

Green tea extract-mediated augmentation of imipenem antibacterial activity against *Enterobacter cloacae* clinical isolates

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ABSTRACT

The emergence of pathogenic bacteria with β -lactam antibiotics-resistant profile has threatened the continued use of such antibiotics in the future. This research was conducted to investigate the antimicrobial activity of green tea ethanol extract (GTE) and its ability to improve the antibacterial action of several β -lactam antibiotics against *Enterobacter cloacae* clinical isolates. The simplicia of green tea was extracted by sonication for 30 minutes using 50% ethanol solvent, and the total phenolic content of the GTE was subsequently determined. Next, the GTE used in testing against two clinical isolates of *E. cloacae* was obtained from the Pathology Laboratory of Wahidin Sudiro Husodo Hospital in Makassar. The sensitivity of bacteria to GTE was confirmed using the agar diffusion method, the Vitek® rapid method, and the double-disk synergistic test. Antibacterial activity of antibiotics, GTE, and combination of antibiotics with GTE were then tested against clinical isolates of *E. cloacae* using the checkerboard microdilution assay. The results showed that GTE contained 51.64 ± 0.21 % measured as gallic acid equivalent and $37.95 + 5.17$ % Epigallocatechin gallate (EGCG). The confirmatory test results indicated that one clinical isolate of *E. cloacae* (code 13/04) was resistant to amoxicillin-clavulanate but did not produce an extended-spectrum β -lactamase (ESBL). Another clinical *E. cloacae* isolate (code 275B/06) was indicated to produce ESBL and demonstrated to yield resistance to amoxicillin-clavulanate and cefotaxime. The minimum inhibitory concentration of GTE against the two clinical isolates of *E. cloacae* was >8000 ppm (8 mg/ml). In conclusion, GTE could not increase the antibacterial activity of amoxicillin and cefotaxime, but it was sufficient to improve the activity of imipenem against the tested isolates of *E. cloacae*.

Keywords: β -lactam antibiotics, *Camellia sinensis* L., Antibacterial modulation, *E. cloacae*

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INTRODUCTION

In the last decade, many *Enterobacter cloacae* complex (ECC) that were initially classified as opportunistic have been regarded as infectious-causing bacteria in hospitals and turned out to be resistant to conventional or even newer generations of antibiotics (Annavaajhala et al., 2019). *Enterobacter cloacae*, one of the Enterobacteriaceae species, is frequently isolated from patient specimens taken from the blood and the respiratory-, urinary-, and digestive tracts (Mezzatesta et al., 2012; Davin-Regli and Pagès, 2015). In addition, this pathogen has also been reported to contaminate medical equipment in hospitals (Davin-Regli and Pagès, 2015). Clinical isolates of *E. cloacae* were previously reported to be resistant against carbapenem, tigecycline, colistin (Huang et al., 2017). It was found that out of 385 patient specimens, 64.68% were found to be positive for pathogenic bacteria, including *E. cloacae* (2%). Unfortunately, third-generation cephalosporins were also inefficient against most of these bacteria (Radji et al., 2011).

Researchers are trying to find appropriate solutions in response to the increased cases of infection by antibiotic-resistant bacteria, the scarcity of effective antibiotics, and the high cost required to discover new antibiotics (Towse et al., 2017). One of the suggested ways is by maintaining the use of existing antibiotics in combination with bioactive compounds from medicinal plants that could further improve the antibacterial activity of those antibiotics (Coutinho et al., 2008; Matias et al., 2011). It has been recently reported that certain phenolic compounds can increase the antibacterial activity of conventional antibiotics (Sartini et al., 2019). For example, the MICs of kanamycin, gentamycin, amikacin, neomycin were decreased as much as 87.5% or even 99.9% when they were used in combination with the flavonoid-rich ethanolic extract of *Croton campestris* leaves (Júnior et al., 2011).

One of the natural resources demonstrated to incite antimicrobial activity against some pathogenic bacteria is green tea (Sartini et al., 2019). The antibacterial properties of green tea are mainly attributed to the catechin and caffeine compounds (Scoparo et al., 2012; Bermejo et al., 2015). In fact, epigallocatechin gallate (EGCG), a major catechin present in the green tea, was reported to increase gentamycin activity against multi-drug resistant (MDR) *Escherichia coli* (Parvez et al., 2019). However, it is essential to note that the combination of caffeine with several antibiotics yielded no improvement or even antagonistic activity towards some pathogenic bacteria (Bazzaz et al., 2016). Therefore, it is essential to note that the clinical combination of certain bioactive plant compounds and the existing antibiotics does not always improve the corresponding antibiotics' antibacterial activity. This research was carried out aiming to investigate whether green tea extract (GTE) could yield antibacterial activity and/or to improve the antibacterial activity of several β -lactam antibiotics (amoxicillin, cefotaxime, and imipenem) against clinical isolates of *E. cloacae*. Once it is proven, the use of existing β -lactam antibiotics can be further explored, in combination with certain bioactive compounds or plant extracts, for the clinical management of antibiotic-resistant pathogenic bacteria.

