Synergistic effect of the combination triclosan with 2–phenylphenol against Pseudomonas aeruginosa and fungi

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ABSTRACT

Objective: Triclosan is active against Gram-positive and Gram-negative bacteria but lesser against *Pseudomonas aeruginosa (P.aeruginosa).* 2-phenylphenol is considered as an effective agent against fungi but its antifungal action is more important than its antibacterial activity. The aim of this study is to evaluate the bactericidal and fungicidal activities of triclosan and 2-phenylphenol alone, and in combination against standard strains of bacteria and fungi.

Methods: The study was carried out in the Research Laboratory of Pharmaceutical Microbiology, Faculty of Pharmacy, Jordan University of Science and Technology, Irbid, Jordan, during the year 2004. The standard French method of in-vitro dilution quantitative suspension test, according to the Association Françoise de Normalization (AFNOR) guideline, has been used to determine the antibacterial and antifungal activity of each agent alone as well as the combination of the agents.

Results: The antibacterial activity of the combination triclosan-2-phenylphenol was significantly enhanced over that of each agent used alone against *P.aeruginosa*. Synergistic effect was observed against all tested strains of fungi.

Conclusion: This synergistic effect that was exhibited by triclosan-2-phenylphenol combination can be considered to enhance the antibacterial activity against *P.aeruginosa* and the antifungal activity of the 2 agents, to reduce the in-use concentration of each agent used alone, that may minimize any possible side effect of the 2 agents, and to avoid the occurrence of bacterial resistance to one of the 2 agents.

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Triclosan, 2,4,4'-trichloro-2'-hydroxydiphenyl ether, is a broad-spectrum antimicrobial agent, with low activity against *Pseudomonas aeruginosa (P. aeruginosa)*. Its efficacy against gram-negative bacteria and yeast can be enhanced by formulation.[1] Triclosan is used in many contemporary consumer and professional health care products. These include medicated soaps, surgical scrubs, deodorant products, hand lotion and creams, toothpastes, mouthwashes, and other dermatological formulations.[2-5] The concentration of triclosan used in most preparations ranges from 0.5-2%.[6-8] Many recent studies, showed triclosan acts on a defined bacterial target in the bacterial fatty acid biosynthetic pathway, NADH-dependent enoyl-[acyl carrier protein] reductase (FabI).[9-12] The fatty acid biosynthetic (Fab) pathway is an excellent target for antibacterial agents. It plays a pivotal role in providing metabolic precursors for several important cellular functions, including cell wall biogenesis (phospholipids, lipopolysaccharides and lipoproteins) and the synthesis of acylated

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homoserine lactones required for virulence factor gene expression.[13,14] The antimicrobial action of 2-phenylphenol, such as the most phenol derivatives, has a broad-spectrum, is widely used, and has become one of the most important phenolic biocides for application in hospital-type disinfectants, cosmetics, and in industrial preservation. However, its antifungal action is more important than its antibacterial action.[15] 2-phenylphenol nonspecifically denatures microbial cell wall component and inhibits various enzyme systems, including NADH-oxidase.[16]

In this study, the antibacterial and antifungal activities of the combination of triclosan and 2-phenylphenol were evaluated in accordance with the French Standards (NF) of the Association Française de Normalisation (AFNOR) guideline.[17]

Methods. Antibacterial activity of the 2 compounds and combination were tested against: *Staphylococcus aureus* (*S. aureus)* CIP 53.154, *Enterococcus hirae* CIP 5.855, *Pseudomonas aeruginosa* CIP A22 and *Escherichia coli* (*E. coli)* CIP 54.127. Antifungal activity of the 2 compounds and combination were tested against: *Candida albicans* (*C.albicans)* IP 1180.79, *Aspergillus versicolor* (*A. versicolor)* IP 1187.79, *Penicillium verrucosum var. cyclopium* (*P. verrucosum var. cyclopium)* IP 1231.80, and *Absidia corymbifera (A. corymbifera)* IP 1129.75. All were obtained from the culture collection of the Pasteur Institute, Paris, France. Stock solutions were prepared from pure substances of both chemical compounds, triclosan was obtained from Ciba-Geigy, Greensboro, NC, USA and 2-phenylphenol was obtained from Aldrich Chemical Company, Inc, Milwaukee, USA. Both agents were solubilized in 95% ethanol, at double concentrations, and then diluted to give a final concentration of 1% in the reaction mixture for each agent tested alone and 0.5% of each agent in the combination. All solutions were used within 2 hours of preparation.

