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Phytochemical Constituent, Antibacterial, and Antioxidant Leaves of Dracaena trifasciata (Prain) Mabb.

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Abstract. Sansevieria, commonly known as Dracaena trifasciata. Apart from serving as an ornamental and air-purifying plant, it also exhibits medicinal properties, making it a potential candidate for developing of novel natural drugs. The demand for natural antibacterial agents has significantly increased due to the rising incidence of resistance among pathogenic bacteria, such as Staphylococcus aureus. This research conducted to determine the antibacterial and antioxidant activities of D. trifasciata leaf extracts. The D. trifasciata leaf material was sequentially extracted using sonication, beginning with chloroform and then followed by ethanol. The Kirby-Bauer method was employed to conduct the antibacterial test, wherein the inhibition zones generated by the chloroform and ethanolic extracts were measured. The test bacteria used in this study was Staphylococcus aureus. The assessment of the antioxidant activity was conducted using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method. Phytochemical compound identification in the extracts was conducted using GC-MS analysis. The ethanolic extract showed a higher yield percentage compared to the chloroform extract. Both extracts exhibited moderate antibacterial activity. The chloroform extract exhibited an inhibition zone of 73.33 mm2, while the ethanolic extract showed an inhibition zone of 110 mm2. The chloroform extract had a minimum inhibitory concentration (MIC) of 500 mg/mL and a minimum bactericidal concentration (MBC) of 1000 mg/mL. The ethanolic extract had an MIC of 31.25 mg/mL and an MBC of 62.50 mg/mL. In terms of antioxidant activity, the chloroform extract had an IC50 of 370.8±0.07 µg/mL, while the ethanolic extract had an IC50 of 647.4±0.12 µg/mL. GC-MS analysis revealed 47 compounds in the chloroform extract and 49 compounds in the ethanolic extract. Based on the results of yield, antibacterial and compound identification, the ethanolic extract of D. trifasciata leaves exhibited greater potential compared to the chloroform extract. Its antibacterial activity showed more promise than its antioxidant activity.

Keywords: antibacterial, antioxidant, dracaena, phytochemical

Citation

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INTRODUCTION

Sansevieria, also known as "motherin-law's tongue" or Dracaena trifasciata, is a common name for this plant species, is currently being explored for its potential as a medicinal plant. In addition to its ornamental value, this plant has long been used as a traditional medicine in Asia and Africa, including for treating snake bites, burns, flu, cough, and respiratory ailments (Rwawiire & Tomkova, 2015; Tallei et al., 2016). Its leaves have been known for absorbing pollutants and other harmful substances (Li & Yang, 2020). Its high concentration of bioactive substances in both its leaves and roots has been proposed to be contributing to its ability to treat a variety of ailments (Babu & Prabhu, 2023).

In traditional medicine, the leaves and rhizomes of this plant are employed for the treatment of various conditions such as bronchitis, asthma, coughs, snake bites, insect bites and also utilized to address influenza, coughs, and respiratory infections. Saponins are present in the roots and leaves as secondary metabolites, which have healing properties for cough, snake bites, sprains, bruises, boils, abscesses, respiratory inflammation, and hair tonics (El Hawary et al., 2021). This herb also has anti-diabetic, anti-allergic, anaphylactic, and thrombolytic properties. Leaf extracts exhibit antibacterial action against Escherichia coli and Staphylococcus aureus (Febriani et al., 2019; W. F. Dewatisari & To'bungan, 2023). Recent studies have demonstrated that its leaves possess antialopecia activity (Kasmawati et al., 2022). This plant contains phytoconstituents such as flavonoids, sapogenin steroids including 25S-ruscogenin and sansevierigenin, pregnancy glycosides, and steroid saponins. Additionally, it contains various compounds, including and Methyl gallate, Methyl

pyrophaeophorbide A, Oliveramine,(2S)-3',4'-Methylenedioxy-5,7-dimethoxyflavan, 1-Acetyl-β-carboline, Digiprolactone, and Tricosanoic acid (Abdullah et al., 2018; Kanimozhi, 2011; Tinggi, 2018)

