on a surface of a biomaterial. However, other natural components studied by Chang et al., used plasma pre-treatment to promote the grafting of chitosan on polyester fabrics to obtain antibacterial activity (Chang, Y. et al., 2008). According to the authors, fabrics are previously pre-treated by an argon/oxygen (Ar/O$_2$) dielectric barrier discharge (DBD) plasma for surface activation, subsequently exposed to the atmosphere for further oxidation and finally immersed in a chitosan solution for chitosan grafting (Chang, Y., et al., 2008). Other natural compounds, like nisin peptides, thymol and herbs, have also been grafted onto plasma-treated polymer surfaces to obtain an antibacterial material (Duday, D., et al., 2013; Karam, L. et al., 2013; Nikiforov, A., et al., 2016). They employed different types of plasma pre-treatments, namely N$_2$ and Ar/O$_2$ plasma modifications, and plasma-induced grafting of acrylic acid (AA), in order to incorporate Nisin peptides onto the surface of low density poly-ethylene (LDPE) films (Karam, L., et al., 2013). Other interesting developments using whey protein formulations as coating strategies polyethylene terephthalate (PET) films pre-treated by corona discharge of CAP resulted in excellent barrier properties, making the packaging efficacy comparable with other layers conventionally used in food packaging composites (Schmid, M., et al., 2012). Similarly, another study fabricated a novel corn– zein coating on corona-discharge-treated polypropylene (PP) for flexible packaging industry (Tihminlioglu, F., et al., 2010).

Thus, it is expected to replace conventional sterilization/decontamination processes that use a high energy with processes with lower environmental impact (McKeen, L., 2012).

1.3 Tissue Engineering

Novel solutions are required to overcome the limitations of current grafting approaches through tissue engineering (TE) or regenerative medicine which offer promising strategies for treating biomedical

solutions. TE offers an alternative approach to the restoration of injured tissue while circumventing the drawbacks associated with autologous and allogeneic tissue transplantation (Rose, F.R., 2002). To fabricate three-dimensional (3D) tissue, TE involves knowledge of cell biology, chemistry, materials science, nanotechnology, and micro- and nanofabrication including the combination of cells, biomaterial scaffolds and biochemical and physical stimuli to encourage in vitro tissue formation (Figure 10). The development of responsive biomaterials capable of a modulated functionality in response to the dynamic physiological and mechanical environments found in vivo remains an important challenge in bone tissue engineering to achieve long-term repair and good clinical outcomes (Rose, F.R., 2002). TE scaffolds are biomaterial-based matrices that require several key features:

i) Biocompatibility, to be accepted by the body without causing toxicity, carcinogenicity, and inflammation.

ii) Porosity, to host cells and ECM secretion, and to facilitate diffusion phenomena.

iii) Pore interconnectivity, to allow cell migration, scaffold colonization and nutrient/waste trafficking.

iv) Biodegradation, to be replaced by new tissue.
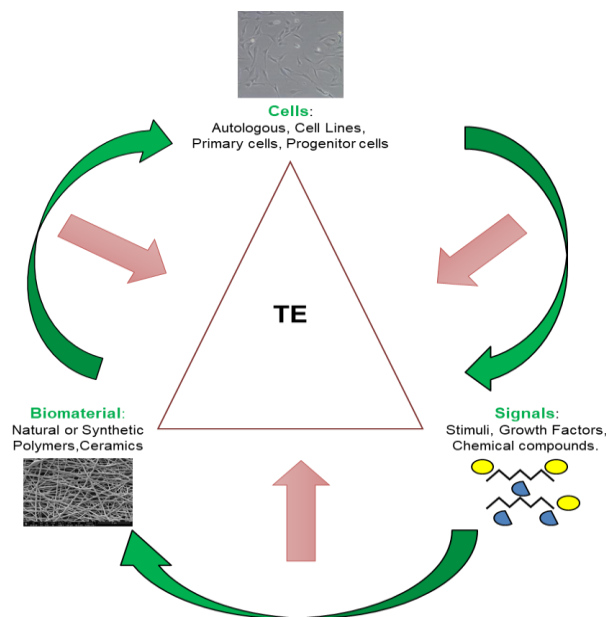
Figure 10. *The TE triad.*

Many polymers synthetic and natural have been used in the design of nanofibrous scaffolds with different structural properties. Synthetic polymers have the characteristic to provide high flexibility in synthesis, processing, and modification and are more cost-effective than natural biomaterial. However, synthetic polymers lack bioactivity and thus require more modifications than natural ones. In contrast, polymers of natural origin are inherently bioactive, presenting cell-interactive domains on their backbones, and scaffolds prepared using them offer better adhesion, proliferation, and

differentiation of cells than is available with synthetic polymers. Actually, to take advantage of both synthetic and natural polymers, researchers have fabricated hybrid scaffolds with the physical properties and high bioactivity favorable for tissue regeneration (Holzwarth, J.M. and Ma, P.X., 2011). Also, novel application of TE involve naturals compounds or bioactive molecules incorporated into the scaffold nanofabrication to improve untoward issues associated with hydrophilicity, cell attachment, and biodegradability. Moreover, the scaffold surfaces can be more functionalizing using specific ligands such as protein molecules that help enhance cellular responses. Also, the incorporation of drug for release and delivery or bioactive agent encapsulation. (Sttanton, S. et al., 2016). Scaffold nanofabrication in tissue regeneration is a novel strategy in biomedical sciences. The aim of developing novel wound dressings is to employ the wound environment in purposeful ways to reach an ideal wound dressing. The characteristic of this must be non-toxic and non-allergenic to promote absorption of wound exudates, provide exchange gaseous mechanisms, insulate the wound against thermal extremes, protection against microorganisms, comfortable with body and prevent of dehydration and formation of eschar (Pilehvar-Soltanahmadi, Y., et al., 2018).

Even though many conventional and non-conventional technologies are available to produce scaffolds, electrospinning offers several advantages in the field of tissue engineering. Indeed, this technique allows the simple and cost-effective production of ultrafine fiber meshes, which for their nature, mimic the fibrous ECM of biological tissues, usually formed by collagen and elastic fibers, with similar size (Dong, Y., et al., 2020). Electrospinning exploits a simple key principle: a charged polymer solution jet is formed and collected on a collector when the applied electrostatic charge overcomes the surface tension of the polymer solution. As such, this technique operates at room temperature using an electric field as a driving force for fiber formation. For this reason, electrospinning is particularly suitable to incorporate bioactive molecules and drugs into polymeric fibers (Cristallini, C., et al., 2019). Electrospun fibrous scaffolds are typically assembled into nonwoven networks, which are deposited with an anisotropic (i.e., random) fiber orientation if a static collector is used (Azimi, B., et al., 2020). These randomly deposited fibrous scaffolds have potential applications as temporary substitutes for skin tissue engineering because they replicate the microstructure of the dermal connective tissue (Jun, I. ,et al., 2018). Moreover, the high surface area of electrospun fibrous scaffolds allows oxygen permeability at the wound site, making these scaffolds suitable substrates for wound dressings (Azimi, B., et al., 2020).

Recently, different bioactive molecules have been incorporated into electrospun nanofibers such as growth factors, vitamins, Antibiotics and antimicrobial agents, analgesics, and anti-inflammatory drugs (Azimi, B., et al., 2020). Also, there is a focus on design and fabrication of novel bioactive nanofibrous mats via electrospinning of natural compounds (e.g., plant and animal origin) into appropriate biomaterials. Utilization of natural substances provide therapeutic properties such as

demulcent, softening, re-epithelializing, astringent, antimicrobial, antioxidant, and anti-inflammatory activities that can accelerate and improve the wound healing process (Pilehvar-Soltanahmadi, Y., et al., 2018). Natural, or synthetic polymers or in combination of these are being applied in the preparation of nanofiber-based natural substances incorporated with electrospun nanofibrous scaffold is a valid and novel strategy to wound healing or ulcers issues. These natural substances are renewable resources, biodegradable and biocompatible promoting induction and stimulation of the wound healing process, reparation, and regeneration of damaged skin.

Based on unique features of electrospun nanofibers, up to now, some natural products have been explored including crude bark extract of *Garcinia cowa, Garcinia mangostana, Tecomella undulate, Indigofera aspalathoides, Azadirachta indica, Memecylon edule, Lawsonia inermis (henna), Myristica andamanica, Aloe vera, Ginko biloba*. Other bioactive molecules derived from plants such as curcumin, chamomile, gum tragacanth, Gin, tea tree oil, olive oil, emu oil and honey have been incorporated with polymer-based electrospun nanofibers for tissue regeneration applications (Serrano, A. et al., 2018).

Currently, some studies including biomaterials and OLE have been explored. Erdogan et al., (2014) studied OLE as a crosslinking agent for the preparation of electrospun zein fibers. Zein is the prolamine, comprising 60% of protein content in corn and consisting of one-third hydrophilic and two-thirds hydrophobic amino acid residues in its primary structure. Glutamic acid, leucine, proline, and alanine comprise the amino acid composition of zein. Although, in a publication titled "S*ilk Fibroin Nanofibers Loaded with hydroxytyrosol from hydrolysis of Oleuropein in Olive Leaf Extract*", the authors prepared an antimicrobial silk fibroin nanofibers from the aqueous formic acid solutions of silk fibroin and HT with the *in-situ* hydrolysis of oleuropein present in OLE using electrospinning method, in this case the nanofibers exhibited antibacterial properties due to HT loaded nanofibers. The authors suggest that Silk Fibroin nanofibers loaded with HT may offer a new alternative biomaterial to be used in wound dressing or medical textile applications.

1.3.1 Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHAs) are biopolyesters and biopolymers synthesized by various microorganisms as lipid inclusion stored within cells as energy storage materials in granular forms. More than 90 genera of both Gram-positive and Gram-negative bacteria have been identified as PHAs producers under both aerobic and anaerobic conditions (Philip, S., et al., 2007; Rinat, N., et al., 2015). Many microorganisms can store intracellular inorganic and/or organic inclusions which are surrounded by phospholipids, in Figure 11 is exposed the general structure of PHA and granules formation in the bacterial cell.
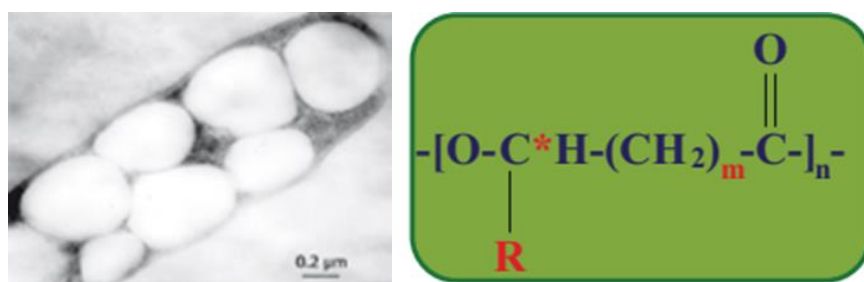
Figure 11. Granules of PHA generate by the bacteria and its general structure. Source: Chen, G.-Q. (2010) Plastics from Bacteria Natural Functions and Applications (Microbiology Monographs Volume 14).

The biosynthesis of PHA has been studied over past many years. Acetyl-CoA isthe key component to supply the 3-hydroxyalkanoyl-CoA of different lengths as substrates for PHA synthesis of various specificities. Also, many genes encoding various enzymes PhaA, PhaB, PhaC, PhaZ being identified in *R. eutropha*. Have been characterized 8 pathway of Biosynthesis but 3 are considered relevants. The first pathway involves the three key enzymes β-ketothiolase, NADPH-dependent acetoacetyl-CoA reductase, and PHA synthase encoded by genes phaA, phaB, and phaC, respectively. *R. eutropha* is the representative of this pathway. An associated pathway involving PHA degradation catalyzed by PHA depolymerase, dimer hydrolase, 3-hydroxybutyrate dehydrogenase, and acetoacetyl-CoA synthase helps regulate PHA synthesis and degradation. The associated pathway was found in strains of *A. hydrophila*, *P. stutzeri, R. eutropha*, and *P. oleovorans* (Sudesh et al. 2000). The second PHA synthesis pathway (pathway II) is related to fatty acid uptake by microorganisms. After fatty acid b-oxidation, acyl-CoA enters the PHA monomer synthesis process. Enzymes including 3-ketoacyl-CoA reductase, epimerase, (R)-enoyl-CoA hydratase/enoyl-CoA hydratase I, acyl-CoA oxidase (putative), and enoyl-CoA hydratase I (putative) were found to be involved in supplying the PHA precursor 3-hydroxyacyl-CoA for PHA synthesis. *Pseudomonas putida, Pesudomonas aeruginosa*, and *A. hydrophila* can use pathway II to synthesize medium-chain-length (mcl) PHA or copolymers of (R)-3-hydroxybutyrate (R3HB) and (R)-3-hydroxyhexanoate (PHBHHx). Pathway III involves 3-hydroxyacyl-ACP-CoA transferase (PhaG) and malonylCoA-ACP transacylase (FabD), which help supply 3-hydroxyacyl-ACP to form PHA monomer 3-hydroxyacyl-CoA, leading to PHA formation under the action of PHA synthase (Sudesh, et al., 2000; Zheng, et al., 2005; Taguchi, et al., 1999; Chen, 2010). In the illustration 12 is appreciated the principals pathways involved in the PHA Biosynthesis
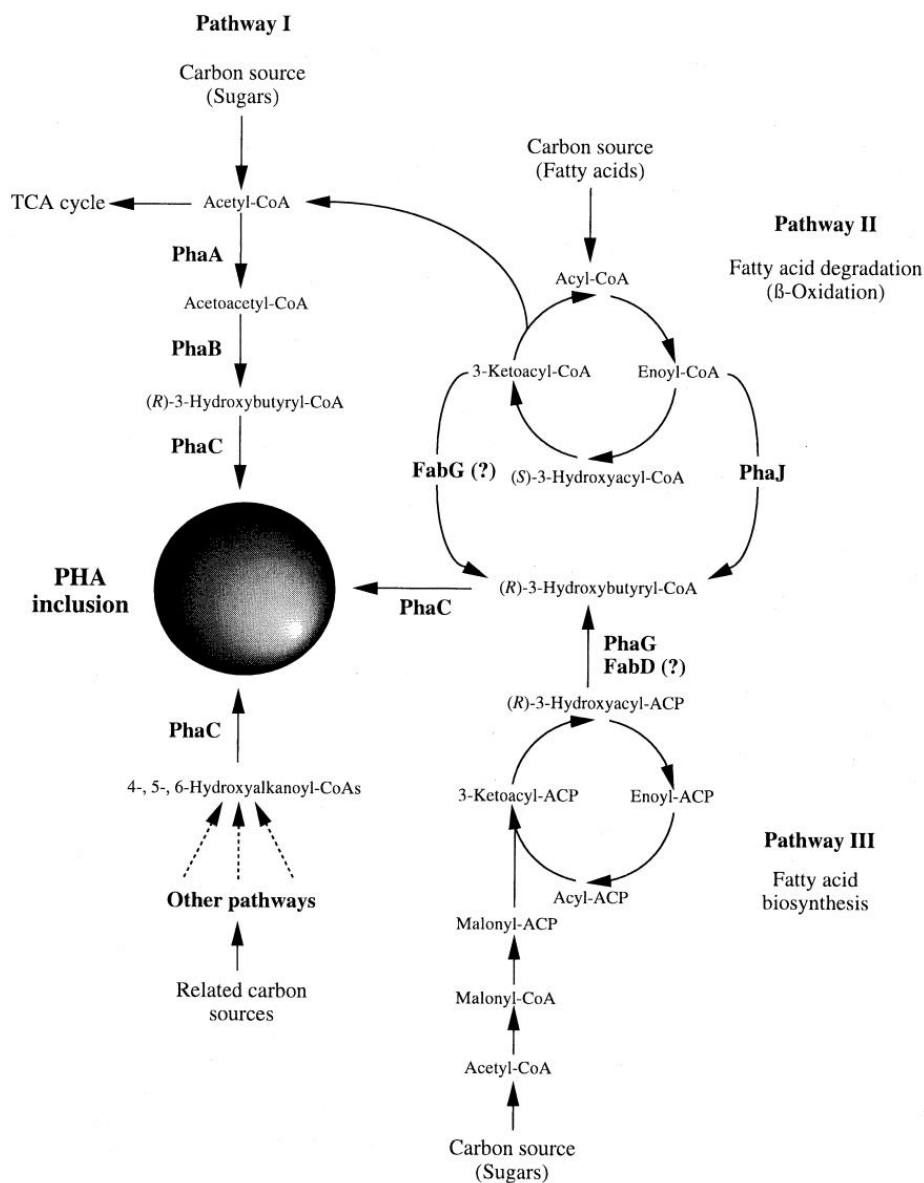
Figure 12. Metabolic Pathway of PHA Biosynthesis. Source: Sudesh, K., Abe, H. and Doi, Y. (2000) 'Synthesis, structure and properties of polyhydroxyalkanoates: Biological polyesters', Progress in Polymer Science (Oxford), 25(10), pp. 1503–1555. doi: 10.1016/S0079-6700(00)00035-6.

Recently, numerous studies have been performed using PHAs as biomaterials for synthesis of medical and pharmaceutical devices due to their biocompatibility and biodegradability, as well as for tissue engineering scaffolds, as matrices for the *in vitro* proliferous of different human cells, cardiovascular patches, drug delivery, surgery, nanotechnology applications and wound dressing (Cristallini, C. et al., 2014; Francis, L. et al., 2011; Lacoste, C. et al., 2020). PHAs have the chemical structure and molecular interaction between material components give different properties to the substrate including hydrophilicity/hydrophobicity, roughness, porosity, chemical compatibility, mechanical characteristics and degradation kinetics associated to the material in analogy to those of the native tissue (Akaraonye, E., et al., 2010; Cristallini, C., et al., 2014).

Being a very wide family of biopolymers, PHAs possess diverse chemical structure enabling different molecular interactions, which give rise to a tunable range of material properties, including mechanical behavior and degradation kinetics (Akaraonye, E., et al., 2010; Cristallini, C., et al., 2014). Among PHAs, poly(hydroxybutyrate-co-hydroxyvalerate) (PHBHV), is a copolymer investigated in the biomedical field. The presence of hydroxyvalerate (HV) imparts better processability to the rigid polyhydroxybutyrate (PHB). As such, PHBHV shows great potential for fabricating biomedical micro- and nanocarriers, being highly soluble in organic solvents, such as chloroform and dichloromethane and insoluble in water (Francis, L., et al., 2011). In fact, PHBHV can be processed from the molten state, blended, and be electrospun using copolymer solutions (Lacoste, C. et al., 2020; Azimi, B., et al., 2020).

Other PHAs are under development to achieve tunable mechanical properties, such as the poly(3-hydroxyoctanoate-co-3-hydroxydecanoate) P(HO3HD). Are also highly biocompatible and naturally occurring bacteria-derived polyesters and are produced by bacterial fermentation (Azimi, B., et al., 2020). Some interesting studies have successfully fabricated innovative surface-functionalized PHA fibers with P(3HB)/P(3HO-co-3HD) that would enable the development of novel bio-based products for potential use for skin-related applications, such as wound healing or skin contact due to shows anti-inflammatory capacities (Azimi, B., et al., 2020).

Fibers based on PHBHV and PHOHD have a wide range of applications in various industries such as biomedical sector including tissue engineering, bio-implant patches, drug delivery, surgery and wound dressing (Raza, Z.A., et al., 2018). Table 5 summarizes some important studies of PHAs in regenerative medicine.

