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Thymol: antibacterial, antifungal and antioxidant activities

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SUMMARY: Timolo: antibatterico, antifungo e antiossidante attivo.

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Adhesion is the essential prerequisite for the pathogenesis of bacterial and fungal infections because the micro-organisms must adhere to host mucosal cells in order to multiply and create colonies before specific symptoms allow the disease to be detected. This is particularly true in the case of female urogenital infections such as urinary tract infections, bacterial vaginosis and vaginitis. It has been reported that tymol, a component of thyme essential oil, has interesting antimicrobial effects on various micro-organisms and can interact with adhesiveness, a major determinant of bacterial and fungal virulence. It significantly reduces Escherichia coli adhesion to human vaginal epithelial cells (VECs) at concentrations ranging from 1/2 MIC to 1/32 MIC, and that of Staphylococcus aureus *at concentrations ranging from 1/2 MIC to 1/16 MIC. Candida albicans is particularly capable of adhering to VECs endothelial cells, soluble factors and extracellular matrix, but its incubation with thymol reduces its adhesion to VECs with a significant linear relationship from 1/2 MIC to 1/8 MIC.*

Phenolic compounds such as thymol also play an important role as a result of their anti-oxidant activity. During respiratory bursts and the killing of micro-organisms, neutrophils generate reactive oxygen species whose release can induce oxidative stress and tissue injury. The use of luminol amplified chemiluminescence has shown that thymol incubated with human neutrophils significantly reduces oxidative bursts down to 2.73 μg/ml, a very low concentration.

Together with the other findings concerning bacterial and fungal adhesion, the antioxidant activity of thymol is useful for the strategy of protecting against vaginosis or vaginitis using new compounds other than antibiotics or antimycotics, because the presence in only one molecule of antibacterial, antifungal and anti-oxidant activities may have sinergistic effects.

RIASSUNTO: Thymol: antibacterial, antifungal and antioxidant activities.

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L'adesione è un prerequisito essenziale per la patogenesi delle infezioni batteriche e fungine perchè i micro-organismi devono aderire alle cellule della mucosa ospite per potersi moltiplicare e creare colonie prima che compaiano i sintomi specifici che indichino la presenza della malattia. Questo è particolarmente vero nel caso di infezioni urogenitali femminili quali infezioni del tratto urinario, vaginosi batterica e vaginiti. È stato riportato che il timolo, un componente dell' olio essenziale di timo, possiede interessanti effetti antimicrobici su vari micro-organismi e può interagire con l'adesività, un importante determinante di virulenza batterica e fungina. Il timolo riduce significativamente l'adesione di Escherichia coli alle cellule epiteliali vaginali umane (VECs) a concentrazioni tra 1/2 MIC e 1/32 MIC, e quella di Staphylococcus aureus a concentrazioni tra 1/2 MIC e 1/16 MIC. La Candida albicans è particolarmente abile nell'aderire a cellule VECs, a fattori solubili ed alla matrice extracellulare, ma la sua incubazione con il timolo riduce la sua adesione a VECs con una correlazione lineare significativa da 1/2 MIC a 1/8 MIC.

Composti fenolici quali il timolo giocano un ruolo importante anche sulla base della loro attività anti-ossidante. Durante il "respiratory burst" *e il* "killing" *di micro-organismi, i neutrofili generano varie specie reattive dell'ossigeno la cui liberazione può indurre stress ossidativo e lesioni tissutali. L'impiego della chemiluminescenza amplificata con luminol ha mostrato che il timolo incubato con neutrofili umani riduce significativamente il* burst *ossidativo già a 2.73 μg/ml, una concentrazione molto bassa.*

Unitamente alle altre evidenze sull'adesione di batteri e funghi, l'attività antiossidante del timolo appare utile nella strategia di protezione dalle vaginosi o vaginiti usando nuovi composti diversi dagli antibiotici o antimicotici, in quanto la presenza in una sola molecola come quella del timolo di attività antibatterica, antifungina e anti-ossidante può avere effetti sinergici.

KEY WORDS: Thymol - Bacteria - Fungi - Anti-adhesive effect - Anti-oxidant effect.
Timolo - Batteri - Funghi - Effetto anti-adesivo - Effetto anti-ossidante.

