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Antibacterial Activity of Tannic Acid and Tannic Acid/Amphiphilic Cationic Polymer Mixtures

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In this study, the antibacterial activity of tannic acid/amphiphilic cationic polymer (poly{2-[(methacryloyloxy)ethyl]trimethyl-ammonium chloride}, PMADQUAT) and tannic acid mixtures was examined on the strains of Gram-positive (*S. aureus*) and Gram-negative (*E. coli* CI2, *E. coli* K12, *Klebsiella pneumonia* and *P. aeruginosa*) bacteria. Tannic acid exhibited the antibacterial activity against all the studied bacterial strains. The ester linkage between glucose and gallic acid is vital for the antimicrobial activity of tannic acid. Tannic acid inhibited the growth of *S. aureus and E. coli* K12 (1 wt%) and reduced the growth of *P. aeruginosa* to 23%. Mixing cationic polymers having different structures (statistical copolymer, homopolymer and diblock polymer) with tannic acid lead to an increase in antibacterial activity of tannic acid and the stability and clarity of mixtures was higher than that of a pure tannic acid solution. Tannic acid/diblock polymer and tannic acid/homopolymer mixtures (0.1 wt%) were excellent for inhibiting the growth of planktonic *E. coli* K12 bacteria, and a low concentration (0.0001 wt%) of tannic acid/diblock polymer reduced its growth to 19%. By contrast, the tannic acid/statistical polymer mixture (0.0001 wt%) was excellent for inhibiting the growth of Gram-positive *S. aureus* bacteria.

Keywords: Cationic polymers, Tannic acid, Antibacterial activity, Amphiphilic cationic polymer mixtures.

INTRODUCTION

Microbial contaminations induced by natural pathogens presents a severe health problems, leading to a financial burden on resources. Antimicrobial agents can prevent pathogen growth. Tannins are polyphenolic compounds that occur in two types: hydrolyzable and condensed. Tannins are prevalent in plants, such as grape, cranberry, green tea, *etc*. Tannic acid (gallotannin or tannin) is the commonest hydrolyzable tannin [1,2]. Tannins obtained from plants exhibit bactericidal effects on numerous types of bacteria, such as *Streptococcus sobrinus, Streptococcus mutans*, *Actinomyces viscois* and *Streptococcus Salivarius* [3]. Chung *et al.* [4] showed the inhibition of the growth of several food-borne bacteria by using some plant extracts.

Numerous mechanisms for the effect of tannins on bacterial growth were suggested, including the inhibition of the extracellular-microbial enzymes, dispossession of substrates required for microbial growth, and interference with microbial metabolism by using oxidative phosphorylation [5]. Withholding the metal ions essential for bacterial growth through complexation with tannins is an envisaged mechanisms, through which tannins inhibit microbial growth [6,7]. In many tannins, especially condensed type tannins, such as proanthocyanidins present in grapes and berries, antibacterial effects are caused by the antiadhesion properties. Consuming cranberry products prevents the adhesion of *Escherichia coli* strains to the uroepithelium [8,9] and thus interrupts growth and propagation [10]. Howell *et al*. [11] studied an antiadhesion activity of proanthocyanidins of different fruit juices such as grape, cranberry, and apple and concluded that only cranberry juice exhibited A-type linkages in proanthocyanidins and was associated with antiadhesion activity. Moreover, the antiviral activity of many tannins has been reported and both hydrolyzable and condensed tannins exhibited the antiviral activity. A radio-labelled study reported that the antiviral effect of both the types of tannins is induced by inhibiting virus absorption [12]. Several hydrolyzable tannins exhibit an antihuman immunodeficiency virus (anti-HIV) activity. The inhibition of virus absorption may cause

by the binding of tannins with viral envelope, which prevents penetration of the plasma membrane and viral adherence [13].

The multifunctional polar groups and amphiphatic nature of natural polyphenolic compounds allows them to complex with a various macromolecules through not only intermolecular and intramolecular H-bonding interactions but also π-cation and hydrophobic interactions [6]. The production of new colloidal structures that use interactions between methylcellulose and polyphenol epigallocatechin gallate, obtained from green tea, for encapsulation applications has been reported [14,15]. Soluble complexes were formed from interactions between macromolecules and polyphenols. These complexes can self-associate and continue to grow, resulting in its eventual sedimintation [14].

