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Antimycoplasmal Activity of Hydroxytyrosol

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The aim of this study was to investigate the in vitro antimycoplasmal activity of hydroxytyrosol. Twenty strains of *Mycoplasma hominis*, three strains of *Mycoplasma fermentans*, and one strain of *Mycoplasma pneumoniae* were used. For *M. pneumoniae*, *M. hominis*, and *M. fermentans*, the MICs were 0.5, 0.03 (for 90% of the strains tested), and 0.25 μg/ml, respectively.

Typical components of the Mediterranean diet, such as olive oil and red wine, contain high concentrations of complex phenols, which are endowed with strong antioxidant activity. Also in the Mediterranean basin, olive oil, along with fruits, vegetables, and fish, are important constituents of the diet and are considered major factors in preserving a healthy and relatively disease-free population. Epidemiological data show that the Mediterranean diet has significant protective effects against cancer and coronary heart disease.

The increasing resistance to antibiotics represents the main factor justifying the need to find and develop new antimicrobial agents. Thus, many studies are focused on the antimicrobial properties of plant-derived active principles (such as spices and essential oils) that have been used for a long time in traditional medicine to overcome infections (7).

The fruit and leaves of the olive (*Olea europaea* L.) contain a series of compounds that represent multichemical mechanisms of defense against microbe and insect attacks. There is clear evidence concerning the antimicrobial activity of compounds contained in olives, olive oil, and leaves and vegetation waters. In particular, the possible use of *O. europaea* biocompounds against human pathogenic bacteria has been suggested (4, 5, 9, 17).

The major phenolic compounds identified and quantified in olive oil belong to three different classes: simple phenols (hydroxytyrosol and tyrosol), secoiridoids (oleuropein, the aglycone of ligstroside, and their respective decarboxylated dialdehyde derivatives), and lignans [(+)-1-acetoxypinoresinol and pinoresinol]. Recently, oleuropein (the bitter molecule present in large amounts in olives) and hydroxytyrosol (which derives from oleuropein by acidic or enzymatic hydrolysis and is responsible for the high stability of olive oil) (4, 21) have been demonstrated to inhibit or delay the rate of growth of a range of bacteria and fungi, and so they might be efficiently used as alternative food additives or in integrated pest management programs (3, 6, 11, 14–16). Moreover, we have demonstrated the good antimicrobial activity of oleuropein and hydroxytyrosol against American Type Culture Collection and clinically isolated gram-positive and gram-negative bacterial strains (*Salmonella* sp., *Vibrio* sp., and *Staphylococcus aureus*) (4). Since oleuropein has also been shown to inhibit mycoplasmas (8), we carried out the study described here with the aim of determining the in vitro susceptibilities of *Mycoplasma pneumoniae*, *Mycoplasma hominis*, and *Mycoplasma fermentans* to hydroxytyrosol.

Hydroxytyrosol was synthesized as previously described by Bisignano et al. (4). A working solution was prepared in 0.1 M phosphate buffer, and the final pH was the same as that of the assay medium.

Nineteen low-passage clinically isolated strains of *M. hominis* (vagina, urethra, and cervix isolates), one reference strain (PG 21) of *M. hominis*, one reference strain (FH) of *M. pneumoniae*, and one low-passage strain (vagina isolate) and two reference strains (PG18 and K7) of *M. fermentans* were investigated.

M. hominis was grown in 10-B broth (pH 6.0) (13) containing 1% arginine instead of urea. *M. pneumoniae* and *M. fermentans* were grown in SP-4 (18). All of the strains were maintained frozen (−80°C) until assayed against the drug.

The MIC was determined by a broth microdilution assay that was essentially equivalent to a metabolism inhibition test as previously described (12). Mycoplasma broth (0.025 ml of the specific broth) was inoculated into microtiter wells. The stock solution (0.025 ml) of each drug was added to the first well, and serial twofold dilutions (0.025 ml) were made with a multichannel pipette beginning with the second well; the final 0.025 ml was discarded, and a total of 11 drug concentrations were prepared. A suspension of organisms (0.175 ml) was added to each well containing the drugs. Plates were sealed with transparent acetate and incubated at 37°C under atmospheric conditions.

