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Synergistic effect of commercial mangosteen extract (*Garcinia mangostana* L.) and amoxicillin against methicillin-resistant *Staphylococcus aureus* (MRSA)

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Abstract

Antibiotic resistance occurs worldwide, and has become a threat to humankind. Previous data have shown that antimicrobial resistance is a global issue demanding immediate resolution because it threatens the environment and society. The present work thus investigated the synergistic effects of commercial *Garcinia mangostana* L. (GML) extract and amoxicillin on the growth of methicillin-resistant *Staphylococcus aureus* (MRSA) bacterial cells. A commercial GML extract was screened for phytochemical properties, and the presence of α-mangostin was detected using high-performance liquid chromatography (HPLC). The antibacterial activity of the commercial GML extract with amoxicillin was analysed by minimum inhibitory concentration (MIC) and checkerboard assays. The morphology ultrastructure of bacteria was observed using transmission electron microscopy (TEM), after treatment with commercial GML extract, either single or in combination with amoxicillin. The MICs of amoxicillin and commercial GML extract against MRSA bacteria were 250.00 and 137.50 µg/mL, respectively. The checkerboard assay showed synergistic activity in the combination of commercial GML extract (34.38 µg/mL) and amoxicillin (62.50 µg/mL) at fractional inhibitory concentration (FIC) index of < 0.5. Damage to the structure of bacteria occurred due to the commercial GML extract plus amoxicillin. It was observed that the loss of bacterial cell membranes led to an irregular bacterial structure. These findings provided evidence that the combination of commercial GML extract and amoxicillin could reverse bacterial resistance in order to determine the susceptibility of traditional drugs.

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Introduction

Patients with infection of antibiotic-resistant bacteria are at high risk of a worse clinical outcome, or even death. In addition, they require more health resources than patients infected by the same strain of bacteria that is not resistant to antibiotics (WHO, 2020). New research into, and the development of antibiotics or herbal antimicrobials, are crucial in providing new hope for further study. Plant-derived antimicrobials are believed to have minimum side effects. Drug combination is one strategy that has been widely tested in the development of antimicrobials, especially between plant/natural materials as adjuvants and antibiotics such as amoxicillin. Several existing plant-derived materials have been identified, such as mangosteen (Ansori *et al.*, 2020), mango (Ediriweera *et al.*, 2017), and *Jatropha* leaves (Rahu *et al.*, 2021). Mangosteen (*Garcinia mangostana* L.) is known for its traditional healing properties. As a commercial fruit, it has significant consumer demand due to its innocuous

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behaviour and ability to be consumed orally, whether as a preventative dietary strategy or a treatment plan (Marzaimi and Aizat, 2019).

Methicillin-resistant *Staphylococcus aureus* (MRSA) is now a commonly isolated bacterium from nosocomial infections, and can lead to fatalities. MRSA has also shown resistance to other antibiotics such as tetracycline, erythromycin, and gentamicin (Amin *et al*., 2015). Owing to MDR (multidrug-resistance), the only remaining option is vancomycin, which is also experiencing resistance amid reports of the emergence of vancomycin-intermediate *S. aureus* (VISA) and vancomycin-resistant *S. aureus* (VRSA). The present work therefore aimed to investigate the synergistic effects of commercial *G. mangostana* L. (GML) extract and amoxicillin against MRSA strains.

Materials and methods

Bacterial strains

Staphylococcus aureus ATCC BAA-38 (MRSA) was obtained from PT Multiredjeki Kita, Indonesia. The bacterial stock was stored in a Tryptic Soy Broth medium with 10% glycerol. Afterward, each strain was preserved in a stock tube, and stored in the freezer before use.

Preparation of α-mangostin standard and commercial GML extract

The α-mangostin powder (analytical standard, Sigma Aldrich) with purity of ≥ 98.0% was purchased from PT Elo Karsa Utama Jakarta, Indonesia. Next, 10 mg of α-mangostin powder were dissolved in 1 mL of dimethyl sulfoxide (DMSO) before the mixture was filtered through a 0.2 μm diameter filter. A final concentration of 10 mg/mL of this solution was thus obtained.