Numerous mixtures of tannic acid and non-ionic polymers, including polyethylene glycol (PEG) [16], polyvinyl pyrrolidone (PVP) [17], methyl cellulose [18] and a block copolymer, namely poly(ethylene oxide)-blockpoly(2-hydroxylethyl methacrylate) (PEOb-PHEMA) [19] have been reported. The mixtures of tannic acid and cationic polymers was assembled layer by layer with two different cationic polymers, namely weak poly(allylamine) and strong poly (dimethyl diallylamide), to develop a polyelectrolyte microcapsule with an adjustable drug-loading-release profile [20]. However, to the best of our knowledge, the antimicrobial behaviour of tannic acid/amphiphilic cationic polymer (poly{2-[(methacryloyloxy)ethyl]-trimethylammonium chloride}, PMADQUAT) mixtures has not been investigated.

EXPERIMENTAL

Tannic acid purchased from Sigma-Aldrich was used without purification. Cationic polymers (homo, statistical and diblock) were synthesized using RAFT polymerization or free radical polymerization and the details are shown in Table-1.

Bacterial strains: In this study, two types of bacterial strains, Gram-positive and Gram-negative were used. Bacterial strains were obtained from Central Manchester Foundation Trust (Clinical Sciences Building 2, Manchester, UK). In 80% glycerol (obtained from Fisher Scientific Ltd.), bacterial glycerol stocks were prepared and stored at 80 ºC. Luria-Bertani (LB) agar plates were used to culture stock bacterial strains through overnight incubation at 37 ºC. To store working culture plates, a temperature of 4 ºC was employed. To prepare cultures, five colonies of bacteria obtained from working culture plates were inoculated into 10 mL of LB broth (50 mL Falcon tube) and then were incubated at 37 ºC for 16-18 h and shaking at 200 rpm.

Bacterial growth assay: An overnight culture of each bacterial strain with 1/100 dilution was prepared in LB medium and then, 200 µL of each dilution sample was aliquoted into a flat-bottomed untreated polystyrene 96-well microtitre plate (Greiner Bio-one Ltd., UK, ref. code 655161). For each concentration of inhibitor (0.0001, 0.001, 0.01, 0.1, 0.25, 0.5 and 1 wt%), 16 replicate wells were used and eight wells were inoculated only with 100 µL inoculum to obtain positive growth control, and remaining eight wells were inoculated with 100 µL of LB medium without any organism to obtain negative control. A multi-channel pipette was employed to perform all experiments with microtitre plates. All microtitre plates were incubated at 37 ºC overnight and then a microplate reader was used to quantify the optical density (OD at 595) of bacterial cells. Microtitre plate assay was performed according to the procedure given by Govindji *et al.* [21] to study biofilm formation.

Minimum inhibitory concentration (MIC) determination: For determining the minimal inhibitory concentration (MIC) value of every inhibitor, various samples were prepared. The MIC is the minimum concentration of a polymer solution that inhibits bacterial growth after overnight incubation [22].

Tannic acid sample preparation: A tannic acid stock solution (3 wt%) was prepared in water and used to obtain dilutions with desired concentrations. The pH used for each tannic acid solution was 7. For higher concentrations (> 0.1 wt%), a colour change to darker brown and precipitation were observed in tannic acid solutions, because at pH of > 6, carbohydrates of tannic acid (*i.e.* polyhydric alcohol and glucose) occupy the central core position in the tannic acid structure tannic acid hydrolyzes in water and hydroxyl groups attach to one or more phenolics (*i.e.* ellagic acid and gallic acid) that partially hydrolyze into glucose and gallic acid moieties at extreme pH [2,23,24].

Preparation of PMADQUAT and tannic acid mixture: Addition of poly{2-[(methacryloyloxy)ethyl]trimethylammonium chloride} (PMADQUAT) homopolymer provided a clearer and more stable solutions even at high concentrations, because the multifunctional polar groups and amphiphatic nature of natural polyphenolic compounds allow them to complex with cationic polymers through not only intramolecular and intermolecular H-bonding interactions but also hydrophobic and π-cation interactions [6].

