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ORIGINAL ARTICLE

Combination of essential oil and ciprofloxacin to inhibit/eradicate biofilms in multidrug-resistant *Klebsiella pneumoniae*

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Abstract

Aim: This study aimed to test biofilm inhibition activities of each of essential oils (EOs), main compounds of EOs and enzymes against pathogenic *Klebsiella pneumoniae*.

Methods and Results: The effect of seven EOs and three enzymes was tested on formation and eradication of *K. pneumoniae* biofilm. Peppermint oil showed a robust biofilm inhibitory effect, causing inhibition that ranged from 69·2 to 98·2% at 5 μl ml^{-1}. Thyme oil was found to have the best biofilm eradication ability, causing eradication that ranged from 80·1 to 98·0% at 10 μl ml^{-1}. The most effective EOs were analysed by GC/MS, to determine the major chemical constitutes of each oil. Pure menthol was found to cause 75·3–97·5% biofilm inhibition at 2·5 μg ml^{-1}, whereas thymol caused 85·1–97·8% biofilm eradication at 5 μg ml^{-1}. However, moderate inhibition activity was detected for α-amylase and bromelain, while poor activity was detected for β-amylase. Ciprofloxacin combination with thyme oil and thymol was found to enhance antibiotic activity, and affect biofilm cell viability. The observed inhibitory/eradication activity on *K. pneumoniae* biofilms was confirmed by scanning electron microscopy.

Conclusions: Thyme and peppermint EOs, and their active components are promising antibiofilm agents alone and/or in combination with ciprofloxacin to inhibit/eradicate biofilms of *K. pneumoniae*.

Significance and Impact of the Study: The presented results suggest the potential application of EOs against infections, caused by biofilm-producing *K. pneumoniae*, to prevent biofilm formation or decrease their resistance threshold. Moreover, the combination of EOs with ciprofloxacin minimizes the antibiotic concentration used and accordingly the potential accompanying toxic side effects.

Introduction

Klebsiella pneumoniae (KP) is a common human pathogen associated with nosocomial chronic infections. The infections caused by multidrug-resistant (MDR) strains of KP are spreading widely all over the world (Abdelaziz *et al.* 2013; Trivedi *et al.* 2015). MDR-KP is one of the leading causes of healthcare-associated infections worldwide and poses significant treatment challenges as they demonstrated increased tolerance to antibiotics, chemical disinfectants and the human defence system such as phagocytosis (Lam *et al.* 2018).

The formation of biofilm in KP has also been hypothesized to contribute to bacterial persistence, these biofilms have been shown to resist killing with prolonged exposure to antibiotics (Chung 2016). The bacteria within the

biofilm are 10–1000 times more resistant to antibiotics than planktonic cells, but their resistance mechanism is still unexplained, therefore, it is difficult to eradicate bacterial biofilms causing numerous infections (Abdulhasan *et al.* 2016).

Prevention of biofilms can be achieved by early intensive antibiotic prophylaxis or therapy as well as by chronic suppressive therapy. During the last few years, the appearance of side effects of antibiotics has led to the search for new antimicrobial agents to overcome the foregoing disadvantage (Mohsenipour and Hassanshahian 2015). Hence, alternative strategies or effective agents to act against biofilm-producing micro-organisms are of great interest (Millezi *et al.* 2016). The discovery of anti-infective agents which are active against planktonic micro-organisms as well as microbial biofilms represents an ultimate objective (Ceylan *et al.* 2014).

The antimicrobial activity of EOs has long been recognized and has been extensively tested *in vitro* against a wide range of pathogenic bacteria. However, more research is urgently needed to validate their antibiofilm activity (Oulkheir *et al.* 2017). The use of biological enzymes that can dissolve the bacterial biofilm matrix, which consists of DNA, protein and polysaccharide, represents a promising approach which is still in the nascent phase of development (Sadekuzzaman *et al.* 2015). All these approaches may eventually lead to effective prevention and control of biofilms.

Therefore, the attention of our study was focused on the possibility of using natural compounds, such as EOs and biological enzymes, in the fight against KP biofilms. Moreover, we evaluate the ability of selected antibiofilm compounds to minimize the effective dose of ciprofloxacin, consequently minimizing their cost and potential toxic side effects.

Materials and methods

Essential oils, major compounds and enzymes

Caraway oil (*Carum carvi*), cinnamon oil (*Cinnamomum verum*), clove oil (*Syzygium aromaticum*), ginger oil (*Zingiber officinale*), nigella oil (*Nigella sativa*), peppermint oil (*Mentha piperita*) and thyme oil (*Thymus vulgaris*) were obtained from the Arab Co., and Al-Andalus Co. (Cairo, Egypt). Menthol (99% pure) and thymol (≥98·5% pure) were obtained from Sigma-Aldrich (St. Louis, MO). Tween 60 was used at a concentration of 0·5% to enhance the solubility in medium. α-amylase from *Aspergillus oryzae* (30 U mg^{-1}), and bromelain from pineapple stem (≥3 U mg^{-1}) were obtained from Sigma-Aldrich. β-amylase from barley flour (20–80 U mg^{-1}) were purchased from Roche (Mannheim, Germany).

Bacterial strains

Ten clinical biofilm-producing KP strains were randomly collected during the period of October 2015 until March 2016 from the main laboratory of Kasr Alaini Hospital, Cairo, Egypt from different sources (Table 1). Strains were identified by conventional microbiological methods and confirmed by MALDI-TOF/MS with score values >(1·9) using Bruker Biotyper 3.1 software. The antibiotic susceptibility of KP to different types of antibiotics was performed by disc diffusion method according to CLSI (2016) recommendations using commercially available antibiotic discs (Oxoid, Basingstoke, UK) (Table 1).

