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Antibiofilm activity of papain enzyme against pathogenic *Klebsiella pneumoniae*

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ABSTRACT

Infections caused by biofilm-embedded pathogens decrease the efficacy of traditional treatments and increase antibiotic tolerance. Most of the human bacterial infections are biofilm-associated. Therefore, this study aimed to investigate the antimicrobial as well as the antibiofilm activity of papain enzyme on drug-resistant biofilm producing *Klebsiella pneumoniae*. Effect of papain enzyme was tested on *Klebsiella pneumonia* planktonic cells as well as on the formation, eradication and cells viability of biofilm. Although no antimicrobial activity of papain enzyme was detected against planktonic cells, significant biofilm inhibition and eradication were recorded. Biofilm inhibition of different *K. pneumonia* strains ranged from (10.6-56.2%) at concentration 50 mg/mL and increased to (21.4-59.0%) at 100 mg/mL papain. Furthermore, noticeable biofilm eradication was recorded (7.7-54.9%) and (9.6-55.6%) at 50 mg/mL and 100 mg/mL papain concentration respectively. Nevertheless, no significant activity was detected on biofilm cells viability. Scanning electron microscopy (SEM) confirmed the inhibitory and eradication activity of papain on *K. pneumoniae* biofilm. This study demonstrated for the first time that papain enzyme exerts an antibiofilm effect against drug-resistant *K. pneumoniae*, but no antibacterial activity was detected, suggesting its potential application as an antibiofilm agent in combination with traditional antibacterial agents.

INTRODUCTION

Antimicrobial resistance is an emerging global healthcare crisis with significant impact on human health (Jana *et al.,* 2017). The spread of this resistance and the emergence of nosocomial infections caused by resistant bacteria are factors that adversely affect the course, duration and cost of hospitalizations and mortality (Karampatakis *et al.,* 2016). The prevalence of such resistance among Enterobacteriaceae is a threat observed in the last decades (Swathi *et al.,* 2016). *Klebsiella* is a Gram-negative, non-motile, encapsulated, lactose fermenting, facultative anaerobe belonging to the Enterobacteriaceae family, the second most popular member of the aerobic bacterial flora of the human intestine, and the most common causative agent of nosocomial and community-acquired infections, at which three to seven percent of hospital-acquired bacterial infections are related to *Klebsiella pneumoniae* (Khan *et al.,* 2015; Pereira and Vanetti, 2015; Shahraki-Zahedani *et al.,* 2016).

Currently, most bacterial infections (60–80%) are linked to microbial biofilm formation, a lifestyle in the bacterial community that presents inherent resistance to antibiotics and to host immune defense, and organisms which present in biofilm can be 10-1000 times more resistant to antimicrobials compared to their planktonic stage (Patel *et al.,* 2014; Ribeiro *et al.,* 2016). Capsular polysaccharides, type 1 and type 3 pili, are the most important virulence factors contributing to *K. pneumoniae* pathogenesis, and also found to contribute to biofilm formation (Seifi *et al.,* 2016).

The fact that the natural medicines were found to be much safer than synthetic drugs, has led to a resurgence of scientific interest in their biological effects (da Silva *et al*., 2010). Plants contain various biologically active compounds such as phenolics, alkaloids, carbohydrates, proteins, essential oils, and enzymes, to mention a few of which have antimicrobial and medicinal activities (Abdelhadi and Mohamed, 2016; Mohamed *et*

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al., 2017; Mohamed *et al*., 2018a). Plant proteolytic enzymes have received attention in the field of medicine and biotechnology due to their proteolytic properties, and also have been widely used in the medical-pharmaceutical, cosmetic and other industries (Malek *et al.,* 2016).

Papain is an important peptidase enzyme derived from the papaya plant (*Carica papaya* L), including family Caricaceae, known for its proteolytic and anti-inflammatory action and adopted as a topic debridement agent in skin wounds (Gartika *et al.,* 2014; dos Anjos *et al.,* 2016). Usage of papain is indicated for clean and infected wounds at different phases of the healing process (De Siqueira Mota *et al*., 2015). It is also bactericidal, bacteriostatic, anti-inflammatory and debridement material and shows a broad proteolytic activity against the protein, short chain peptides, amino acid ester and amid (Gartika *et al.*, 2014).

