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Antimicrobial potential of *Aspergillus oryzae* secondary metabolites against carbapenem-resistant *Klebsiella pneumoniae*

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ABSTRACT

The antibacterial potential of *Aspergillus oryzae* (*A. oryzae*) EO product (AOEP) extract against *Klebsiella pneumoniae* (*K. pneumoniae*) strains BAA-1706 and BAA-1705 was investigated through minimal inhibitory concentration (MIC), biofilm inhibition assays, electron microscopy, and gene expression analysis. AOEP extract exhibited inhibitory activity with MIC_{50} values ranging from 3.1 % to 50 % for both strains. Notably, AOEP suppressed biofilm formation at low concentrations (3.1 %–12.5 %), outperforming the positive control, kanamycin, at 6.25 %. Morphological examination revealed significant alterations upon AOEP treatment, including reduced colony size and fragmented cells, distinct from kanamycin-induced changes. qRT-PCR demonstrated that AOEP significantly downregulated key virulence genes *ompA*, *lppA*, and *mrkA* in strain BAA-1706, and *ompA* and *lppA* in strain BAA-1705, while *pal* and *wzi* expression remained unaffected. LC-MS/MS profiling identified several lactone-related secondary metabolites, including acyl homoserine lactone (AHL) analogs and butyrolactone I analogs, suggesting possible quorum sensing interference as a mechanism of action. These findings highlight AOEP extract as a promising multifunctional antibacterial agent that disrupts *K. pneumoniae* growth, biofilm formation, and virulence, with potential applications in combating antibiotic-resistant infections.

1. Introduction

The rapid rise of multidrug-resistant (MDR) pathogens has emerged as one of the most pressing global health crises of the 21st century. *K. pneumoniae*, a major causative agent of nosocomial infections, has been classified as a critical priority pathogen by the World Health Organization due to its ability to acquire resistance to a broad range of antibiotics, including the β-lactam class of drugs [1]. Particularly concerning is the emergence of *K. pneumoniae* strains that produce *K. pneumoniae* carbapenemase (KPC), an enzyme capable of hydrolyzing the β-lactam ring of carbapenems, thereby rendering them ineffective [2]. Carbapenem-resistant *K. pneumoniae* (CRKP) infections are associated with high morbidity and mortality rates, particularly in immunocompromised individuals or those with comorbidities, compounded by limited treatment options and the ability of these pathogens to acquire additional mechanisms of resistance [3,4]. Consequently, there is an urgent need to develop novel antimicrobial agents that can effectively target these resistant pathogens and mitigate the public health threat posed by carbapenem resistance.

In response to the growing challenge of antibiotic resistance, the exploration of natural products, particularly microbial secondary metabolites, has gained substantial attention as a promising strategy for discovering new therapeutic agents [5]. Secondary metabolites are organic compounds produced by microorganisms, such as fungi, bacteria, and actinomycetes, which are not essential for their primary metabolic processes but often confer ecological advantages, including antimicrobial activity [6]. *A. oryzae*, a filamentous fungus renowned for its use in traditional food fermentation processes, has emerged as a rich source of secondary metabolites, including peptides, polyketides, and terpenoids, many of which exhibit potent antibacterial, antifungal, and anticancer properties [7–10]. Despite its extensive use in food production, *A. oryzae* has not been thoroughly investigated for its potential to combat antibiotic-resistant pathogens, particularly those producing β-lactamases such as KPC. Notably, our previous study demonstrates that *A. oryzae* secondary metabolites exhibit the ability to disrupt cell envelope integrity and inhibit quorum sensing-associated virulence factors and biofilm formation in *K. pneumoniae* extended-spectrum β-lactamase (KP-ESBL) strains [9,10]. Nonetheless, the molecular mechanisms underlying the antibacterial effects of these metabolites remain poorly understood, making *A. oryzae* as an attractive candidate

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for further investigation. Additionally, *A. oryzae* is recognized as a safe organism, classified as Generally Recognized as Safe (GRAS) by regulatory bodies like the US Food and Drug Administration (FDA) [11]. Its well-established safety profile, combined with its potential to produce novel antimicrobial agents, presents a unique opportunity to bridge the gap between traditional food-based applications and contemporary pharmaceutical needs. Herein, in this study, we aim to investigate the antimicrobial activity of secondary metabolites isolated from *A. oryzae* against carbapenem-resistant *K. pneumoniae* (CRKP) strains harboring KPC. Specifically, our objectives are to (1) isolate and characterize the bioactive metabolites produced by *A. oryzae* and (2) evaluate their antibacterial activity against CRKP strains.