D. trifasciata's roots and leaves were phytochemical subjected to screening, which detected the presence of alkaloids, tannins, terpenoids, saponins, flavonoids, steroids, and phenols. These compounds possess the capacity to hinder the growth of pathogenic bacteria, including the Grampositive bacterium S. aureus. S. aureus is recognized for its virulence, toxin production, invasiveness, and resistance to antibiotics. It can result in a variety of illnesses, from minor skin infections to systemic infections and also food poisoning. Therefore, extracts from D. trifasciata have the potential to serve as an alternative approach to combat drug-resistant S. aureus infections, which have become a major concern due to the increasing resistance rates (Tallei et al., 2016).

Polyphenols and flavonoids are the most common and important phytoconstituents in plants, particularly for their pharmacological activities, including antioxidant activity. Terpenoids and alkaloids, on the other hand, are bioactive components that play a role in antibacterial activity (Abdullah et al., 2018). Several investigations have confirmed its antibacterial efficacy, including against Pseudomonas aeruginosa. The ethanolic extract of D. trifasciata contains an active component that has been found to inhibit the expression of genes associated with biofilm formation in P. aeruginosa (Dewatisari et al., 2023). The antioxidant properties of this plant has not been thoroughly investigated.

Several studies investigating the potential of D. trifasciata commonly used maceration as the extraction method. In this study, the researchers employed sonication as

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the extraction method, as it is believed to be more effective and rapid compared to maceration. Furthermore, the application of ultrasonic vibration allows the simple extraction of chemicals contained within plant cells by the solvent. In addition, less solvent is used for the sonication extraction procedure than for the maceration method. (Sholihah et al., 2017; Umoh et al., 2020).

Numerous pharmacological studies have been carried out on D. trifasciata. Its antibacterial action is restricted, and research on its antioxidant qualities is currently inadequate. Thus, the purpose of this study is to investigate D. trifasciata's potential as an antioxidant and antibacterial agent through a multistage sonication extraction method, where chloroform is used as a solvent in the first step and followed with ethanol. This study identified and analyzed the phytochemical components of D. trifasciata's leaf extract and assessed the plant's antibacterial and antioxidant qualities. Furthermore, the study conducted to pinpoint the precise substances that could be accountable for its antimicrobial and antioxidant properties.

MATERIALS AND METHODS.

The leaves of D. trifasciata were utilized in this study, and the bacterial strain S. aureus ATCC 25923. The extraction was conducted at the Biotechno-Industry Laboratory of Universitas Atma Jaya Yogyakarta. Antibacterial, antioxidant, and The Integrated Research and Testing Laboratory (LPPT) of Universitas Gadjah Mada in Yogyakarta conducted the GC-MS analysis.

Extraction (Sonication)

The powdered D. trifasciata herbal material was sieved using a mesh size of 60. The herbal material was extracted using

a multistage sonication process, starting with chloroform as the solvent followed by ethanol. The ratio of extract to solvent used was 1:10. Sonication was performed using a pulse mode, a frequency of 37, a power of 100, at a temperature of 50 °C for 20 minutes, followed by filtration. Filtration yielded a filtrate, and the solvent was removed by evaporation using a rotary evaporator. The remaining chloroform solvent was evaporated from the herbal material, and the extraction process was continued with ethanol solvent using the same procedure. The percentage yield of the extraction results is determined using the following formula (Mabruroh et al., 2019). :

$$Yield = \frac{The total weight of the extract in the form of paste(g)}{The total dry weight(g)} \times 100\%$$

Antibacterial Activity Analysis

The Kirby Bauer method was employed to conduct the antibacterial test. The test Staphylococcus aureus ATCC bacteria, 25923, were inoculated onto NB medium for 18-24 hours at a temperature of 36±1 °C. The bacteria were centrifuged, and the pellet was suspended using sterile saline. The test bacteria used were equivalent to a McFarland turbidity of 0.5 (108 CFU/mL) or its equivalent. A bacterial suspension of 1000 µL and 15-20 mL of MHA medium were poured into a petri dish. Then, a blank paper disc containing 25 µL of extract was placed onto the bacteria and solidified MHA medium, followed by incubation at 37°C for 24 hours. The clear zone was measured and expressed as bacterial activity. A negative control was prepared using sterile distilled water, while a positive control was established using an antibiotic.

