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Original Article

Synergistic mechanism of *Phyllanthus emblica* extract and tetracycline against multidrug-resistant *Acinetobacter baumannii*

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Abstract

The rising incidence of multidrug-resistant (MDR) *Acinetobacter baumannii* infections underscores the urgent need for novel antimicrobial strategies. The aim of this study was to investigate the synergistic effects between a polyphenol-rich extract from *Phyllanthus emblica* fruit and tetracycline against MDR *A. baumannii* strains. The extraction process was optimized using the Box-Behnken design approach to maximize the total phenolic content (TPC) of the *P. emblica* extract. Key variables, including ethanol concentration, extraction time, and solid-to-liquid ratio (w/v), were determined through single-factor experiments. The antimicrobial activity of the extract, both alone and in combination with tetracycline, was evaluated against *A. baumannii*. Mechanistic studies focusing on bacterial lysis and efflux pump inhibition were conducted to assess the extract's effects and its combined potential with tetracycline. The Box-Behnken design successfully optimized the extraction conditions, yielding the highest TPC at 68.92% ethanol concentration, 1.85 days of extraction time, and a 1:9.58 w/v ratio. The predicted and experimentally verified TPC values of the extract were 129.19 and 130.76±2.46 mg GAE/g samples, respectively, with no significant difference ($p>0.05$). The extract contained several phenolic compounds identified using liquid chromatography–high-resolution mass spectrometry (LC-HRMS). It exhibited antimicrobial activity against MDR *Acinetobacter baumannii*, either alone or in combination with tetracycline. The combination demonstrated a synergistic effect against MDR *A. baumannii*, with a fractional inhibitory concentration index (FICI) of 0.37. Moreover, the combination showed superior bacteriolytic effects against MDR *A. baumannii* cells, as evidenced by increased release of nucleic acid components and membrane destabilization, compared to the extract or tetracycline alone ($p<0.0001$ for all comparisons). Additionally, the combination significantly enhanced the efflux pump inhibition effect compared to the extract or tetracycline alone ($p<0.05$ for both). These findings support the potential use of polyphenol-rich *P. emblica* extracts as adjuncts to conventional antibiotics in treating drug-resistant bacterial infections.

Keywords: *Acinetobacter baumannii,* multidrug-resistant, *Phyllanthus emblica*, total phenolic content, antimicrobial activity

Introduction

Acinetobacter baumannii is a prominent nosocomial pathogen responsible for diverse infections, especially in critically ill patients [1]. Its capacity to acquire multidrug resistance

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(MDR) and last on surfaces for prolonged durations has developed as a worldwide health threat [2]. *A. baumannii* infections are often linked to high mortality rates and prolonged hospital stays, with risk factors including prior antibiotic use, mechanical ventilation, and extended intensive care unit admissions [3,4]. The bacterium's significant genetic adaptability enables the acquisition of resistance genes, further complicating the treatments. Consequently, therapeutic options for MDR *A. baumannii* remain limited, and the efficacy of existing treatments is often uncertain [5]. Effective control strategies include stringent hand hygiene, reduction of biofilm formation, and the judicious use of antibiotics [6,7].

Recent advancements in genomic studies have shed light on *A. baumannii* evolution and adaptation as a human pathogen, emphasizing the urgency of developing innovative treatment strategies [8,9,10]. Among these, plant-derived polyphenols have emerged as promising candidates due to their antibacterial properties against MDR pathogens [11]. Compounds such as catechins, theaflavins, and phenolic acids have demonstrated potent antibacterial and antibiofilm activities in both in vitro and in vivo settings [12-14]. For instance, epigallocatechin-3-gallate from green tea has shown bactericidal effects against resistant strains [15], while ellagic and tannic acids enhance antibiotic efficacy by inhibiting efflux pumps [16]. Additionally, molecular docking studies have identified plant-derived metabolites as potential inhibitors of *A. baumannii* proteins, further underscoring their therapeutic potential [17]. Phenolic acids, including caffeic, gallic, and protocatechuic acids, have also been shown to amplify colistin-induced lethality by inducing oxidative stress [18]. These findings suggest that plant-derived polyphenols could play a crucial role in developing novel therapies against antibiotic-resistant *A. baumannii* [19,20].

Phyllanthus emblica L., commonly known as Indian gooseberry or amla, is a medicinal plant rich in bioactive compounds, particularly polyphenols and vitamin C [21]. Renowned for its antioxidant, anti-inflammatory, anticancer, antidiabetic, and hepatoprotective properties, *P. emblica* has been extensively utilized in traditional medicine systems, including Ayurveda, for treating various ailments [22-24]. The fruit contains diverse phytochemicals, such as tannins, flavonoids, alkaloids, and terpenoids, which contribute to its broad pharmacological activities [25,26,27]. A recent study highlighted its chemopreventive potential and ability to modulate biological pathways, with extracts demonstrating antibacterial efficacy against multiple pathogens [28]. Notably, its potential to combat drug-resistant microbes and serve as an adjunct to conventional antibiotics has garnered increasing interest [29,30,31].

Combination therapy, leveraging synergistic interactions between antimicrobial agents, has emerged as a promising approach to managing complex infections [32]. Synergistic combinations occur when the joint action of two or more agents produces a cumulative effect greater than the sum of their individual activities [33]. This strategy offers multiple advantages, including reduced therapeutic dosages, lowered minimum inhibitory concentrations (MICs), and enhanced antimicrobial efficacy [34]. The concept of synergy has gained attraction among researchers and pharmaceutical developers seeking to expand antimicrobial spectra and counteract resistance in pathogens like *A. baumannii* [35,36]. For instance, synergistic effects have been observed between tetracycline and natural products, such as coriander oil, as well as between phenolic extracts from *Ficus nitida* and tetracycline against various bacteria [37,38]. Despite these advancements, the potential interactions between *P. emblica* and antibiotics remain underexplored. The aim of this study was to evaluate the synergistic effects of polyphenol-rich *P. emblica* fruit extract in combination with tetracycline against *A. baumannii* strains.

Methods

Study design and setting

This study was designed as a comprehensive laboratory-based investigation to explore the synergistic antibacterial effects of a polyphenol-rich extract from *P. emblica* fruit in combination with tetracycline against MDR *A. baumannii*. The research focused on optimizing extraction methods, characterizing bioactive compounds, and evaluating antimicrobial mechanisms. The extraction process was conducted in the Biology Laboratory at Universitas Sumatera Utara, Medan, Indonesia. The chemical analyses and antimicrobial assessments were conducted at the National Research and Innovation Agency, Indonesia. The ethical approval was deemed

unnecessary, as the study involved in vitro experiments without direct involvement of human or animal subjects.

Plant material

P. emblica fruits were collected from Deli Serdang, North Sumatra, Indonesia. The samples were identified as *P. emblica* fruit by the Medanese Herbarium (MEDA) of Universitas Sumatera Utara in Medan, Indonesia, and assigned voucher ID 255/UN.5.1.2.8.2/PPM/2024. Following collection, the fruits were thoroughly washed under running water to remove debris. The fruits were then sliced into smaller pieces and subjected to drying in a controlled drying cabinet at a consistent temperature. Once fully dried, the fruits were ground into a fine powder using a conventional blender Philips HR 2116 (Philips, Amsterdam, Netherlands). The resulting dry powder was stored in an airtight glass container at room temperature until needed for extraction.

