ANTIMICROBIAL ACTIVITY OF TURMERIC, GINGER, AND GALANGAL RHIZOME ETHANOL EXTRACTS IN COMBINATION USING THE CHECKERBOARD METHOD

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ABSTRACT

Bacterial infection remains a significant problem around the globe and worsens with the emergence of antibiotic-resistant strains. Indonesia is rich in natural resources, such as its flora diversity, with various benefits, including medicinal use. This study investigated the potency of turmeric, ginger, and galangal rhizome ethanol extracts in single and combination using the checkerboard method against several pathogens. The most potent inhibitory activity in a single form was shown by turmeric extract against Streptococcus mutans and Cutibacterium acne with the minimum inhibitory concentration (MIC) value of 16 µg/mL. Meanwhile, the bactericidal activity was shown best by turmeric extract against Staphylococcus aureus and Enterococcus faecalis with both MIC and minimum bactericidal concentration (MBC) of 32 µg/mL. The synergistic combination was shown by turmeric-ginger (TGi), turmeric-galangal (TGa), and ginger-galangal (GiGa) against Staphylococcus aureus with the FICI (fractional inhibitory concentration index) of 0.15625, 0.1328, and 0.15625, respectively, and TG\textsubscript{i} against Streptococcus mutans with the fractional inhibitory concentration index (FICI) of 0.1875. This preliminary study showed the potency of those rhizomes for further research to investigate the active compounds with a synergistic activity that may be used as an alternative therapy in the future.

Keywords: combination, galangal, ginger, synergistic, turmeric

INTRODUCTION

As one of the most populated countries in the world, Indonesia, with its 275,5 million population, faces a series of challenges in the health sector, one of which is infection\textsuperscript{1,2}. Infection diseases have contributed significantly to the death toll in the last decade, ranked at several levels in Indonesia's top 10 causes of death, along with cardiovascular diseases, neoplasms, and diabetes\textsuperscript{3}. Dealing with this kind of situation must involve thorough action, including control of antibiotic usage, management of healthcare, and national policy regarding preventing and combating infectious diseases. Though antibiotics can solve this problem, it is still difficult to completely avoid death due to infections. It is due to insufficient healthcare facilities are not available and properly accessible,
especially in remote areas, and antibiotic abuse worsens the problem, resulting in many cases of antibiotic-resistant bacteria that narrow the antibiotic therapy options, resulting in high mortality\(^4\).

The strategy for controlling antibiotic-resistant infections can be carried out by using three approaches: a combination of antibiotics, antibiotic prescribing management and monitoring, and investigation of alternatives to the current treatment\(^5,6\). The combination of active substances has several properties: synergistic, additive, and antagonist. Combinations of antimicrobials are commonly used as a strategy in treating infectious cases with several purposes, including obtaining a superior effect than the single-therapy regimen, preventing the development of antimicrobial resistance, and improving the effectiveness of certain antibiotics against less-susceptible strains. However, antimicrobial combinations must be carefully considered because unwanted combinations may negatively impact the therapy outcome. In practice, the expected combination is synergistic and additive, where these two properties have more beneficial activities than single-therapy\(^7,8\).

Despite dreadful health challenges constantly haunting Indonesia, it is also blessed with abundant natural resources widely distributed in the land to marine environments, enabling the possibility to discover alternative treatments in addition to current modern therapies for infectious diseases. Traditional medicines have been used since the ancestors of Indonesia, long before Western medicine was widely used in Indonesia. This involves using various indigenous herbs and medicinal plants processed traditionally and empirically used in daily activities, especially in rural areas\(^9\).

Several rhizomes are commonly used in Indonesia, not only as spices but also for medicinal use. Ginger (Zingiber officinale Rosc.), turmeric (Curcuma domestica Valeton), and Alpinia galanga (L.) Willd.) are some of the plants belonging to the Zingiberaceae family. These three plants are widely found in various regions in Indonesia and are commonly used in everyday life as cooking spices, herbal medicine, and traditional medicine\(^10\)–\(^13\). Galangal has been widely used in alternative medicine, including as an antifungal, anti-inflammatory, and antibacterial\(^14\). Ginger, also in the same family as galangal, has many properties that are used empirically in Indonesia, including as a medicine for coughs, diarrhea, and bacterial infections\(^15\). Turmeric has curcumin as the main content, which is an antibacterial, including against Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, and Pseudomonas aeruginosa\(^16\). The combination of turmeric and several antibiotics shows synergistic properties against Staphylococcus aureus and Methicillin-resistant Staphylococcus aureus\(^17,18\).