MATERIALS AND METHOD

Materials

Green tea used in this study was one of the commercial products marketed in Makassar, South Sulawesi, Indonesia. All antibiotic preparations and gallic acid were procured from Sigma-Aldrich. The Vitek[®]2 AST-G07 cassette (bioMe'rieux, Durham, NC), Brain Heart Infusion Broth (Merck), Muller Hinton Agar (Merck), and clinical isolates of *E. cloacae* were obtained from the Clinical Pathology Laboratory of Wahidin Sudiro Husodo Hospital in Makassar.

Preparation of green tea ethanol extract

Green tea ethanolic extract, or referred to as green tea extract (GTE), was prepared based on the previously established method (Sartini et al., 2019) with slight modifications. Briefly, GTE was prepared by firstly extracting the coarse powder of green tea by ultrasound-assisted maceration

method for 15 minutes using hexane (1:10) to eliminate non-polar compounds. The resulting extract was subsequently subjected to ultra-sonication-assisted maceration using 50 % ethanol with ratio of 1:10 for 15 minutes, and the resulting liquid GTE was kept at room temperature for 1×24 hours. At the designated time, the liquid GTE was processed using rotary evaporator at 60 °C and freeze dried to eliminate the water residue.

Calculation of total phenolic content and epigallocatechin gallate content

Total phenolic content (TPC) was confirmed and calculated by Folin–Ciocalteu spectrophotometry method based on the established protocol (Sartini et al., 2020), with slight modifications. Briefly, 10 mg of GTE was dissolved in methanol to obtain GTE concentrations of 600, 300, 150, and 75 µg/mL, respectively. Afterward, 100 µL of the obtained solution was immediately added to 2.5 mL of 7.5% w/v Folin-Ciocalteu reagent and homogenized. Following this step, 2.4 mL of 1% w/v NaOH was subsequently added to the mixture. The reaction was maintained at ambient temperature for 60 minutes, and the resulting absorbance was determined using UV spectrophotometer -1800 (Shimadzu). Gallic acid was used as the standard solution.

Next, the amount of epigallocatechin gallate (EGCG) in the GTE was determined using Ultra-Fast Liquid Chromatography (UFLC). Initially, serial dilutions of standard EGCG were prepared in methanol to obtain a calibration curve with concentrations ranged from 3.25 µg/mL to 200 µg/mL. Afterward, 10 mg of GTE was dissolved in methanol, and the resulting solution was injected into the UFLC to precisely quantify the EGCG concentration.

Antibiotic susceptibility test

Susceptibilities of clinical isolates of *E. cloacae* (codes 13/04 and 275B/06) to antibiotics were tested using both Vitek[®]2 automatic test and disk diffusion methods (Stone et al., 2007), with slight modifications. For the Vitek[®]2 automatic test, bacterial suspension was freshly prepared in 0.45% saline to achieve equal turbidity with 0.5 McFarland standard of the Densi-Chek 2 system (bioMérieux, Durham, NC). Subsequently, an antibacterial susceptibility testing card (AST-GN07) was inoculated with the prepared bacterial suspension. Results obtained from Vitek[®]2 were interpreted categorically based on the Advanced Expert System when available. For disk diffusion assay, plates of sterile Mueller-Hinton Agar were inoculated with 10 µL of bacterial suspension, by spread methods, equal to the turbidity of 0.5 McFarland standard. Commercially available antibiotic disks were immediately placed on the Mueller-Hinton Agar and incubated at 37°C for 1×24 hours. Interpretations of disk diffusion tests were carried out for *Enterobacteriaceae* based on the CLSI guidance, and categorical interpretations (susceptible, intermediate, or resistant) were subsequently assigned. The production of extended-spectrum β-lactamases (ESBL) was investigated by the double-disc synergy test (DDST) method, using cefotaxime and ceftazidime, either alone or in combination with clavulanic acid according to the previous study (Khalaf et al., 2008).