2. Materials and methods

2.1. Bacterial and fungal isolates

K. pneumoniae carbapenemase (KPC)-producing strain (BAA-1705) and *K. pneumoniae* strain AIS 2007023 [6179], lacking the *blaKPC* gene (BAA-1706), were obtained from the Laboratory of Microbiology, Faculty of Medicine, Brawijaya University, Indonesia, while *A. oryzae* was provided by the Indonesian Culture Collection (Ina-CC). All experimental procedures were approved by the Ethical Committee of the Faculty of Medicine and Health Sciences, Maulana Malik Ibrahim State Islamic University (Ref. No. 027/EC/KEPK-FKIK/2020).

2.2. Preparation of A. oryzae extracellular protein (AOEP)

A 100 mL volume of potato dextrose broth (PDB) medium was inoculated with 8-mm A. *oryzae* mycelium and incubated in a 250 mL Erlenmeyer flask containing 2 % glucose for 72 h at 27 °C under static conditions (OD600 = 1.2). After incubation, the culture was filtered through 0.22-μm filter paper (Whatman, Sigma Aldrich). The supernatant, containing extracellular proteins, was centrifuged at 12000 rpm for 15 min at 4 °C. To precipitate the proteins, ammonium sulfate was added to 80 % saturation, followed by stirring in an ice bath for 1 h. The mixture was incubated overnight at 4 °C, and the precipitate was collected by centrifugation at 12000 rpm for 15 min at 4 °C. The crude protein extract was then dialyzed for 24 h in 0.01 M phosphate buffer (pH 7). Protein concentration was determined using the Bio-Rad Protein Assay (Bradford method), as previously described [12].

2.3. Determination of minimal inhibitory concentration

Minimum inhibitory concentration (MIC) was determined using microdilution broth [13]. Fresh bacterial cultures were inoculated into LB medium at a turbidity of 0.5 McFarland standard. Each well of a 96-well plate was inoculated with 100 μL of bacterial suspension and treated with either AOEP with various concentrations (3.1, 6.25, 12.5, 25, and 50 %). After 24 h of incubation at 37 °C, absorbance was measured at 600 nm. The MIC was defined as the lowest concentration reducing absorbance by >90 % compared to the negative control. All experiments were performed in triplicate.

2.4. Biofilm inhibition assay

The biofilm inhibition assay was performed following previously established protocols [10]. Briefly, 100 μL of AOEP at varying concentrations (3.1 %, 6.25 %, and 12.5 %) was added to each well of a 96-well microplate, followed by the addition of 100 μL of BAA-1706 or BAA-1705 bacterial suspension. The negative control well contained LB medium supplemented with 1 % glucose, while the positive control well contained either 64 μg/mL kanamycin or J8-C8 (Sigma), an AHL analogs (AHL synthase inhibitor, ProbeChem), at a concentration of 50 μM. The plate was then incubated at 37 °C for 24 h, and stained with 0.1 % crystal violet. After several washes, the stain was dissolved in 96 % ethanol, and the absorbance was measured at 570 nm using a microplate reader.

2.5. Scanning electron microscopy (SEM)

To evaluate the effect of AOEP on the morphology of both of BAA-1706 and BAA-1705, scanning electron microscopy (SEM) was performed. Briefly, the bacteria were cultured in LB broth at 37 °C for 24 h under aerobic conditions. At concentration of 1×10^8 CFU/mL, 1 mL of the bacterial suspension was treated with 12.5 % AOEP for 2 h. Simultaneously, another 1 mL sample was used as control. Samples were centrifuged at 1400 rpm for 3 min and washed with PBS. Cells were then fixed in 2.5 % glutaraldehyde at 4 °C for 2 h, followed by dehydration by a series of ethanol, centrifuged at 1400 rpm for 10 min, and air-dried. Finally, the samples were coated with an 80:20 ratio of gold and palladium and examined under SEM (model Zeiss 224 EVO 50 VP, Germany) at 20 kV with a working magnification of less than 10 mm for optimal focus. Bacterial length was measured using ImageJ, and the number of fragmented cells was quantified and expressed as a percentage.