This study aimed to evaluate the antibacterial and antifungal activity of turmeric, ginger, and galangal rhizome ethanol extracts in single and combination against Escherichia coli ATCC 8739, Salmonella typhi ATCC 6539, Enterococcus faecalis ATCC 29212, Staphylococcus aureus ATCC
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6538, *Streptococcus mutans* ATCC 35668, *Pseudomonas aeruginosa* ATCC 9027, *Neisseria meningitidis* ATCC 13090 Serogroup B, *Cutibacterium acne* ATCC 6919, and *Candida albicans* ATCC 10231. The activity of extracts in single form was evaluated using the microdilution method, and in combination form, was assessed using the checkerboard method. To our knowledge, no study has reported the antibacterial activity of all those extracts in single and combination form against the tested microbes in this study concomitantly.

**METHODS**

**Plant Determination and Extraction**

Turmeric, ginger, and galangal rhizomes were obtained from the Manoko Lembang Experimental Garden, Bandung. Determination was carried out at the Jatinangor Herbarium, Padjadjaran University, Bandung. The rhizomes are dried and finely chopped. A total of 1 kg of powder was macerated using 4 L of 96% ethanol for 3x24 hours. Every 24 hours, the solvent was changed. The liquid extract is then evaporated using a rotary evaporator with a rotation speed of 65 rpm at 40° C until it thickens.

**Phytochemistry Screening**

Phytochemical screening was carried out for all simplicia and dry extracts of ginger, turmeric, and galangal using the Farnsworth method.

**Antimicrobial Assay in Single Form**

Minimum inhibitory concentration (MIC) was carried out using the microdilution method with a microplate consisting of 96 wells. In each well, the first to the twelfth column was filled with 100 μL of liquid media (Mueller Hinton Broth/MHB for bacteria and Saboraud Dextrose Broth for *Candida albicans*) and carried out in triplicate. The first column was a negative control containing liquid media, and then 100 μL of extract was added to the twelfth column. After mixing, 100 μL of the mixture was transferred from the twelfth to the eleventh column. This dilution process is continued until the third column so that it has the smallest extract concentration. The remaining volume in the third column is discarded. About 10 μL of bacterial inoculum suspension was added to each dilution column, except for the first column as a negative control (5x10⁵ cfu/mL as the final cell concentration for each well for bacteria and 0.5x10³ cfu/mL for *Candida albicans*). Microplates were incubated at 35 ± 2 °C for 18 hours. The MIC value obtained is the smallest concentration, indicating no bacterial growth or sediment at the bottom of the microdilution plate. The minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were carried out by taking aliquots from all wells that did not show growth and then streaking them on the agar surface. After 24 hours of incubation, microbial growth was observed on the Mueller Hinton Agar for bacteria and...
Saboraud Dextrose Agar for *Candida albicans*. MBC and MFC are determined from the well with the lowest concentration, which shows no bacterial growth on the agar surface.

**Antimicrobial Assay in Combination**

The combination assay was performed using the checkerboard method with modification\(^{20}\). Briefly, all extracts were series diluted from 2-4x MIC to 1/8 – 1/16 MIC. The 100 µL of each extract were combined (turmeric-ginger/TGi, turmeric-galangal/TGa, and ginger-galangal/GiGa) to obtain 200 µL per well for each series of concentrations, then 10 µL of bacterial inoculum suspension was added to each dilution column (10\(^5\) cfu/mL as the final cell concentration for each well) as shown in **Figure 1**.

**Figure 1.** Checkerboard scheme

The observation was done visually to evaluate the pattern of bacterial growth and combination. MIC of extract A can be observed from row H since there is no extract B (concentration B = 0 µg/mL) in that row, and MIC of extract B can be observed from column 12 since there is no extract A (concentration A = 0 µg/mL) in that column. Combination activity is determined by observing the patterns as shown in **Figure 2** and calculating the fractional inhibitory concentration index (FICI) using the formula below:

\[
FICI\ of\ extract\ A\ and\ B = \frac{MIC\ A\ in\ combination}{MIC\ A\ in\ single} + \frac{MIC\ B\ in\ combination}{MIC\ B\ in\ single}
\]

The synergistic activity was shown if the FICI is < 0.5, additive if the FICI is > 0.5 to < 1.0, and indifference to antagonist if the FICI is > 2.0.\(^7,8\)
RESULTS AND DISCUSSION

Plant Determination

The results of the determination carried out at the Jatinangor Herbarium, Plant Taxonomy Laboratory, Department of Biology FMIPA UNPAD with document numbers No.18/HB/07/2020, No.19/HB/07/2020, and No.20/HB/07/2020 show that the ginger rhizome species is *Zingiber officinale* Roscoe, the turmeric rhizome is *Curcuma domestica* Valeton, and the galangal rhizome is *Alpinia galanga* (L.) Willd.