Determination of antibacterial and modulation assay of green tea extract (GTE)

The MIC of GTE, amoxicillin, cefotaxime, imipenem, and their combination was carried out by using the checkerboard microdilution assay (Fankam et al., 2017; Parvez et al., 2019), with slight modifications. Briefly, two-fold serial dilutions of each sample were freshly prepared in the BHIB in 96-well microtiter plates. Then, the suspension of *E. cloacae* was inoculated to each well to achieve 10⁶ CFU/mL of final concentration. Sequentially, the microplates were incubated at 37°C for 1×24 hours, and the MIC was subsequently determined by adding 10 µL solution of 0.1 % triphenyl tetrazolium chloride (TTC) to each well prior to incubation at ambient temperature for 30 minutes. The lowest concentration of the tested samples with unobservable TTC reduction to red formazan after a 30-minute incubation at 37°C was defined as the MIC value. In addition, the modulation factor of the GTE was calculated based on the ratio of the MIC for the antibiotic alone

and the MIC of the antibiotics in the presence of GTE. Modulation factor ≥ 2 was used as the cut-off value to indicate substantial effects of the corresponding sample(s) in the reduction of the antibiotic resistance profiles of the tested pathogens (Fankam et al., 2017).

RESULTS AND DISCUSSION

Extraction yield, total phenolic, and EGCG contents

Extraction processes used in this research were carried out based on the method described in established publications (Veillet et al., 2010; Sartini et al., 2020). A summary of extraction yield, TPC, and EGCG content calculated from the obtained GTE are shown in Table 1.

Table 1. Extraction yield (%), total phenolic content (TPC), and EGCG content of green tea ethanolic extract

Extraction Yield (%)	TPC (%)	EGCG (%)
27.87	51.64 + 0.21	37.95 + 5.17

As shown in Table 1, TPC obtained in this study was approximately doubled compared to the previous study results (Sartini et al., 2019). In addition, we also found that the GTE prepared in this study contained higher EGCG than our previous result (Sartini et al., 2019). While the true nature of such discrepancy remains unknown, we hypothesized that the high content of polyphenols in the extract obtained in this study is due to an additional pre-treatment in the extraction process. As described in the method section, we performed GTE extraction using hexane with ultrasound-assisted extraction (UAE) to remove the non-polar components from GTE prior to the final extraction procedure using 50% ethanol. The mechanical effect of UAE accelerated the release of organic compounds that contained within the plant body by disrupting cell walls, enhancing mass transfer, and facilitating solvent access to the cell content (Pasrija and Anandharamakrishnan, 2015).

Susceptibility profiles of *E. cloacae* clinical isolates to β -lactam antibiotics

Susceptibility profiles of *E. cloacae* clinical isolates were determined using disk diffusion (Table 2), Vitek[®]2 (Table 3), and DDST (Figure 1 and Table 4) methods. Based on the results obtained from the agar disk diffusion test (Table 1) and Vitek[®]2 rapid test (Table 2), it appears that the clinical isolates of *E. cloacae* code 13/04 were resistant to the penicillin derivative (amoxicillin-clavulanate) and sensitive to other antibiotics used in the assay: the cephalosporins (cefotaxime, ceftazidime, ceftriaxone) and the carbapenems (imipenem, meropenem, doripenem). However, the susceptibility profile of *E. cloacae* clinical isolate code 275B/06 was quite different from the 13/04. While the 275B/06 *E. cloacae* were still sensitive to the carbapenems (imipenem, meropenem, and doripenem), this clinical isolate appeared to be resistant to the penicillin derivative (amoxicillin-clavulanate) and the cephalosporins (cefotaxime, ceftazidime, cefazoline, cefoxitin). Our results suggest that the two clinical isolates of *E. cloacae* might have different profiles in the availability of antibiotic resistance genes encoded in either the plasmid or the chromosome.

Table 2. Susceptibility patterns of *E. cloacae* clinical isolates to β -lactam antibiotics by disk diffusion method

<i>E. cloacae</i> isolates	Inhibition zone diameter (mm)					
	Penicillin	Cephalosporines			Carbapenems	
	AMC	CAZ	CRO	CTX	IMI	MEM
13/04	8 R	31 S	33 S	34 S	29 S	27 S
275B/06	8 R	17 R	8 R	8 R	27 S	25 S

Note: AMC: amoxicillin-clavulanate (20/10 μ g); CAZ: ceftazidime (30 μ g); CTX: cefotaxime (30 μ g); CRO: ceftriaxone (30 μ g); IMI: imipenem (10 μ g); MEM: meropenem (10 μ g); S: sensitive, I: intermediate, R: resistant

