Chapter 5
The Antimicrobial Activities of Oleuropein and Hydroxytyrosol

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Abstract Several studies have reported that olive leaf extract and its constituents, particularly oleuropein and hydroxytyrosol, have health benefits including antioxidant and antimicrobial properties. Oleuropein and hydroxytyrosol have significant *in vitro* activity against fungi including opportunistic pathogen *Candida albicans*. Both compounds target virulence factors essential for the establishment of *C. albicans* infection. Both biomolecules express wide antibacterial activity *in vitro*. On the bacterial model *Staphylococcus aureus*, different targets have been detected. Oleuropein and hydroxytyrosol also interact with biofilm formation and could potentiate the activity of ampicillin. Considering the growing resistance to existing therapeutics has triggered the need for the development of new antimicrobial drugs, based on the presented results in this chapter, it seems that oleuropein and its derivative hydroxytyrosol could be considered as promising candidates for the treatment and/or prevention of candidiasis, and local infections caused by bacteria.

Keywords Antimicrobial activity · Oleuropein · Hydroxytyrosol · *Olea europaea* · Olive leaf extract

Abbreviations

ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BC	Bactericidal concentration
CSH	Cell surface hydrophobicity
DMPG	Dimyristoylphosphatidylglycerol
DNA	Deoxyribonucleic acid

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EUCAST	European Committee on Antimicrobial Susceptibility Testing
EVOO	Extra virgin olive oil
GAE	Gallic acid equivalent
HPLC-DAD	High-performance liquid chromatography with a diode-array detector
IC	Inhibitory concentration
MFC	Minimum fungicidal concentration
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
SAP	Secreted aspartyl proteinases

1 Introduction

Vegetation native to Mediterranean region due to its substantial sun exposure has been correlated to the high antioxidant content in plants. Epidemiologic studies have established an inverse relationship between intake of fruit- and vegetable-based antioxidants and mortality rates from chronic diseases (Huang and Sumpio 2008). Olive oil and olive leaf extract have been used in folk medicine within European Mediterranean countries and islands since ancient times and are well known for their broad health benefits associated with high levels of antioxidants (Medina et al. 2007; Khan et al. 2007; Zorić et al. 2021). The most widespread species of olive is the *Olea europaea* L. and its genus includes 35 species of evergreen shrubs and trees (Boskou 1996). It is scientifically accepted that *O. europaea* products, such as fruits and virgin olive oil, have beneficial health effects when they are a regular part of the human diet (Thielmann et al. 2017). The biological and pharmacological properties of olive oil and olive leaf extract have been attributed to its high content of biophenols (oleuropein, hydroxytyrosol, and their derivatives) (Ortega-Garcia and Peragon 2010). Previous studies on the composition of olive (*Olea europaea* L.) varieties, organs, and olive products have led to the identification of a plethora of phenolic compounds, including phenolic alcohols, secoiridoid derivatives, phenolic acids, lignans, and flavonoids (Suarez et al. 2010). The phenolic profiles of olive leaves and fruits are dominated by phenolic acids (e.g., ferulic, vaillic, coumaric acid), phenolic alcohols (e.g., hydroxytyrosol and tyrosol), flavonoids (e.g., luteolin-7-glucoside, cyanidin-3-glucoside, cyanidin-3-rutinoside, rutin, apigenin-7-glucoside, quercetin-3-rhamnoside, luteolin), and secoiridoids (e.g., oleuropein, ligstroside) (Thielmann et al. 2017). The content of phenolic compounds varies depending on environmental conditions including region, climate and is also affected by the variety, organ, olive product, ripeness of the olives at harvesting, and the processing system employed (Hassen et al. 2015). The bitter tasting secoiridoid oleuropein (Fig. 5.1) is one of the most abundant bioactive components contained in the *Olea europaea* L. and is exclusive to the plants of *Oleaceae* family (Servili et al. 2004). Oleuropein consists of a polyphenol, namely, 4-(2-hydroxyethyl) benzene-1,2-diol, commonly known

