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Original Article

Antimicrobial Activity of *Olea europaea* Fatty Oil against Multi-Drug Resistant and Biofilm Forming Microorganisms

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Abstract

The aim of the present study was to investigate the potential antimicrobial activity of *Olea europaea* fatty oil against a collection of bacterial (*Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa*) and fungal (*Candida albicans*) clinical isolates. The antimicrobial and antibiofilm activity were evaluated by broth microdilution method for establishing the minimal inhibitory concentration (MIC) and microtiter assay for determining the minimal biofilm eradication concentration (MBEC). Some of the potential microbial targets of the fatty oil were investigated by flow cytometry (FCM). The results obtained hereby revealed that *Olea europaea* fatty oil inhibited microbial planktonic growth (MICs values of 5.23-41.8 mg/ mL) and biofilm development on inert substrata (MBEC values of 1.31-20.9 mg/ mL). The FCM measurements confirmed that the analyzed oil induced microbial membrane damages and inhibited microbial efflux pump activity.

Keywords: antimicrobial activity; anti-biofilm activity; efflux pump; fatty oil; flow cytometry

Introduction

Nowadays, microbial resistance is recognized as a top 5 priority worldwide problem (Perveen *et al*., 2012), generating an acute need for searching new antimicrobial agents. The use of medicinal plants for the treatment of diseases dates back to the history of human life, that is, since human beings have sought a tool into environment for combating diseases (Halberstein, 2005). *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* are opportunistic nosocomial pathogens responsible for a wide range of acute or chronic infections (Telcian *et al*., 2017). Evidence shows that medicinal plants have been cultivated approximately 60 000 years ago (Solecki and Shanidar, 1975). Scripts about medicinal plants date back to about 5000 years ago in China, India, Egypt, and at least 2500 years in Greece and Central Asia (Ang-Lee *et al*., 2001). Almost 80% of the people around the world have used herbal plants in primary health care (Mehrotra and Srivastava, 2010). Herbal drugs could represent promising solutions for fighting antibiotic resistant infections (Rahman and Hossain, 2010). Many researchers have investigated the antimicrobial properties of several vegetal active compounds obtained from spices, herbs and extracted oils (Burt, 2004; Proestos *et al*., 2005), some of the most studied being the phenolic compounds, which are components of plant defense mechanisms against microbial pathogens (Pereira, 2007; Slobodníková *et al*., 2016), such as thymol, catechins, carvacrol, chlorogenic acid, oleuropein and cinnamaldehyde (Friedman *et al*., 2006; Almeida *et al*., 2006). In the present study, the purposes were to investigate the antimicrobial and anti-biofilm activities of *Olea europaea* fatty oil. At the end of the sixties the researchers began to correlate the antimicrobial activity of olive oil to the phenolic compounds (Fleming and Etchells, 1967; Zanichelli *et al*., 2005; Pereira, 2007). The major phenolic compound in olive fruits is oleuropein, a bitter glucoside composed of glucose, polyphenol hydroxytyrosol and elenolic acid.

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Antimicrobial inhibitory effects are more pronounced in case of virgin olive oils, followed by olive oils and pomace olive oils, which is in accordance with their decreasing content in phenolic compounds. The olive oils were demonstrated to possess antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus, Listeria monocytogenes*, *Clostridium perfringens, Lactobacillus acidophilus*, *Bifidobacterium bifidum, Escherichia coli, Salmonella enterica*, *Yersinia sp.*, *Shigella sonnei* (Medina *et al*., 2006). The studies revealed that Gram-positive bacteria are more susceptible to olive oil polyphenols than Gram-negative ones, mainly due to differences in the cell wall structure and to the presence of an additional layer represented by the outer membrane in Gram-negative bacteria, which can act as a barrier towards macromolecules penetration (Nikaido, 1996). Spanish virgin olive oils were demonstrated to possess antimicrobial activity against *Helicobacter pylori* strains (Romero *et al*., 2007). Also, the extract of olive leaf exhibited growth inhibition ability against *Candida albicans* (Markin *et al*., 2003). The main antimicrobial mechanisms described for phenolic compounds include plasma membrane damage, interference with cell wall synthesis, reduction of membrane fluidity, inhibition of nucleic acid synthesis (Gyawali and Ibrahim, 2014). Antibiofilm activity was correlated with the ability of phenolic compounds to affect the bacterial regulatory mechanisms such as quorum sensing or other global regulator systems (Silva *et al*., 2016). The aim of the study was to investigate the potential antimicrobial activity of *Olea europaea* fatty oil against multi-drug resistant (MDR) microorganisms.