The bactericidal and fungicidal activities were assessed by means of AFNOR guidelines. Preliminary test was carried out to validate the method and prove the efficacy of the neutralizing solution (3% polysorbate 80 v/v, 0.4% w/v sodium lauryl sulfate, and 0.3% lecithin w/v). The combination of triclosan-2-phenylphenol was tested at a ratio of 1:1 (one part triclosan to one part 2-phenylphenol). For testing chemical disinfectants against vegetative bacteria, the quantitative suspension test involving dilution-neutralization was used (NF T 72-150). Homogeneous suspensions of $3x10^8$ cfu/mL of each test strain were prepared. The suspension (1mL) was pipetted into a tube containing 4 mL distilled water and after 5 minutes at 23°C; 5 mL disinfectant prepared at double concentration was added. After 5 minutes contact at 23°C, 1 mL of the tested mixture was pipetted into a tube containing 9 mL of neutralizer. After 10 minutes neutralization at 23°C, 2 samples of 1mL of the mixture were transferred into separate Petri dishes and 15mL of the melted medium was added (2.5 g/L yeast extract, 1 g/L glucose, 5 g/L tryptic peptone of casein and 15 g/L agar).

For testing chemical disinfectants against fungi, the quantitative suspension test involving dilution-neutralization was used (NF T 72-200). The inoculum for fungicidal activity was adjusted to a concentration of $3x10^8$ cfu/mL to ascertain the synergistic effect of the combination. The procedure was as described for bactericidal activity except for a contact time of 15 min at 23°C. The recovery medium was 5 g/L of yeast extract, 20 g/L of glucose and 15 g/L of agar.

Results. Preliminary tests were directed towards a search for an effective neutralizer, which would protect the microorganisms exposed to the disinfectant. The neutralizer had to be harmless to the bacteria. Once the preliminary test conditions were met, and when neutralizer was effective in the inhibition of antimicrobial activity of 1% of the 2 compounds alone and 0.5% triclosan plus 0.5% 2-phenylphenol of the combination in each test carried out for all microorganisms tested. The actual tests consisted of placing the microbial suspension in contact with the disinfectant and determining the number of surviving cfu/mL after contact with the disinfectant, 5 minutes for bacteria and 15 minutes for fungi, and then to be compared with the number of cfu/mL in an inoculum dilution, as described in AFNOR guidelines.

Table 1 shows the results of 1% triclosan+2-phenylphenol that had a basic bactericidal activity after 5 min exposure against *S. aureus*, *E. herae*, *P. aeruginosa*, and *E. coli* more than 5-log (99.9%) reduction (spectrum 5 bactericidal activity), when tested using NF T 72-150. **Table 1** also shows that a contact time of 15 min was achieved at <4-log reduction against *C. albicans* and against mould spores. The 1% concentration is therefore considered fungicidal according to guideline NF T 72-200.

The results shown in **Table 2** indicate that triclosan had a bactericidal activity after 5 min exposure against *S. aureus* (≤1.5 µg/mL), *E. hirae* (7 µg/mL), *P. aeruginosa* (500 µg/mL), and *E. coli* (3 µg/mL). Moreover, the fungicidal activity of triclosan was after 15 min exposure 30 µg/mL against *C. albicans*, *A. versicolor, A. corymbifera,* and 60 µg/mL against *P. verrucosum var. cyclopium*.

Table 2 shows also the bactericidal activity of 2-pheylphenol after 5 min exposure against *S. aureus*, *E. coli* (30 µg/mL), *E. hirae* (60 µg/mL), and *P. aeruginosa* (250 µg/mL). **Table 2** shows the

Table 1 - Basic bactericidal and fungicidal with 1% triclosan+2-phenylphenol in the quantitative suspension test.

Micro-organism	Contact time (minute)	N	N*	n†	n	-Log reduction
Staphylococcus aureus	5	210	170	181	0	>5
Enterococcus hirae	5	240	227	204	0	>5
Pseudomonas aeruginosa	5	230	273	289	0	>5
Escherichia coli	5	240	229	232	0	>5
Candida albicans	15	240	251	244	0	>4
Aspergillus versicolor	15	220	215	210	0	>4
Penicillium verrucosum	15	230	218	212	0	>4
Absidia corymbifera	15	250	254	261	0	>4

N - number of cfu/ml in an inoculum dilution; N* - number of cfu/mL survivors after 10 min contact time with the neutralizer;
n† -number of cfu/ml survivors after being in contact with the neutralized disinfectant (5 min for bacteria, 15 min for fungi);
n - number of cfu/ml survivors after contact with the disinfectant and its subsequent neutralization.

Table 2 - Antimicrobial activities of triclosan, 2-phenylphenol, and their combination.