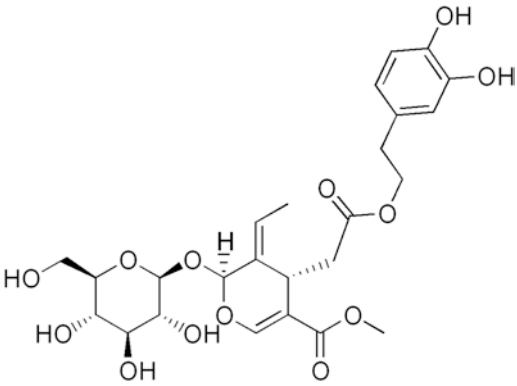

Fig. 5.1 Chemical structure of oleuropein

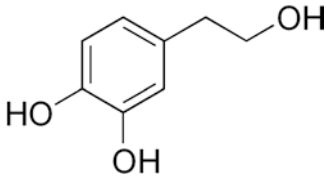

Fig. 5.2 Chemical structure of hydroxytyrosol

as hydroxytyrosol, a secoiridoid called oelenolic acid and a glucose molecule (Fig. 5.2). Phytoalexins and their precursors such as oleuropein, accumulated during fruit and leaf maturation, act as defense molecules against herbivores and microbial pathogens (Thielmann et al. 2017; Kubo et al. 1985). Although the main biological activities demonstrated so far are antioxidant and anti-inflammatory effects of oleuropein and its derivative hydroxytyrosol including their ability to treat oxidant and inflammatory-related diseases (i.e., cancer, cardiovascular disease, diabetes, etc.) (Hassen et al. 2015), in this chapter, available scientific data on their antimicrobial activities will be discussed.

2 Antifungal Activity

Natural compounds are potential source of antimycotic agents either in their nascent form or in the form of their more effective derivatives (Jacob and Walker 2005). Often these natural compounds are phenolics found in edible plants and are innately safe for humans (Faria et al. 2011). Interest in medicinal plants and their isolated constituents has increased due to the efficacy of new plant-derived drugs and in general the growing interest in natural products. Also, because of the concerns about the side effects of conventional medicine, the use of natural products as an alternative to conventional therapy in the healing and treatment of various diseases has been on the rise in the last few decades (Zuzarte et al. 2011). Additionally, the progression of drug resistance to conventional therapeutics, partially as a consequence of rising overprescription and overuse of conventional antifungals, triggered a need for more

effective treatment. As an interim solution, antibiotic resistance could be "broken" by coadministering appropriate nonantibiotic drugs with failing antibiotics. Some of these compounds can either directly kill microorganisms, reduce the antibiotic minimum inhibitory concentration when used in combination with existing antibiotics, and/or modulate host defense through effects on host innate immunity (Brown 2015). There has also been an increase in serious human infections in immunocompromised patients caused by fungi. The range of severity of these infections is a consequence of the host reaction to the metabolic products produced by fungi, the virulence of the infecting strain, the site of infection, and environmental factors (Zuzarte et al. 2011). Available antifungals predominantly include azoles, echinocandins, polyenes, and allylamines. They have a distinct mode of action; for example, azoles target heme protein, cytochrome P450 lanosterol-14-α-demethylase, thereby impending conversion of lanosterol to fecosterol and subsequently blocking ergosterol biosynthesis. Echinocandins interfere with cell wall synthesis by inhibiting ß(1–3)-glucan-synthase. Polyenes have an affinity to bind membrane sterols that results in the formation of aqueous pores, ensuring the leakage of crucial cellular components and subsequent cell death. Allylamines are a relatively newer class of antifungals that also inhibit ergosterol biosynthesis but by specifically targeting squalene epoxidase (Dhamgaye et al. 2014).