To determine the tannic acid optimal concentration for mixing with homopolymer solutions of various concentrations, 0.001, 0.01, 0.1, 0.25, 0.5 and 1 wt% tannic acid were mixed with 0.01 wt% PMADQUAT and LB medium.

Preparation of 0.5 wt% of PMADQUAT with 0.1 wt% of tannic acid: Tannic acid (2 mL of 0.4 wt%) was mixed with 5 mL of 2 wt% polymer and then 100 µL inoculum was mixed with of 100 µL of the resulting solution in each well.

RESULTS AND DISCUSSION

Antimicrobial activity of tannic acid and the prepared mixtures against a planktonic laboratory *S. aureus*, *E. coli* K12, and *P. aeruginosa* strains was tested. Tannic acid and prepared mixtures exhibited the antimicrobial activity against all isolates

TABLE-1
SUMMARY OF M_w, M_n AND PDI OF HOMO, STATISTICAL AND DI-BLOCK POLYMERS OBTAINED BY AQUEOUS GPC

Polymers	Mw (g mol^{-1})	Mn (g mol^{-1})	PDI
PMADQUAT (*via* RAFT polymerization)	15000	9000	1.6
Poly(MADQUA T_{50}-*s*-MMA_{50}) *via* free radical polymerization)	35000	11000	3.0
Poly(MADQUA T_{50}-*s*-MMA_{50}) (*via* RAFT polymerization)	4800	3400	1.3

with MICs of 1 wt% for both *S. aureus* and *E. coli* K12. Moreover, the growth of *P. aeruginosa* was reduced to 23% by using an MIC of 0.1 wt%. When tannic acid was mixed with different-structured cationic polymers, such as statistical co-polymer, homopolymer and diblock polymer, the antibacterial activity improved. Subsequently, crystal violet staining of adhered bacterial cells was employed for determining the amount of a biofilm formed after 18 h by remaining viable planktonic cells.

Tannic acid as antibacterial agent: Fig. 1 presents the results of the preliminary study of tannic acid and revealed its excellent antibacterial activity against all tested bacterial strains, and for *P. aeruginosa*, *E. coli* K12 and *S. aureus*, MICs were 0.1, 0.01 and 0.01 wt.%, respectively. The results can be explained by two phenomena: (i) tannic acid solutions at pH 7 and low concentration (< 0.1 wt%) are not influenced by the pH adjustment and at high concentrations (≤ 0.1 wt%) they can break down and lead to precipitation. Interference of optical density in bacterial cells and such precipitates may result in errors; (ii) the optical density of tannic acid solutions is high, and when it is abstracted from the total optical density of bacterial cells and tannic acid, the optical density of bacterial cells could be reduced after a treatment.

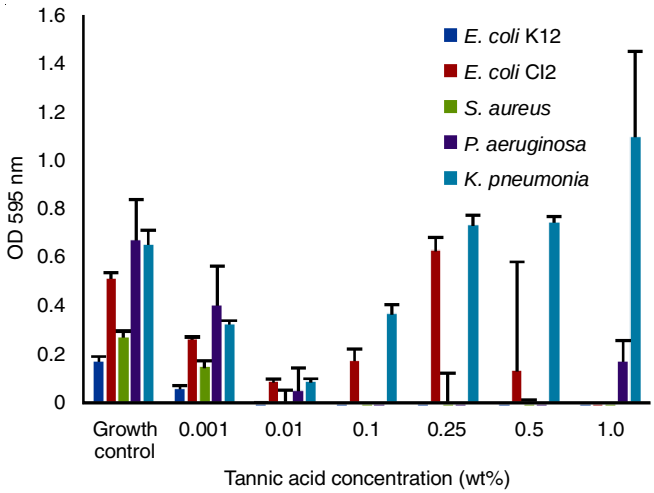

Fig. 1. Preliminary study of tannic acid on planktonic growth of studied bacteria. The results are expressed as the mean of 24 replicate wells involving three biological replicate for each strain. Error bars indicate the standard error of the mean

MICs of tannic acid at unadjusted pH for planktonic bateria: Without adjusting the pH, the experiment was repeated to prove the antibacterial activity of tannic acid. Therefore, precipitation at higher concentrations was avoided and only three strains with low MIC values were used in the repeated experiment. The antibacterial effectiveness of tannic acid with unadjusted pH was lower than that of tannic acid with pH adjusted to 7. However, a similar trend was observed for concentration > 0.1 wt%, where the antibacterial activity of tannic acid with unadjusted pH started to decrease. This result may have been caused by interactions between the optical density of tannic acid solutions at higher concentrations and that of bacterial cells.