Antibiotics and antimycotics generally work well against bacterial or fungal infections, but the progressive increase in bacterial and fungal resistance has stimulated the search for new therapeutic approaches. Many researchers have concentrated on investigating the essential oils of plants because, first empirically but now scientifically, it has been found that they interfere with micro-organisms in various ways (1-5).

Over the last 20 years, more than 600 studies have investigated and confirmed the antimicrobial effects of essential oils on various different bacteria and fungi using modern pharmacological techniques (1). The essential oils of plants are very complex mixtures of various components (therpenes, aldehydes,

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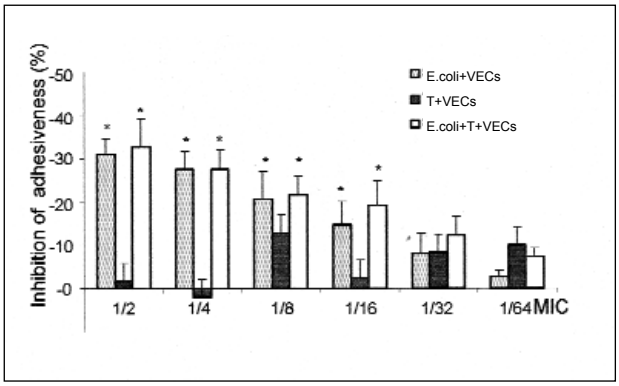

Fig. 1 - Comparative plot of the percentages of inhibition of *E. coli* adhesion to human vaginal cells at different sub-MICs of thymol.

alcohols, acyclic esters, etc.) with different chemotypes, and a number of investigations have found that those of thyme are highly antimicrobial (6-10) mainly because of their high phenol content (7, 11, 12).

Thymol, one of the major components of thyme oil with a phenolic structure, has useful antimicrobial activity against various micro-organisms (1, 13, 14), but its ability to interact with such an important determinant of virulence as bacterial and fungal adhesiveness has not yet been investigated. The adhesion of bacteria and fungi to human mucosae is important because it offers many advantages for the survival of the enormous number of micro-organisms present in the environment. It has now been clinically confirmed that, if there is no adhesion, bacteria and fungi cannot grow and form colonies and, if there is no colonisation, there is no infection and no disease (15,16).

As the mucosal surfaces of the genitourinary, respiratory and gastrointestinal tracts are the most common portals of entry for micro-organisms, and the sites where they make contact with and adhere to the human body, the pathogenic mechanisms of bacterial and fungal adhesion have been widely studied.

It is now generally accepted that bacterial and fungal adhesion of mucosal epithelial cells is a prerequisite and obligatory first step in the pathogenesis of many bacterial and fungal infections. This is particularly true in the case of female urogenital infections, such as urinary tract infections, bacterial vaginosis and vaginitis (17).

Under normal conditions, the microbial flora in the vagina of healthy women is a natural ecosystem in which pathogenic and non-pathogenic micro-organisms are balanced. Urinary tract infections, bacterial vaginosis and yeast vaginitis afflict an estimated 1,000,000,000 people in the world every year (17) as a result of the disturbed ecological balance of local microflora and micro-organism colonisation of the perineum, the vagina and periurethra extending to the bladder. One common approach to counteracting this situation is the use of antibiotics or antimycotics which can still inhibit bacterial or fungal adhesion to human mucosal cells (15,16) even at subinhibitory concentrations.

The effect of thymol on bacterial and fungal adhesion to human vaginal epithelial cells (VECs) has been investigated using the same method.

In a first study of Gram-negative and Gram-positive bacteria adhesion, thymol MIC values for three *Escherichia coli* strains ranged from 125 to 245 μg/ml (means ±SD = 181±60 μg/ml), and the MIC for three strains of *Staphylococcus aureus* was 175 μg/ml. These MICs are in the same order of magnitude as the 100 μg/ml (13-15) to 250 μg/ml (18) found by other authors.