Extraction of *P. emblica* fruit using Box-Behnken design approach

The extraction of *P. emblica* was obtained using the maceration method, following the response surface methodology (RSM) with the Box-Behnken design approach. This approach evaluated the impact of extraction factors, including ethanol concentration (%), extraction time (days), and solid-to-liquid ratio (w/v). To obtain information related to the optimum point of each factor, an analysis of the influence of the extraction factor in the single experiment on the response was carried out. The provisions of the extraction factor in the single experiment were as follows: ethanol concentration ranging from 40 to 90%, extraction time from 1 to 5 days, and solid-to-liquid ratio from 1:5 to 1:25 (w/v). The experiment was carried out by varying certain factors while other factors were maintained under constant conditions. The responses obtained were evaluated to determine the conditions of each factor that would be interacted with, using the Design Expert software version 13.0 (State-Ease, Inc., Minneapolis, USA). Therefore, the optimization of the extraction process to obtain the optimum response was established following the instructions from calculations by the software. A total of 17 running extraction conditions were performed to produce crude extract. The crude extracts were stored in a glass container at 2–8°C before being used to determine the total phenolic content (TPC) [39]. The optimum conditions from the optimization process using the Box-Behnken design approach were adopted to produce the extract of *P. emblica* fruit.

Determination of total phenolic content

The TPC in the fruit extract was quantified spectrophotometrically using the Folin–Ciocalteu reagent, with gallic acid serving as the standard (Sigma-Aldrich, Ltd. Co St. Louis, Missouri, US). The assay mixture consisted of 0.5 mL of the extract, 3.0 mL of distilled water, and 0.25 mL of Folin–Ciocalteu reagent. The mixture was thoroughly shaken and allowed to react for 5 minutes in the dark. Subsequently, 1.0 mL of 7.5% sodium carbonate (Na_2CO_3) solution was added, and the mixture was incubated at room temperature for 90 minutes in the dark to facilitate color development. A reagent blank was concurrently made using distilled water. Absorbance readings were recorded at 760 nm against the reagent blank using a double-beam UV/Vis spectrophotometer (Shimadzu, Kyoto, Japan). The TPC was expressed as milligrams of gallic acid equivalent (mg GAE) per gram of sample, calculated from a standard calibration curve [40].

Liquid chromatography high-resolution mass spectrum (LC-HRMS) analysis of phenolic compounds

The phenolic compounds were analyzed using a liquid chromatography system (Orbitrap Q-Exactive, Thermo Fisher Scientific, Waltham, USA), equipped with a reversed-phase C18 column. The mobile phases consisted of water with 0.1% formic acid (phase A) and acetonitrile with 0.1% formic acid (phase B). A gradient elution program was applied, starting with 5% phase B and gradually increasing to 95% over a 30-minute period. The flow rate was maintained at 0.3 mL/min, with the column held at a constant temperature of 25°C. A 10 µL sample was injected into the system for each analysis. For detection, high-resolution mass spectrometry (HRMS) was employed, utilizing electrospray ionization (ESI) in both positive and negative ionization modes. The mass spectrometer was operated at a resolution greater than 30,000 at m/z 200, covering a mass range from m/z 100 to 1,000. Ionization conditions were optimized to enhance sensitivity,

with capillary voltage set between 3.0 and 4.0 kV, a desolvation temperature ranging from 250 to 300°C, and a nebulizer gas flow of nitrogen. The mass spectral data were processed by matching the observed molecular ions and fragmentation patterns against established chemical databases, including Metlin, PubChem, and MassBank, to identify and characterize the phenolic compounds present in the sample [41].

Antimicrobial activity using the microdilution method

The MDR *A. baumannii* was obtained from a clinical isolate and handled by the Marine Education and Research Foundation (MERO) in Bali, Indonesia. The used isolate of MDR *A. baumannii* was confirmed to be resistant to ceftazidime, meropenem, gentamicin, and amikacin. The antimicrobial activity test was carried out using the microdilution method to obtain the minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC). This study used four test groups consisting of 0.5% v/v DMSO (control), optimized extract of *P. emblica* fruit (group 1) and tetracycline (group 2) in various concentrations ranging from 3.90 to 500 µg/mL, and a combination of optimized extract and tetracycline (group 3). In brief, 1.5×10^8 colony forming unit (CFU)/mL of test bacteria were prepared by cultivating bacteria using brain heart infusion broth (BHIB) on a well plate. Then, the bacterial suspension was adjusted to obtain 1.5×10^6 CFU/mL bacterial colonies by adding NaCl (0.9% v/v). Furthermore, the bacterial suspension was mixed with each test group at concentrations between 500 µg/mL and 3.9 µg/mL. The mixtures were incubated at 37°C for 24 hours. Following incubation, the MIC was determined by the absence of visible bacterial growth, indicated by a colorless solution. The MBC was determined by subculturing samples from the MIC wells onto fresh media and assessing for bacterial growth [42].

Evaluation of synergistic interactions via checkerboard testing

The checkerboard test was performed to evaluate the combinatory effect of the optimized extract from *P. emblica* fruit and tetracycline against MDR *A. baumannii*. The concentrations of the test samples were prepared in BHIB to ensure consistent dilution. The optimized extract of *P. emblica* fruit was serially diluted along the x-axis of a 96-well microtiter plate, while tetracycline was diluted along the y-axis. Bacterial suspensions at a concentration of approximately 1×10^6 CFU/mL were added to each well, and the plates were incubated at 37°C for 24 hours to allow for sufficient interaction and bacterial growth. The combination effects were assessed using the fractional inhibitory concentration index (FICI), which was calculated using the equation (Equation 1) [43]:

$$\text{FICI} = \frac{\text{MIC of optimized extract in combination}}{\text{MIC of optimized extract alone}} + \frac{\text{MIC of tetracycline in combination}}{\text{MIC of tetracycline alone}}$$

Based on the FICI values, the interactions were categorized into four types: synergistic (FICI ≤0.5), additive (0.5≤ FICI ≤1), indifferent (1≤ FICI ≤4), and antagonistic (FICI >4) [42].

Evaluation of bacteriolytic activity

The bacteriolytic activity of the test samples was carried out using bacterial suspensions with an optical density (OD) of 0.4 (equal to 4×10^8 CFU/mL). The activity was assessed by measuring nucleic acid release and crystal violet uptake (% absorption) in the presence of various test conditions. Briefly, a total of 500 µL of bacterial suspension was mixed with 500 µL of a test sample in Eppendorf tubes, divided into the following groups: 0.5% v/v DMSO as the control, optimized *P. emblica* fruit extract (group 1) and tetracycline (group 2) in their each MIC concentrations, and a combination of optimized *P. emblica* fruit extract with tetracycline (group 3) at their MIC combination concentration. The mixtures were centrifuged at 13,000 rpm for 1 hour. For nucleic acid release, the supernatant was collected and its absorbance was measured at OD_{260}. For crystal violet uptake, a parallel experiment was conducted with the addition of 500 µL of 0.001% crystal violet to the reaction mixture. The resulting supernatant was analyzed for % absorption at OD_{590} [43].

The inhibitory activity of efflux pumps was assessed by quantifying the accumulation of fluorescence from ethidium bromide (EtBr). Briefly, the MDR *A. baumannii* was cultured in 10

mL of BHIB. After incubation, the bacterial suspension was centrifuged at 3000 rpm for 15 minutes, and the resulting pellet was washed with phosphate-buffered saline (PBS) until a pH of 7.3 was achieved. The pellet was then resuspended in normal saline to an OD_{600} of 0.4. Subsequently, 50 µL of test solution from each group was combined with 100 µL of the cultures, and then incubated at 37°C for 30 min. Following this, 50 µL of 0.5 mg/L EtBr was added to each well under conditions that ensured the stability of the EtBr solution. Fluorescence was measured at 37°C with an excitation wavelength of 530 nm and emission wavelength of 600 nm. Measurements were taken at 0, 5, 15, and 45 minutes, with the fluorescence intensity was recorded as ratio fluorescence units (RFU) of EtBr [44].