Table 3. Susceptibility patterns of *E. cloacae* clinical isolates to β -lactam antibiotics by Vitek[®]2 rapid test method

<i>E. cloacae</i> isolates	Antibiotics MIC (ppm) determined by Vitek-2 rapid test assay						
	Penicillin	Cephalosporines			Carbapenem		
	AMC	CAZ	CTX	CRO	IMI	MEM	DORI
13/04	> 32 R	< 1 S	< 1 S	< 1 S	< 0.25 S	< 0.25 S	< 0.12 S
275B/06	> 32 R	> 64 R	> 64 R	> 64 R	0.5 S	< 0.25 S	< 0.12 S

Note: AMC: amoxicillin-clavulanate; CAZ: ceftazidime; CTX: cefotaxim; CRO: ceftriaxone; IMI: imipenem; MEM: meropenem; DORI: doripenem; S: sensitive, I: intermediate, R: resistant

Further analysis using the DDST method revealed that the 276B/06 isolate was an extended-spectrum β -lactamase (ESBL) *E. cloacae*, while the 13/04 isolate was not (Figure 1 and Table 4). This is because the inhibitory zone diameter of cefotaxime-clavulanate was more than 5 mm compared to the result shown by cefotaxime alone. Since many bacteria in the Gram-negative *Enterobacteriaceae* family have been shown to be ESBL-producers (Rodríguez-Baño et al., 2018), our DDST result was already anticipated. Moreover, the same report (Rodríguez-Baño et al., 2018) suggested that the profile of clinically resistant *E. cloacae* was achieved not only by producing ESBL alone but may also involve the production of AmpC β -lactamase. This remains an interesting event to be explored in future experiments.

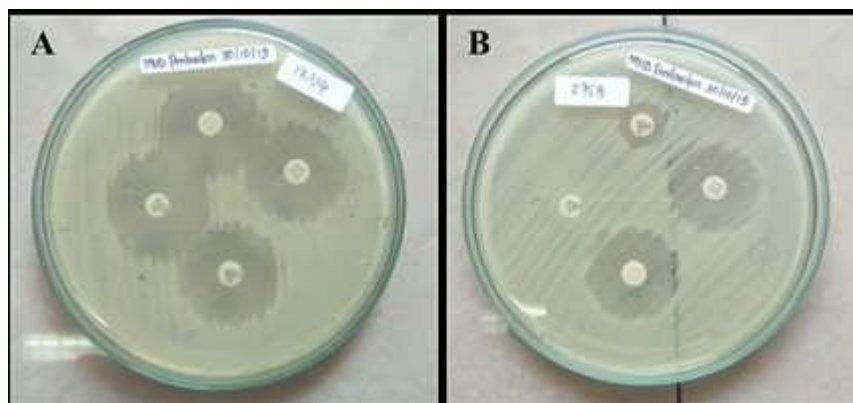


Figure 1. Susceptibility profiles of *E. cloacae* clinical isolates to cefotaxime, ceftazidime, cefotaxime-clavulanate, and ceftazidime-clavulanate by DDST method. A. *E. cloacae* code 13/04 (ESBL negative) B. *E. cloacae* code 275B/06 (ESBL positive)

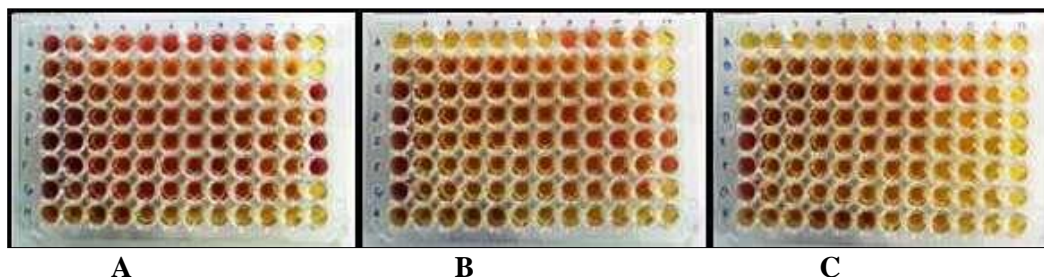
Table 4. Inhibition zone diameter (mm) of several antibiotics against *E. cloacae* clinical isolates

<i>E. cloacae</i> isolates	Ceftazidim - clavulanate	Ceftazidim	Cefotaxim – clavulanate	Cefotaxim	Interpretation
13/04	26	25	28	28	ESBL Negative
275B/06	24	12	24	8	ESBL Positive

