



Phenolic-rich green tea extract increases the antibacterial activity of amoxicillin against *Staphylococcus aureus* by *in vitro* and *ex vivo* studies

[Extracto de té verde rico en fenoles aumenta la actividad antibacteriana de la amoxicilina contra *Staphylococcus aureus* mediante estudios *in vitro* y *ex vivo*]

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Abstract

Context: Methicillin-resistant *Staphylococcus aureus* (MRSA) has been a significant challenge in health problems.

Aims: To investigate synergistic properties of green tea extract that can potentially enhance the antibacterial activity of amoxicillin against methicillin-resistant *Staphylococcus aureus* (MRSA) *in vitro* and *ex vivo*.

Methods: Green tea coarse powder was extracted by distilled water using high-pressure extraction. The total of phenolic and tannin contents was determined using spectrophotometry, and epigallocatechin gallate (EGCG) content was analyzed using Ultra-Fast Liquid Chromatography. Minimum Inhibitory Concentration (MIC) against MRSA was determined using a microdilution assay. Antibacterial assay of the combination of the extract and amoxicillin was performed using employing-checkerboard microdilution and agar diffusion assays.

Results: The results exhibited that the green tea extract (GTE) contained total phenolic content of 545.6 ± 4.7 mg GAE/g extract; total tannin content of 356.6 ± 4.1 mg EGCG equivalent /g extract and EGCG of 221.1 ± 11.6 mg/g extract. The MICs combinatorial of GTE and $\frac{1}{4}$ MIC of amoxicillin was $75 \mu\text{g/mL}$. Fractional Inhibitory Concentration Index value was 0.28, indicating that there was a synergy effect between amoxicillin and green tea extract. Essentially, the inhibitory zone diameter obtained by amoxicillin combined with $\frac{1}{4}$ MIC of GTE was two-fold than the zone produced by only amoxicillin. Although not significant, in *ex vivo* study, this combination was able to enhance the antibacterial activity of rat's plasma against MRSA *in vivo* after the oral administration of GTE and amoxicillin.

Conclusions: The results presented here serve as proof of concept for the enhancement of the antimicrobial activity of synthetic antibiotics when combined with a natural product.

Keywords: amoxicillin; antibacterial; *Camelia sinensis*; green tea; methicillin-resistant *Staphylococcus aureus*.

Resumen

Contexto: *Staphylococcus aureus* resistente a metilina (MRSA) ha sido un desafío importante en problemas de salud.

Objetivos: Investigar las propiedades sinérgicas del extracto de té verde que potencialmente pueden mejorar la actividad antibacteriana de la amoxicilina contra *Staphylococcus aureus* resistente a la metilina (MRSA) *in vitro* y *ex vivo*.

Métodos: El polvo de té verde se extrajo mediante agua destilada usando extracción a alta presión. El total de contenido fenólico y de tanino se determinó mediante espectrofotometría, y el contenido de galato de epigallocatequina (EGCG) se analizó mediante cromatografía líquida ultrarrápida. La concentración inhibitoria mínima (MIC) contra MRSA se determinó usando un ensayo de microdilución. El ensayo antibacteriano de la combinación del extracto y la amoxicilina se realizó utilizando ensayos de microdilución de tablero de ajedrez y difusión de agar.

Resultados: Los resultados mostraron que el extracto de té verde (GTE) tuvo un contenido fenólico total de $545,6 \pm 4,7$ mg de extracto de GAE/g; contenido total de taninos de $356,6 \pm 4,1$ mg de equivalente de EGCG/g de extracto y EGCG de $221,1 \pm 11,6$ mg/g de extracto. La MIC combinatoria de GTE y $\frac{1}{4}$ MIC de amoxicilina fue de $75 \mu\text{g/mL}$. El valor del índice de concentración inhibitoria fraccional fue de 0,28, lo que indica que hubo un efecto de sinergia entre la amoxicilina y el extracto de té verde. Esencialmente, el diámetro de la zona inhibitoria obtenida por amoxicilina combinada con $\frac{1}{4}$ MIC de GTE fue dos veces mayor que la zona producida por solo amoxicilina. Aunque no es significativo, en el estudio *ex vivo*, esta combinación fue capaz de mejorar la actividad antibacteriana del plasma de rata contra MRSA *in vivo* después de la administración oral de GTE y amoxicilina.