Determination of minimal inhibitory and minimal bactericidal concentrations of planktonic cells

The effect of essential oils (EOs) and enzymes against planktonic cells was determined in Müller–Hinton broth using a broth microdilution method in a 96-well microplate (Tutar *et al.* 2016). Briefly, 100 μl (0·5 MacFarland bacterial cultures) was dispersed into each well of the microplate, and was then mixed with 100 μl of twofold dilutions of treatment concentrations for 24 h at 37°C. Final concentration was 0·03–4% v/v for EOs, 0·007–4% v/v for cinnamon and clove EOs and 1·56–100 mg ml^{-1} for enzymes. Well containing 100 μl of brain heart infusion (BHI) inoculated with 100 μl of bacterial culture mixed with Tween 60 (final concentration 0·5%) was used as a positive control, whereas negative control was BHI broth only. Minimum inhibitory concentration (MIC) considered the lowest concentration of an antimicrobial agent that inhibited the visible growth of KP. Minimum bactericidal concentration (MBC) was evaluated by plating 5 μl from each well on nutrient agar medium after incubation at 37°C for 24 h. The lowest concentration of EO at which no visible bacterial growth was detected on the subculture agar medium was defined as MBC (Santurio *et al.* 2014).

Biofilm inhibition assay

Inhibition of biofilm formation was assessed using tissue culture plate method adopted by Adukwu *et al.* (2012) with minor modifications. Briefly, cultures were grown overnight in BHI broth, 100 μl (0·5 MacFarland bacterial culture) was dispensed into each well of 96-well polystyrene flat-bottomed microtitre plates in the presence of 100 μl of twofold dilutions of EOs (0·03–4% v/v) or enzymes (1·56–100 mg ml^{-1}). After incubation for 24 h at 37°C, the medium was discarded and each well was washed twice with phosphate-buffered saline (PBS) pH 7·4, dried, stained for 20 min with 1% (w/v) crystal violet and washed with water. The stained biofilms were

Table 1 The antibiotic resistance pattern of *Klebsiella pneumoniae* strains (KP1–KP10) isolated from different clinical samples

		Antimicrobial agents														
		Carbapenems		Cephalosporins				Cephamycins	Aminoglycosides		Fluoroquinolones			Penicillins	Penicillins/β-lactamase inhibitors	Sulphonamides
Isolates ID	Clinical source	IPM	MEM	CAZ	CXM	CTX	FEP	FOX	GM	AK	NA	CIP	LEV	AMP	TZP	SXT
KP1	Urine	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R
KP2	Urine	S	R	R	R	R	R	R	S	R	R	R	R	R	R	R
KP3	Sputum	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
KP4	Urine	I	R	R	R	R	R	S	S	R	R	R	R	R	R	S
KP5	Sputum	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R
KP6	Sputum	S	R	R	R	R	R	R	R	R	R	R	R	R	R	S
KP7	Urine	S	R	R	R	R	R	S	R	R	R	R	R	R	R	R
KP8	Wound	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R
KP9	Urine	S	S	R	R	R	SDD	S	R	S	R	R	R	R	S	R
KP10	Urine	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R

IPM, imipenem (10 μg); MEM, meropenem (10 μg); CAZ, ceftazidime (30 μg); CXM, cefuroxime (30 μg); CTX, cefotaxime (30 μg); FEP, cefepime (30 μg); FOX, cefoxitin (30 μg); GM, gentamicin (10 μg); AK, amikacin (30 μg); NA, nalidixic acid (30 μg); CIP, ciprofloxacin (5 μg); LEV, levofloxacin (5 μg); AMP, ampicillin (10 μg); TZP, piperacillin–tazopactam (110 μg) and SXT, cotrimoxazole (1·25/23·75 μg). S, susceptible; I, intermediate; SDD, susceptible dose-dependent; R, resistant. KP, *Klebsiella pneumoniae*.

resuspended in 200 μl absolute ethanol. The optical density (OD) was measured at 630 nm using a microplate reader (Stat Fax-2100; GMI, Inc., Miami, FL, USA). The negative control was BHI broth only, whereas the positive control for each strain contained cell cultures of the same strain without treatment. All determinations were performed in triplicates.

Biofilm eradication assay

Cultures were grown overnight in BHI broth, 100 μl (0·5 MacFarland bacterial culture) was dispensed into each well of 96-well polystyrene flat-bottomed microplates. After 24 h of incubation at 37°C, the planktonic cells were gently removed and the wells were washed three times with PBS pH 7·4, and filled with 100 μl of twofold dilutions of EOs (0·03–4% v/v) or enzymes (1·56–100 mg ml^{-1}). The plates were incubated for 24 h at 37°C, then the medium was discarded and each well was washed twice with PBS, dried, stained for 20 min 1% (w/v) crystal violet and washed with water. The stained biofilms were resuspended in 100 μl absolute ethanol. The OD was measured at 630 nm using a Microplate reader (Stat Fax-2100; GMI, Inc.). The negative control was BHI broth only, whereas the positive control contained biofilm cells alone with no treatment. All determinations were performed in triplicates (Adukwu *et al.* 2012).

Biofilm cells viability assay

The biofilm cells viability was tested after 24 h from biofilm eradication treatment. The medium was discarded and the wells were rinsed gently twice with PBS. A total of 100 μl of fresh BHI broth was added and incubated. After 24 h, 5 μl of planktonic culture were transferred from each well to the surface of the nutrient agar plate. MBC was identified after 24 h of incubation (Mah 2014).

Gas chromatography/mass spectrometry

Mass spectra were recorded using Shimadzu GCMS-QP2010 (Tokyo, Japan) equipped with Rtx-5MS fused bonded column (30 m × 0·25 mm × 0·25 μm film) (Restek Corporation, Bellafonte, PA) equipped with a split–splitless injector. The capillary column was directly coupled to a quadrupole mass spectrometer (SSQ 7000; Thermo-Finnigan, Bremen, Germany). The initial column temperature was kept at 45°C for 2 min and programmed to 300°C at a rate of 5°C min^{-1}, and kept constant for 5 min. Injector temperature was 250°C. Helium carrier gas flow rate was 1·41 ml min^{-1}. All the mass spectra were recorded applying the following condition: filament emission current, 60 mA; ionization voltage,

70 eV; ion source, 200°C. Diluted samples were injected with split mode (split ratio 1 : 15).