The prime requirement at the moment would be finding an agent with a broad-spectrum of activity against susceptible species (Odds et al. 2003). This chapter summarizes the current knowledge on oleuropein and hydroxytyrosol activities against fungi as potentially promising targets and inhibitors in continuous research for effective antifungal therapy. Mainly, available literature on the effects against fungi for these compounds is directed toward members of genus *Candida* including *C. albicans* as one of the most important human pathogens (Kosalec et al. 2011). The outgrowth of *C. albicans* results in superficial mycoses of the skin, nails, and mucous membranes. However, in individuals with immune deficiencies caused by underlying disease, chemotherapy treatment, or immunosuppression following transplantation, *C. albicans* can cause severe, life-threatening invasive candidiasis. Recently, targeting virulence rather than essential process, as with conventional drugs, has been postulated as a new paradigm for the development of antifungal agents, following the successful development of drugs targeting bacterial virulence in antimicrobial therapy. So, instead of being killed, a pathogen is maintained in a harmless form by blocking virulence attributes that contribute to its pathogenicity. In addition, resistance to drugs that target virulence instead of growth is less likely to develop, given that selective pressure is reduced on nonessential targets that are required only to colonize host environments (Shareck and Belhumeur 2011). The majority of published reports are describing antimicrobial properties of olive leaf extracts with identified composition and with quantified phenolic compounds present in the extract. Pereira et al. (2007) showed *in vitro* activity of olive leaf aqueous extracts against several microorganisms including fungi *C. albicans* and *Cryptococcus neoformans*. Seven phenolics were identified and quantified by HPLC-DAD analysis of olive leaf extract: caffeic acid, verbascoside, oleuropein, luteolin, 7-*O*-glucoside, rutin, apigenin 7-*O*-glucoside, and luteolin 4'-*O*-glucoside.

Oleuropein was a compound present in the extract in the highest amount, representing approximately 73% of total identified compounds. The extract showed antimicrobial activity of olive leaf extracts in a concentration-dependent manner and *C. albicans* was found to be one of the most sensitive microorganisms with IC_{25} value lower than 1 mg/ml (IC_{25} = 0.85 mg/ml). *C. neoformans* was found to be less susceptible to olive leaf extract activity with IC_{25} = 3.00 mg/ml. Previously, also Pereira et al. (2006) performed comparative studies using extracts from olives that did not show activity against *C. albicans* (up to 100 mg/ml). Authors have concluded that cultivar and processing technology-dependent changes in phenolic composition have a considerable impact on the antimicrobial potential of crude olive extracts. Also, Medina et al. (2006) assessed the antimicrobial activity of virgin olive oil (50% v/v) where prevalent phenolic compounds were oleuropein aglycone, hydroxytyrosol, and tyrosol and they observed that *C. albicans* was unaffected. In 2014, Karygianni et al. (2014) examined dried extract from *Olea europaea* obtained by extraction with acetone (containing 60% oleuropein), table olive processing wastewater extract (contained as its main compound, the degradation product of oleuropein, hydroxytyrosol, in a percentage around 15%) against bacterial and one *Candida albicans* strain. In general, table olive extract was more active than olive leaf extract. Olive leaf extract showed a milder inhibitory effect against investigated oral pathogens. Although the extract was found to be active against each of the tested microorganisms; however, it was less active against *C. albicans* than against bacterial strains (minimum inhibitory concentration (MIC) value for *C. albicans* strain was 10.00 mg/ml). The authors concluded that the conflicting outcomes of investigated activity of olive leaf extract against *C. albicans* could be attributed to different extraction methods, which result in different chemical profiles, so which phenolic or other compound was responsible for this favorable effect remains unknown. In that study, two main compounds of the extracts were oleuropein in olive leaves and hydroxytyrosol in table olive processing wastewater. Halawi et al. (2015) performed a comparative study to evaluate the antifungal activity of olive leaves and cake samples extracted differently to obtain three categories of extracts: ethanolic extract, cold aqueous, and hot aqueous against five strains of *C. albicans* isolated in hospital. The antifungal activity was tested using well-diffusion method. Cold aqueous extract of olive cake (total phenolic content was 91.76 GAE mg/g dry matter) and ethanolic extract from leaves (total phenolic content was 98.03 GAE mg/g dry matter) showed antifungal activity against the growth of all isolates with the lowest recorded MIC and minimum fungicidal concentration (MFC) of 2.5 and 15 mg/ml, respectively, for both extracts. Also, the time-kill assay showed that fungal cells died within 6 hours after their treatment with both selected extracts. The ultrastructure of treated *C. albicans* with the two selected extracts revealed the presence of deformed cells with disintegrated protoplasm and even ruptured cell wall and cell membrane. An additional study was performed by Zorić et al. (2016b) to evaluate activity of olive leaf water extract against *C. albicans* and *C. dubliniensis*. MIC values of the extract were determined by several *in vitro* assays. The water extract showed concentration-dependent effect on the viability of *C. albicans* with MIC value of 46.875 mg/ml and *C. dubliniensis* with MIC value of 62.5 mg/ml. The