Statistical analysis

The statistical analysis for the optimization study was carried out using the Design Expert software (State-Ease, Inc., Minneapolis, USA). The design comprised 17 experimental combinations, including five replicates of the central point to facilitate the estimation of pure error and ensure robustness. The Brown-Forsythe test was conducted to evaluate the homogeneity of variance, whereas the analysis of variance (ANOVA) test at a significance level of $p<0.05$ was utilized to observe the significant effects of the tested variable combinations [45]. The experimental data were fitted using the quadratic model, represented by the following equation (Equation 2):

$$Y = a_0 + \sum_{j=1}^{3} a_j x_J + \sum_{j=1}^{3} a_{jj} x_{jj}^2 + \sum_{i} \sum_{<j=2}^{3} a_{ij} x_i x_j + e_i$$

The dependent variable (Y) represents the response, while x_i and x_j denote the coded independent variables. The regression coefficients a_o, a_j, a_{jj}, and a_{ij} correspond to the intercept, linear, quadratic, and interaction effects, respectively, with the residual error represented by e_i. The performance of the generated mathematical models was evaluated using key statistical parameters, including the determination coefficient (R^2), adjusted (adj) R^2, predicted (pred) R^2, coefficient of variation (CV%), and adequate precision. These metrics ensured the model's reliability and predictive capability for optimizing the response [45].

The experiments, including single-factor experiments and antimicrobial tests, were conducted in triplicate and reported as mean ± standard deviation (SD). The differences in data from these experiments between groups were evaluated using ANOVA followed by a post hoc Tukey HSD test, with a p-value<0.05. The data were analyzed using SPSS version 26 (IBM, New York, USA).

Results

Preliminary single-factor experiments on ethanol concentration, extraction time, and solid-to-liquid ratio

The impact of ethanol concentration (40–90%) on the TPC extracted from *P. emblica* fruit powder was evaluated under fixed conditions of two days extraction time and a solid-to-liquid ratio of 1:10 (w/v). The results indicated that the TPC was significantly increased with higher ethanol concentrations ($p<0.001$) (**Figure 1A**). This suggests that the ethanol concentrations affect the TPC of the extract. The best TPC yield was obtained when the ethanol concentration increased from 50% to 70%, while beyond 70%, no additional improvement in extraction yield was observed. The highest TPC obtained using 70% ethanol was 121.86±1.62 mg GAE/g sample (**Figure 1A**). Based on these findings, ethanol concentrations of 50%, 70%, and 90% were selected for subsequent RSM experiments.

The effect of extraction time (1–5 days) on TPC yield was further evaluated using 70% ethanol and a solid-to-liquid ratio of 1:10 (w/v) (**Figure 1B**). The extraction time demonstrated a significant impact on TPC yield, with an extension extraction time from 1 to 2 days significantly enhancing the TPC yield, reaching 109.50±0.95 mg GAE/g sample. However, further prolonging the extraction period beyond two days led to a significant decrease in TPC yield (**Figure 1B**). Consequently, extraction times of 1, 2, and 3 days were selected for the RSM experiments.

The influence of varying the solid-to-liquid ratio (1:5 to 1:25, w/v) was also assessed while keeping the extraction time and ethanol concentration constant (**Figure 1C**). Our data indicated that increasing the solid-to-liquid ratio from 1:5 to 1:10 (w/v) improved the TPC yield from 84.57±1.05 to 118.45±1.04 mg GAE/g sample (**Figure 1C**). However, the elevating of the solid-to-liquid ratio beyond 1:10 resulted in a decrease of TPC yield. Therefore, solid-to-liquid ratios of 1:5, 1:10, and 1:15 (w/v) were selected for the RSM design.

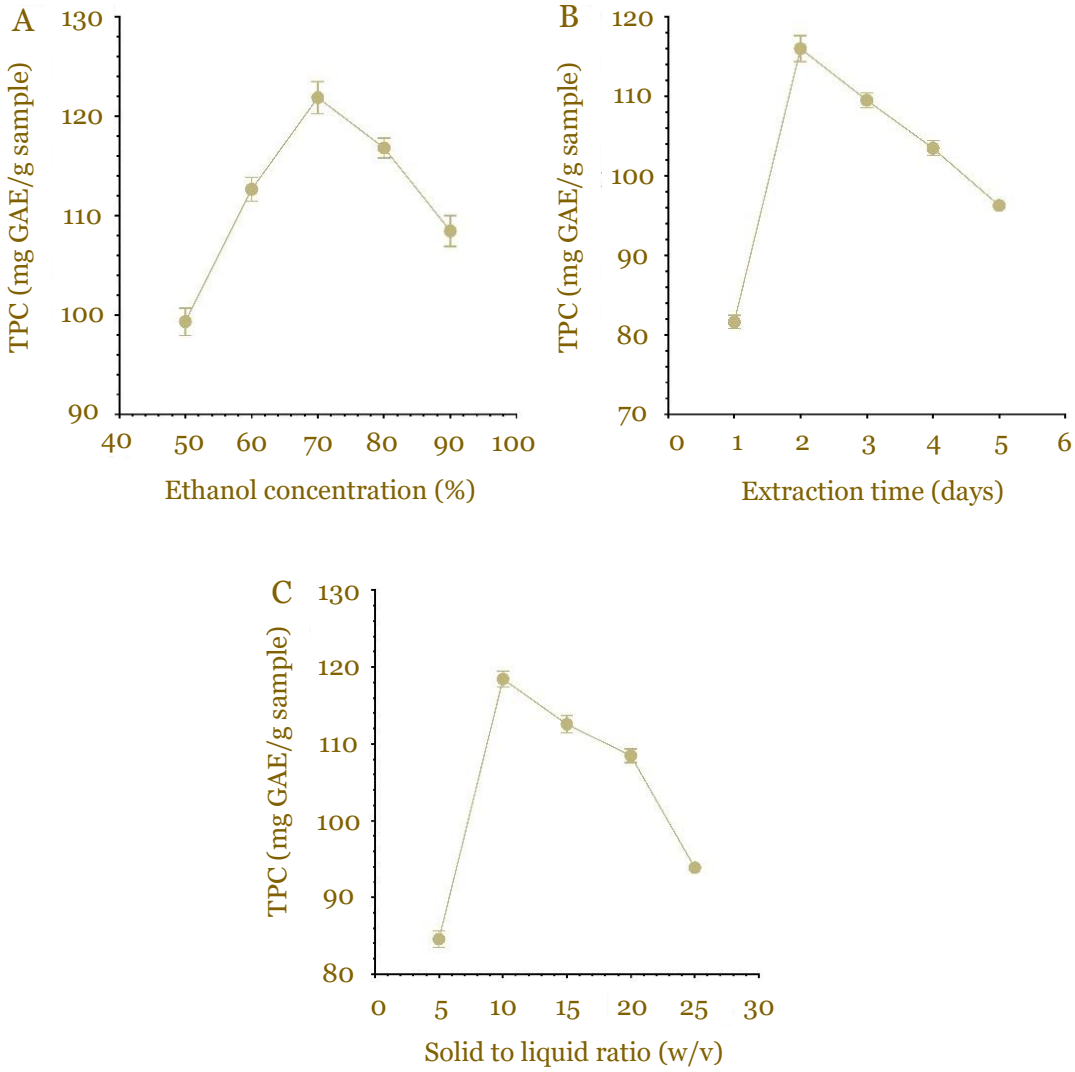

Figure 1. Determination of total phenolic content (mg GAE/g sample) of *Phyllanthus emblica* fruit under single-factor analysis. The effect of ethanol concentration (%) (A); effect of extraction time (days) (B); and effect of solid-to-liquid ratio (w/v) (C).