Effect of menthol and thymol on planktonic and biofilm cells

The activity was determined using broth dilution method described by Nostro *et al.* (2007) with some modifications. Briefly, thymol and menthol were dissolved in dimethylsulphoxide (DMSO) and diluted to 40 μg ml^{-1} in BHI broth. Then, twofold serial dilutions were prepared. In order to avoid the antibacterial effects of DMSO, the final DMSO concentration never exceeded 1% (v/v). Effect of thyme and peppermint oils on biofilm inhibition/eradication was repeated using the same conditions used for major components.

Scanning electron microscopy

Scanning electron microscopy (SEM) was employed for investigating the effect of thyme oil, peppermint oil, thymol, menthol and bromelain enzyme on KP biofilm. Sections of the interior of polystyrene tubes coated with bacterial biofilm were processed similarly with the method described by Ansari *et al.* (2013) with modifications. Briefly, samples were fixed for 2 h in equal volumes of 4% (v/v) glutaraldehyde and 0·2 mole ml^{-1} caccodylate, and washed in equal volumes of 0·4 mole ml^{-1} saccharose and 0·2 mole ml^{-1} caccodylate for 2 h and then postfixed in 2% (w/v) osmium tetroxide (Carl Roth, Karlsruhe, Germany) and 0·3 mole ml^{-1} caccodylate for 1 h. The samples were washed with deionized water and finally dehydrated in ascending grades of ethanol for 5 min each (30, 50, 70, 90%) and finally 100% absolute ethanol for 10 min three times, then examined with Philips XL30 scanning electron microscope (Eindhoven, the Netherlands) operated at 20 kV.

Synergistic effect of antibiofilm agents and ciprofloxacin

Synergistic effect of antibiofilm agents and ciprofloxacin has been studied by the broth microdilution method to determine MIC of ciprofloxacin (Andrews 2001; Rath and Padhy 2014). Serial dilutions of ciprofloxacin concentrations ranging from 0·25 to 64 μg ml^{-1} were used against planktonic and biofilm cells with and without peppermint oil, thyme oil, menthol and thymol at final concentrations 5, 10, 2·5 and 5 μg ml^{-1} respectively.

Statistical analysis

The EOs' and enzymes' antibiofilm activities were performed in triplicate independent experiments ($n = 3$). The calculated mean values are presented ± standard error (SE) and compared using the Duncan test using SPSS software (Statistical Package for the Social Sciences, ver. 12.0, New York) and the difference was considered statistically significant ($P < 0{\cdot}05$).

Results

In this study, seven EOs and three biological enzymes were investigated for their antimicrobial activity, as well as their ability to inhibit and eradicate biofilms of strong biofilm-producing KP strains. The strains were characterized as MDR according to Magiorakos *et al.*'s (2012) categories as resistant to at least one agent in three or more antimicrobial categories (Table 1). The results revealed that, cinnamon oil recorded the best antibacterial activity against planktonic cells with MIC values ranging from 0·06–0·25 v/v, followed by clove oil with MIC values (0·12–0·25 v/v) in all tested KP strains (Table 2). On the other hand, no observable antibacterial activity could be recorded for ginger, nigella and peppermint oils in all tested concentrations up to 4% (v/v) which was considered as ½ MIC for the following biofilm inhibition experiments.

Although peppermint oil did not seem to have any antimicrobial activity upon KP planktonic cells, it showed significant biofilm inhibiting ability at 1/16 and 1/8 MIC (Fig. 1a), with an inhibition percentage 98·2% which was the highest inhibitory percentage reached in all tested KP strains (Fig. S1). A further increase in peppermint concentration (¼, ½ MIC) demonstrated no significant difference. Following peppermint oil, thyme oil showed inhibitory effect at subinhibitory concentration (95·2%) followed by nigella oil (77·2%). However, no significant inhibitory effect was recorded for caraway, cinnamon, clove and ginger oils (Fig. 1a).

Thyme oil was found to have the best biofilm eradication ability at 1, 2 and 4% (v/v) concentrations, causing eradication percentage to range from 80·1 to 98·0% (Fig. 1b) followed by peppermint and cinnamon oils against all tested strains (Fig. S2).

On the other hand, relative antibiofilm ability was recorded for biological enzymes, inducing inhibition ranging from 1·4 to 85·5% and eradication ranging from 1·3 to 74·6% (Fig. 2a,b). Bromelain was recorded the best biofilm eradicator enzyme, followed by α-amylase and β-amylase, while no clear activity was observed for biofilm inhibition (Figs S3 and S4).

Gas chromatography/mass spectrometry (GC/MS) analysis of the two most effective oils (Table 3) shows that 40 compounds are identified in peppermint oil, representing 99·9% of the oil. Menthol (41·6%) was predominant followed by eucalyptol (12·2%) and isomenthone (10·0%)

Table 2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of essential oils used against planktonic *Klebsiella pneumoniae* strains

	Essential oils (%v/v)*													
	Caraway		Cinnamon		Clove		Ginger		Nigella		Peppermint		Thyme	
Isolates	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
KP1	0·5	0·5	0·125	0·125	0·25	0·25	>4	>4	>4	>4	>4	>4	4	>4
KP2	0·5	0·5	0·125	0·25	0·125	0·25	>4	>4	>4	>4	>4	>4	2	2
KP3	0·5	0·5	0·125	0·125	0·25	0·25	>4	>4	>4	>4	4	>4	0·25	0·5
KP4	0·5	1	0·125	0·125	0·25	0·25	>4	>4	>4	>4	>4	>4	1	2
KP5	0·5	0·5	0·06	0·125	0·125	0·25	>4	>4	>4	>4	>4	>4	4	>4
KP6	0·5	0·5	0·125	0·125	0·25	0·25	>4	>4	>4	>4	>4	>4	4	>4
KP7	0·5	0·5	0·25	0·25	0·125	0·25	>4	>4	>4	>4	>4	>4	1	1
KP8	1	1	0·25	0·25	0·25	0·25	>4	>4	>4	>4	>4	>4	>4	>4
KP9	0·5	0·5	0·125	0·25	0·25	0·25	>4	>4	>4	>4	>4	>4	2	2
KP10	0·5	0·5	0·125	0·125	0·125	0·25	>4	>4	>4	>4	>4	>4	4	>4

*Essential oils' final concentrations ranged from 0·03–4 (%v/v).