Note: ESBL: extended-spectrum β -lactamase

Minimum inhibitory concentration of β -Lactam antibiotics and/or GTE against *E. cloacae* clinical isolates

To determine the antibacterial activity of β -lactam antibiotics or GTE in a single form or combination, we performed a checkerboard broth microdilution assay. This method has been suggested as one of the proper methods in determining the synergetic effect of two antibacterial or antibiotics used in combination (Jain et al., 2011; El-Azizi, 2016). As shown in Figure 2 and Table 5, the MIC of GTE against both *E. cloacae* clinical isolates (13/04 and 275B/06) were higher than 8,000 ppm, suggesting that the antibacterial activity of GTE is ineffective to both β -lactam-sensitive (13/04) and β -lactam-resistant (275B/06) *E. cloacae*. In other words, the absence of GTE antibacterial activity observed in this study was not due to the resistance profile of *E. cloacae*. The reason for such results remains to be determined in the future.

**Figure 2. Checkerboard broth microdilution assay for antibiotics and/or GTE against *E. cloacae* clinical isolates**

Notes: The sample test using two-fold dilution. A. Amoxicillin (2000 - 2 ppm); B. Cefotaxime (8000 - 8 ppm); C. Imipenem (250 - 0,25 ppm).

Antibiotics (Row A1-A11); GTE (column 1/ B1-F1); Antibiotic + GTE combination (Row B2-G11). Row H (control media + bacteria; control media); Column 12 (control media + GTE).

Red color in the media denotes bacterial growth, yellow color in the media indicates no bacterial growth

Table 5. Minimum inhibitory concentration (MICs) of amoxicillin, cefotaxime, imipenem, green tea extract (GTE), and combination of antibiotics with sub-inhibitory GTE against *E. cloacae* clinical isolates

Test Samples	MIC (ppm)	
	<i>E. cloacae</i> 13/04	<i>E. cloacae</i> 275B/06
Amoxicillin	> 2, 000	> 2, 000
Cefotaxime	125	> 8,000
Imipenem	7.5	15
GTE	> 8,000	> 8,000
Amoxicillin + sub inhibitory GTE	>2,000	>2,000
Cefotaxime + sub inhibitory GTE	125	> 8,000
Imipenem + sub inhibitory GTE	0.2	7.5

Note: concentration of sub-inhibitory GTE used in the assay was 8,000 ppm

Figure 2 (B1 – F1) and Table 5 show that the ethanolic extract of green tea up to a concentration of 8% (8000 ppm) has not shown antibacterial activity against *E. cloacae*, which means that GTE is not effective as an antibacterial against *E. cloacae*. In the previous report, GTE was reported to constrain *Escherichia coli* with MIC value < 4000 ppm (Reygaert, 2014). We previously reported that GTE could impede *Staphylococcus aureus* growth at a concentration of 300 ppm (Sartini et al., 2019). This is in line with the notion that the antimicrobial properties of green tea catechins are effective, to a greater extent, to Gram-positive bacteria than the Gram-negative ones (Reygaert, 2014). EGCG, the major catechin of green tea, has been considered a promising component in the GTE responsible for GTE's broad antibacterial activity against some Gram-negative and Gram-positive bacteria. This catechin can inhibit bacterial growth by several mechanisms, i.e., inhibition of bacterial cell wall synthesis, impairment of fatty acid synthesis, and inhibition of important enzymatic activities (Nakayama et al., 2013; Reygaert, 2014; Mikłasińska et al., 2016).

From Table 5, it can be seen that the combination of antibiotics with GTE did not change the MIC values of amoxicillin and cefotaxime on the clinical isolates of *E. cloacae* tested in this study, suggesting that GTE did not augment the antibacterial activity of the corresponding antibiotics. However, it is essential to note that the antibacterial activities of imipenem against the non-ESBL *E. cloacae* (code 13/04) and the ESBL strain of *E. cloacae* (code 275B/06) were increased by forty-fold and two-fold, respectively. Our results were in line with the previous research results that demonstrated the enhanced bactericidal activity of imipenem against imipenem resistant-*Klebsiella pneumonia* in the presence of EGCG, a major component of GTE (Cho et al., 2011).

CONCLUSION

Green tea ethanolic extract (GTE) was able to augment the antibacterial activity of imipenem against both the non-ESBL and the ESBL isolates of *E. cloacae*. However, it did not provide any beneficial effect on the antibacterial activities of amoxicillin and cefotaxime.

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