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In Vitro Antibacterial Activity of Curcumin and Protocatechuic Acid Against Extended-Spectrum β-Lactamase–Producing Klebsiella pneumoniae Isolated from Chronic Cough Sputum

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ABSTRACT

Introduction: Curcumin and protocatechuic acid are naturally occurring phenolic compounds that have been investigated for their potential antimicrobial properties. Extended-spectrum beta-lactamase-producing Klebsiella pneumoniae is a major clinical concern because of the limited therapeutic options. This study aimed to isolate and identify K. pneumoniae ESBL from the sputum of patients with chronic cough and evaluate the antibacterial effects of curcumin and protocatechuic acid.

Methods: A laboratory-based experimental study was conducted using sputum specimens from 100 patients with chronic cough. Bacterial isolation and identification were performed using standard microbiological procedures, followed by the confirmation of ESBL production. The antibacterial activities of curcumin and protocatechuic acid at 25 %, 50 %, and 75% concentrations were assessed using the disc diffusion method. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by post hoc testing.

Results: Nine bacterial species were identified in the sputum samples. Klebsiella pneumoniae accounted for 27.5% of isolates, of which 7.5% were confirmed as ESBL-producing strains. Curcumin, at all tested concentrations, produced inhibition zones of approximately 6 mm, with no statistically significant differences between the concentrations tested. In contrast, protocatechuic acid demonstrated concentration-dependent antibacterial activity, producing mean inhibition zones of 20.6, 16.0, and 10.8 mm at 25%, 50%, and 75% concentrations, respectively, with statistically significant differences. The antibacterial effect of 75% protocatechuic acid was comparable to that of 10 µg meropenem.

Conclusion: Curcumin showed no significant antibacterial activity against ESBL-producing K. pneumoniae, while protocatechuic acid exhibited significant and concentration-dependent inhibitory effects. These findings suggest that protocatechuic acid is a potential natural antibacterial agent against ESBL-producing K. pneumoniae.

Keywords

Curcumin, Protocatechuic acid, Klebsiella pneumoniae, Extended-spectrum beta-lactamase, Antibacterial activity, ESBL

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INTRODUCTION

Naturally derived polyphenolic compounds have gained considerable attention as potential alternative antibacterial agents. Curcumin, the principal phenolic constituent of turmeric (Curcuma domestica Valeton), exerts antibacterial effects by disrupting the integrity of bacterial cell membranes through lipid bilayer modification, leading to impaired cellular homeostasis and inhibition of essential metabolic processes [1]. Similarly, roselle (Hibiscus sabdariffa Linn) contains bioactive polyphenols with antimicrobial properties,

among which protocatechuic acid is the key compound [2]. Protocatechuic acid (3,4-dihydroxybenzoic acid) can be oxidatively converted to p-hydroxybenzoic acid, a metabolite associated with bactericidal activity against several gram-negative bacteria, including Vibrio and Pseudomonas species [3,4]. Experimental studies have indicated that curcumin and protocatechuic acid share common antimicrobial mechanisms, including the disruption of bacterial membrane function and inhibition of intracellular protein synthesis, even at relatively low concentrations [5-8].

Bacterial pathogens frequently infect humans via the respiratory tract, which serves as the primary portal of entry into the body [8]. Respiratory tract infections commonly present with cough, and symptoms persisting for more than eight weeks are classified as chronic cough. Chronic bacterial respiratory infections remain a significant clinical concern because of their prolonged course, recurrence, and negative impact on quality of life. Several bacterial species, including Klebsiella pneumoniae, Streptococcus pneumoniae, Haemophilus influenzae, Pseudomonas aeruginosa, Staphylococcus aureus, and members of the Enterobacter genus [9-11]. Among these pathogens, Klebsiella pneumoniae is of particular concern because of its ability to colonize the lower respiratory tract, cause persistent infections, and develop resistance to multiple antimicrobials.