PT Borobudur produced the commercial GML extract in capsule form, comprising 550 mg of mangosteen extract per α-mangostin capsule. The extract was determined using high-performance liquid chromatography (HPLC). The commercial GML extract powder was added to 1 mL of DMSO.

Phytochemical screening of commercial GML extract

A qualitative phytochemical screening study was undertaken for significant bioactive chemicals such as alkaloids, tannins, flavonoids, saponins, glycosides, steroids, terpenoids, and anthocyanins (Selvaraj *et al*., 2016).

Test for tannins

Aqueous mangosteen extract (0.5 g) was mixed with 10 mL of bromine water. The decolourisation of the bromine water revealed the presence of tannin.

Test for saponins

In a test tube, 5.0 mL of distilled water was combined with mangosteen extract, and the mixture was thoroughly mixed. The foam was combined with several drops of olive oil, and thoroughly mixed again. Saponins were observed in the foam's appearance.

Test for flavonoids

Full yellow colour was created when 2 mL of 2.0% NaOH was mixed with mangosteen extract; this faded to colourless when two drops of diluted acid were added, thus indicating the presence of flavonoids.

Test for terpenoids

Approximately 2.0 mL of chloroform was mixed with 5 mL of aqueous mangosteen extract, and evaporated on a water bath before being heated with 3 mL of concentrated H_2SO_4. Grey colour indicated the presence of terpenoids.

Test for steroids

Into 5 mL of mangosteen extract, 2 mL of chloroform and concentrated H_2SO_4 were added. Red colour indicated the presence of steroids in the lower chloroform layer.

Test for quinones

Around 3 mL of each extract was treated with 3 mL of chloroform in Borntragers test, and the chloroform layer was separated. To this, 5% potassium hydroxide dissolution was added. Red colour in the alkaline phase indicated the presence of quinones. Samples showing yellow colour with green fluorescence were treated with one drop of 6% hydrogen peroxide; the formation of a red shade was considered positive for anthrone derivatives.

Test for alkaloids

Each extract (10 mg) was dissolved in 2 mL of 5% hydrochloric acid; after mixing and filtering, three aliquots were taken. Each extract was added with drops of Wagner, Mayer, Bouchard, and Dragendorff reagents. A red-brown precipitate (Wagner), yellowish-white precipitate (Mayer),

brown precipitate (Bouchard), and red-orange precipitate (Dragendorff) indicated the presence of alkaloids.

Determination of α-mangostin concentration in commercial GML extract by HPLC

The commercial GML extract (100 µL) was pipetted into a 10.0 mL volumetric flask, and methanol was added to adjust the volume to the mark. A 0.45 µm membrane was used to filter the solutions. The chromatographic analysis was carried out at room temperature at a 1.5 mL/min flow rate, with the eluate monitored at 319 nm. Methanol-water solution (95:5% v/v) was used as the mobile phase. After the crude extract was injected into the HPLC column, the amount of α-mangostin in the crude extract was calculated using the calibration curve (Muchtaridi *et al.*, 2016).

Determination of killing curves

The killing curve was determined to confirm the combination of synergistic activity of commercial GML extract and amoxicillin, based on Clinical and Laboratory Standard Institute (CLSI, 2019) methods with slight modifications (Tran and Rybak, 2018). The MIC of each molecule that generated the combinations synergism by the FIC index was chosen for exploration once the FIC index had been obtained. *Staphylococcus aureus* ATCC BAA-38 (MRSA) was used to determine the half-MICs of amoxicillin alone, and the MICs of this combination that generated the synergistic FIC index values. Concentrations of 125.00 µg/mL amoxicillin, 68.75 µg/mL GML extract, amoxicillin at 31.25 µg/mL plus commercial GML extract at 17.19 µg/mL combinations, and a control (no amoxicillin or GML extract) were prepared. The cultures were grown on Mueller Hinton Broth (MHB) for 24 h at 37°C. At log phase, 2 mL of culture was inoculated into 98 mL MHB and shaken at 100 rpm at 37°C for 4 h. The concentration of bacterial cultures in saline was increased to 5×10^6 CFU/mL. MHB plus amoxicillin GML extracts, either single or mixed, was applied to the log phase cultures at the above-mentioned concentration. The bacterial suspensions were incubated at 37°C, and viable numbers were determined after 0, 4, 8, and 24 h of incubation. Following colony counts, dilution plating on MH agar plates in triplicate was conducted, followed by incubation at 37°C for 16 - 18 h.

