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On the In-vitro Antimicrobial Activity of Oleuropein and Hydroxytyrosol

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Abstract

Secoiridoides (oleuropein and derivatives), one of the major classes of polyphenol contained in olives and olive oil, have recently been shown to inhibit or delay the rate of growth of a range of bacteria and microfungi but there are no data in the literature concerning the possible employment of these secoiridoides as antimicrobial agents against pathogenic bacteria in man.

In this study five ATCC standard bacterial strains (*Haemophilus influenzae* ATCC 9006, *Moraxella catarrhalis* ATCC 8176, *Salmonella typhi* ATCC 6539, *Vibrio parahaemolyticus* ATCC 17802 and *Staphylococcus aureus* ATCC 25923) and 44 fresh clinical isolates (*Haemophilus influenzae*, eight strains, *Moraxella catarrhalis*, six strains, *Salmonella* species, 15 strains, *Vibrio cholerae*, one strain, *Vibrio alginolyticus*, two strains, *Vibrio parahaemolyticus*, one strain, *Staphylococcus aureus*, five penicillin-susceptible strains and six penicillin-resistant strains), causal agents of intestinal or respiratory tract infections in man, were tested for in-vitro susceptibility to two olive (*Olea europaea*) secoiridoides, oleuropein (the bitter principle of olives) and hydroxytyrosol (derived from oleuropein by enzymatic hydrolysis and responsible for the high stability of olive oil). The minimum inhibitory concentrations (MICs) calculated in our study are evidence of the broad antimicrobial activity of hydroxytyrosol against these bacterial strains (MIC values between 0·24 and 7·85 $\mu g\,mL^{-1}$ for ATCC strains and between 0·97 and 31·25 $\mu g\,mL^{-1}$ for clinically isolated strains). Furthermore oleuropein also inhibited (although to a much lesser extent) the growth of several bacterial strains (MIC values between 62·5 and 500 $\mu g\,mL^{-1}$ for ATCC strains and between 31·25 and 250 $\mu g\,mL^{-1}$ for clinical isolates); oleuropein was ineffective against *Haemophilus influenzae* and *Moraxella catarrhalis*.

These data indicate that in addition to the potential employment of its active principles as food additives or in integrated pest-management programs, *Olea europaea* can be considered a potential source of promising antimicrobial agents for treatment of intestinal or respiratory tract infections in man.

Despite the wide availability of clinically useful antimicrobial drugs, several arguments (limited antimicrobial spectrum and serious side-effects of major antibiotics, increasing clinical resistance of previously sensitive microorganisms, emergence of previously uncommon infections) stimulate the development of new plant molecules with antibacterial activity.

Because the olive (*Olea europaea*) leaf is known to be resistant in nature to microbial and insect attack, much research has focused on the antimicrobial activity of compounds contained in olives, olive oil and vegetation waters.

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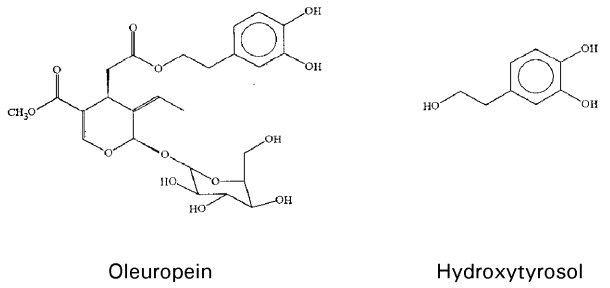

Figure 1 The structural formulae of oleuropein and hydroxytyrosol.

Secoiridoides (oleuropein and derivatives), one of the major classes of polyphenol contained in olives and olive oil, have recently been shown to inhibit or delay the rate of growth of a range of bacteria and microfungi, and so they might be efficiently used as alternative food additives or in integrated pest-management programs (Ruiz-Barba et al 1991; Tassou et al 1991; Tassou & Nychas 1994, 1995; Capasso et al 1995; Aziz et al 1998). However, there are no data in literature about the possible employment of *Olea europaea* secoiridoides as antimicrobial agents against pathogenic bacteria in man.

In this study several bacterial strains which are causal agents of intestinal or respiratory tract infections in man were tested for in-vitro susceptibility to oleuropein (the bitter principle of olives) and hydroxytyrosol (derived from oleuropein by enzymatic hydrolysis and responsible for the high stability of olive oil; Figure 1).