Response surface methodology (RSM) analysis

The average TPC values observed under different experimental conditions during RSM analysis are presented in **Table 1**. The Brown-Forsythe test confirmed the homogeneity of variances across groups (p=0.854; exceeding the cut-off p>0.6). The test confirmed that variances were consistent across groups, ensuring that the data met the assumptions required for further analysis (**Table 1**). One-factor ANOVA analysis identified statistically significant differences in the TPC production yield (p=0.013) (**Table 1**). This finding indicated that every combination of independent variables (ethanol concentration, extraction time and solid-to-liquid ratio) in the extraction process had a significant impact on TPC production. The TPC was obtained in varied yields between 110.38 and 130.93 mg GAE/g sample, indicating substantial variation influenced by the experimental factors (**Table 1**).

Table 1. Design experiment by Box-Behnken design approach for optimization of the extraction parameters from *Phyllanthus emblica* fruit

Samples	Ethanol concentration (A) (%)	Extraction time (B) (days)	Soli-to-liquid ratio (C) (ratio)	TPC (mg GAE/g sample)
1	70	3	15	115.72
2	70	2	10	128.91
3	70	1	15	120.67
4	70	2	10	125.75
5	70	1	5	121.54
6	70	3	5	117.74
7	90	3	10	110.38
8	90	1	10	113.75
9	90	2	5	116.43
10	70	2	10	130.93
11	70	2	10	128.54
12	50	2	15	118.25
13	50	2	5	117.03
14	70	2	10	130.87
15	90	2	15	112.68
16	50	3	10	111.79
17	50	1	10	115.23
p-value (Brown-Forsythe)				0.854
p-value (ANOVA)				0.013

Our analysis indicated that the quadratic model was the most suitable representation of the experimental data (**Table 2**). The R^2, adjusted R^2 and predicted R^2 of the quadratic model exhibited the highest values and were supremely significant ($p<0.0001$) compared to other models, with the exception of the cubic model, which was subject to aliasing or confounding effects. Consequently, the subsequent second-order polynomial equation incorporating interaction terms was employed to illustrate the impact of independent variables on the response. The equation was derived using the following formula:

$$Y_{TPC}\ (\text{mg GAE/g sample}) = 129.00 - 1.13A - 1.94B - 0.6775C + 0.0175AB - 1.24AC - 0.2875BC - 9.52A^2 - 6.70B^2 - 3.39C^2$$

Table 2. Adequacy of the model tested

Model	SD	R^2	Adjusted R^2	Predicted R^2	PRESS	Prob <F	Remark
Linear	7.36	0.0591	-0.1581	-0.3984	1046.52	0.8445	
2FI	8.35	0.0678	-0.4916	-1.4150	1807.30	0.9922	
Quadratic	1.68	0.9735	0.9394	0.9233	57.43	<0.0001	Suggested
Cubic	2.12	0.9759	0.9038	ND	ND	0.9337	Aliased

2FI: two-factor interaction; F: F-statistic; ND: not detected; PRESS: predicted residual error sum of squares; SD: standard deviation.
Quadratic model is suggested by the Design-expert software. The proposal relies on a subjective scoring system that integrates various selected metrics to indicate that the quadratic model is superior in comparison to alternative models.

The capability of the derived equation to characterize the variability of the response was assessed through multiple regression analysis and ANOVA (**Table 3**). The derived model demonstrated high significance, as evidenced by a low probability value ($p<0.0001$). However, the regression coefficients for factors A, C, and their interaction terms (AB, AC, and BC) were excluded from the final model because their contributions were statistically insignificant (**Table 3**). By excluding these coefficients, the model was simplified without compromising its predictive accuracy or interpretability, focusing only on the factors and interactions that significantly influenced the response. The R^2 and adj R^2 values exceeded 0.9735 and 0.9384, respectively. These high values indicated that the model accounted for 97.35% of the variability in the experimental data, with the adj R^2 value confirming the model's robustness by compensating for the number of predictors. The closeness of these values suggested that the model was not overfitted and was capable of accurately predicting the experimental outcomes. The $p=0.9337$ indicated that the lack of fit was not significant. The probability of obtaining a "lack of fit *p*-value" of this magnitude due to random noise was merely 0.01%. Adequate precision quantifies the

signal-to-noise ratio. In a similar manner, the precision for the response (**Table 3**) exceeded 13, further indicating the adequacy of the signals. Additionally, the model's coefficient of variation (CV) was less than 10, suggesting that the model sufficiently elucidated the reaction and that the experimental data exhibited an elevated level of precision (**Table 3**).

Table 3. ANOVA and statistical observation of the optimization model

Source	Sum of square	Degrees of freedom	Mean square	F-value	*p*-value
Model	728.54	9	80.95	28.57	<0.001*
A (ethanol concentration, %)	10.26	1	10.26	3.62	0.0988
B (extraction time, days)	30.26	1	30.26	10.68	0.0137*
C (soli-to-liquid ratio, w/v)	3.67	1	3.67	1.30	0.2924
Interaction A and B	0.0012	1	0.0012	0.0004	0.9840
Interaction A and C	6.18	1	6.18	2.18	0.1834
Interaction B and C	0.3306	1	0.3306	0.1167	0.7427
A^2	381.30	1	381.30	134.56	<0.001*
B^2	188.80	1	188.80	66.63	<0.001*
C^2	48.28	1	48.28	17.04	0.004*
Residual	19.84	7	2.83		
Lack of fit	1.83	3	0.6105	0.1356	0.9337
Pure error	18.00	4	4.50		
Cor total	748.38	16			
Fit statistic					
Standard deviation	1.68				
Mean	119.78				
CV (coefficient of variation, %)	1.41				
PRESS (predicted residual error sum of squares)	57.43				
R^2	0.9735				
Adj R^2	0.9384				
Pred R^2	0.9233				
Adeq precision	14.9276				

*Statistically significant difference at p=0.05

Effect of independent variables on total phenolic content (TPC)

The second-order polynomial equation (Equation 2) has facilitated the generation of three-dimensional plots to demonstrate the interaction phenomena between independent variables (ethanol concentration (A), extraction time (B), and solid-to-liquid ratio (C)) on TPC yield (**Figure 2A-C**). The increase in A and B reached a particular point of 70% and two days, respectively, increasing the TPC. Increasing two factors above beyond the crucial point will decrease the TPC (**Figure 2A**). On the other hand, the interaction effect of A and C on the TPC indicated that the TPC reached a maximum of 130.93 mg GAE/g sample when the A and C reached the particular point of 70% and 1:10 (w/v), respectively (**Figure 2B**). Increasing each factor reduced TPC, but the maximum TPC was still achieved when only factor C was varied, while factor A remained constant at 70%. Additionally, the interaction between factors B and C was not significantly different from the interactions of the other factors (**Figure 2C**). The TPC reached its optimum when factor B was set at two days and factor C at a ratio of 1:10 (w/v). Beyond these points, further increases in the factors led to a decline in TPC, although the reduction was less pronounced compared to other interactions.