PHA applications in Rigenerative Medicine

Tecniques	Aim of the study/biomaterial	Outcomes	Referehences
Electrospray	-To develop completely bio-based and bioactive meshes intended for wound healing. *P(3HB)/P(3HO-co-3HD) fiber* (Chitin nanofibril (CN) and nanolignin (NL) be loaded with bioactive factors, such as the glycyrrhetinic acid (GA) and CN-NL/GA (CLA))	- The presence of CN and CLA in the fiber improved the indirect antimicrobial and anti-inflammatory activity of the electrospun fiber meshes by downregulating the expression of the most important pro-inflammatory cytokines and upregulating human defensin 2 expression.	Azimi, B., et al., 2020
Electrospinning	Poly(3 hydroxybutyrate), P(3HB), and poly(3-hydroxyoctanoate), P(3HO), combined to form blend fibres for directional guidance of neuronal cell growth and differentiation.	PHA blend fibre groups were able to support growth and guide aligned distribution of neuronal cells, and there was a direct correlation between the fibre diameter and neuronal growth and differentiation	Lizarraga-Valderrama, L., et al., 2019
Electrospinning	Regeneration of damaged articular cartilage.	The study permitted to produce a cartilage repair kit for clinical use to	Ching, K.Y., et al.,2016

	Poly(3hydroxybutyrate)/poly(3-hydroxyoctanoate)	reduce the risk of developing secondary osteoarthritis.	
Emulsification-diffusion method	Anti-tumor medicines against colorectal cancer. Anticancer nanocarrier using biocarriers able to both bind and easily release their load in a controlled manner. Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) carriers	-*in vitro* studies clearly demonstrated that while the nanocarriers themselves slightly alter HT-29 cell viability. -Decreasing cell viability	Radu, I.C., et al., 2019
Soft lithography	Fabrication of PHBHV/gelatin constructs for mimicking myocardial structural properties.	- Viability and proliferation assays demonstrated adhesion and growth of mesenchymal stem cells (MSCs) and cardiac resident non myocytic cells (NMCs). - Constructs adopted a distribution mimicking the three-dimensional cell alignment of myocardium.	Critallini, C., et al., 2014
Electrospinning	The use of keratin-loaded-poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) electrospun fibrous meshes for wound dressing applications	-Review aims to improve awareness on the use of bio-based polymers in medical devices	Azimi, B., et al., 2020
Electrospinning	Curcumin-loaded electrospun PHBV nanofibers as potential wound dressing material.	-Fiber no toxic. - Enhanced cell attachment and proliferation of L929 mouse fibroblast.	Mutlu, G., et al., 2018
Film formation	Injectable implant system development by dissolving microbial PHBHV.	-PHBHV injectable implant system can be developed as a tissue adhesion prevention film for surgical operation.	Dai, Z.W., et al., 2009
Powder form	PHBHHV for tarsal repair in eyelid reconstruction in the rat.	-Good tissue compatible, elastic mechanical properties, satisfactory repair results.	Zhou, J., et al., 2010

Table 5. *Studies of PHA applications in Tissue Engineering and regenerative medicine.*

1.3.2 Poly(vinylidene fluoride–trifluoroethylene) biopolymer in electrospun nanofiber

Poly(vinylidene fluoride–trifluoroethylene) [P(VDF–TrFE)] has been known for many studies as a biopolymer with excellent dielectric, ferroelectric and piezoelectric performances (Zhang, W., et al., 2017). In fact, ferroelectric properties of the polymers are because of their high spontaneous polarization and chemical stability caused by the dipoles in crystalline or polycrystalline materials that spontaneously polarize and align with an external electric field (Mao, D., et al., 2014). P(VDF–TrFE) copolymer show fast response speeds, giant electrostriction, high electric energy density, a large electrocaloric effect and strain levels far above those of traditional piezoceramic materials (Xia, et al., 2002; Bornar, V., et al.,2011) which have considerable interest in the expansion of electronic devices, including wearable smart devices, sonars, sensors, transistors, actuators, and energy storage

capacitors due to their good sensitivity, flexibility, mechanical strength and displacement (Lui Y. et al., 2010; Ting, Y. et al., 2013). PVDF is a crystalline polymer, contains a monomer unit of $-CH_2-CF_2-$, in between polyethylene (PE) ($-CH_2- CH_2-$) and polytetrafluoroethylene (PTFE) ($-CF_2-CF_2-$) monomers (Figure 14).
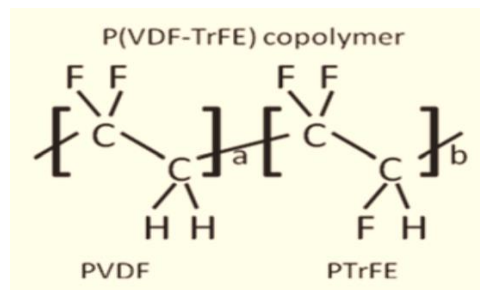
Figure 13. Chemical structure of P(VDF–TrFE). Source: Mao, D., E., B. and A., M. (2011) 'Ferroelectric Properties and Polarization Switching Kinetic of Poly (vinylidene fluoride-trifluoroethylene) Copolymer', Ferroelectrics - Physical Effects, (August). doi: 10.5772/17147.

Other characteristics of this polymer is your high melting point, which gives them some potential applications as high-temperature dielectric materials but probably the disadvantage of P(VDF–TrFE) is the differences in the reactivities between TrFE and PVDF monomers, e.g. r_1(TrFE) = 0.5 and r_2 (VDF) = 0.7 access to these copolymers often suffers from an inhomogeneous composition distribution (Zhang, W., et al., 2017). However, the fiber mesh of P(VDF-TrFE) has other important and advantageous characteristic such biocompatibility used for biomedical devices and tissue engineering (Azimi, B., et al., 2019). The fibrous character of the scaffold produced via electrospinning had the purpose of mimicking natural ECM, thus helping the maintenance of native cell morphology and function (Jun, I., et al., 2018; Stocco, T., et al., 2018). For our proposes P(VDF-TrFE) was specifically chosen for scaffold fabrication for its chemical inertia and non-degradability, to prevent any biomaterial damage following hydrogen peroxide administration. P(VDF-TrFE) possesses additional peculiar characteristics, such as piezoelectricity, which can be exploited to obtain truly functional 3D models of blood vessels (Azimi, B. et al. 2019; Fukuda, et al.,1969; Lenz, T., et al., 2017). Some interesting and promising studies have been published; in table 6 are visualized.

[P(VDF-TrFE)] applications in Rigenerative Medicine

Tecniques	Aim of the study/biomaterial	Outcomes	Refererences
Nanoimprinting	-Characterization of the [P(VDF-TrFE)] for functional biomedical devices.	The imprinted patterns show excellent ferroelectricity and piezo response without post- imprinting annealing, enabling ferroelectric nonvolatile memory cells for data storage.	Liu, Y., et al., 2010
Electrospinning	Evaluate if OLE extracted from the Tuscan Olea europaea protects endothelial cells against oxidative stress generated by reactive oxygen species (ROS) in 2D and 3D model	-Protects endothelial cells against oxidative stress generated by reactive oxygen species (ROS). -Using [P(VDF-TrFE)] 3D scaffold could be the best predictive model to mimic the physiological conditions of vascular tissue reaction.	De la Ossa, J.G., et al., 2019
Electrospinning	-Piezoelectric nanocomposites based on ultrafine PVDF fibers and barium titanate nanoparticles (BTNPs), as a strategy to improve the PVDF (simple conformation) performance for cochlear stimulation	-The BTNP/PVDF fibers were not cytotoxic towards cochlear epithelial cells. Neural-like cells adhered to the composite fibers and, upon mechanical stimulation, showed enhanced viability	Mota, C., et al., 2017
Bulk polymerization process	Description of [P(VDF-TrFE)] characteristics for applications, such as sensors, actuators, artificial muscles, and integrated micro electromechanical systems	-Higher elastic energy density and electromechan- ical coupling factor in the P(VDF-TrFE-CFE) terpolymer. -Higher elastic energy density and electromechan- ical coupling factor in the P(VDF-TrFE-CFE) terpolymer.	Xia, F., et al.,2002
Casting-etching process	Progress on Piezoelectric and Triboelectric Energy Harvesters in Biomedical Systems	Chemical stability and biocompatibility of the polymer is beneficial to the biomedical application for in vivo biological sensors and energy harvester.	Zheng, Q., et al., 2017
Electrospinning	[P(VDF-TrFE)] fiber fabrication as a scaffold in combination with Schwann cells (SCs) for spinal cord repair.	The scaffold supported SC growth and neurite extension which was further enhanced by coating the scaffold with Matrigel.	Wu, S., et al.,2018
Electrospinning	Electrospun PVDF and P(VDF-TrFE) scaffold can produce electrical charges during mechanical deformation for stimulation and repair bone defect and damaged nerve cells.	Fibrous mats can enhance neurite growth along the fiber orientation.	Li, Y., et al., 2019

Table 6. *Studies conducted with P(VDF-TrFE) in biomedical applications.*

Research Objectives

In light of the biological and antimicrobial properties of OLE, as well as the importance of the use of tissue engineering, it is of great interest to study and characterize autochthonous olive extract to improve their antioxidant properties and to elaborate new therapeutic strategies.

OLE can, in fact, be used as an adjuvant in wound healing, thanks to its antimicrobial, antioxidant and anti-inflammatory properties, in order to produce new biomedical devices and/or for tissue engineering applications. Furthermore, OLE can be exploited in a new combined strategy that uses the CAP as a system against bacteria for applications in the food, as well as biomedical industries.

To this end, the research objectives of the present thesis was to:

- ✓ Characterize and evaluate *in vitro* antioxidant properties of OLE, obtained from Tuscan olive trees, in different culture condition (2D cultures and 3D scaffolds), to show the potential of natural extract in biomedical applications.

- ✓ Investigate the antimicrobial effect of OLE versus CAP technology or their combination against pathogens;

- ✓ To characterize electrospun OLE-loaded PHBHV based composite fibers for wound healing applications.

- ✓ To evaluate the ability of OLE incorporated in Polyhydroxyalkanoates fibers to modulate the release of cytokines from healthy Human Keratinocytes.

CHAPTER 2

EXPERIMENTAL SECTION I

2. AUTOCHTHONOUS TUSCAN OLE AS ANTIOXIDANT SOURCE FOR BIOMEDICINE

Considering the biological properties of OLE, recent studies have elucidated its biological effects in several fields with particular attention on their antioxidant activity due to the presence of molecules such as: oleuropein, tyrosol and hydroxytyrosol. Thus, as well as the importance of the use of tissue engineering, it is of great interest to study and characterize autochthonous OLE to improve their antioxidant properties and to elaborate new therapeutic strategies.

To this end, the main research objective of the present chapter was to:

- Characterize and evaluate *in vitro* antioxidant properties of OLE, obtained from Tuscan olive trees, in different culture condition (2D cultures and 3D scaffolds), to show the potential of this natural extract in biomedical applications.

2.1 MATERIALS AND METHODS

2.1.1 Materials

HPLC-grade acetonitrile and methanol were purchased from Labscan (Dublin, Ireland). Acetic acidof analytical grade (assay >99.5%) was purchased from Fluka (Buchs, Switzerland). Water was purified by using a Milli-Q System (Millipore, Bedford, MA, USA). Other reagents unmarked were of analytical grade. Methanol (MeOH) was purchased from Carlo Erba (Rodano, MI, Italy), H_2O_2 was bought from Farmac-Zabban S.p.a. (Calderara di Reno, BO, Italy), $NaCO_3$, gelatin and Hoechst 33342 were obtained from Sigma-Aldrich (Milan, Italy), poly(vinylidenefluoride-*co*-trifluoroethylene) [P(VDF-TrFE)] powder (70:30 % mol) was purchased from Piezotech Arkema (Pierre-Benite, France), Methyl ethyl ketone (MEK) was bought from Merck (Darmstadt, Germany). Medium 199 (M199), fetal bovine serum (FBS), penicillin-streptomycin solution, L-glutamine, HEPES buffer were supplied by Hospira S.r.l. (Naples, Italy). 4-[3-(4-iodophenyl)-2-(4nitrophenyl)-2H-5-tetrazolium]-1,3-benzenedisulfonate (WST-1 assay), was purchased from Roche Applied Science (Mannheim, Germany), 5-(and-6)-chloromethyl-2',7'-dichloro-di-hydro-fluorescein diacetate, acetyl ester (CM-H_2DCFDA) was supplied by Invitrogen. EGM-2 medium bullet kit was bought from Lonza (Verviers, Belgium), alamarBlue® was obtained from Thermo Fisher Scientific (Waltham, MA, USA).

2.1.2 Sample Preparation

Olive leaves were obtained from *Olivastra seggianese* cultivar. The collection was performed at CNR-IVALSA, Follonica (GR), Italy. Leave collection was carried out manually and at different months between February 2018 and March 2019. The leaves were stored at 25 °C after harvesting. For each lot, 20 g of leaves were placed in liquid nitrogen and crushed manually. A final weight ratio of 0.9 g OLE/g (namely, 18 g of OLE powder per 20 g of olive leaves) was obtained and used for future experiments.

2.1.3. OLE Polyphenol Characterization

To measure TP content, 1 mg of OLE powder was dissolved in water and therefore analyzed with Folin–Ciocalteau's method (Aisnworth, E.A., 2007), as per the manufacturer's instructions. The results were expressed in gallic acid equivalent (GAE), a universally accepted standard for polyphenols to determine the value of the TP in 100 g of sample.

2.1.4. HPLC Characterization

A high-performance liquid chromatography analysis (HPLC) was carried out to identify and quantify the major phenolic compounds of the OLE using a slightly modified method developed in our previous study (Palla, M. et al., 2018). The retention times and UV absorbance spectra of phenolic compounds present in OLE were compared with those of the commercial standard and quantified at 278 nm, using p-hydroxyphenyl acetic acid as the internal standard, according to the previously reported method. Sample concentrations were determined by linear regression. For each calibration curve, the correlation coefficients were >0.99. HPLC analysis was performed using an HPLC instrument (Beckman, Ramsey, MN) equipped with a System Gold Solvent Delivery module (Pumps) 125 and a System Gold UV/Vis Detector 166, set to 280 nm, and using a Phenomenex Gemini reverse-phase C18 column (250 × 4.6 mm, 5 μm particle size; Phenomenex, Castel Maggiore, Italy). The mobile phase was a mixture of H_2O/AcOH (97.5:2.5 *v/v*) (A) and MeOH/ACN (1:1 *v/v*) (B), programmed as follows: a gradient from 5% (B) to 30% (B) in 45 min; 30% (B) for 5 min and then from 30% (B) to 5% (B) in 5 min. The flow rate was 1 ml/min, and the injected volume was 50.0 μl.

2.1.5. Endothelial Cell Isolation and Culture

Tissue samples were collected and treated anonymously and in conformity with the Declaration of Helsinki. Human umbilical vein endothelial cells (HUVECs) were isolated from the umbilical vein endothelium of healthy human donor cords, following the procedure described by Jaffe et al. Isolated HUVECs were centrifuged, the cell pellet was plated on gelatin pre-coated flasks and incubated for 24 h at 37 °C, 5% CO_2 using growth culture medium consisting of M199, supplemented with 10% FBS, penicillin-streptomycin solution, L-glutamine, HEPES buffer. After 24 h, the growth medium was replaced to remove excess red blood cells.

2.1.6. Scaffold Fabrication and Characterization

P(VDF-TrFE) was dissolved in MEK at a concentration of 20% *w/v* and stirred at 300 rpm for 12 h at room temperature to allow complete dissolution. The polymer solution was loaded into a 10 mL glass syringe, fitted with a blunt tip stainless steel needle (21G × 3/4"), and placed into a syringe pump (NE-300, New Era Pump Systems, Inc., NY, USA). The ground terminal of high voltage supply (S1600079 Linari High Voltage, Linari Engineering s.r.l., Pisa, Italy) was connected to the metal needle, whereas the positive terminal was connected to the collector; 35 kV potential was applied. A cylindrical collector (diameter 8 cm, Linari Engineering s.r.l.), was placed at 15 cm from the tip of the needle. The polymer solution was injected from the needle in the presence of the electric field at a constant flow rate of 0.016 mL/min and collector velocity of 500 rpm, room temperature, and 46% relative humidity. The fiber meshes were kept in an oven at 60 °C overnight to remove solvent traces. The morphology of the scaffolds was evaluated qualitatively by scanning electron microscopy (SEM) using an FEI FEG-Quanta 450 instrument (Field Electron and Ion Company, Hillsboro, OR, USA). The samples were sputtered with gold (Gold Edwards SP150B, UK) before analysis. All parameters have been standardized in the Engineering Department at the University of Pisa, Italy.

2.1.7. Investigation of OLE Effects

2.1.7.1. 2D HUVEC Model

HUVECs between passage P2–P4 were treated for 2 and 24 h with different polyphenol concentrations of OLE (2 μg/ml, 5 μg/ml, 10 μg/ml, 25 μg/ml, 50 μg/ml, 100 μg/ml, 250 μg/ml, 500 μg/ml, 1000 μg/ml) in growth medium with 5% FBS ($n = 3$).

Cell viability was determined by colorimetric assay with WST-1, based on the cleavage of tetrazolium salt by mitochondrial dehydrogenases, present in viable cells. Briefly, at the end of the treatment, HUVECs were incubated with tetrazolium salt at 10 μl/well for 3 h at 37 °C, in 5% CO_2 cell culture

incubator. Thereafter, the formazan dye formed was quantified by measuring the absorbance at 450 nm, with a multiplate reader (Thermo Scientific Multiskan FC microplate photometer). The absorbance was directly correlated to the number of metabolically active cells, and viability was expressed as a percentage of viable cells.

The protective effect of OLE was evaluated pre-treating HUVECs with different OLE polyphenol concentrations for 2 and 24 h and then with 100 μM of H_2O_2 for 1 h. At the end of each treatment, cells were analyzed for viability and ROS production.

Intracellular ROS production was evaluated by ROS fluorescent probe CM-H2DCFDA, a cell-permeable indicator for these compounds. Briefly, during the last 30 min of treatment with H_2O_2, the HUVECs were co-incubated with CM-H2DCFDA at 10 μM/well dissolved in PBS, in the dark at room temperature. ROS production was detected by measuring the increase in fluorescence over time by microplate reader (Thermo Scientific Fluoroskan Ascent Microplate Fluorometer), using excitation at 488 nm and emission at 510 nm.

2.1.7.2. 3D HUVEC Model

The P(VDF-TrFE) scaffolds were cut into 0.8×0.8 mm squares and sterilized in absolute ethanol overnight. The scaffolds were coated with a sterile-filtered 2% (*w/v*) gelatin aqueous solution for 30 min and seeded with HUVECs at a density of 1×10^5 cells/sample. Cell/scaffold constructs were cultured in EGM-2 medium bullet kit (CC-4176) for 1 week in a standard incubator (37 °C, 5% CO_2) by replacing the medium every 48 h.

The *in vitro* effect of OLE on metabolic activity and ROS production was tested on HUVEC/scaffold constructs following H_2O_2 administration, according to the following experimental design ($n = 3$):

- Cell/scaffold construct (control);
- Cell/scaffold construct + OLE (100 μg/mL GAE) for 24 h;
- Cell/scaffold construct + H_2O_2 (100 μM) for 1 h;
- Cell/scaffold construct + OLE (100 μg/mL GAE for 24 h) + H_2O_2 (100 μM for 1 h).

Cell metabolic activity was assessed using the alamarBlue® assay, according to the manufacturer's instructions and expressed as the percentage of reduced alamarBlue® (%ABred). Data were acquired after 3 days of culture without treatments to evaluate cytocompatibility and post-treatment to evaluate the antioxidant activity of OLE. The absorbance of supernatants was measured with a spectrophotometer (Victor 3; PerkinElmer, Waltham, MA, USA) under a double-wavelength reading (570 and 600 nm). Finally, %ABred was calculated by correlating the absorbance values and the molar extinction coefficients of the dye at the selected wavelengths, following the protocol provided by the manufacturer.

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At the endpoint, the cell/scaffold constructs were washed twice with 1× PBS and then fixed in 1% (*w/v*) neutral buffered formalin for 10 min at 4 °C. SEM was used on cell/scaffold constructs to image cell attachment, for morphology, and for spreading onto the scaffolds. The biological samples were dehydrated with 70% ethanol for 30 min and finally dried in oven at 37 °C for two h prior to sputter coating. For examination, the samples were mounted on aluminum stubs, sputter-coated with gold, and observed under SEM.

Intracellular ROS production was evaluated by ROS fluorescent probe CM-H$_2$DCFDA. Briefly, after cell treatment, the cells/scaffold constructs were washed twice with 1× PBS and then stained with Hoechst 33342 (5 µM for 10 min) for nuclei visualization. The constructs were then washed twice with 1 × PBS and incubated with 10 µM CM-H$_2$DCFDA in PBS for 30 min in the dark at room temperature. The cells/scaffold constructs were then washed three times with PBS and visualized under a fluorescent microscope (Carl Zeiss MicroImaging GmbH, Germany). ImageJ software (Version 1.52r) was used to measure the integrated optical density (OD) of the stained cells, which was normalized for green + blue integrated OD ($n = 3$).

2.1.8. DAPI Staining

Formalin-fixed samples were washed with 1× PBS and incubated with 10 µg/mL 4',6-diamidino-2-phenylindole (DAPI-Life Technologies, acquired by Thermo Fisher Scientific, Waltham, MA, USA) in 1x PBS for 10 min RT, to detect cell nuclei (fluorescent in blue) and washed in 1× PBS. Specimens were observed by an inverted fluorescence microscope equipped with a digital camera (Nikon Eclipse Ti, Amsterdam, The Netherlands).

2.1.9. Statistical Analysis

Data are presented as mean ± standard deviation (SD). Three independent replications were evaluated for each treatment with OLE. The in vitro experiments were performed in three separate series of three independent replicates for each well. The difference among groups of values was evaluated by a one-way ANOVA, and a post hoc analysis was performed by Tukey's or Bonferroni's multiple comparisons test when appropriated. Differences with a *p*-value < 0.05 were considered statistically significant.

2.2 RESULTS

2.2.1 Characterization of OLE from Tuscan *Olea europaea*

The total polyphenol (TP) content of OLE was determined by the method of Folin–Ciocalteu assay, thus obtaining 23.29 mg GAE/g. Moreover, the identification of phenolic compounds of OLE was performed by high performance liquid chromatography (HPLC) analysis. For this experiment, the results showed that oleuropein was the major compound present in the extract with a concentration of 14.69 ± 0.92 mg GAE/g of OLE, corresponding to 1.47 % (w/w%). Furthermore, the OLE contained luteolin 7-O-glucoside but at lower concentrations of 3.60 ± 0.25 mg GAE/g of OLE, corresponding to 0.36% (w/w%). We obtained different quantification of TP in diverse sampling periods (Table 1). TP content obtained by olive leaves harvested between February 2018 and March 2019 ranged between 14.99–27.83 mg GAE/g of OLE extract, the highest content being during springtime.