Given the different starting adhesion values of the different strains, the baseline adhesion values were normalised to 100 and the results were expressed in percentages. Figure 1 shows the effects of thymol sub-MICs on the adhesion of *E. coli* to human VECs. The concentration of 1/2 MIC induced the greatest inhibition, with values gradually returning to baseline from 1/2 to 1/64 MIC, and the differences were statistically significant between 1/2 MIC and 1/32 MIC. Fig. 1 also shows the findings obtained when the VECs were incubated with different thymol sub-MICs before being challenged with untreated *E. coli*. In this case, bacterial adhesion was not significantly different from that of the controls, thus indicating that thymol did not interfere with vaginal cell receptors.

In a third series of experiments, in which *E. coli*, vaginal cells and thymol were incubated together there was a significant inhibition of adhesion at thymol concentrations of between 1/2 MIC and 1/32 MIC (Fig. 1) (19). Figures 2A and 2B are scanning electron micrographs showing examples of the adhesion of *E. coli* to human VECs under baseline conditions (without thymol), and when the bacteria and the cells were incubated with 1/2 MIC of thymol (19).

Inhibition of adhesion was also observed in the case of *S. aureus* strains. As expected, the concentrations of 1/2 MIC (Fig. 3) induced the greatest inhibition, but the degree of inhibition remained statistically significant down to 1/16. The effect of thymol on VECs subsequent challenged with untreated *S. aureus* were not different from its effect on controls and, when *S. aureus*, vaginal cells and thymol were incubated together, the inhibition of adhesion was significant from 1/2 MIC to 1/16 MIC of thymol (Fig. 3) (19). Figures 4A and 4B are scanning electron micrographs showing examples of the adhesion of *S. aureus* to human VECs under baseline conditions (without

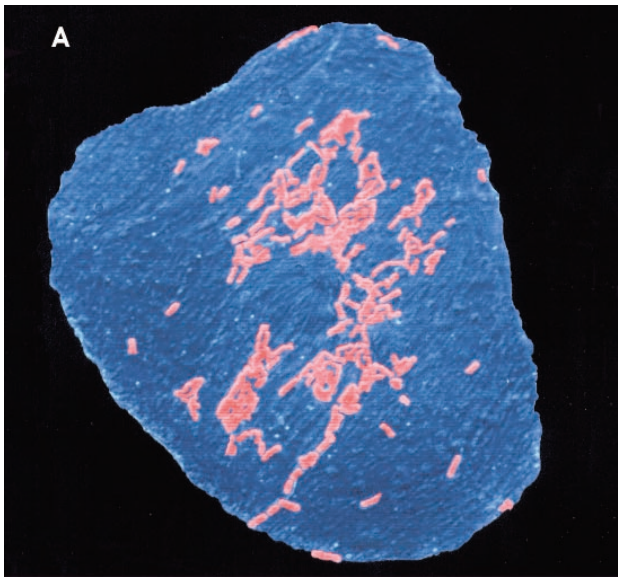

Fig. 2 - A) Scanning electron micrograph (SEM) of human vaginal epithelial cells showing the large number of adherent *E. coli* in the absence of thymol.

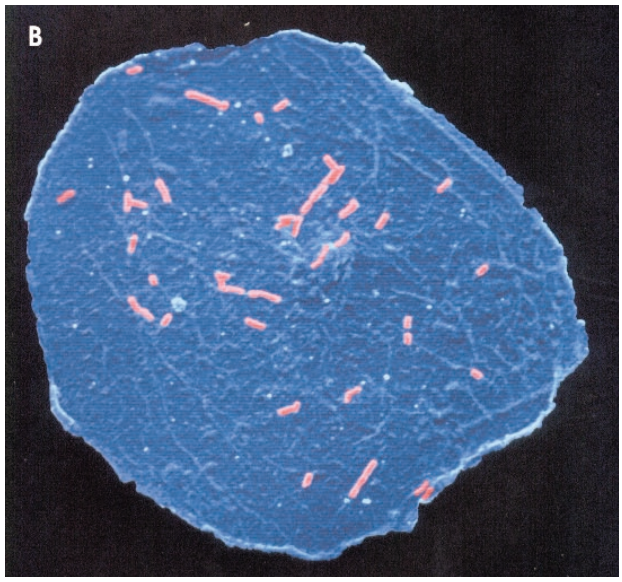

Fig. 2 - B) Example of the large reduction in the number of adherent *E. coli* after incubation with 1/2 MIC of thymol.