Conclusiones: Los resultados presentados aquí sirven como prueba de concepto para mejorar la actividad antimicrobiana de los antibióticos sintéticos cuando se combinan con un producto natural.

Palabras Clave: amoxicilina; antibacteriano; *Camelia sinensis*; té verde; *Staphylococcus aureus* metilina resistente.

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INTRODUCTION

The use of antibiotics to handle infectious diseases has increased significantly, along with cases of bacteria with resistance to prescribed antibiotics. Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the bacteria, which has been well-known as a dangerous antibiotic-resistant bacterium. MRSA is resistant to methicillin and other β -lactam antibiotics, resulting in the ineffectiveness of medical therapy required these antibiotics (Stryjewski and Corey, 2014). Resistance is caused by the addition of a nonnative gene encoding a penicillin-binding protein (PBP2a), which is encoded by the *mecA* gene, significantly decrease affinity for β -lactams. This resistance allows cell-wall biosynthesis, the target of β -lactams, to continue even in the presence of typically inhibitory concentrations of antibiotics (Peacock and Paterson, 2015). Therefore, the new solutions to solve this emergence problem is required.

Due to the significant increase of the number of pathogen bacteria with resistance to antibiotics, several studies have been carried out using bioactive compounds from plants that potentially have a synergistic effect that can potentially increase the activity of antibiotics (Gibbons, 2005; Hemaiswarya et al., 2008; Chusri et al., 2014; Stefanović, 2018). Studies so far showed that the polyphenol derived from plants had been found to have antibacterial properties and a synergy effect with several antibiotics (Lin et al., 2008; Daglia, 2012; Sanhueza et al., 2017). To highlight just one natural compound, green tea, a product from *Camelia sinensis* has been reported to have a high concentration of phenolic compounds (Lorenzo and Munekata, 2016; Nibir et al., 2017). The main phenolic compounds contained in green tea are epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epigallocatechin (EGC), epicatechin (EC), gallic acid (GC), and gallic acid gallate (GCC) (Bhagwat and Beecher, 2003; Perva-Uzunalić et al., 2006). Radji et al. (2013) reported that green tea extract has antibacterial activity against *S. aureus* ATCC 25923 and MRSA with the MIC of 400 $\mu\text{g}/\text{mL}$ for each bacterium. According

to Zhao et al. (2001), EGCG green tea showed synergistic properties with several β -lactam, including ampicillin, oxacillin, methicillin, and cephalixin. However, the study, as mentioned above, used a direct extraction method to extract the bioactive compounds in *Camelia sinensis*, which led to an extremely crude extract obtained from the plants.

In this study, for the first time, we initially removed the nonpolar compounds from the green tea using a hexane solvent. Following this, the non-polar-free green tea was re-extracted using high-pressured extraction using distilled water as a solvent. The water extract obtained was evaluated to determine total phenolic, tannin, and EGCG contents. Finally, the effect of green tea extract on the antibacterial activity of amoxicillin in *in vitro* and *ex vivo* studies. This proof of principle study points towards the potential synergy effect between green tea extract and amoxicillin, leading to an increase in the latter's antibacterial activity towards MRSA.

MATERIAL AND METHODS

Chemical and materials

Amoxicillin trihydrate (PHR1127) was purchased from Supelco. Methanol for liquid chromatography, Folin-Ciocalteu reagent, sodium hydroxide (NaOH), acetic acid, Muller-Hinton broth (MHB), Muller-Hinton agar (MHA) media, 2,3,5-triphenyl-tetrazolium chloride were purchased from Merck, Germany. Gallic acid (G7384) and epigallocatechin gallate (E4268) were purchased from Sigma-Aldrich, amoxicillin 25 μg disc (Oxoid). All other reagents were of analytical grade and purchased from standard commercial suppliers.

