RESEARCH ARTICLE

# Evaluations of Antibacterial Properties of *Zingiber purpureum* Essential Oil Against 13 Different Gram-positive and Gram-negative Bacteria

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## Abstract

**BACKGROUND:** Indonesia's tropical forest is home to around 80% of the world's medicinal plants. One of these is *Zingiber purpureum*, which have traditionally been used to treat joint discomfort, the common cold, and jaundice. The rhizomes of this plant have been suggested to possess antibacterial action in the treatment of infections. In this study, *Z. purpureum* was screened for antibacterial activity against 13 bacteria (Gram-positive and Gram-negative).

**METHODS:** *Z. purpureum* rhizomes were obtained and the distillated extracts were made to generate essential oil. The minimum inhibitory concentration (MIC) and Kirby Bauer disk diffusion methods were used to determine the antibacterial activity.

**RESULTS:** All bacteria activity were inhibited by the essential oil of *Z. purpureum* at concentrations ranging from 2.5 vol% to 10 vol%. However, several bacterias (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Enterobacter cloacae*) were inhibited at the lowest concentration (0.63 vol %), with the inhibition zones ranging from 6.7 mm to 8.0 mm. Meanwhile, the widest inhibition zone (13.3 mm) was reported on *E. cloacae* at 10 vol% concentration.

**CONCLUSION:** A 10 vol% *Z. purpureum* rhizome extract inhibits Gram-positive and Gram-negative bacteria, particularly those that are resistant to a variety of antibiotics.

**KEYWORDS:** *Zingiber purpureum*, antibacterial agents, susceptibility test, infection

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## Introduction

Indonesia is a megadiverse country teeming with diverse plant species. Numerous plant species in Indonesia are utilized medicinally or in traditional herbs, for instance, *Zingirberaceae*, found in Indonesia and other countries. (1) Numerous *Zingerbeacea* species exist, one of which is *Zingiber purpureum* (Rosc), with some other synonyms as *Zingiber cassumunar* (Roxb), *Zingiber cliffordiae* (Andrews), *Zingiber luridum* (Salisb), and *Zingiber xantorrhizon* (Steud) which can be found throughout Southeast Asia, most notably in East Kalimantan, Indonesia. The Dayak ethnic group has long used this herb to relieve joint pain, the common cold, and jaundice. The extracts from stems, leaves, flowers, and rhizomes are used to cure conditions.(2,3)

Previously published research on the rhizomes isolated a variety of phenylbutenoids, curcuminoids, and terpenoids with anti-inflammatory, analgesic, ovicidal,

insecticidal, and enzyme inhibitory properties. Additionally, the rhizomes of these plants had considerable antibacterial action.(4) Zingiberaceae family was found to be reactive with *Bacillus subtilis* (ATCC6633), *Enterococcus faecalis* (ATCC2921), *Escherichia coli* (ATCC25922), *Klebsiella pneumoniae* (TISTR1843), *Pseudomonas aeruginosa* (ATCC741), *Staphylococcus aureus* (ATCC25923), *Salmonella typhi*.(5)

Meanwhile, the oil plant's have been revealed as antimicrobial activity against *Escherichia coli*, *Propionibacterium acnes*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Candida albicans*, *Cryptococcus neoformans*, *Epidermophyton occosum*, *Microsporum gypseum*.(6) The oil contained a minimum bactericidal concentration (MBCs) of 0.62-2.5%. However, the oil was tested for antibacterial activity as well as yeast and dermatophytes, and it was discovered that dermatophytes were the most responsive, followed by yeast and bacteria.(7,8)

Numerous herbs, spices, and plants have been reported to be potential sources of antibacterial, especially against multi drug resistant bacteria.(9) However, few have been studied concerning levels and range of activity. *Hence, the aim of this study wants to screen the antibacte*rial activity of *Z. purpureum* against 13 kinds of bacteria, including Gram-positive and gram-negative, using minimum inhibitory concentration (MICs) and Kirby Bauer disk diffusion method because both are considered the gold standard for determining the antimicrobial susceptibility.

