Antimicrobial activity and time-kill kinetics of Boesenbergia rotunda essential oil and geraniol alcohol against oral bacterial pathogens

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ABSTRACT
Boesenbergia rotunda (L.) Mansf. demonstrates remarkable antimicrobial activity against various pathogenic bacteria. However, there have been no reports on the bactericidal activity of the essential oils from B. rotunda (BREO) with active components against oral pathogenic bacteria. In this investigation, the antibacterial potency of BREO against oral pathogenic bacteria was comprehensively assessed. The evaluation of antibacterial activities was performed using agar disc diffusion, broth microdilution, and time-kill kinetics assays. Active antibacterial compounds were determined by thin-layer chromatography (TLC)-bioautography. The chemical composition was determined through gas chromatography-mass spectrometry. Inhibition zones against Streptococcus pyogenes, Streptococcus mutans, and Streptococcus sobrinus ranged between 19.81 ± 0.62 mm and 22.22 ± 5.59 mm. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were found to be between 6.25 and 12.5 mg/ml. A bactericidal effect was indicated by the MIC/MBC ratio. The time-kill assay revealed that a concentration of 4 × MIC could kill 99.99% of the tested bacteria after 1 minute of treatment. The major constituents of BREO were beta-ocimene (40.82%), followed by geraniol (16.79%) and camphor (16.01%). The TLC-bioautography of the bacteria identified geraniol as an active antibacterial compound. These results suggest that BREO is a viable ingredient for the development of natural oral care products.

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INTRODUCTION
Oral diseases, such as dental caries and pharyngitis, are major health concerns caused by various factors, including bacterial overgrowth. The oral microbiome is a collection of microorganisms that reside in the mouth, with the genus Streptococcus being the most prevalent [1]. Certain Streptococcus species, such as Streptococcus mutans and Streptococcus sobrinus, are prevalent in the progression of dental caries [2,3]. Additionally, orthodontic treatment has been linked to an increasing S. mutans and Candida albicans population in the oral cavity [4]. Streptococcus pyogenes can cause pharyngitis and tonsillitis, as well as more serious, potentially fatal diseases [5–7]. Various antibacterial products such as chlorhexidine, fluorides, and phenol derivatives have been widely used in dentistry to control bacterial growth in the mouth. However, excessive use of these products can lead to microbial resistance, tooth discoloration, erosion, and abrasion [8].

Extensive utilization of Boesenbergia rotunda (L.) Mansf. (commonly known as krachai or fingerroot), classified under the family Zingiberaceae, is observed as traditional medicine in Southeast Asia for the treatment of cough, stomach ache, and postnatal care in women. It displays antimicrobial, anti-allergic, antioxidant, and anti-tumor activities [9,10]. Boesenbergia rotunda demonstrates remarkable antimicrobial activity against various pathogenic bacteria, including Helicobacter pylori, Escherichia coli, S. mutans, S. sobrinus, S.
pyogenes, Staphylococcus aureus, Staphylococcus epidermidis, and Bacillus subtilis [11–13]. Essential oils in B. rotunda (BREO) contain geranyl formate, geranyl propionate, geraniol, neral, myrcene, isoborneol, and beta-pinene [14]. There have been no reports of BREO with active components identified against oral pathogenic bacteria [15]. In this study, we isolated BREO components and assessed their antibacterial activities. These findings can help in the development of an effective natural product to control oral pathogenic bacteria.

MATERIALS AND METHODS

Preparation of essential oil

Boesenbergia rotunda was sourced locally from the Muang district, Chiang Mai province. The authentication of plant rhizomes was conducted at the Faculty of Science, Maejo University, Chiang Mai, Thailand, with the assignment of a specimen voucher number MJU 2102-005. Cleaned rhizomes were chopped using a kitchen blender. The chopped rhizomes were then placed into a distillation flask along with distilled water and then heated. The steam with essential oil is cooled and condensed to produce a liquid mixture of water for 6 hours and essential oil was separated by using a Clevenger-type apparatus [16]. The obtained essential oils were dried over anhydrous sodium sulfate and subsequently stored at −20°C before analysis.