Morphology ultrastructure analysis with transmission electron microscopy (TEM)

The ultrastructure morphology of bacteria was observed using TEM. TEM preparations were performed in line with a previously reported study with slight modifications (Liu *et al.*, 2015). *Staphylococcus aureus* ATCC BAA-38 (MRSA) strain was spectrophotometrically adjusted to a final concentration of approximately 5×10^5 CFU/mL after pre-incubation at 35°C for 18 h. The culture was cultivated for 4 h in a water bath at 37°C with no antibacterial agent (control), but in the presence of commercial GML extract (68.75 µg/mL), amoxicillin (125.00 µg/mL), and a combination of commercial GML extract (17.19 µg/mL) and amoxicillin (31.25 µg/mL). The culture was then harvested by centrifugation at 6,000 *g* for 15 min at 4°C, and the pellets were fixed for 12 h in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). The samples were then washed twice with 0.1 M phosphate buffer. Post-fixation was performed for 2 h at room temperature with 1% osmium tetroxide in 0.1 m phosphate buffer (pH 7.2). After washing the buffer, the samples were gently dehydrated for 15 min with graded ethanol (20, 40, 60, 80, and 100%, respectively). The infiltration and embedding were then sectioned and placed on copper grids using an ultramicrotome with a diamond knife. Finally, the ultrathin sections were counterstained for 3 min with 2% uranyl acetate, and 2 min with 0.25% lead citrate. After staining, the specimen was examined under a 120 kV Tecnai G2 electron microscope. The half-MICs of one commercial GML extract and amoxicillin were used. The cell area from micrographs was examined by measuring cell width multiplied by cell length to confirm the effects of the commercial GML extract, and amoxicillin was used separately or in combination with cell size (nm^2).

Statistical analysis

All experiments were performed at least three times in parallel. SPSS version 17.0 (SPSS Inc., Chicago, USA) for Windows was used for the analysis of variance (ANOVA), and Duncan's multiple range test was used to evaluate the level of significance ($p < 0.05$). Kaplan-Meier Estimate measured the fraction of subjects who survived for a certain amount of survival under the same circumstances.

Results

Phytochemical screening of commercial GML extract

The preliminary phytochemical characteristics of commercial GML extract indicated that flavonoids, alkaloids, tannins, saponins, quinones, steroids, and triterpenoids were detected. Table 1 shows the phytochemical screening analysis of commercial GML extract. The total α-mangostin in the extract was 68.59 mg/g.

Table 1. Phytochemical screening of commercial GML extract.

Phytochemical compound	Result	Commercial GML
Flavonoids	Yellow colour	+
Alkaloids	Not precipitate	-
Tannins	Greenish black	+
Saponins	Foam appearance	+
Quinones	Brown	-
Steroids	Yellow	-
Triterpenoids	Grey	+

+ = presence; - = absence.

Determination of α-mangostin concentration in commercial GML extract by HPLC

HPLC chromatograms of this isolated α-mangostin exhibited a significant peak of isolated α-mangostin from the commercial GML extract, which was practically the same as the peak of α-mangostin standard shown in Figure 1.

Determination of killing curves

The MIC results for commercial GML extracts and amoxicillin against *S. aureus* ATCC BAA-38 (MRSA) were 137.50 and 250 µg/mL, respectively. These results indicated that these MRSA strains were resistant to amoxicillin. Checkerboard assays showed commercial GML synergistic activity (34.38 µg/mL) plus amoxicillin (62.50 µg/mL). The FIC indices of amoxicillin plus commercial GML against MRSA strains were 0.50, thus indicating synergistic activity against *S. aureus* ATCC BAA-38 (MRSA).