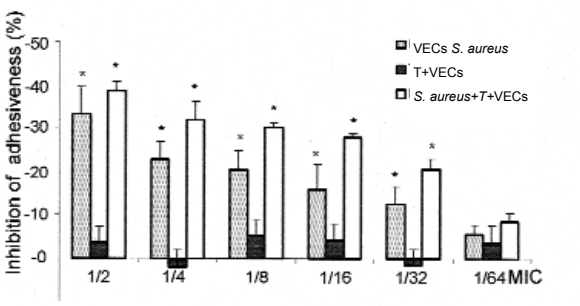

Fig. 3 - Comparative plot of the percentages of inhibition of *S. aureus* adhesion to human vaginal cells at different sub-MICs of thymol

thymol), and when the bacteria and the cells were incubated with 1/2 MIC of thymol (19).

The same methodology was used to assess whether thymol interferes with the adhesion of four strains of *Candida albicans* to human vaginal epithelial cells. The thymol MIC values were 125 μg/ml for three strains, and 150 μg/ml for one (mean value 131.25 ± 12.5 μg/ml). When thymol sub-MICs were incubated with the VECs and then challenged with untreated *Candida albicans*, the adhesion of all the strains was not significantly different from that of the controls, thus indicating that thymol did not interfere with surface vaginal cell receptors (Fig. 5). In a second set of experiments, the reduction in adhesiveness was maximal at 1/2 MIC and progressively returned to mean control values. There was a significant reduction in the mean number of Candida cells adhering to VECs at up to 1/8 MIC (Fig. 5). In a final set of experiments, in which Candida strains, VECs and thymol were incubated together, 1/2 MIC concentrations induced the greatest inhibition, but the degree of inhibition remained statistically significant down to 1/8 MIC (Fig. 5) (20).

Figures 6A and 6B are scanning electron micrographs showing examples of the adhesion of *Candida* spp to human VECs under baseline conditions (without thymol), and when the fungi and cells were incubated with 1/2 MIC of thymol (20).

Products containing essential oil of plants and designed for the treatment of vaginal conditions are now available in the UK, USA, Australia and Europe (21, 22, 23).

Monoterpenes are substances derived from isoprene hydrocarbure (2-methyl-1,3-butadiene) and originated by attaching two or more isoprene molecules (24). They can be found as components of many essential oils and include thymol. Interest in isolated monoterpenes has been growing because of their pharmacological usefulness, and this is particularly true of thymol, which is widely known as antimicrobial and antifungal agent (24).

From a biophysical point of view, thymol has amphipatic and/or hydrophobic behaviour, which suggests its ability to affect the structure of cell membranes and surface electrostatics, and thus generate asymmetries in membrane tensions (24). This assumption has been confirmed by the observation that terpenes enter between the fatty acyl chains making up the membrane lipid bilayer (25,26), disrupt lipid packing, and cause changes to membrane properties and functions (26,27,2) by increasing membrane fluidity and altering permeability (28, 29). Terpenes also inhibit respiration in Candida, thus suggesting adverse effects on mitochondria (28).

As bacterial and fungal cells must keep their struc-

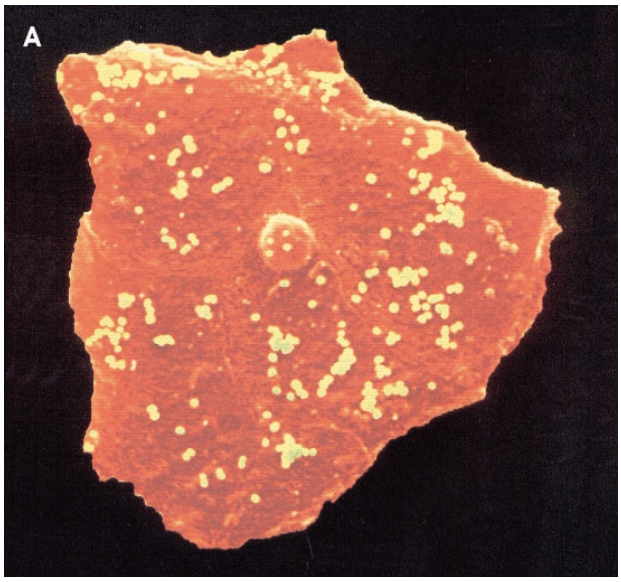

Fig. 4 - A) Scanning electron micrograph (SEM) of human vaginal epithelial cells showing the large number of adherent *S.aureus* in the absence of thymol.

