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Antibacterial effects of theaflavin and synergy with epicatechin against clinical isolates of *Acinetobacter baumannii* and *Stenotrophomonas maltophilia*

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ABSTRACT

Stenotrophomonas maltophilia and *Acinetobacter baumannii* are recognised as important nosocomial pathogens; however, due to their intrinsic resistance to multiple antibiotics, treatment options are limited. Polyphenols from black tea have been shown to possess antibacterial action. In this study, the antibacterial effects of various concentrations of theaflavin as well as combinations of theaflavin and epicatechin were determined using the disk diffusion assay. The results showed strong antibacterial activity of theaflavin against eight clinical isolates of *S. maltophilia* and *A. baumannii*. Significant synergy ($P \leq 0.05$) was also observed between theaflavin and epicatechin against all isolates. Although the mechanisms for this activity and synergy are not well understood, the clinical potential is clear and further research is recommended to determine the modes of action.

1. Introduction

Until recently the clinical importance of *Acinetobacter baumannii* has been underestimated, but it is now widely acknowledged as a common bacterium in hospital irrigation and intravenous solutions. It possesses inherent multidrug resistance and the ability to rapidly colonise and infect patients [1–3]. Similarly, *Stenotrophomonas maltophilia* has become a troublesome opportunistic pathogen. This aerobic, Gram-negative bacterium is also intrinsically multidrug-resistant and can cause pneumonia, urinary tract infections and bloodstream infections, particularly in immunocompromised patients [4,5].

New treatment regimens are under investigation owing to the increasing number of resistant micro-organisms and their widening resistance profiles. Some resistant strains of bacteria can be treated effectively with combinations of antibiotics in contrast to the poor results provided by monotherapy [6]. These new approaches include the use of naturally occurring antibacterial compounds, such as polyphenols, in formulations with traditional antibiotics.

The antimicrobial effects of tea polyphenols have been well documented. Most investigations have used the very abundant polyphenol epigallocatechin gallate (EGCG) found in green tea [7–9]. The antifolate activity of EGCG against *S. maltophilia* has also been reported [10]. Black tea polyphenols are also known to have antibacterial effects against *Shigella* spp. [11] and *Bacillus cereus* [12]. However, little research has been published regarding the antibacterial properties of the black tea polyphenol theaflavin against clinical isolates of *S. maltophilia* and *A. baumannii*. An investigation has shown that epicatechin has an antibacterial effect

[13], which according to another study [14] can be attributed to membrane disruption properties.

Tea polyphenols not only exhibit independent antibacterial effects but also show significant antibacterial synergy with more common antibiotics, e.g. tetracycline against staphylococci [15] and oxacillin against methicillin-resistant *Staphylococcus aureus* (MRSA) [16], and also increase the susceptibility of *Mycobacterium* to isoniazid [17]. Black tea mixtures show synergy and antagonistic properties with different clinical antibiotics when used against *Streptococcus pyogenes* [18].

Interestingly, earlier research also revealed that tea polyphenols can invoke antibacterial synergy with other antioxidants such as ascorbic acid [19]. It was proposed that ascorbic acid inhibits the oxidative structural change that would otherwise cause a decline in antibacterial activity. However, there has been little research describing synergy between black and green tea polyphenols. The aim of this research was therefore to assess the antibacterial effect of theaflavin against hospital isolates of *A. baumannii* and *S. maltophilia* and to determine whether any significant synergistic effect would be produced with the addition of epicatechin.

2. Materials and methods

2.1. Clinical isolates

Acinetobacter baumannii and *S. maltophilia* strains were isolated from sputum samples of respiratory patients at the Northern General Hospital (Sheffield, UK) and

Hull Royal Infirmary (Hull, UK), respectively. Bacteria were cultured on blood agar and were characterised by antibiotic susceptibility and biochemical profiles.

2.2. Preparation of polyphenol stock solutions

Epicatechin (purity $\geq 90\%$) was sourced from Sigma-Aldrich (Gillingham, UK) and theaflavin (purity $\geq 95\%$) was donated by Unilever (Shanghai, China). Theaflavin stock solution was prepared by adding 0.5 g of theaflavin to 10 mL of ethanol and mixing for 10 min to provide a 50 mg/mL solution. A 25 mg/mL stock solution of epicatechin was prepared by adding 0.25 g of epicatechin to 10 mL of ethanol and mixing for 15–20 min until the compound had dissolved. A combination solution of theaflavin and epicatechin was prepared using 0.5 g of theaflavin and 0.25 g of epicatechin added to 10 mL of ethanol with subsequent mixing until dissolved (15–20 min).

2.3. Preparation of polyphenol disks

Blank antibiotic susceptibility disks (MASTDISCSTM) were purchased from Mast (Bootle, UK). All materials and media were autoclaved prior to use.