Materials and Methods

Microbial strains

A collection of 43 clinical microbial strains belonging to different species of bacteria (*Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*) and fungi (*Candida albicans)* were included in the present study. They were isolated form patients admitted to "Prof. C. C. Iliescu" Institute of Cardiovascular Diseases, Bucharest, Romania, Fundeni Hospital and identified at species level using Vitek II. The isolates were maintained in the laboratory on TSA (Tryptone Soy Agar) medium.

Broth microdilution assay

The antimicrobial activity of the commercial *Olea europaea* fatty oil (Salvadori, Italy) was investigated by broth microdilution assay carried out in 96 multi wells plates (Saviuc *et al*., 2011). The plant fatty oil was solubilized in dimethylsulfoxide (DMSO) in 1:1 ratio (v/v). Serial two-fold dilutions were prepared in Luria Broth broth starting with 41.8 mg/mL to 0.08 mg/mL. Then the wells containing different concentrations of the tested fatty oil were inoculated with microbial suspensions (1.5×10^8 cfu/ml) prepared from 18-24 h solid cultures. Negative controls, represented by uninoculated broth and positive controls represented by untreated bacterial cultures were included for each of the tested microbial strain. Additionally, the solubilizing agent, DMSO, was also examined for antimicrobial activity. After incubation at 37 °C for 24 h, the minimal inhibitory concentrations (MICs) values of the fatty oil against the microbial isolates were assessed by measuring the optical density at 620 nm (EZ Read 400, Bichrom). All experiments were carried out in triplicate.

Evaluation of the potential antibiofilm activity of Olea europaea fatty oil

The influence of *Olea europaea* fatty oil on biofilm development at inert substratum was evaluated by microtiter method. The microbial cultures exposed to different concentrations of fatty oil in 96 well plates were discarded after 24 hours of incubation at 37 °C. The wells were washed gently three times with PBS. The remaining, well adherent microbial cells were then fixed with cold methanol for 5 minutes and colored with 1% crystal violet for 20 minutes at room temperature. The wells were gently washed three times with tap water and the resulting colored biofilms were resuspended by adding 33% acetic acid. The colored suspensions were quantified spectrophotometrically at 492 nm (EZ Read 400 reader, Bichrom) to establish the minimal biofilm eradication concentration (MBEC). The experiments were performed in triplicate.

Flow cytometric (FCM) assay for detection of plant EOs antimicrobial action mechanisms

The tested microbial strains treated with olive oil at 1/2 × MIC were analyzed by FCM, using the acid nucleic intercalating dyes: propidium iodide (PI) and ethidium bromide (EB). The dyes do not penetrate and accumulate inside viable cells, therefore the fluorescence (median fluorescence intensity (MFI)) measured in channel 2 (ethidium bromide) and respectively in channel 3 (propidium iodide) is low. If the microbial cell wall is affected (loss of integrity / affected efflux pump activity), the dyes enters the cell and bind to nucleic acid, resulting in an increased fluorescence.

Samples were stained at room temperature with 10 μL/ml for PI and 5 μg/mL EB, in the dark, for 10 minutes. The fluorescence measurements were performed using the FACSCalibur flow cytometer (BD, Sparks, USA) equipped with an argon laser with an excitation wavelength of 488 nm. For each sample, a total of 10,000-30,000 events were acquired. CellQuest Pro software was used for statistical analysis. Microbial populations were gated based on the FSC / SSC characteristics.

The antimicrobial activity of olive oil

Antimicrobial resistance emerged as a result of overuse and inappropriate use of antibiotics in the treatment of infectious diseases. The high resistance rates are threating the efficiency of the current treatment strategies and encourage us to search novel strategies to combat increasing MDR organisms (Llor and Bjerrum, 2014). Since ancient times, plants extracts are known to possess antimicrobial properties being used in the treatment of different diseases. In this context, we investigated the potential antimicrobial activity of *O. europaea* oil against a collection of pathogenic bacteria and fungi. Broth microdilution assays showed that *O. europaea* oil exhibited a variable antimicrobial activity