Bacterial strains and culture conditions

All tests were performed using S. pyogenes Department of Medical Science, Ministry of Public Health, Thailand (DMST) 30563, S. mutans DMST 18777, and S. sobrinus DMST 35719. The test strains were obtained from the DMST, of Medical Science, Ministry of Public Health, Thailand [16]. The obtained essential oils were standardized to the 0.5 McFarland standard turbidity and diluted without BREO as a positive control. The tested bacterial strains were adjusted to a 0.5 McFarland standard turbidity and diluted 1:100 in beta-hydroxybutyrate. A 50 µl bacterial suspension was added to each well of the microtiter plate. The plates were gently tapped at the corners to ensure thorough mixing of the solutions and then incubated at 37°C for 24 hours in an anaerobic jar. The lowest BREO concentration that showed no visible growth of the tested organisms was determined as the MIC. The MIC assay wells showing no observable proliferation were subjected to culturing on MHA, followed by a 24-hour incubation period at 37°C. The minimal bactericidal concentration (MBC) was identified as the lowest concentration of BREO that could inhibit microbial proliferation on MHA plates.

Thin layer chromatography-bioautography

The BREO active compounds were separated, and their antibacterial effect was evaluated using TLC plates. Previously described methods [18] were modified and performed in parallel on three TLC plates (I, II, and III) made of Silica Gel 60 F$_{254}$ (from MERCK, Germany). One microliter of BREO was added to each plate. All chemicals used for TLC were of analytical grade. BREO was separated using a mobile phase of toluene:ethyl acetate (95:5). To eliminate the solvent, the plates were left to dry in a fume hood for 15 minutes.

Plate I was visualized under ultraviolet (UV) light to identify the separated compounds. Plate II was sprayed with a 5% sulfanilic acid spray, while Plate III was coated with bacteria-infused molten BHA for bioautography purposes. After a 24-hour humid incubation at 37°C, Plate III was treated with a 1% solution of thiazolyl blue tetrazolium bromide, followed by a 10-minute room temperature incubation. The presence of an antibacterial agent is indicated by clear spots on a purple background.

Plates I and II were compared to plate III to identify spots with the same retention factor (Rf) values as the antibacterial zone on plate III. These spots were scraped off and eluted from silica gel plates with dichloromethane. The eluted compounds were analyzed using gas chromatography–mass spectrometry (GC-MS).

Gas chromatography mass spectrometry

The BREO and active compounds extracted from the TLC plate were analyzed by GC-MS using an Agilent 6890 Plus instrument with an HP-5MSI column (0.25 mm x 30 m x 0.25 µm) and a Hewlett Packard 5973 detector. The procedures were modified from those previously described [19] with a slight alteration in the temperatures. The oven temperature was set to 60°C for the first 3 minutes, increased to 200°C at 3°C/minutes, and further increased to 280°C at 15°C/minutes. The temperature was maintained at 280°C for an additional 5 minutes. Helium was used as the carrier gas at a flow rate of 1 ml/minute, and 1 µl of BREO (diluted 1:100 with dichloromethane) was injected into the system. To extract the active compounds obtained from TLC, a 200 µl volume of dichloromethane was used for elution. The eluate was then filtered using a 0.2 µm membrane and subsequently injected into the GC-MS instrument. For the GC-MS analysis, the transfer line temperature was maintained at 150°C, while the ion source temperature was set to 230°C. To calculate the Kovats retention indices, a homologous series of C9–C20 n-alkanes were injected into the GC-MS instrument. To calculate the Kovats retention indices, a homologous series of C9–C20 n-alkanes were injected into the GC-MS instrument. Compound identification was verified by comparing the results with the Wiley 7 and Nist 05 libraries, as
well as relevant literature data [20]. Geraniol (Sigma-Aldrich, St. Louis, MO) served as the standard compound.