These explanations are supported by the statistical analyses (**Table 3**). The linear term of factor B was significant (p=0.0137) and negative, whereas the two factors, including factors A and C, were not significant (p=0.0988 and p=0.2924). Additionally, all interaction factors, including AB, AC, and BC, were not significant. Meanwhile, all terms of quadratic involve A^2, B^2, and C^2 were significant and negative values with each p<0.0001, p<0.0001, and p=0.0044, respectively.

Validation of the model

The optimum model was generated after interaction analysis between independent factors on TPC. The optimum conditions were obtained around 68.92 % (A), 1.85 days (B), and 1:9.58 w/v (C) with desirability of 0.916 and predicted TPC of 129.19 mg GAE/g sample.

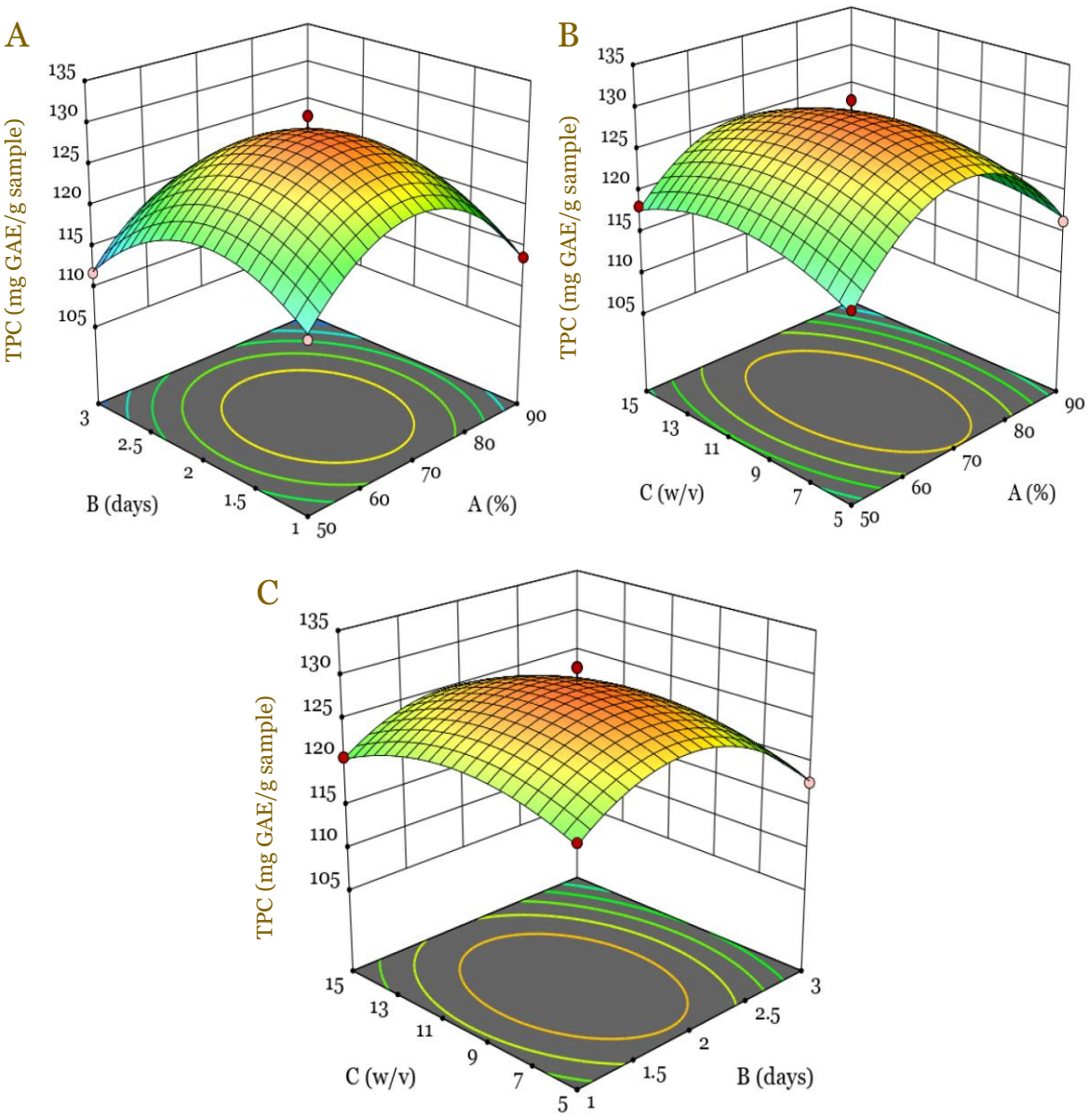

Figure 2. Response surface plots illustrate the interaction impact of independent factors (ethanol concentration (A, %), extraction time (B, days), and solid-to-liquid ratio (C, w/v) on total phenolic content (TPC, mg GAE/g sample). The relationship between ethanol concentration and extraction time (A); the relationship between ethanol concentration and solid-to-liquid ratio (B); and the relationship between extraction time and solid-to-liquid ratio (C).

The model was validated through experiments conducted under the optimized conditions identified by the Box-Behnken design, with results presented in **Table 4**. The verification test showed the TPC of *P. emblica* fruit extract under the optimized conditions of 130.76±2.46 mg GAE/g sample. According to the T-Test, the prediction and verification of TPC were not significantly different (p=0.80). This result showed the extraction condition derived from the Box-Behnken design is appropriate for applied to produce polyphenol-rich extract from *P. emblica* fruit.

Table 4. Verification of the total phenolic content model for *Phyllanthus emblica* fruit based on optimized extraction conditions

	A (%)	B (days)	C (ratio)	TPC (mg GAE/g sample)
Prediction	68.92	1.85	1:9.58	129.19
Verification	68.92	1.85	1:9.58	130.76±2.46
T-test (p-value)	-	-		0.80 (NS)

A: ethanol concentration; B: extraction time; C: solid-to-liquid ratio; TPC: total phenolic content; GAE: gallic acid equivalent; NS: not significant

Phenolic compounds analysis

The phenolic compounds of *P. emblica* fruit extract under the optimized condition were determined using LC-HRMS. The optimized extract had six phenolic compounds, including quercetin (retention time (RT) of 7.59 min), kaempferol (6.11 min), caffeic acid (4.70 min), protocatechuic acid (2.00 min), rutin (5.49 min), and hyperoside (5.73 min) (**Table 5**). Of the total six phenolic compounds, four compounds were flavonoids, including quercetin, kaempferol, rutin, and hyperoside.

Table 5. Phenolic compounds from the optimized extract of *Phyllanthus emblica* fruit

Name	Formula	Retention time (min)	IUPAC (International Union of Pure and Applied Chemistry)	Molecular weight (g/mol)
Quercetin	$C_{15}H_{10}O_7$	7.59	2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one	302.04
Kaempferol	$C_{15}H_{10}O_6$	6.11	3,5,7-Trihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one	286.04
Caffeic acid	$C_9H_8O_4$	4.70	3-(3,4-Dihydroxyphenyl)-2-propenoic acid	180.04
Protocatechuic acid	$C_7H_6O_4$	2.00	3,4-Dihydroxybenzoic acid	154.01
Rutin	$C_{27}H_{30}O_{16}$	5.49	2-(3,4-Dihydroxyphenyl)-3-[Î±-L-rhamnopyranosyl(1â†'6)-Î²-D-glucopyranosyloxy]-5,7-dihydroxy-4H-1-benzopyran-4-one	610.14
Hyperoside	$C_{21}H_{20}O_{12}$	5.73	2-(3,4-Dihydroxyphenyl)-3-[Î²-D-galactopyranosyloxy]-5,7-dihydroxy-4H-1-benzopyran-4-one	464.09

Antibacterial activity of the samples

The optimized *P. emblica* fruit extract had antibacterial activity against MDR *A. baumannii* bacteria with a MIC value of 125 µg/mL and an MBC of 250 µg/mL (**Table 6**). In comparison, tetracycline, as a standard antibiotic, showed a MIC value of 31.25 µg/mL, indicating stronger antibacterial activity than the extract individually. However, the combination of the optimized extract with tetracycline produced a combined MIC value of 31.25 µg/mL with a FICI value of 0.37. Based on this FICI value, the relationship between the optimized extract and tetracycline was synergistic, meaning that the combination significantly increased antibacterial activity compared to the use of each agent separately.

