Effects of Sauropus androgynus extract and its combination with ampicillin against Methicillin-resistant Staphylococcus aureus: An in vitro study

Asih Rahayu, Chylen Setiyo Rini, Yos Adi Prakoso, Bagus Uda Palgunadi and Muhammad Aris Munandar

1. Faculty of Veterinary Medicine, University of Wijaya Kusuma Surabaya, East Java, Indonesia; 2. Integrated Laboratory, Faculty of Health, University of Muhammadiyah Sidoarjo, East Java, Indonesia.

Corresponding author: Bagus Uda Palgunadi, e-mail: bagusuda24@gmail.com

Received: 23-04-2020, Accepted: 22-07-2020, Published online: 14-09-2020


Abstract

Background and Aim: The massive utilization of antibiotics has increased resistant genes produced by bacteria. Many bacteria, including Methicillin-resistant Staphylococcus aureus (MRSA), have become resistant against ampicillin (AMP). The combination of an herbal extract with AMP is expected to generate synergistic effects and may restore the susceptibility of MRSA against AMP. This study aimed to analyze the potency of Sauropus androgynus extract (SAE) as a single extract and combination with AMP against MRSA.

Materials and Methods: Sauropus androgynus was extracted using 60% ethanol. SAE biochemical compounds were analyzed qualitatively and quantitatively. SAE, AMP, and SAE+AMP were tested against MRSA isolates to determine the minimum inhibitory concentration and fractional inhibitory concentration. The inhibition of penicillin-binding proteins 2a (PBP2a) was analyzed using a latex agglutination test. Further, the disruptive membrane effects of SAE, AMP, and SAE+AMP were analyzed using a scanning electron microscope. The analysis of data was conducted using SPSS version 16 with p=0.01.

Results: SAE contained bioactive compounds such as phenolics and flavonoids. Further, 2 mg/mL of SAE could be used as the potential concentration against MRSA isolates in vitro. In addition, the utilization of SAE+AMP generated synergistic effects, restored the susceptibility of isolates against AMP, decreased the synthesis of PBP2a by the MRSA, and induced ultrastructural changes in the bacterial membrane.

Conclusion: This study indicated that the utilization of SAE potentially inhibits the growth of MRSA through decreasing of PBP2a expression, disruption of the MRSA membrane, while the combination of SAE+AMP showed synergistic effects against MRSA.

Keywords: ampicillin, herbal extract, Methicillin-resistant Staphylococcus aureus, Sauropus androgynus, ultrastructure.

Introduction

Antibiotics have been developed massively since they were first discovered in the late 19th century. From a positive standpoint, a large number of antibiotic derivatives are currently available for the treatment of many bacterial species causing infections. However, the development of antibiotics has also influenced the formation of a resistant gene among the microbes [1]. Resistance to the new antibiotics commonly occurs within 2-3 years after they are first utilized as therapeutic agents [2].

Furthermore, antimicrobial resistance (AMR) has spread widely over the world and has become a global concern. Many strategies have been developed by scientists, microbiologists, and clinicians to control the resistance phenomenon. One important strategy is the utilization of a combination drug [3]. Antibiotic combinations to treat an infection are often practiced for many reasons, including to broaden the antimicrobial spectrum and to prevent polymicrobial infection, and are expected to generate synergistic effects [4].

An extended-spectrum antibiotic is ampicillin (AMP). AMP is an active antibacterial agent against Gram-positive as well as Gram-negative bacteria [5]. Clinicians generally use AMP to treat many types of infection, including soft tissue and skin infections [6]. However, several species of bacteria affecting the skin have been transforming a resistant gene against AMP. Methicillin-resistant Staphylococcus aureus (MRSA) is a resistant bacterium against AMP through the synthesis of penicillin-binding proteins (PBPs) [7]. A previous study described that the combination of AMP and Coptis chinensis extract may have synergistic effects on inhibiting MRSA colonization because of its berberine content [8]. Another study reported that Acalypha wilkesiana extract exerts a...
synergistic effect when combined with AMP to inhibit MRSA colonization and restores MRSA susceptibility to AMP [9]. This shows that herbal extracts have potential effects of increasing the potency of synthetic antimicrobial agents, especially AMP. Further, the utilization of herbal extracts is expected to promote healing during bacterial infection without promoting resistant genes of these bacteria.