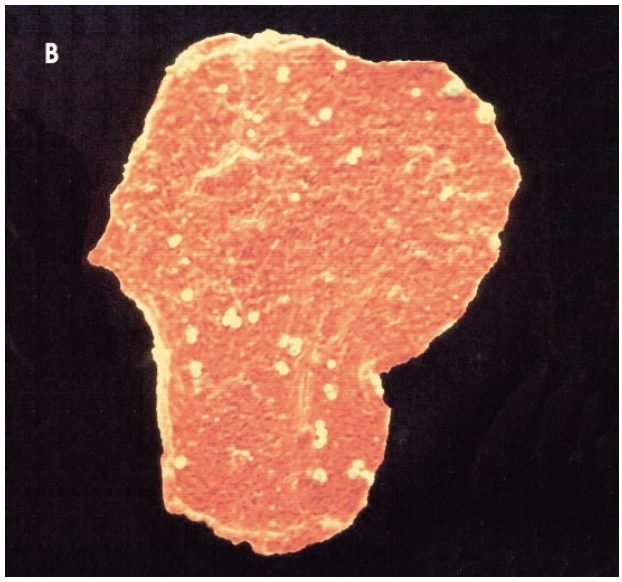

Fig. 4 - B) Example of the large reduction in the number of adherent *S.aureus* after incubation with 1/2 MIC of thymol.

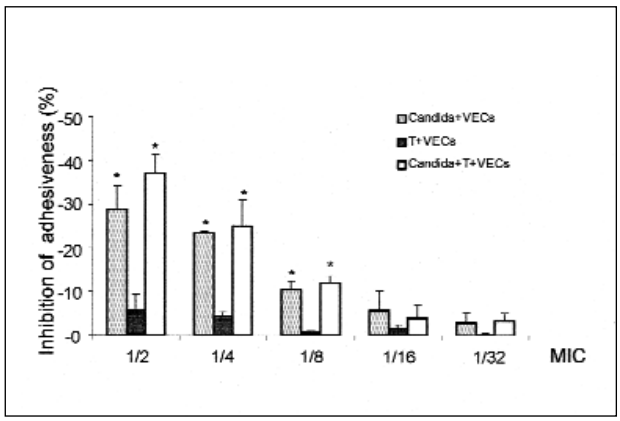

Fig. 5 - Comparative plot of the percentages of inhibition of *C. albicans* adhesion to human vaginal cells at different sub-MICs of thymol.

ture intact and have a proper surface fibrillar layer in order to adhere (30, 31, 32), any compound that alters their structure (thymol in this case) is also capable of modifying their adhesiveness.

Our findings indicate that subinhibitory concentrations of thymol can interfere with the mechanisms of bacterial and fungal adhesion to human VECs (19, 20).

Phenolic compounds such as thymol also have anti-oxidant activity. This is important because bacterial or fungal infection generally lead to the recruitment of poymorphonuclear neutrophils (PMNs), the major cell mediators of inflammation. During respiratory bursts and the killing of micro-organisms, PMNs generate reactive oxygen species (ROS) whose release can induce oxidative stress and tissue injury.