Materials and Methods

Materials

Oleuropein was obtained from Extrasynthese (Genay, France). Hydroxytyrosol was synthesized according to the method described by Le Tutour & Guedon (1991). Stock solutions ($10\,mg\,mL^{-1}$) of each compound in dimethylsulphoxide (DMSO), were diluted with DMSO (1 : 10 at least) and subsequently diluted in sterile buffer (1 : 1).

Ampicillin and erythromycin were purchased from Sigma (Milan, Italy); working solutions were prepared according to procedures of the National Committee for Clinical Laboratory Standards (NCCLS; 1991). All culture media and supplements were obtained from Oxoid (Unipath s.p.a., Milan, Italy).

Bacterial isolates

A total of 44 fresh clinical isolates (*Haemophilus influenzae*, eight strains, *Moraxella catarrhalis*, six strains, *Salmonella* species, 15 strains, *Vibrio cholerae*, one strain, *Vibrio alginolyticus*, two strains, *Vibrio parahaemolyticus*, one strain, *Staphylococcus aureus*, five penicillin-susceptible strains and six penicillin-resistant strains), identified by conventional procedures (confirmed by API system, Bio Merieux) and five American Type Culture Collection (ATCC) standard strains (*Haemophilus influenzae* ATCC 9006, *Moraxella catarrhalis* ATCC 8176, *Salmonella typhi* ATCC 6539, *Vibrio parahaemolyticus* ATCC 17802 and *Staphylococcus aureus* ATCC 25923) were used.

An inoculum of each bacterial strain was suspended in Triptic Soy broth (3 mL) and incubated overnight at 37°C. Before use the overnight cultures were diluted 1 : 10 with nutrient broth. To ensure that the density of diluted cultures were all within the range 10^7-10^8 colony-forming units mL^{-1}, serial dilution plate counts were performed for each culture.

Susceptibility test

The drugs were tested by the disc-diffusion method (Bauer et al 1966). Briefly, diluted bacterial cultures ($100\,\mu L$) were spread on sterile Muller–Hinton agar plates, after which 6-mm-diameter discs (sterile blank), impregnated with the drug to be tested ($100\,\mu g$), were placed on the plates. The plates were incubated for 24 h at 37°C under aerobic conditions and the diameter of the inhibition zone around each disc was then measured and recorded.

If the drugs were found to be active in the disc-diffusion test (inhibition zone ≥ 10 mm), they were evaluated to determine minimum inhibitory concentration (MIC) values. MICs were calculated by the broth-microdilution test, as described by Sahm & Washington (1991), using Mueller–Hinton medium. Final concentrations from 0·015 to $500\,\mu g\,mL^{-1}$ were tested for each drug, using a standard 1×10^5 to 5×10^5 colony-forming units mL^{-1} inoculum. The plates were incubated at 37°C for 18 h. MIC was defined as the lowest concentration of drug that inhibits visible growth after 18 h incubation. All determinations were performed in triplicate.

Results and Discussion

Olives and olive oil contain a variety of minor bioactive components responsible for their unique flavour and taste. Among these compounds, oleuropein and hydroxytyrosol are endowed with several biological properties, particularly antioxidant and anti-inflammatory activity (Visioli et al 1995; Ghisalberti 1998; Saija et al 1998). In our current

work we have shown that these biophenols might be good candidates for employment as antimicrobial agents against pathogenic bacteria in man.

The MICs reported in Table 1 are evidence of the antimicrobial activity of oleuropein and, particularly, hydroxytyrosol against the ATCC bacterial strains tested in our study and are consistent with those reported by other authors (Tassou & Nychas 1994, 1995; Aziz et al 1998). Interestingly, hydroxytyrosol and, to a much lesser extent, oleuropein also proved cytotoxic to the clinically isolated bacterial strains tested (Table 2). In fact, the growth of all tested strains of *Haemophilus influenzae* (eight penicillin-resistant strains) and *Moraxella catarrhalis* (six strains) was significantly inhibited by hydroxytyrosol (MIC values between 1·9 and 15·6 $\mu g\,mL^{-1}$); conversely, oleuropein was completely ineffective. When assayed against *Salmonella* species (15 strains), *Vibrio* species (four strains) and *Staphylococcus aureus* (five penicillin-susceptible strains and six penicillin-resistant strains), hydroxytyrosol seemed much more active than oleuropein—MIC values were between 0·97 and 31·25 $\mu g\,mL^{-1}$ for hydroxytyrosol and between 31·25 and 250 $\mu g\,mL^{-1}$ for oleuropein.