Figure 2 shows the effects of the commercial GML extracts and amoxicillin, either single or in combination, on viable MRSA counts. The viable count of the cells treated with commercial GML in single 137.50 µg/mL showed a dramatic decrease in antibacterial activity, where the growth of bacteria continued to decline at 4 h, and was stable until 24 h (from 222 to 92 CFU/mL), and where amoxicillin in single 250.00 µg/mL, showed a significant increase. The control cells showed no drop in viable counts, and continued to rise in the log phase for the whole 24 h period. In contrast, cells treated with a commercial GML extract and amoxicillin combination showed no significant change, though the viable count reduced from 194 to 60 CFU/mL.

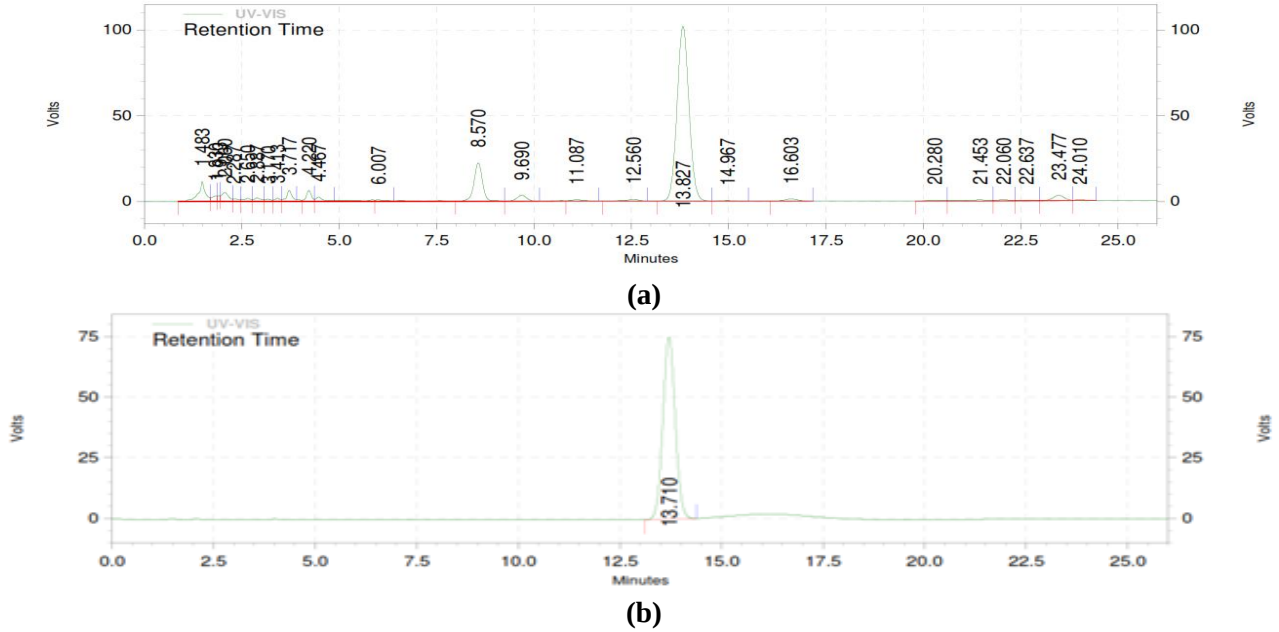

Figure 1. HPLC chromatograms of α-mangostin: **(a)** α-mangostin extract from commercial GML, and **(b)** α-mangostin standard from Sigma Aldrich.