**Time-kill kinetics assay**

In the time-kill assay, the antibacterial effects of BREO and geraniol were evaluated against *S. pyogenes, S. mutans,* and *S. sobrinus*. A suspension of bacteria was prepared in a normal saline solution to match the turbidity of a 0.5 McFarland standard. To the bacterial suspension, either BREO or geraniol was added, with final concentrations ranging from 0.5 to 4 times the MIC. Subsequently, the mixture was incubated at 37°C in a reciprocal shaker operating at 150 rpm. Viable counting was performed in triplicate at 0, 1, 5, 10, 30, and 60 minutes, and the cultures were additionally observed for 24 hours on BHA. The procedure was performed in triplicate, and the log$_{10}$ colony-forming units (CFU)/ml were plotted against time. The extract-free medium was used as a non-treated control [21]. The percentage kill was calculated as follows:

**Statistical analysis**

The experiments were performed in triplicate, and the inhibition zones were analyzed using a one-way analysis of variance followed by Duncan’s multiple range test to identify significant differences. International Business Machines Statistical Package for the Social Sciences Statistics 2 software was used with a $p$-value < 0.05 regarded as significant.

**RESULTS AND DISCUSSION**

**Chemical composition analysis**

The yield of BREO from fresh rhizomes was 4.5 ml per 1,000 g at a density of 0.863 g/ml. GC-MS revealed that BREO comprised 22 chemical components (Table 1). The major constituents were beta-ocimene (40.82%), geraniol (16.79%), and camphor (16.01%), which together accounted for more than 70% of the essential oil. These findings provide insights into the specific chemical compounds present in BREO and their potential role in its medicinal properties. A previous study [22] on the BREO also used GC-MS to analyze its chemical composition and identified nerol (39.6%) and L-camphor (36.0%) as the main constituents. While both essential oils contain high levels of camphor, there are notable differences in their chemical compositions, with BREO in this study having higher concentrations of beta-ocimene and geraniol.

**Antibacterial activity**

This study investigated the antibacterial activity of BREO against three oral pathogenic bacteria, *S. pyogenes, S. sobrinus,* and *S. mutans*. The agar disc diffusion assay revealed that the BREO had significant inhibitory effects on the growth of these bacteria (Table 2, Fig. 1). The MIC and MBC values for

![Image](image-url)

**Figure 1.** Inhibition zone of BREO against tested bacteria (1), tetracycline (2) and erythromycin (3) against *S. pyogenes* (A) *S. mutans* (B) and *S. sobrinus* (C).
BREO (Table 3, Fig. 2) were 6.25–12.5 mg/ml, indicating the bactericidal effects of the extract on all tested bacteria. These findings are consistent with those of a previous study [23], demonstrating the antimicrobial properties of *B. rotunda* extracts against *S. pyogenes* and other oral pathogens with suggested mechanisms of action associated with the cell wall and cell membrane damage [24]. In addition to its antibacterial effects, *B. rotunda* extracts have inhibitory effects on biofilm formation by oral bacteria and anti-periodontitis activity. Furthermore, *B. rotunda* extracts have anti-inflammatory properties, inhibiting the synthesis of pro-inflammatory cytokines and the expression of certain enzymes involved in inflammation [25].

**TLC-bioautography and GC-MS**

The TLC-bioautography analyses revealed that the crude extract of *B. rotunda* comprised a single antibacterial component with an Rf value of 0.28 against the tested bacteria (Fig. 4). This component was subsequently identified as geraniol.