most sensitive methods for testing the antifungal effect of the extract were trypan blue exclusion method and fluorescent dye exclusion method, while MIC could not be determined according to the EUCAST recommendation, suggesting that herbal preparations contain compounds that may interfere with this susceptibility testing. The fluorescent dye exclusion method was also used for the assessment of morphological changes in the nuclei of treated cells. Necrosis predominated over apoptosis (1 h and 18 h of incubation) in the *C. albicans* sample treated with the highest concentration of olive leaf extract (46.875 mg/ml) and in the *C. dubliniensis* sample treated also with the highest concentration of olive leaf extract (187.5 mg/ml). In other samples, apoptosis was the predominated type of cell death. It has to be mentioned that 46.875 mg/ml to *C. albicans* and 187.5 mg/ml to *C. dubliniensis* were highly cytotoxic after 18 h of incubation. Induction of apoptosis in that sample was comparable to positive controls (amphotericin B and H_2O_2). Even though there are research studies dealing with antimicrobial including antifungal effects of olive leaf extract, there is far less studies dealing with oleuropein and hydroxytyrosol activities against fungi. There are reports published in 1998 (Aziz et al. 1998; Koutsoumanis et al. 1998) regarding antimicrobial activity of oleuropein against yeasts, fungi, molds, and other parasites. According to Bisignano et al. (1999) and later Khan et al. (2007), hydroxytyrosol demonstrated broader antimicrobial activity than oleuropein and is comparable to ampicillin and erythromycin in spectrum and potency. In 2009, Rahioui et al. (2009) have shown that polyphenols, hydroxycinnamic derivatives, oleuropein derivatives, tyrosol derivatives, and flavonol monoglucosides, were responsible for olive tree resistance to the leaf-spot disease caused by *Fusicladium oleagineum*. Resistance to *F. oleagineum* was related positively to tyrosol derivatives, oleuropein and rutin contents and negatively to verbascoside and apigenin contents. Recently, Khan and Murphy (2021) performed study with *Cunninghamella elegans*, a filamentous fungus that is of biotechnological interest as it catabolizes drugs and other xenobiotics in an analogous manner to animals. The authors reported that 3-hydroxytyrosol is a novel signaling molecule that regulates fungal biofilm growth. The cultures were grown planktonically and as biofilms for 72 h. Planktonic cultures have higher concentrations of the metabolite. In the presence of exogenous hydroxytyrosol (at concentrations 0.3, 0.5, and 0.8 mg/ml), the growth of aerial mycelium was inhibited and there was selective inhibition of biofilm when it was added to culture medium. The compound was not biotransformed by the fungus, when it was added to 72-h-old cultures. In the presence of 0.8 mg/ml, biofilm of *C. elegans* was approximately 75% less than the control, but planktonic growth was 30% lower.

Karygianni et al. (2019) have reported that MIC value of 1.25 mg/ml has been observed for the strain of *C. albicans*, while 99.9% of *C. albicans* was eradicated with 2.5 mg/ml. In 2013, a study was performed (Zorić et al. 2013) to test antifungal activity of hydroxytyrosol against medically important yeasts and dermatophyte strains using several *in vitro* approaches. MIC values were as follows: 6.25 mg/ml for *C. albicans*, *C. dubliniensis*, *C. tropicalis* and *Saccharomyces cerevisiae*, 1.5625 mg/ml for *C. parapsilosis* and *C. kefyr*, 0.1953 for mg/ml *Blastoschizomyces capitatum* and 0.0976 mg/ml for *C. curvata*, while for dermatophyte strains, they were 1.5625 mg/