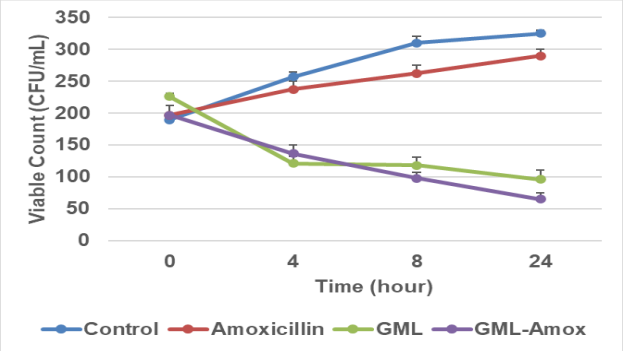

Figure 2. Time killing curve of MRSA after exposure to commercial GML extract and amoxicillin, either single or in combination.

Morphology ultrastructure analysis with transmission electron microscopy (TEM)

An electron microscope can be used to identify the cytoplasmic membrane and cell wall of MRSA cultivated in the absence of an antibiotic agent (control). This revealed that there was no damage to the ultrastructure (which was observed to be intact) (Figure 3a). Figure 3d shows MRSA cells that have been treated with amoxicillin. The peptidoglycan and cytoplasmic membrane were both disrupted. Figure 3b shows a micrograph of these cells following exposure to a single commercial GML extract. The peptidoglycan and cytoplasmic membrane were also damaged. The isolated commercial GML extract plus amoxicillin-treated cells showed the most extensive damage to the peptidoglycan and cytoplasmic membrane, thus resulting in intracellular material leakage and overall morphological abnormalities (Figure 3c). These cells had larger average cross-sectional areas than the control cells, although there was no statistically significant difference.

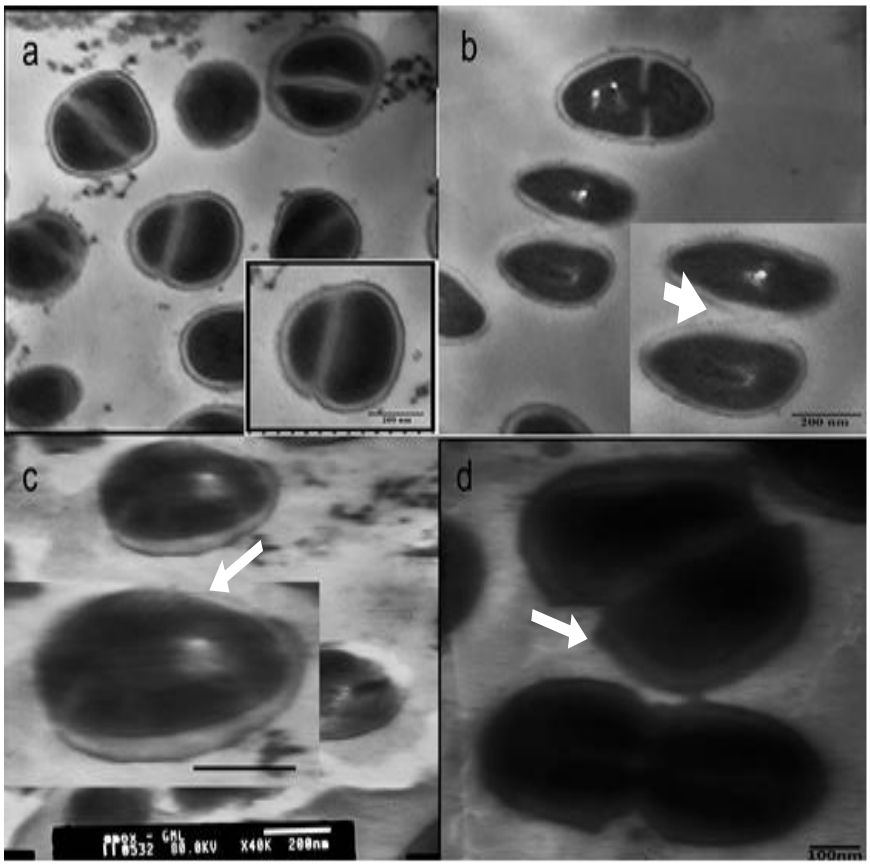

Figure 3. Ultrathin sections of log-phase methicillin-resistant *S. aureus* grown in CAMHB containing: **(a)** control (drug-free); **(b)** commercial GML extract at 137.50 µg/mL; **(c)** commercial GML extract at 34.38 µg/mL plus amoxicillin at 62.50 µg/mL; and **(d)** amoxicillin at 250.00 µg/mL. Damage to the structure of bacteria was due to commercial GML extract plus amoxicillin. Loss of bacterial cell membranes was indicated by the fact that the bacterial structure became irregular, as shown by the white arrow (the possibility of attack which led to peptidoglycan).