against the tested isolates, with MIC values ranging from 5.23 mg/ml to > 41.8 mg/ml. *O. europaea* oil was more active against *S. aureus* isolates (Table 1). The least susceptible microorganism was *P. aeruginosa,* the resistance of this bacterium to a wide range of natural and synthetic antimicrobials being partly due to its hydrophilic outer membrane and exopolysaccharidic capsule. Different geographical conditions (Ronalli, 2006) and the method of preparation influence the content of antimicrobial components present in the extracts. Janakat *et al*. (2015) found that MIC and minimum bactericidal concentration (MBC) of olive oil leaves ranged from 60 to 80 µl/ml. Pereira *et al*. (2007) found the MICs of a Portuguese table olive extract against many Gram-positive and Gram-negative bacterial strains, ranged from 10 to 100 µl/ml at 37 °C. The results of the study of Sudjana *et al*. (2009) revealed that the MICs of commercial extract derived from the leaves of *Olea europaea* against *Campylobacter jejuni*, *Helicobacter pylori* and *Staphylococcus aureus* [including meticillin-resistant *S. aureus* (MRSA) were as low as 0.31-0.78% (v/v). In contrast, the extract showed little activity against many other tested organisms, with MICs for most ranging from 6.25% to 50% (v/v). The antibacterial activity of olive oil is due to the phenolic compounds, which can induce damages of the bacterial membrane and disrupt the cell wall peptidoglycan, causing the loss of structural solidity and leakage of intracellular cytoplasmic components (Caturla *et al*., 2005). Furthermore, the hydroxyl group in phenolic compounds can bind to the active sites of enzymes, changing their substrate affinity. Additionally, the lipid solubility and the degree of steric hindrance they may cause contribute to their overall antimicrobial activity (Ceylan and Fung, 2004).

O. europaea oil activity on microbial adhesion to inert substrata

Biofilm-associated infections accounting for up to 80% of the total number of human infections (Fleming and Rumbaugh, 2017) are considered a big challenge to the medical community, as antibiotic tolerance is more than 1000-fold increase in biofilm embedded pathogens (Rogers *et al*., 2010); moreover, the biofilm also protects microbial cells from the host immune response). The study of Slobodníková *et al*. (2016) revealed that the phenolic compounds are also very active against biofilm embedded bacteria.

Regarding the antibiofilm activity, the results indicated that the *O. europaea* oil was able to inhibit adherence of the microbial cells at inert substrate at 1/2 × MIC. Inhibition of biofilm formation was detected at concentrations ranging from 1.31 mg/ml (*S. aureus*) to > 41.8 mg/ml (*P. aerugionsa, C. albicans*). The antimicrobial and antibiofilm activities of the tested oil could be attributed to the high amounts (73.41%) of the phenolic compound oleuropein present in *O. europaea* oil (Pereira, 2007).

Investigation of some of the possible mechanisms of the antimicrobial activity of O. europaea oil by flow cytometry

The possible mechanisms of the antimicrobial action of vegetal oils were previously reported to be the membrane damage potential and efflux pumps inhibitor (EPI) activity (Gellatly and Hancock, 2013). In our study, microbial cells exposed to *O. europaea* oil, at 1/2 × MIC exhibited an increased fluorescence after staining with PI and EB, respectively. The MFI of the treated cells was significantly higher than the MFI of the controls (two-fold or higher values than of viable control cells) (Table 3) suggesting an increase in the fraction of microbial cells with permeabilized cell envelope, at 1/2 × MIC. The PI and EB dyes were able to enter the permeabilized cells, bind nucleic acids resulting in an enhanced fluorescence of the cells. Therefore, the FCM results confirm that one of the mechanisms by which the *O. europaea* oil extert its antimicrobial activity is represented by inducing damages to the microbial cell wall. This mechanism was detected in all tested microbial isolates. Engel (2003), Martins *et al*. (2013) proposed a relation between the fluorescence intensity (FI) of the cells labeled with EB and the ratio net influx of EB/EB extracellular ejection via the efflux pump activity ratio (Martins *et al*., 2013) (Figs. 1-2).

Table 1. MIC values (mg/ml) of the tested fatty oil

Microbial strains	*O. europaea* min - max
E.coli (n=7)	20.9 - 41.8
P. aeruginosa (n=10)	41.8
S. aureus (n=10)	5.23 - >41.8
C. albicans (n=16)	10.45 - >41.8

Table 2. MBEC values (mg/ml)

Microbial strains	*O. europaea* min - max
E. coli (n=7)	5.23 - 20.9
P. aeruginosa (n=10)	10.45 - >41.8
S. aureus (n=10)	1.31 - >41.8
C. albicans (n=16)	2.61 - >41.8

Table 3. The values of MFI for PI stained microbial cells exposed to *O. europaea* oil at 1/2 × MIC

Plant EOs Microbial strains	*O. europaea* (n= number of isolates)	Viable cells control	Dead cells control
C. albicans	16.14 (n=5)	6.78	323.57
E. coli	10.46 (n=1)	4.53	15.68
P. aeruginosa	14.41 (n=5)	4.53	15.68

Table 4. The values of MFI for EB stained microbial cells exposed to *O. europaea* oil at 1/2 × MIC

Plant EOs Microbial strains	*O. europaea* (n= number of isolates)	Viable cells control	Dead cells control
C. albicans	31.92 (n=11)	6.78	323.57
E. coli	36.84 (n=3)	4.53	24.62
P. aeruginosa	20 (n=2)	4.53	24.62