Therefore, we are in need of searching out newer infection-fighting strategies against newer worse strains of pathogenic microbes with higher resistance. This exploration of newer strategies is a global challenge taking by many research institutions, pharmaceutical companies, and academic institutions, because of resistance of pathogens to a commercially available antibiotic (Islam *et al.*, 2015).

According to our previous study we had concluded that among biological enzymes proteolytic enzyme (bromelain) was the best to achieve inhibition and eradication of biofilms of *K. pneumoniae* (Mohamed *et al.*, 2018b), and considering the proteolytic characteristic described for papain and the biopolymer matrix composition of bacterial biofilms, this study aimed to evaluate the ability of papain to act as an inhibitor for *K. pneumoniae* biofilms in different concentrations.

MATERIALS AND METHODS

Microbial strains and enzyme

Ten biofilm-producing multi-drug resistant (MDR) *K. pneumoniae* strains from our previous study were used (Mohamed *et al.*, 2018b). Strains were previously identified by conventional microbiological methods and confirmed by MALDI-TOF/MS using Bruker Biotyper 3.1 software. Antibiotic susceptibility testing was done according to CLSI (2016). Papain from *Carica papaya* (≥3 U/mg) was obtained from Sigma-Aldrich (St. Louis, USA). Dilutions of papain ranged from 3.125-100 mg/ml.

Effect of papain on planktonic cells

The bi-fold serial concentrations of papain enzyme (100, 50, 25, 12.5, 6.25 and 3.125 mg/mL) were tested against planktonic cells of *K. pneumoniae* in Mueller-Hinton broth using the broth dilution method according to Doughari and Manzara (2007) with minor modification. In each well of the sterile 96-tissue culture plate, 100 µl of bacterial suspension (0.5 MacFarland) and 100 µl of papain dilution were added and then incubated at 37°C for 24 hours. The lowest concentration of enzyme that can inhibit the visible growth of *K. pneumoniae* after overnight incubation considered minimal inhibition concentration (MIC), while the lowest enzyme concentration needed to kill bacteria as defined by the inability to re-culture on agar medium considered as minimum bactericidal concentration (MBC), that was evaluated by plating on Nutrient Agar after incubation at 37°C for 24 hours.

Effect of papain on biofilm formation ability

Inhibition of biofilm formation was assessed using a method mentioned by Nostro *et al*. (2007) with minor modifications. In each well of sterile 96 tissue culture plate, 100 µl of bacterial suspension (0.5 MacFarland) and 100 µl of papain dilution were added and then incubated at 37°C for 24 hours. After aspiration of planktonic cells, plates are washed twice with phosphate buffer saline (PBS) or sterile saline water and air-dried. Then, 200 µl of crystal violet solution (1%) was added to all wells. After three minutes, the excess dye was removed and plates were washed twice and air dried. The negative control was uninoculated Brain Heart Infusion (BHI) broth, whereas the positive control contained cell cultures alone with no treatment. Biofilm growth was monitored in terms of $O.D_{630}$ nm using microplate reader (STAT FAX 2100) in triplicate.

Effect of papain on established biofilms

The method used was similar to that described by Nostro *et al*. (2007). After biofilm formation for 24 hours, the medium was discarded, and the wells gently rinsed twice with PBS. A total of 200 µl of each treatment concentration were added into the wells. The negative control was biofilm without treatment. After incubation for 24 hours at 37°C wells were aspired and the plates are washed twice with phosphate buffer saline or sterile saline water and air-dried. Then, 200 µl of crystal violet solution (1%) was added to all wells. The OD was measured at 630 nm using a Microplate reader (STAT FAX 2100, USA) in triplicate.

Effect of papain on biofilm cells viability

Biofilm cell viability assay was done according to the method mentioned by Mah (2014) using BHI broth, to test the ability of biofilm cells to recover after treatment. After formation of the biofilm for 24 hours, the medium was discarded, and the wells gently rinsed twice with PBS. A total of 100 µl of each treatment concentration were added into the wells and incubated 24 hours at 37°C, the MBC was evaluated by plating on Nutrient Agar after incubation at 37°C, 24 hours.