Preparation phenolic-rich green tea extract

Green tea utilized in this study was one of the market products in Makassar, South Sulawesi, Indonesia. A total of 100 g coarse powder of green tea was extracted by 1 L hexane to remove the nonpolar compounds over three days. The non-dissolved hexane powder was extracted using a

high-pressured extraction (PT. IFI, Makassar, Indonesia) with a pressure of 700 Bar, employing distilled water as a solvent (1:10). The resultant water extract was then lyophilized using a freeze-drier (Buchi L-200) to obtain the dried extracts.

Total phenolic, tannin contents and EGCG determination

The determination of total phenolic content (TPC) and total tannin content (TTC) was performed using the Folin-Ciocalteu method analyzed using a spectrophotometer. Gallic acid was used as the standard solution for TPC and EGCG for TTC with slight modification (Kemenkes, 2011). Briefly, 10 mg green tea extract was dissolved in methanol to obtain 75, 150, 300 and 600 µg/mL, respectively. Afterward, 100 µL sample was reacted with 2.5 mL of 7.5% w/v Folin-Ciocalteu and homogenized. Following this step, 2.4 mL of 1% w/v NaOH was added to the mixture. The reaction was maintained at room temperature for 60 minutes, and the absorbance was determined using UV spectrophotometer -1800 (Shimadzu). Initially, serial dilutions of EGCG as a standard were prepared in methanol, obtaining the calibration curve with concentrations ranging from 3.25 µg/mL to 200 µg/mL. Afterward, 10 mg of green tea extract was dissolved in methanol. The solutions were injected into UFLC, and the EGCG concentration was quantified.

Bacterial culture

Methicillin resistance *Staphylococcus aureus* was one of the collections of Microbiology Laboratory of Hasanuddin University Hospital, Makassar, Indonesia, obtained from pus specimen from the wound of the patient.

Antibacterial assay using checkerboard microdilution assay

The effect of green tea extract on the antibacterial activity of amoxicillin was evaluated based on the checkerboard method assay (Choi et al., 2015; Akinyele et al., 2017) by quantifying the minimum inhibitory concentrations (MICs) of green tea,

amoxicillin, and the combination of both. MICs assay was performed based on the microdilution method using Muller-Hinton broth (MHB) media. Various concentrations of amoxicillin (0.25, 0.5, 1, 2, 4, 8, 16 µg/mL) and green tea extract (75, 150, 300, 600 µg/mL) and their combination were prepared. A 48-well was established on the growth media and inoculated with MRSA colonies with 10⁶ CFU/mL in MHB media. Samples were incubated for 24 hours at 37°C. Afterward, 2,3,5-triphenyl-tetrazolium chloride was added into the media and was incubated for 30 minutes. In the presence of viable bacteria, triphenyl-tetrazolium chloride was reduced to form formazan (red color). Based on the MIC, the synergistic effect was determined using the Fractional Inhibitory Concentration Index (FICI). FICI was calculated and interpreted as follows (Teethaisong et al., 2014) [1]:

$$FICI = FIC_A + FIC_B = \frac{\text{Conc. of A in MICs of A+B}}{\text{MIC of A alone}} + \frac{\text{Conc. of B in MICs of A+B}}{\text{MIC of B alone}} \quad [1]$$

Where: FICI ≤ 0.5 was denoted to possess synergistic effect; FICI > 0.5-4.0 was denoted to possess no interaction; FICI > 4 was denoted to possess antagonist effect. In this study, the concentration of GTE combined with MIC of amoxicillin was ¼ of GTE MIC, and the concentration of amoxicillin combined with MIC of GTE was ¼ of amoxicillin MIC.