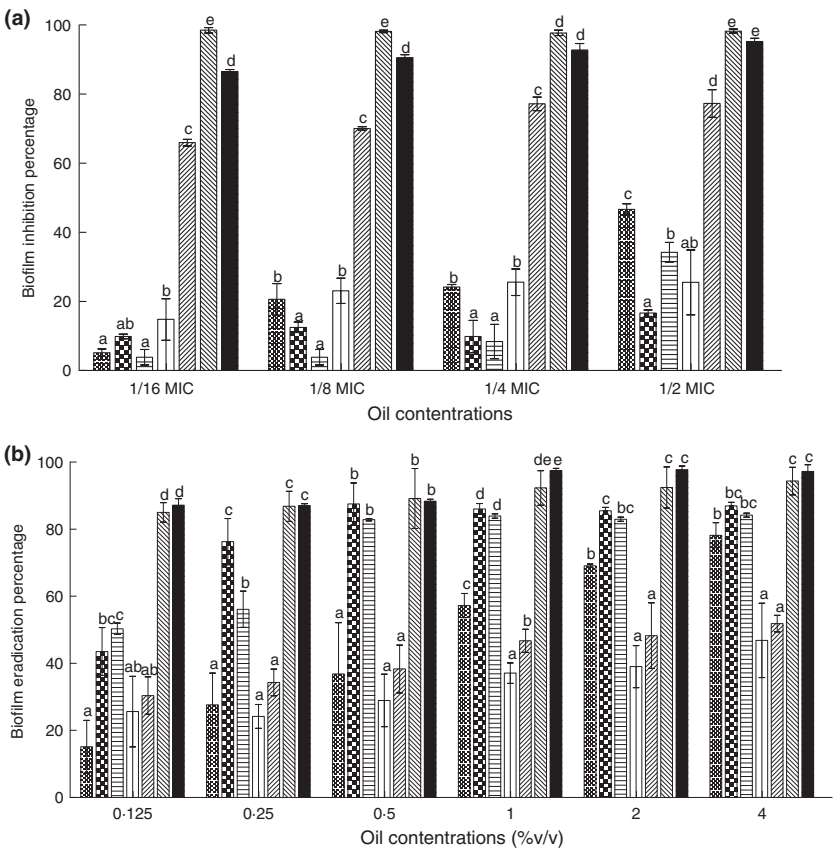

Figure 1 Effect of different concentrations of tested essential oils on *Klebsiella pneumoniae* (KP10). (a) Biofilm inhibition at subinhibitory concentrations of essential oils ½ MIC, ¼ MIC, ⅛ MIC and 1⁄16 MIC. (b) Biofilm eradication at concentrations of essential oils ranging from 0·125–4% v/v. The bars on the graph represent mean ± SE as a percentage of biofilm inhibition/eradication of triplicate independent experiments (n = 3). The different letters above the bars indicate significant differences ($P < 0·05$) within the same concentration. Mean values sharing at least one common letter are not significantly different (▩ caraway; ▣ cinnamon; ▤ clove; ▥ ginger; ▨ nigella; ▧ peppermint; ■ thyme).

with relatively low concentrations. Thyme oil was found to be qualitative but not rich, with 19 identified compounds, representing 99·9% of the oil. It is mainly composed of thymol (51·8%), *p*-Cymene (29·1%) and linalool (5·2%).

Menthol was investigated for its antimicrobial and biofilm inhibitory ability, showing that no antimicrobial activity was detected even in high concentrations, while strong biofilm inhibitory activity was detected causing biofilm inhibition 75·3–97·5% at 2·5 μg ml^{-1}. Thymol

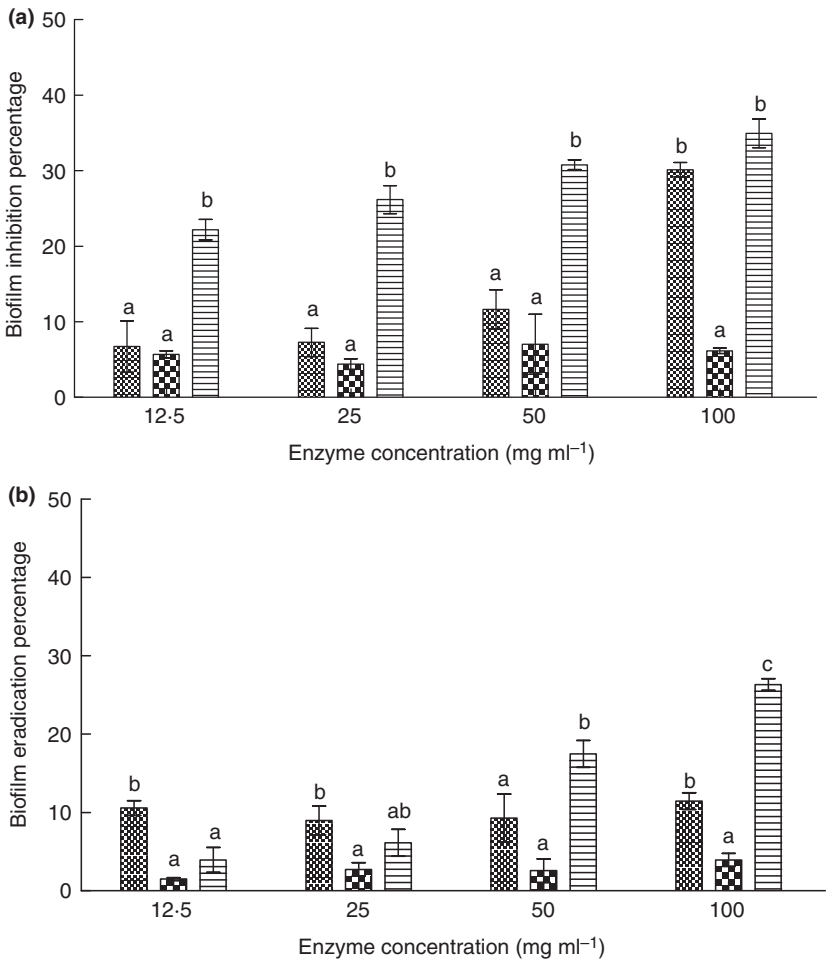

Figure 2 Effect of α-amylase, β-amylase and bromelain enzymes different concentrations (12·5–100 mg ml^{-1}) on *Klebsiella pneumoniae* (KP10). (a) Inhibition of biofilm by enzymes at different concentrations and (b) eradication of biofilm by enzymes at different concentration. The bars on the graph represent mean ± SE as a percentage of biofilm inhibition/eradication of triplicate independent experiments ($n = 3$). The different letters above the bars indicate significant differences ($P < 0·05$) within the same concentration. Mean values sharing at least one common letter are not significantly different (▩ alpha amylase; ▩ beta amylase; ▤ bromelain).