ml for *Trichophyton mentagrophytes* var. *mentagrophytes* and 0.7812 mg/ml for *Trichophyton mentagrophytes* var. *interdigitale*. It was also observed that below MIC value, hydroxytyrosol showed potent damage of *C. albicans* cell wall using the fluorescent dye exclusion method. At subinhibitory concentrations (sub-MIC), hydroxytyrosol caused disturbances in cell surface hydrophobicity (CSH) of *C. albicans* and influenced dimorphic transition of the same strain, which is considered as one of the most important virulence factors of *C. albicans* (Ishida et al. 2006). Also, in 2016, additional *in vitro* study was performed (Zorić et al. 2016a), in which authors have investigated antifungal activity against *C. albicans*. Oleuropein was found to have antifungal activity with MIC value of 12.5 mg/ml. Morphological changes in the nuclei after staining with fluorescent DNA-binding dyes revealed apoptosis as a primary mode of cell death in the analyzed samples treated with sub-MIC concentrations of oleuropein. Results suggest that this antifungal agent targets virulence factors an essential for establishment of the fungal infection. It was noticed that oleuropein modulates morphogenetic conversion and inhibits filamentation of *C. albicans*. The hydrophobicity assay showed that oleuropein in sub-MIC values has significantly decreased, in both aerobic and anaerobic conditions, the CSH of *C. albicans*, a factor associated with adhesion to epithelial cells. It was also demonstrated that the tested compound inhibits the activity of SAPs, cellular enzymes secreted by *C. albicans*, which are reported to be related to the pathogenicity of fungi (Costa et al. 2010). Additionally, it was noted that oleuropein accomplishes its antifungal activity by altering total sterol content and subsequently affecting the membrane of *C. albicans* cells. Based on these findings, authors report that oleuropein *Candida*-cidal activity involves mechanisms at the level of the cell membrane, so this compound could potentially serve in treatment and/or prevention of candidiasis.

3 Antibacterial Activity

Since ancient years, olive products such as oil and different extracts prepared from leaves were used as remedies against many maladies, especially in Mediterranean area. Chemical composition analysis of olive product has confirmed presence of phytochemicals in extracts with antibacterial activity. The abundant group of chemicals present in olive products (such as cake) are biophenolics, and antibacterial activities against Gram-positive bacterial *Staphylococcus aureus*, *Bacillus cereus*, Gram-negative bacteria *Klebsiella pneumoniae*, and *Escherichia coli* were performed with MIC values up to 0.4 mg/ml for oleuropein (Aziz et al. 1998; Korukluoglu et al. 2010). Different authors found higher MIC values of commercially available olive leaf extracts (main active compound oleuropein 12–16 mg/capsule) against *S. aureus* ATCC 25923 and *E. coli* ATCC 25922, 100 and 400 mg/mL, respectively (Lim et al. 2016).

Ethanol-obtained extracts from olive leaves showed antibacterial activity against Group B *Streptococcus* (*Streptococcus agalactiae* from vaginal swabs) isolated

from woman with inhibition zones 28 mm (concentration of olive extract 0.5 mg/ml) and MIC values 0.02 mg/ml in microdilution assay (Mukesi et al. 2019).

Antimicrobial susceptibility of tested bacterial strains largely depends on method of extraction performed. Aqueous extract of olive leaf expresses antibacterial activity using agar well diffusion assay with dose-dependent inhibition zones using from 10 to 50 mg/ml extracts (Aliabadi 2012). In more extensive study Korukluoglu et al. (2010) using ethyl alcohol, acetone, and diethyl ether extracts of olive leaf showed that all extracts have potent antibacterial activity against Gram-positive pathogens (*B. cereus, Enterococcus faecalis, S. aureus*), with MIC ranging from 50 to 105 μg/ml; and against Gram-negative pathogens (*Salmonella typhimurium, S. enteritidis, E. coli, Pseudomonas aeruginosa, Klebsiella pneumoniae*), with MICs ranging from 25 to 178 μg/ml.

