Bioactivity assessment of *Zingiber zerumbet* Linn rhizome extract for topical treatment of skin diseases

Napapat Rattanachitthawat¹, Pornsak Sriamornsak², Vivek Puri³, Inderbir Singh¹, Kampanart Huanbutta⁴, Tanikan Sangnim¹*

¹Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Burapha University, Chonburi, Thailand.
²Department of Pharmaceutical Technology, Faculty of Pharmacy, Silpakorn University, Nakhon Pathom, Thailand.
³Chitkara College of Pharmacy, Chitkara University, Patiala, India.
⁴Department of Pharmaceutical Industry, School of Pharmacy, Eastern Asia University, Rangsit, Thailand.

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**ABSTRACT**

Fungal and bacterial skin diseases are still major public health concerns in Thailand. Despite the accessibility of numerous antimicrobial medications, antibiotic resistance remains a problem. Furthermore, only a few new medicines have been developed, and their side effects are a cause for concern. Identifying and utilizing natural chemicals is an alternative resolution for these issues. The rhizome of *Zingiber zerumbet* (L.) is one of the herbs used as an antibiotic in Eastern Thailand. This research aimed to develop an appropriate extraction method for *Z. zerumbet* (L.) rhizome using different solvents. The extract's biomarkers and various pharmacological activities were also analyzed. Results showed that the ethanol extract had no effect on the growth of *Staphylococcus aureus*, *Staphylococcus epidermidis*, or *Candida albicans*. Meanwhile, the hexane and dichloromethane extracts had minimal bactericidal concentration (MBC) of 0.01562 and 0.03125 mg/ml, respectively, for *S. aureus*. Their MBC and minimal fungicidal concentration for *S. epidermidis* and *C. albicans* were 0.03125 and 0.0625 mg/ml, respectively. The ethanol extract outperformed the dichloromethane and hexane extracts in terms of antioxidant activity as measured by 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) and 2,2-diphenyl-1-picrylhydrazyl assays. Furthermore, the ethanol extract showed anti-inflammatory properties as determined by the protein denaturation test. Finally, the LC₅₀ values of ethanol, dichloromethane, and hexane extracts were 122.78, 220.76, and 67.96 g/ml, respectively, according to the cytotoxicity assay. These findings suggest that the extract from *Z. zerumbet* (L.) rhizome could be employed to develop an antimicrobial medicine in the future.

**INTRODUCTION**

Skin, hair, and nail fungal infections are a widespread public health concern worldwide but are seldom addressed according to a population-based survey (dos Santos et al., 2010). Cutaneous fungal infections are projected to be prevalent in 20%–25% of the world's population (Male, 1990). Dermatophytes, including *Trichophyton*, *Microsporum*, and *Epidermophyton*, could infect the stratum corneum and keratinized tissues and are the major culprits of skin fungal infections (Kwon-Chung and Bennett, 1992). Even though fungal infection epidemiology has become a remarkable global concern, issues regarding access to antifungal drugs and their variable cost are continuously faced by the world (Kneale et al., 2016). Several antifungal compounds have been recently discovered and developed. In particular, those from plants exhibit low toxicities and contain abundant bioactive secondary metabolites, such as tannins, terpenoids, saponins, alkaloids, and flavonoids (Arif et al., 2009). Numerous plants, including *Inga dulcis*, *Schinus terebinthifolius* Raddi, *Alternanthera brasiliiana* Kuntze, *Piper regnellii*, *Herissantia crispa*, *Rubus ulmifolius*, *Rumex acetosa*, and *Baccharis dracunculifolia*, have antifungal properties (Johann*
et al., 2007). However, the antifungal activity of Zingiber zerumbet (L.) rhizome extract in humans has rarely been documented. Zingiber zerumbet is a ginger species that has leafy stems and reaches a height of 1.2 m (Roskov et al., 2014). Although it originated in Asia, this plant can now be found in various tropical nations. Zingiber zerumbet is also known as awapuhi, bitter ginger, shampoo ginger, and pinecone ginger (Akbar, 2020). Its rhizomes have been used as a flavoring agent and appetizer in various cuisines, and its rhizome extracts have been employed in traditional herbal medicine for anti-inflammatory properties, pain relief, and treatment of worm infestation and diarrhea (Yob et al., 2011). Zingiber zerumbet rhizome extract has been recently reported as an antifungal agent in plants. The primary constituent of edible wild ginger rhizome, Z. zerumbet (L.), is zerumbone, which exhibits various pharmacological properties, including antitumor activity, anti-inflammatory, antioxidant, antibacterial, antinociceptive, hepatoprotective, and immunomodulatory ones (Kitayama et al., 2003). Thus, this investigation examined the novel antifungal activity of Z. zerumbet rhizome extract.