Each strain was cloned three times before the test and then used for MIC determinations. The number of organisms added was verified by making serial 10-fold dilutions in order to ensure an adequate (10^3 CFU/ml) but not an excessive ($>10^5$ CFU/ml) amount of inoculum for the test system. All micro-

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TABLE 1. Susceptibilities of *M. hominis*, *M. fermentans*, and *M. pneumoniae* to hydroxytyrosol

Microorganism (no. of strains)	MIC (μg/ml)		
	50% of strains	90% of strains	Range
Mycoplasma hominis (20)	0.03	0.03	0.03–0.12
Mycoplasma fermentans (3)			0.25
Mycoplasma pneumoniae (2)			0.5

plates were examined after 18 h of incubation and then once daily until growth in the organism control tube occurred. The MIC was defined as the lowest concentration of antibiotic that inhibited a color change in the broth caused by a given *Mycoplasma* strain at the time when the color of the control tube changed, that is, when the pH of the medium decreased from 7.5 to 7.0 (*M. pneumoniae* and *M. fermentans*) or increased from 6.0 to 6.5 (*M. hominis*). The required incubation times were 24 to 48 h for *M. hominis* and *M. fermentans* and 3 to 5 days for *M. pneumoniae*. Further incubations were not carried out. Each mycoplasma strain was tested six times against each antimicrobial agent. The strains were tested six additional times, on different days, with the drug to ensure the reproducibility of the results.

A positive control (growth) consisting of organisms in broth, a negative control (sterility) consisting of uninoculated broth, and a drug control consisting of broth with the highest drug concentrations were included for each mycoplasmal strain tested. *S. aureus* ATCC 29213 was included as a control; the MIC of the drug obtained in Muller-Hinton broth was compared to that obtained in mycoplasma medium. This reference strain was inoculated into microtiter plates containing 10-B broth, SP-4 broth, Muller-Hinton broth II (Becton Dickinson & Co., Sparks, Md.), and the appropriate dilutions of the drug tested for MIC determination. These control procedures were repeated each time that an assay was performed.

The results of the susceptibility tests are given in Table 1. Hydroxytyrosol inhibited mycoplasmas at concentrations of 0.03 to 0.5 μg/ml. The MICs for *M. pneumoniae*, *M. hominis*, and *M. fermentans*, were 0.5, 0.03, and 0.25 μg/ml, respectively. As regards reproducibility, no variations among the MIC results were observed in the separate assays. Therefore, 10-B and SP-4 both yielded a hydroxytyrosol MIC of 4.0 μg/ml for *S. aureus*, which is equal to that obtained for this strain in Mueller-Hinton broth.

The antimicrobial activity of naturally occurring compounds has been reviewed recently in the literature, and there is considerable interest in the use of these compounds as new antimicrobial agents in humans (7). Of course, safety and bioavailability are primary considerations for antimicrobial agents to be used for therapy in humans. Several papers report the good bioavailability of hydroxytyrosol following ingestion of olive oil or of the pure active principle in humans (10, 19, 20). Although plasma concentrations of hydroxytyrosol and/or its metabolites in olive oil-consuming people have not been described yet, when hydroxytyrosol levels were measured in plasma samples from volunteers after a single ingestion of 25 ml of virgin olive oil (a dose close to the daily oil intake in Mediterranean countries), a maximum concentration of 25 μg/liter was reached (10). Moreover, the disposition of hydroxytyrosol in humans has been reported to be dose dependent (19, 20).

Furthermore, olive oil has proven its safety through many years of use and consumption by humans. Aeschbach et al. (1) and Aruoma et al. (2) observed a slight in vitro pro-oxidant activity of hydroxytyrosol on DNA, but only at nonphysiological, millimolar concentrations. Moreover, olive polyphenols (including hydroxytyrosol) are generally claimed to be free of toxicity against mammalian cells (7).

The present findings indicate that hydroxytyrosol might be considered as a promising antimicrobial agent for treating human infections; its safety (7) and good bioavailability (10, 19, 20) represent additional advantages for its possible therapeutic use.

Therefore, one might speculate that dietary intake of the polyphenols contained in olives and olive oil could reduce the risk of mycoplasmal infection. We believe that mycoplasmas could be an interesting tool to study and better characterize the interaction of hydroxytyrosol with bacteriological membrane. Further studies are needed to clarify these two points.

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