Test susceptibility disks were prepared by pipetting 10 μ L volumes of the individual polyphenol solutions directly onto blank disks. Disks were then dried for 20 min prior to the addition of any further solutions, if required. For each different polyphenol solution, disks were made by pipetting a volume of stock solution that would provide 0.5, 0.75, 1, 2, 3 and 4 mg of polyphenol and 2:1 (mg ratio) combination disks. Control disks of ethanol were prepared using an identical volume of ethanol as the

maximum load of polyphenol(s) on test disks. All of the disks were then allowed to dry in a sterile Petri dish before use in experiments in order to purge them of solvent and thus of any ethanol-mediated antibacterial activity.

2.4. Disk diffusion assay

Eight clinical isolates were each independently inoculated onto six Iso-Sensitest agar plates (Oxoid Ltd., Basingstoke, UK) using the standardisation method [20]. Plate 1 contained separate disks having 0.5, 0.75 or 1 mg of epicatechin plus an ethanol control disk. Plate 2 contained separate disks having 2, 3 or 4 mg of epicatechin plus an ethanol control disk. Plates 3 and 4, and plates 5 and 6, were similar to plates 1 and 2 but with theaflavin or a theaflavin:epicatechin combination, respectively. The experimental plates were incubated at 37 °C for 24 h after which the zones of inhibition were recorded.

2.5. Statistical analysis

Six-fold replicates of all experimental plates were performed, enabling the results to be provided as mean \pm standard error. Significant differences between data sets for each combination of polyphenols were determined using the Wilcoxon Mann–Whitney test, and results showing $P \leq 0.05$ were considered as significant.

3. Results

3.1. Susceptibility to epicatechin

All isolates of *A. baumannii* and *S. maltophilia* showed no sensitivity when exposed to epicatechin alone (Tables 1 and 2; Figs 1 and 2).

3.2. Susceptibility to theaflavin

Theaflavin provided an antibacterial effect against all isolates of *A. baumannii* and *S. maltophilia* (Figs 1 and 2). This was significantly higher ($P \leq 0.05$) than that of epicatechin (Tables 1 and 2). However, the antibacterial activity of theaflavin against *A. baumannii* or *S. maltophilia* was significantly lower when a concentration of <3 mg was used.

3.3. Susceptibility to theaflavin:epicatechin combination

The combination of theaflavin:epicatechin (2:1) above a concentration level of 1 mg resulted in significantly higher antibacterial activity ($P \leq 0.05$) compared with that of theaflavin alone against all but one isolate of *A. baumannii* and *S. maltophilia*. A possible exception was against isolate 2 of *A. baumannii* where the increase in activity was observed at a slightly higher concentration of the theaflavin:epicatechin combination, i.e. above a concentration of 2 mg. The antibacterial activity of the theaflavin:epicatechin combination was lower at concentrations <3 mg (see Figs 1a–c and Fig. 2d, whereas Figs 1d and 2a–c show that a large reduction in antibacterial effectiveness occurred when the concentration of the theaflavin:epicatechin combination was <2 mg in these tests).

4. Discussion

In the present study, epicatechin showed no antibacterial effects against any isolate, whereas previous research [13,14] found activity against periodontal bacteria and *Vibrio cholera*. However, the current findings are consistent with another report [21] where epicatechin was found to be an ineffective antibacterial agent against *S. aureus*. A variation in the activity of antibacterial agents against different bacteria is not uncommon.

Theaflavin has a strong antibacterial effect that potentially could be of significant clinical relevance when dealing with microorganisms resistant to conventional antibiotics. *Stenotrophomonas maltophilia* isolates are most susceptible to the antibacterial action of theaflavin. The mechanism of its antibacterial activity is thought to be related to membrane interaction [22]. Further research is required to determine whether this is a bactericidal or bacteriostatic effect and whether any other mechanisms are involved.

It is apparent from the results reported here that there is significant synergism in the antibacterial activity between theaflavin and epicatechin against all isolates of *A. baumannii* and *S. maltophilia*. From the increase in antibacterial activity, the results suggest that the mechanism responsible for the synergy is similar to that shown for ascorbic acid and EGCG [19]. In the present case, epicatechin possibly inhibits the oxidation of theaflavin thereby prolonging its antibacterial effect. Although synergy was observed when low concentrations of the compounds were used, the greatest levels of synergy were seen when the concentration of the compounds was in

excess of 2 mg per susceptibility disk. This could be due the impact of the concentration of the polyphenols on bacterial osmolality or a saturation point of polyphenol expulsion by the bacteria.