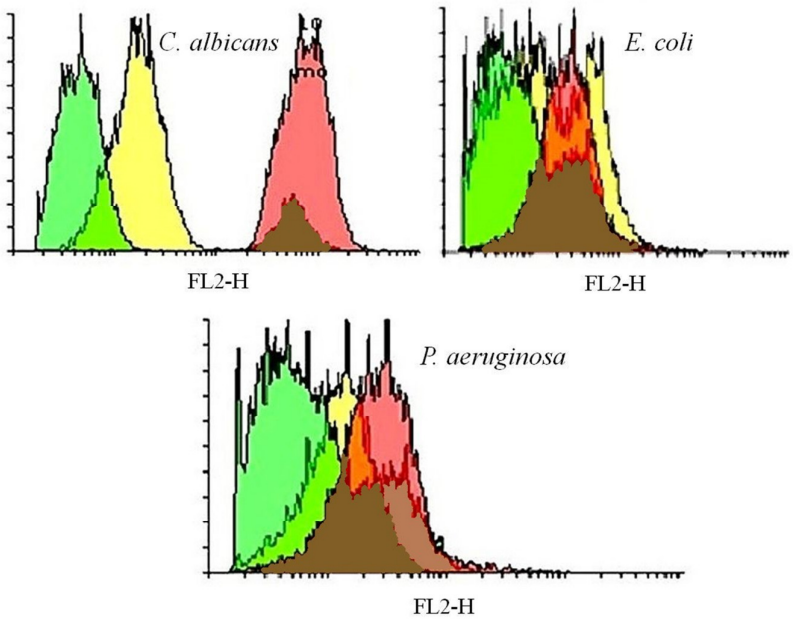

Fig. 1. Overlay of the histogram for the median of the fluorescence intensity of efflux pumps, viable cells control (green) and heat treated cells control (red), cells treated with *O. europaea* fatty oil (yellow); a, b, c - typical aspects of the histograms obtained at MIC/2 concentration values for tested bacteria

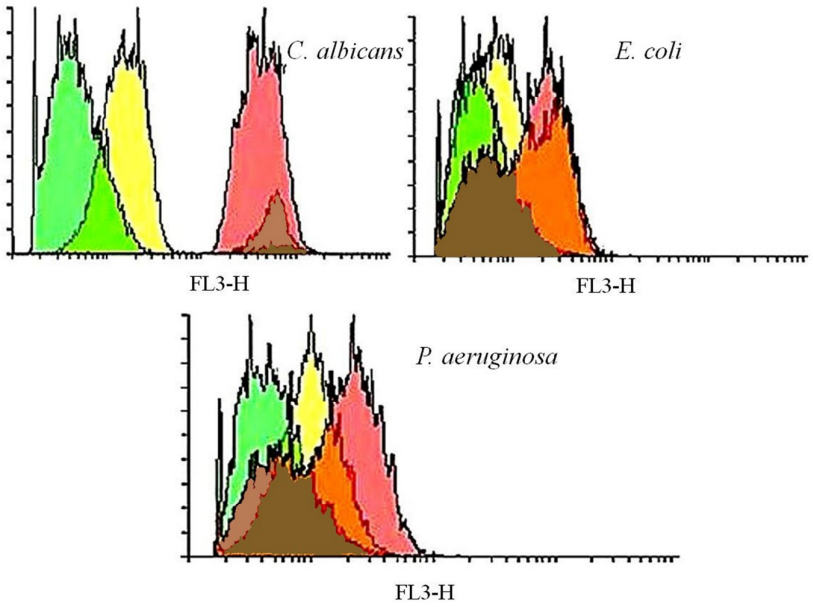

Fig. 2. Overlay of the histogram for the median of the fluorescence intensity of cell membrane integrity, viable cells control (green) and heat treated cells control (red), cells treated with *O. europaea* fatty oil (yellow); a, b, c, d - typical aspects of the histograms obtained at MIC/2 concentration values for *C. albicans*, *E. coli*, *P. aeruginosa*, *S. aureus*, respectively

Conclusions

The investigation of the influence of *O. europaea* fatty oil on drug resistant bacterial and fungal strains growth has shown that the tested oil has been active against planktonic and biofilm embedded cells, by actively interacting with microbial surface structures essential for growth and survival, such as membrane and efflux pumps. These results are demonstrating the potential of *O. europaea* as a source of bioactive antimicrobial compounds for controlling antibiotic resistant pathogens. However, for drug development additional tests are needed to determine their toxicity and pharmacokinetic and pharmacodynamic properties.

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