was investigated for its biofilm eradication ability and biofilm cell viability, and the results showed high biofilm eradication ability at 5 μg ml^{-1} causing 85·1–97·8% eradication, but no activity against biofilm cells viability was recorded. Therefore, the biofilm inhibitory ability of peppermint oil and menthol as well as the biofilm eradication ability of thyme oil and thymol was further characterized by SEM.

SEM was employed to determine the inhibitory and eradication effects of the best tested compounds on biofilms (Figs 3 and 4). The SEM micrographs for untreated KP experimental groups show many tightly packed cells encased in a thick matrix, alongside fewer number of regular free cells with well-defined rod shape and normal smooth surfaces (Fig. 3a–c). The KP cells treated with 0·5% peppermint oil showed the predominant release of cells from biofilms with a significant change in the morphology of the cells (Fig. 3d–f). Remarkable shrinkage of the cells treated with menthol was observed (Fig. 3g–i).

Significant eradications in the established biofilm of the KP cells have also been found compared with untreated control (Fig. 4a,b). The thyme oil-treated and thymol-treated biofilms were found to be completely dispersed in all tested fields (Fig. 4c,d) and (Fig. 4e,f) respectively. However, partial eradication was found in bromelain-treated biofilm as protein hydrophilic water sheath could be observed in some collected cells (Fig. 4g,h).

In order to be tested for their antibacterial and antibiofilm activity, pure thymol and menthol were tested in combination with ciprofloxacin. Results showed strong synergistic activities with ciprofloxacin which affect the viability of biofilm cells, but limited effect was recorded for peppermint oil and menthol on ciprofloxacin MIC of KP biofilm (Table 4). Thymol antibiofilm concentration was found to decrease ciprofloxacin effective dose by (1–6) bifolds against planktonic cells, and by (1–4) bifolds against biofilm cells (except KP2 and KP3), followed by thyme oil which cause 1–5 and 1–2 bifolds decrease in ciprofloxacin dose against planktonic and biofilm cells respectively (except KP2, KP3 and KP9) (Table 4).

Table 3 Chemical composition of peppermint and thyme essential oils

ID	Compound	RI	Peak area (%)		Identification method
			Peppermint oil	Thyme oil	
1	α-Thujene	921	0·32	–	MS, RI
2	α-Pinene	924	3·58	0·08	MS, RI
3	cis-3-Methylcyclohexanol	936	0·04*	–	MS
4	Camphene	941	0·07	–	MS, RI
5	Cyclohexanone, 3-methyl-, (R)-	944	0·11	–	MS
6	trans-p-Menthane	969	–	0·04*	MS
7	Sabinene	970	1·32	–	MS, RI
8	β-Pinene	974	2·00	1·28	MS, RI
9	Menthomenthene	980	0·04*	–	MS
10	Myrcene	987	0·34	0·55	MS, RI
11	3-Octanol	995	0·35	–	MS, RI
12	trans-beta-Ocimene	1003	0·04*	–	MS
13	Pseudolimonene	1004	–	0·02*	MS, RI
14	m-Cymene	1021	–	0·15	MS
15	p-Cymene	1024	0·69	29·05	MS, RI
16	Limonene	1028	4·59	3·68	MS, RI
17	Eucalyptol	1031	12·20	0·13	MS, RI
18	γ-Terpinene	1059	0·15	5·03	MS
19	cis-Linalool oxide	1073	–	0·05*	MS, RI
20	trans-Linalool oxide (furanoid)	1088	–	0·05*	MS, RI
21	Tetrahydrolinalool	1097	0·04*	–	MS, RI
22	Linalool	1100	–	5·24	MS, RI
23	cis-p-mentha-1(7),8-dien-2-ol	1134	–	0·06	MS
24	neo-Isopulegol	1146	0·69	–	MS, RI
25	Menthone	1154	13·34	–	MS, RI
26	d-Menthol	1158	0·12	–	
27	3-Butyl-cyclohexanone	1163	0·53	–	MS
28	Isomenthone	1165	10·03	–	MS, RI
29	trans-Verbenol	1168	–	0·17	RI
30	**Menthol, cis-1,3,trans-1,4-**	**1178**	**41·61**	**–**	**MS, RI**
31	trans-Sabinenehydrate	1179	–	0·91	MS
32	Levomenthol	1185	0·73	–	MS
33	dl-Menthol	1191	0·25	–	MS
34	α-Terpineol	1191	0·65	–	MS, RI
35	1,3,6-Heptatriene, 2,5,6-trimethyl-	1199	0·04*	–	MS
36	Pulegone	1244	0·91	–	MS, RI
37	Carvone	1248	0·03*	–	MS, RI
38	Piperitone	1257	0·63	–	MS, RI
39	trans-p-Mentha-2,8-dien-1-o	1260	–	0·11	MS
40	n-Decanol	1272	0·12	–	MS, RI
41	Menthyl acetate	1297	3·24	–	MS, RI
42	**Thymol**	**1302**	**–**	**51·80**	**MS, RI**
43	Carvacrol	1307	–	1·58	MS, RI
44	gamma-Elemene	1341	0·04*	–	MS
45	β-Bourbonene	1388	0·12	–	MS
46	β-Elemene	1393	0·03*	–	MS, RI
47	Isocaryophyllene	1425	0·65	–	MS
48	β-Copaene	1433	0·03*	–	MS, RI
49	α-Amorphene	1460	0·11	–	MS
50	beta-ylangene	1506	0·07	–	MS
51	δ-Cadinene	1530	0·03*	–	MS, RI
52	Caryophyllene oxide	1592	0·03*	–	MS, RI
	Total		99·91	99·98	

Compounds listed in order of elution on HP-5MS column (RI—experimentally determined retention indices on the mentioned column by co-injection of a homologous series of n-alkanes C7-C29); MS: constituent identified by mass spectra comparison; RI: constituent identified by retention index matching; *: trace (<0·05%). Bold text represents the major component in each essential oil.