Authors noted differences in antimicrobial activity using microdilution assay depending on the type of solvents used. Another study showed that 0.6% (w/v) olive leaf extract after 3 hours of exposure in "kill-time" assay expressed bactericidal activity against *E. coli, K. pneumoniae* and *S. aureus* with MBC of 0.3%, 0.3%, and 0.6%, respectively (Markin et al. 2003). Contrary to the findings of Aliabadi (2012), the results of Korukluoglu et al. (2010) were negative using aqueous extract of olive leaf. However, due to differences in phytochemical profiles, pH of water for extraction, and sample preparation, these variables could change the outcomes of *in vitro* susceptibility testing (Korukluoglu et al. 2010). Olive leaf products are commercially available, and health claims include cardioprotective, antioxidative, anti-inflammatory, and antimicrobial activities (Romani et al. 2019). Sudjana et al. (2009) conducted extensive *in vitro* survey of antimicrobial activities by broth microdilution assay on 122 microbial strains (both Gram-positive, Gram-negative bacterial pathogens and yeasts as well). The results showed big differences between MIC values and strains tested, and interestingly, the most susceptible were *Campylobacter jejuni* (MIC_{range} 0.3–2.5% v/v), *Helicobacter pylori* (MIC_{range} 0.6–1.2% v/v), and MRSA strains (MIC_{range} 0.8–12.5 V) (Sudjana et al. 2009). Data presented indicate that olive leaf extracts do not possess broad-spectrum activity, but only potent activity was in the case of *C. jejuni, H. pylori,* and MRSA. Since the products derived from olive were widely used in diet and as food-supplements, after ingestion there may be a direct or local activity of bioactive compounds from oil or leaf that have antimicrobial activity against *H. pylori*. Olive oil reduces the gastric acid production, and it suppresses the serum gastrin level and higher levels of peptide YY in cholecystectomized patients (Serrano et al. 1997). Olive oil significantly reduces the size of ulcers (Taits 1986), the role of small-molecule compounds presents in olive oil, besides the fatty acid content could be a key role in pharmacological effect (Romero et al. 2007). Furthermore, dialdehydic form of decarboxymethyl ligstroside aglycon is the most potent polyphenol from olive oil, and after diffusion into gastric juice could inhibit the growth of *H. pylori* at concentrations, which are bactericidal (Romero et al. 2007). The pilot clinical study with virgin olive oil on 60 *H. pylori*-positive patients showed that *H. pylori* was eradicated in 27–40% of individuals, and 23% after 1 month of intervention, which is promising result and good base for future studies (Castro et al. 2012). Some of the

phenolics present in olive oil could easily penetrate in acid phase of gastric juice, such as hydroxytyrosol but not in the case of oleuropein, which cannot hydrolyze in lower pH (Romero et al. 2007). Due to its presence in hydrophilic phase and in acid environment of gastric juice, anti- *H. pylori* activity of hydrohytyrosol could be predicted.

The antimicrobial activity of olive oil's compounds, namely, maslinic and oleanic acid, exhibited more potent antibacterial activity against oral pathogens *Streptococcus mutans, S. sobrinus, S. oralis, Prevotella intermedia, Porphyromonas gingivalis, Fusobacterium nucleatum, Parvimonas micra* than oleuropein, hydroxytyrosol, olocanthal, and oleacin (Karygianni et al. 2019).

Furthermore, olive mill waste waters rich in biophenols showed antibacterial activity against *S. aureus, B. subtilis, E. coli*, and *P. aeruginosa* using disk diffusion assay at 5 mg/ml (Obied et al. 2007), which imply the use of waste waters as by-products as source of possible bioactive compounds.

The extracts of olive leaves present in the market as nutraceuticals can decrease the count of food-borne bacteria, such as *E. coli* O157:H7*, Salmonella enterica, Listeria monocytogenes*, and *S. aureus* with different range of quantitative bactericidal 50% value (BC_{50}) (Friedman et al. 2013). The authors presented data of BC_{50} value, suggested a broad spectrum of antibacterial activity of olive juice powder (12% olive polyphenolics of which 4-hydroxytyrosol is approximately 6% total) against *E. coli* O157:H7 (0.829 ± 0.019%), *Salmonella enterica* (0.318 ± 0.006%), *Listeria monocytogenes* (0.284 ± 0.031%), and *S. aureus* (0.252 ± 0.014%). The olive pomace also possesses antibacterial activity, expressed as BC_{50} as follows against *E. coli* O157:H7 (0.178 ± 0.006%), *S. enterica* (0.070 ± 0.001%), *L. monocytogenes* (0.039 ± 0.001%), and *S. aureus* (0.008 ± 0.001%) (Friedman et al. 2013).

In 2020, Menchetti et al. (2020) published study about the influence of phenolic extract from olive vegetation water (PEOVW) on the survival of *Salmonella enteritidis* on mayonnaise. Phenolic extract from olive vegetation water has antibacterial effect on mayonnaise. The most abundant phenolic compound identified in PEOVW was the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol. *S. enteritidis* is reduced by 9.5%/h in mayonnaise added with polyphenols at 4 °C, while lower elimination rate of *S. enteritidis* was found at room temperature.