Another experiment was conducted for only *P. aeruginosa*, because it exhibited higher bacterial growth rate than other strains did, to determine the reason of increase in MICs at higher concentrations. After treatment with different concentrations of tannic acid, on LB agar plates, *P. aeruginosa* was streaked and those were then incubated for 8 h at 37 ºC.

Fig. 2 reveals that the treatment with 0.25 wt% tannic acid led to a considerable reduction in bacterial growth and that no growth was observed at 0.5 wt% concentration, which explained the increase in MICs at higher concentrations, which must have been caused by the high optical density of tannic acid solutions. Subsequently, the antibacterial activity of mixtures of cationic polymers with different structures and tannic acid against the three bacterial strains (*P. aeruginosa*, *E. coli* K12, and *S. aureus*) was investigated, in both planktonic and biofilm forms.

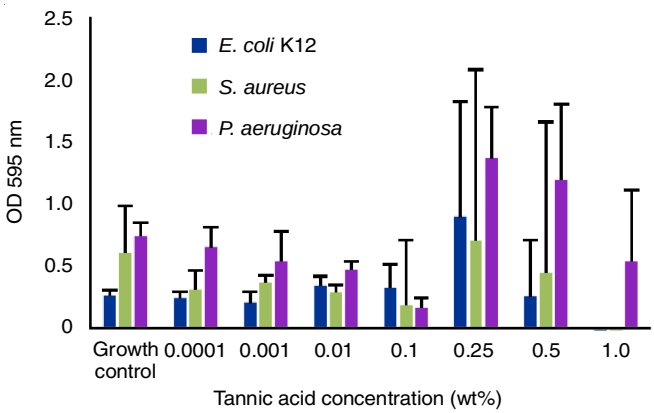

Fig. 2. Effect of tannic acid unadjusted for pH on planktonic bacterial cell grown under static condition for 18 h. The results are expressed as the mean of 24 replicate wells involving three biological replicates for each strain. Error bars indicate the standard error of the mean

Effect of tannic acid/PMADQUAT mixtures on planktonic and biofilm: Tannic acid with a concentration of 0.1 wt% was mixed with the different concentrations of PMADQUAT homopolymer. Fig. 3 (Table-2) present the effects of the resulting mixtures on the three planktonic bacterial strains. Fig. 4 (Table-2) present the effects of tannic acid/PMADQUAT

TABLE-2
COMPARING MICs (wt.%) of PMADQUAT, TANNIC ACID AND TA/PMADQUAT MIXTURES FOR PLANKTONIC BACTERIA AND BACTERIA IN BIOFILMS

Bacteria	Planktonic bacteria			Bacteria in biofilms		
	PMADQUAT	Tannic acid	Tannic acid/ PMADQUAT mixtures	PMADQUAT	Tannic acid	Tannic acid/PMADQUAT mixtures
E. coli K12	1.0	1.0	0.01	0.25	1.0	0.1
P. aeruginosa	0.1	1.0	0.10	Reduced to 2%	At 0.1 wt% reduced to 23%	0.1
S. aureus	Reduced to 2%	0.5	0.01	0.25	1.0 (at 0.1 reduced to 31%)	0.01 (at 0.0001 reduced to 3%)

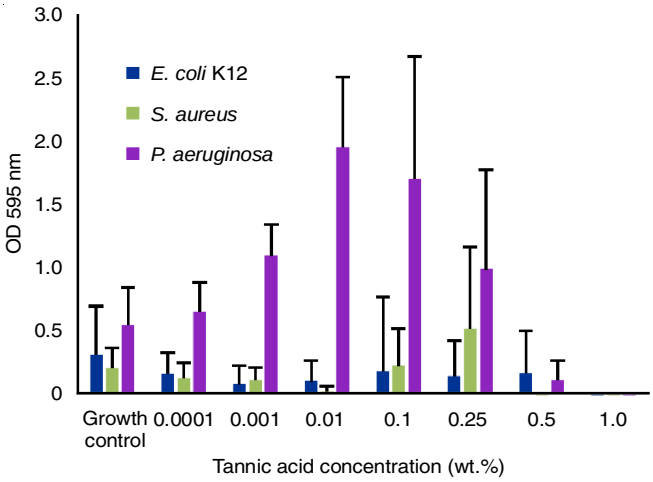

Fig. 3. Effect of tannic acid on biofilm development under static condition for 18 h. The results are expressed as the mean of 24 replicate wells involving three biological replicates for each strain. Error bars indicate the standard error of the mean