Klebsiella pneumoniae is a Gram-negative bacillus capable of producing β-lactamase enzymes that hydrolyze a broad range of β-lactam antibiotics, including penicillins, cephalosporins, and aztreonam. Strains expressing extended-spectrum β-lactamase (ESBL) represent a major therapeutic challenge in respiratory tract infections [12]. The global prevalence of ESBL-producing bacteria has increased markedly, with Klebsiella pneumoniae being the most prevalent ESBL-producing species (31.1%), followed by Escherichia coli (8.6%) and Proteus mirabilis (6.3%) [13]. In Asia, the prevalence of ESBL ranges from 5% to 8%, whereas in Indonesia, it reaches 12%–24%, indicating a substantial regional burden [14]. Given the limited treatment options and growing resistance associated with ESBL-producing K. pneumoniae, the exploration of alternative antibacterial agents is urgently needed. Therefore, this study aimed to evaluate the antibacterial effectiveness of curcumin and protocatechuic acid against ESBL-producing Klebsiella pneumoniae isolated from sputum samples of patients with chronic cough.

METHOD

This study was conducted as a laboratory-based experimental investigation using a completely randomized design. Two natural compounds, curcumin and protocatechuic acid, were assessed for their antibacterial activities. Each compound was assigned to five experimental groups, consisting of two control groups and three treatment groups with different concentrations. All experiments were performed in five independent replicates to ensure reproducibility and reliability of the results.

All laboratory procedures were performed using standard microbiological and analytical equipment. General laboratory equipment included analytical balances, glassware (beakers, graduated cylinders, Erlenmeyer flasks, volumetric flasks, and test tubes), Petri dishes, inoculating loops, forceps, spatulas, and microscope slides with staining racks. Sterilization and incubation were performed using a Bunsen burner, spirit lamp, autoclave, incubator, oven, and laboratory heater. Microbiological analyses were performed using light microscopy and sterile sputum containers under aseptic conditions. Sample processing and compound purification involved the use of maceration equipment, desiccators, column chromatography apparatuses, vacuum systems, and cutting instruments. All procedures used continuously supplied laboratory-grade distilled water.

The materials used in this study included sputum samples collected in sterile containers, distilled water, and standard laboratory solvents. The microbiological reagents comprised methylene blue, immersion oil, xylene, sterile blank antibiotic discs, and meropenem discs, which were used as a positive control. The culture media included MacConkey agar for bacterial isolation and Mueller–Hinton agar for antimicrobial susceptibility testing (AST). Analytical materials used for compound separation included silica gel, chromatography plates, activated charcoal, and column chromatography. The chemical reagents used were ethyl acetate, n-hexane, ethanol, calcium chloride, ferric chloride, sulfur-containing reagents, hydrochloric acid, lead acetate, gasoline, and distilled water. Supporting consumables, such as lens paper, labeling materials,

and tissue paper, were also used. All equipment, media, and heat-resistant materials were sterilized before use to prevent microbial contamination. Sterilization was performed using moist heat in an autoclave at 121 °C for 15 min under standard pressure conditions. This procedure ensured aseptic conditions during the experiment.

MacConkey agar was prepared by dissolving 18.89 g of powdered medium in 400 mL of distilled water, followed by heating until completely dissolved. The medium was sterilized by autoclaving at 121 °C for 15 min, cooled to approximately 45 °C, and aseptically poured into sterile Petri dishes (≈20 mL per plate). After solidification, the plates were inverted and incubated at 35 ° C for 24 h prior to use. Mueller‐Hinton agar was prepared by dissolving 15.2 g of powdered medium in 400 mL of distilled water and heating until fully dissolved. After autoclaving at 121 °C for 15 min, the medium was cooled in a water bath at approximately 45 °C, poured into sterile Petri dishes, and allowed to solidify. The plates were inverted and incubated at 35 °C for 24 h before use.