A traditional Indonesian herbal extract is *Saurus androgynus* (SA). This herbal preparation contains several bioactive compounds such as alkaloids, tannins, saponins, and flavonoids that can be used as antibacterial agents as well as antioxidants [10]. A previous study elucidated that the SA extract (SAE) could be utilized as an antifungal agent [11]. As an antibacterial agent, SAE can potentially inhibit the growth of Gram-negative bacteria, including *Edwardsiella tarda*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Vibrio spp.*, and *Aeromonas hydrophila* [12]. Another study showed that SAE promoted wound healing on the wound in a diabetic mouse model infected with MRSA both as a single and combination treatment [13].

Those potencies are caused by the antioxidant activity of SAE activating the healing promoter. However, these previous studies have not explored the potency of SAE in promoting AMP effects against MRSA. Based on this background, this study aimed to explore the potency of SAE against MRSA through the observation of its effects as a single treatment and in combination with AMP.

**Materials and Methods**

**Ethical approval**

The ethical approval was not applicable because of no animal or human experimentation in this study.

**Study location and period**

All the research procedures were carried out in the Integrated Laboratory, Faculty of Health, University of Muhammadiyah Sidoarjo, East Java, Indonesia. The study was conducted from April to October 2019. Further, the scanning electron microscope (SEM) examination was conducted in the Biological Research Center, Indonesian Institute of Sciences, Bogor, Indonesia.

**Extract preparation**

SA leaves were purchased from a local botanical garden, Mojokerto, East Java, Indonesia. The plant species was authenticated by a botanist at the Indonesian Institute of Sciences, Purwodadi, Indonesia, with voucher specimen: 0277/IPH.06.HM/II/2019. The SA was dried in an oven (Memmert UN30, custom tariff number 84198998, Germany) for an hour at 80°C. The dried SA was pulverized using an electric blender (Waring, MX100XTS, Indonesia). The SA powder was soaked using 60% ethanol (50 g/L) and was heated using a microwave (Toshiba, EM245A5C-BS, Japan) with 110°C, 1250 W, for 10 min. Next, the SA extract (SAE) was filtered through Whatman filter paper number 4 (Whatman, catalog number 1441-090, United States) and was dried at 40°C using a rotary evaporator (Buchi, catalog number 6.268.005, Switzerland) to remove residual ethanol [14].

**Phytochemical screening**

SAE was tested to determine levels of biochemical compounds, including alkaloids, flavonoids, glycosides, phenolics, saponins, and tannins. The biochemical compounds of SAE were screened using qualitative and quantitative methods. Qualitative tests were performed using the following methods: The Mayer test for alkaloids, magnesium+hydrogen chloride+ethanol test for flavonoids, Borntrager’s test for glycosides, 10% natrium chloride+1% gelatin for phenolics, saponins detected by the presence of foams, and tannins were detected using 1% ferric chloride. Quantitative phytochemical screening was tested in triplicate repetitions. Both qualitative and quantitative tests were performed in accordance with a previous study [15].

**Bacterial strain**

The MRSA isolate was obtained from the American Type Culture Collection (ATCC) encoded (ATCC® BAA-29213) and from a clinical isolate from the Department of Microbiology, Faculty of Medicine, University of Airlangga encoded: 53/UN3.1.1/MK/LL/2019. The stability of bacterial characterization was reconfirmed using several tests, including coagulase, DNase, hemolysis, colony pigmentation, alkaline phosphatase, urease, mannitol, maltose, esculin hydrolysis, novobiocin, polymyxin B, and ceftoxitin. The MRSA isolate was stocked in Todd Hewitt agar (Merck, catalog number 146598, Germany) for further research.