Phytochemistry Screening

The results of phytochemical screening showed that the simplicia and ethanol extracts of galangal, turmeric, and ginger rhizomes were positive for alkaloid, flavonoid, saponin and quinone compounds, phenols, tannins, steroids, and triterpenoids. These results are in line with previous research regarding phytochemical screening of simplicia and ethanol extracts of galangal, turmeric, and ginger rhizomes\textsuperscript{14,21–23}.

Antimicrobial Assay in Single Form

The antimicrobial activity of each extract is shown in Table 1.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Activity in µg/mL</th>
<th>Activity in µg/mL</th>
<th>Activity in µg/mL</th>
<th>Activity in µg/mL</th>
<th>Activity in µg/mL</th>
<th>Activity in µg/mL</th>
<th>Activity in µg/mL</th>
<th>Activity in µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Escherichia coli</td>
<td>Salmonella typhi</td>
<td>Enterococcus faecalis</td>
<td>Staphylococcus aureus</td>
<td>Streptococcus mutans</td>
<td>Neisseria meningitidis</td>
<td>Pseudomonas aeruginosa</td>
<td>Cutibacterium acne</td>
</tr>
<tr>
<td>Turmeric</td>
<td>512</td>
<td>&gt;8192</td>
<td>512</td>
<td>4096</td>
<td>32</td>
<td>32</td>
<td>16</td>
<td>128</td>
</tr>
<tr>
<td>Ginger</td>
<td>256</td>
<td>&gt;8192</td>
<td>1024</td>
<td>4096</td>
<td>512</td>
<td>1024</td>
<td>512</td>
<td>&gt;8192</td>
</tr>
<tr>
<td>Galangal</td>
<td>256</td>
<td>&gt;8192</td>
<td>256</td>
<td>4096</td>
<td>256</td>
<td>2048</td>
<td>256</td>
<td>128</td>
</tr>
<tr>
<td>Tetracycline/</td>
<td>0.5</td>
<td>0.5</td>
<td>2</td>
<td>32</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>0.5</td>
<td>0.5</td>
<td>2</td>
<td>32</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>4</td>
</tr>
</tbody>
</table>

The activity of the extract can be categorized as good (MIC < 100 µg/mL), moderate (MIC > 100 to < 500 µg/mL), weak (MIC > 500 to < 1000 µg/mL), and inactive (MIC > 1000 µg/mL)\textsuperscript{24}. In single form, turmeric extract showed the best activity of all extracts against *Enterococcus faecalis*, *Salmonella typhi*, and *Staphylococcus aureus*. The extracts showed a wide range of activities against the tested microbes, with turmeric extract showing the best activity against *Enterococcus faecalis*.
Staphylococcus aureus, and Cutibacterium acne, which were categorized as good activity, while ginger and galangal mostly were also active but with the tendency to moderate activity and some even were considered to be inactive. Curcumin, the most abundant component contained in turmeric, plays a pivotal role in the antibacterial activity. Several studies reported the synergistic activity of curcumin with some antibiotics with several possible mechanisms, including enzyme inhibition, reducing lysis and hydrolyzation of antibiotics.

All extracts showed weak activity to inactive against all tested Gram-negative bacteria and Candida albicans. This may be due to the unique structures of Gram-negative outer membranes, which predispose hydrophobic surfaces that hamper the entrance of active substances to the target inside the cell. Gram-negative has two layers of membrane (outer and inner membrane), making most antibacterial compounds to be extremely difficult to penetrate and reach the target. Hence, very few compounds are active against Gram-negative compared to Gram-positive.

Based on the MIC value of each extract against the tested microbes, a combination activity assay was performed by combining turmeric and ginger (TGi), turmeric and galangal (TGa), and ginger and galangal (GiGa) in a specific range of MIC, typically starting from 2–4x MIC to 1/8–1/16 MIC. The results of the combination assay using the checkerboard method are shown in Table 2.