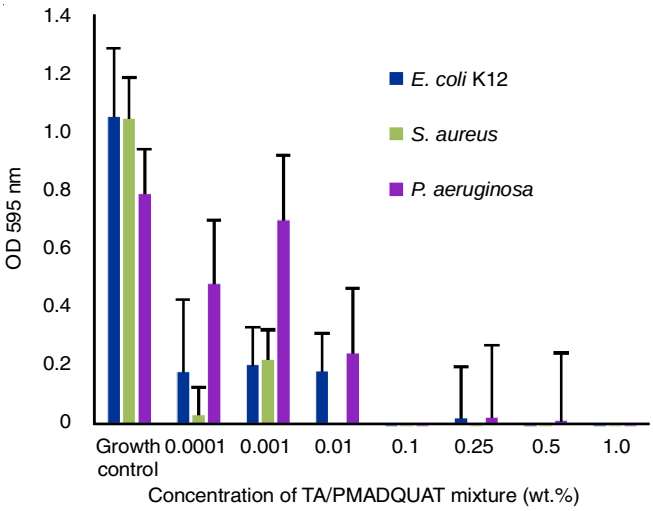

Fig. 4. Effect of tannic acid (0.1 wt%)/PMADQUAT mixtures on planktonic bacterial cell grown under static condition for 18 h. The results are expressed as the mean of 24 replicate wells involving three biological replicates for each strain. Error bars indicate the standard error of the mean

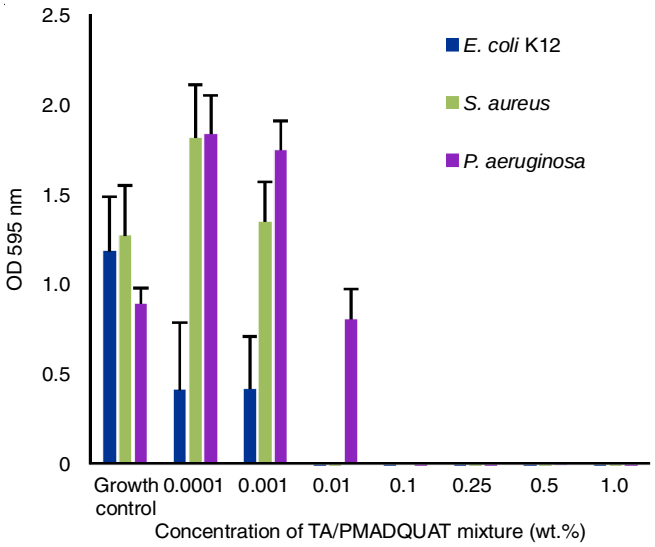

Fig. 5. Effect of tannic acid (0.1 wt%)/PMADQUAT on biofilm development under static condition for 18 h. The results are expressed as the mean of 24 replicate wells involving three biological replicates for each strain. Error bars indicate the standard error of the mean