**Minimum inhibitory concentration (MIC) test**

The MIC determinations of AMP (Sigma-Aldrich) and SAE against MRSA were conducted according to the methods used in a previous study [16]. The 0.1 mL of MRSA suspension (5×10⁵ CFU/mL) was added to 0.9 mL of Mueller-Hinton broth (Merck, catalog number 1.10293.0500, Germany). Further, SAE, AMP, and SAE+AMP were diluted using sterile distilled water to obtain stock solutions of 1024 μg/mL (AMP) and 1024 mg/mL (SAE). The antibiotic and extract stock solution was serially diluted 3-fold to achieve respective treatment concentrations. The bacterial suspension was added to SAE, AMP, and SAE+AMP solution and was incubated at 37°C for 18 h. The lowest concentration that indicated no visible bacterial growth after incubation was recorded as the MIC.

**Checkerboard synergy test**

The synergistic effects of SAE+AMP against the MRSA isolates were tested using the checkerboard synergy test, which was performed similarly to that described above to determine the MIC. However, the SAE+AMP condition was combined and incubated at 37°C for 18 h. The lowest concentration of SAE+AMP combination that had the potential to
inhibit bacterial growth was defined as the MIC. The fractional inhibitory concentrations (FICs) of SAE and AMP were used to determine the FIC index (FICI) using the following formulae:

\[
FICI = \frac{FIC_A + FIC_B}{2}
\]

\[
FIC_A = \frac{MIC_A \text{ after combination}}{MIC_A \text{ alone}}
\]

\[
FIC_B = \frac{MIC_B \text{ after combination}}{MIC_B \text{ alone}}
\]

The combination of SAE+AMP was classified as synergistic when the FICI was ≤0.5; no interaction when FICI was >0.5 ≤2; and antagonistic when FICI was ≥2 [17].

**PBP2a latex agglutination test**

PBP2a latex agglutination test using MRSA biofilm was analyzed semi-quantitatively as follows: -- (absence); + (weak); ++ (moderate); and +++ (strong). All the test protocols were conducted following the method described in product protocols (PBP2, Oxoid, Latex Agglutination Test, DR0900).

**SEM**

The disruptive effects of SAE, AMP, and SAE+AMP on the MRSA membrane were analyzed using an SEM. SEM was conducted in accordance with the MIC. After the incubation, the bacterial suspension was centrifuged (Scoligex, SC1412S, United States) at 1500 rpm for 5 min. Further, 2% of glutaraldehyde was added and incubated for 3 h, and it was replaced with 2% of tannin acid and incubated for 6 h. Graded alcohol was used as a dehydrated reagent. The bacteria were then coated with gold and carbon using a sputter coater. The ultrastructure of the MRSA membrane was analyzed using SEM (JEOL NeoScope, JSM5000, Japan), and the diameter and area of the membrane were measured using Image J software (NIH Public Domain, BSD-2, United States).

**Statistical analysis**

The experiments were conducted in triplicate. The collected data were compared using one-way ANOVA followed by Bonferroni’s post hoc test for multiple comparisons. The results of the analyses were expressed as mean±standard deviation. p=0.01 was considered statistically significant.

**Results**

Based on the phytochemical analysis, the ethanolic extract of SA contained alkaloids, flavonoids, glycosides, phenolics, saponins, and tannins. The highest concentration of the biochemical compound of SAE was in the order phenolic > glycoside > flavonoid > alkaloid > saponin > tannin, respectively (Table-1).