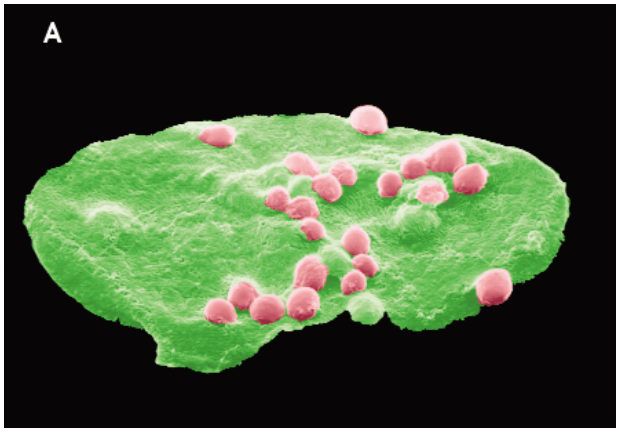

Fig. 6 - A) Scanning electron micrograph of human vaginal epithelial cell showing the large number of adherent *C. albicans* in the absence of thymol.

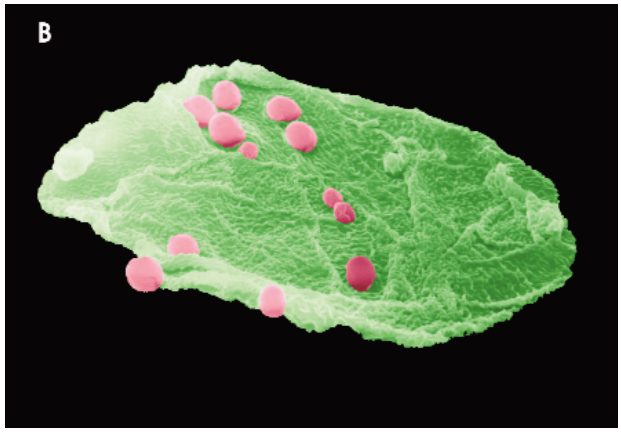

Fig. 6 - B) Example of the large reduction in the number of adherent C. *albicans* cells after incubation with *1/2* MIC of thymol.

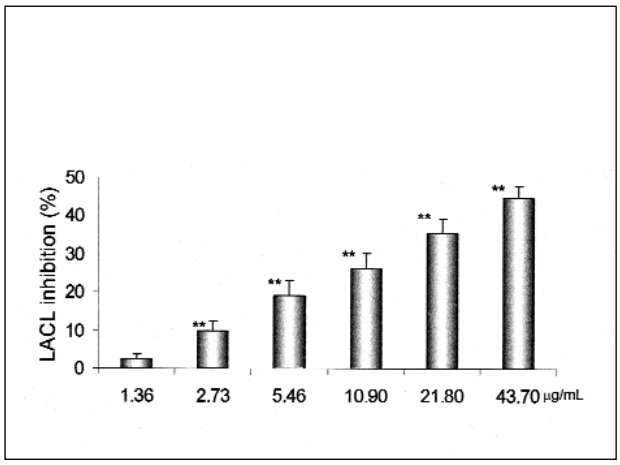

Fig. 7 - Effect of various concentrations of thymol on LACL of PMN respiratory burst by fMLP stimulation.

The therapeutic strategies for decreasing oxidant damage to cells during inflammation and PMN respiratory bursts therefore include removing specific cause, increasing intracellular anti-oxidant systems, and administering anti-oxidant agents. This last approach is interesting because it can be easily adopted, and so we investigated whether thymol can interfere with the production of $O_2^{-\bullet}$ and derived reactive oxygen species during neutrophil respiratory bursts using luminol amplified chemiluminescence and the soluble N-formyl-methionyl-leucyl-phenylalanine stimulant.

Thymol incubated with human neutrophils significantly reduced oxidative bursts down to 2.73 µg/ml, a very low concentration (Fig. 7) (33).

The anti-oxidant activity of thymol has been attributed to its phenolic structure (34, 35, 36, 37), and the anti-oxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralising free radicals or decomposing peroxides (37). Carvacrol, another molecule with phenolic structure, is found in various concentrations in the essential oils of the Thymus species, and also possess anti-oxidant activity (34, 35, 36, 37). Together with the other findings concerning bacterial and fungal adhesion, the anti-oxidant activity of thymol is useful for the strategy of protecting against vaginosis or vaginitis using new compounds other than antibiotics or antimycotics because the presence in only one molecule of antibacterial, antifungal and anti-oxidant activities may have synergistic effects.

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