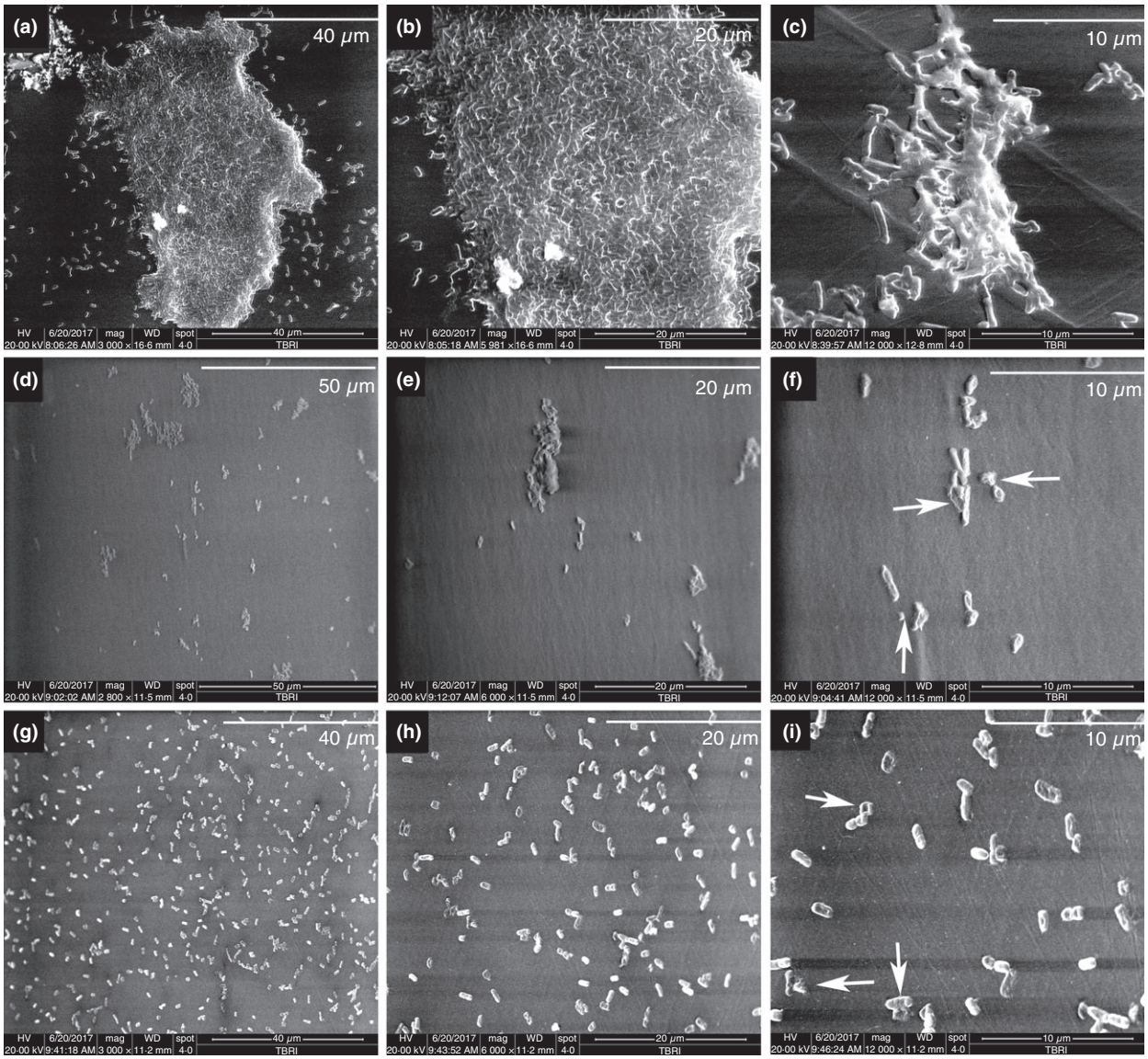

Figure 3 Scan electron micrographs of *Klebsiella pneumoniae* in BHI culture medium after 24 h without (a–c) or supplemented with peppermint oil at concentration of 5 μl ml^{-1} (d–f) or menthol at concentration of 2·5 μg ml^{-1} (g–i). White arrows indicate abnormal cells morphology. a, 3000×; b, 5981×; c, 12 000×; d, 2800×; e, 6000×; f, 12 000×; g, 3000×; h, 6000×; i, 12 000×.

Discussion

KP are emerging as one of the primary opportunistic pathogens causing a significant rate of mortality and morbidity in hospitals and the fact that most of these organisms are MDR causes serious concern in eradication of these infections (Dsouza *et al.* 2017). This high resistance to antibiotics is directly correlated with reduced drug penetration due to the presence of a physical barrier that is formed by the extracellular polymeric substance (EPS)-like capsule and biofilms (Abdulhasan *et al.* 2016). Recently, there has been an increased interest in the therapeutic properties of some medicinal plants and natural compounds which have demonstrated antibiofilm activities (Ceylan *et al.* 2014).