Antibacterial assay using disc diffusion methods

The effect of green tea extract on the antibacterial activity of amoxicillin was evaluated by disc diffusion as per the method described by Abreau et al. (2014) with slight modification. Briefly, 20 mL of sterile Muller-Hinton agar (MHA) were mixed with green tea extract to obtain the final concentration of extract in media of 0.5- and 0.25-folds of extract MIC, and negative control (MHA media without extract) were poured into glass Petri dishes. Afterward, 10 µL of MRSA (10⁶ CFU/mL) were spread into agar plates. Following this, the amoxicillin discs (25 µg/disc) were placed onto the medium growth and incubated for 24 hours at 37°C. The inhibitory zone diameter was measured by a digital caliper (Krisbow®)

Detection of antibiotic activity in body fluids by disc diffusion bioassays

The studies were approved by ethical clearance no. 95/UN4.6.4.5.31/PP36/2019. Healthy, male Sprague-Dawley rats were acclimatized to the laboratory conditions for 1 week before the experiments. The animals were divided into three groups (n = 3 per group). The first group received an oral aqueous of green tea extract (25 mg/kg), the second group received an oral suspension of amoxicillin (25 mg/kg), and the third group received an oral suspension of the combination of green tea extract (25 mg/kg) and amoxicillin (25 mg/kg), as described in a previous study (Ali et al., 2012). Specifically, in the third group, the green tea extracts were administrated after 30 min of the amoxicillin administration. After 2 hours, the blood was collected via tail vein bleeds into Eppendorf tubes containing sodium citrate. The blood collected was centrifuged at $3000 \times g$ for 20 min and the plasma was then collected in the supernatants. The concentration of ECGC and amoxicillin in each group were further analyzed. These compounds were extracted with a one-step protein precipitation method (Permana et al., 2019). Amoxicillin was extracted using the mixture of acetonitrile and water (5:95), and ECGC was extracted using methanol. Briefly, 500 μL of each organic solvent was added to an aliquot of 100 μL rat plasma and vortexed for 5 min. The samples were then centrifuged at $12,000 \times g$ for 20 min. The supernatant was collected into an Agilent® HPLC vial and analyzed using UFLC (Shimadzu Corporation, Kyoto, Japan).

The antibacterial activities in body fluids were carried out as described by Driscoll et al. (2012), with slight modifications. In brief, 10 mL of MHA was poured into a glass Petri dish, forming a base layer. Afterward, 10 μL of MRSA (10^6 CFU/mL) was spread on the top of the base layer. Posteriorly, filter paper discs (6 mm diameter, Oxoid) containing 20 μL of plasma from each group were placed into solidified media containing MRSA and incubated for 24 hours at 37°C . The inhibitory zone diameter was measured by a digital caliper (Krisbow®).

UFLC assays

The determination of ECGC concentration in green tea was carried out using Shimadzu UFLC (Series columns Shim-pack Vp-ods). The mobile phases used were phosphate buffer pH 5:acetonitrile (95:5, v/v) and methanol:water:acetic acid (27:70:5, v/v) for amoxicillin and EGCG, respectively. The injection volume and flow rate was 20 μL and 1 mL/min. The quantification was carried out at room temperature with UV detection of 254 nm for amoxicillin and 280 nm for EGCG.

Statistical analysis

All data were presented as mean \pm standard deviation (SD). The calculation of SD was carried out using Microsoft® Excel® 2016 (Microsoft Corporation, Redmond, USA). Statistical analysis was performed using GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA). Where appropriate, an unpaired t-test was performed to compare two different cohorts. The Kruskal-Wallis test with post-hoc Dunn's test performed to compare multiple cohorts. In all cases, $p < 0.05$ was denoted as a significant difference.

RESULTS AND DISCUSSION

Total phenolic, tannin contents and EGCG determination

The green tea extraction process was initially carried out by removing the nonpolar compounds with hexane extraction (1:10). Furthermore, the green tea compounds that did not dissolve in hexane were extracted by distilled water (1:10), using a high-pressure extraction method, producing a yield of 21.4% green tea extract (GTE). The high-pressure extraction method was selected in this study due to several advantages, namely short extraction period, less solvent, and had a higher yield than the conventional extraction procedure (Huang et al., 2013; Xi et al., 2015). The results of quantitative analysis of TPC, TTC and EGCG content in GTE are shown in Fig. 1A. The TPC, TTC and EGCG contents of GTE were found to be 545.6 ± 4.7 mg GAE/g extract, 356.6 ± 4.1 mg EGCG

equivalent/g extract and 221.1 ± 11.6 mg/g extract, respectively. The chromatogram of EGCG in GTE analysis is shown in Fig. 1B.