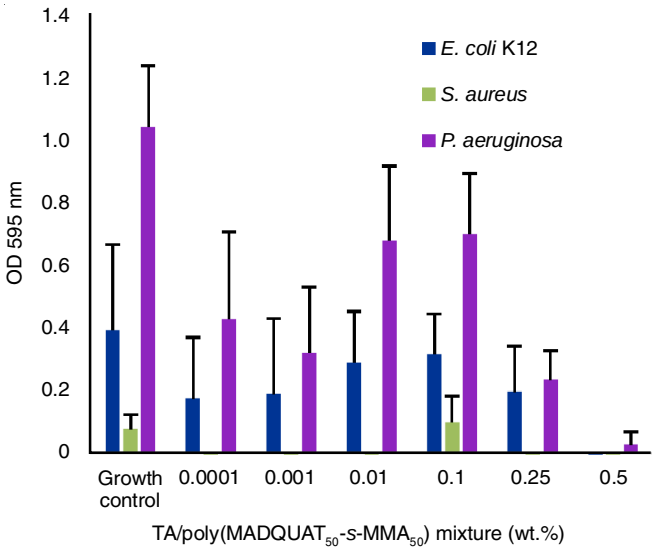

Fig. 6. Effect of tannic acid (0.1 wt%)/poly(MADQUAT$_{50}$-*s*-MMA$_{50}$) mixtures on biofilm developed under static condition for 18 h. The results are expressed as the mean of 24 replicate wells involving three biological replicates for each strain. Error bars indicate the standard error of the mean

on biofilm growth. Tannic acid with a concentration of 0.1 wt% was mixed into the statistical polymer with different concentrations. Fig. 5 (Table-3) present the effects of the resulting mixtures on the three planktonic bacterial strains. Fig. 6 (Table-3) presents the effect of the mixture on biofilm growth.

Tannic acid/poly(MADQUAT$_{50}$-*s*-MMA$_{50}$) mixtures: Tannic acid with a concentration of 0.1 wt% of was mixed with the different concentrations of diblock polymers. Fig. 7 (Table-4) present the effects of the resulting mixtures on the three planktonic bacterial strains. Fig. 8 (Table-4) present the effect of the mixture on biofilm growth. The biofilm inhibitory and antimicrobial properties of tannic acid/cationic polymers and tannic acid with different concentrations were studied.

TABLE-3
COMPARING MICs OF POLY(MADQUAT$_{50}$-s-MMA$_{50}$), TANNIC ACID AND TA/POLY(MADQUAT$_{50}$-*s*-MMA$_{50}$) MIXTURES FOR PLANKTONIC BACTERIA AND BACTERIA IN BIOFILMS

Bacteria	Planktonic bacteria			Bacteria in biofilms		
	Poly (MADQUAT$_{50}$-*s*-MMA$_{50}$)	Tannic acid	TA/poly (MADQUAT$_{50}$-*s*-MMA$_{50}$) mixtures	Poly (MADQUAT$_{50}$-*s*-MMA$_{50}$)	Tannic acid	TA/poly (MADQUAT$_{50}$-*s*-MMA$_{50}$) mixtures
E. coli K12	0.001	1.0	0.5	0.1	1.0	0.0001
P. aeruginosa	0.100	0.1 reduced to 23%	Reduced to 3%	0.1	1.0	0.5000
S. aureus	0.010	1.0 (at 0.1 reduced to 31%)	0.0001	Reduced to 2%	0.5	0.0001

TABLE-4
COMPARING MICs OF POLY(MADQUAT$_{50}$-*b*-MMA$_{50}$), TANNIC ACID AND TA/POLY(MADQUAT$_{50}$-*b*-MMA$_{50}$) MIXTURES FOR PLANKTONIC BACTERIA AND BACTERIA IN BIOFILMS

Bacteria	Planktonic bacteria			Bacteria in biofilms		
	Poly (MADQUAT$_{50}$-*b*-MMA$_{50}$)	Tannic acid	TA/poly (MADQUAT$_{50}$-*b*-MMA$_{50}$) mixtures	Poly (MADQUAT$_{50}$-*b*-MMA$_{50}$)	Tannic acid	TA/poly (MADQUAT$_{50}$-*b*-MMA$_{50}$) mixtures
E. coli K12	0.10	1.0	0.1 (at 0.0001 reduced to19%)	0.0001	1.0	0.0001
P. aeruginosa	0.10	At 0.1 wt% reduced to 23%	0.25	0.1000	1.0	0.2500
S. aureus	0.01	1.0 (at 0.1 reduced to 31%)	0.1 (at 0.0001 reduced to 4 %)	0.0001	0.5	0.0001