Table 1. *Quantification of TP in lyophilized OLE in different periods.*

Sampling Period (Month-Year)	TP (mg/g)
02–2018	27.83
05–2018	23.06
10–2018	14.99
03–2019	23.29

2.2.2 Scaffold Characterization

Figure 1 shows the electrospinning process and a representative SEM image of the obtained P(VDF-TrFE) scaffolds. Uniformly distributed and bead-less ultrafine fibers were formed under the applied working conditions. Trapped Methyl Ethyl Ketone (MEK) with moisture due to ambient relative humidity (46%) led to the formation of surface nanoporosity (lens in Figure 4), which is desirable in TE, as it offers enhanced surface area for protein deposition and cell adhesion. In addition, shifting from smooth to nanoporous surface in P(VDF- TrFE) ultrafine fibers is produced by changing the environmental conditions, such as relative humidity (Azimi, B et al., 2020).The 500 rpm rotative collector resulted in randomly oriented fibers with an average diameter of 1.9 ± 0.5 µm and $94\% \pm 2\%$ porosity.
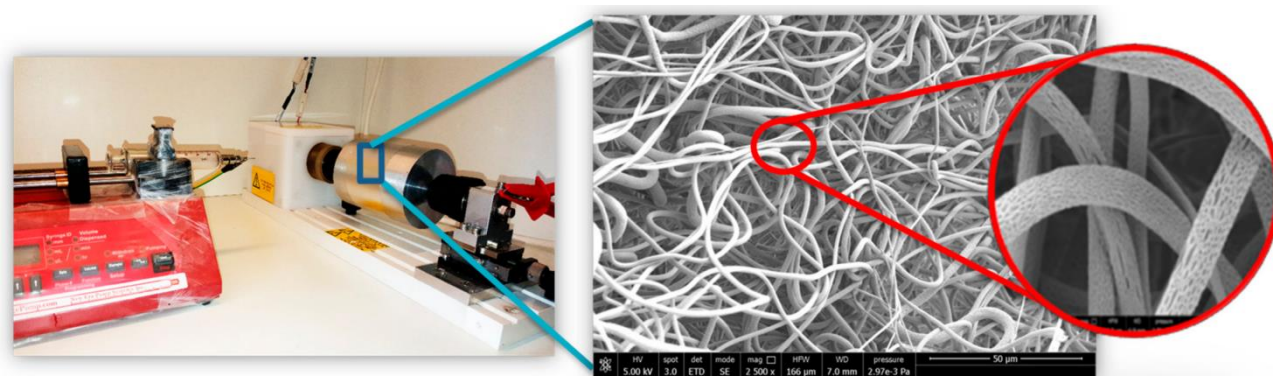
Figure 1 *Electrospinning process and fiber morphology. Lens on the right shows nanoporosity on the fiber surface.*

2.2.3 Dose- and Time-Dependent Effect of OLE on 2D Culture Model

The metabolic cell activity of HUVECs cultured on tissue culture plastics (2D model) was evaluated by WST-1 assay, as described in Section 4.7.1. The dose-dependent metabolic curve showed no significant cytotoxic effects at OLE concentration below 250 µg GAE/ml TP (Figure 2A). To evaluate the time-dependent metabolic activity of HUVECs, a WST-1 assay was performed after 24 h of treatment (Figure 2B). OLE also resulted as non-toxic after a long period of treatment below 250 µg GAE/ml TP.
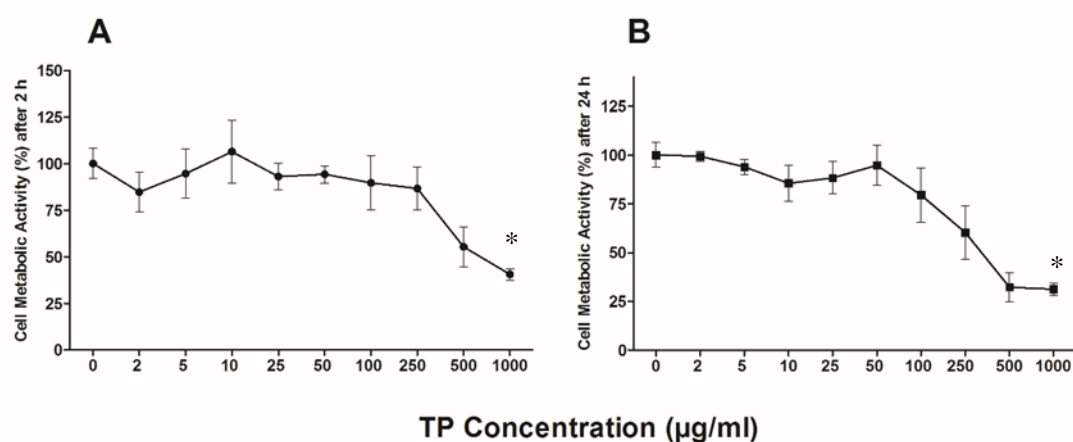
Figure 2. *Dose- and time-dependent cell metabolic activity. HUVECs were cultured for 2 h (A) and 24 h (B) in the presence of different concentrations of TPs from OLE. Cell metabolic activity was determined by WST-1 colorimetric assay and expressed as metabolic activity percentage compared to control (untreated cells). Graphical data are represented as mean ± SD of three separate experiments run in triplicate. *p < 0.05 vs control*

2.2.4 Antioxidant activity of OLE

To evaluate the antioxidant activity of OLE, cells were pre-treated for 2 h and 24 h with different OLE concentrations and thereafter an oxidative stress insult was applied by treating the cells with

100 µM H_2O_2 for 1 h. As shown in Figure 3, only 24 h pre-incubation at a concentration range between 50 µg/ml and 250 µg/ml of TP prevented H_2O_2-induced viability reduction of HUVECs ($p < 0.05$), thus demonstrating to possess an antioxidant effect.
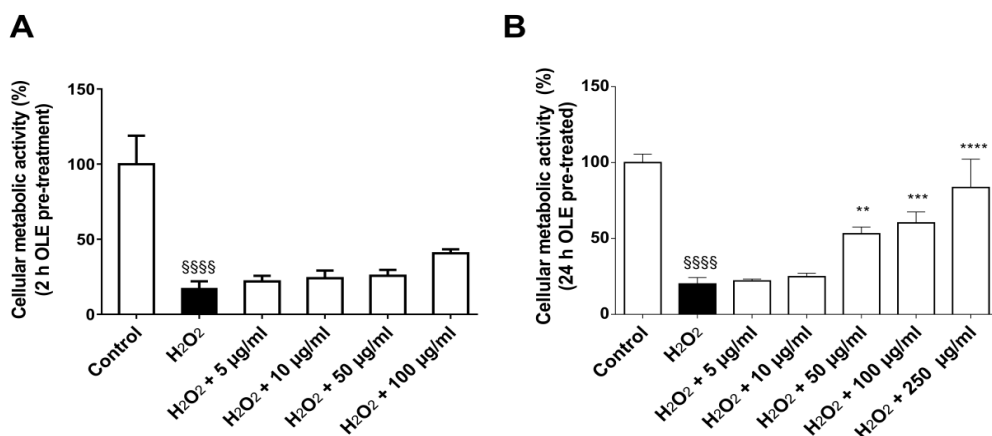
Figure 3. *Antioxidant effect of OLE. HUVEC viability was evaluated after 2 h (**A**) and 24 h (**B**) of pre-treatment with different concentrations of OLE (i.e., 0, 5, 10, 50, 100, up to 250 µg/ml of TPs) followed by treatment with 100 µM H_2O_2 for 1 h. Data are expressed as metabolic activity percentage compared to control (untreated cells) and are representative of 3 separate experiments run in triplicate. **p < 0.005 and *** p < 0.0005 vs. H_2O_2; §§§§p < 0.00001 vs. control.*

2.2.5   ROS production

ROS accumulation in HUVECs was evaluated after 24 h pre-treatment of OLE at different concentrations (ranging in 10-250 µg GAE/ml of TP). Figure 4 shows OLE effect on ROS accumulation in HUVECs, as determined by CM-H_2DCFDA. Results highlighted a significantly protective effect by OLE polyphenols at a concentration below 250 µg GAE/ml TP ($p < 0.0001$ vs. H_2O_2).

2.2.6 OLE effect in 3D culture model

The cytocompatibility of HUVECs grown on P(VDF-TrFE) fiber meshes was analyzed after 3 days and 7 days of culture using the alamarBlue® assay. As displayed in Figure 5 (white bars), the HUVECs were viable on the scaffolds, showing a metabolic activity above 40%. HUVECs were grown on the scaffolds for 6 days, then they were preconditioned with OLE for 24 h and subsequently to administrated with 100 µM H_2O_2 for 1 h. The results demonstrated a metabolic activity higher than 80% (grey bars) and confirmed the protective effect of OLE at a TP dose of 100 µg/ml (*$p < 0.05$ vs. H_2O_2). This model represents one of the first studies to demonstrate the cytocompatibility of P(VDF-TrFE) fibers with HUVECs. Previously Augustine, R., et al., with incorporation of zinc oxide (ZnO) nanoparticles into the P(VDF-TrFE) matrix studied the biocompatibility in HUVECs, exhibiting higher cell viability, adhesion, and proliferation compared to cells cultured on tissue culture plates or neat P(VDF-TrFE) scaffolds (Agustine, R., et al., 2017). Other studies used P(VDF-TrFE) fibrous mats to enhance neurite growth along the fiber orientation direction and bone regeneration (Li, Y et al.,2019)
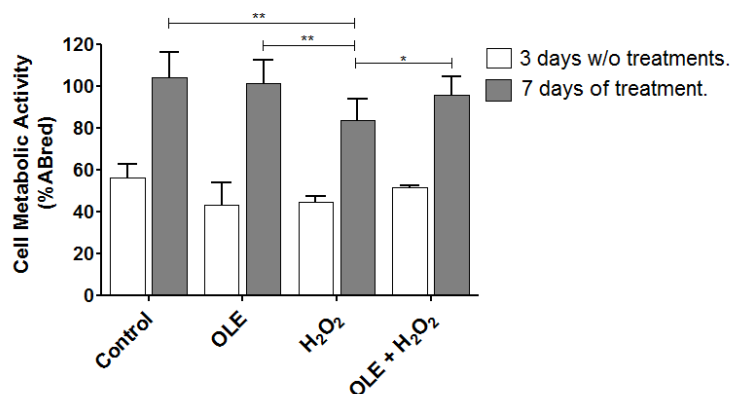
Figure 5. Metabolic activity of HUVECs on P(VDF-TrFE) scaffolds and antioxidant activity of OLE on cell/scaffold constructs. HUVECs after 3 days of culture were viable in the 3D model (white bars) before OLE and H_2O_2 treatments. Gray bars represent cell/scaffold constructs incubated with OLE (100 µg/ml of TPs) for 24 h and 100 µM H_2O_2 for 1 h. Data are expressed as % alamarBlue® reduction and are representative of 3 separate experiments in triplicate. *p < 0.05 and **p < 0.005 vs. H_2O_2.

ROS production in cell/scaffolds construct was evaluated after 24 h pre-treatment of OLE (100 µg GAE/ml of TP) followed by 100 µM H_2O_2 for 1 h. Figure 6 shows a significant protective effect of OLE on ROS accumulation in H_2O_2-stressed HUVECs ($p < 0.05$). The generation of ROS was qualitatively detected using by fluorescent microscopy, as described in methods section.
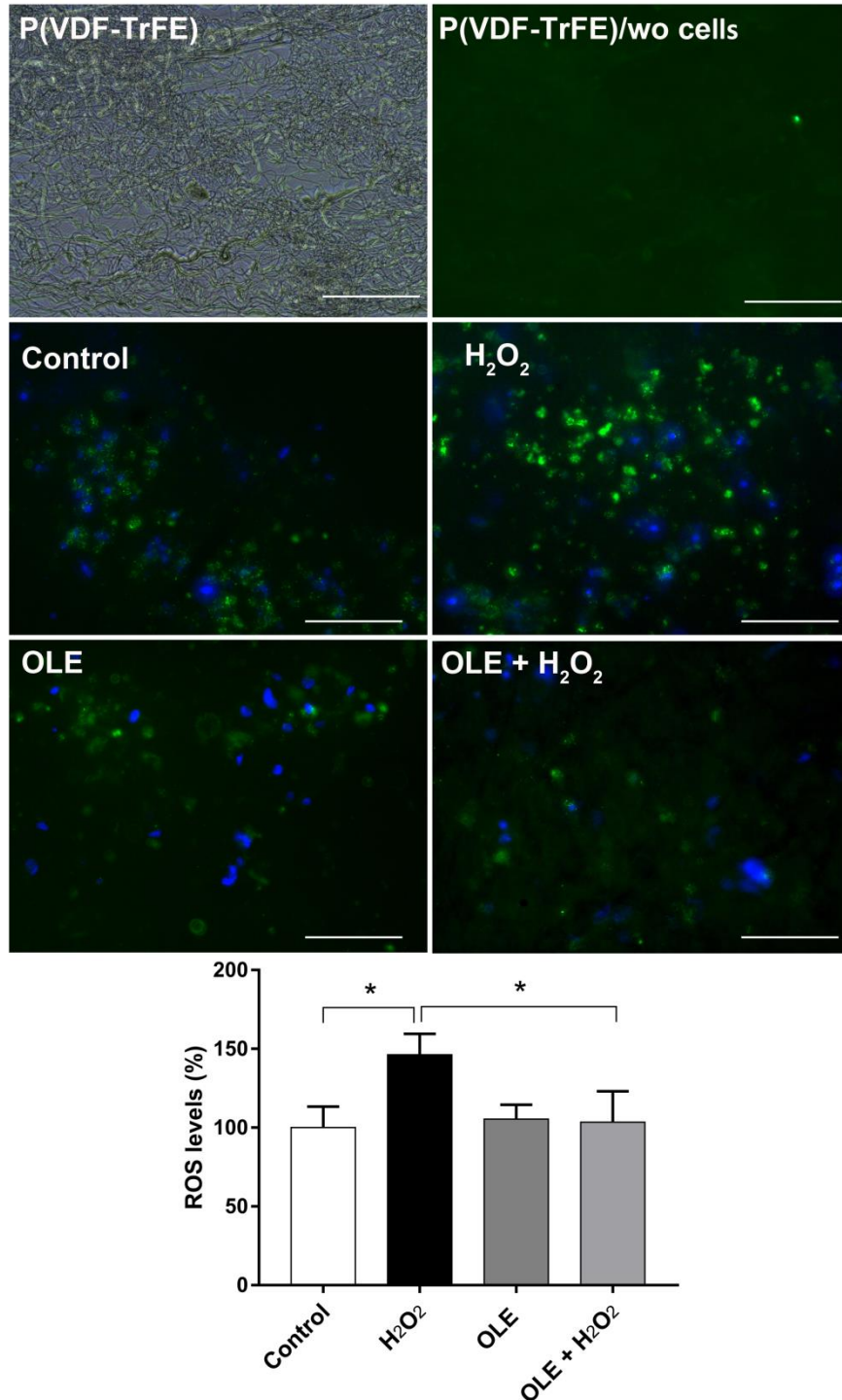
Figure 6. *Results of ROS analysis performed on the 3D model treated with OLE for 24 h prior to H2O2 incubation for 1 h. (**A**) Imaging of fibers without (wo) cells under transmission and fluorescence microscopy modes. (**B**) Panel of fluorescence micrographs of the 3D models under the different treatments showing nuclei in blue and ROS in green. Magnification is 20×; scale bar is 100 μm. (**C**) ROS induction percentage as from integrated OD (* p < 0.05 vs. control, H2O2, and OLE + H2O2).*

2.2.7. 3D model characterization

SEM was used to evaluate HUVEC morphology and fluorescence microscopy to assess the cellular colonization of the scaffolds by detecting blue-stained cell nuclei (Figure 7). Endothelial cells were grown within the fibrous matrix of the scaffold and showed a good spatial distribution. Cell nuclei were observed at different depths, without any signs of pyknosis, thus corroborating the results obtained with the alamarBlue® test.
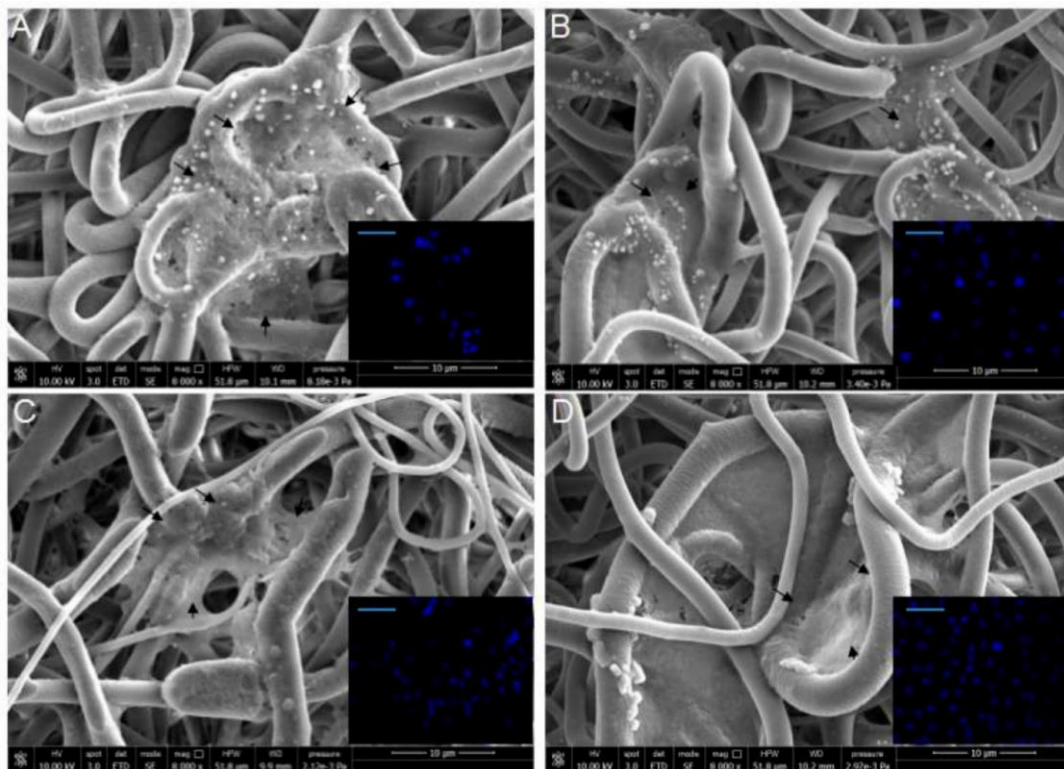
Fi*gure 7. SEM and fluorescence analyses of P(VDF-TrFE)/HUVEC construct: (**A**) cell/scaffold construct (control); (**B**) cell/scaffold construct + OLE; (**C**) cell/scaffold construct + H$_2$O$_2$; and (**D**) cell/scaffold construct + OLE + H$_2$O$_2$. Black arrows indicate the HUVECs in the construct. Fluorescence analysis is reported in the inserts. Scale bar in inserts is 100 μm.*

2.3 DISCUSSION

OLE can be extracted from the waste leaves of the olive tree and represents a valid source of polyphenols. Several studies are currently evaluating the possible applications of OLE, not only for nutrition as a food supplement but also as a pharmaceutical agent, mainly for cardiovascular diseases. Indeed, the antioxidant and cytoprotective effects of OLE have been recognized at the endothelium level. They are of great importance since tissue damage at the vascular wall leads to stress in the

vascular endothelium and induces complications in the vascular system by oxidative stress (Woywodt, A., et al., 2008).

The mechanism by which OLE can exert such a strong antioxidant activity is a subject of investigation. OLE is rich in polyphenols, mainly in oleuropein, which is its best-known polyphenol. Other polyphenols in OLE have been reported to exert pro-oxidant effects, due to their iron-and-copper-reducing activities. These reduced metals, in turn, can catalyze the production of OH· radical by the Fenton reaction. Moreover, the capacity of dietary polyphenols to act as antioxidant/prooxidants under in vitro and in vivo systems is dependent on a number of factors, such as their concentration and structure (Kouka, P., et al., 2017; Micucci, M., et al., 2015). These bioactive compounds have shown cell reinforcement, antihypertensive, antiatherogenic, anti-inflammatory, hypoglycemic, and hypocholesterolemic properties (Micucci, M., et al., 2015). Therefore, there is a growing scientific interest in disclosing, with improved specificity, the potential of OLE in biomedicine. In particular, the Tuscan autochthonous variant of the olive tree (*O. europaea var. Olivastra seggianese*) is studied for its peculiar properties. Olive oil plantations produce several wastes, mostly unexploited, such as olive tree prunings, which produce, upon burning by farmers, a considerable amount of greenhouse gases (Salomone, R., et al., 2012). Under a circular economy and life cycle analysis point of view, a sustainable use of agri-food waste to produce high-value products is greatly encouraged in place of arbitrary disposal and lop burning. Solvents commonly used, such as ethanol or aqueous-based ethanol or methanol solutions are generally used for obtaining extracts with high phenol content and antioxidant activity. However, these popular extraction methods suffer from some drawbacks, such as insufficient recovery of extracts and long extraction times, and intensive heating and/or mixing results in high levels of energy consumption (Rombaut, N., et al., 2014). In order to have a fully green process, we opted for water-based extraction. Indeed, the use of water alone has given results comparable to those obtained with ethanol (Lafka, T., et al., 2013).

The aim of this study is to characterize OLE from Tuscan olive trees and assess its antioxidant properties. In particular, we set-up a 3D in vitro model by using a biocompatible fibrous scaffold to obtain more reliable results about OLE effects. 3D in vitro tissue models offer ethically sustainable alternatives for drug testing, largely catching the attention of the scientific community. The protective effect of OLE could widen the possibility of the application of agrifood waste derivatives to biomedical devices and TE.