Table 6. Antimicrobial activity of samples against multidrug-resistant *Acinetobacter baumannii*

Samples	MIC (µg/mL)	MBC (µg/mL)	MIC combination (µg/mL)	FICI	Interaction
Optimized extract	125	250	31.25	0.37	Synergistic
Tetracycline	31.25	31.25	3.90		

FICI: fractional inhibitory concentration index; MBC: minimum bactericidal concentration; MIC: minimum inhibitory concentration

Bacteriolytic activity of the samples

The antibacterial activity of the samples was determined through their bacteriolytic effects. The bacteriolytic effects of the samples and their combinations were measured through the number of nucleic acid components released from *A. baumannii* cells due to sample exposure. Our data indicated significant variations in the amount of nucleic acid released in each treatment group described by OD values (**Figure 3A**). In the optimized extract (group 1), the OD values were significantly elevated compared to the control group (0.27±0.01 vs 0.05±0.00, $p<0.0001$). This indicated that the optimized extract could stimulate the release of nucleic acids from *A. baumannii* cells. Meanwhile, group 2, which was treated with tetracycline, also demonstrated an increase in OD values compared to the control group (0.09±0.00 vs 0.05±0.00, $p=0.03$). It examined the tetracycline in MIC concentration that could trigger the release of nucleic acid from *A. baumannii* and also similar to the administration of the extract against *A. baumanii*. Our data also indicated that the bacteriolytic effect of tetracycline (group 2) was lower than the effect of administration of optimized extract (group 1) with 0.09±0.00 and 0.27±0.01, respectively and $p<0.0001$. Group 3, which was a combination of optimized extract and tetracycline, had the

highest OD values, and significantly higher compared to control group, tetracycline only group or optimized extract only group, all had $p<0.0001$. These findings suggested that treatment with the optimized extract, tetracycline, and a combination of both can increase the release of nucleic acid components from *A. baumannii*. The use of a combination of the optimized extract and tetracycline produced the strongest effect, indicating the potential synergy between the two substances in affecting bacterial cell integrity.

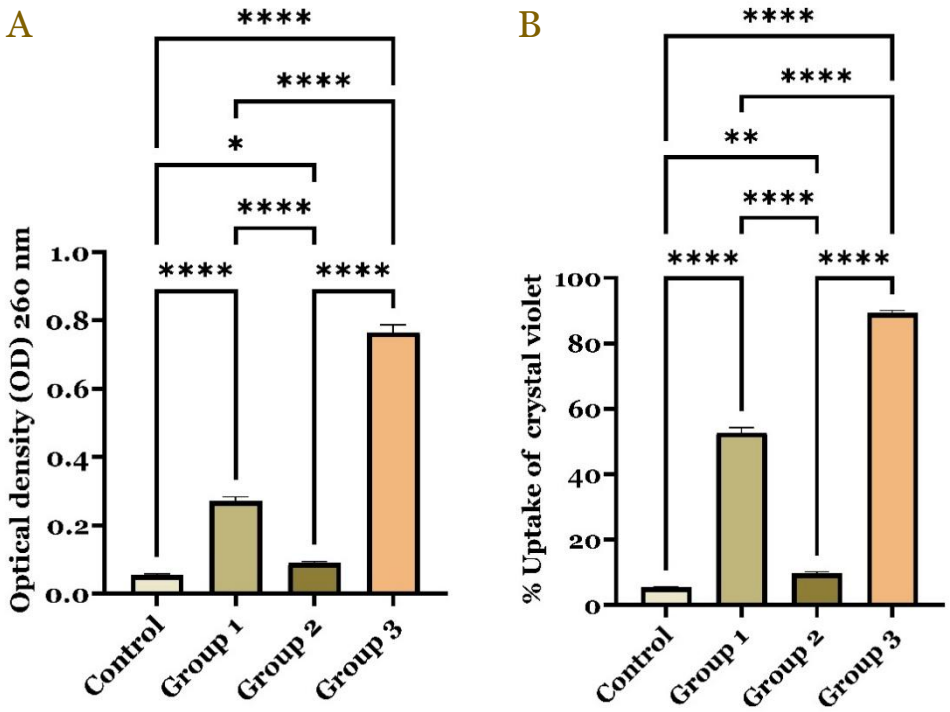

Figure 3. Bacteriolytic effects of the tested samples against multidrug-resistant *Acinetobacter baumannii*. The bacteriolytic effects are expressed as the number of nucleic acid components released from the bacteria (A) and percent uptake of crystal violet (B). Control treated with 0.5% v/v DMSO; Group treated with 125 µg/mL of the optimized extract only; Group 2 treated with 31.25 µg/mL of tetracycline only, and Group 3 treated with 31.25 µg/mL of the optimized extract and 3.90 µg/mL of the tetracycline. *Significantly different with $p<0.05$, **Significantly different with $p<0.01$, ****Significantly different with $p<0.0001$.

To support the bacteriolytic effect of sample exposures, a crystal violet uptake test was carried out. This test was conducted to assess the effect of the sample on membrane destabilization and the results are presented in **Figure 3B**. The results showed significant variation in the level of crystal violet absorption between the three treatment groups. In group 1 (optimized extract), the percentage of crystal violet absorption was recorded at 52.75±1.54%, which was higher compared to the control group ($p<0.0001$). This suggests that the optimized extract could affect the permeability of the *A. baumannii* cell membranes, allowing more crystal violet to enter the cells. Group 2, which was treated with tetracycline, had 9.70±0.50% crystal violet absorption, indicating that tetracycline increased the permeability of the bacterial cell membrane, although the absorption rate was slightly lower compared to the optimized extract ($p<0.0001$) (**Figure 3B**). In group 3, which received a combination of extract and tetracycline, the crystal violet absorption reached 89.28±0.77%, which was the highest value among the three treatment groups (all comparisons had $p<0.0001$). These results indicated that the combination of extract and tetracycline provided a strong synergistic effect in increasing the permeability of the cell membrane, allowing more crystal violet to be absorbed into the bacterial cells. Overall, the results of this study demonstrated that the optimized extract, tetracycline, and the combination of both could increase crystal violet uptake by *A. baumannii* cells. The combination of the extract and tetracycline demonstrated the most significant effect, suggesting a synergistic potential for enhancing the bacteriolytic effect.

Efflux pump inhibitor assay

The effect of the sample on inhibiting the efflux pump was assessed based on the accumulation of EtBr and the results are presented in **Figure 4**. EtBr accumulation was measured using relative fluorescence unit (RFU) to evaluate the effect of treatment on bacterial efflux pump activity. In group 1 which was treated with an optimized extract only, EtBr accumulation was recorded to increase starting from 5 min, 15 min, and 45 min by 155621.66, 171723.33, and 193655.00 RFU, respectively. This showed a significant increase compared to the control with values of 113770.00, 120005.00, and 126866.66 RFU, respectively ($p<0.01$; $p<0.01$; and $p<0.0001$) (**Figure 4**). This suggests that the optimized extract could inhibit the activity of the *A. baumannii* efflux pump, allowing more EtBr to accumulate in the cells.