Scanning electron microscopy (SEM)

SEM was employed for investigating the effect of papain enzyme on *K. pneumoniae* biofilm. Sections of the interior of polystyrene tubes coated with bacterial biofilm were fixed for 2 hours in equal volumes of 4% glutaraldehyde and 0.2 M cacodylate and washed in equal volumes of 0.4 M saccharose and 0.2 M cacodylate for 2 hours, then post-fixed in 2% (w/v) osmium tetroxide and 0.3 M cacodylate for 1 hour. The samples were washed with deionized water and finally dehydrated in ascending grades of ethanol for five minutes each and finally 100% absolute ethanol for 10 minutes, then examined with Philips XL30 scanning electron microscope (Eindhoven, Netherlands) operated at 20KV (Ansari *et al.*, 2013).

Cluster analysis

Hierarchical cluster analysis was carried out based on the results of biofilm inhibition and eradication of different *K. pneumoniae* strains at different enzyme concentrations using SPSS software (SPSS Inc. v. 12). The positive results of biofilm inhibition or eradication for each strain were coded as ‘1; more

than 50%' and negative results coded as '0; less than 50%'. The hierarchical cluster was presented graphically to find the strains that are most similar, clustering of the samples was performed based on average linkage, the branch length represents the distance between the strains.

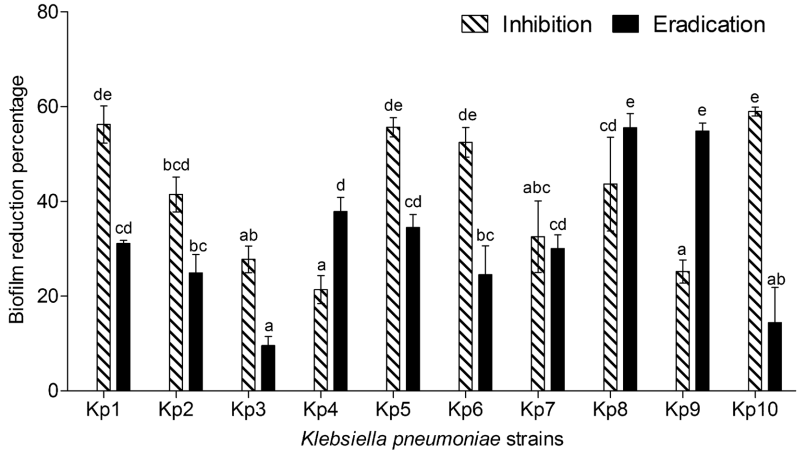

Fig. 1: Biofilm inhibition and eradication percentages of papain enzyme against biofilm-producer *Klebsiella pneumoniae* strains at concentration 100 mg/ml.

Statistical analysis

All of the data were analyzed by SPSS statistical software, version 12.0. One-Way ANOVA and a post hoc multiple comparisons (Duncan test) were used to compare the effect of various concentrations of the enzyme on bacterial strains. Values were considered statistically significant at $P < 0.05$. Quantitative variables were expressed as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Recently the most alarming is that bacteria with regular sensitivity to antibiotics are even able to develop a strategy to survive, and the appearance of side effects of antibiotics has led to search for new antimicrobial agents to overcome more disadvantage. Hence, the discovery of anti-infective agents which are active against planktonic microorganisms as well as microbial biofilms represents an ultimate objective (Ayukekbong *et al*., 2017). Identification of the bacterial strains used in this study was previously confirmed using MALDI-TOF technology, which allows accurate bacterial identification of a large variety of species in reduced time using only a small amount of microbial biomass (De Carolis *et al.*, 2014; Kang *et al.*, 2017).

The current study indicates that no antibacterial effect against planktonic cells was detected when using papain even in high enzyme concentration (100 mg/mL). In this context, several studies tried to test the antibacterial activity of papain on Gram-positive and Gram-negative bacteria, as a novel strategy against bacteria. Lima *et al.* (2009) verified that papain has no antibacterial activity for *E. faecalis, P. aeruginosa, S. aureus, K. pneumoniae* and *S. typhi* indifferent concentrations. In addition, de Oliveira *et al*. (2014) stated that no antibacterial activity of papain was observed for any isolates of methicillin-resistant *S. epidermidis* and methicillin-resistant *S. haemolyticus* tested, which is in line with our results. In contrast, other study reported that papain has antibacterial activity against *Streptococcus mutans* (Gartika *et al.*, 2014). Papain was reported to have more significant activity against bacteria than fungi, recorded zone of inhibition against *P. aeruginosa, B. subtilis, E. coli,* and *S. aureus* was 25, 22, 24 and 18 mm, respectively (Seenivasan *et al.*, 2010).