Hydrogen sulfide gas was generated through a controlled reaction between FeS and hydrochloric acid. Briefly, 200 mL of hydrochloric acid was placed in a sealed glass reaction vessel, followed by the careful addition of iron sulfide (FeS). The vessel was tightly closed to prevent gas leakage. The generated hydrogen sulfide gas was directed into a reaction flask containing the test sample and allowed to react as required. Petroleum ether was separated from gasoline using a distillation technique based on the differences in their boiling points. Distillation was performed at a temperature range of 30–40 °C, allowing the petroleum ether fraction to vaporize and condense for collection, effectively separating it from the higher boiling components. Sputum samples were obtained from patients with chronic cough at the Clinical Microbiology Laboratory of the Department of Clinical Pathology at RSUDZA. Prior to sample collection, the patients were instructed to rinse their mouths with water to reduce oral contamination. Sputum was collected following deep inhalation and forceful coughing to ensure sampling from the lower respiratory tract. Samples were collected in sterile wide-mouthed containers. Adequate specimens were defined as thick, purulent sputum with a volume of 3–5 mL and minimal saliva contamination.

Sputum specimens were aseptically inoculated onto MacConkey agar plates and incubated at 37 °C for 24 h. Distinct colonies were selected and subcultured onto fresh MacConkey agar plates to obtain pure isolates for further analyses. The subcultures were incubated at 37 °C for 24 h before further analyses. The initial identification was based on macroscopic colony characteristics, including morphology, color, surface texture, and margin appearance. Microscopic identification was performed using Gram staining. Klebsiella pneumoniae was identified as a Gram-negative, rod-shaped bacterium. Biochemical identification included an oxidase test, with results interpreted based on color change within the specified time frame. Definitive identification was performed using the API 20E system. A bacterial suspension adjusted to 0.5 McFarland turbidity was prepared and inoculated into API 20E strips according to the manufacturer's instructions. The strips were incubated at 30 °C for 24–48 h before use. Only pure cultures were included in the interpretation of the results.

Extended-spectrum β-lactamase production was confirmed using the antibiotic disc diffusion method, in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines. Antibiotic-impregnated discs were placed on Mueller–Hinton agar plates inoculated with standardized bacterial suspensions. After incubation at 37 °C for 24 h, the inhibition zone diameters were measured and interpreted using the CLSI breakpoints. Isolates resistant to β-lactam antibiotics but susceptible to carbapenems and monobactams were classified as ESBL-producing Klebsiella pneumoniae. Dried turmeric rhizomes (Curcuma domestica Valeton) were macerated in absolute ethanol (100%) to obtain a crude curcuminoid extract. Curcumin was purified by column chromatography using silica gel GF254 as the stationary phase and n-hexane–ethyl acetate (6:4) as the mobile phase. Fractions were analyzed using thin-layer chromatography, and curcumin-containing fractions were collected and evaporated to obtain purified curcumin. Protocatechuic acid was isolated from dried roselle (Hibiscus sabdariffa Linn) calyces using a precipitation-based extraction method. The extract was subjected to sequential purification steps, including hydrogen sulfide treatment, vacuum filtration, concentration, solvent partitioning, and desiccation using calcium chloride. The presence of phenolic compounds was confirmed using a ferric chloride reagent, and the isolated compound was identified as protocatechuic acid (PCA).

The antibacterial activities of curcumin and protocatechuic acid were evaluated using the agar diffusion method on Mueller–Hinton agar. ESBL-producing Klebsiella pneumoniae suspensions were adjusted to 0.5 McFarland turbidity and evenly inoculated onto agar plates. Sterile discs impregnated with different concentrations of each compound were placed on the agar surface and incubated at 37 °C for 24 h. Antibacterial activity was assessed by measuring the diameter of the inhibition zone around each disc.

RESULTS

A total of 100 sputum samples obtained from patients presenting with chronic cough were analyzed at the Clinical Microbiology Laboratory of RSUDZA. Microbial growth was detected in 39 specimens. Of these, bacterial isolates were identified in 33 samples (33%), fungal isolates in 5 samples (5%), and concurrent bacterial and fungal growth in one sample (1%). The remaining 61 samples (61%) showed no detectable microbial growth under the culture conditions employed in this study (Table 1).

Table 1. Microbiological Isolation Results from Chronic Cough Sputum Samples

Microorganism Category	Frequency (n)	Percentage (%)
Bacteria	33	37
Fungi	5	4
Bacteria + Fungi	1	1
No Growth	61	58
Total	100	100

Note: Sputum cultures were obtained from patients with chronic cough. “No growth” indicates the absence of identifiable microorganisms after the standard incubation period.