Additional study by Shiry et al. (Shiry et al. 2020) evaluated changes in cutaneous mucosal immunity in the intestine of rainbow trout (*Oncorhynchus mykiss*) orally administrated florfenicol (FFC) and/or olive leaf extract (obtained by methanol), experimentally infected with *Streptococcus iniae*. The most obvious active component of olive leaf extract, found by HPLC analysis, is oleuropein (0.496 mg/l). The juvenile fish (55 ± 7.6 g) were divided into different groups according to the use of added olive leaf extract (80 g/kg food), the presence/absence of FFC (15 mg/kg body weight for 10 consecutive days), and the streptococcal infectivity (2.87×10^7 CFU/ml as 30% of LD_{50}–96 h). Authors report that the combined use of olive leaf extract and FFC could lower some skin mucous immunological indices (e.g., TP, TIg, and ALP) and the gene expression of inflammatory cytokines (e.g.,

Table 5.1 Spectrum of antibacterial activity of oleuropein and hydroxytyrosol

Microbes	Oleuropein MIC (μg/ml)	Hydroxytyrosol MIC (μg/ml)	References
S. aureus	62.5–3200	7.85–400	Bisignano et al. (1999) Furneri et al. (2002) Tuck and Hayball (2002) Tafesh et al. (2011) Medina-Martínez et al. (2015) Lim et al. (2016) Karygianni et al. (2019)
Streptococcus mutans *S. sobrinus* *S. oralis* *S. pyogenes*	625 625 1250	312 625 1250 400	Tafesh et al. (2011) Karygianni et al. (2019)
Enterococcus faecalis	1250	1250	Karygianni et al. (2019)
Pseudomonas aeruginosa	ND	1000–>1000	Furneri et al. (2002) Medina-Martínez et al. (2015)
E. coli	ND	400	Bisignano et al. (1999) Medina-Martínez et al. (2015)
Klebsiella pneumoniae	ND	400–1000	Bisignano et al. (1999) Tafesh et al. (2011) Medina-Martínez et al. (2015)
Haemophilus influenzae	500	0.97	Bisignano et al. (1999) Tuck and Hayball (2002)
Moraxella catarrhalis	>500	1.92	Bisignano et al. (1999) Tuck and Hayball (2002)
Salmonella typhi *S. typhimurium*	125 ND	3.94 >1000	Tassou and Nychas (1995) Bisignano et al. (1999) Tuck and Hayball (2002) Medina-Martínez et al. (2015)
Vibrio spp.	62.5–125	0.97–7.8	Bisignano et al. (1999) Tuck and Hayball (2002)
Mycoplasma hominis *M. fermentans* *M. pneumoniae*	20 20 160	0.03–0.12 0.25 0.5	Furneri et al. (2002) Furneri et al. (2004) Furneri and Bisignano (2010)

Legend: *ND* not determined

TNF-*a* and IL-*1ß*) of rainbow trout. Furthermore, lysozyme and protease activities were invigorated by the FFC and olive leaf extract treatment. Use of olive leaf extract induced the gene expression of hepcidin-like antimicrobial peptides.

As stated in the introduction section, olive oil, leaves, and other products are source of bioactive polar components in EVOO or leaf extracts, and oleuropein (as secoiroidoids) and hydroxytyrosol (as phenolic) were scientifically explored in more details than other compounds belonging to non-fatty fraction of the olive oil. As it can be seen in Table 5.1, both oleuropein and hydroxytyrosol exhibited wide spectrum of antimicrobial activity against food-borne, respiratory, and nosocomial bacterial pathogens. The oleuropein and 4-hydroxytyrosol possess more potent bactericidal activity than olive pomace or olive juice powder, suggesting that some

Table 5.2 Effects of oleuropein and hydroxytyrosol on *Staphylococcus aureus* cells

