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REGULAR ARTICLE

MINIMUM INHIBITORY AND BACTERICIDAL CONCENTRATIONS OF THEAFLAVIN AND SYNGERGISTIC COMBINATIONS WITH EPICATECHIN AND QUERCETIN AGAINST CLINICAL ISOLATES OF *STENOTROPHOMONAS MALTOPHILIA*

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ABSTRACT

Stenotrophomonas maltophilia is an important nosocomial pathogen with intrinsic resistance to multiple antibiotics. Previous investigations have shown flavanols from black tea to possess antibacterial activity. This study describes the determination of minimum inhibitory concentrations and minimum bactericidal concentration for theaflavin independently and in formulations with the polyphenols epicatechin and quercetin against nine clinical isolates of *Stenotrophomonas maltophilia* and the control isolate NCTC 130141 via the microtitre assay. The results demonstrate that theaflavin has strong antibacterial activity and also shows significant synergism with epicatechin and quercetin. The minimum inhibitory concentrations of the isolates range between 200-400 μg/mL for theaflavin and 100-200 μg/mL for both theaflavin:epicatechin and theaflavin:quercetin combinations. The minimum bactericidal concentrations were discovered to be a 2 fold increase on those of the

minimum inhibitory concentrations. The research highlights the potential use of polyphenols for the clinical treatment of highly antibiotic resistant bacteria.

Keywords: resistance, synergy, antibacterial, polyphenol, theaflavin, epicatechin, quercetin

INTRODUCTION

The gram negative bacterium *Stenotrophomonas maltophilia* is an emergent hospital pathogen of significant importance due to its intrinsic and acquired multi-antibiotic resistance (Alonso and Martinez, 1997). As a nosocomial pathogen the bacterium typically affects patients who are immunocompromised (Looney *et al*., 2009) or those subjected to invasive procedures and prosthetic devices that circumvent normal protections. Numerous resistance genes have been identified in *S. maltophilia* enabling the bacterium to disable the activity of several important antibiotics via mechanisms including a multidrug efflux system (Zhang *et al*., 2000) and β-lactamases (Gould *et al.,* 2006). Surveillance studies (Betriu *et al.,* 2001) have indicated the importance of good infection control to avert the cross-transmission of *S. maltophilia* within hospitals.

To combat the growing problem of resistance to antibiotics, much research is being undertaken into the antibacterial properties of other natural compounds. One group that show antibacterial effects against a variety of bacterial species are polyphenols and investigations have already established that polyphenols such as epigallocatechin gallate (EGCG) and theaflavins are active against pathogens including *S. maltophilia* (Gordon and Wareham, 2010), *Shigella* spp. (Vijaya *et al.*, 1995) and *Bacillus cereus* (Friedman *et al.,* 2006).

Not only have polyphenols been shown to be effective independent antibacterial agents, but research has revealed their synergy with other antimicrobials. In a study with methicillin-resistant *Staphylococcus aureus* (MRSA), synergy was observed between EGCG and ampicillin/sulbactam (Hu *et al.,* 2001). Other studies have demonstrated that polyphenols act synergistically with other antioxidants such as ascorbic acid (Hatano *et al.,* 2008) where a protective effect of EGCG is exhibited by the protection of natural structural change.

The antibacterial action of theaflavin against *S. maltophilia* and *Acinetobacter baumannii,* and the synergy between theaflavin and epicatechin, has been previously reported (Betts *et al.,* 2011). However, the minimum inhibitory concentrations (MICs) and minimum

bactericidal concentrations (MBCs) have not been established against *S. maltophilia*. Any synergy of theaflavin with other common polyphenols has also yet to be identified.

The objective of our study was to determine the MIC and MBC of theaflavin and a theaflavin:epicatechin combination against hospital isolates of *S. maltophilia* and to establish whether any significant synergy occurs between theaflavin and another common polyphenol, quercetin.

MATERIAL AND METHODS

Clinical isolates

Nine strains of *S. maltophilia* were isolated on blood and MacConkey agar from sputum samples of respiratory patients at Hull Royal Infirmary, Hull, UK. They were identified using gram staining, antibiotic susceptibility evaluation and biochemical profiling using API 20E testing kits (BioMérieux, France.) A control strain of *S. maltophilia* (NCTC 13014) was purchased from the Health Protection Agency Culture Collections, Porton Down, UK.

Tea polyphenols, antibiotic discs and media

Epicatechin (EC) with purity ≥ 90% and quercetin (Q) with purity ≥ 98% were purchased from Sigma-Aldrich, UK. Theaflavin (TF) with purity ≥ 95% was donated by Unilever, Shanghai, China. Iso-Sensitest broth, agar powders and antibiotic discs were purchased from Oxoid, UK.