There are differences in the levels and types of phenolic compounds in *O. europaea* L. leaves, fruits, and seeds. TP and the chemical composition of olive leaves change according to several conditions, such as origin, the proportion of branches on the tree, storage conditions, climatic conditions, moisture content, and degree of contamination with soil (El, S.N., et al.,2009). Our OLE polyphenol characterization showed a TP content of 23.29 mg GAE/g, with oleuropein as a main component. In

line with other varieties of *O. europaea*, the TP content in aqueous extraction was around 9–17 mg GAE/g (Ozcan, M., et al., 2017). These results are in accordance with those reported in other studies of polyphenol quantification in OLE (Ozcan, M., et al., 2017; Zheng, Q., et al., 2017). However, we obtained different quantification of TPs in diverse sampling periods, with early spring OLE being the richest in TPs.

We investigated the protective effect of OLE from Tuscan olive trees on endothelial cells, using a conventional culture (2D model) and a novel tissue-engineered culture (3D model) of HUVECs. Our past work has, in fact, highlighted the importance of scaffold architecture in generating complex vascularized in vitro models using HUVECs (De la Ossa, J.G., et al., 2017). In this study, a new 3D model was obtained by fabricating a fiber mesh of P(VDF-TrFE), a biocompatible polymer used for biomedical devices and tissue engineering (Azimi, B., et al.,2019). The fibrous character of the scaffold produced via electrospinning had the purpose of mimicking natural ECM, thus helping the maintenance of native cell morphology and function (Jun, I., et al., 2018; Stocco, T., et al., 2018). P(VDF-TrFE) was specifically chosen for scaffold fabrication for its chemical inertia and non-degradability, in order to prevent any biomaterial damage following hydrogen peroxide administration. P(VDF-TrFE) possesses additional peculiar characteristics, such as piezoelectricity, which can be exploited to obtain truly functional 3D models of blood vessels (Fukada, E et al., 1969; Lenz, T., et al., 2017; Azimi, B., et al., 2019). It has been outlined that tissue-engineered in vitro models are more reliable than conventional 2D cell cultures on tissue culture plastics (Jensen, G et al., 2018). Therefore, we tested the non-cytotoxic dose and ROS protective effect of OLE in 2D and 3D cellular cultures.

In the 2D model, we demonstrated a dose-dependent effect of OLE on cell metabolic activity: a concentration of 250 µg/mL TPs was sufficient both to reduce the effect of oxidative stress induced by hydrogen peroxide and to produce cytoprotective effects in HUVECs.

In the 2D and 3D models, we investigated if HUVECs were still protected by OLE after exposure to oxidative stress induced by H_2O_2. Following treatment with OLE at 100 µg/mL, we also showed that in the 3D model, OLE exerted cytoprotective effects. The metabolic activity of cells grown on the scaffolds and treated with OLE, followed by H_2O_2 treatment, was indeed significantly higher than that observed in OLE-untreated samples. These results demonstrate the indirect antioxidant potential of OLE, possibly acting through the augmentation of cellular antioxidant capacity by enhancing specific genes encoding antioxidant proteins (Jung, K.A., et al., 2010). Moreover, studies have suggested that the combination of olive phenolic compounds, such as those present in OLE, exhibit a synergistic behavior towards free radical elimination, superior to the antioxidant capacity of the vitamin C and E (Benavente-Garcia, O., et al., 2000). On the other hand, the drop-in metabolic activity

experienced by the same cells after the H_2O_2 insult in the 2D model was much more enhanced than in the 3D model. This outcome is meaningful since it shows that the 3D model is better predictive of real tissue response, including disease tissue conditions (Benam, K.H., et al 2015). Ultimately, our results performed on the 3D models confirmed a significantly protective effect of OLE on ROS accumulation in HUVECs, the detected ROS level in OLE pre-treated H_2O_2-stressed samples being in the same range of non-stressed control samples, where it was significantly lower than that of H_2O_2-stressed samples. Moreover, different from the 2D model in which a 250 µg/mL OLE dose was necessary, in the 3D model, a 100 µg/mL OLE dose was sufficient to have a ROS-protective effect. This can be linked to the reduced metabolic activity drop and may deal with diffusion gradients and other complex phenomena occurring in 3D microenvironments, which are still a subject of investigation (Tabandeh, M.R.., et al., 2014).

The strategy of using natural polysaccharides and proteins to reduce free radical production has been explored in wound healing using plant and milk derivatives such as *Aloe vera* and whey protein (Tabandeh, M.R., et al 2014; Kerasioti, E., et al., 2014). Scaffolds loaded with palm extracted polyphenols have shown the ability to reduce free radical-derived inflammation, thus maintaining homeostasis and endothelial cell function (Zaden, K., et al., 2019). These encouraging results also highlight the increasing scientific interest in the application of characterized natural extracts to biomedicine.

Our results indicate that OLE sustains endothelial cell viability in 3D scaffolds, thus suggesting a potential use in TE applications. For example, many TE scaffolds are obtained by processing aliphatic polyesters, such as polylactic acid, which are highly hydrophobic. No studies of hydrophobicity or hydrophilicity were conducted because the absorption of the extract in the biomaterial was no the goal of this first investigation, as OLE was added directly in the culture media. On the other hand, polyphenols have been demonstrated to enhance cell proliferation on those materials by virtue of their hydrophilic properties. From our results, the polyphenols in the 2D and 3D models might have played an active role in improving cell viability and proliferation. We show that the antioxidant property of polyphenols can both minimize ROS-induced cell damage and improve cell viability. As a next step, biobased scaffolds, incorporating OLE inside the polymer to enable a controlled polyphenol release, could be developed to minimize the oxidative stress and be used in different tissue regeneration fields, such as cardiovascular disease and wound healing.

CHAPTER 3

EXPERIMENTAL SECTION II

3 COMBINED ANTIMICROBIAL EFFECT OF COLD ATMOSPHERIC PLASMA AND BIO-WASTE OLE AGAINST BACTERIAL PATHOGENS

The overall health beneficial action of olive tree phenolic components is well established being the antioxidant properties of the olive tree have been the best known. Nevertheless, recently there has been increased attention on its antibacterial properties showing inhibition effects against microorganisms. Many studies demonstrated the combination of OLE phenolics composition possess not only antioxidant activity but also antimicrobial activities. This new approach valorizes the olive leaves in a valuable bioactive source. In this chapter a new strategy is proposed:

- Investigate the antimicrobial effect of OLE versus CAP technology or their combination against pathogens.

3.1. MATERIALS AND METHODS

3.1.1 CAP Set-Up

A DBD reactor was used for CAP inactivation provided by Fourth State Medicine Ltda. To generate CP, the instrument was set up at a flow rate of 5 L/min air for 1 min. The samples were treated at room temperature (namely, 20°C). The samples plasma treated were *Listeria innocua* (ATCC 33090), *Escherichia coli* (ATCC 47076) and *Staphylococcus aureus* (ATCC 25923) all with and without OLE incubation. The plasma power supply was 8 kV of voltage and frequency of AC 20 kHz.

3.1.2 Inoculum of OLE

Stock cultures of *Listeria innocua* (ATCC 33090), *Escherichia coli* (ATCC 47076) and *Staphylococcus aureus* (ATCC 25923) were stored at −80 °C in Tryptic Soy Broth (TSB, Oxoid Ltd., UK), supplemented with 15% v/v glycerol. A loopful of thawed culture was inoculated in 15 ml TSB for 24 h at 37 °C. 20 μl was subsequently transferred to fresh 20 ml TSB and cultured for 24 h at 37 °C for either 6h or 24h to obtain bacterial cells in the exponential or stationary phase, respectively some described or modified in (Costello et al., 2019; Dessauw, E et al., 2019).

3.1.3 Effect of OLE and CAP/OLE on *Escherichia coli*, *Staphylococcus aureus* and *Listeria innocua*

A volume of 1 ml from the 6h and 24h culture was centrifuged (10000g, 10 min, 23°C) and re-suspended in 1 ml of PBS. For each resuspended *E. coli*, *S. aureus* and *L. innocua* cells were separately treated with OLE (100 mg GAE/ml TP) that according to the literature is a good concentration (Liu, Y., et al., 2017). In a separate 24-well plate 900 ml of treatment solution (composed by 100 mg/ml TP of OLE dissolved in TBS) or TSB (used as controls) was added to 100 µL of bacterial inoculum. The effects of the OLE on the strains were evaluated at different time points for a total duration of 24 h by plate counting of colony forming units (CFU)/ml using the spread plate or drop plate method and by measuring optical density (O.D.) 600 nm. The experiments were carried out in triplicate. CAP and OLE was assessed after the OLE treatment at 0,4,6,12 and 24h. Every time point was treated with CAP during 1 min and 5 LMP and consequently the effect was evaluated by spread plating and measuring CFU/ml.

3.1.4 Statistical analysis

The data was collected on excel and average values and standard deviations determined. Statistical analysis was performed using SPSS 22.0 (SPSS Inc., Chicago, United States) by conducting one-way analysis of variance (ANOVA) and the Tukey's HSD post hoc test to determine whether there was statistical difference among samples and differences were considered significant at $P < 0.05$.

3.2 RESULTS AND DISCUSSION

3.2.1 Polyphenol Characterization

Furthermore, HPLC analysis allowed the main polyphenols present in OLE to be determined, which were reported in Table 1 and given as mg/g in table 1. They were oleuropein with a concentration of 14.69 ± 0.92; moreover, OLE contained apigenin-7-O-glucoside with a concentration of 1.97 ± 0.17 mg/g of OLE, rutin with a concentration of 3.37 ± 0.33 mg/g of OLE and other compounds (HT, caffeic acid and *p*-coumaric acid) with a concentration lower than 1 mg/g of OLE.

Bioactive Molecule	Concentration (mg/g OLE)
Apigenin-7-O-glucoside	1.97 ± 0.17
Rutin	3.37 ± 0.33
Hydroxytyrosol	0.85 ± 0.08
Caffeic Acid	0.18 ± 0.02
Oleuropein	14.69 ± 0.92

Table 1. Concentrations of Polyphenols in OLE

3.2.2 Effect of OLE on *E. coli*, *S. aureus* and *L. innocua*

The antibacterial effects of OLE against *E. coli*, *S. aureus* and *L. innocua* were determined and there was an antimicrobial activity against *S. aureus* while *in L. innocua* and *E. coli* antimicrobial effect was not observed.The values are reported as mean ± standard error of the mean (SEM) (n = 3), significance at $p < 0.05$ by One-way ANOVA and Tukey's HSD post hoc test; *** $p < 0.0001$). The bactericidal activities of OLE represent in activities of oleuropein, tyrosol and HT have been investigated, showed positive effects on bacteria strains (Djaname, et al., 2019).
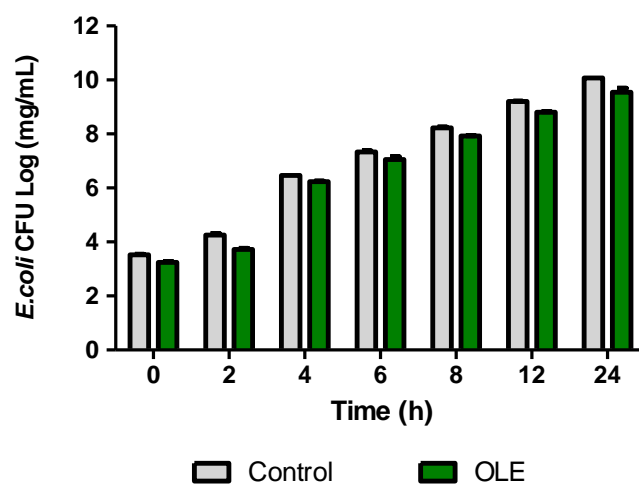
Figure 1. Effects of OLE on *Escherichia coli.* The values are reported as mean ± standard error of the mean (SEM) (n = 3), significance at $p < 0.05$ by One-way ANOVA and Tukey's HSD post hoc test; *** $p < 0.0001$, n.s. not significant.
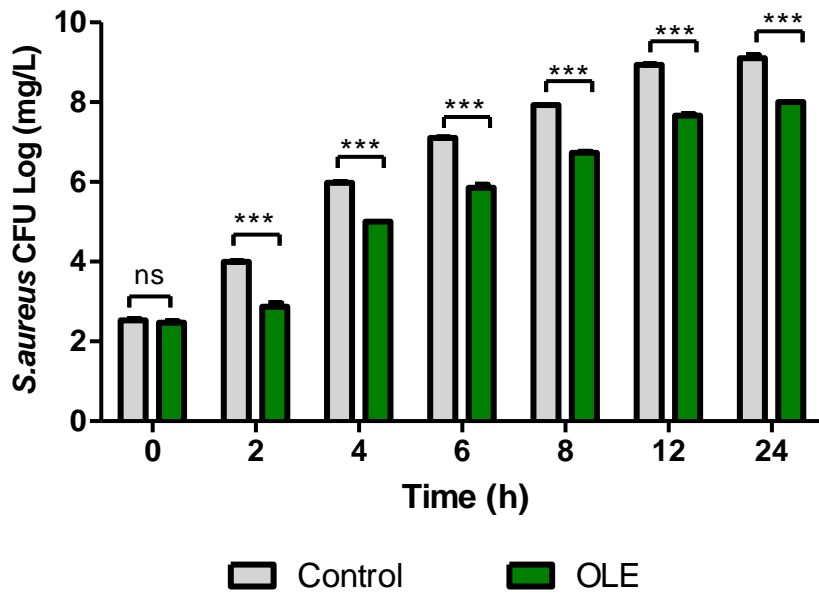
Figure 2 *Effect of OLE on Staphylococcus aureus.* The values are reported as mean ± standard error of the mean (SEM) (n = 3), significance at p <0.05 by One-way ANOVA and Tukey's HSD post hoc test; *** p < 0.0001, n.s. not significant.
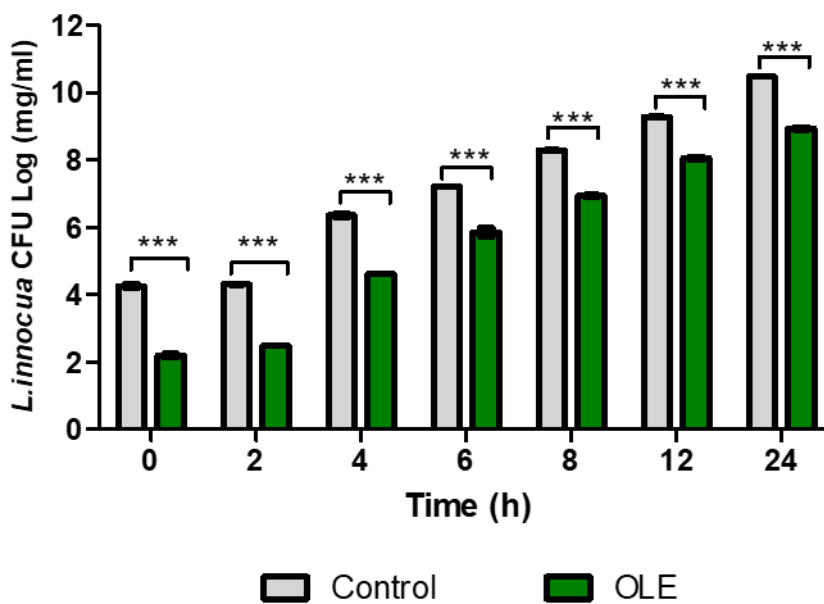
Figure 3. Effect of OLE on *Listeria innocua.* The values are reported as mean ± standard error of the mean (SEM) (n = 3), significance at p <0.05 by One-way ANOVA and Tukey's HSD post hoc test; *** p < 0.0001, n.s. not significant.

3.2.3 Effect of atmospheric cold plasma on inactivation of *Escherichia coli*, *Staphylococcus aureus* and *Listeria innocua*

Ours method employing CAP/OLE have a synergistic antimicrobial activity against the *S.aureus, E.coli* and *L. innocua* at 6 h. The *S. aureus* counts of samples with CAP treatment decreased significantly. CAP/OLE was effective against the number of S. *aureus* samples but were not effective against *L. innocua* at 24 h. *E.coli* had total inhibition at 6 h while 24 h or exponential phase the bacteria are more resistant at plasma and CAP and OLE (n = 3), significance at p <0.05 by One-way ANOVA and Tukey's HSD post hoc test; *** $p < 0.0001$).
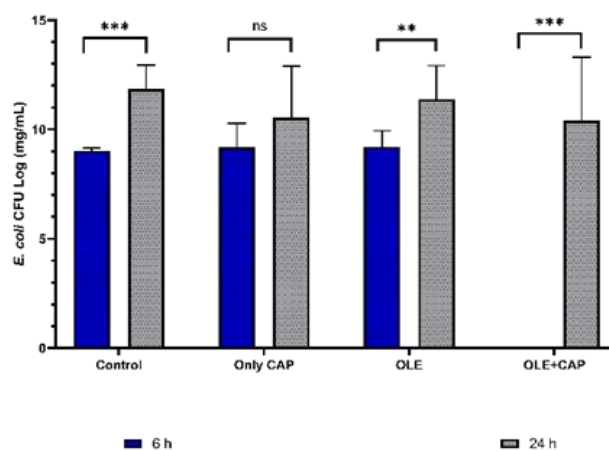
Figure 4*. The effects of CAP (1 min exposure time) and/or OLE on E. coli. The values are reported as mean ± standard error of the mean (SEM) (n = 3), significance at p < 0.05 by One-way ANOVA and Tukey's HSD post hoc test; ** p < 0.001, *** p < 0.0001, n.s. not significant.*

The influence of CAP on *E. coli* have not differences between controls (P < 0.05) but interesting results were found in the treatment with OLE and CAP, in this synergic effect there was no bacterial growth. The effect of CAP in synergy with OLE is effective against *S. aureus* inhibition. However, for this strain an inhibition with OLE without plasma treatment were sufficient to inhibit the bacteria after 24h. (Statistical analysis: mean ± standard error of the mean (SEM) (n = 3), significance at p <0.05 by One-way ANOVA and Tukey's HSD post hoc test; *** p < 0.0001).
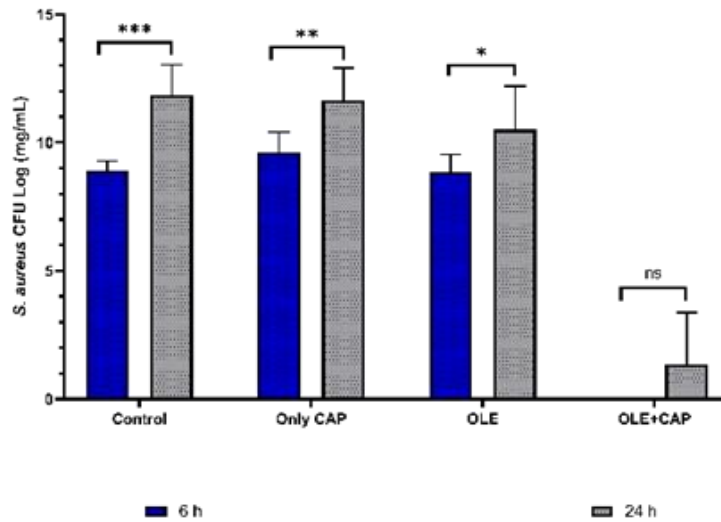
Figure 5. *The effects of CAP (1 min exposure time) and/or OLE on S. aureus. The values are reported as mean ± standard error of the mean (SEM) (n = 3), significance at p < 0.05 by One-way ANOVA and Tukey's HSD post hoc test; * p < 0.01, ** p < 0.001, *** p < 0.0001, n.s. not significant.*
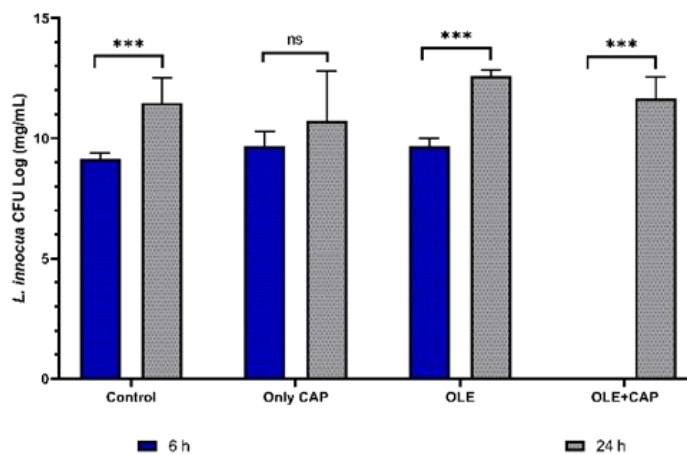
Figure 6. *The effects of CAP (1 min exposure time) and/or OLE on L. innocua. The values are reported as mean ± standard error of the mean (SEM) (n = 3), significance at p < 0.05 by One-way ANOVA and Tukey's HSD post hoc test; * p < 0.01, ** p < 0.001, *** p < 0.0001, n.s. not significant.*

3.3. DISCUSSION

The effect of OLE on the strains is illustrated in Figures 1-3 For *E. coli*, OLE did not exhibit any antimicrobial effect (Figure 1), whereas in *S. aureus* and *L. Innocua*, statistically significant antimicrobial effects were observed over time (Figures 2, 3). In particular, for *L. innocua*, OLE showed a reduction in the number of colonies even if an inactivation was not achieved (Figure 3).