Furthermore, the SAE was utilized as the additive agent with AMP for further studies. As single agent therapy against either the MRSA clinical or ATCC isolate, the AMP showed highest resistance profile at 1024 µg/mL (based on the CLSI standard). Further, SAE at the concentration of 2 mg/mL was potent against MRSA isolates in vitro. Surprisingly, the combination of SAE+AMP had increased potency against both MRSA isolates. As shown by the FICI of SAE+AMP was ≤0.5, which indicates a synergistic mechanism of action (Table-2). The results suggested that the increasing level of PBP2a within the MRSA biofilms along with the combination of AMP (1024 µg/mL) as the therapeutic agents in vitro was more effective than the untreated controls. Conversely, the SAE (2 mg/mL) could inhibit PBP2a formation by MRSA, and the synergistic test showed that SAE+AMP combination decreased PBP2a formation against either the MRSA clinical isolate or the ATCC isolate (Table-3).

The SEM analysis showed that there were no significant differences between the MRSA clinical isolate and the ATCC isolate, in terms of the diameter and membrane area in the untreated group. However, the SAE promoted an increase in diameter of either the clinical (p=0.01 or p≤0.01) or the ATCC isolate (p=0.005 or p≤0.01). Similar effects by SAE were observed on the membrane area of the clinical isolate (p=0.01 or p≤0.01) and ATCC isolate (p=0.006 or p≤0.01). In contrast, the AMP generated a decreasing diameter of MRSA (p≤0.01). A similar result was obtained by the combination treatment group (p≤0.01) (Table-4). The ultrastructural representation of the MRSA following the treatment is shown in Figure-1.

**Discussion**

Herbal extracts contain several bioactive compounds that can be utilized as antioxidant and antimicrobial agents. Bioactive compounds present in Table-1: Phytochemical screening of SAE.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Qualitative screening</th>
<th>Quantitative screening</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloid</td>
<td>++</td>
<td>10.13±0.12</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>++</td>
<td>12.16±0.06</td>
</tr>
<tr>
<td>Glycoside</td>
<td>+++</td>
<td>19.18±0.43</td>
</tr>
<tr>
<td>Phenolic</td>
<td>+++</td>
<td>23.88±0.15</td>
</tr>
<tr>
<td>Saponin</td>
<td>+++</td>
<td>9.40±0.39</td>
</tr>
<tr>
<td>Tannin</td>
<td>+</td>
<td>3.96±0.05</td>
</tr>
</tbody>
</table>

The results of quantitative screening were obtained from three repetitions. SAE=Sauropus androgynous extract

**Figure-1:** The ultrastructure of MRSA without and with the administration of SAE+AMP. The uniform in size and shape of the MRSA in a group without treatment (a); and the diverse size and shapes of the MRSA after the therapy using a combination of SAE+AMP in vitro (b). JSM5000, SEM, 10,000×. MRSA=Methicillin-resistant Staphylococcus aureus, SAE=Sauropus androgynous extract, AMP=Ampicillin.
**Table 2:** Potency of SAE, AMP, and SAE+AMP against MRSA.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Bacterial strain</th>
<th>SAE (mg/mL)</th>
<th>AMP (µg/mL)</th>
<th>SAE+AMP (mg/mL+µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC</td>
<td>MRSA clinical isolate</td>
<td>2&lt;sup&gt;ND&lt;/sup&gt;</td>
<td>1024&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.5+4</td>
</tr>
<tr>
<td></td>
<td>ATCC® 29213</td>
<td>1024&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NT</td>
<td>≤0.5&lt;sup&gt;v&lt;/sup&gt;</td>
</tr>
<tr>
<td>FICI</td>
<td>MRSA clinical isolate</td>
<td>NT</td>
<td>NT</td>
<td>≤0.5&lt;sup&gt;v&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>ATCC® 29213</td>
<td>NT</td>
<td>NT</td>
<td></td>
</tr>
</tbody>
</table>

SAE=Sauropus androgynus extract, AMP=Ampicillin, ND=No data provided, R=Resistant, NT=Not tested, Sy=Synergistic, MRSA=Methicillin-resistant Staphylococcus aureus