Antibacterial assay using checkerboard microdilution assay and disc diffusion methods

This study was performed by quantifying the MICs of green tea, amoxicillin, and the combination of both. The results of this study are detailed in Table 1. The results depicted that the MIC of amoxicillin was found to be $8 \mu\text{g/mL}$, and the MIC of GTE was $300 \mu\text{g/mL}$. After the combination of amoxicillin and GTE, the MIC amoxicillin combined with $75 \mu\text{g/mL}$ ($\frac{1}{4}$ MIC) of GTE was $0.25 \mu\text{g/mL}$, and the MIC GTE + $\frac{1}{4}$ MIC amoxicillin was $75 \mu\text{g/mL}$. These findings indicated that GTE was able to decrease MIC of amoxicillin and, therefore, improve the antibacterial activity of amoxicillin against MRSA. According to the MIC values, the FICI was then determined. As shown in Table 2, the combination of amoxicillin with $\frac{1}{4}$ MIC of GTE resulted in FICI value of 0.28, which could potentially indicate a synergetic effect. However, to further understand the synergetic

effect, further studies should be conducted. Considering the EGCG content in GTE (221.1 ± 11.6 mg/g), the MIC of EGCG itself was found to be $6.63 \mu\text{g/mL}$.

It has been reported that EGCG, a major phenolic in green tea, has antibacterial activity. In addition, EGCG and β -lactam antibiotics have also been reported to have a synergistic effect on MRSA by disrupting the integrity of the cell wall via direct binding to peptidoglycan (Zhao et al., 2001). EGCG is able to inhibit the penicillinase activity of bacterial, which might decrease the effectiveness of amoxicillin and, thus, maintain the activity of amoxicillin (Zhao et al., 2002). Additionally, it has been postulated that phenolic compounds possess the ability to break down the structure of the cytoplasmic membrane of the bacterium, leading to loss of integrity and subsequent cell death (Xie et al., 2014). The phenolic compounds contained in GTE would enable the entry of the amoxicillin to the cell cytoplasm, therefore enabling the entry of amoxicillin, which possess its site action within the bacterial cell, and less antibiotic dose would be required.