To understand and compare the obtained results, it is important to consider that OLE has a high variability in the concentration of bioactive compounds, as a consequence of various factors, such as the raw material and the extraction process, among others (Difonzo, G., et al., 2017). Moreover, the final composition of the OLE has a great importance on the antimicrobial effect. In fact, the bacterial inactivation was more evident in *S. aureus* and *L. innocua* using the surface spread method, while *E. coli* did not show a considerable susceptibility to OLE. The amounts of TPs and other compounds indicate that the presence of phenolic compounds at 100 mg GAE/ml TPs can exert an antimicrobial activity, but still insufficient for obtaining a total or significant bacterial inactivation. It is reported that the polyphenols in OLE, or possibly the synergistic effects between them (Lee, O.H., and Lee, B.Y., 2010), may be responsible for OLE antimicrobial activity by inducing membrane permeability in bacteria, further inhibition of biochemical pathways and finally disintegration of the outer membrane causing the bacteria death (Mai-Prochnow, A., et al., 2016). Furthermore, a broad-spectrum polyphenols have been found in OLE at small concentrations (Table 1), that probably can exert an antimicrobial effect together with oleuropein.

The comparison of results obtained after 6 h and 24 h using the single treatment and their combination is reported in Figures 4-6. As shown, the combined treatment (OLE + CAP) demonstrated a synergistic antimicrobial effect against the *E. coli*, *S. aureus* and *L. innocua* at 6 h (Figure 7).
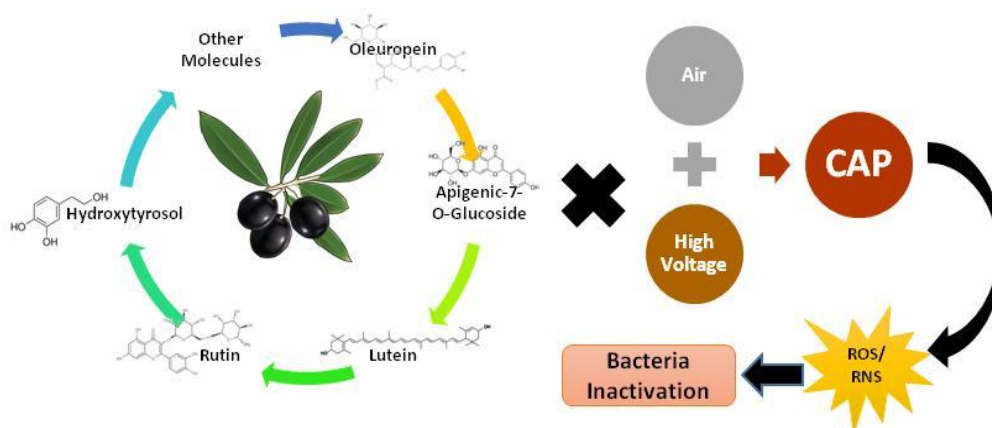
Figure 7. *The characteristics of OLE and CAP produce bacteria inactivation.*

However, the remaining bacteria in small numbers were able to grow in longer time.

Using the combined OLE + CAP treatment, *E. coli* had significant inhibition at 6 h, while at 24 h the bacteria reproduced but still with a significant reduction with respect to the control and individual treatments (Figure 4). As a gram-negative bacterium, *E.coli* is more resistant to eradication (Albertos, I. et al., 2017). The most remarkable effect was obtained against S. aureus (Figure 5). *S. aureus* samples subjected to OLE + CAP treatment significantly decreased after 6 h and 24 h if compared to control and single treatments. The effectiveness of the combined treatment was confirmed by a very

low replication of residual bacteria at 24 h. The combined OLE + CAP technique allowed a remarkable bacterial inactivation during the exponential phase (6 h) for all the strains. In the stationary phase (24 h) the treatment OLE + CAP was sufficiently effective on *E. coli*, which grew by reaching ~7Log[CFU] and still remarkably effective on S. aureus, which grew up to ~3Log[CFU], thus confirming the overall synergistic effect with OLE and CAP treatments. In contrast, the other individual treatments did not demonstrate any relevant antibacterial effects.

The case of *L. innocua* was more complex. After a high eradication at 6 h, the bacteria were reproducing very fast and grew up to untreated control levels (Figure 6). For this strain, the plain OLE seems to be able to control bacterial growth over time. *L. innocua,* bacterial decontamination was observed after 6 h, but the effect was nullified after 24 h, reaching ~7Log[CFU]. Interestingly, and in accordance with the results reported in Figure 3, OLE alone seems to be best effective in containing bacterial growth. Especially in this case, higher time exposure to CAP or more concentrated OLE could be needed to show a more prolonged effect of bacterial eradication. In addition, the precise antimicrobial mechanism of polyphenols in olive leaf extract in unknown. According to some studies, in somehow, the activity of OLE inhibits several enzymes, micrococcal nuclease, lysozyme and cause damages to cell membrane (Borjan, D et al., 2020). However, other investigation notes that phenolic product causes changes in intracellular ATP concentration and changes in membrane potential depolarizing the bacterial membrane and leakage of intracellular components (Barbosa et al., 2015). Some signs about the influence of phenolic compounds as antimicrobial resource are showed in L. monocytogenes, indicating the inhibition of DNA synthesis or promote the cleavage of DNA of the bacterium (Guo, L et al.,2019). Wang et al., 2017 suggested that the phenolic component not only reduced the amount of DNA in cells by increasing cell membrane permeability and destroying the cell morphology but also bound to the minor groove of genomic DNA, resulting in changes in the secondary structure and morphology of DNA.

Our CAP technology involves chemical components, such as ROS/RNS, which in combination with the polyphenolic compounds of OLE, such as oleuropein, is supposed to inactivate the microorganisms. Recent studies (Han, L., et al., 2016; Dobrynin, D., et al., 2009) showed that CAP efficacy is directly correlated to bacterial cell wall thickness in several species. Gram-negative species, such as *P. aeruginosa*, were almost completely eradicated due to their thin cell membrane (2.4 nm); while Gram-positive species, such as *B. subtilis*, showed the highest resistance to CAP due to their thicker membranes (55.4 nm cell wall). *E. coli* belongs to Gram-negative bacteria, with a thinner outer membrane compared to the Gram-positive *S. aureus* and *L. innocua*. However, none clear trend is apparent from this and other studies, since complex interactions with the system, process, surface or medium may also impact on CAP efficacy in combination with cell type.

However, in combination with OLE, a better inactivation was obtained also with *S. aureus*, a Gram-positive bacterium, probably due to ROS-enhanced intracellular damage (Dobrynin, D., et al., 2009). Another significant role in the mechanical disruption of bacterial cell membrane is the effect of charged particles that could accumulate on surface and cause electrostatic stress (Dobrynin, D., et al., 2009). Due to reactive species produced in plasma which react with the protein amino-acids and further cause structural changes in proteins, finally destroying the quiescent cells (Surowsky, B et al.,2014). It is hypothesized that such morphological changes overcome the tensile strength of cell membrane (Yang, D.C., et al., 2016). In fact, *S. aureus* displayed a size reduction of the colonies (results not shown). Cell membrane perforation caused by etching enhances the diffusion of secondary reactive species that might be formed in the plasma discharge inside the cell (Bourke, P., et al., 2017). Due to its complex composition and multiple different reactive components, CP is expected to play a role, independently or in synergy, in the inactivation of microbial targets.

Generally, the good efficacy of CP depends on the device design and system operating parameters, such as gas composition, flow rate, moisture, temperature, voltage, and frequency (Bourke, P., et al., 2017; Difonzo, G., et al., 2017; Metelmann, H.R., et al., 2018). In addition, the ozone, O_3, present in CAP effluent could break structural bonds in the peptidoglycan component of cell wall, like C-O, C-N bonds, leading to cell wall destruction and, consequently, cell death (Stoffels, E., et al., 2006; Metelmann, H.R., et al., 2018). On the basis of the results, it can be deduced that CP, containing among other ROS/RNS (Yusupov, M., et al., 2013), combined with the action of the OLE polyphenols exerts an enhanced antimicrobial activity by an efficient damage and disruption of bacterial membrane. A recent study has shown an efficacy of the combined effect of CAP and nisin against *L. innocua*, grown planktonically or as surface colonies in a food model (Costello, K.M., et al., 2021), which supports our findings. CAP is thus a useful means to possibly synergize with other anti-bacterial and antifouling strategies inherently provided by biomaterials (Milazzo, M., et al., 2020). Finally, further research can be developed with this strategy; we can hypothesize the use of OLE as a coating or polymer blend in food or medical packaging followed by CAP treatment to ensure a green and safely decontaminated environment.

CHAPTER 4
EXPERIMENTAL SECTION III

3. POLYHYDROXYALKANOATES: ELECTROSPUN FIBERS INCORPORATING OLE AS BIO-BASED SCAFFOLDS FOR WOUND HEALING.

Tissue engineering has shown promise in improving methods of tissue regeneration, drug delivery, drug release, 3D tissue modeling, among others. A promising potential biomaterials candidate in scaffold manufacturing for tissue regeneration is PHA PHAs are biopolyesters produced by bacteria with prospective biomedical applications because they have possess tunable properties, are biodegradable and biocompatible.

This chapter is aimed at incorporating OLE inside PHBHV via electrospinning to obtain bioactive bio-based composites useful in wound healing.

4.1 MATERIALS AND METHODS

4.1.1 Fabrication of electrospun PHBHV/OLE fiber meshes.

PHBV (HV content 8% w/v) was purchased from Sigma-Aldrich (Milan, Italy). The mix solution was prepared using Dichoromethane (DCM)/methanol (MeOH) (10:1 w/w) bought from Carlo Erba (Rodano, Milan). OLE at 16.5%, w/w respect to PHBHV was dissolved in the solvent mix by stirring overnight at room temperature. The obtained PHBHV/OLE solution was poured in a 10 mL glass syringe and placed into a syringe pump (NE-300, New Era Pump Systems, Inc., NY, USA). The ground terminal of high voltage supply (S1600079 Linari High Voltage, Linari Engineering s.r.l, Pisa, Italy) was connected to the metal needle, whereas the positive terminal was connected to the collector. The fibers were collected onto a rotatory collector set at quasi-zero velocity, in order to collect randomly oriented fibers with a uniform mesh thickness, recovered of aluminum foil with a tip-to-collector distance of 15 cm at a flow rate of 0.0016 ml/min and 30kV after 1 h to have self-standing samples. Humidity was around 40% and temperature was 20°C throughout. PHVBH fiber meshes without OLE were collected in the same way to be used as controls. The parameters for the fiber fabrication were standardized at the Department of Civil and Industrial Engineering, University of Pisa. Image J software (version 1.52t) was used to evaluate the size of fibers. An average of 100 measurements has been reported for each sample.

4.1.2 OLE characterization by HPLC

The TP content was determined using the Folin-Ciocalteu method and gallic acid as the standard equivalent (ug GAE /mg) (from Merk, Darmstadt, Germany), following a reported procedure (Alessandri, S., et al., 2014). About 10 mg of OLE were dissolved in 10 mL of CH_3OH-H_2O (80:20 v/v) and then an aliquot of this solution (1 mL) was mixed with 0.25 mL of Folin-Ciocalteu reagent (FCR) and 1.5 mL of Na_2CO_3 (20% w/v). After, purified water was added to reach the volume of 10 mL, and the resulting mixture was kept for 45 min at the controlled temperature of 25 °C. Spectrophotometric analysis was performed at $\lambda = 725$ nm (Ainsworth, E., et al., 2007).

HPLC analysis was carried out to identify and quantify the major phenolic compounds of OLE using a slightly modified method developed in our previous study (Benavente-Garcia, et al., 2000). The retention times and UV absorbance spectra of phenolic compounds present in OLE were compared with those of the commercial standard and quantified at 278 nm, using p-hydroxyphenyl acetic acid as the internal standard, according to the previously reported method. Sample concentrations were determined by linear regression. For each calibration curve, the correlation coefficients were > 0.99.

HPLC analysis was performed with a HPLC instrument (Thermo Finnigan-Spectra System SCM1000) equipped with a Spectra System P2000 (Pumps), Spectra System UV2000, set to 280 nm, and using a Phenomenex Gemini reverse-phase C18 column (250×4.6 mm, 5 μm particle size; Phenomenex, Castel Maggiore, Italy). The mobile phase was a mixture of H_2O/AcOH (97.5:2.5 v/v) (A) and ACN (B). A linear gradient was run from 5% (B) to 25% (B) in 20 min; it changes to 50% (B) during 20 min (40 min total time); in 10 min it changes to 80% (B) (50 min total time), after re-equilibration in 5 min (55 min, total time) to initial composition. The flowrate was 1 mL/min and the injected volume was 50.0 μL. Samples was injected as a mixture of methanol/PBS (1:1 v/v). To assess the phenol content, 10 mg of OLE powder was dissolved in PBS and therefore analyzed with HPLC. The amount of phenolic compounds incorporated inside the polymer fibers was investigated using 4 cm^2 square meshes dissolved in MeOH and the resulting solution was analyzed by HPLC. Solvents used for HPLC analysis were purchased from Sigma-Aldrich. Tyrosol, HT, caffeic acid, p-coumaric acid, as analytical standards, were purchased from TCI (Zwijndrecht, Belgium). Oleuropein and p-hydroxyphenylacetic acid were purchased from Sigma-Aldrich. Luteolin-7-O-glucoside, apigenin-7-O-glucoside and rutin were purchased from Extrasynthese (Lyon, France). PBS for each analysis was diluted 10 times.

4.1.3 Characterization of PHBHV/OLE electrospun fibers

After production, the fibers were let dry under the laminar hood and stored at 4 °C for further use. The morphology of the fiber meshes was evaluated by scanning electron microscopy (SEM) using a FEI FEG-Quanta 450 instrument (Field Electron and Ion Company, Hillsboro, OR, USA). The samples were sputtered with gold (Gold Edwards SP150B, UK) before analysis. FT-IR ATR was used to characterize the presence of specific chemical groups in the tested samples. Analysis of the bulk samples was performed using Nicolet T380 instrument (Thermo Scientific, Waltham, MA, USA) equipped with a Smart ITX ATR attachment with a diamond plate. FT-IR analysis Chemical Imaging was then used to evaluate the surface distribution of components in the biomaterial, obtaining chemical and correlation maps to visualize their distribution. The micro-ATR and spotlight FTIR spectra and maps were carried out by means of a Perkin Elmer Spectrum One FTIR Spectrometer, equipped with a Universal ATR Sampling Accessory and a Spectrum Spotlight 300 FT-IR Imaging System (Perkin Elmer, Norwalk, CT) using the image mode of the instrument. For each sample, areas of 1 mm × 1 mm were analyzed. Afterward, an IR image was generated using a Nitrogen-cooled, 16-pixel mercury cadmium telluride line detector at a resolution of 25 μm per pixel. An absorbance spectrum was recorded for each pixel in the µATR mode. The spotlight software used for the acquisition was also employed to pre-process the spectra. Consequently, the spectra images were analyzed with a reference correlation image. Additionally, the full spectral maps were analyzed by Principal Component Analysis (PCA). For this analysis, the data set was composed by the spectra that make up the full spectral image. The spectra data grouping of similar variability is represented with the same color allowing the identification of different spectral group.

4.1.4 Degradation study of PHBHV/OLE electrospun fibers

A complete degradation analysis was carried out in vitro on PHBHV/OLE electrospun fibers. The tests were conducted soaking samples of the fiber meshes (size 1×1 cm^2; weight 5 mg) in phosphate buffer saline (PBS, from Sigma-Aldrich) up to 2 months. To understand the sample susceptibility to enzymatic degradation, a set of samples were soaked in PBS auditioned with metalloproteinase 9 (MMP-9; from Sigma-Aldrich), stock concentration 50 µg dissolved at 10 µg in 200 µl of PBS for a final concentration of 1.5 mL, room temperature (RT). The solution was replaced every week. Every week, the loss mass was weighted in an analytic balance (Alfa Precision, CONACOM, Italy) and the morphology of the samples was observed via SEM. The analysis of degraded samples was investigated by molecular loss mass, using GPC (Perkin Elmer, USA) and molecular weights of samples were determined using a GPC apparatus Agilent 1260 (Agilent, USA). Chloroform was used as the mobile phase and its constant flow-rate maintained at 1.0 mL/min. Polymer sample solutions were prepared by dissolving samples (0.5% w%) in chloroform (1 mL) and sterile filtered (0.45 µm).

A total volume of 100 μl was used for GPC analysis. Calibration was performed using the standards of polystyrene to determine the number-average and weight-average molecular weights (Mn and Mw) of samples.

4.1.5 *In vitro* phenol release study

In order to investigate the release of phenols from PHBHV/OLE, the diffusion of phenolic compounds in a release medium (PBS) was determined. A 4 cm^2 square of PHBHV/OLE was inserted into a 3 cm diameter well of a multi-well plate and 2 mL of PBS was added to each well. Then the plate containing the solutions was incubated in an oven at a controlled temperature of 37°C. At defined time intervals (0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 24, 48, 72, 144 h) medium was removed, the well was washed with 1 mL of PBS and an equal amount of the fresh medium (2 ml) was replaced each time (Doğan, G., et al., 2016; Chuysinuan, P., et al., 2018). The qualitative-quantitative evaluation of the phenols released from the polymer was carried out by HPLC analysis.

4.1.6 Cytocompatibility of PHBHV/OLE fiber scaffolds

Human Caucasian foreskin fetal fibroblasts (HFFF2) (ICLC #Catalog HL95002), were kindly provided from IRCCS AOU San Martino, IST, Italy. The cells were cultured with Dulbecco's Modified Eagle Medium (DMEM; Lonza, Walkersville, MD, USA) added with 10% fetal bovine serum (FBS), 2 mM L-Glutamine, 1% penicillin-streptomycin solution in T75 tissue culture flasks (all from Primo ®Cell Culture Flasks, Euroclone S.p.A, Pero, MI, Italy). The cells were seeded at 2-3×10^4 cells/cm^2, confluent cultures spitted 1:2-1:4 using Trypsin 0.25% (v/v)/EDTA (Euroclone S.p.A). Cell cultures were performed in a humidified incubator set at 37°C, 5% CO_2/95% air. The scaffolds were cut into 1× 1 cm squares and sterilized under UV light for 30 min. Before seeding, the scaffolds were coated with a sterile-filtered 2% (w/v) gelatin (type B, from bovine skin; Sigma-Aldrich) aqueous solution for 30 min. HFFF2 cells were trypsinized and seeded on PHBHV and PHBHV/OLE fiber meshes at a density at 200.000 cells/20 µl in 2% gelatin solution per scaffold, placed in 24 well plate. Cell/scaffold constructs were cultured in 1 ml growth medium replaced every 1-2 days for 1 week in a standard incubator. The metabolic activity of cell/scaffold constructs was evaluated using resazurine dye (Sigma-Aldrich) dissolved at a concentration of 0.5 mg/ml and incubated for 4 hours for 72 h after seeding. The absorbance (λ) of supernatants was measured with a spectrophotometer (Victor 3; PerkinElmer, Waltham, MA, USA) under a double wavelength reading (570 nm and 600 nm). Finally, dye reduction percent (%red) was calculated correlating the absorbance values and the molar extinction coefficients of the dye at the selected wavelengths. The equation applied is shown below, in which: λ = absorbance, s = sample, and c = control.

$$\%red = 100 \cdot \frac{(117{,}216 \cdot \lambda_{S(570\,nm)} - 80{,}586 \cdot \lambda_{S(600\,nm)})}{(155{,}677 \cdot \lambda_{c(600\,nm)} - 14{,}652 \cdot \lambda_{c(570\,nm)})} \qquad \text{(eq. 1)}$$

4.2 RESULTS

4.2.1 OLE characterization

The TP content of OLE was estimated Using the Folin-Ciocalteu method, resulting in 58.47 µg GAE/mg. Furthermore, HPLC analysis allowed the main polyphenols present in OLE to be determined, which were reported in Table 1 and given as percentages in Figure 1. They were oleuropein and luteolin-7-O-glucoside, with a concentration of 32.64 ± 3.06 mg GAE/g of OLE (3.26 w/w %) and 6.97 ± 0.24 mg GAE/g of OLE (0.70 w/w %), respectively; moreover OLE contained apigenin-7-O-glucoside with a concentration of 1.97 ± 0.17 mg/g of OLE (0.20 w/w %), rutin with a concentration of 3.37 ± 0.33 mg/g of OLE (0.34 w/w %) and other compounds (HT, caffeic acid and *p*-coumaric acid) with a concentration lower than 1 mg/g of OLE.

Table 1 *– Content of main phenols in OLE*

Hydroxy-tyrosol	Caffeic acid	*p*-Coumaric acid	Rutin	Luteolin-7-O-glucoside	Apigenin-7-O-glucoside	Oleuropein
0.85 ± 0.08	0.18 ± 0.02	0.085 ± 0.007	3.37 ± 0.33	6.97 ± 0.24	1.97 ± 0.17	32.64 ± 3.06

Data are expressed as means $\pm$ SD (mg GAE/g) of two independent experiments, each performed in duplicate.
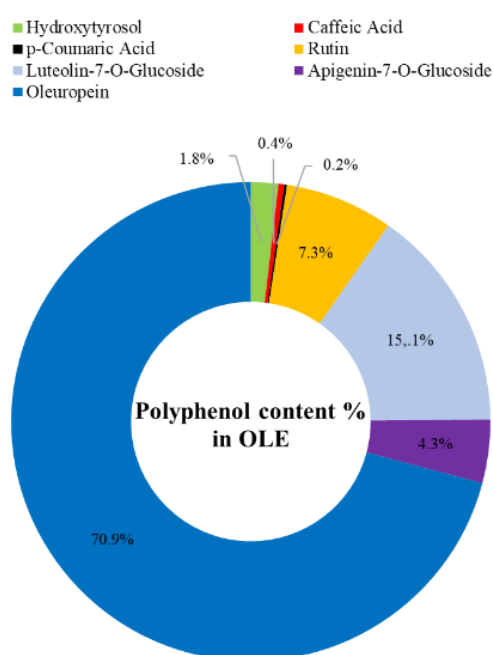
Figure 1. Anulus graph showing the percentage of different polyphenols in OLE.