2.6. qRT-PCR

RNA extraction was performed using the hot-phenol method, followed by cDNA synthesis. A total of 1 μg of total RNA was reverse transcribed using M-MulV-RT (Thermo Fisher Scientific) [14,15]. Quantitative real-time PCR (qRT-PCR) was performed on a LightCycler 480 instrument (Roche) using SYBR Green I Master Mix (Roche) with the following primer sequences listed in Table 1. The 16*S* rRNA gene served as an internal reference control.

2.7. LC-MS/MS

LC-MS/MS analysis was performed as previously described [16]. Briefly, 5 μL of the extract were injected into an LC-MS/MS system with a Phenomenex Gemini Column C18 (150 × 2 mm, 5 μm particle size) at 50 °C. The mobile phase consisted of 0.1 % formic acid and 200 μM EDTA in water (phase A) and 0.1 % formic acid in acetonitrile (phase B). The gradient profile was: isocratic for 1 min, linear increase from 10 % to 50 % B over 0.5 min, further increase to 99 % B over 4 min, hold at 99 % B for 1.5 min, and re-equilibration for 2.9 min at 0.45 ml/min flow rate. Mass spectrometry (MS) analysis was performed on a 4000 QTRAP hybrid triple-quadrupole linear ion trap mass spectrometer in positive ion electrospray mode using Analyst 1.4.1 for data acquisition and processing.

2.8. Statistical analysis

All data are presented as mean ± SEM. Statistical significance was examined using one-way analysis (ANOVA), followed by a post-hoc test using GraphPad Prism ver. 10. Group was considered significant difference if $p < 0.05$.

3. Results

3.1. Antibacterial and antibiofilm activities of AOEP extract

The antibacterial and antibiofilm activities of AOEP extract was evaluated using two assays: determination of the minimal inhibitory concentration (MIC) and biofilm inhibition. The results showed that the AOEP extract inhibited both *K. pneumoniae* strains, with relatively uniform absorbance across different concentrations of the extract. The MIC threshold of 90 % inhibition was not achieved for either strain, with maximal inhibition reaching approximately 83 % in BAA-1706 and ~75 % in BAA-1705. Consequently, the MIC_{50} was determined to fall within the range of 3.1 %–50 % for both strains (Fig. 1A and B). Notably, a stronger inhibitory effect of the AOEP extract was observed in BAA-1706

Table 1
Primer used for qRT-PCR experiments.

No.	Gene	Forward	Reverse
1	*wzi-3*	GCTTAYGCRGCYGGGTTAGTRGT	GGCCASGTCGACARGCTCAG
2	*pal*	GAGAATTGCATGAGCAGTAAC	TTCTTTGATTCCTTTAATAATC
3	*lppA*	TAGATTGAGTTAATCTCCATG	GAGTTCTGGTAATAAAAATGGCGC
4	*ompA*	GTTTTTTTATCGGTTATAAC	TTAACAACAAGTCACGCCCCCG
5	*mrkA*	CGGTAAAGTTACCGACGTATCTTGTACTG	GCTGTTAACCACACCGGTGGTAAC