The direct activity of all tested EOs on planktonic cells of KP showed that cinnamon oil was the most effective oil followed by clove oil, caraway and then thyme oil. The recorded MIC is in line with the MIC values reported from previous studies against KP clinical isolates (Shenoy *et al.* 2014; Zenati *et al.* 2014; Sakkas *et al.* 2016; Oulkheir *et al.* 2017). On the other hand, no activity was detected for nigella, ginger and peppermint oils. Likewise, no antimicrobial activity of enzymes was detected in our

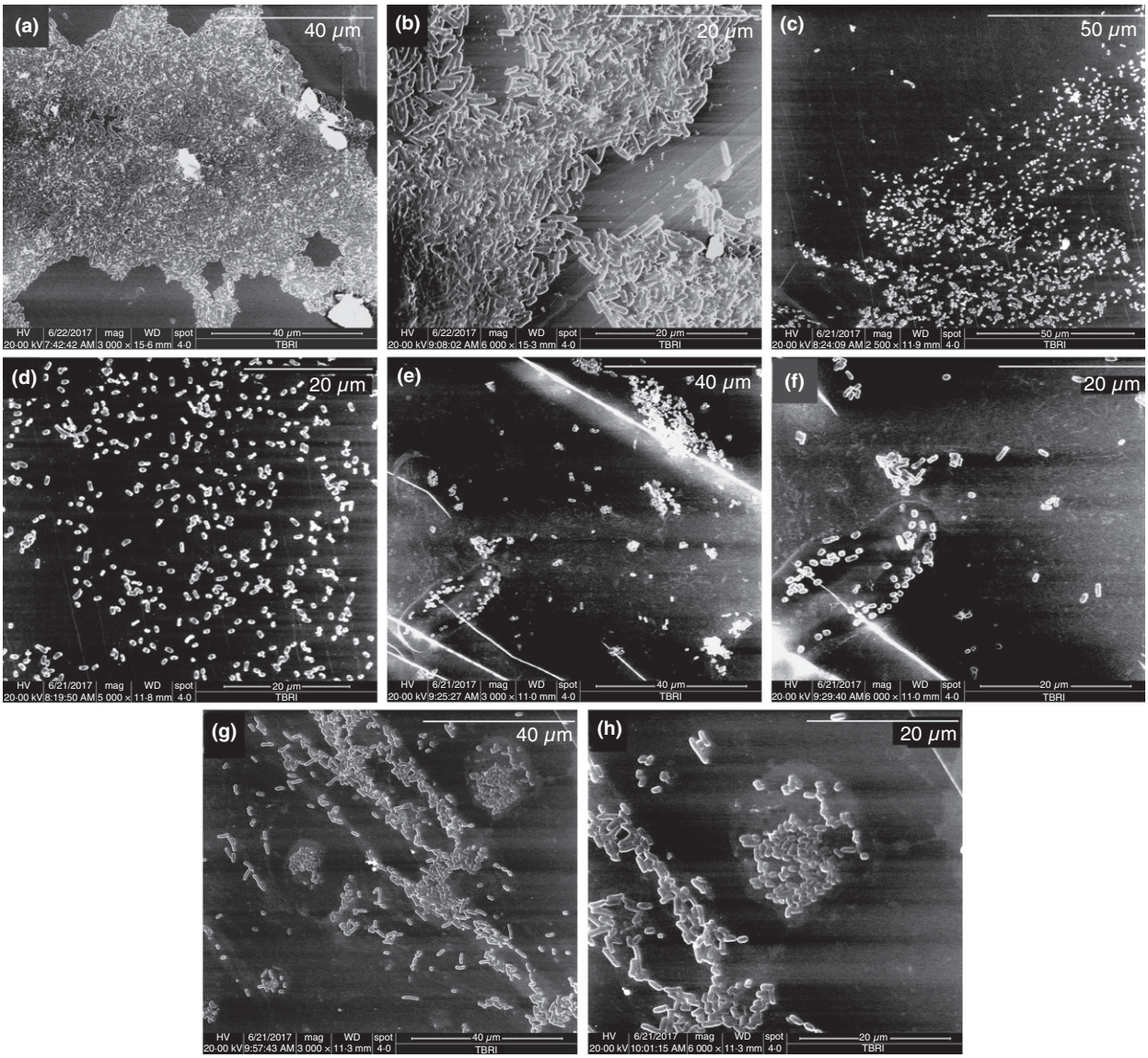

Figure 4 Scanning electron micrographs of the established biofilm of *Klebsiella pneumoniae* after 24 h without treatment (a, b); exposed to thyme oil at a concentration of 10 μl ml^{-1} (c, d); to thymol at a concentration of 5 μg ml^{-1} (e, f) or to bromelain at a concentration of 100 mg ml^{-1} (g, h). a, 3000×; b, 6000×; c, 2500×; d, 5000×; e, 3000×; f, 6000×; g, 3000×; h, 6000×.

study (Table 2), despite the antibacterial and antifungal activity of bromelain enzyme that have been reported before (López-García *et al.* 2012; Praveen *et al.* 2014). Screening studies of EOs against MDR-KP demonstrated no antimicrobial activity for ginger and peppermint EOs (Hammer *et al.* 1999; Prabuseenivasan *et al.* 2006). This suggests that there is a basal level of resistance provided by EPS in KP, or else, by efflux mechanism that expels the active compounds of EOs (Fadli *et al.* 2011).

Peppermint and thyme oil was found to have the best biofilm inhibition/eradication ability, respectively, at concentrations below the MIC, compared with other tested oils. In the literature survey, only few reports on inhibitory activity of natural products against biofilm-producing KP were available. It was reported that eucalyptus oil and thyme oil had strong biofilm inhibitory activity against biofilm-producing isolates including *Klebsiella*, while clove oil showed weak inhibition (Mathur *et al.* 2013; Al-Shuneigat *et al.* 2014a). Eradication of biofilms is difficult to achieve because of host defences and inherent resistance to antibiotics (Romero *et al.* 2016). Biofilm eradication activity of oregano, lemongrass and grapefruit EOs was demonstrated against *Staphylococcus* biofilms (Nostro *et al.* 2007; Adukwu *et al.* 2012). Recently,

Table 4 MIC of ciprofloxacin (μg ml^{-1}) and in combination with antibiofilm agents against *Klebsiella pneumoniae*