This is believed to be the first evidence of the antibacterial effects of theaflavin against clinical isolates of *A. baumannii* and *S. maltophilia*. It is also the first report of antibacterial synergy between theaflavin and epicatechin against such hospital isolates. Although the mechanism underlying the antibacterial activity and synergy is not yet clear, the findings indicate that there may be an important clinical potential for these polyphenols, especially when used in combination. Further studies are recommended to determine the mechanisms involved and the prospective synergy of theaflavin with other polyphenols from natural sources.

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Competing interests

None declared.

Ethical approval

Not required.

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Fig. 1. Antibacterial effects of epicatechin (EC) and theaflavin (TF) and synergy between TF and EC against isolates 1–4 of *Acinetobacter baumannii*. Error bars are the standard error of the mean of six measurements.

Fig. 2. Antibacterial effects of epicatechin (EC) and theaflavin (TF) and synergy between TF and EC against isolates 1–4 of *Stenotrophomonas maltophilia*. Error bars are the standard error of the mean of six measurements.

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Table 1

Mean (\pm standard deviation) zones of inhibition for isolates 1–4 of *Acinetobacter baumannii* caused by epicatechin (EC), theaflavin (TF) and a 2:1 (mg ratio) TF:EC combination

Mass of compound (mg)	EC				TF				TF:EC (2:1)			
	1	2	3	4	1	2	3	4	1	2	3	4
0.5	6 (0)	6 (0)	6 (0)	6 (0)	6 (0)	7.1 * (0.5)	6 (0)	6 (0)	6 (0)	6.5 * (0)	6.5 * (0)	6 (0)
0.75	6 (0)	6 (0)	6 (0)	6 (0)	6 (0)	6.5 * (0.5)	6.5 * (0)	6 (0)	6.5 * (0)	9.5 * (0.5)	7 * (0.29)	6.5 * (0)
1	6 (0)	6 (0)	6 (0)	6 (0)	7 * (0)	10.5 * (0.67)	9 * (0.8)	6 (0)	7 * (0.29)	9 * (0)	9 * (1)	7.5 * (0.5)
2	6 (0)	6 (0)	6 (0)	6 (0)	9 * (0)	11.5 * (0.5)	10.5 * (0.5)	8.5 * (0.4)	11 * (0)	12.5 * (0.5)	12.5 * (0.41)	11 * (0.82)
3	6 (0)	6 (0)	6 (0)	6 (0)	14 * (0)	14 * (0.58)	13 * (0.29)	10 * (0)	16.5 * (0.5)	17 * (0)	15.5 * (0.5)	12 * (0)
4	6 (0)	6 (0)	6 (0)	6 (0)	14.5 * (0.41)	15.5 * (0.41)	14 * (0)	10 * (0)	17 * (0)	17.5 * (0.41)	16.5 * (0.41)	12 * (0)

* Significant Mann–Whitney results ($P \leq 0.05$) compared with mean zones for EC or TF:EC.

Table 2

Mean (\pm standard deviation) zones of inhibition (mm) for isolates 1–4 of *Stenotrophomonas maltophilia* caused by epicatechin (EC), theaflavin (TF) and a 2:1 (mg ratio) TF:EC combination

Mass of compound (mg)	EC				TF				TF:EC (2:1)			
	1	2	3	4	1	2	3	4	1	2	3	4
0.5	6 (0)	6 (0)	6 (0)	6 (0)	6 (0)	6.5 * (0.5)	6.1 (0.19)	6 (0)	6 (0)	7 * (0.29)	6.5 * (0)	6.5 *
0.75	6 (0)	6 (0)	6 (0)	6 (0)	6 (0)	7.5 * (0.75)	7.5 * (0.41)	8.5 * (0.41)	6.5 * (0.29)	9 * (0)	8.5 * (0.91)	9 * (0)
1	6 (0)	6 (0)	6 (0)	6 (0)	9 * (0)	11.5 * (0.41)	10 * (0)	10 * (0)	9 * (0)	13.5 * (0.41)	11 * (0)	11 * (0)
2	6 (0)	6 (0)	6 (0)	6 (0)	12 * (0)	14 * (0)	13 * (0)	14.5 * (0.41)	14.5 * (0.41)	19 * (0)	17 * (0)	18 * (0)
3	6 (0)	6 (0)	6 (0)	6 (0)	14 * (0.29)	16.5 * (0.41)	16 * (0.29)	15 * (0)	16.5 * (0.5)	20 * (0.58)	19 * (0.82)	24 * (0)
4	6 (0)	6 (0)	6 (0)	6 (0)	14.5 * (0.41)	15 * (0.29)	16 * (0)	16 * (0.29)	17.5 * (0.41)	21.5 * (0.41)	20 * (0.65)	25.5 * (0.5)

* Significant Mann–Whitney results ($P \leq 0.05$) compared with mean zones for EC or TF:EC.