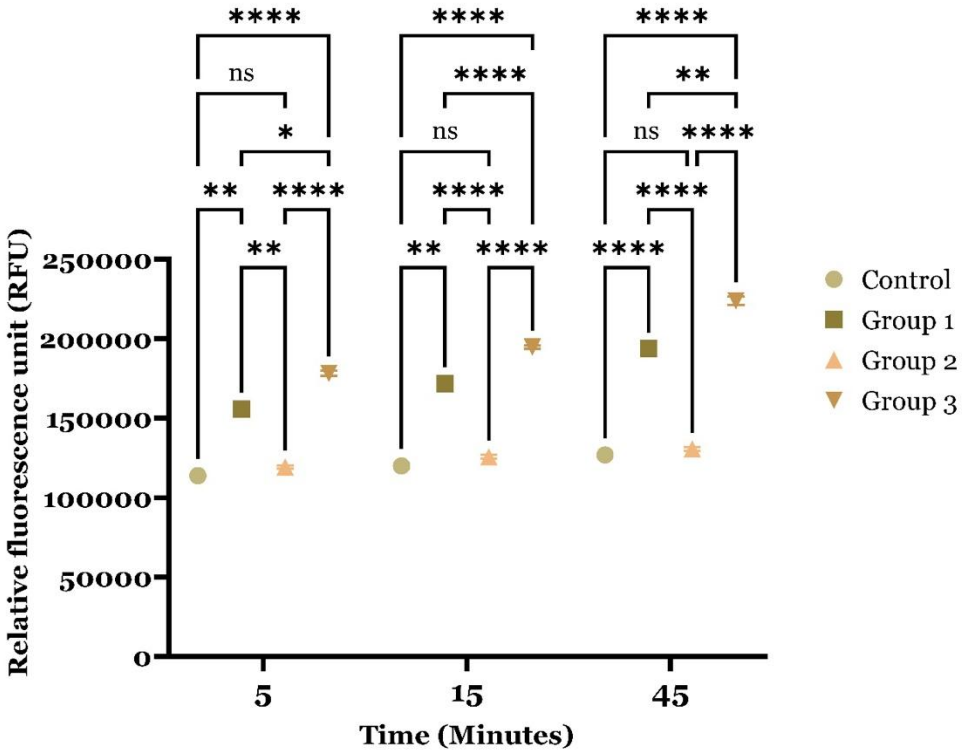

Figure 4. Effects of tested samples in inhibiting the efflux pump of multidrug-resistant *Acinetobacter baumannii*. Control was treated with 0.5% v/v DMSO; Group 1 was treated with 125 µg/mL of the optimized extract only; Group 2 was treated with 31.25 µg/mL of tetracycline only, and Group 3 was treated with 31.25 µg/mL of the optimized extract and 3.90 µg/mL of the tetracycline. *Significantly different at $p<0.05$, **Significantly different at $p<0.01$, ****Significantly different at $p<0.0001$, ns: not significantly different at $p>0.05$.

Group 2, which was treated with tetracycline, showed higher EtBr accumulation compared to the control at each observation time, with RFU values of 119241.66, 125685.00, and 130548.33 (**Figure 4**). This increase indicates that tetracycline also has the potential to inhibit efflux pumps in *A. baumannii*, although at a lower level compared to the optimized extract and is known to be not significantly different from the control where $p>0.05$ in each observation time. In Group 3, which was given a combination of the optimized extract and tetracycline, EtBr accumulation was recorded as the highest RFU values at each observation time, with 178185.00, 194675.00, and 223809.66 RFU (**Figure 4**). These results indicate that the combination of the optimized extract and tetracycline provides a stronger synergistic effect in inhibiting efflux pump activity, which allows for higher EtBr accumulation in bacterial cells compared to a single treatment of optimized extract ($p<0.05$, $p<0.0001$, and $p<0.01$). Overall, the results of this study indicate that both optimized extract, tetracycline, and a combination of both can increase EtBr accumulation in *A. baumannii* cells, indicating inhibition of efflux pump activity. The combination of the optimized extract and tetracycline provides the most significant synergistic effect, which has the potential to increase the effectiveness of antibiotic therapy by overcoming resistance caused by efflux pumps.

Discussion

This study demonstrated that the extraction of polyphenols from *P. emblica* fruit was significantly affected by ethanol concentration, extraction time, and solid-to-liquid ratio. In the preliminary single-factor experiments, ethanol concentration proved to be a key factor in enhancing the TPC, with concentrations of 50–70% yielding the highest extraction efficiency. This finding aligns with previous research suggesting that binary solvent systems, such as ethanol/water mixtures, enhance the solubility of polyphenolic compounds due to their relative polarity, thereby improving the extraction process [46]. Beyond 70%, increasing the ethanol concentration did not lead to a significant improvement in TPC, indicating that the optimal ethanol concentration for extracting polyphenols from *P. emblica* is around 70% [47]. Similarly, the effect of extraction time demonstrated that extending the extraction process beyond two days resulted in a decrease in TPC, which could be attributed to the saturation of polyphenols in the solvent after this period. These findings are consistent with the saturation hypothesis in solvent extraction, where further exposure does not significantly enhance the extraction yield [48]. Consequently, an extraction time of two days was considered optimal for further experimentation. The solid-to-liquid ratio also exhibited a significant effect on extraction efficiency, with the highest TPC observed at a ratio of 1:10 (w/v). This is likely due to the optimal mass transfer between the solvent and the solid material, as further increases in the ratio beyond 1:10 did not significantly improve extraction. These results are consistent with the principle that increasing the solid-to-liquid ratio enhances the extraction process only up to a certain point, after which the mass transfer of polyphenols reaches its limit [49].

The analysis using RSM further revealed the complex interaction between the three independent variables, with a quadratic model providing the best fit for the experimental data most adequately [50]. The RSM analysis demonstrated that the optimal conditions for polyphenol extraction from *P. emblica* fruit were 68.92% ethanol, an extraction duration of 1.85 days, and a solid-to-liquid ratio of approximately 1:9.58. These findings were validated through experimental verification, where the predicted TPC of 129.19 mg GAE/g was closely matched by the actual value of 130.76±2.46 mg GAE/g, indicating the robustness and reliability of the model. This result exceeds a previous study that identified a TPC of 128.10 mg GAE/g in dried *P. emblica* fruit [51]. The observed difference can be attributed to the location of harvest, the solvent type, and the extraction method employed [52]. Further analysis of the phenolic compounds in the optimized extract revealed the presence of several bioactive compounds, including quercetin, kaempferol, and rutin, all known for antimicrobial properties. This is consistent with a previous study that reported the presence of these compounds in *P. emblica* extracts [53].