## Methods

### Collection and Identification of *Z. purpureum*

The rhizome of *Z. purpureum* was collected from Muara Badak village, Kutai Kertanegara, East Kalimantan, Indonesia, and then identification by Laboratory of Dendrology and Forest Ecology, Faculty of Forestry, Universitas Mulawarman, Indonesia (Identification Letter No. 123/UN17.4.08/LL/2022).

### Preparation and Distillation of *Z. purpureum*

The fresh rhizomes of *Z. purpureum* were washed, removed from the outer skin, and then chopped. About 2 kg flesh of rhizome was put in the distillation apparatus. The distillation process was done in 6 hours under 100°C. The essential oil obtained from this process was 1% and then the oil was placed in a bottle and stored at room temperature until used for the experiment.(3)

### Bacteria Strain Preparation

All process of antimicrobial susceptibility testing was done at Microbiology Laboratory of Faculty of Medicine, Universitas Mulawarman which referred to CLSI M-100-S25.(4,10) The testing process required bacteria to be isolated in pure culture and identified to the genus and species level. Various organisms were accessible from the laboratory of the Abdul Wahab Sjahranie Hospital, Samarinda, Indonesia, under the approval of Health Research Ethics Committee, Faculty of Medicine, Universitas Mulawarman (Ethical Clearance Letter No. 71/KEPK-FK/XII/2020) and from The American Type Culture Collection (ATCC). In this study, the *Z. purpureum* was used against both bacterial Gram-positive (Methicillin-resistant *S. aureus* ATCC 33591, *S. aureus* ATCC 25923, *S. epidermidis* ATCC 14990, *Staphylococcus pyogenes* ATCC 19615, *Streptococcus mutans* ATCC 35668) and Gram-negative bacteria (*E. coli* 35218, *S. typhi* ATCC 14028, *Campylobacter jejuni* ATCC 33291, *P. aeruginosa* ATCC 15442, *Shigella sonnei* ATCC 25932, *E. cloacae* ATCC 13047, locally *E. coli*, *Porphyromonas gingivalis* ATCC 33277).

### Inoculum

It was important to standardize and find the optimum bacterial cell number employed in susceptibility testing to produce reliable and consistent results. A 0.5 McFarland standard was prepared to obtain final inoculum size. The recommended final inoculum size for broth dilution experiments was $1x10^8$ colony-forming units (CFU)/mL.(10)

### Evaluation of MICs

The extract of *Z. purpureum* on the 96-well microplates was incubated at 37°C for 24 hours. The MICs of plant extracts were defined as the lowest maximum dilution concentration at which no measurable bacterial growth occurred after 24 hours in microdilution wells. These experiments were repeated three times. This technique was used to determine the minimal inhibitory concentration.(11)

### Kirby Bauer Disk Diffusion Method

Kirby Bauer Method with disk diffusion method was conducted as described as follows. Ten microliters of the extracts dissolved in ethanol were added to sterile filter paper discs (Whatman No.1). The discs were dried at 70°C overnight. The plates of Mueller-Hinton agar were applied with a 200 µL culture of bacteria. The discs contained extracts seeded on those plates. Five µg ciprofloxacin and 30 µg oxacillin were used as positive controls, while 0.4

µg DMSO was used as a negative control. The plates were then incubated at 37°C for 18-24 hours. The experiments performed in duplicate and the means of the diameters of the inhibition zones were calculated.(12)

**Statistical Analysis**

Antibacterial activity was described as the mean±standard error median (SEM) and statistical analysis was carried out by Kruskal Wallis test at a confidence level of 95 % (α level of 0.05) using SPPS ver. 23 (IBM Corporation, Armonk, NY, USA). The Mann Whitney multiple range test was also used for each subgroup.

## Results

The potential of *Z. purpureum* rhizomes to inhibit Gram-positive and Gram-negative bacteria was revealed in Table 1, with extract concentrations ranging from 0.32 to 10 vol%. Result of Mann-Whitney test following Kruskal Wallis showed that *Z. purpureum* significantly inhibited both Gram-positive and Gram-negative bacteria with a $p$=0.000. Some of the lowest MIC range was reportedly found in Gram-negative bacterias (*E. cloacae*, *E. coli*, *C. jejuni*, and *P. aeruginosa*).