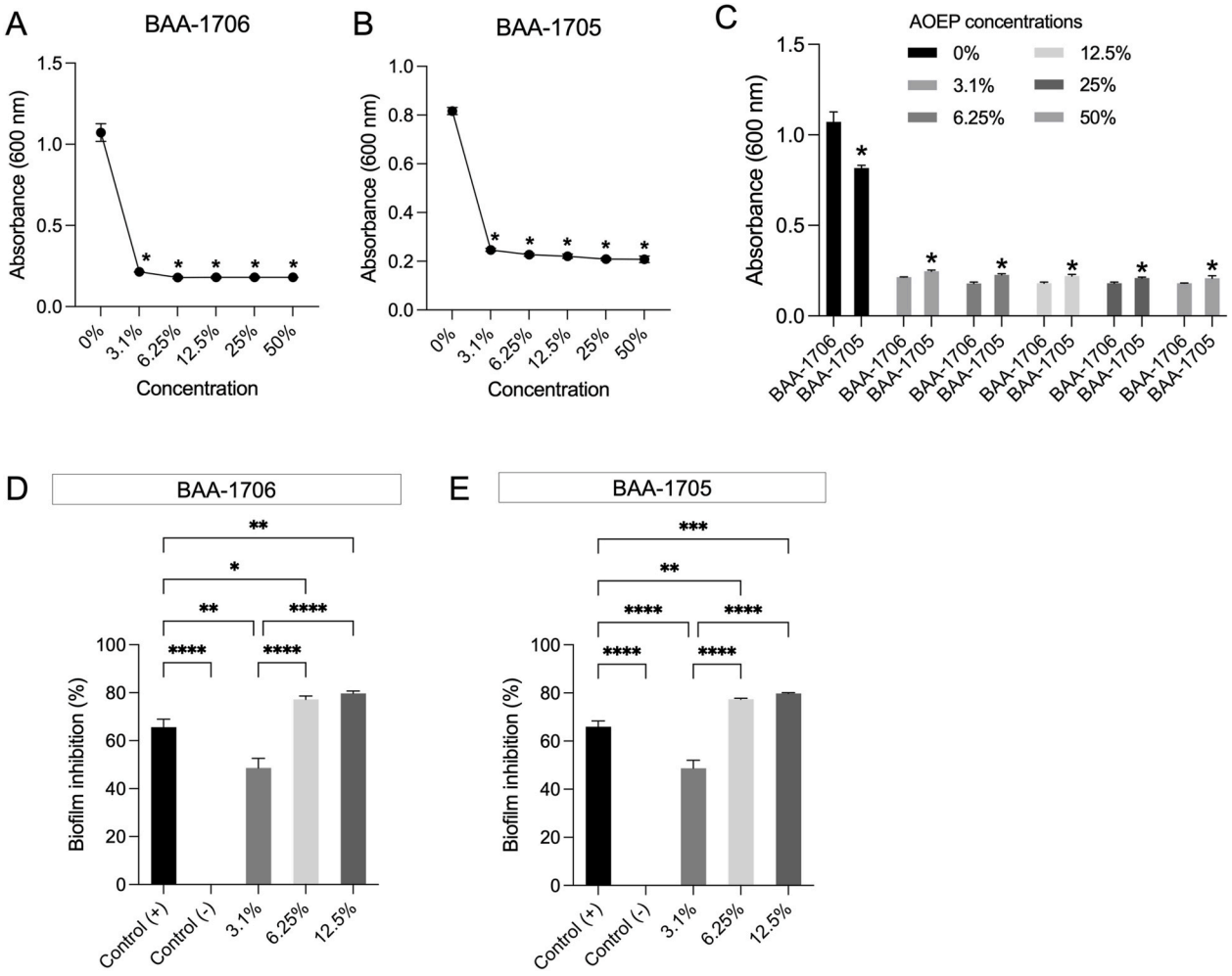

Fig. 1. (A–C) Effects of *A. oryzae* extracellular protein (AOEP) on the minimum inhibitory concentration (MIC) and **(C–D)** biofilm formation, in non-KPC-producing BAA-1706 and KPC-producing BAA-1705. Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

than in BAA-1705 across all tested concentrations (Fig. 1C). At concentrations ranging from 3.1 % to 12.5 %, AOEP extract inhibited biofilm formation in both BAA-1706 and BAA-1705 strains, with concentrations as low as 6.25 % showing greater inhibitory activity than the positive control (kanamycin) (Fig. 1D and E).

3.2. Morphological alterations induced by AOEP extract

We further examined whether AOEP extract affected the morphology of BAA-1706 and BAA-1705 strains using electron microscopy. In untreated samples, bacterial colonies were evenly distributed in both strains. Upon treatment with positive control (kanamycin), colonies of BAA-1706 appeared more separated, whereas those of BAA-1705 remained less dispersed. In contrast, treatment with AOEP extract resulted in notable morphological changes in both strains, characterized by smaller colony sizes and the presence of fragmented cells compared to the positive control (kanamycin). No significant morphological differences were observed between BAA-1706 and BAA-1705 across all measured parameters, except that BAA-1706 exhibited more fragmentation than BAA-1705 after treatment with AOEP (Fig. 2A and B).