<table>
<thead>
<tr>
<th>Sample</th>
<th>MIC/MBC (mg/ml)</th>
<th><em>Streptococcus pyogenes</em> DMST 30563</th>
<th><em>Streptococcus mutans</em> DMST 18777</th>
<th><em>Streptococcus sobrinus</em> DMST 35719</th>
</tr>
</thead>
<tbody>
<tr>
<td>BREO</td>
<td>6.25/6.25</td>
<td>6.25/6.25</td>
<td>12.5/12.5</td>
<td></td>
</tr>
<tr>
<td>Geraniol</td>
<td>6.25/6.25</td>
<td>6.25/6.25</td>
<td>6.25/6.25</td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
<td>&lt;0.06/0.06</td>
<td>&lt;0.06/0.06</td>
<td>&lt;0.06/0.06</td>
<td></td>
</tr>
<tr>
<td>Erythromycin</td>
<td>&lt;0.06/0.06</td>
<td>&lt;0.06/0.06</td>
<td>&lt;0.06/0.06</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 2.** MIC microtiter plate of BREO against tested bacteria [*S. pyogenes* (a), *S. mutans* (b), *S. sobrinus* (c)] Row A-C (BREO) Row D-F (geraniol) Well 12A - 12F (positive control) Well 12G (negative control).

**Figure 3.** GC-MS analysis of the BREO (A), components with antibacterial zone from TLC (B), and standard geraniol (C).
through GC-MS analysis of BREO separation on a TLC plate (Fig. 3A–C). Although geraniol was not the most concentrated component in the BREO, the TLC-bioautography results demonstrated its antibacterial activity against all tested bacterial strains. A previous study [10] showed that *B. rotunda* possessed promising bioactive compounds exhibiting antioxidant and antibacterial properties. However, the specific active components responsible for these properties have not yet been identified. Our study suggests that geraniol is a key active antibacterial component of BREO against *S. pyogenes*, *S. sobrinus*, and *S. mutans*. These findings further support the promise of BREO as a source of antimicrobial substances and highlight the importance of investigating its potential medicinal uses.

**Time-kill kinetics assay**

A time-kill kinetics assay of BREO and geraniol against the tested bacteria was performed to characterize their bactericidal action (Fig. 5A–F). The cell survival patterns and reduction in
cell numbers using varied concentrations of BREO, and geraniol were similar across the bacteria used in this study. BREO and geraniol displayed concentration-dependent kinetics. Higher concentrations resulted in rapid bacterial death, suggesting a dose-dependent action. The 1 × MIC of BREO lowered the CFU count against all examined bacteria by ≥99% of all tested bacteria in 1 minute, whereas geraniol was not considered bactericidal at this time.

Table 4 shows the percentage kill of bacteria over 60 minutes using 4 × MIC of the tested bacteria. BREO reduced ≥99% of all tested bacteria in 1 minute. Geraniol reduced ≥99% of S. pyogenes and S. mutans in 10 minutes and ≥99% of S. sobrinus in 5 minutes. BREO exhibited bactericidal activity at 1 minute and demonstrated a higher percentage kill of all tested bacteria than geraniol at 5 and 10 minutes. In a previous study, Geraniol increased the rate of potassium leaked from intact cells and increased the membrane fluidity of C. albicans as measured by fluorescence polarization [26]. The findings of our study demonstrate that BREO exhibits considerably greater efficacy in killing the tested bacteria than geraniol alone. The potential synergistic effects of other components in BREO must be further investigated as BREO contains several components that may contribute to its bactericidal activity.

CONCLUSION

The BREO has potent antibacterial activity against S. pyogenes, S. mutans, and S. sobrinus. A time-kill assay demonstrated the superior efficacy of BREO against these bacteria compared to geraniol at an equivalent MIC. As these bacteria are common pathogens associated with oral diseases, BREO has the potential to serve as a valuable source of antimicrobial agents for oral hygiene. Furthermore, the outcomes of this investigation suggest that BREO is more effective than geraniol alone, suggesting that further investigation of the synergistic effects of other components in BREO could be valuable.

AUTHORS’ CONTRIBUTION

N.W. and D.S. conceived and designed the project. K.B. and R.W. performed the experiments. N.W., D.S., and P.S. analyzed the results. N.W. drafted the manuscript. N.W., D.S., and P.S. revised and finalized the manuscript which was reviewed and approved by all authors.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVAL

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

This research article encompasses all the data that has been generated and analyzed, which is readily available within this document.

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