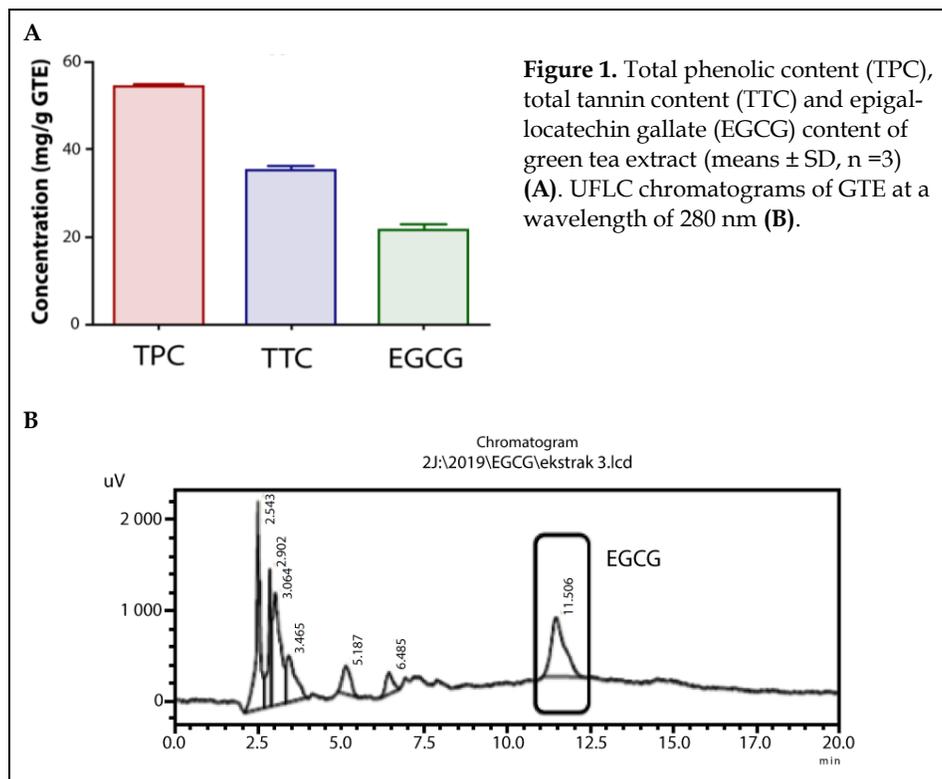


Figure 1. Total phenolic content (TPC), total tannin content (TTC) and epigallocatechin gallate (EGCG) content of green tea extract (means \pm SD, n =3) (A). UFLC chromatograms of GTE at a wavelength of 280 nm (B).

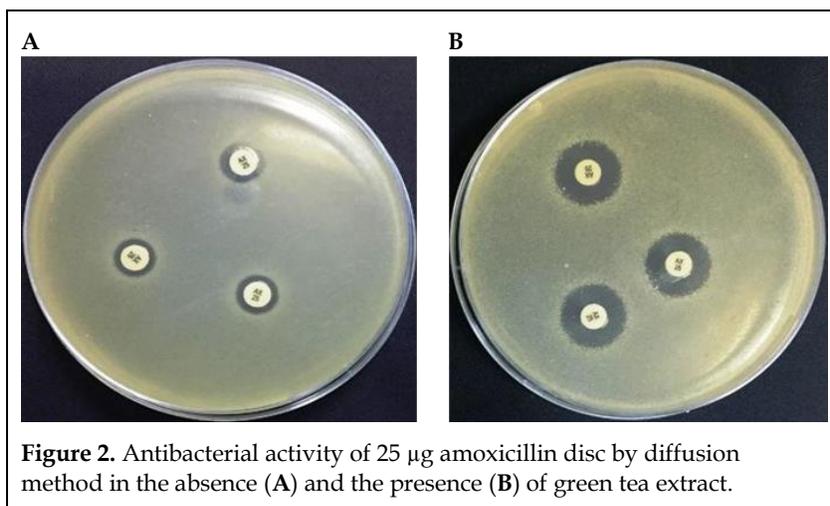
Table 1. Minimum inhibitory concentrations (MICs) of samples test, fraction inhibitory concentration (FIC) and FIC index (FICI).

Samples	MIC ($\mu\text{g/mL}$)	FIC _{Amox}	FIC _{GTE}	FICI
Amoxicillin (Amox)	8	N/A	N/A	N/A
GTE	300	N/A	N/A	N/A
Amox + 1/4 of GTE MIC	0.25	0.03	N/A	0.28
GTE + 1/4 of amox MIC	75	N/A	0.25	

Table 2. The diameter of the inhibition zone of amoxicillin and the combination of amoxicillin and green tea extract (GTE) against MRSA *in vitro*.

Sample	Inhibition zone diameter (mm)
Amoxicillin	8.75 \pm 0.05
Amoxicillin + 1/4 MIC GTE	16.22 \pm 1.10*

Data are presented as mean \pm standard deviation (n = 3). MIC: Minimal inhibitory concentration.

**Figure 2.** Antibacterial activity of 25 μg amoxicillin disc by diffusion method in the absence (A) and the presence (B) of green tea extract.

The synergistic effect by diffusion method was in agreement with the results from microdilution assay. Fig. 2 and Table 2 represent the inhibition zone and the diameter of the inhibition zone of amoxicillin against MRSA, in comparison with the combination of amoxicillin and GTE. The diameters of the inhibition zone were found to be 8.75 \pm 0.05 mm and 16.22 \pm 1.10 mm for amoxicillin and the combination of amoxicillin and GTE, respectively. Analyzed statically, the diameter of the inhibition zone of the combination approach was

significantly higher ($p < 0.05$) compared to amoxicillin.