**Table 1. MIC range of *Z. purpureum* against Gram-positive and Gram-negative bacteria.**

| Bacteria | MIC Range (vol %) |
|---|---|
| Gram-Positive | |
| *MRSA* ATCC 33591 | 2.08±0.42 |
| *S. aureus* ATCC 25923 | 1.25±0.00 |
| *S. pyogenes* ATCC 19615 | 5.00±0.00 |
| *S. epidermidis* ATCC 14990 | 5.00±0.00 |
| *S. mutans* ATCC 35668 | 4.17±0.83 |
| Gram-Negative | |
| *E. coli* 35128 | 1.04±0.21 |
| *C. jejuni* ATCC 33291 | 1.04±0.21 |
| *S.typhi* ATCC 14028 | 2.08±0.42 |
| *P. aeruginosa* ATCC 15442 | 1.04±0.21 |
| *S. sonnei* ATCC 25923 | 2.50±0.00 |
| *E. cloacae* ATCC 13047 | 0.63±0.00 |
| Locally *E. coli* | 3.33±0.83 |
| *P.gingivalis* ATCC 33277 | 2.50±0.00 |

Values represent in n=3 experiments. Data was expressed as mean±SEM.

The extract was shown to be able to inhibited bacteria with inhibition zones ranging from 13.3 mm to 6.7 mm (Table 2). Only *P. aeruginosa* inhibited bacteria at the lowest dose (0.32 vol%). Whila, at a 10 vol% concentration of extract *Z. purpureum* rhizomes, the highest zone inhibition was seen against *E. cloacae* (13.3±0.3 mm), followed by *P. aeruginosa* (13.0±0.6 mm), *S. aureus* (12.7±0.7 mm), and *C. jejuni* (12.7±0 mm). The minimum diameter of the zone of growth inhibition against *MRSA* (8±0 mm) and *S. pyogenes* (8±0 mm) was then determined. Additionally, only *E. coli*, *P. aeruginosa*, and *E. cloacae* preserved zone inhibition at a concentration of 0.63 vol%.

According to Table 2, bacteria were inhibited at the concentration ranging from 0.63 to 5 vol%. *E. cloacae* (0.63 vol%) had the lowest MIC on the *Z. purpureum* at 10 vol%, followed by *S. pyogenes* (5 vol%), *S. epidermidis* (5 vol%), and *S. mutans* (5 vol%). Other bacteria have a MIC of between 1.25 and 2.5 vol%. Only *C. perfringens* was unable to be inhibited by 10 vol% of *Z. purpureum*. We estimated that there was a significant different in the resistance of Gram-positive and Gram-negative bacteria to *Z. purpureum* rhizomes extract with $p$<0.001 (Table 3).

## Discussion

The ancient and traditional use of plants as medicine, the emergence of multidrug-resistant pathogens, and the increasing use of essential oils make the study of their antibacterial activity timely and relevant. Some plants, that have long been used in traditional medicine for various illness, were already proven to have antibacterial properties in some research.(13-18) *Z. purpureum* or *Z. cassumunar*, which has traditionally been used to treat joint discomfort, the common cold, and jaundice, might have an antibacterial properties both for Gram-positive and Gram-negative bacterias.

Based on our results, we found that both Gram-positive and Gram-negative bacterias are affected and inhibition zone is reported from all bacterias. The lowest MIC scores are reported from four Gram-negative bacterias (*E. cloacae*, *E. coli*, *P. aeruginosa*, *C. jejuni*), indicating that from this study, *Z. purpureum* might has a stronger antibacterial activity against Gram-negative bacteria as compared to Gram-positive bacteria, especially in the lowest concentration (0.63 vol%). All these bacteria are always present in severe or nosocomial infections, and contribute to immunocompromised patients. However, further research and analysis is needed.