**Table 3:** Potency of SAE, AMP, and SAE+AMP against the PBP2a of the MRSA.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Bacterial strain</th>
<th>Control</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SAE</td>
<td>AMP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2 mg/mL)</td>
<td>(1024 µg/mL)</td>
</tr>
<tr>
<td>PBP2a</td>
<td>MRSA clinical isolate</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>ATCC® 29213</td>
<td>++</td>
<td>-</td>
<td>+++</td>
</tr>
</tbody>
</table>

SAE=Sauropus androgynus extract, AMP=Ampicillin, −=Absence/no agglutination, +=weak agglutination, ++=Moderate agglutination, +++=Strong agglutination, MRSA=Methicillin-resistant Staphylococcus aureus

**Table 4:** Potency of SAE, AMP, and SAE+AMP on the ultrastructure of the MRSA's membrane.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Bacterial strain</th>
<th>Control</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SAE</td>
<td>AMP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2 mg/mL)</td>
<td>(1024 µg/mL)</td>
</tr>
<tr>
<td>Diameter of membrane (µm)</td>
<td>MRSA clinical isolate</td>
<td>0.73±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.87±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>ATCC® 29213</td>
<td>0.76±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.85±0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Membrane area (µm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>MRSA clinical isolate</td>
<td>0.54±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.76±0.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>ATCC® 29213</td>
<td>0.57±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.73±0.10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>The different superscript in the same row showed significant differences at p≤0.01. SAE=Sauropus androgynus extract, AMP=Ampicillin, MRSA=Methicillin-resistant Staphylococcus aureus

Herbal extracts potentially interfere with intracellular mechanisms within bacteria. It may cause an imbalance in the molecular transport mechanism or interfere with membrane permeability of the bacteria. These mechanisms are similar to those achieved by synthetic antibiotics such as AMP. AMP inhibits bacterial growth through its ability to bind to the PBPs produced by bacteria, inhibits transpeptidation reactions, and blocks peptidoglycan synthesis, leading to cell death [18]. However, following the widespread use of AMP in public health services, many pathogens develop resistance to the β-lactam antibiotics by modifying one or more of those mechanisms [19]. This study demonstrated that MRSA has a high resistance profile against AMP, as reflected by the high concentration of AMP (1024 µg/mL) on the inhibition of MRSA growth. Conversely, SAE inhibited MRSA at a concentration of 2 mg/mL, which was similar to that of a previous study [20]. The combination of SAE+AMP induced stronger bacteriostatic activity, which was supported by a decreased concentration of SAE+AMP to 0.5 mg/mL+4 µg/mL for MRSA inhibition, respectively.

Furthermore, the synergistic checkerboard test indicated that the SAE+AMP combination was synergistic, as shown by the FICI < 0.5. The synergistic effects of the combination of SAE+AMP were suspected to be caused by several biochemical mechanisms, including the degradation of matrix protein of the bacteria wall and the inhibition of the protein-mediated flux [21]. The combination of SAE+AMP inhibited the synthesis of peptidoglycan that gradually leads to the degradation and depletion of the membrane. Furthermore, the inhibition of protein-mediated flux led to a chaos in the molecular transport system, whereby carbon, which is potentially required by the bacteria to maintain its existence, was no longer available. The blocking of the molecular transport chain allowed binding of the bacterial membrane and the antimicrobial agents, which inhibited the growth of bacteria in vitro [22]. However, these synergistic mechanisms were not clearly demonstrated in the present study; thus, further investigations including in-vitro utilization of AMP against MRSA increased the synthesis of PBP2a semi-quantitatively. Moreover, PBP2a synthesis was increased by MRSA to one level higher than normal conditions without any drug intervention. Following SAE intervention, inhibition of PBP2a synthesis by MRSA occurred in both
isolates. Surprisingly, the combination of SAE+AMP indicated the effect on PBP2a synthesis by MRSA, and it demonstrated that AMP facilitated the synthesis of PBP2a, albeit at low levels.