4.2.2 Characterization of PHBHV/OLE fiber meshes

Morphological properties of the PHBHV and PHBHV/OLE fibers were investigated via SEM. The fibrous meshes resulted with smooth fiber surfaces in both electrospun types. The PHBHV/OLE fibers showed some beads. The samples resulted to have fiber diameters of 1287 ± 343 nm while that PHBHV revealed a diameter of 935 ± 230 nm. Representative micrographs of PHBHV and PHBHV/OLE fibers are reported in Figure 2.
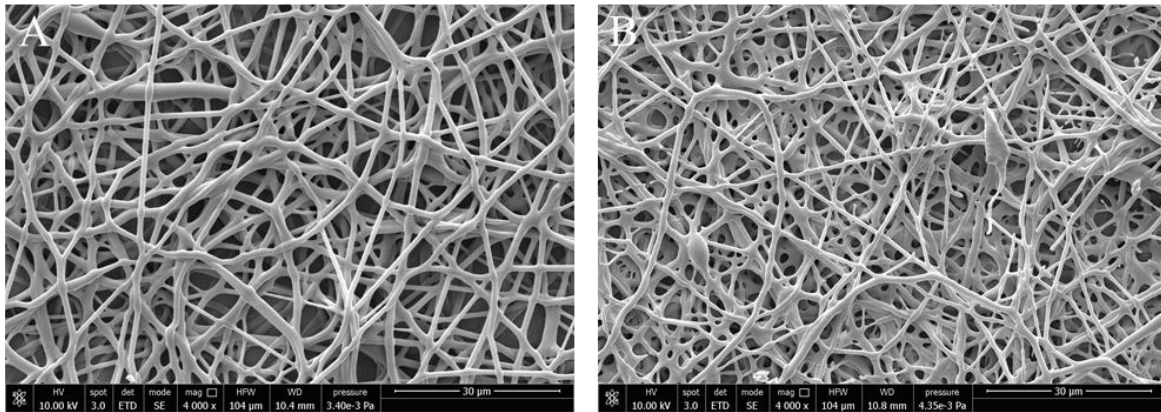
Figure 2. SEM micrographs of (A) PHBHV, and (B) PHBHV/OLE electrospun fibers.

FT-IR spectra, acquired for plain OLE, PHBHV and PHBHV/OLE fiber samples are shown in Figure 3. The band at 3300 cm^{-1} evidenced in the spectra of OLE-containing samples is indicative of molecular groups -OH related to the hydrophilicity of the extract. In the spectra were also indicated the absorption bands between 1700-1738 cm^{-1} related the C=O groups of PHBHV which is confirmed in literature (Nwinyi, O.C and Owolabi, T.A., 2019).
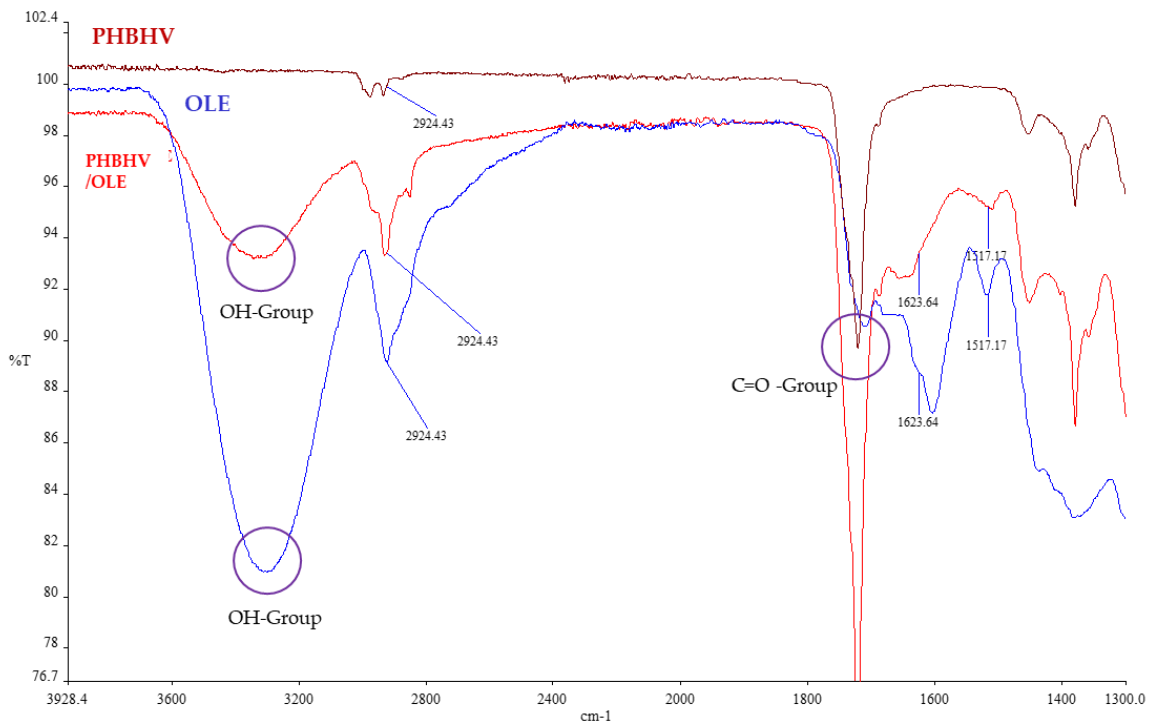
Figure 3. *FT-IR spectra of OLE, PHBHV and PHBHV/OLE fibers. Figure shows the characteristic bands.*

Chemical imaging technique is a useful and innovative tool to investigate about the chemical composition of polymeric material surfaces and the molecular interactions between material components. Maps acquired in FT-IR showed the homogeneous presence of -OH groups on the mesh surface, thus indicating an optimally dispersed composite material (Figure 4).
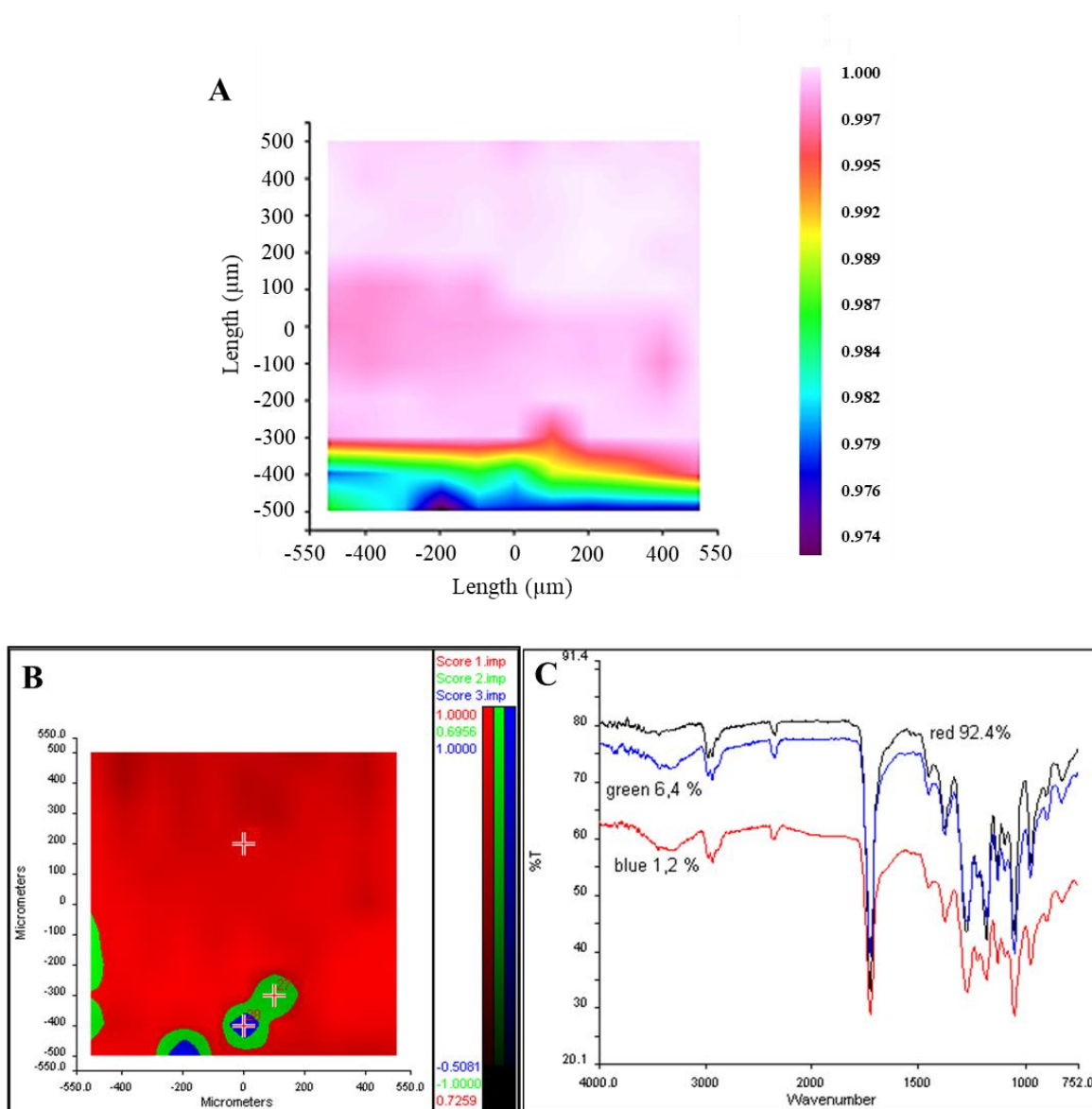
Figure 4. *Results of Chemical imaging analysis showing: (A) The correlation map with the characteristic spectrum in the absorption region of the -OH groups. The correlation map shows values between 0.974 and 1.000 over the entire analyzed surface; (B) Statistical elaborated by PCA to identify the zones on the map with the same spectral variability. The PCA analysis showed the presence of 3 spectral groups, identified by blue, green and red; (C) The spectrum of each zone is represented the graph of FT-IR spectra.*

A degradation analysis was carried out in vitro on PHBHV/OLE scaffolds, using PBS and PBS added with MMP-9. The mass loss weighted using an analytical balance up to 56 days is reported in Table 2. The trend of mass loss due to hydrolytic degradation is shown in Figure 5. The maximum percentage of degradation was revealed in the fibers treated with MMP-9 at 56 days reaching 9.6% ± 0.282.

Table 2 – *Weight loss (mg/mg %) of PHBHV/OLE fibers in different media: PBS and PBS added with MMP-9.*

Time (days)	Weight loss (%)	
	PBS	**PBS + MMP-9**
14	3.33 ±0.106	2.44 ±0.070
28	2.76 ±0.074	3.75 ±0.106
42	3.85 ±0.106	7.14 ±0.017
56	4.21 ±0.141	9.64 ±0.282

Data are expressed as means ± SD of two independent experiments, each performed in duplicate

The loss of molecular weight of PHBHV and PHBHV/OLE fiber meshes was performed by GPC. The results are reported in Figure 5. Molecular weight reduction highlighted the rupture of the copolymer chains, without substantial differences due to the enzymatic activity of MMP-9.
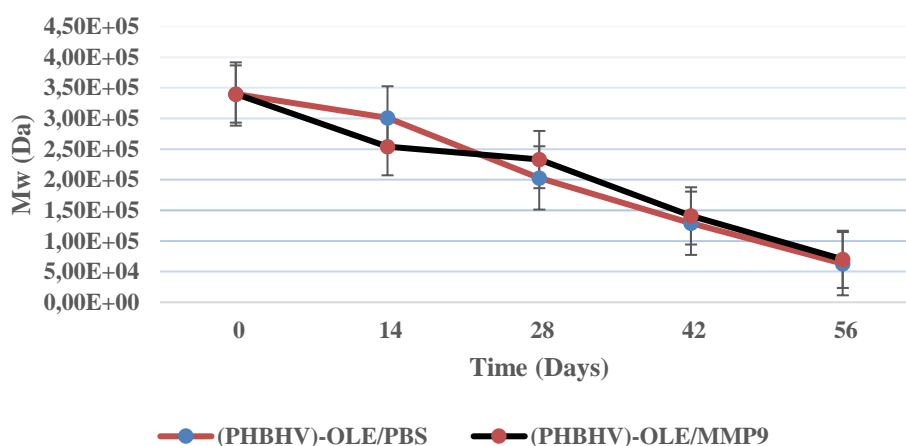
Figure 5. *Graph showing molecular weight loss as obtained from GPC of PHBHV for 28 days and PHBHV/OLE fiber meshes up to 56 days in different media: plain PBS.*
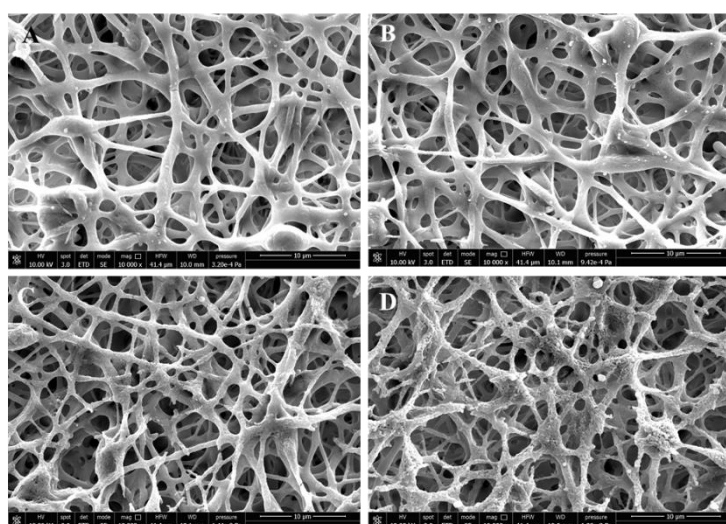
Figure 6. *SEM micrographs of PHBHV/OLE under degradation in PBS. for (A) 2 weeks, (B) 4 weeks, (C) 6 weeks, and (D) 8 weeks*

Morphological analysis conducted by SEM showed changes in the surface of the fibers including cracks, holes, gradual loss of parts were evidenced independently from degradation with MMP-9, after 4 weeks (Figure 6).

4.2.3 Polyphenol release from PHBHV/OLE fiber meshes

First, the phenolic content of PHBHV/OLE was analyzed. The results of HPLC analysis indicated that the amount of main phenols present was 21.84 ± 0.14 µg for oleuropein, 7.22 ± 0.78 µg for luteolin-7-O-glucoside and 3.54 ± 0.13 µg for apigenin-7-O-glucoside. Subsequently, we evaluated the release profile of these phenols (i.e., oleuropein, luteolin-7-O-glucoside and apigenin-7-O-glucoside) from the copolymer. The quantity of these released phenolic compounds during the submersion time are reported in Table 3 and Figure 8 and expressed as percentage of initial amount. At the first 30 minutes, the amount of oleuropein released was about 60% of the total oleuropein content in polymer. Then the release became more gradual, reaching about 90% after 6 h. As concerns luteolin-7-O-glucoside and apigenin-7-O-glucoside after the first 30 minutes the release was about 40% and 30%, respectively, and was about 80% after 6 h in both cases. Thus, the phenolic compounds incorporated on the PHBHV/OLE were effectively released into the medium.

Table 3 – *Cumulative release (µg) of the main OLE phenolic compounds in a 4 cm² square of PHBHV/OLE fiber mesh. Data are expressed as means ± SD of an experiment performed in duplicate.*

Time (h)	Luteolin-7-O-Glucoside	Apigenin-7-O-Glucoside	Oleuropein
0	0.00	0.00	0.00
0.5	3.44 ± 0.85	0.95 ± 0.04	12.29 ± 1.30
1	3.48 ± 1,45	1.58 ± 0.49	14.53 ± 2.60
1.5	4.01 ± 1.58	1.86 ± 0.71	15.70 ± 2.96
2	4.37 ± 1.75	2.10 ± 0.94	16.53 ± 2.79
3	4.68 ± 1.95	2.25 ± 0.98	17.63 ± 2.52
4	4.81 ± 1.93	2.31 ± 0.90	18.49 ± 2.15
6	5.05 ± 2.01	2.52 ± 1.20	19.38 ± 1.92
24	5.64 ± 2.20	2.95 ± 1.37	20.93 ± 2.45
48	5.87 ± 2.22	3.07 ± 1.43	21.75 ± 2.85
72	6.08 ± 2.23	-	-
144	6.47 ± 2.21	-	-
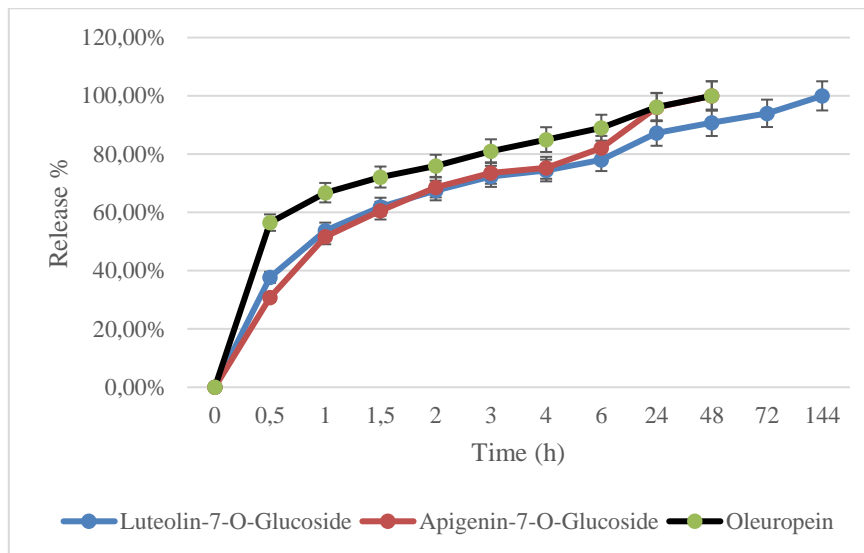
Figure 7. *Graph showing the cumulative release of polyphenols from PHBHV/OLE fiber meshes up to 144 h.*

4.2.4 Cytocompatibility of the scaffolds using HFFF2 cells

The cytocompatibility of PHBHV and PHBHV/OLE was studied in vitro using HFFF2 cells.
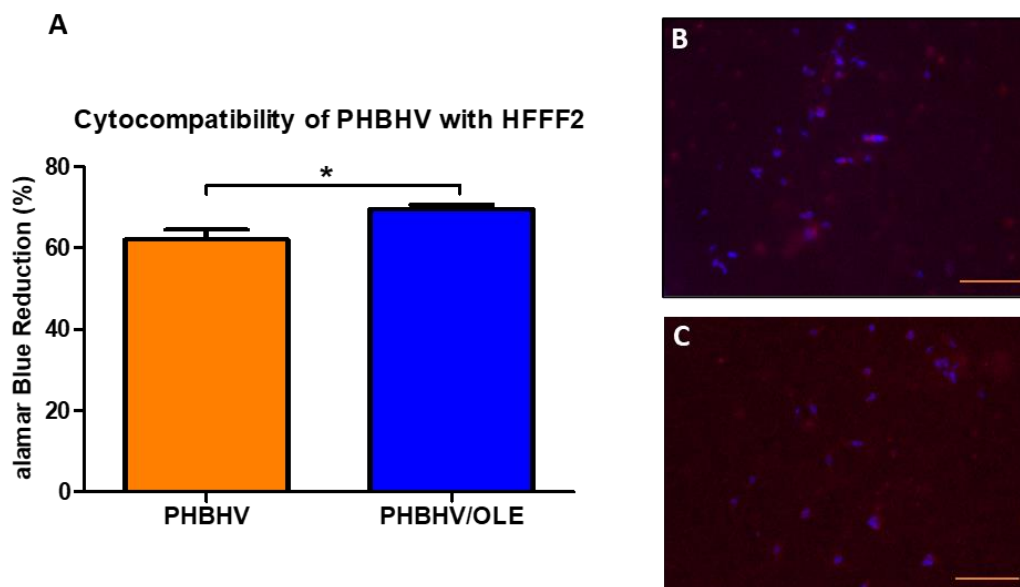
Figure 8. *Cytocompatibility of PHBHV and PHBHV/OLE scaffolds. The scaffolds were culture with HFFF2 cells. (A) Bar graph showing %reduction of Resazurine dye, indicating metabolic activity of the cells after 72 h. (n = 3; t.test; one way, p 0.01). (B-C) Florescence staining of f-actin in red and nuclei in blu for: (B) PHBHV and (C) PHBHV/OLE . The scale bar in insert is 100 µm.*

4.3 DISCUSION

We prepared new ultrafine fiber meshes made of PHBHV and OLE having morphological, physico-chemical, biocompatibility and functional properties that make them suitable as scaffolds for skin repair. The extraction phase of OLE allowed to obtain a blend of active principles which can be entrapped in the fibers performing functions as: controlled release, interaction, and cell stimulation. (Larry L.H et al., 2002), features like these converts to OLE in a set of biomolecules suitable for incorporation into fibers. The procedure of electrospinning was performed using a blend of the polymer and the extract allowing the production of meshes with uniform fibers and highly porous structures, the addition of OLE during electrospinning process did not substantially alter the fiber dimension and mesh porosity as observed by SEM analysis. In vitro tests carried out on PHBHV/OLE meshes have demonstrated the capability of OLE of inducing a favorable biodegradation and an improved cytocompatibility. In addition, the electrospun system can serve as drug carrier for a controlled release of antioxidant principles able to treat human tissue alterations.