ID	Cip.	+Peppermint oil	+Menthol	+Thyme oil	+Thymol
Planktonic cells					
KP1	16	8 (1)	4 (2)	4 (2)	2 (3)
KP2	ND	16 (>3)	16 (>3)	8 (>4)	4 (>5)
KP3	16	8 (1)	4 (2)	8 (1)	2 (3)
KP4	16	16 (0)	16 (0)	8 (1)	8 (1)
KP5	32	16 (1)	2 (4)	8 (2)	2 (4)
KP6	16	16 (0)	16 (0)	8 (1)	4 (2)
KP7	64	32 (1)	32 (1)	8 (3)	2 (5)
KP8	16	8 (1)	2 (3)	4 (2)	1 (4)
KP9	16	16 (0)	16 (0)	8 (1)	4 (2)
KP10	ND	16 (>3)	16 (>3)	4 (>5)	2 (>6)
Biofilm cells					
KP1	32	32 (0)	32 (0)	8 (2)	4 (3)
KP2	ND	ND	ND	ND	ND
KP3	ND	ND	ND	ND	ND
KP4	ND	ND	ND	32 (>1)	32 (>1)
KP5	ND	ND	ND	32 (>1)	16 (>2)
KP6	32	32 (0)	32 (0)	16 (1)	8 (2)
KP7	ND	ND	ND	32 (>1)	8 (>3)
KP8	ND	32 (>1)	32 (>1)	32 (>1)	16 (>2)
KP9	ND	ND	ND	ND	16 (>2)
KP10	ND	ND	ND	16 (>2)	8 (>4)

Final concentration: Cip; ciprofloxacin (0·25–64 μg ml^{-1}), peppermint oil (0·5% v/v), thyme oil (1% v/v), menthol (2·5 μg ml^{-1}) and thymol (5 μg ml^{-1}). Values in parentheses represent number of bifold reduction of ciprofloxacin effective dose. ND (not detected).

Mentha pulegium EO effective biofilm eradication activities against MDR *Acinetobacter baumannii* was reported at concentration 10 μl ml^{-1} (Tutar *et al.* 2016).

Scarce research was conducted to test the inhibitory/eradiation activity of biological enzymes against KP biofilms. α-amylase extracted from *Bacillus* species tested against authenticated KP ATCC 14579 strain reported inhibition of biofilm ranged from 19 to 24·5% (Gomaa 2013). The mechanism by which enzymes act on the physical integrity of the EPS may be through disruption of the proteins, carbohydrate and lipid making up the structures of the EPS through the degradation process (Kalpana *et al.* 2012). In our study, the relatively poor biofilm inhibition activity of β-amylase may be explained by the fact that β-amylase hydrolyses the α-1,4-glucosidic linkages of polysaccharide only from the nonreducing end (Craigen *et al.* 2011). However, α-amylase can act simultaneously anywhere on the substrate, thus biofilm inhibition activity of α-amylase (11·7–84·8%) tends to be faster-acting than β-amylase (1·4–11·3%).

There is commonly observed correlation between the chemical structures of the prevalent constituents in the EOs under investigation and the antimicrobial/antibiofilm activities. Therefore, GC/MS analysis was performed and thymol was found to be the major compound that characterized the chemotype of *T. vulgaris* oil representing the highest percentage of thymol in comparison with all other studies from different countries (Miladi *et al.* 2013; Al-Shuneigat *et al.* 2014b; Santurio *et al.* 2014; Satyal *et al.* 2016). It is well known that the components and chemical characteristics of EOs vary with plant maturity, variety, geographical region, growth and processing conditions which affect their antimicrobial/antibiofilm activity. While menthol in *M. piperita* EO was found to be higher than those from Morocco, India and Taiwan, it was lower than those from Iran and Washington (İşcan *et al.* 2002; Derwich *et al.* 2010; Saharkhiz *et al.* 2012; Al-Shuneigat *et al.* 2014b).

The components with phenolic structures, such as thymol and menthol exhibit strong antimicrobial activity against MDR bacteria (Sakkas *et al.* 2016). This could explain the enhanced antibiofilm activity of peppermint and thyme oils. The antibiofilm activities have been justified by the SEM micrograph observations of KP in which both biofilm and cell morphology of treated bacteria were significantly affected (Figs 3 and 4). The cell abnormalities could be attributed to the phenolic constituents, especially menthol, of peppermint oil. Supporting this hypothesis, the phenolic compounds are capable of altering cell permeability due to their lipophilicity allowing them to penetrate the plasma membranes, while uncoupling the oxidative phosphorylation may be accomplished by the hydroxyl groups (Mohamed *et al.* 2017).

The enhanced activity of ciprofloxacin recorded in this study is in line with the results demonstrated in a previous study of thyme and peppermint oils' combination with ciprofloxacin against KP planktonic cells (Van Vuuren *et al.* 2009).

In conclusion, our results demonstrate that thyme oil, peppermint oil and their major components are promising antimicrobial and antibiofilm agents alone and/or in combination with ciprofloxacin. The use of these combinations is a suggested potential application against infections caused by *K. pneumoniae,* to minimize ciprofloxacin's effective dose cost and the potential accompanying toxic side effects. However, further work is required to assess the therapeutic application of these combinations.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical approval

Not required.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Effects of the tested essential oils (caraway, cinnamon, clove, ginger, nigella, peppermint and thyme) on biofilm inhibition of different biofilm-producing *Klebsiella pneumoniae* strains KP1-KP9 (a–i) using the microtitre plate test and crystal violet assay.

Figure S2 Effects of the tested essential oils (caraway, cinnamon, clove, ginger, nigella, peppermint and thyme) on biofilm eradication of different biofilm-producing *Klebsiella pneumoniae* strains KP1-KP9 (a–i) using the microtitre plate test and crystal violet assay.

Figure S3 Effects of the enzymes α-amylase, β-amylase and bromelain on biofilm inhibition of biofilm-producing *Klebsiella pneumoniae* strains KP1-KP9 (a–i) using the microtitre plate test and crystal violet assay.

Figure S4 Effects of the enzymes α-amylase, β-amylase and bromelain on biofilm eradication using the microtitre plate test and crystal violet assay on biofilm-producing *Klebsiella pneumoniae* strains KP1-KP9 (a–i).