**Table 2. Antibacterial zone of inhibition of *Z. purpureum* against Gram-positive and Gram-negative bacteria.**

| Bacteria | Zone of Inhibition in Diameter (mm) | | | | |
|---|---|---|---|---|---|
| | 0.63 vol% Concentration | 1.25 vol% Concentration | 2.5 vol% Concentration | 5 vol% Concentration | 10 vol% Concentration |
| Gram-Positive | | | | | |
| *MRSA* ATCC 33591 | 0.0±0.0 | 8.0±0.0 | 8.0±0.0 | 9.0±0.0 | 8.0±0.0 |
| *S.aureus* ATCC 25923 | 0.0±0.0 | 10.7±0.3 | 11.0±0.0 | 12.3±0.3 | 12.7±0.7 |
| *S.pyogenes* ATCC 19615 | 0.0±0.0 | 0.0±0.0 | 6.0±0.0 | 7.3±0.3 | 8.0±0.0 |
| *S. epidermidis* ATCC 14990 | 0.0±0.0 | 0.0±0.0 | 6.0±0.0 | 8.0±0.0 | 8.3±0.3 |
| *S. mutans* ATCC 35668 | 0.0±0.0 | 6.0±0.0 | 8.0±0.0 | 8.7±0.3 | 9.3±0.3 |
| Gram-Negative | | | | | |
| *E. coli* ATCC 35128 | 8.0±0.0 | 8.0±0.0 | 9.0±0.0 | 10.0±0.7 | 11.0±0.6 |
| *C. jejuni* ATCC 33291 | 0.0±0.0 | 9.3±0.3 | 10.3±0.3 | 12.0±0.0 | 12.7±0.3 |
| *P. aeruginosa* ATCC 15442 | 7.0±0.0 | 9.7±0.7 | 12.0±0.0 | 12.3±0.9 | 13.0±0.6 |
| *S. sonnei* ATCC 25923 | 0.0±0.0 | 0.0±0.0 | 8.0±0.6 | 8.3±0.3 | 10.0±0.0 |
| *E. cloacae ATCC 13047* | 6.7±0.3 | 8.3±0.3 | 11.3±0.3 | 13.0±0.6 | 13.3±0.3 |
| Locally *E. coli* | 0.0±0.0 | 0.0±0.0 | 6.7±0.3 | 8.0±0.0 | 9.3±0.3 |

Values represent in n=3 experiments. Data was expressed as mean±SEM.

*Z.purpureum* contains phenylbutenoids, curcuminoids, sesquiterpenoids, benzaldehydes, and quinones, as well as essential oils containing monoterpenoids, neocassumunarin A, neocassumunarin B, 6-gingerol, and 12-gingerol.(19,20) Due to its phytochemical composition, *Z. purpureum rhizomes* extract possesses antibacterial, antifungal, antiviral, and antioxidant properties. Antibacterial activity was demonstrated for the phytochemical compounds against *P. aeruginosa*, *S. aureus*, *A. baumannii*, *E. coli*, *B. subtilis*, and *S. typhi.* Additionally, 6- and 12-gingerol showned antibacterial action against periodontal microorganisms. (21-23)

According to previous studies, *S. aureus*, *S. epidermidis*, and *S. mutans* were more reactive to *Z. purpureum* oil than other bacteria, owing to the fact that *Z.== purpureum* oil contained 32 vol% terpinene-4-ol as the primary active compound and demonstrated activity against a broad range of Gram-positive bacteria.(24,25) Gram-negative organisms are marginally less sensitive to oil-related contamination than Gram-positive bacteria due to the presence of hydrophilic lipopolysaccharides in their outer membrane that functions as a barrier to the hydrophobic compounds found in essential oils.(21-23) That result is contrary to our study which implies that the hydrophobic components of essential oils enable them to enter the periplasm of Gram-negative bacteria via porin proteins in the outer membrane. Bacterias which reported to have the lowest MIC scores are Gram-negative bacterias except *S. aureus* (Table 1). This might be due to the structure of the *S. aureus* cell wall, which allows hydrophobic molecules to flow through easily.(26-28)

**Table 3. Difference of Gram-positive and Gram-negative resistance against *Z. purpureum*.**

| Concentration | *p*-value |
|---|---|
| 0.63 vol% | 0.000 |
| 1.25 vol% | 0.001 |
| 2.5 vol% | 0.001 |
| 5 vol% | 0.001 |
| 10 vol% | 0.001 |

Significant if $p<0.001$.