3.3. AOEP suppresses virulence-associated genes in K. pneumoniae

Several genes associated with the virulence of *K. pneumoniae* were assessed. qRT-PCR analysis revealed that the expression of *ompA*, *lppA*, and *mrkA* was significantly suppressed in strain BAA-1706 following AOEP treatment. In contrast, in strain BAA-1705, only *ompA* and *lppA* showed a significant reduction, while *mrkA* exhibited a non-significant downward trend. Notably, the reduction in *lppA* expression was slightly stronger in BAA-1706 compared to BAA-1705, although the difference did not reach statistical significance ($p = 0.06$). No changes were observed in the expression levels of *pal* and *wzi* in either strain

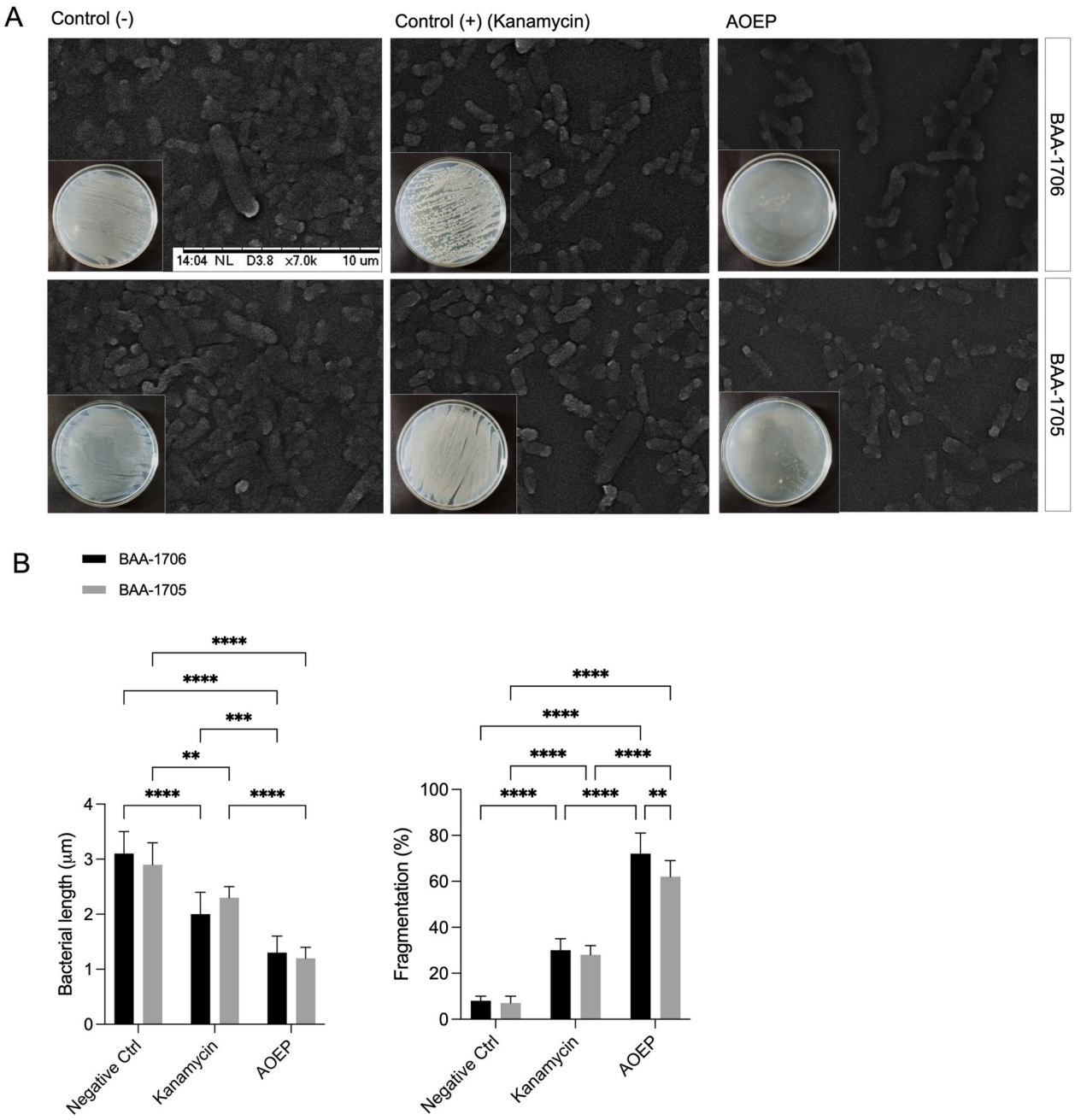

Fig. 2. (A–B) Electron microscopy and quantitative analysis of bacterial length and fragmentation reveal the effects of *A. oryzae* extracellular protein (AOEP) on the morphology of non-KPC-producing *K. pneumoniae* BAA-1706 and KPC-producing strain BAA-1705. Data are presented as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

(Fig. 3A).