Target sites	Results of interaction	References
Inhibition of enterotoxin B production	0.6% of oleuropein inhibited growth of *S. aureus* and toxin production *in vitro* Low pH and low inoculum enhance anti-*S. aureus* activity	Tassou and Nychas (1994)
Cell membrane integrity	Disruption caused leakage of glutamate, potassium, and inorganic phosphate (on *E. coli* as a model) Oleuropein interacts with phosphatidylglycerol (PG) as a model membrane for *S. aureus* Oleuropein possibly promotes pores and disruption in membranes in interaction with dimyristoylphosphatidylglycerol (DMPG) Leakage of intracellular proteins with irreversible damage of *S. aureus* cells with oleuropein at conc. 20 mg/ml	Juven et al. (1972) Tranter et al. (1993) Caturla et al. (2005) Cinar (2009)
Interaction with antibiotics	Oleuropein with ampicillin decreases MIC values against *S. aureus* Additive effects of oleuropein and hydroxytyrosol according to FIC index Strong synergistic activity in combination ampicillin with hydroxytyrosol	Lim et al. (2016)
Interaction with biofilm formation	Hydroxytyrosol-polymer (polyacrylate) nanoparticles significantly reduce adhesion of *S. epidermidis* Reduction of ROS production in biofilm	Crisante et al. (2015)
Interaction with H_2O_2	Hydrogen peroxide triggers antibacterial activity of oleuropein against *Staphylococcus aureus, S. epidermidis,* and *S. saprophyticus*	Zanichelli et al. (2005)

antagonistic effect could be seen in extracts (Friedman et al. 2013). For example, in case of *S. aureus*, 4-hydroxytyrosol has more potent bactericidal activity with BC_{50} 0.057 ± 0.004% than oleuropein BC_{50} 0.141 ± 0.013%), respectively (Friedman et al. 2013). For the same pathogen, BC_{50} value of olive juice powder is 0.252 ± 0.014% (Friedman et al. 2013).

The activity is not exclusively connected within Gram-positive or Gram-negative bacterial species. Important nosocomial pathogen *S. aureus* is more susceptible to hydroxytyrosol than oleuropein (Table 5.1), which encourage to elucidate mechanism of action on different target sites inside bacterial cells, which include cell wall and membrane structure, toxin production, and biofilm formation. Early works on mechanisms reveal that oleuropein has surface-active properties and could disrupt the structure of bacterial cell membranes (Juven et al. 1972). Furthermore, the effect of interaction of oleuropein with membrane structures in bacteria has been provided by leakage of cytoplasmic molecules potassium and inorganic phosphate together with decreased level of ATP at a concentration 2 mg/ml (Juven et al. 1972). Leakage of potassium outside the bacterial cell was induced with damage of membrane physical structure, and the leaked of potassium is good marker of damage of lipophilic structures in lipid bilayer of membranes. More precise research in integrity of bacterial cells found that oleuropein has affinity to membrane-based phosphatidylglycerol and promotes pores, which lead to leakage of intracellular

molecules and consequently lead to cell death (Caturla et al. 2005; Cinar 2009). Based on early works on mechanisms of bactericidal activity, new research data revealed more complex activity of oleuropein and hydroxytyrosol. As shown in Table 5.2, there are several targets in planktonic and biofilm cells of *S. aureus*. Both compounds from olive leaf, olive oil and extracts, interact with enterotoxin production, and could potentiate the antibacterial activity of antibiotics.

These data, demonstrated *in vitro*, could lead to more extensive research into translation of data from *in vitro* to the *in vivo* conditions. Data of antibacterial activity of hydroxytyrosol and oleuropein are in favor of local treatment of infections with products rich in hydroxytyrosol and oleuropein.

4 Conclusions

In conclusion, findings of so far conducted studies have shown that both oleuropein and hydroxytyrosol have promising *in vitro* antifungal activity including activity against opportunistic fungal pathogen *C. albicans*. These antifungal agents target virulence factors of *C. albicans*, which are essential for the establishment of opportunistic infection. However, additional studies are necessary to further investigate the mechanism of action of oleuropein and hydroxytyrosol and the possible development of new antifungal therapeutics. Both oleuropein and hydroxytyrosol possess a wide range of antibacterial activity, as well. Based on bacterial models (such as *S. aureus*), targets of bactericidal activity of oleuropein and hydroxytyrosol include several sites. Both compounds interact also with bacterial biofilm formation and enterotoxin production. To potentiate activity of some antibiotics is also positive outcome of interaction of oleuropein and hydroxytyrosol with bacterial cells. As very interesting biomolecules, the research on antimicrobial activities of both oleuropein and hydroxytyrosol could lead to translation of data to *in vivo* conditions.

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