Gram-negative bacteria have a more complex cell wall. It possesses a 2-3 nm thick peptidoglycan layer, which is thinner than the cell wall of Gram-positive bacteria and accounts for around 20% of the cell's dry weight. Outside of the thin peptidoglycan layer is an outer membrane. (27,29) Braun's lipoprotein forms a strong bond between the peptidoglycan and the outer membrane; this protein is covalently attached to the peptidoglycan and entrenched in the outer membrane. One of the characteristics that distinguish Gram-negative bacteria from Gram-positive bacteria is the existence of an outer membrane. It is made up of two layers of phospholipids connected by lipopolysaccharides (LPS) to the inner membrane. (30,31) The peptidoglycan layer is surrounded by an outer membrane composed of various proteins and LPS. This

LPS is composed of two polysaccharides: lipid A, the core polysaccharide, and the O-side chain, which provides the quid that enables Gram-negative bacteria to be more resistant to essential oils penetrating the cell wall and acting on both the cell wall and the cytoplasm.(32,33)

As a result, we propose that there was a change in the surface of Gram-negative bacteria's cell wall and that the mechanisms were dependent on chemical components found in the plant's essential oil. Furthermore, Table 2 indicated that the minimum inhibitory concentration of *E. cloacae* was 0.63 vol%. It was established that *E. cloacae* is more susceptible to *Z. purpureum* than other species, and a positive control of 5 µg ciprofloxacin was used. This finding may provide a prospective option for the treatment of antibiotic-resistant Gram-negative bacteria, such as those that produce extended-spectrum beta-lactamase (ESBL). Some studies found that *E. cloacae* is a common nosocomial organism that is resistant to carbapenem, meropenem, imipenem, ertapenem, penicillin, aztreonam, and first-, second-, and third-generation cephalosporins; the fact that our study discovered that 10% *Z. purpureum* could inhibit *E. cloacae* could be a promising future for future treatment of *E. cloacae* infection.(34-36)

Essential oils work by disrupting the cell wall and cytoplasmic membrane, resulting in the lysis and leaking of intracellular chemicals. Increased disruption of the cell membrane disrupts a variety of essential processes, including energy conversion, nutrition processing, structural macromolecule synthesis, and growth regulator secretion. (37) The essential oil of *Z. purpureum* has been observed to promote cellular component leakage and ion loss across the membrane. As a result, Gram-negative bacteria have a larger inhibitory zone than Gram-positive bacteria, with the exception of *S. aureus* (Table 1).Three bacterias which being suppressed by 10 vol% *Z. purpureum* rhizomes the most were *E. coli* (ATCC 35128), *P. aeruginosa* (ATCC 15442), *E. cloacae* (ATCC 13047). These bacterias are opportunistic human pathogens that are usually associated with infections of the lower respiratory tract and urinary tract acquired in hospitals.(26) These microorganisms can colonize and grow on the epithelial lining of the urinary tract in response to acidic conditions and the presence of adhesin or fimbriae and exhibit cytotoxic activity, implying the production of bacterial toxins by the cell host. However, that the essential oil of Z. purpureum (Rosc) may be capable of reversing this process. We hypothesize that the plant could neutralize the urinary organ's acidity and so prevent these bacteria from adhering, thereby flushing them from the body.(38-40)

## Conclusion

The essential oil of *Z. purpureum* rhizome has shown the ability to inhibit Gram-positive and Gram-negative bacteria, particularly those resistant to a variety of antibiotics, including beta-lactams, carbapenem, and first-, second-, and third-generation cephalosporins. Based on these results, this oil can be used for application in humans with safety being a priority concern, therefore further experiments need to be done.

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## Authors Contribution

NT and ETA were involved in concepting and planning the research, NT and SP performed the data acquisition/collection, NT and SA calculated the experimental data and performed the analysis, SA and SP drafted the manuscript and designed the table and figures, NT and YK aided in interpreting the results. All authors took parts in giving critical revision of the manuscript.

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