The results indicated that chronic cough–associated infections were predominantly caused by a single type of microorganism, either bacterial or fungal. The absence of growth in a large proportion of specimens may be attributed to the presence of fastidious organisms that require specialized culture media for growth. From 38 sputum samples with positive cultures, 40 bacterial isolates were obtained. Nine bacterial species were identified. Klebsiella pneumoniae was the most frequently isolated organism (11 isolates; 27.5%), followed by Staphylococcus aureus (9 isolates; 22.5%), Acinetobacter spp. (6 isolates; 15%), Pseudomonas aeruginosa (5 isolates; 12.5%), Streptococcus spp. (4 isolates; 10%), Streptococcus viridans (2 isolates; 5%), Enterobacter spp. (1 isolate; 2.5%), Klebsiella ozaenae (1 isolate; 2.5%), and Staphylococcus spp. (1 isolate; 2.5%) (Table 2).

Table 2. Bacterial Isolates Identified from Chronic Cough Sputum (n = 40)

Bacterial Species	n	%
Klebsiella Pneumoniae	11	27.5
Staphylococcus Aureus	9	22.5
Acinetobacter spp.	6	15.0
Pseudomonas Aeruginosa	5	12.5
Streptococcus spp.	4	10.0
Streptococcus Viridans	2	5.0
Other Bacteria	3	7.5
Total	40	100

Note: Other bacteria include *Enterobacter* spp., *Klebsiella ozaenae*, and *Staphylococcus* spp.

Antimicrobial susceptibility testing of the 11 Klebsiella pneumoniae isolates revealed resistance to ceftazidime (CAZ) in three isolates (27.3%) and cefotaxime (CTX) in four isolates (36.4%). Three isolates (2.5% of total bacterial isolates) exhibited concurrent resistance to CAZ and CTX and were classified as ESBL-producing Klebsiella pneumoniae. These ESBL-positive isolates were subsequently used as test organisms in the antibacterial activity assays (Table 3).

Table 3. Antibiotic Susceptibility of *Klebsiella pneumoniae* (n = 11)

Antibiotic	S	R
AMP	1	10
AMC	2	9
CAZ	8	3
CTX	7	4
MEM	9	2

Note: S = Sensitive; R = Resistant.

Column chromatography of turmeric extract yielded 53 fractions. Based on similar chromatographic profiles, these fractions were pooled into three combined fractions (Table 4), as follows: fractions 3–19 exhibited the highest Rf values and visible yellow spots consistent with curcumin and were therefore selected for further analysis.

Table 4. n-Hexane:Ethyl Acetate (6:4) Fractionation of Turmeric Rhizome Curcumin

Combined Fraction	Color	Rf Value
A (Fractions 3-19)	Dark yellow	0.36–0.40
B (Fractions 20-33)	Yellow	0.30–0.34
C (Fractions 34-53)	Pale yellowish	0.10–0.14

Figure 1. Results of Analysis with TLC

The inhibition zones produced by curcumin against ESBL-producing Klebsiella pneumoniae at concentrations of 75%, 50%, and 25% were identical, with a mean diameter of 6 mm for all three concentrations. The positive control (meropenem) produced a mean inhibition zone of 32.4 mm, whereas the negative control produced a zone of 5 mm (Tables 5 and 6).

Table 5. Mean Inhibition Zone Diameter of Curcumin Against *Klebsiella pneumoniae* ESBL

Treatment	Mean ± SD (mm)	Significance (α = 0.05)
PC-0	5.0 ± 0.00	a
PC-1	6.0 ± 0.00	a
PC-2	6.0 ± 0.00	a
PC-3	6.0 ± 0.00	a
PC-4	32.4 ± 1.52	b

Note: Different superscript letters indicate statistically significant differences (P < 0.05).

Table 6. Inhibition Zone Diameter of Curcumin Against *Klebsiella pneumoniae* ESBL

Treatment	Replicates (n = 5), mm	Mean ± SD (mm)
PC-0	5, 5, 5, 5, 5	5.0 ± 0.00
PC-1	6, 6, 6, 6, 6	6.0 ± 0.00
PC-2	6, 6, 6, 6, 6	6.0 ± 0.00
PC-3	6, 6, 6, 6, 6	6.0 ± 0.00
PC-4	30, 34, 33, 33, 32	32.4 ± 1.52

The presence of protocatechuic acid was confirmed by a positive ferric chloride test, indicated by the development of a dark yellow–black coloration (Figure 2).