Antibiotic susceptibility testing

Individual plates of Iso-Sensitest agar was innoculated with each isolate of *S. maltophilia* using the standardized method by Moosden *et al.,* (1988). To each plate one disc of ampicillin (AMP) 25 μg, gentamycin (CN) 10 μg, imipenem (IPM) 10 μg, cefoxitin (FOX) 10 μg, tetracycline (TE) 10 μg and ciprofloxacin (CIP) 1 μg was added. Zones of inhibition were measured after 24 hours incubation at 30°C and antibiotic susceptibility of each isolate was determined.

Determination of minimum inhibitory and bactericidal concentrations

A microtitre assay based on that of Andrews (2001) was performed to determine the MICs of EC, theaflavin (TF) and a (theaflavin:epicatechin) TF:EC and theaflavin:quercetin (TF:Q) combination against the nine clinical isolates and one control strain (NCTC 13014) of *S. maltophilia*. All antibiotics were diluted in DMSO to prepare stocks solutions of 80 mg/ml of TF and Q, 100 mg/ml of EC, and 80 mg:40 mg/ml for the TF:EC and TF:Q combinations.

A serial dilution of each stock solution was then performed into sterile ISO-sensitive broth. A 40 µL volume of stock solution was added to a sterile tube containing 2 mL of ISO-sensitive broth. From this solution a double dilution was performed 4 times, providing 5 different concentrations of the polyphenol in ISO-sensitive broth and a tube containing ISO-sensitive broth only to use as a control.

Table 1 Final polyphenol concentrations in the 96 well plate.

TF	Q	EC	TF:EC combi (2:1)	TF:Q combi (2:1)
800 µg/mL	800 µg/mL	1000 µg/mL	800:400 µg/mL	800:400 µg/mL
400 µg/mL	400 µg/mL	500 µg/mL	400:200 µg/mL	400:200 µg/mL
200 µg/mL	200 µg/mL	250 µg/mL	200:100 µg/mL	200:100 µg/mL
100 µg/mL	100 µg/mL	125 µg/mL	100:50 µg/mL	100:50 µg/mL
50 µg/mL	50 µg/mL	62.5 µg/mL	50:25 µg/mL	50:25 µg/mL
0 µg/mL	0 µg/mL	0 µg/mL	0 µg/mL	0 µg/mL

TF - theaflavin, Q - quercetin, EC - epicatechin, TF:EC - theaflavin:epicatechin combination and TF:Q - theaflavin:quercetin combination

For each concentration of TF in broth, 75 µL was pipetted into 10 horizontal wells of a 96 well cell culture plate (1 well per concentration per bacterial isolate). An isolate of *S. maltophilia* was pipetted into each well as a 75 µL volume of 0.5 MacFarland suspension. The procedure was repeated for each compound and the combinations, giving final well concentrations as shown in Table 1. Each plate was incubated in a rocking incubator at 30°C for 24 h. Following incubation wells were observed for turbidity. MICs were taken as the lowest concentrations not showing any visible growth.

Minimum bactericidal concentration was also determined by removing 2 µL volume of the medium from each microtitre plate well (containing polyphenol) and spotting onto ISO-

sensitive agar. Agar plates were incubated for a further for 18 h at 30°C. Any growth observed from the spots was designated as an ineffective bactericidal concentration of compound. For each test 6 replicates were undertaken.

RESULTS AND DISCUSSION

The results from the susceptibility testing shows various degrees in antibiotic resistance (Table 2). All isolates showed resistance to ampicilin and imipenem and with the exception of the control, all isolates prestented resistance to cefoxin. High numbers of isolates were also resistant to tetracycline and mixed results were observed with gentamycin and ciprofloxacin. Isolate 7 presented the highest level of resistance, only being susceptible to ciprofloxacin.

Table 2. Antibiotic susceptibility profiles for *S. maltophilia* isolates

Isolate	AMP	CN	IPM	TE	FOX	CIP
1	R	S	R	R	R	R
2	R	S	R	R	R	R
3	R	S	R	R	R	R
4	R	S	R	S	R	S
5	R	S	R	R	R	R
6	R	S	R	S	R	S
7	R	R	R	R	R	S
8	R	S	R	R	R	S
9	R	S	R	R	R	S
Control	R	S	R	R	S	R

AMP - ampicillin (25 μg), CN - gentamycin (10 μg), IPM - imipenem (10 μg), TE - tetracycline (10 μg), FOX - cefoxitin (10 μg) and CIP - ciprofloxacin (1 μg). R - resistant, S - Susceptible. Control - NCTC 13014

The results from the MIC determination confirm that EC has no antibacterial effect against the strains of *S. maltophilia* used in this study. Concentrations of up to 1 mg/mL of EC did not inhibit the growth of *S. maltophilia* and therefore no MIC was achieved. Quercetin also showed no antibacterial effect against any strain of *S. maltophilia*. Concentrations of up to 800 μg/mL of Q did not inhibit *S. maltophilia* growth.