Although several naturally occurring compounds have antimicrobial properties, few are active against Gram-negative bacteria. Interestingly, as would be expected from previous studies (e.g. Tranter et al 1993), our current findings show the high antimicrobial activity of olive catechol derivatives (such as oleuropein and hydroxytyrosol) against both Gram-negative and Gram-positive bacteria. These results also confirm the high activity of olive phenols against *Staphylococcus aureus*, a microorganism extensively studied because of its capacity to produce enterotoxins and its exceptional resistance (among Gram-positive bacteria) to natural phenolic compounds.

With regard to the mechanism of action of the antimicrobic activity of olive polyphenols, our findings clearly show that they penetrate the structurally different cell membranes of both Gram-negative and Gram-positive bacteria. Phenolic and antioxidant compounds are known to cause disruption of cell peptidoglycans or damage the cell membrane, or both (Tranter et al 1993). However, although both these biophenols have an o-diphenol system (responsible for the antibacterial activity of olive polyphenols) on their backbone structure, oleuropein was significantly less toxic to bacterial cells than hydroxytyrosol. One can speculate that the glycosidic group of oleuropein might render the drug unable to penetrate cell membranes or to reach the target site.

Table 1. Minimum inhibitory concentrations of oleuropein, hydroxytyrosol and ampicillin (as reference drug) against ATCC bacterial strains.

Strain	Minimum inhibitory concentration ($\mu g\,mL^{-1}$)		
	Oleuropein	Hydroxytyrosol	Ampicillin
Haemophilus influenzae ATCC 9006	500	0·97	1·91
Moraxella catarrhalis ATCC 8176	> 500	1·92	0·48
Salmonella typhi ATCC 6539	125	3·94	1·93
Vibrio parahaemolyticus ATCC 17802	62·5	0·24	3·90
Staphylococcus aureus ATCC 25923	62·5	7·85	0·48

Table 2. Range of minimum inhibitory concentrations of oleuropein and hydroxytyrosol, and of ampicillin and erythromycin as reference drugs, against clinical bacterial strains.

Strain	n[a]	Range of minimum inhibitory concentrations ($\mu g\,mL^{-1}$)			
		Oleuropein	Hydroxytyrosol	Ampicillin	Erythromycin
Haemophilus influenzae	8	> 500	1·90–15·60	> 500	0·50–15·60
Moraxella catarrhalis	6	> 500	3·80–15·60	0·60–1·95	0·12–0·48
Salmonella spp.	15	125–250	1·90–7·80	0·97–31·25	n.a.
Vibrio parahaemolyticus	1	125	0·97	1·95	n·a.
Vibrio alginolyticus	2	125–125	0·97–1·90	1·95–1·95	n.a.
Vibrio cholerae	1	125	1·90	0·24	n.a.
Staphylococcus aureus[b]	5	62·5–125	3·9–31·25	0·48–15·60	0·24–3·90
Staphylococcus aureus[c]	6	31·25–125	3·9–31·25	> 500	0·97–125

[a]The number of bacterial strains tested. n.a. = not active. [b]Penicillin-susceptible. [c]Penicillin-resistant.

A recent paper reported that oleuropein dose-dependently enhanced the production of nitric oxide (known to have cytotoxic effects against several pathogens) by endotoxin-challenged mouse macrophages (Visioli et al 1998). This effect of oleuropein and, perhaps, of its derivatives might contribute to their potential in-vivo protective effect against bacterial infections.

Finally, safety is a primary consideration for antimicrobial agents to be used for therapy in man. Olive oil has proven its safety through many years of use and consumption by man. Although Aeschbach et al (1994) observed slight pro-oxidant activity for hydroxytyrosol, olive polyphenols (including hydroxytyrosol) are generally claimed to be free from toxicity against mammalian cells (Capasso et al 1995); this should be an additional advantage in the possible employment of olive polyphenols as antimicrobial agents in man.

These data indicate that *Olea europaea* might be considered a potential source of promising antimicrobial agents for treatment of intestinal or respiratory tract infections in man and one might speculate that dietary intake of polyphenols contained in olives and olive oil (major components in the Mediterranean diet) reduces the risk of bacterial infection, particularly of the intestinal tract. Because information on the pharmacokinetics of oleuropein and hydroxytyrosol (particularly gastrointestinal stability, absorption and metabolism) is sparse, we are unable to suggest whether these compounds retain their antimicrobial properties in-vivo. Further studies are needed to clarify this point.

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