Concerning physicochemical characterization of PHBHV and PHBHV/OLE by FT-IR, the analysis indicated an absorption band at 1700-1738 cm^{-1} corresponding to C=O group of PHAs in both samples and a band between 3300 cm^{-1} and 3200 cm^{-1} containing several vibration modes, present only in the sample with OLE, associated to hydroxyl groups including those of adsorbed water. The latter band can be considered an index of material hydrophilicity degree due to the presence of OLE into the PHBHV fibers. The hydrophilicity of OLE is a very important factor because promotes processes like the cellular adhesion and affinity with biological components (e.g., OLE); is essential for subsequent tissue regeneration application. In addition, the chemical map associated to -OH groups confirm the efficiency of loading procedure of the extract into the PHBHV fibers and a homogeneous distribution of OLE over the whole mesh. In vitro biodegradation study was carried out on the PHBHV/OLE samples for a period of two months by immersing the samples in PBS and PBS with the addition of MMP-9, in order to closely resemble the conditions of a normal tissue remodeling process. It is known, in fact, that MMP-9 is a proteolytic enzyme highly expressed during wound healing and its proteolytic activity is to degrade the ECM leading to delay the wound healing (Sahana, T.G., and Rekha, P.D., et al.,2018). At different times of incubation in two distinct media, samples were analyzed by measuring the weight loss over the time. The results of weight loss showed a slow degradation with a modest reduction of mass at the end of incubation period both in PBS (4 %) and in PBS in the presence of MMP-9 (9.6%).In general, materials in form of film made up with members of the PHA family (i.e PHB, PHBHV) are characterized by a slow hydrolytic degradation in vitro, remaining more than 95% of their initial weight after 240 days in PBS (Han, J., et al., 2017). In our study, also if modest, a higher erosion was observed respect to literature data; this can be attributed

to the hydrophilic contribution of OLE to the PHBHV fiber structure. In any case, this modest reduction does not represent great implications for our goals since it would guarantee a long-lasting product for a potential wound dressing model for the healing of skin wounds. However, this data give light for future research where degradation is an important point for its use in biomedical applications. PHBHV has a low biodegradation rate that depending on the application can limit its use to some implant applications requiring fast degradation. In fact, Gogolewski, et al., observed that PHBHV polymers degraded much less than polylactic acid (PLA) following 6 months of *in vivo* implantation (Gogolewski, et al.,1993). To accelerate its biodegradation was proposed the MMP-9 use, that additionally the presence of OLE can favor the surface adsorption of MMP-9 molecules at level of ester groups of PHBHV which could result in an increased degradation due to a possible enzymatic effect in addition to that purely hydrolytic one. In this respect, GPC results showed an evident reduction of molecular mass indicating the breakdown of ester bonds in the polymeric chains without an evident bulk erosion, as observed also by SEM analysis. The trend of molecular weight change showed how the hydrolytic process is responsible for the rupture of copolymeric chains showing a similar behavior in two different media, even if in the first two weeks the degradation rate was higher due to effect of active enzyme molecules. The effect of enzyme on the corresponding erosion process was observed in the following weeks as pointed out by weight loss and morphological aspect of PHBHV-OLE fibers after 8 weeks. In addition, is important remark that OLE does not have a molecular weight comparable to PHBHV; because of that is not possible analyze differences of OLE loss weight in the samples.

Comparing PHBHV with another member of PHA family, PHB studies conducted by Salgero, et al., using our same protocol of degradation in abiotic conditions submitted PHB to a hydrolytic degradation *in vitro*, in a saline solution of phosphate with pH 7, at a temperature of 37 °C for 40 days. After this period, the results showed that the PHB lost little mass during the incubation period, a value that was between 1% and 1.5%. This small percentage may be explained due to micro porosity of polymer that reduced the rate of water adsorption into the polymeric matrix and at the same time represented a barrier to the flow of the products of degradation to the solution. Finally, the importance of the use of enzyme degradation permits a major time control (Salguero, N.G., et al., 2012) while MMP-9 reaches the polymer to produce hydrolytic catalysis. During the degradation enzymes break the long polymeric chain, forming oligomers and after some time, these oligomers may be hydrolyzed to monomeric units of the polymer (Shah, A.A., et al., 2008; Michel, A.T., 2012; Costa, C.Z., et al., 2015).The principle and the importance of the process happens through the hydrolysis of connections esters of polymer.

The release study showed an interest release profile of OLE. The bioactive OLE components loaded onto the fiber were released after 144 h, i.e. biomolecules such as luteolin-7-O-glucoside and apigenin-7-O-glucoside released the fiber of PHBHV after 48h while Oleuropein the principal compound of the extract was released after 144h. Hence, this scenario allows us to elucidate the application of PHBHV/OLE for wound healing issues due to the properties of the extract. Usually, patients suffering from skin ulcers take drugs orally or directly applied to the skin. Although, the drug is delivered to the damaged site but is necessary a high concentration of the drug or an excessive amount of drug for several times, with expensive treatment to treat the wounds, which may induce unpleasant effect for the patient. The rapid release of the biomolecules in OLE can be considered an ideal strategy to deliver the minimum required amount of polyphenols of the extract a "natural drug" to the damage site, especially in chronic skin wounds (Balta, A.B., 2010; Song, R., et al., 2018). Moreover, the direct release of the OLE components by the matrix allows concentrating the amount of the substance at the wound site. We hypothesize that it is better to act locally because it would have a long-term bioavailability of the bioactive molecules, presenting a rapid effect at the beginning to protect the tissue or said in another way, rapid but prolonged over time. Additionally, the absorption of substances in the human body can be faster with the smaller size of the drug and its coating material (Balta, A.B., 2010). We can say that such bioactive vegetable compounds and bio functional fibers are potential candidates for the medical applications of the new generation.

Chapter 5

Experimental Section IV

5. IMMUNOMODULATORY EFFECTS OF OLE INCORPORATED IN ELECTROSPUN POLYHYDROXYALKANOATE FIBERS.

Until now the properties of OLE and its polyphenols were investigated for endothelial dysfunction, cytocompatibility with biomaterials for wound healing and antibacterial capacity. According to the results reported, OLE shows a broad range of biological activities even anti-inflammatory and immunomodulatory properties. Now, this final chapter relied on the understanding about how polyphenols in OLE can act on multiple inflammatory components and lead to anti-inflammatory mechanisms. In many studies polyphenols showed that they can regulate immunity by interfering with immune cell regulation, proinflammatory cytokine synthesis and gene expression. Taking advantage of these features is proposed,

- To produce fiber meshes of PHBHV and PHB/PHOHD incorporating OLE, using human dermal keratinocytes (i.e., HaCaT cells) to evaluate the expression of a panel of cytokines involved in the inflammatory process, such as the antimicrobial peptide human beta defensin 2 (HBD-2).

5.1 MATERIALS AND METHODS

5.1.1 Scaffolds fabrication

PHAs were used to prepare blends with OLE to be processed via electrospinning: Poly(3-hydroxyoctanoate-co-3-hydroxydecanoate) P(3HO-3HD) and poly(3-hydroxybutyrate) P(3HB) (Roylab, UoS). Different solutions were set up for PHA electrospinning: (1) P(3HO-3HD)/(PHB) 10:1 (w/w) was dissolved in chloroform/2-butanol (7:3 w/w) mixture and 0.002 g/ml LiBr at 11% w/w%. (2) PHBHV was dissolved in a dichloromethane/methanol (10:1 w/w) mixture at 15% w/w%. OLE (16.8 w%) was added to both solutions. Parameters of electrospinning were 40 kV, 0.5 ml/h flow rate and a distance from needle tip (ground charge) to static aluminum collector (positive charge) of 40 cm for 1 h. Humidity was maintained around 40% and temperature 20°C throughout. The parameters for the fiber fabrication has been standardized at the Department of Civil and Industrial Engineering, University of Pisa. The morphology of the fiber meshes was evaluated by scanning electron microscopy (SEM) using a FEI FEG-Quanta 450 instrument (Field Electron and Ion Company, Hillsboro, OR, USA).

5.1.2. Epidermal cell culture and viability assay

Immortalized human keratinocyte Hacat cell line (purchased from CLS–Cell Lines Service, Eppelheim, Germany), were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% Penstrep, 1% glutamine and 10% fetal calf serum (Invitrogen, Carlsbad CA) at 37°C in air and 5% CO_2. The HaCat cells, seeded in 12-well plates until 80% of confluence, were incubated for 24 hours with the films BM and E-BM5. At the end of this time, resazurine (AlamarBlue dye) was added to the concentration of 0,5 mg/ml and incubated for 4 hours. The absorbance (l) of supernatants was measured with a spectrophotometer (Victor 3; PerkinElmer, Waltham, MA, USA) under a double wavelength reading (570 nm and 600 nm). Finally, $\%AB_{red}$ was calculated correlating the absorbance values and the molar extinction coefficients of the dye at the selected wavelengths, following the protocol provided by the manufacturer.

5.1.3. Evaluation of Immunomodulatory Properties

The immunomodulatory properties of BM and E-BM5 films were assayed using HaCaT cell.The cells, cultured as described above, were seeded inside 12-well TC plates until 80% of confluence, were incubated for 24 h with the films for 6 h and 24 h (n = 3). At these endpoints, total RNA was isolated with TRizol and 1 µm of RNA was reverse-transcribed into complementary DNA (cDNA) using random hexamer primers, at 42 °C for 45 min, according to the manufacturer's instructions. Real time polymer chain reaction (PCR) was carried out with the LC Fast Start DNA Master SYBR Green kit using 2 µL of cDNA, corresponding to 10 ng of total RNA in a 20 µL final volume, 3 mM MgCl2 and 0.5 µM sense and antisense primers (Table 3). Real-Time PCR was used to evaluate the expression of interleukins IL-1α, IL 1β, IL-6 and IL-8, TNF-α and TGF-β.

Gene	Primer Sequence	Conditions	Size (bp)
IL-1 α	5′-CATGTCAAATTTCACTGCTTCATCC-3′	5 s at 95 °C, 8 s at 55 °C,	421
	5′-GTCTCTGAATCAGAAATCCTTCTATC-3′	17 s at 72 °C for 45 cycles	
IL-1 β	5′-GCATCCAGCTACGAATCTCC-3′	5 s at 95 °C, 14 s at 58 °C,	708
	5′-CCACATTCAGCACAGGACTC-3′	28 s at 72 °C for 40 cycles	

TNF-α	5′-CAGAGGGAAGAGTTCCCCAG-3′	5 s at 95 °C, 6 s at 57 °C,	324
	5′-CCTTGGTCTGGTAGGAGACG-3′	13 s at 72 °C for 40 cycles	
IL-6	5′-ATGAACTCCTTCTCCACAAGCGC-3′	5 s at 95 °C, 13 s at 56 °C,	628
	5′-GAAGAGCCCTCAGGCTGGACTG-3′	25′ s at 72 °C for 40 cycles	
IL-8	5-ATGACTTCCAAGCTGGCCGTG-3′	5 s at 94 °C, 6 s at 55 °C,	297
	5-TGAATTCTCAGCCCTCTTCAAAAACTTCTC-3′	12 s at 72 °C for 40 cycles	
TGF-β	5′-CCGACTACTACGCCAAGGAGGTCAC-3′	5 s at 94 °C, 9 s at 60 °C,	439
	5′-AGGCCGGTTCATGCCATGAATGGTG-3′	18 s at 72 °C for 40 cycles	

Table 1. Real-time PCR conditions for HaCaT cells

5.2 RESULTS

5.2.1. PHBHV and PHB/PHOHD Scanning Electronic Microscopy

Morphological properties of the PHBHV/OLE fibers were investigated by SEM. With the magnification between 1000x - 4000x resulted in a smooth composite fiber and a more uniform fiber size than P(3HO-3HD)/(PHB). The fibers were prepared without the occurrence of bead defects. It was remarkable to note that for the PHB/PHOHD electrospun fiber, some bulges could be detected but it can be explained due to the phenolic group showing the shape of spindle and sphere. Conditions such as supply voltage and electric field were constant, the solvents result suitable for the fibers fabrication. Other Parameters of electrospinning were 40 kV, 0.5 ml/h flow rate and a distance from needle tip (ground charge) to static aluminum collector (positive charge) of 40 cm for 1 h. Humidity was maintained around 40% and temperature 20°C throughout.
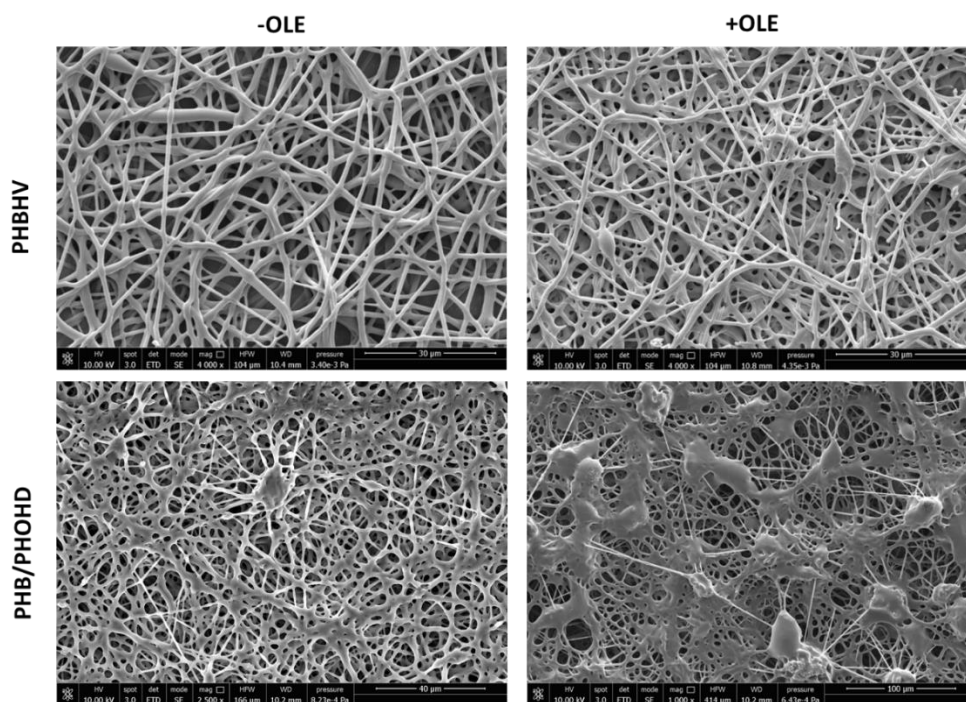
Figure 1. SEM micrographs of PHA electrospun fibers with and without OLE. Specifically, 2 different PHAs were used: PHBHV (commercial) and PHB/PHOHD (produced by UoS).

5.2.2 Cell metabolic activity.

Results of metabolic activity (AlamarBlue test) performed using human dermal keratinocytes (HaCaT cells) after 24 h showed that all the samples were non-cytotoxic, being dye reduction percentage above 70% (Table 2). Pure OLE, tested in monolayer (scaffold-less) cultures increased the metabolic activity in these cells, although it was tested in monolayer cultures. Overall, these results confirmed the high cytocompatibility of the PHAs and OLE.

SAMPLE	%AB$_{RED}$
PHBHV fibers	74
PHBHV fibers + Olive extract (OLE)	77
PHB/P(3HO-3HD) fibers	76
PHB/P(3HO-3HD) fibers + Olive Extract (OLE)	75
Pure Olive Extract (OLE)	94

Table 2. Results of metabolic activity (AlamarBlue test). All the samples were non-cytotoxic. From the percentage of reduction seems that OLE induced an increase in metabolic activity in the cells.

5.2.3 Cytokine expression

The results for the different pro-inflammatory ILs expressed at mRNA level from HaCaT cells in contact with the scaffolds as well as pure OLE after 6 h and 24 h are reported in Figures 2-6. OLE alone was administrated to the cells in standard culture (without fibers) at the same dose loaded in 1 cm^2 of fibers. Figure 4 shows the expression of IL-1 (α and β isoforms) by HaCaT cells in contact with the different PHA fibers and with OLE up to 24 h.
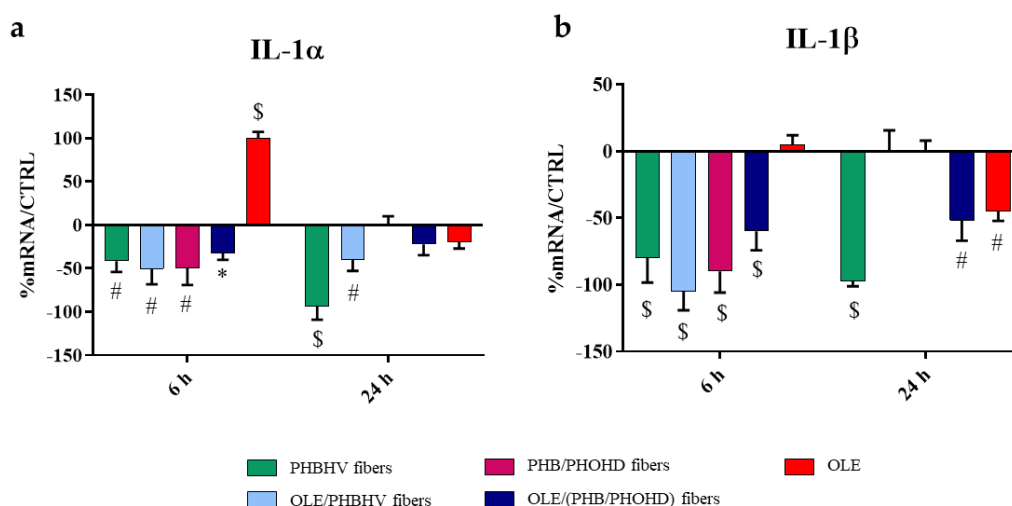
*Figure 2. Bar graphs showing IL-1 expression by HaCaT cells at 6 and 24 h: a) IL-1α, and b) IL-1β. Comparisons between samples were analyzed by multifactor ANOVA (Tukey's HSD for unequal values). Data are mean ± SD and are expressed as percentage of increment relative to unstimulated cells (used as). Significant differences are indicated by * (p < 0.05), # (p < 0.01), and $ (p < 0.001) for comparison between each sample and its respective control.*

Comparing to the basal expression conditions of HaCaT cells, both IL-1α ($p < 0.01$) and IL-1β ($p < 0.001$) expressions were significantly downregulated by all the fibers in 6 h, independently of OLE incorporation. OLE alone was able to modulate IL-1α and IL-1β expression in 24 h, showing statistical significance in IL-1β ($p < 0.01$). The specific action of OLE in long term downregulation of IL-1β was observed in OLE/P(PHB/PHOHD) fibers at 24 h ($p < 0.01$). Differently, plain PHBHV fibers maintained a significantly reduced expression over time ($p < 0.001$).

The expression of IL-6 is displayed in Figure 5. IL-6 resulted strongly downregulated by all the PHA fiber formulations with statistical significance (all $p < 0.001$, but OLE/PHBHV $p < 0.01$). OLE alone increased the IL-6 expression level at 6 h ($p < 0.05$), thus reporting it to the basal expression level of HaCaT cells (p = n.s.). A specific effect of OLE incorporation in the fibers could not be revealed for IL-6 expression.
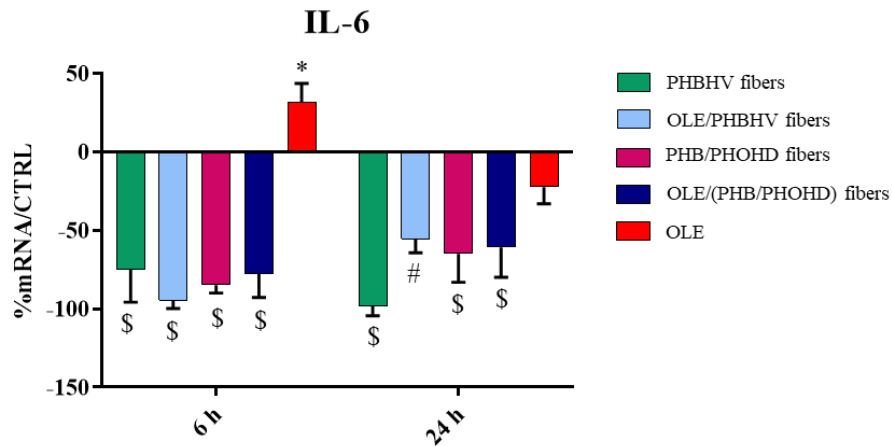
Figure 3. *Bar graph showing IL-6 expression by HaCaT cells at 6 and 24 h. Comparisons between samples were analyzed by multifactor ANOVA (Tukey's HSD for unequal values). Data are mean ± SD and are expressed as percentage of increment relative to unstimulated cells (used as). Significant differences are indicated by * (p < 0.05), # (p < 0.01), and $ (p < 0.001) for comparison between each sample and its respective control.*

The outcomes of IL-8 expression are reported in Figure 4.
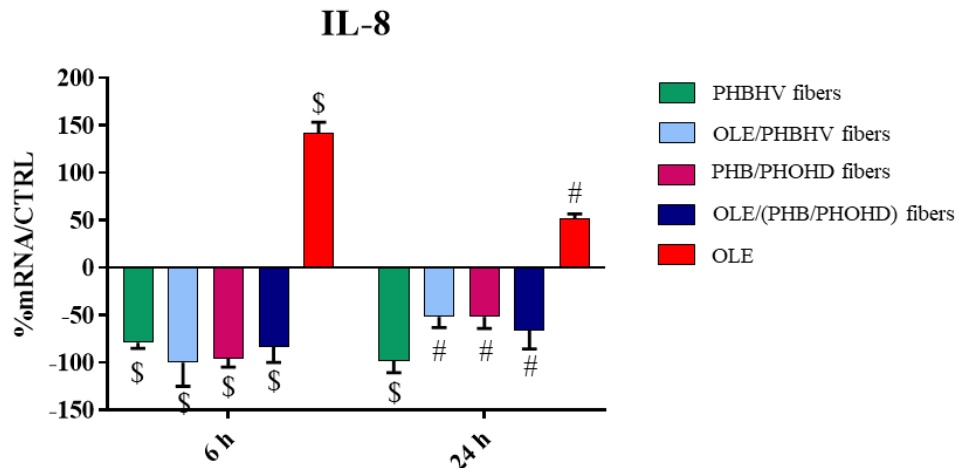
Figure 4. *Bar graph showing IL-8 expression by HaCaT cells at 6 and 24 h. Comparisons between samples were analyzed by multifactor ANOVA (Tukey's HSD for unequal values). Data are mean ± SD and are expressed as percentage of increment relative to unstimulated cells (used as). Significant differences are indicated by # (p < 0.01), and $ (p < 0.001) for comparison between each sample and its respective control.*

Also in this case, a strong downregulation of this cytokine was exerted by all the fibers independently of OLE incorporation. Differently from OLE alone, which instead caused a marked upregulation after 6h ($p < 0.001$), which was reduced after 24 h, remaining still significant ($p < 0.01$). The results of TNF-α expression are shown in Figure 7. In a similar fashion of the findings reported for the other cytokines, all the PHA fibers downregulated the expression of TNF-α independently of OLE incorporation. It is interesting to note that plain OLE was able to modulate the expression of this pro-inflammatory cytokine by inducing an initial raise at 6 h ($p < 0.001$), followed by a drop after 24 h ($p < 0.001$).