Micro-organism	N			Concentrations of triclosan, 2-phenylphenol, and their combination (% w/v)																							
	10			n																							
	10-30			0.00015			0.0003			0.0007			0.0015			0.003			0.006			0.0125			0.025		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Staphylococcus aureus	20	22	21	2	+	1	0	+	0	0	+	0	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterococcus hirae	23	24	24	+	+	18	6	+	0	0	+	0	0	+	0	0	8	0	0	0	0	0	0	0	0	0	0
Pseudomonas aeruginosa	22	21	23	+	+	+	+	+	+	+	+	7	+	+	0	+	+	0	+	+	0	+	+	0	500 µg/ml	0	0
Escherichia coli	24	20	24	19	+	3	0	+	0	0	+	0	0	14	0	0	0	0	0	0	0	0	0	0	0	0	0
Candida albicans	23	19	24	+	+	2	+	+	0	+	0	0	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aspergillus versicolor	21	22	22	+	+	1	+	+	0	+	+	0	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Penicillium verrucosum	23	21	23	+	+	11	+	+	0	+	+	0	+	+	0	9	0	0	0	0	0	0	0	0	0	0	0
Absidia corymbifera	20	23	25	+	+	1	+	+	0	+	+	0	13	0	0	0	0	0	0	0	0	0	0	0	0	0	0

+ = More than 300 colonies, 0 = absence of colonies, A = antimicrobial activities of triclosan;
B = antimicrobial activities of 2-phenylphenol, C = antimicrobial activities of the combination.

fungicidal activity of 2-phenylphenol after 15 min exposure against *C. albicans* (7 µg/mL), *A. versicolor*, *A. corymbifera* (15 µg/mL), and *P. verrucosum var. cyclopium* (30 µg/mL).

Table 2 indicates as well the bactericidal activities of the combination (triclosan+2-phenyphenol). Considerable synergistic effect was observed at 15 µg/mL against *P. aeruginosa*, 33-fold of triclosan and 17-fold of 2-phenylphenol when the 2 agents used alone against this microorganism, at concentrations tested. Synergistic effect of the combination was observed after 15 min exposure at the concentrations of ≤1.5 µg/mL against *C. albicans*, *A. versicolor*, *A. corymbifera* and 3 µg/mL against *P. verrucosum var. cyclopium* (**Table 2**).

Discussion. In the current study, the 2 agents were selected as both of them are widely used in many preparations. Triclosan is a broad-spectrum against all bacteria except *P. aeruginosa* that requires higher concentration of 100-1000 µg/mL.[18] Due to its favorable safety profile, triclosan was incorporated into a variety of many antimicrobial products alone or in combination with other agents such as zinc citrate, sodium fluoride, ethylenediamine tetra-acetic acid (EDTA), chlorhexidine, pyrophosphate, and povidone-iodine.[8,19-22] On the other hand, 2-phenylphenol is active against fungi rather than bacteria, it may be formulated alone or in combination with alkyl, halogenated phenolic derivatives and other agents.[15] The 2 agents were also selected as they exert different mechanisms of action: 2-phenylphenol denatures microbial cell wall, causes enzymes whose normal role is to synthesize the cell wall to reverse their role in some way and effect its disruption; whereas triclosan causes disorganization of the cytoplasmic membrane, resulting in leakage of a group of characteristic chemical species, such as amino acids, purines, and pyrimidines that are essential for microbial survival.[23] According to those mechanisms and to the synergism of their combination, 2-phenylphenol may render *P. aeruginosa* species more sensitive, to the action of triclosan, possibly by altering the permeability of the outer envelope.

Recently, many authors reported the prevalence of triclosan-resistant bacteria, and the links of cross-resistance between triclosan and antibiotics due to widespread use of numerous antiseptics, disinfectants, and other preparations containing triclosan that may aid in development of microbial resistance, particularly cross-resistance to antibiotics.[4,24-27] Although *P. aeruginosa* is already triclosan resistant, its resistance could be further increased by additional multidrug resistance efflux systems.[4] Chuanchen et al[25] have demonstrated that exposure of triclosan-sensitive mutants of *P. aeruginosa* to triclosan confers an efflux-mediated resistance to many antibiotics such as ciprofloxacin. Other studies demonstrated that *P. aeruginosa* strains resistant to the antibiotics were also resistant to phenols.[28] Synergistic effect exhibited by this combination can be used to avoid the emergence of bacterial resistance to one of the 2 agents and other antibiotics, as well as to increase the bactericidal activity against *P. aeruginosa*. However, the 2 agents are not considered as antipseudomonal when each agent used alone. This combination can be considered also to reduce the in-use concentration of each agent used alone, that may minimize any possible side effect of the 2 agents. In addition, the combination of triclosan and 2-phenylphenol can be used to enhance the fungicidal activities at lower concentrations.

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