This study determined the feasibility of developing a new antifungal agent from Z. zerumbet rhizome extract. Three solvents (hexane, dichloromethane, and ethanol) were used to extract the rhizome. The obtained extracts were discovered to have antifungal, antibacterial, antioxidant, and anti-inflammatory properties. Finally, the cytotoxicity of the extracts was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test.

MATERIALS AND METHODS

Chemicals and materials

Ethanol (Batch No. 20060068), dichloromethane (Batch No. 21080094), and hexane (Batch No. 21070007) were purchased from RCI Labscan, Thailand. Standard gallic acid (Batch No. A0422721) and zerumbone (Batch No. S00321110) were obtained from Acras Organics and ChemFaces (China), respectively, and 2,2' azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Batch No. Z10G016) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Batch No. Q16G021) were purchased from Alfa Aesar, UK. All other chemicals and reagents were of analytical grade.

Extraction of Z. zerumbet (L.) rhizome

Fresh rhizomes of Z. zerumbet (L.) were collected in February 2022 from Chanthaburi Province. The rhizomes were cleaned by washing with tap water, and a portion was dried in a hot air oven at 50°C for 48 hours. The dried rhizomes were ground using a cutting mill (SM100, Retsch GmbH, Haan, Germany) until they can pass through a 0.5 mm sieve, kept in sealed containers, and protected from light until further use. The specimens were identified by Dr. Boonyadist Vongsak, Faculty of Pharmaceutical Sciences, Burapha University, Thailand. The voucher specimens (KM No. 0415001, KM No. 0415002, and KM No. 0415003) were deposited at the Faculty of Pharmaceutical Sciences, Burapha University, Thailand. For the extraction, the Z. zerumbet (L.) rhizomes were soaked in hexane, dichloromethane, and ethanol as presented in Figure 1.

Biomarker monitoring by high-performance liquid chromatography (HPLC)

Zerumbone, the major active compound of Z. zerumbet rhizomes, was selected as the biomarker from the extract (Kitayama et al., 2003). This compound was monitored and quantified using HPLC (DGU-20A5R, Shimadzu, Kyoto, Japan) following the validated method of Rahman (Rahman et al., 2013). Stainless steel with 5 µm particle size (4.6 mm internal diameter × 250 mm length) analytical symmetry column (Merek, Darmstadt, Germany) packed with a dimethyl octysilane- (C18-) bonded amorphous silica stationary phase was used. The mobile phase was composed of a binary mixture of HPLC-grade acetoniitrile and purified water at a gradient ratio of 65%:35%−75%:25% (v/v) that was blended within 26 minutes, freshly prepared for each run, and degassed before use. The injection volume was 10 µl, and the flow rate was 1 ml per minute. The samples were examined by ultraviolet (UV) detection at a wavelength of 250 nm. For the standard curve, the reference standard was injected and determined at five concentration levels.

Antibacterial activity test

Minimal inhibitory concentration (MIC) of Z. zerumbet rhizome extract

The MIC of Z. zerumbet rhizome extract was calculated through macrobroth dilution to determine its appropriate loading dose for topical formulation. Staphylococcus aureus and Staphylococcus epidermidis were transferred to Mueller–Hinton broth (MHB) and incubated at 37°C for 3–4 hours. The extracts of Z. zerumbet rhizomes were diluted to concentrations of 1,000, 500, 250, 125, 62.5, 31.25, 15.6, 7.8, and 3.9 µg/ml in test tubes. Afterward, 5 ml of MHB was added to each test tube containing the extract. The extracts of Z. zerumbet rhizomes and microbe mixes were then incubated at 37°C for 20–24 hours. Microbe growth was monitored by determining the turbidity of the mixture in the test tubes following their incubation. The lowest concentration of Z. zerumbet rhizome extract that can inhibit microorganism growth was defined as the MIC (Wiegand et al., 2008).

Minimal bactericidal concentration (MBC) of Z. zerumbet rhizome extract

The MBC of Z. zerumbet rhizome extract was determined by streaking the turbid mixture from the MIC test onto the surface of Mueller–Hinton agar (MHA) and incubating it at 37°C for 20–24 hours. The lowest concentration of Z. zerumbet rhizome extract that can inhibit the formation of microbial colonies on the MHA plate was defined as the MBC.