3.4. LC-MS/MS profiling reveals lactone-related metabolites in AOEP

Based on the total solids and protein quantification of AOEP, the protein fraction accounted for approximately 16.5 %, while the remaining 83.5 % consisted of non-protein components, likely metabolites. Thus, to characterize the chemical profile of secondary metabolites produced by *A. oryzae*, LC-MS/MS analysis was performed. LC-MS/MS analysis of AOEP extracts revealed several lactone-related compounds with precursor ions from *m/z* 214 to 288 and diagnostic fragment ions at *m/z* 101.5 and 158.5. Compounds 1–3 are likely isomers of 3-oxo-C6-acyl homoserine lactone (AHL) analogs, molecules typically associated with bacterial quorum sensing. Compounds 4 and 7 correspond to butyrolactone I analogs, well-known fungal secondary metabolites involved in regulating development and secondary metabolism in *Aspergillus* species. Compound 5, an aromatically substituted AHL-like molecule, and compound 6, a heavier, more hydrophobic lactone derivative (see Table 2, Fig. 3B).

3.5. Comparative effects of J8-C8 and AOEP on biofilm inhibition and virulence gene expression

While we demonstrated that AOEP contains AHL analogs, to confirm that AHL secondary metabolites are responsible for quorum sensing inhibitor, four experimental groups were conducted: negative control, positive control treated with J8-C8, AOEP, and heated AOEP (to denature proteins). We observed that all treated groups exhibited inhibition

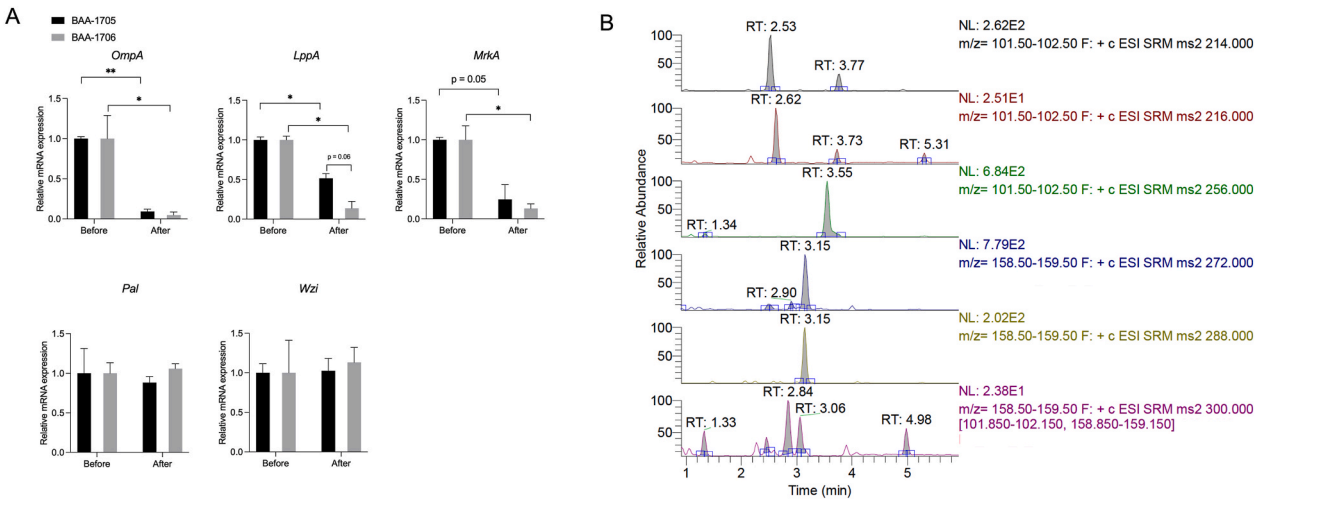

Fig. 3. (A) Effects of *A. oryzae* extracellular protein (AOEP) on the expression of virulence-associated genes in *K. pneumoniae* strains before and after AOEP treatment. **(B)** Chemical characterization of AOEP metabolites by LC-MS/MS. Data are presented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$.

Table 2
Preliminary compound identification based on LC-MS/MS.