Discussion

Medicinal plants are an essential source of valuable bioactive secondary metabolites necessary for health in individuals and societies. Plants' medicinal values are due to the chemical substances that produce a definite physiological action on the human body (Manandhar *et al*., 2019). Antibiotic resistance in MRSA has become more common due to changes to drug target site, enzyme modifications, and changes in membrane permeability. As a result, there are fewer medication options available every day to treat multidrug-resistant MRSA.

Phytochemical screening of commercial GML extract

Table 1 shows the qualitative analysis of various commercial GML extracts. It can be observed from Table 1 that the extracts contained amounts of flavonoids, tannins, triterpenoids, and saponins.

Determination of α-mangostin concentration in commercial GML extract by HPLC

The isolated α-mangostin chromatograms showed a significant peak of isolated α-mangostin of commercial GML, nearly identical to that of the α-mangostin standard (Sigma Aldrich). Isolated α-mangostin had a purity of 98.0% (HPLC). These findings were very similar to those reported by Phitaktim *et al*. (2016). The α-mangostin standard chromatogram showed a retention time (RT) for α-mangostin standard (Sigma Aldrich) of 13.710 min, with an area of 6,376,482. It was represented as the only peak on the chromatogram, thus indicating 100% α-mangostin, and no other active substances.

In contrast, the commercial GML extract had an RT of 13.827 min, with an area of 8,791,103. Many of the active substances in the commercial GML extract were marked by the formation of peaks around 35.26%. However, every peak was in insignificant concentration, and therefore did not affect this study.

Determination of killing curves

The killing curve assay confirmed the synergistic effect of amoxicillin and commercial GML extract against *S. aureus* ATCC BAA-38 (MRSA). These findings were remarkably similar to those of Phitaktim *et al*. (2016), where α-mangostin isolated from *G. mangostana* plus oxacillin exhibited synergistic activity against an oxacillin-resistant *S. saprophyticus* strains at FIC index of 0.37. The findings showed that these combinations of commercial GML extract and amoxicillin were effective against the strain when used synergistically. In another study, Amin *et al*. (2015) reported on the effect of flavonoids combined with antibiotics (amoxicillin, ampicillin, cephradine, ceftriaxone, imipenem, and methicillin) against MRSA bacteria. They were found to improve each other's activity against test microorganisms when administered with antibiotics (Amin *et al*., 2015).

Morphology ultrastructure analysis with transmission electron microscopy (TEM)

The untreated cells seemed normal with peptidoglycan, and the cytoplasmic membranes were intact. Based on the TEM data, the cells treated with amoxicillin plus commercial GML extract displayed much more morphological damage, and thin peptidoglycan and cytoplasmic membrane as compared to the control cells. The results from these studies matched a previous result where the combination of oxacillin plus α-mangostin isolated from *G. mangostana* caused damage to the ultrastructure's of the cells, affected the integrity of the cell walls, and led to an increase in the cell size of oxacillin-resistant *S. saprophyticus* (Phitaktim *et al.*, 2016). Confocal microscopic pictures confirmed the TEM findings, indicating that the peptidoglycan of the combination-treated cells had been destroyed. Furthermore, the finding of this research resembled those of Phitaktim *et al*. (2016) who discovered that α-mangostin had a substantial morphological effect on *S. aureus* (MRSA), including cell lysis and wall damage. Similarly, the findings were consistent with those of Joung *et al*. (2016) who reported septa formation and midline disruption in MRSA treated with oxyresveratrol (ORV).

Conclusion

The present work demonstrated that commercial GML extract and amoxicillin in combination could act as an antibacterial agent against MRSA.

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