Antifungal activity test

MIC of Z. zerumbet rhizome extract

The MIC of Z. zerumbet rhizome extract was determined through broth dilution. Candida albicans was incubated in Sabouraud dextrose broth at 37°C for 20–24 hours and then diluted to a turbidity of 0.5 McFarland at 1 × 10⁶ CFU/ml C. albicans. The specimens were then incubated in Z. zerumbet rhizome extracts at different concentrations (1,000, 500, 250, 125, 62.5, 31.25, 15.6,
7.8, 3.9, and 1.9 µg/ml) at 37°C for 20–24 hours. Microbe growth was monitored by determining the turbidity of the mixture in the test tubes following their incubation. The lowest concentration of Z. zerumbet rhizome extract that can inhibit microorganism growth was defined as the MIC (Anyanwu, 2012).

**Minimal fungicidal concentration (MFC) of Z. zerumbet rhizome extract**

The MFC of Z. zerumbet rhizome extract was determined by streaking the turbid mixture from the MIC test onto the surface of Sabouraud dextrose agar (SDA), which was then incubated at 37°C for 20–24 hours. The lowest concentration of Z. zerumbet rhizome extract that can inhibit the formation of fungal colonies on the SDA plate was defined as the MFC (Anyanwu, 2012).

**Antioxidant activity test**

The antioxidant activity of Z. zerumbet rhizome extract was determined by a radical-scavenging assay using 2,2'-azino-bis-ABTS and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals. Zingiber zerumbet rhizome extract was prepared at concentrations of 0.3125–1 mg/ml. The antioxidant activity of Z. zerumbet rhizome extract was then benchmarked with the gallic acid standard in the concentration range of 2.5–0.1953 µg/ml.

**ABTS assay**

ABTS radical-scavenging assay is a widely used method for determining the antioxidant activity of hydrophilic and lipophilic compounds. This technique was adopted in this study following the modified version by Arumugam et al. (2014). In brief, 7 mM ABTS solution was prepared and kept in the dark at room temperature (25°C) for 18 hours. Different concentrations of Z. zerumbet rhizome extracts (0.3125–1 mg/ml) were mixed with 160 µl of 7 mM ABTS solution in 96-well microplates for testing. The final absorbance (Abs) was determined at 734 nm. ABTS radical-scavenging activity was calculated using the following equation:

\[
\text{ABTS radical-scavenging activity} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100
\]  

where Abs control is the Abs of ABTS radical and methanol and Abs sample is the Abs of ABTS radical and Z. zerumbet rhizome extracts. All experiments were performed in triplicate.

**DPPH assay**

DPPH free radical-scavenging assay is a simple method for determining the antioxidant activity of extracts in a short period of time. This technique is also used to measure the antiradical activity of an antioxidant by measuring the decrease in DPPH Abs at 517 nm. This decrease appears as a change in color from purple to yellow due to hydrogen being donated to form a stable DPPH-H molecule. This study used the modified version of the DPPH free radical-scavenging assay by Omokhua-Uyi et al. (2020). Six different concentrations (0.5, 0.25, 0.125, 0.625, and 0.3125 mg/ml) of the extract were evaluated. The DPPH solution was prepared at a concentration of 60 µM and then added with 40 µl of the ethanol extract. The mixture was vigorously shaken and allowed to stand in the dark for 45 minutes. A spectrophotometer was used to determine the decrease in Abs at 517 nm relative to a blank sample (ethanol). The DPPH radical-scavenging activity was calculated using a calibration curve that was obtained with various extract concentrations and described in the following equation:

\[
\text{DPPH radical-scavenging activity} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100
\]

where Abs control is the Abs of DPPH radical and methanol and Abs sample is the Abs of DPPH radical and Z. zerumbet rhizome extracts. All experiments were replicated three times.

**Anti-inflammatory activity test**

Antiprotein denaturation assays were used to determine the anti-inflammatory activity of Z. zerumbet rhizome extracts. In brief, 0.2% bovine serum albumin (BSA) was dissolved in Tris-HCl buffer (pH 6.8). Zingiber zerumbet rhizome extracts were then added and mixed with the BSA solution, and the mixture was heated to 75°C for 10 minutes and allowed to cool for 20 minutes. The turbidity of the sample was then determined using a UV/Vis spectrometer set to 660 nm. The inhibition of protein denaturation (BSA) was calculated as a percentage using the following equation:

\[
\text{Inhibition percent} = \frac{\text{Abs control} - \text{Abs treated}}{\text{Abs control}} \times 100
\]

where Abs control is the Abs of the sample before heat and Abs treated is the Abs of the sample after heat treatment. Diclofenac sodium was applied as a positive control. All experiments were performed in triplicate.