The ultrastructural study was conducted using SEM at 10,000× to evaluate any morphological changes at the cellular level of the MRSA following treatment with AMP, SAE, or its combination. Based on the results, there was a change in the membrane structure in the MRSA after treatment. Morphological changes were induced in the membrane by SAE and SAE+AMP treatment as indicated by the swelling and the contraction of the MRSA membrane, respectively. The area of the membrane of MRSA in both isolates without treatment was between 0.54 and 0.57 µm². However, following the administration of SAE, there was an increase in membrane area, and further, a reduction of the membrane area occurred following exposure to SAE+AMP. The cell swelling caused an increase in the membrane area of the MRSA following the utilization of SAE. These effects were due to the chaos of the membrane transport system induced in the bacteria. This ultrastructural study also indicated that SAE and the combination of SAE+AMP generated a disruptive membrane mechanism of the MRSA in vitro. As the most prominent part of the bacteria, its membrane supports the viability of bacteria against extreme environments and any changes in the membrane impacts on the existence of bacteria.

Many studies have shown that herbal remedies have beneficial effects as antioxidant and antimicrobial agents because of the generation of secondary metabolite compounds and its derivatives [24]. This is supported by the present study that showed that the alkaloid, phenolic, glycoside, flavonoid, saponin, and tannin were also components of the SAE. All these compounds are potential antimicrobial agents [25].

MRSA is a Gram-positive bacterium with a thick membrane that consists of lipoprotein and peptidoglycan. Furthermore, the lipoprotein and peptidoglycan within the MRSA membrane facilitate the phenolic components of SAE to induce interactions, leading to changes in osmotic pressure and subsequent membrane disruption. A low osmotic pressure (osmotic downshift) promotes the fluid release from the cytoplasm leads to membrane contraction. Conversely, high ionic pressure promotes the fluid diffusion that causes cell swelling [26]. Furthermore, these mechanisms were also demonstrated in our study. A previous study reported that phenolic compounds decrease the permeability and rigidity of the membrane of Gram-positive bacteria [27]. Phenolic compounds also have been shown to bind the cations present on the bacterial membrane and cause an imbalance in the ion charges of the bacteria; furthermore, these mechanisms result in high positive charges that promote the lysis of the membrane [28].

Besides, phenolic compounds, other metabolites of the SAE having potential as antimicrobial agents, are tannin and flavonoids. Tannins can neutralize the lipopolysaccharides within the fibrils and pili of the bacteria, which lead to a weakening of the bacteria to attach on the surface of the host’s cells [29]. In addition, flavonoids decrease the fluidity of the MRSA membrane and create hydroxy groups (-OH) during the synthesis of ATP, which cause perforation and decrease the rigidity of the membrane to promote membrane lysis [30].

Conclusion

This study demonstrated that the SAE exerts potential effects on the growth of MRSA in vitro. Further, the combination of SAE extract and AMP generated synergistic inhibitory effects on PBP2a synthesis and membrane disruption. As a future perspective, herbal medicines should gain more widespread interest in the treatment of microbial resistance because of its potency to restore the susceptibility of bacteria against commercial antibiotics, such as ampicillin.

Authors’ Contributions

AR, CSR, YAP, BUP, and MAM designed the research. YAP analyzed the data and interpretation. YAP, AR, and BUP performed the research. AR, CSR, YAP, MAM, and BUP collaborated during the writing and revising of the manuscripts and approved the manuscript’s final version. All authors read and approved the final manuscript.

Acknowledgments

All the technicians from Integrated Laboratory, the University of Muhammadiyah Sidoarjo and Biological Research Center, Indonesian Institute of Sciences, Bogor, were acknowledged for their support during the study. The authors did not receive any funds for this study.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on request.

Competing Interests

The authors declare that they have no competing interests.

Publisher’s Note

Veterinary World (Publisher of International Journal of One Health) remains neutral with regard to jurisdictional claims in published institutional affiliation.

References


**********