One of the most important findings of this study was the significant antibacterial activity exhibited by the optimized *P. emblica* fruit extract, particularly against MDR *A. baumannii*. The extract demonstrated notable antibacterial effects with a MIC value of 125 µg/mL and an MBC value of 250 µg/mL. When compared to the standard antibiotic tetracycline, which exhibited a MIC of 31.25 µg/mL, the extract alone showed weaker antibacterial activity. However, this result is not unexpected, as polyphenolic compounds are generally less potent than antibiotics when used in isolation [54]. Nevertheless, the combination of the *P. emblica* extract with tetracycline resulted in a significantly improved antibacterial effect, with a combined MIC value of 31.25 µg/mL, matching the MIC of tetracycline alone. The fractional inhibitory concentration index (FICI) of 0.37 indicates a synergistic interaction between the extract and tetracycline, highlighting the potential of polyphenol-rich extracts to enhance the effectiveness of conventional antibiotics against resistant strains [55]. This result, supported by previous studies, showed that various polyphenols enhance the efficacy of tetracyclines. For example, quercetin synergized with tetracycline against MDR *Escherichia coli* [56], while *Ficus nitida* phenolic extract exhibited synergistic effects with tetracycline against several pathogens [38]. Similarly, baicalin demonstrated synergy with oxytetracycline and tetracycline against *Staphylococcus aureus* [57]. These findings suggest that polyphenols could be valuable adjuvants in combating antibiotic-resistant infections.

The synergistic effect observed between the optimized extract and tetracycline is particularly significant in the context of MDR bacteria. Resistance to antibiotics, particularly through mechanisms such as efflux pumps, enzymatic degradation, and altered target sites, has led to the

emergence of difficult-to-treat infections [58]. The combination therapy approach using natural plant extracts like *P. emblica* could serve as a promising strategy to overcome these resistance mechanisms and restore the efficacy of antibiotics, especially in combating resistant pathogens like *A. baumannii*. Further supporting the antibacterial potential of the extract, the bacteriolytic effects of the optimized *P. emblica* extract were evaluated by measuring the release of nucleic acid components from *A. baumannii* cells. The optimized extract showed a significant increase in the OD values, indicating a substantial release of cellular material, which is a typical indicator of membrane disruption [59]. The results were comparable to those observed with tetracycline, although the effect of the extract was stronger ($p<0.0001$), suggesting that the optimized extract could significantly affect the integrity of the bacterial cell membrane.

Interestingly, the combination of the optimized extract and tetracycline produced the most significant bacteriolytic effect, further supporting the idea that the two substances work synergistically. This enhanced membrane disruption could be due to the polyphenolic compounds in the extract acting on the bacterial cell membrane, increasing its permeability [60], while tetracycline targets bacterial protein synthesis [61], providing a two-pronged approach to bacterial eradication. These findings are aligned with previous studies that have demonstrated the membrane-disrupting activity of plant polyphenols [62]. In addition, the crystal violet uptake test, which measures the permeability of the bacterial cell membrane, showed that the optimized extract can disrupt the *A. baumannii* cell membrane. Group 1, which was treated with the optimized extract, demonstrated a 52.75% absorption rate of crystal violet, significantly higher than the control group, indicating that the extract increased the permeability of the bacterial membrane. Tetracycline, which showed a lower absorption rate (9.7%), likely exerts its antibacterial effect through a different mechanism, primarily by inhibiting protein synthesis. The combination of the extract and tetracycline showed the highest crystal violet uptake (89.28%), suggesting a synergistic effect that further compromised the bacterial cell membrane. When the optimized extract of *P. emblica* fruit and tetracycline are combined, the polyphenolic compounds likely compromise the cell membrane by increasing its permeability [63], while tetracycline simultaneously weakens the bacterial cell's ability to repair or maintain that membrane [64]. As a result, the bacterial cell becomes more vulnerable to the destabilizing effects of the polyphenols, allowing more crystal violet to enter and bind to the bacterial cell's internal structures. This is in contrast to the individual treatments, where the effects on the membrane are less pronounced.

Additionally, the efflux pump inhibitor assay revealed that the optimized *P. emblica* extract significantly inhibited the efflux pump activity in *A. baumannii*. Efflux pumps are a major mechanism by which bacteria expel antibiotics, rendering many drugs ineffective [65]. By inhibiting these pumps, the extract increases the intracellular concentration of antibiotics like tetracycline, potentially restoring their efficacy [66]. The optimized extract was shown to increase the accumulation of EtBr in the MDR *A. baumannii* cells over time, suggesting the inhibition of efflux pump activity. The combination of the extract and tetracycline resulted in the highest EtBr accumulation, suggesting that this combination could effectively counteract one of the primary resistance mechanisms in MDR bacteria. In this study, the optimized extract of *P. emblica* likely interferes with the efflux pump's ability to expel EtBr from the bacterial cell. As a result, EtBr accumulation inside the bacterial cell increased significantly. Polyphenolic compounds, such as those found in the optimized extract of *P. emblica*, have been shown to inhibit bacterial efflux pumps [67]. By disrupting the function of these pumps, polyphenols increase the intracellular accumulation of substances that would otherwise be expelled. Moreover, the presence of tetracycline can lead to the downregulation of certain efflux pump genes or further damage the bacterial cell's ability to maintain the pump function, making the bacteria even more susceptible to the effects of the optimized extract of *P. emblica*. Thus, the combination treatment reduces the ability of the efflux pump to expel not only tetracycline but also EtBr, a marker for efflux activity.

The combined antibacterial, bacteriolytic, and efflux pump-inhibiting effects of the optimized *P. emblica* extract are highly promising, particularly in the context of combating antibiotic resistance. The synergistic interaction between the polyphenolic extract and tetracycline suggests that polyphenols in the extract not only enhance the effectiveness of the antibiotic but also assist in overcoming bacterial resistance mechanisms, such as efflux pump activity and membrane integrity [68]. This synergy could be crucial in the treatment of infections

caused by resistant pathogens like *A. baumannii*, a major nosocomial pathogen known for its multidrug resistance. The potential of *P. emblica* fruit extract as an adjuvant in antibiotic therapy could pave the way for new approaches to treating resistant infections. Moreover, the use of plant-derived polyphenols as adjunctive therapies offers a natural, less toxic alternative to traditional antibiotic treatments, which often come with severe side effects [69]. Future studies are supposed to focus on further elucidating the molecular mechanisms through which these polyphenolic compounds exert their antibacterial effects and exploring their clinical applications in combination with existing antibiotics.

Conclusion

The RSM with Box-Behnken design approach successfully optimized the extraction condition to generate a polyphenol-rich extract of *P. emblica* fruit. The optimum TPC of *P. emblica* fruit was gained under conditions of 68.92% ethanol, 1.85 days of extraction, and a solid-to-liquid ratio of approximately 1:9.58. The predicted and experiment results for TPC were not significantly different: 129.19 mg GAE/g and 130.76±2.46 mg GAE/g, respectively. The optimized extract of *P. emblica* fruit had at least six phenolic compounds, including quercetin, kaempferol, caffeic acid, protocatechuic acid, rutin, and hyperoside. In addition, the optimized extract of *P. emblica* fruit showed antimicrobial activities (both as bacteriolytic and efflux pump inhibitor) against MDR *A. baumannii,* whether administered alone or in combination with tetracycline. Synergistic antimicrobial activity was observed in the combination of the optimized extract from *P. emblica* fruit and tetracycline against MDR *A. baumannii*. This synergistic action counters one of the primary resistance mechanisms in MDR bacteria, offering a promising strategy to improve the effectiveness of antibiotics against resistant strains.

Ethics approval

Not required.

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Competing interests

All the authors declare that there are no conflicts of interest.

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Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

Declaration of artificial intelligence use

This study used artificial intelligence (AI) tools and methodologies during manuscript writing. ChatGPT and Quillbot were employed for language refinement and technical writing assistance. We confirm that all AI-assisted processes were critically reviewed by the authors to ensure the integrity and reliability of the results. The final decisions and interpretations presented in this article were solely made by the authors.

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