The bacterial ability to adhere and form biofilms on biotic and abiotic surfaces causes an increase in virulence, as well as bacterial pathogenicity, where bacteria in biofilms are already up to thousands of times more resistant to antibiotics when compared to their free-floating, planktonic counterparts (Schroeder *et al.,* 2017). In our study, different biofilm reduction percentage was detected for inhibition and eradication of established biofilms when treated with different concentrations of papain enzymes (Table 1). The best reduction was achieved at the highest enzyme concentrations 100 mg/ml, at which the effect of papain on *Klebsiella* strains significantly different (Figure 1), causes biofilm inhibition and biofilm eradication ranged from 21.51% to 58.85% and 9.45% to 55.07%, respectively. Followed by enzyme concentration 50 mg/ml, causing biofilm inhibition and biofilm eradication ranged from 11.07% to 55.99% and from 7.83% to 53.82%, respectively (Figure 2). Despite the antibiofilm activity, no effect of biofilm cell viability was detected for any strain. Recently, it was reported that enzymatically active papain induces the immune and/or allergic responses *in vitro* in human primary keratinocytes through degradation of tight junctional proteins leading to permeabilize the skin barrier (Stremnitzer *et al.*, 2015). On the other hand, clinical studies on *Carica papaya* aqueous leaf extract with a dose of 2000 mg/kg body weight indicated no signs of toxicity on tested rats (Halim *et al.*, 2011). Papain enzyme has shown immunomodulatory and anti-inflammatory activities by influencing the levels of inflammatory markers (Mohr and Desser 2013; Pandey *et al.*, 2016). Therefore, papain should be handled with care and more *in vivo* studies still need to be performed.

Cluster analysis of converted biofilm inhibition and eradication data was performed, and similarities between strains were presented in a dendrogram (Figure 3). The resulting dendrogram showed two main clades. The upper one is comprised of two subclades. *K. pneumoniae* with different susceptibility are separated alone to the bottom whereas comparable strains

were clustered together. In this context, the clustering analysis tool was applied to bacterial antibiotic susceptibility to survey the prevalence of known and/or unknown bacterial antibiotic susceptibility and complete phenotypic classification (Berrazeg *et al.*, 2013).

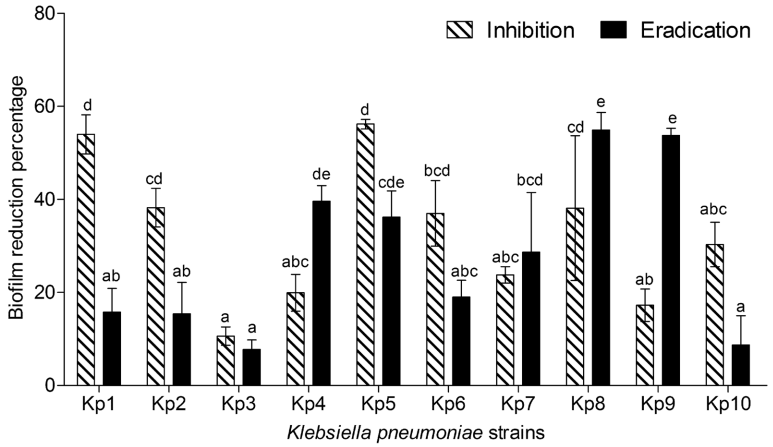

Fig. 2: Biofilm inhibition and eradication percentages of papain enzyme against biofilm-producer *Klebsiella pneumoniae* strains at concentration 50 mg/ml.

Table 1: Biofilm reduction percentages of *Klebsiella pneumoniae* by different papain concentrations.