No.	RT (min)	*m/z* Precursor	*m/z* Fragment	Compound	Possible structure/Function	Notes
1	3.15	214.0	101.5	3-oxo-C6-HSL analog	Acyl-homoserine lactone is a short chain with a 3-oxo group.	QS mimic, similar to AHL
2	2.62	214.0	101.5	Isomers of compound No. 1	Possible stereo-isomers or structural isomers	Similar biological activity
3	2.53	214.0	101.5	Other isomers	Early retention, likely more polar compound	
4	3.55	256.0	101.5	Butyrolactone I analog	Lactones with larger side chains	Possible *Aspergillus -specific metabolites*
5	3.73	272.0	158.5	Aromatic-substituted AHLs	Homoserine lactone with phenolic/aromatic substituents	Potential as a QS inhibitor
6	5.31	288.0	158.5	Heavy AHLs or lactone derivatives	It may be a long chain AHL, a complex lactone, or a heavy phenolic compound.	Slow elution, more nonpolar compounds
7	3.06	256.0	101.5	Duplicate compound No. 4	Butyrolactone-like from different fractions	

of biofilm formation in both BAA-1706 and BAA-1705 strains (Fig. 4A) compared to the control. Additionally, three genes previously shown to be significantly downregulated by AOEP treatment were reassessed. All treated groups again displayed significant reduction in expression in both strains, with a greater reduction observed in BAA-1706 than in BAA-1705, although the difference was not statistically significant (Fig. 4B). Our analysis thus demonstrates that AOEP's heat-stable lactones function as AHL synthase inhibitors similar to J8-C8, effectively suppressing quorum sensing, biofilm formation, and virulence gene expression in both KPC– and KPC + *K. pneumoniae strains*.

4. Discussion

Our results demonstrated that the AOEP extract exerts notable antibacterial properties against *K. pneumoniae*, encompassing inhibition of bacterial growth, biofilm formation, and virulence factor expression. These findings corroborate previous evidence supporting the therapeutic potential of fungal secondary metabolites as alternatives or adjuncts to conventional antibiotics [17,18], particularly against problematic pathogens such as *K. pneumoniae*, which is known for its multidrug resistance. The MIC_{50} range of 3.1 %–50 % observed in this study is

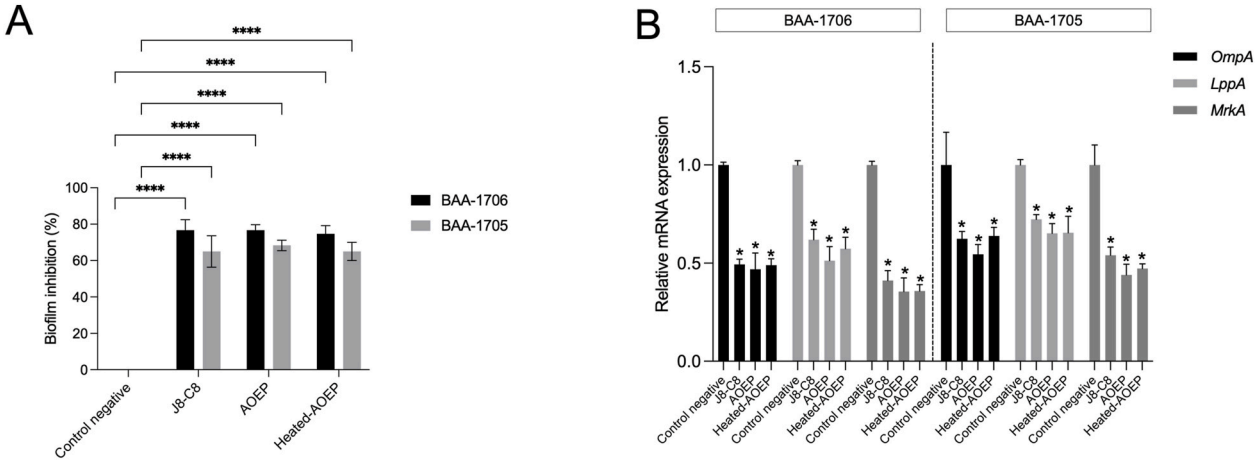

Fig. 4. (A–B) Comparative analysis of biofilm inhibition and virulence gene xxpression by J8-C8 and AOEP in in non-KPC-producing BAA-1706 and KPC-producing BAA-1705, respectively. Data are presented as mean ± SEM. $^{*}p < 0.05$, $^{****}p < 0.0001$.