**Cytotoxicity test**

Cell viability was assayed using the colorimetric method with. Medical research council cell strain 5 (MRC-5) MRC-5 cells were seeded at a density of 10,000 cells per well in a 96-well plate and incubated at 37°C in a 5% CO₂ atmosphere for 24 hours. The cells were then treated in triplicate with 15.625, 31.25, 62.5, 125, 250, 500, and 1,000 µg/ml Z. zerumbet rhizome extract for 24 hours. Afterward, 10 µl of 5 mg/ml MTT in phosphate buffered saline solution was added directly to each well, followed by incubation at 37°C for 3 hours. After the cultured medium was aspirated, 100 µl aliquot of absolute dimethyl sulfoxide was added. Abs (optical
density (OD]) values were determined at a wavelength of 570 nm using a microplate reader (Metertech, Taiwan). The cells treated with various concentrations of *Z. zerumbet* rhizome extract were photographed to monitor their viability. The following equation was used to determine the percentage of viable cells:

\[
\% \text{ Cell viability} = \frac{OD \text{ sample} - OD \text{ blank}}{OD \text{ control} - OD \text{ blank}} \times 100.
\]

### Statistical analysis

Data were statistically analyzed using one-way ANOVA, followed by post hoc Tukey’s honestly significant difference test for the data from three independent experiments.

### RESULTS AND DISCUSSION

#### Extraction yield and appearance

The yield percentage and physical appearance of *Z. zerumbet* rhizome crude extracts obtained using various solvents are shown in Table 1. The yield from hexane extraction was markedly higher than that from dichloromethane and ethanol extractions. This phenomenon occurred because *Z. zerumbet* rhizome crude extract contains a high proportion of sesquiterpenes (15-carbon skeletons), a low-polar chemical group, which caused the high extraction efficiency from hexane, a low-polar solvent (Koga et al., 2016). The viscosity and thickness of the extracts were recorded with (+) symbols, where (+) signifies low viscosity and (++++) signifies extremely thick and viscous. According to Table 1, the viscosity and thickness of the ethanol extract were noticeably higher than those of dichloromethane and hexane. -Compared with the dichloromethane and ethanol extracts, the hexane extract showed a liquid appearance.

#### Biomarker monitoring by HPLC

Zerumbone was chosen as the biomarker for extraction monitoring because it is the predominant active component of *Z. zerumbet* rhizome (Kitayama et al., 2003). The retention time of standard zerumbone at concentrations ranging from 10 µg/ml to 500 µg/ml was around 11 minutes, which is consistent with a previous finding (Akhtar et al., 2019). As shown in Table 1, the hexane extract contained a higher zerumbone concentration than the dichloromethane and ethanol extracts. This result is consistent with the findings on extraction yield: zerumbone has a low polarity and hence dissolves more readily in hexane than in the other solvents (Chia et al., 2021).

#### Antibacterial activity test

The MIC of *Z. zerumbet* rhizome extracts obtained using various solvents was determined using ABTS and DPPH assays as illustrated in Figure 2a and b, respectively. The IC₅₀ (concentration of antioxidant required to reduce the initial ABTS or DPPH concentration by 50%) of each extract was determined using the graph of radical-scavenging activity as shown in Table 3. Among the extracts, the ethanol extract had the strongest antioxidant activity (IC₅₀ values of 28.52 ± 5.03 and 221.03 ± 6.74 g/ml for ABTS and DPPH, respectively) possibly due to its high concentration of flavonoids, which act as antioxidants by scavenging free radicals in an organism (Saija et al., 1995). The

### Table 1. Extraction yield percentage, viscosity, thickness, and zerumbone concentration of *Z. zerumbet* rhizome extracts obtained using hexane, dichloromethane, and ethanol.

<table>
<thead>
<tr>
<th>Type of solvent</th>
<th>Extraction yield (%)</th>
<th>Viscosity and thickness</th>
<th>Zerumbone concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>3.81</td>
<td>+++</td>
<td>410.05 ± 0.13</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>2.46</td>
<td>++++</td>
<td>276.06 ± 0.23</td>
</tr>
<tr>
<td>Ethanol</td>
<td>2.14</td>
<td>++</td>
<td>45.23 ± 14.10</td>
</tr>
</tbody>
</table>
antioxidant activity of the dichloromethane and hexane extracts was lower than that of the ethanol extract. Sambou et al. (2020) successfully extracted phenolic compounds, the phytochemicals responsible for the majority of the antioxidant activity in plants, using various solvents in the following order: methanol, ethanol, dichloromethane, and hexane (Sambou et al., 2020).