Finally, TGF-β was not modulated by any PHA and/or OLE formulation tested in this study.

TNF-α
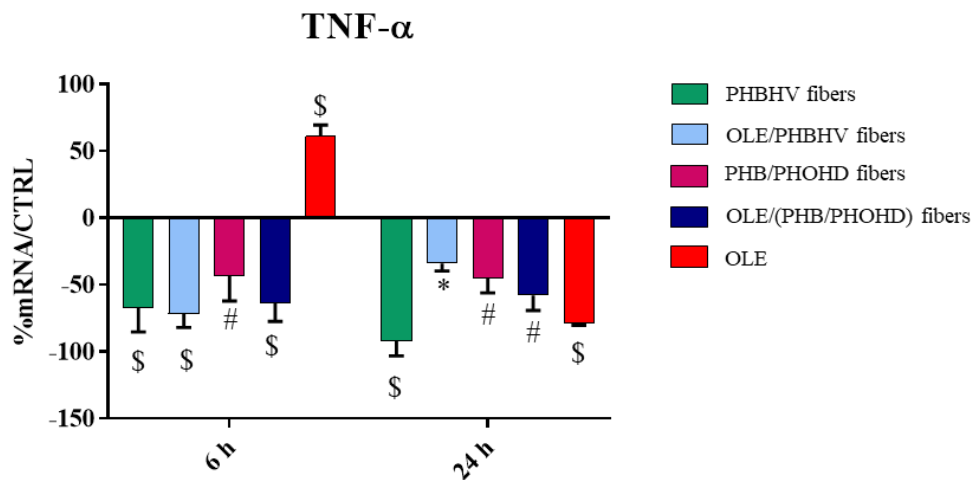
*Figure 5. Bar graph showing TNF-α expression by HaCaT cells at 6 and 24 h. Comparisons between samples were analyzed by multifactor ANOVA (Tukey's HSD for unequal values). Data are mean ± SD and are expressed as percentage of increment relative to unstimulated cells (used as). Significant differences are indicated by * ($p < 0.05$), # ($p < 0.01$), and $ ($p < 0.001$) for comparison between each sample and its respective control.*

5.2.4 Indirect antimicrobial activity
The expression of the antimicrobial peptide HBD-2 is reported in Figure 6.
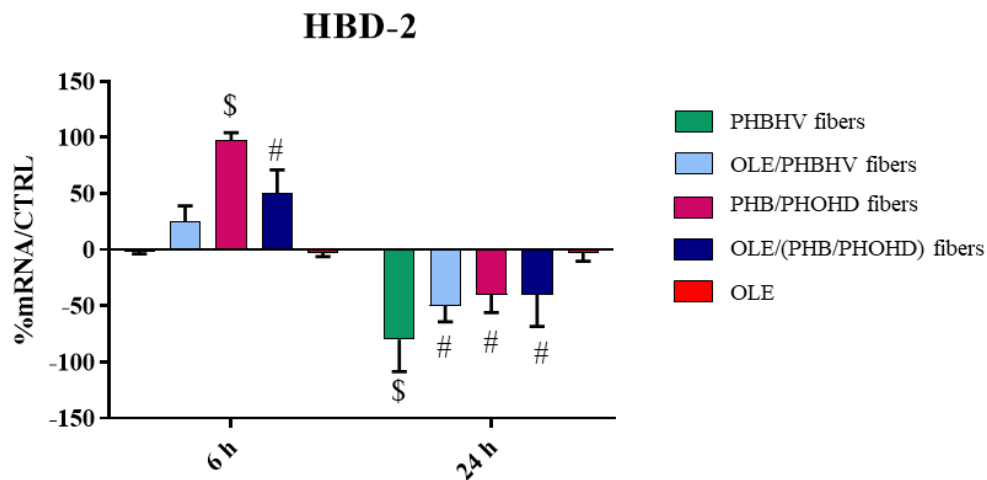
HBD-2

Figure 6. Bar graph showing HBD-2 expression by HaCaT cells at 6 and 24 h. Comparisons between samples were analyzed by multifactor ANOVA (Tukey's HSD for unequal values). Data are mean ± SD and are expressed as percentage of increment relative to unstimulated cells (used as). Significant differences are indicated by # ($p < 0.01$), and $ ($p < 0.001$) for comparison between each sample and its respective control.

In this case, it was observed that only two PHB/PHOHD fiber formulations were able to promote the upregulation of HBD-2 with statistical significance, after 6 h, specifically PHB/PHOHD containing OLE ($p < 0.01$) and plain PHB/PHOHD ($p < 0.001$). OLE/PHBHV was able to increase the expression of this peptide without statistical significance after 6 h. OLE alone, at the same concentrations, did not show any effect on HaCaT cells.

5.3 DISCUSSION

Chronic wounds result from a widespread group of pathologies, including, among others, ulcers from diabetic, vascular and pressure diseases (Frykberg, R.G. and Banks, J., 2015). Such wounds still represent a healthcare challenge around the globe by affecting several millions of people, as they remain unhealed for months or years. Even if the underlying pathologies play a key role in wound establishment and perpetuation, chronic wounds possess some common features, defined as: "prolonged or excessive inflammation, persistent infections, formation of drug-resistant microbial biofilms, and the inability of dermal and/or epidermal cells to respond to reparative stimuli" (R.G. Frykberg and J. Banks, 2015). The possibility of modulating the inflammatory processes and improving the self-defense towards microorganisms, can thus facilitate a more efficient regeneration operated by skin cells. Dressings are usually applied at the wound site to enable a protection, homeostasis and absorb exudate if needed (Dhiyya, S., et al., 2015). The modern wound dressings also aim to facilitate the reparatory processes by releasing growth factors or providing material-intrinsic antimicrobial properties (Danti, S., et al., 2017). Bioresorbable natural-origin materials offer remarkable opportunities in this field; in fact, if these biomaterials are spun into micrometric or submicrometric fibers, a structure similar to the fibrous components of the extracellular matrix (ECM) is provided for the neighboring cells to repair a wound (Azimi, B., et al., 2020). To this purpose, biomaterial scaffolds stimulating immunomodulatory and antimicrobial activity in the skin cells, would offer a relevant alternative to current treatments. We hypothesized that the polyphenols present in OLE could provide a beneficial role in skin immunomodulation and that their presence in a fibrous dressing could promote their function by means of a better controlled local release to the cells within/from a 3D structure.

We considered a novel family of polyesters, i.e., PHAs, as a bio-based polymer source to produce ultrafine fiber nonwovens via electrospinning. Previous studies with plain PHAs confirmed the good electro-spinnability of this polymers (Azimi, B., et al., 2020; Lizzarraga, L.R., et al., 2016; Ching, K.Y., et al., 2016). In this study, by using identical solvents and solution parameters, two types of fiber meshes were obtained: one based on commercial PHBHV and one based on a blend of two laboratory-produced PHAs, i.e., PHB/PHOHD, which were previously tested under distinct electrospinning modalities and apparatus (Azimi, B., et al., 2020; Lizzarraga, L.R., et al., 2016; Ching, K.Y., et al., 2016)). Both of them were loaded with the same amount of OLE and used for in vitro tests performed using human dermal keratinocytes (HaCaT cells). We observed that the presence of OLE differently affected the final architecture of two PHA formulations, by favoring the formation of beads and molten fibers in particular in the PHB/PHOHD nonwovens. In electrospun manufacts, beads are considered as morphological defects due to nonoptimal electrospinning conditions, as the

fiber diameter is not consistent along fiber length (Fong, H., et al., 1999) . Beads result to strongly depend on surface tension and solvents or additives can help to reduce this phenomenon (Liu, Y., et al., 2008). However, it has been reported that in case of fibrous meshes loaded with bioactive agents, beaded structures can act as mini-reservoirs of the drug, thus enabling a better tunable delivery (Gaharwar, A.K., et al., 2014). Similarly to other parts of the olive tree, OLE contains considerable amounts of polyphenols (Ozcan, M.M., and Mathaus, B., 2017); among them, oleuropein is the key phenolic compound found in unprocessed olive fruits and also in leaves, where it reaches about 60-90 mg/g (dry weight) (Nediani, C., et al., 2019).

The well-known antioxidant activity of oleuropein is manifold: it directly relies on its chemical structure owning an ortho-diphenolic group able to scavenge ROS via hydrogen donation and to stabilize oxygen radicals via an intramolecular hydrogen bond. In addition, oleuropein provides an indirect antioxidant activity by stimulating the expression of intracellular antioxidant enzymes and increasing the level of nonenzymatic antioxidant molecules (Nediani, C., et al., 2019). Recently, the anti-inflammatory properties of olive products have also been investigated in relation to dietary foods (Estruch, R., 2006) . Chronic inflammation is linked to ROS production causing oxidative damage and antioxidant depletion. As such, some studies have investigated the influence of oleuropein on macrophages, the key cells involved in inflammatory processes by producing ROS and other pro-inflammatory cytokines, but further able to support healing by releasing anti-inflammatory cytokines, if timely and properly differentiated (Miles, E.A., et al., 2005; Ryu, S.J. et al., 2015; Gordon, S., and Martinez, F.O., 2010). In some studies, OLE is reported to show direct antimicrobial activity, which was primarily attributed to its main polyphenols (Liu, Y., et al, 2017). However, the findings available on the antibacterial properties of OLE are still debated, as they may depend on extraction methods, polyphenol composition and quantity, including dose, on selected bacteria.

In our study, we assayed the release of three main components of OLE, namely, oleuropein, luteolin-7-O-glucoside and apigenin-7-O-glucoside from PHA fiber meshes. Such polyphenols were almost totally released after 24 h, reaching oleuropein about 90% and the two glucosides about 80% in 6 h. Unlike using systemic or even local injections, the scaffold can promote a better confined delivery at the wound site by avoiding the dispersion of the active principle through the blood vessels, thus keeping the drug concentration locally higher (Chew, S.A. and Danti, S., 2017). In addition to OLE release, it is expected that the fiber meshes would provide a suitable support for tissue regeneration. Since re-epithelization is a leading phenomenon in skin repair, we investigated the viability of HaCaT cells by measuring their metabolic activity on the different meshes, with or without OLE, including using OLE alone. We observed that when cultured on the fibrous scaffolds for 24 h, HaCaT cell metabolic activity was very similar, independently of the type of fiber or OLE loading, whereas it was higher when pristine OLE was added at the same amount. The difference of plain OLE effect on

cell metabolic activity can be explained by the subitaneous availability of the bioactive ingredients with respect to a slower release operated by the fibers. However, it is frequently observed that the presence of a three-dimensional substrate affects cell response at early time-points, as adhesion phenomena and cell-cell interactions are more complex events than in bi-dimensional tissue culture plastics (Ricci, C., et al., 2016; Duval, J.K., et al., 2017) .

In addition to macrophages, also epithelial cells, including skin keratinocytes, play a fundamental role in innate immunity, being primarily exposed to injuries and inflammatory stimuli. As such, epithelial cells contribute to healing by secreting several cytokines and defensins (Larsen, S.B., et al., 2020). As well as for metabolic activity, the administration of OLE alone or mediated by PHA fibers after 24 h, gave different outcomes in terms of cytokine expression at mRNA level. Our results showed that all the PHA fiber samples were endowed with immunomodulatory properties. In fact, they were able to downregulate the pro-inflammatory cytokines at 6 h and 24 h, thus showing a suppressive function towards inflammation. Many studies have reported that cell-biomaterial surface interaction is central in biocompatibility as it rules the inflammatory process, including anti-fouling and antibacterial aspects (Milazzo, M., et al., 2020). Differently from the fiber samples, pure OLE displayed a role in the wound healing process, by modulating the expression of the pro-inflammatory cytokines, which were upregulated at 6 h and downregulated at 24 h. Wound healing is a complex process characterized by a series of overlapping events which, starting from an inflammatory state, through reepithelization and matrix formation finally lead to tissue repair and remodeling. Two main phases drive this process: in the first phase the production of pro-inflammatory cytokines occurs. TNF-α and IL-1 represent the primary cytokines of pro-inflammatory response, which are immediately released by keratinocytes upon wound damage. TNF-α is an essential mediator of inflammation; it evokes several responses by the vascular and coagulation systems which end up in recruiting immune cells and molecules, but may also cause tissue necrosis (Esposito, E., et al., 2009). In addition, TNF-α can stimulates the production of fibroblast growth factor (FGF), suggesting that it can indirectly promote reepithelization (Coondoo, A., et al., 2012) IL-1 has two forms, namely, IL-1α and IL-1β, respectively produced by two different genes, with structural homology of 30%, but analogous biological activity. At low doses, IL-1 promotes local inflammation and stimulates coagulation IL-1β is the best known form; it activates an antimicrobial pattern on the neighboring epithelial cells, contributes to the upregulation of IL-6, which recruits and induces the differentiation of immune cells, and/or to the production of IL-8, which ultimately contributes in regulating reepithelization, tissue remodeling and angiogenesis (Larsen, S.B., et al., 2020; Tanaka, T., et al., 2016; Harada, A., et al., 1994). The TGF-β is part of the family of anti-inflammatory cytokines and is considered to be powerful in negatively modulating the great part of inflammatory responses. TGF-

β has different activities, both prohibitory and stimulatory. In our finding, it was not modulated after 24 h by any tested compounds and materials.

Inflammation is characterized by an interplay between pro- and anti-inflammatory cytokines. Usually, chronic wounds have high levels of pro-inflammatory cytokines and underlying infections, conditions that self-sustain each other over long times, thus impeding the second phase of the healing process (Fryberg, R.G. and Banks, J., et al., 2015). We showed that all the produced PHA fibers, either incorporating or not OLE, were able to strongly and persistently downregulate of hundred fold differences the expression of all the above cited ILs, with respect to the basal conditions of HaCaT cells in conventional culture. It is possible that this particular polymer class, i.e., PHAs, and the architecture of the ultrafine fibrous meshes, both concurred to mimic the fibrous ECM of dermal tissue produced by fibroblasts. In fact, it has been demonstrated that the smaller diameter fiber scaffolds (e.g., micro-nano size) the lesser immune response by macrophages using electrospun fibers of another polyester, the polylactide acid (Saino, E., et al., 2011). Regarding OLE alone, Liu, et al., indicated that OLE showed a remarkable inhibitory effect on the growth of several food pathogens when administrated at 62.5 mg/ml OLE, or 25 mg/ml oleuropein. Due to the high volume-to-surface area and porosity of electrospun fiber meshes, the resulting scaffold has a very low surface density, and consistently OLE in the fibers. As such, this kind of OLE encapsulation is more suitable for a diffused, than for a concentrated release. Using electrospun fibers is thus inherently suitable to provide a sustained low dose delivery combined with architectural cues for tissue regeneration. We thus investigated if these scaffolds could promote indirect antibacterial activity by assaying the expression of HBD-2. In response to injury and irritation, epidermal keratinocytes secrete also antimicrobial peptides, along with cytokines and chemokines (Wanke, I., et al., 2011). The family of β-defensins is composed of small cationic peptides produced by several cell types, including epithelial cells, Paneth cells, neutrophils and macrophages. These peptides possess antimicrobial activity and may be secreted constitutively or in response to microorganisms or cytokines, thus contributing to innate immunity. HBD-2 is an inducible antimicrobial peptide with a molecular mass of 4-6 kDa, reported to act as an endogenous antibiotic against Gram-positive and Gram-negative bacteria, fungi and the envelope of some viruses. Being released by epithelial cells, including epidermal keratinocytes, following inflammation, injury or infections, it is involved in the innate immune response of wounds (Donnarumma, G., et al., 2016). The capability of nano/micro-structured materials of inducing HBD-2 expression in HaCaT cells has been recently reported and is considered very appealing for skin contact applications, including medical, sanitary and cosmetics (Coltelli, M.B., et al., 2020; Teno, J., et al., 2020). In particular, some electrospun fibrous structures have shown promise in enabling HBD-2 by remarkably upregulating its gene expression in HaCaT cells already at early time points, i.e., 6 h (Teno, J et al., 2020; Azimi, B et al., 2020). Among them, PHB/PHOHD

fibers displayed interesting outcomes (Azimi, B et al., 2020). In this study, we compared PHB/PHOHD with commercial PHA, i.e. PHBHV, electrospun fibers produced under the same conditions, either incorporating or not OLE. Our finding confirmed that PHB/PHOHD fiber meshes, with and without OLE, can induce HBD-2 upregulation and at the same time can downregulate pro-inflammatory cytokines. This capability was not or not sufficiently shown by OLE alone and PHBHV fibers, with and without OLE.

CHAPTER 6
CONCLUSIONS AND FURTHER PROSPECTIVES

6. CONCLUSIONS AND FUTURES PERSPECTIVES

Conclusions

- OLE was obtained from the leaves of the Tuscan *Olea europaea* after prunings, It resulted highly rich in polyphenols with variation in total polyphenol (14.99 - 23.29 mg GAE/g), among which oleuropein accounted for a concentration of 14.69 ± 0.92 mg/g of OLE corresponding to 1.47% (*w/w*%), and luteolin 7-*O*-glucoside for a lower concentration of 3.60 ± 0.25 mg/g of OLE, corresponding to 0.36% (*w/w*%). However, furthers characterization shown other amounts of TP 58.47 µg GAE/mg and oleuropein and luteolin-7-O-glucoside, with a concentration of 32.64 ± 3.06 mg/g of OLE (3.26 w/w %) and 6.97 ± 0.24 mg/g of OLE (0.70 w/w %), respectively.

- OLE administration to endothelial cells (HUVECs) showed a protective effect against ROS production, which was also confirmed in a 3D culture model. This model was set up under the tissue engineering approach by using electrospun P(VDF-TrFE) scaffolds. OLE showed good cytocompatibility and antioxidant activity, which revealed effectiveness in controlling the oxidative stress on HUVECs upon exposure to an H_2O_2 insult.

- A number of medical applications can potentially benefit from OLE, including cardiovascular disease and wound healing, especially for wounds compromised by ROS stress. Local administration of OLE may thus represent a valid and strategy in biomedicine, which is also compliant with a sustainable use of bio-resources.

- The application of plasma and OLE treatment to reduce microorganism is an innovative strategy for providing microbiological safety for food sector. Nutraceutical properties of OLE has a synergistic effect with CAP proposing so a suitable antimicrobial approach. Additional research needs to be conducted in those areas that are less explored such as scaffold to food safety application.

- The skin compatibility of the scaffolds model has a great potential to apply this material in wound dressings, using in vitro tests based on the cell culture method. Specifically, human keratinocytes (HaCaT cells) were used as a model of epidermis to disclose any cytotoxicity and to assess the immune related response (inflammation and innate immune response) of these cells in contact with these materials. Our results show that all the PHA samples were

endowed with immunomodulatory properties, in fact were able to regulate both pro- and anti-inflammatory cytokines at 6 and 24 h.

- PHVHV and PHBHV+OLE showed of the characteristic that can be apply in medical applications including wound healing related to ulcers skin issues. The utilization of this PHAs family represent a valid and strategy in biomedicine, which is also compliant with a sustainable use, long period of durability, good biocompatibility and economically sustainable.

Futures perspectives

- Future directions of the research will explore other potential novel electrospun treatments, such as antibacterial target.

- Other aspect to future studies is if the phenolic composition of OLE change with CAP treatment, this aspect would be relevant to understand the mechanisms of action between OLE and CAP, an aspect no explored.

- The translation of nanofiber production from laboratory to clinical trial research, so much work is still required to get such therapies into the clinic, this work contributes to promoting experimentation in this direction.

- An aspect not investigated concerns the mechanical characterization of the electrospun fiber loaded with OLE. This represents a future study to fully establish the use of PHBHV as a potential wound healing patch model.

- Other interesting topic to develop in future is to evaluate the immunomodulatory properties of OLE in an inflammatory stimulus.

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