<table>
<thead>
<tr>
<th>Extract Combination</th>
<th>Escherichia coli</th>
<th>Salmonella typhi</th>
<th>Enterococcus faecalis</th>
<th>Staphylococcus aureus</th>
<th>Streptococcus mutans</th>
<th>Neisseria meningitidis</th>
<th>Pseudomonas aeruginosa</th>
<th>Cutibacterium acne</th>
<th>Candida albicans</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGi</td>
<td>0.5</td>
<td>1</td>
<td>0.75</td>
<td>0.15625</td>
<td>0.1875</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>&gt;1</td>
</tr>
<tr>
<td>TGa</td>
<td>&gt;1</td>
<td>0.625</td>
<td>&gt;1</td>
<td>0.1328</td>
<td>&gt;1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>&gt;1</td>
</tr>
<tr>
<td>GiGa</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0.15625</td>
<td>&gt;1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>&gt;1</td>
</tr>
</tbody>
</table>

According to the results, it can be shown that all combinations showed synergistic activity against Staphylococcus aureus. Meanwhile, only TGi showed synergistic activity against Streptococcus mutans. Compared to the individual MIC of ginger and galangal extracts against Staphylococcus aureus, the MIC of those in combination was significantly decreased, indicating that turmeric extracts contain compounds that may enhance the antibacterial compounds contained in ginger and galangal extracts.

Recent studies reported that the synergistic activity from certain combinations might involve several mechanisms, such as cytoplasmic membrane disruption, increased membrane permeability, inhibition of antibiotic-degrading enzymes such as β-lactamase, and damage to the structure of peptidoglycan. Combining extracts or extract with an antibiotic may also result in the enhanced activity of one of the substrates against resistant phenotype. This may happen because some compounds in the extract may attenuate the action of the antibiotic by either promoting the penetration
to the cells and enhancing the effectivity, preventing degradation of the antibiotic, or inhibiting the efflux pump that may interfere with intracellular antibiotic concentration\(^{28}\). Hence, studying the combination of substances is prospective to reveal new alternative therapies and solve certain existing antibiotic-resistant challenges.

In the case that the activity of one substance is merely active in a single form, but when in combination with other compounds, the activity is increased, this may be due to some possible mechanisms. When a protein synthesis inhibitor compound alone was used against Gram-negative, for example, the killing ability of that compound may not be lethal enough due to the possibility of poor penetration to the target. However, when it is combined with a membrane destabilizer, theoretically, this compound may increase the penetration of the protein synthesis inhibitor compound to work more efficiently\(^{29,30}\). However, this speculation only takes into account the directly associated components that play a role in the mechanism of action. When other elements are taken into account, for example, the existence of an efflux pump and structure-modifying enzyme, these elements can negate the action of the said protein synthesis inhibitor compound. Hence, studying the actual mechanism of action of the compound combination is essential to properly justify the benefit of using extracts or compound combinations.

In this study, other combinations that have FICI equal to or more than one were out of interest since they are indicated to be indifferent or even antagonistic, which shows no value in investigating the compounds of interest as a prospective alternative therapy. However, on certain occasions, investigating the antagonistic effects of certain combinations, such as some medicinal herbs and certain antibiotics, may give beneficial insights in order to avoid negative or unwanted impacts from those combinations, hence preventing therapy failure.

![Figure 3. Checkerboard of turmeric extract (column) and ginger extract (row) against (a) *Staphylococcus aureus* and (b) *Streptococcus mutans*. Bordered distinct white dots in the bottom of the wells indicated significant bacterial growth.](image)
CONCLUSION

Most of the extracts tested showed antibacterial activities in a single form, with turmeric extract showing good activity against *Streptococcus mutans* and *Cutibacterium acne* with the MIC value of 16 µg/mL. Meanwhile, the bactericidal activity was shown as a good activity for turmeric extract against *Staphylococcus aureus* and *Enterococcus faecalis* with both MIC and MBC of 32 µg/mL. The synergistic combination shown by turmeric-ginger (TGi), turmeric-galangal (TGa), and ginger-galangal (GiGa) showed activity against *Staphylococcus aureus* with the FICI (fractional inhibitory concentration index) of 0.15625, 0.1328, and 0.15625, respectively and TGi against *Streptococcus mutans* with the FICI of 0.1875. These findings showed interesting results for further investigation to find the actual compounds from each plant that interact synergistically as antibacterial.

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