Enzyme Concentration (mg/ml)	Reduction percentage	*Klebsiella pneumoniae* strains									
		Kp1	Kp2	Kp3	Kp4	Kp5	Kp6	Kp7	Kp8	Kp9	Kp10
3.125	Inh% ± SD	8.20 ± 5.21	30.35 ± 10.32	8.54 ± 6.40	6.42 ± 4.48	24.09 ± 6.06	10.59 ± 7.76	6.60 ± 7.46	7.83 ± 2.13	3.80 ± 1.74	11.92 ± 8.36
	Era% ± SD	8.55 ± 12.25	10.83 ± 8.26	4.34 ± 1.96	11.10 ± 6.57	16.33 ± 4.65	1.67 ± 1.08	11.73 ± 8.01	27.91 ± 5.24	26.06 ± 23.10	1.98 ± 1.24
6.25	Inh% ± SD	8.37 ± 1.79	32.48 ± 6.71	9.87 ± 6.66	7.22 ± 3.84	28.88 ± 4.59	11.95 ± 10.45	10.57 ± 8.33	7.81 ± 2.19	5.74 ± 0.88	17.48 ± 6.34
	Era% ± SD	14.18 ± 3.82	12.65 ± 7.80	6.51 ± 6.64	22.74 ± 15.79	25.58 ± 5.67	4.58 ± 4.51	10.57 ± 2.30	39.88 ± 10.43	42.26 ± 11.70	2.57 ± 0.59
12.5	Inh% ± SD	8.72 ± 2.10	32.58 ± 9.51	10.73 ± 4.31	9.61 ± 4.59	30.68 ± 4.42	11.74 ± 8.07	12.61 ± 4.30	10.64 ± 1.53	9.31 ± 1.73	18.73 ± 10.25
	Era% ± SD	17.75 ± 4.99	15.19 ± 9.01	6.17 ± 3.26	25.15 ± 4.84	27.49 ± 12.70	5.37 ± 2.95	14.87 ± 5.88	48.18 ± 4.85	52.12 ± 2.48	7.03 ± 5.25
25	Inh% ± SD	14.48 ± 5.18	37.94 ± 6.11	11.02 ± 4.44	20.24 ± 9.27	50.06 ± 5.36	31.97 ± 3.62	23.63 ± 4.33	10.14 ± 5.55	15.89 ± 1.28	18.26 ± 11.00
	Era% ± SD	22.44 ± 5.70	15.77 ± 8.22	8.02 ± 3.22	29.55 ± 5.26	34.30 ± 10.03	5.95 ± 1.53	16.28 ± 7.00	53.40 ± 3.06	53.61 ± 1.58	8.95 ± 6.13
50	Inh% ± SD	53.40 ± 7.26	38.96 ± 7.18	11.07 ± 3.45	19.82 ± 6.80	55.99 ± 1.81	38.82 ± 12.22	23.82 ± 3.09	37.69 ± 26.95	17.15 ± 6.01	29.81 ± 8.24
	Era% ± SD	30.10 ± 8.83	16.99 ± 11.73	7.83 ± 3.53	39.36 ± 5.81	36.79 ± 9.72	19.40 ± 6.29	27.95 ± 22.26	54.31 ± 6.53	53.82 ± 2.68	8.59 ± 10.88
100	Inh% ± SD	55.74 ± 6.79	42.27 ± 6.35	28.36 ± 4.86	21.51 ± 5.11	55.90 ± 3.47	53.08 ± 5.41	31.97 ± 13.05	43.38 ± 17.16	25.15 ± 4.24	58.85 ± 1.59
	Era% ± SD	31.12 ± 1.07	25.84 ± 6.65	9.45 ± 3.35	38.20 ± 5.18	35.13 ± 4.76	23.91 ± 10.54	30.07 ± 5.06	55.07 ± 5.16	54.94 ± 2.86	14.24 ± 12.86

Kp: *Klebsiella pneumoniae* strain, Inh%: Inhibition percentage, Era%: Eradication percentage, SD: Standard Deviation. All results are presented as averages of results from three independent replicates in three parallel trials.

Because the biofilm matrix is composed of DNA, proteins, and extracellular polysaccharides, recent studies have indicated that the disruption of the biofilm structure could be achieved via the degradation of individual biofilm compounds by various enzymes (Taraszkiewicz *et al.*, 2013), but only a few studies were found to test the inhibitory/eradiation activity of biological enzymes against bacterial biofilms in the literature survey (Mohamed *et al.*, 2018b). Gomaa (2013) tested alpha-amylase from *Bacillus subtilis* and *Bacillus cereus* against one *Klebsiella pneumonia* ATCC stain, stating the ability of alpha-amylase to inhibit its biofilm by 24.5% and 19% respectively. Also, a poor antibiofilm activity of β-Amylase compared with α-Amylase against *Staphylococcus aureus* was detected by Craigen *et al.* (2011) in addition to the study done by Kalpana *et al.* (2012) which stated the inhibitory activity of α-amylase of *B. subtilis* against *V. cholera*, *P. aeruginosa,* and MRSA.