Detection of antibiotic activity in body fluids by disc diffusion bioassays

In an attempt to evaluate the effect of this combination *ex vivo*, the concentrations of EGCG and amoxicillin in rat plasma were determined after oral administration of the GTE, amoxicillin and the combination of GTE and amoxicillin. As shown in Fig. 3, the concentrations of EGCG after oral ad-

ministration of GTE and the combination of GTE and amoxicillin were found to be $4.06 \pm 0.54 \mu\text{g/mL}$ and $4.19 \pm 0.37 \mu\text{g/mL}$. In terms of amoxicillin plasma concentration, the concentrations of amoxicillin were $52.65 \pm 7.98 \mu\text{g/mL}$ and $59.16 \pm 8.14 \mu\text{g/mL}$ after oral administration of amoxicillin and the combination of GTE and amoxicillin, respectively. There was no significant difference ($p > 0.05$) in the concentration of EGCG and amoxicillin in all cohorts. Therefore, the combination of GTE and amoxicillin did not affect the plasma concentrations of both compounds in rats.

The antibacterial activity of rat's plasma after oral administration of this combinatorial approach, in comparison with the single administration of GTE and amoxicillin, was finally investigated. As shown in Table 3, the diameters of the inhibition zone of rat's plasma following oral administration of GTE, amoxicillin, and combination of GTE and amoxicillin were $7.10 \pm 0.28 \text{ mm}$, 17.20

$\pm 1.81 \text{ mm}$ and $18.85 \pm 1.91 \text{ mm}$. The inhibition zone of the combination approach was significantly higher ($p < 0.05$) than the single administration of GTE. Despite the higher value, the inhibition zone of this approach was not significantly higher ($p > 0.05$) than a single administration of amoxicillin. This behavior might be due to the low concentration of EGCG found in the rat's plasma. The concentrations found in this study ($4.06 \pm 0.54 \mu\text{g/mL}$ and $4.19 \pm 0.37 \mu\text{g/mL}$) were significantly lower than MIC value obtained in the previous section ($6.63 \mu\text{g/mL}$). Therefore, the EGCG was not able to enhance the antibacterial activity of amoxicillin effectively. The low plasma concentration of EGCG after oral administration of GTE and the combination of GTE and amoxicillin might be owing to the poor bioavailability of catechin compounds. Several studies have reported the low oral bioavailability of catechin compounds of several species of tea (Henning et al., 2005; Mereles and Hunstein, 2011; Cai et al., 2018).