**Anti-inflammatory activity test**

The anti-inflammatory efficacy of *Z. zerumbet* rhizome extracts was determined using antiprotein denaturation assays as presented in Figure 3. The extracts were compared with diclofenac sodium (a positive control), a nonsteroidal anti-inflammatory drug. The ethanol extract showed a greater anti-inflammatory effect than the other extracts. This finding is consistent with the antioxidant activity result, which stated that the ethanol extract had a high concentration of flavonoids. Flavonoids could protect against tissue damage or fibrosis. Multiple investigations *in vitro* and in animal models also revealed that flavonoids may suppress the initiation and progression of inflammatory conditions (Costamagna et al., 2016; Maleki et al., 2019).

**Cytotoxicity test**

The cell viability after treatment with *Z. zerumbet* rhizome extracts is presented in Figure 4. All three extracts (ethanol, dichloromethane, and hexane) showed similar viability. The cell viability percentage was higher than 50% when the extract concentration was lower than 15.625 µg/ml. This result was confirmed and corroborated by the microscope images of MRC-5 cells in Figure 5. When the extract (ethanol, dichloromethane, and hexane) concentration was greater than 31.25 µg/ml, the cells appeared to lyse and lose their density. The LC₅₀ (concentration of extract that kills half of the tested cells in culture) of all extracts was determined using the cell viability trendline, and the values for the ethanol, dichloromethane, and hexane extracts were 122.78, 220.76, and 67.96 µg/ml, respectively. These findings suggested that the ethanol and dichloromethane extracts are moderately hazardous (LC₅₀ values of 100–500 µg/ml) according to Clarkson’s toxicity criterion (Clarkson et al., 2004).

**CONCLUSION**

This study demonstrated that *Z. zerumbet* rhizome extract possessed antibacterial and antifungal properties. In particular, the hexane and dichloromethane extracts were successful in inhibiting the growth of *S. epidermidis*, *S. aureus*, and *C. albicans*. HPLC was used to monitor and measure zerumbone, which was selected as a biomarker for *Z. zerumbet* rhizome extracts. Compared with the hexane and dichloromethane extracts, the ethanol extract exhibited greater antioxidant and anti-inflammatory activities. When added to the cell culture, the ethanol and dichloromethane extracts presented a moderate level of toxicity. In summary, the combination extracts of *Z. zerumbet* rhizome (ethanol, dichloromethane, and hexane) show a potential to be developed as oral or topical, antifungal, and antibacterial agents.

**Table 2.** MIC, MBC, and MFC of *Z. zerumbet* rhizome extracts against *S. epidermidis*, *S. aureus*, and *C. albicans*.

<table>
<thead>
<tr>
<th></th>
<th>Ethanol extract (mg/ml)</th>
<th></th>
<th>Dichloromethane extract (mg/ml)</th>
<th></th>
<th>Hexane extract (mg/ml)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MBC</td>
<td>MIC</td>
<td>MBC</td>
<td>MIC</td>
<td>MBC</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>N/A</td>
<td>N/A</td>
<td>0.03125</td>
<td>0.03125</td>
<td>0.03125</td>
<td>0.03125</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>N/A</td>
<td>N/A</td>
<td>0.03125</td>
<td>0.03125</td>
<td>0.01562</td>
<td>0.01562</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>N/A</td>
<td>N/A*</td>
<td>0.0625</td>
<td>0.0625*</td>
<td>0.03125</td>
<td>0.03125*</td>
</tr>
</tbody>
</table>

*MFC*
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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

CONFLICTS OF INTEREST

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

ETHICAL APPROVALS

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

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

<p>| Table 3. IC_{50} values (µg/ml) of Z. zerumbet rhizome extracts determined by ABTS and DPPH assays. |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|</p>
<table>
<thead>
<tr>
<th>IC_{50} (µg/ml)</th>
<th>Ethanol</th>
<th>Dichloromethane</th>
<th>Hexane</th>
<th>Gallic acid (positive control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABTS</td>
<td>28.52 ± 5.03</td>
<td>84.87 ± 7.52</td>
<td>507.96 ± 69.54</td>
<td>6.15 ± 0.08</td>
</tr>
<tr>
<td>DPPH</td>
<td>221.03 ± 6.74</td>
<td>314.37 ± 9.71</td>
<td>1,162.97 ± 148.94</td>
<td>9.64 ± 0.34</td>
</tr>
</tbody>
</table>

Figure 3. Anti-inflammatory activity of Z. zerumbet rhizome extracts tested by antiprotein denaturation assays.

Figure 4. Cell viability after treatment with Z. zerumbet rhizome extracts obtained using various solvents.

Figure 5. Microscope images of MRC-5 cells after treatment with different concentrations of Z. zerumbet rhizome extracts obtained using various solvents.
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