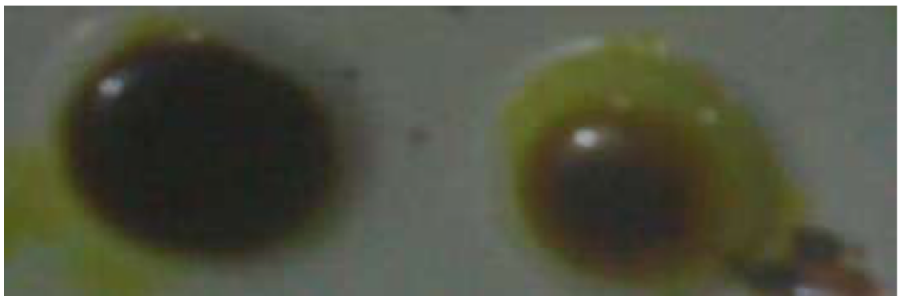

Figure 2. Positive Results for Ferric Chloride

Protocatechuic acid exhibited concentration-dependent antibacterial activity against ESBL-producing Klebsiella pneumoniae. The mean inhibition zone diameters were 20.6, 16.0, and 15.2 mm at 75 %, 50%, and 15.2 mm at 25% RH, respectively. The positive control (meropenem) produced a mean inhibition zone of 32.4 mm, whereas the negative control produced a zone of 5 mm (Tables 7 and 8).

Table 7. Inhibition Zone Diameter of Protocatechuic Acid Against *Klebsiella pneumoniae* ESBL

Treatment	Replicates (n = 5), mm	Mean ± SD (mm)
PP-0	5, 5, 5, 5, 5	5.0 ± 0.00
PP-1	23, 27, 20, 16, 17	20.6 ± 4.50
PP-2	18, 18, 16, 15, 13	16.0 ± 2.12
PP-3	14, 18, 16, 15, 13	15.2 ± 1.92
PP-4	33, 32, 30, 33, 34	32.4 ± 1.52

Table 8. One-Way ANOVA Results of Curcumin Treatment

Source of Variation	DF	SS	MS	F value	F table (5%)
Treatment	4	2844.64	711.16	1546**	2.87
Error	20	9.20	0.46		
Total	24	2853.84			

Note: indicates a highly significant difference ($P < 0.05$).

NOVA demonstrated a significant inhibitory effect of protocatechuic acid ($F = 75$; $p < 0.05$). Post-hoc analysis confirmed the significant differences among the treatment groups (Table 9).

Table 9. One-Way ANOVA Results of Protocatechuic Acid Treatment

Source of Variation	df	SS	MS	F value	F table (5%)
Treatment	4	1974.16	493.54	75**	2.87
Error	20	123.20	6.16		
Total	24	2097.36			

Note: indicates a highly significant difference ($P < 0.05$).

DISCUSSION

The present study demonstrated that Klebsiella pneumoniae was the most frequently isolated bacterial pathogen from sputum samples of patients with chronic cough. This finding reinforces the well-established role of K. pneumoniae as an important etiological agent of lower respiratory tract infections, particularly pneumonia and persistent respiratory symptoms. The ability of this organism to colonize the respiratory tract and cause infection is well documented, especially in hospitalized patients and individuals with underlying comorbidities [15,16]. Similar epidemiological studies from different geographic regions have consistently

reported K. pneumoniae as a dominant pathogen in pneumonia and chronic respiratory infections, supporting the relevance and external validity of our results [17,18].