consistent with previous reports where fungal extracts and metabolites demonstrate broad antibacterial effects at relatively moderate concentrations [19]. The uniform absorbance profile across concentrations further suggests a dose-dependent but stable inhibition, indicating that AOEP exerts a robust effect on both *K. pneumoniae* strains regardless of their antibiotic resistance status. This broad efficacy is crucial given the phenotypic heterogeneity among *K. pneumoniae* clinical isolates, including differences in capsule composition and virulence [20]. The biofilm inhibition data are particularly striking since biofilms represent a major clinical challenge by conferring antibiotic tolerance and protecting bacteria from host immune responses [21]. AOEP's ability to inhibit biofilm formation at low concentrations, even outperforming kanamycin at 6.25 %, implies that it interferes with critical early stages of biofilm development, such as bacterial adhesion or quorum sensing signaling [22]. This antibiofilm activity likely contributes to the extract's overall antibacterial effectiveness, as biofilms increase bacterial tolerance to treatments. Electron microscopy revealed significant morphological changes after AOEP treatment, including smaller colony sizes and cellular fragmentation. These changes may indicate direct damage to bacterial cell envelopes or activation of cell death pathways. This physical disruption contrasts with the more subtle colony dispersal caused by kanamycin, which primarily inhibits protein synthesis. The differing morphological effects suggest that AOEP components may act through unique mechanisms, possibly involving membrane destabilization or interference with the cell division machinery, which is well recognized among natural antimicrobial agents [23,24].

Indeed, molecular analysis of virulence gene expression further elucidates the extract's mechanism. The significant downregulation of *ompA*, *lppA*, and *mrkA* in strain BAA-1706 and selective suppression in BAA-1705 demonstrate that AOEP impairs key determinants of *K. pneumoniae* pathogenicity. OmpA and LppA are outer membrane lipoproteins implicated in bacterial adhesion, serum resistance, and immune evasion [25,26], while MrkA forms the major pilin subunit of type 3 fimbriae essential for robust biofilm formation on abiotic surfaces [27]. Suppression of these genes is consistent with the observed phenotypic inhibition of biofilms and cellular integrity, supporting a possibility that AOEP not only kills bacteria but also attenuates their virulence potential. Strain-specific differences in mrkA repression may reflect underlying genomic or regulatory variations, emphasizing the genetic diversity of *K. pneumoniae* and its complex regulation of virulence factors [28,29]. Using LC-MS/MS, the chemical characterization of AOEP reveals an intriguing profile dominated by lactone-related metabolites, including analogs of acyl homoserine lactones (AHLs) and butyrolactones. AHLs are canonical quorum sensing signals in Gram-negative bacteria, coordinating population-level behaviors such as virulence and biofilm maturation [30]. The presence of AHL analogs in AOEP suggests a plausible quorum quenching mechanism, whereby fungal metabolites disrupt bacterial communication pathways to prevent coordinated expression of virulence traits [31,32]. Butyrolactones, known as fungal autoregulatory molecules that regulate secondary metabolism and development [33], may have unexplored roles in cross-kingdom signaling and antimicrobial activity [34], warranting further investigation.

In conclusion, our results indicate that AOEP acts through a complex antimicrobial mechanism, combining direct bactericidal effects with inhibition of biofilm formation and virulence gene expression, likely by disrupting bacterial quorum sensing. This approach is particularly valuable against persistent pathogens like *K. pneumoniae*, which rapidly develop resistance and form protective biofilms. Future studies should focus on isolating and characterizing the specific active compounds within AOEP, evaluating their individual and synergistic effects, and validating the quorum sensing inhibition hypothesis through molecular and phenotypic assays.

CRediT authorship contribution statement

Lailia Nur Rachma: Methodology, Investigation, Formal analysis, Data curation. **Zulvikar Syambani Ulhaq:** Writing – review & editing, Writing – original draft, Validation, Formal analysis, Supervision.

Ethical approval and informed consent

The study did not involved any human subjects or animals. All experimental procedures were approved by the Ethical Committee of the Faculty of Medicine and Health Sciences, Maulana Malik Ibrahim State Islamic University (Ref.No. 027/EC/KEPK-FKIK/2020).

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Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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None to declare.

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