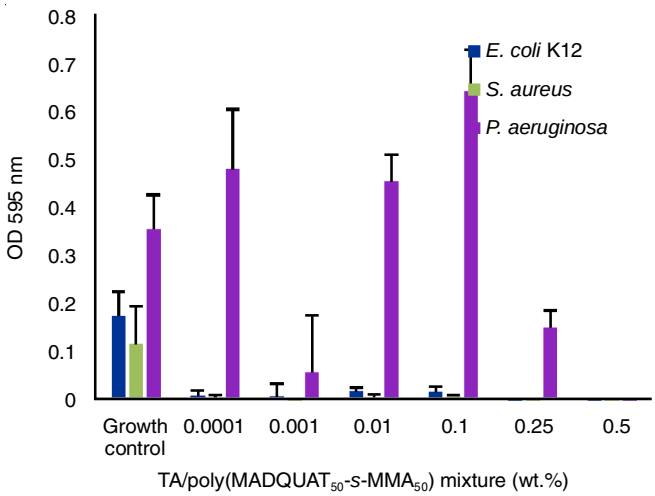

Fig. 7. Effect of tannic acid (0.1 wt%)/poly(MADQUAT$_{50}$-*s*-MMA$_{50}$) mixtures on biofilm developed under static condition for 18 h. The results are expressed as the mean of 24 replicate wells involving three biological replicates for each strain. Error bars indicate the standard error of the mean

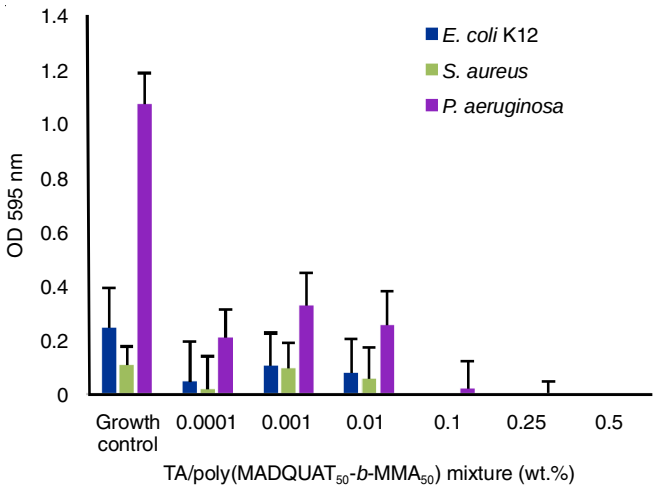

Fig. 8. Effect of tannic acid (0.1 wt%)/poly(MADQUAT$_{50}$-*b*-MMA$_{50}$) diblock polymer mixtures on the biofilm developed under static condition for 18 h. The results are expressed as the mean of 24 replicate wells involving three biological replicates for each strain. Error bars indicate the standard error of the mean

Polymeric-cationic antimicrobial agents are used in food, domestic and medical industries [25,26]. Although both tannic acid and cationic polymers exhibit an excellent antimicrobial activity, this study achieved a higher degree of the antimicrobial activity by using the mixtures of tannic acid/cationic polymers.

A correlation between the MICs of biofilm and planktonic was determined using the microtitre plate assay, where a small number of planktonic cells denoted that few viable cells were able to create the biofilm. The MIC for the planktonic cells of *P. aeruginosa* was less than the biofilm inhibition concentration for those, because of surface factors, including its active drug efflux mechanisms and outer membrane impermeability [27,28], which led to an increase in antibacterial resistant.

The MICs of pure homopolymer, PMADQUAT, pure tannic acid and the mixture of tannic acid/PMADQUAT obtained for both planktonic and biofilm growths were compared. MICs revealed that the mixtures of PMADQUAT homopolymer and tannic acid provided more satisfactory results for all the three bacterial strains than only tannic acid or PMADQUAT did. The mixture inhibited bacterial growth even at a low concentration of 0.1 wt.% and considerably reduced the number of adherent bacterial cells at the same concentration. A comparison of the MICs of poly(MADQUAT$_{50}$-*s*-MMA$_{50}$), pure statistical copolymer, pure tannic acid and the mixture of tannic acid/ poly(MADQUAT$_{50}$-*s*-MMA$_{50}$) obtained foe both planktonic and biofilm growths (Table-3) indicated that mixtures of poly(MADQUAT$_{50}$-*s*-MMA$_{50}$) and tannic acid provided more satisfactory results than only tannic acid or polymer did in both planktonic and biofilm forms for the activity against Gram-positive *S. aureus* bacteria. Tannic acid/statistical polymer mixture (0.0001 wt%) was most favourable for inhibiting the growth of Gram-positive *S. aureus* bacteria. The lowest MIC was obtained for *S. aureus* may be because of the ability of tannic acid to bind directly to a peptidoglycan layer of the bacterial outer membranes, which is an accepted antimicrobial mechanism for polyphenols [29].