The detection of extended-spectrum β-lactamase (ESBL)-producing K. pneumoniae in this study further highlights the growing challenge of antimicrobial resistance in respiratory tract infection treatment. ESBL production enables bacteria to hydrolyze a broad range of β-lactam antibiotics, including penicillins and third-generation cephalosporins, thereby significantly limiting the available therapeutic options [19]. Consequently, carbapenems remain the mainstay treatment for severe infections caused by ESBL-producing organisms. The presence of ESBL-producing K. pneumoniae in patients with chronic cough is clinically concerning, as it may contribute to persistent infection, an increased risk of treatment failure, prolonged hospitalization, and higher health care costs [20]. These findings underscore the importance of antimicrobial stewardship and the urgent need to explore alternative adjunctive antimicrobial strategies.

In this study, curcumin did not exhibit significant antibacterial activity against ESBL-producing K. pneumoniae. The absence of a concentration-dependent increase in the inhibition zone diameter and the comparable inhibitory effects observed across all tested concentrations indicate the limited efficacy against these highly resistant isolates. This result contrasts with those of previous studies that reported the antibacterial activity of curcumin against non-ESBL K. pneumoniae strains [21,22]. This discrepancy may be explained by differences in bacterial resistance profiles, as ESBL-producing strains often exhibit enhanced virulence, reduced outer membrane permeability, and active efflux mechanisms that can limit the intracellular accumulation of antimicrobial agents, including polyphenolic compounds such as curcumin [23].

In contrast, protocatechuic acid exhibited significant, concentration-dependent antibacterial effects against ESBL-producing K. pneumoniae. Higher protocatechuic acid concentrations were associated with larger inhibition zones, indicating an increased antibacterial potency. Although its activity was lower than that of meropenem, statistical analysis showed that meropenem at a dose of 10 µg was comparable to approximately 6.36 µg of protocatechuic acid at a 75% concentration. These findings are consistent with previous reports describing the antibacterial properties of protocatechuic acid derived from Hibiscus sabdariffa against K. pneumoniae and other gram-negative bacteria [24,25]. The antibacterial mechanism of protocatechuic acid is thought to involve intracellular oxidative reactions, leading to membrane destabilization, disruption of essential cellular functions, and eventual bacterial cell lysis [26,27]. Overall, the findings of this study indicate that protocatechuic acid possesses promising antibacterial activity against ESBL-producing K. pneumoniae, whereas curcumin demonstrates limited effectiveness against these highly resistant strains. The contrasting results observed between the two polyphenolic compounds emphasize the importance of selecting antimicrobial agents based on their activity against specific resistance mechanisms. These results support further investigation of protocatechuic acid as a potential complementary or alternative antibacterial agent for managing infections caused by ESBL-producing K. pneumoniae, particularly in the context of the global increase in antimicrobial resistance. This study was limited by its in vitro design and relatively small number of ESBL-producing isolates, which may limit the direct generalizability of the findings to clinical settings.

CONCLUSION

This study demonstrated that Klebsiella pneumoniae was isolated from 11% of sputum samples obtained from patients with chronic cough at RSUDZA, of which 3% were identified as extended-spectrum β-lactamase (ESBL)-producing strains. Curcumin isolated from turmeric rhizomes showed no antibacterial activity against ESBL-producing K. pneumoniae at 25 %, 50 %, or 75% concentrations. In contrast, protocatechuic acid exhibited significant antibacterial activity against ESBL-producing K. pneumoniae at all tested concentrations, with a clear concentration-dependent increase in the diameter of the inhibition zone observed. Notably, 75% protocatechuic acid demonstrated an inhibitory effect statistically equivalent to approximately 6.36 µg meropenem. These findings indicate that protocatechuic acid has promising potential as a natural antibacterial agent against ESBL-producing K. pneumoniae, whereas curcumin appears ineffective against these highly resistant strains.

DECLARATIONS

Ethics approval and consent to participate were obtained. The Ethics Committee of the Universitas Syiah Kuala, Banda Aceh, Indonesia, granted permission for this study.

CONSENT FOR PUBLICATION

The Authors agree to the publication in the Journal of Society Medicine.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest in this report.

AUTHORS' CONTRIBUTIONS

All authors significantly contribute to the work reported execution, acquisition of data, analysis, and interpretation, or in all these areas. Contribute to drafting, revising, or critically reviewing the article. Approved the final version to be published, agreed on the journal to be submitted, and agreed to be accountable for all aspects of the work.

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