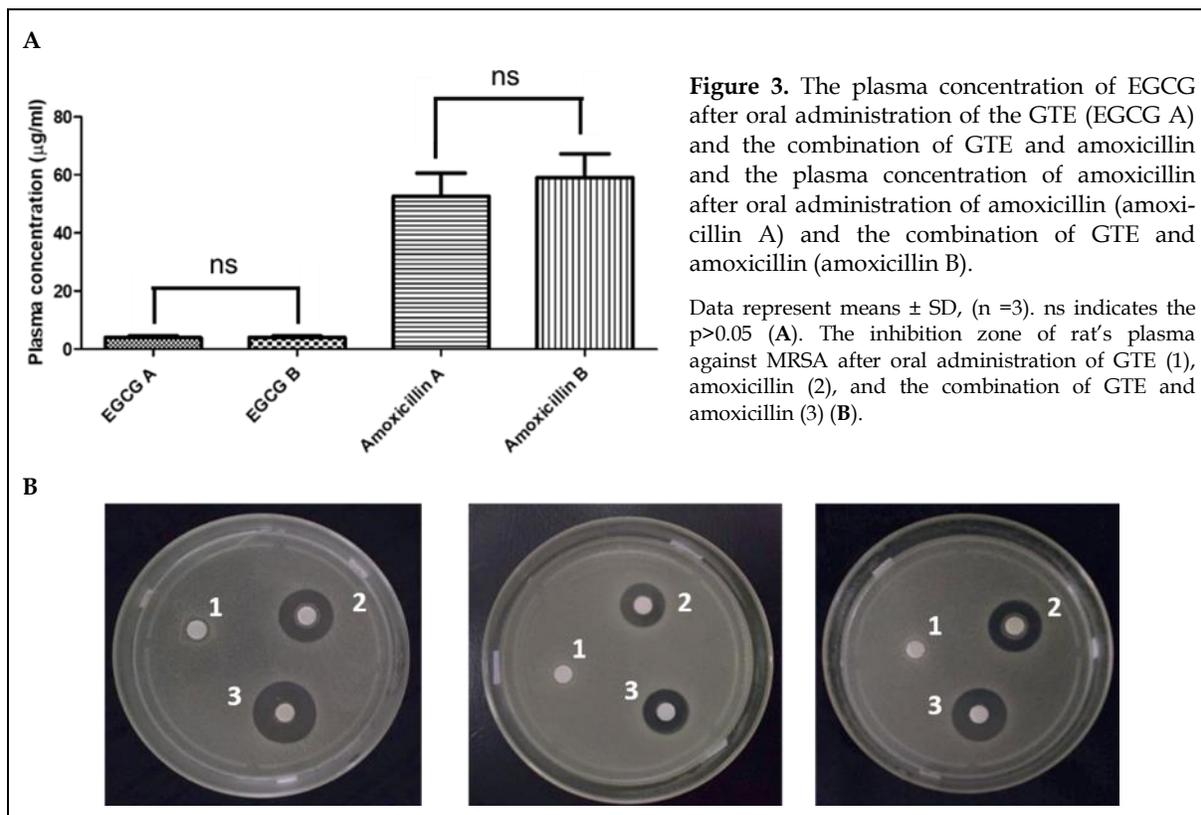


Figure 3. The plasma concentration of EGCG after oral administration of the GTE (EGCG A) and the combination of GTE and amoxicillin and the plasma concentration of amoxicillin after oral administration of amoxicillin (amoxicillin A) and the combination of GTE and amoxicillin (amoxicillin B).

Data represent means \pm SD, ($n = 3$). ns indicates the $p > 0.05$ (A). The inhibition zone of rat's plasma against MRSA after oral administration of GTE (1), amoxicillin (2), and the combination of GTE and amoxicillin (3) (B).

Table 3. The diameter of the inhibition zone of rat's plasma against MRSA after oral administration of GTE (1), amoxicillin (2) and the combination of GTE and amoxicillin.

Samples	Inhibition zone diameter (mm)
GTE	7.10 ± 0.28
Amoxicillin	17.20 ± 1.81*
Combination of GTE and amoxicillin	18.85 ± 1.91*

Data are presented as mean ± standard deviation (n = 3). MRSA: Methicillin-resistant *Staphylococcus aureus*; GTE: Green tea extract.

The results obtained in the present study imply that the GTE was able to enhance the antibacterial activity of amoxicillin *in vitro*. However, due to the poor oral bioavailability of EGCG, in spite of the increase of antibacterial activity, this combination did not significantly improve the antibacterial activity of amoxicillin in *ex vivo* study. Leading on from these results, several approaches are required to improve the oral bioavailability of EGCG following the administration of GTE. Following this, the efficacy of this combinatorial approach should be carried out in a suitable infection animal model. Therefore, in the future, green tea extract could be potentially used as supportive therapy for amoxicillin to treat infectious diseases caused by MRSA.

CONCLUSIONS

This study investigated, for the first time, both *in vitro* and *ex vivo* synergistic effect of green tea extract (GTE) and amoxicillin. The results showed that green tea extract could potentially increase the antibacterial activity of amoxicillin *in vitro* with FICI of 0.28. The oral administration of GTE with amoxicillin did not affect the plasma concentrations of EGCG in GTE and amoxicillin. Additionally, although not significant, the combination of GTE and amoxicillin was able to increase the antibacterial activity of amoxicillin. Accordingly, an effort to enhance the promising antibacterial activity of this combination approach is highly needed. Overall, this study provides a proof of principle of the ability of natural compounds to increase the antibacterial activity of synthetic antibiotics, which could potentially overcome the bacterial resistance

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTION:

Contribution	Sartini S	Djide MN	Amir MN	Permana AD
Concepts or ideas	x	x	x	x
Design	x	x		
Definition of intellectual content	x	x		
Literature search	x	x	x	
Experimental studies	x		x	
Data acquisition	x		x	
Data analysis	x	x	x	x
Statistical analysis	x	x	x	x
Manuscript preparation	x	x		x
Manuscript editing	x			x
Manuscript review	x	x	x	x

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