Effect of tannic acid/poly(MADQUAT$_{50}$-*b*-MMA$_{50}$) mixtures on planktonic and biofilm: The MICs of poly-(MADQUAT$_{50}$-*b*-MMA$_{50}$), pure diblock polymer, pure tannic acid and the mixture of tannic acid/ poly(MADQUAT$_{50}$-*b*-MMA$_{50}$) obtained for both planktonic and biofilm growths indicated that the mixtures of tannic acid and poly-(MADQUAT$_{50}$-*b*-MMA$_{50}$) provided satisfactory results (Table-4), and their activity was intermediate between those of only diblock polymer and tannic acid (Fig. 9). The ester linkage between glucose and gallic acid is crucial for the antimicrobial activity of tannic acid [22] because these polymers contain a cationic moiety, which electrostatically attaches to the outer membrane of bacteria and the hydrophobic moiety (MMA) causes an increase in the antibacterial activity [30] *i.e.* helps an incorporation of polymers into lipid membranes. Thus, the hydrophilic hydrophobic structure of polymers disrupts bacterial membranes, causing the leakage of cytoplasmic contents, break-down of the transmembrane potential, and

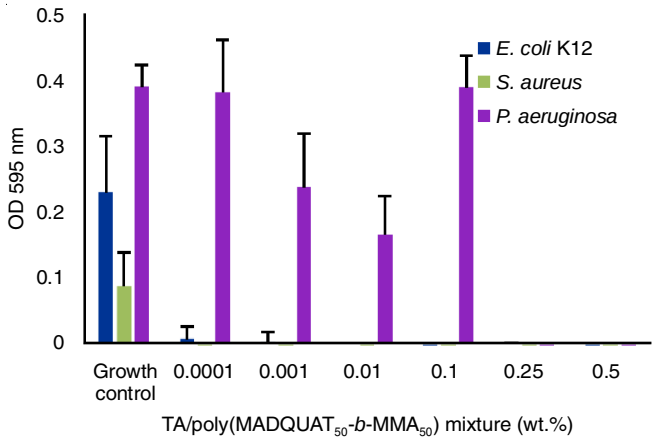

Fig. 9. Effect of tannic acid (0.1 wt%)/poly(MADQUAT$_{50}$-*b*-MMA$_{50}$) mixtures on biofilm growth. The results are expressed as the mean of 24 replicate wells involving three biological replicates for each strain. Error bars indicate the standard error of the mean

finally the death of bacterial cells [31]. Statistical copolymers and homopolymers with MADQUAT exhibited the excellent antibacterial activity. Such a strong effect of polymer structure, which was the same as that of chemical structure on the antibacterial activity was not observed before.

Conclusion

Tannic acid exhibited the antibacterial activity against all the tested bacterial strains. It inhibited *S. aureus* and *E. coli* K12 growth at 1 wt% and reduced the growth of *P. aeruginosa* to 23%. The mixtures of tannic acid and cationic polymers with different structures (statistical copolymer, homopolymer and diblock polymer) led to an increase in the antibacterial activity, and these mixtures exhibited excellent clarity and stability than the pure tannic acid solution did. Tannic acid/diblock polymer and tannic acid/homopolymer mixtures (0.1 wt%) most effectively inhibited the growth of planktonic *E. coli* K12 bacteria, and a low concentration of tannic acid/diblock polymer (0.0001 wt%) reduced growth to 19%. The tannic acid/statistical polymer mixture was most effective for inhibiting the growth of *S. aureus* bacteria (0.0001 wt%). Mixing cationic polymers with tannic acid improved the activity against both planktonic and biofilm bacteria, especially against *S. aureus*. Determining the interaction of tannic acid with cationic polymers having different structures is a new research area. The result indicated that the mixing of tannic acid with statistical polymers and homopolymers improved the antibacterial activity, and mixing tannic acid with diblock polymer provided an average result.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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