The MIC results for TF confirmed that the polyphenol exhibited antibacterial effects when used in a liquid culture. The MIC of TF ranged between 200 μg/mL and 400 μg/mL as shown in Table 3. For the TF:EC and the TF:Q combinations the MIC value ranged between 100 μg/mL and 200 μg/mL (Table 3). These were significantly lower than when using TF alone. The *S. maltophilia* control strain (NCTC 13014) had MICs of 200 μg/mL with TF, 100 μg/mL for the TF:EC combination and 100 μg/mL for the TF:Q combination.

Table 3 Minimum inhibitory concentrations of polyphenols against clinical isolates and a control of *Stenotrophomonas maltophilia* (μg /mL).

Isolate no.	TF	Q	EC	TF:EC combi (2:1)	TF:Q combi (2:1)
1	200	N/A	N/A	100:50	100:50
2	200	N/A	N/A	100:50	100:50
3	400	N/A	N/A	200:100	200:100
4	200	N/A	N/A	100:50	100:50
5	400	N/A	N/A	200:100	200:100
6	200	N/A	N/A	100:50	100:50
7	400	N/A	N/A	200:100	200:100
8	200	N/A	N/A	100:50	100:50
9	200	N/A	N/A	100:50	100:50
Control	200	N/A	N/A	100:50	100:50

N/A - Not applicable, MIC not reached. Control - NCTC 13014

Results from minimum bactericidal concentrations showed that twice the concentration of compound was required for bactericidal activity compared with that for MIC. However, a reduction in antibacterial activity was seen after stock solutions (in DMSO) were stored for 7 days at 4°C. The concentrations of TF, TF:EC and TF:Q needed to be increased 2 fold from previous testing to achieve their MICs. After 14 days MICs remained the same as those after 1 week in storage.

It is clear from the results that TF and the combinations of TF:EC and TF:Q provide a significant antibacterial effect against the clinical isolates of *S. maltophilia* used here and the NCTC 13014 control strain. The result confirms previous research that reported a significant synergistic relationship exists between TF and EC (Betts *et al.,* 2011) and indicates there is a similar effect when EC is substituted for Q. From the results it is apparent that supplementing

TF with EC (itself having no independent antibacterial effect) reduces the MIC of TF by half. This effect is reproduced when Q is added to TF. We believe this to be the first report in which the synergy between TF and Q is described and the first occasion that MICs for TF and combinations of TF with EC and TF with Q are presented.

Possible mechanisms behind the antibacterial activity have yet to be fully determined. However, previous studies have linked the antimicrobial action of flavanols to inhibition of nucleic acid synthesis (Mori *et al.,* 1987) and membrane damage from perforation (Ikaigai *et al.,* 1993). A possible mechanism for the synergistic results observed in this study, is that a protective effect is afforded by EC and Q, as described by (Hatano *et al.,* 2008), where the supplementary compounds reduce the structural change of TF due to natural oxidation. It is possible that EC and Q are more readily oxidised than TF in this situation, allowing the latter to retain its antibacterial activity.

The increase in MIC after 7 days of storage at 4°C is probably due to polyphenol oxidation. However, after an additional 7 days in storage the MIC did not change, indicating no further oxidation occurred. To minimise this it is recommended that all stock solutions should be stored under a nitrogen atmosphere in a freezer. Future research could investigate longer periods of storage to determine if any further reduction in antibacterial action occurs.

In a clinical setting, by using a combination rather than singular antibacterial treatment potential resistance might be avoided. Another benefit is that the cost of treatment could be reduced by minimizing the amount of the more expensive TF used in polyphenol formulations. The findings indicate that there might be important clinical potential for these polyphenols as antibacterial agents, especially when used in combinations. Work is in progress to establish the mechanisms underlying the demonstrated antibacterial activity and synergy, and whether the bioavailability of the polyphenols could be sufficient for use in potential therapies for *Stenotrophomonas* infections at concentrations with tolerable side-effects.

CONCLUSION

The results in this study show that theaflavin alone or in combination with epicatechin or quercetin shows great antibacterial activity against highly resistant isolates of *S. maltophilia*. This highlights the potential application of natural products in the pharmaceutical and medical industry. With the great abundance and variety of natural compounds such as polyphenols, possibilities of synergistic treatments with other

polyphenols, procyanidins and with clinical antibiotics could increase our current arsenal in the fight against resistance. The significant importance of natural compounds for medicinal uses should not be underestimated. Their research and development could open up a new age of chemotherapeutic treatments.

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