SEM was the chosen method for analyzing the surfaces and the morphological changes in the biofilm cells exposed to papain enzyme (Figure 4). Regarding biofilm formation, colonized cells were detected in the untreated biofilm (control) (Figure 4 A), but for biofilm treated with papain, lower number of adhered cells was showed (Figure 4 B-D), confirming the results obtained phenotypically. On the other hand, deformation of *K. pneumoniae* cells was detected in the treated biofilm (Figure 4 D).

This is the first study to test the antibiofilm activity of papain enzyme against pathogenic *K. pneumoniae*, while in literature there is only one study was found to test antibiofilm activity of papain against biofilms of *Staphylococcus species*, stated that papain has been demonstrated as a potential product for reducing biofilm of Methicillin-resistant *Staphylococcus species* (de Oliveira *et al.*, 2014).

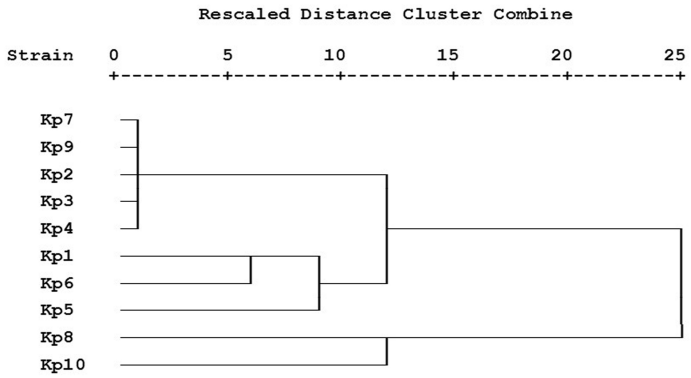

Fig. 3: Hierarchical tree obtained by using data from the biofilm inhibition and eradication percentages of papain enzyme against biofilm-producer *Klebsiella pneumoniae* strains at different papain concentration.

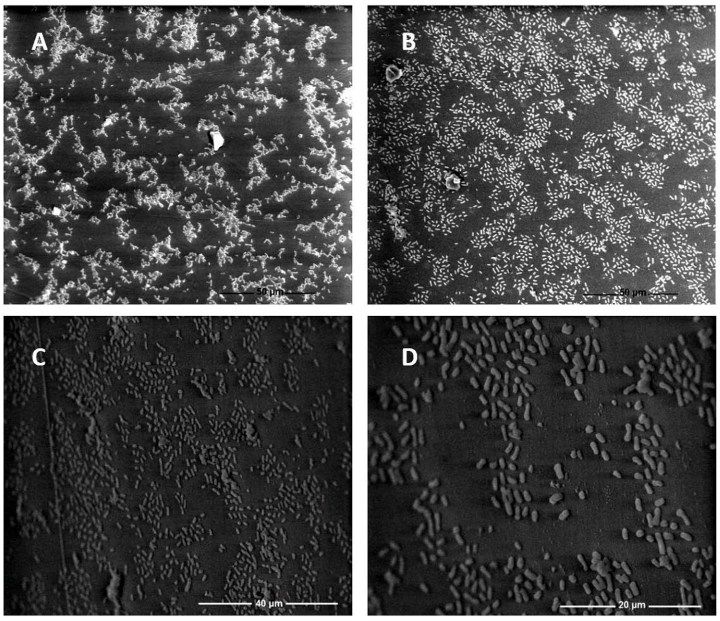

Fig. 4: Scanning Electron Microscopy Imaging (SEM) of *Klebsiella pneumoniae* biofilm in Brain Heart Infusion (BHI) broth after 24 hours incubation at 37 °C, (A) untreated biofilm at magnification 1600×, (B-D) biofilm treated with 100 mg/ml papain enzyme at magnification 1600×, 3000× and 6000×.

Nowadays biofilm is considered a major target for the pharmacological development of drugs. Thus, novel biofilm dispersal strategies that can more effectively release biofilm-associated microbes from the protection of the EPS could serve to bolster the arsenal of anti-biofilm therapeutics (Fleming and Rumbaugh, 2017; Mani and Mahalingam, 2017).

CONCLUSION

Papain enzyme exerts an antibiofilm effect against MDR *Klebsiella pneumoniae*, but no antibacterial activity was detected. Hence, it may be used as a potential antibiofilm agent in combination with traditional antibacterial agents.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

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