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Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Analysis

The microdilution method was used for both MIC and MBC testing. A total of 300 µL of Nutrient Broth (NB) was added to 24 wells. The first well was left empty 600 µL of the sample was added to the first well, then 300 µL of the sample from the first well was transferred to the second well and homogenized. 300 µL of the sample from the second well was transferred to the third well, and this process was repeated until the sixth well, where 300 μ L of the suspension was discarded. The positive control used was NB medium without bacteria and without extract. The suspensions were incubated for 18-24 hours at a temperature of 37 °C. The negative control used was NB medium and extract without bacteria. The MIC and MBC values were then interpreted. Validation was performed by adding 2 mL of sterile NA medium to the wells and incubating them again for 18-24 hours at a temperature of 37 °C. MIC and MBC were determined by confirming bacterial growth on agar medium.

Antioxidant Analysis by DPPH (2,2 diphenyl-1-picrylhydrazyl) Method

To prepare the test sample, 50 μ l of the sample was mixed with 1.0 ml of 0.4 mM DPPH and 3,950 ml of ethanol at different concentrations. The mixture was vortexed and allowed to stand for 30 minutes. The absorbance of the solution was then measured at a wavelength of 517 nm against a blank, which consisted of 50 μ l of extract and 4,950 ml of ethanol. In addition, absorbance measurements were carried out for a mixture of 1.0 ml DPPH and 4.0 ml ethanol as a negative control and ascorbic acid as a positive control. The concentration that resulted in an IC50 value, representing the concentration of the extract that displayed 50% antioxidant activity

compared to the control, was determined using a linear regression equation.

GC-MS-Based Bioactive Compound Identification

The chloroform and ethanolic extract of D. trifasciata was dissolved in 1 mL of ethanol. Subsequently, the solution underwent filtration, and 0.5 µL of the filtered solution was introduced into a Shimadzu GC-MS-QP2010S chromatograph. The chromatograph gas employed a glass column measuring 30 m in length, 0.25 mm in diameter, and 0.25 m in thickness. The stationary phase utilized was CP-Sil 5 CB. The gas chromatograph was programmed with an oven temperature ranging from 70 to 260°C, a temperature increase rate of 10°C/min, a helium carrier gas at a pressure of 12 kPa, a total flow rate of 50 mL/min, and a split ratio of 1:50.

Data Collected

The extraction results were analyzed by calculating the yield from each solvent (chloroform and ethanol). The antibacterial and antioxidant activities were tested with three replications and analyzed using ANOVA, followed by post hoc Tukey test with a significance level of P<0.05. The results of the phytochemical screening using GC-MS were analyzed descriptively.

RESULTS AND DISCUSSION

Extraction Yield

The extraction of D. trifasciata plant material was performed using a sequential extraction method with sonication. The first solvent used for extraction was chloroform, followed by ethanol. The yield obtained from the ethanol solvent was higher compared to the yield obtained from the chloroform solvent (Table 1).). It is assumed this contains numerous polar compounds, as many of them

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are soluble in ethanol, which is more polar than chloroform. According to Kiswandono (2017), a high yield value indicates the presence of a higher amount of bioactive components. The yield value is related to the amount of bioactive constituents present in the plant material. A higher extraction yield indicates a higher content of the desired substances in the raw material (Senduk et al., 2020). Therefore, due to the high percentage yield obtained from the ethanolic extract of D. trifasciata, it is likely that the extract contains a significant amount of bioactive compounds (Dewatisari, 2019).

Table 1. Yield of *D. trifasciata* extraction using chloroform and ethanol solvents.

Sample	Yield (%,w/w)
Chloroform extract	0.35
Ethanolic extract	1.4

Antibacterial Activity

antibacterial activity The testing showed inhibition of bacterial growth, indicated by the formation of inhibition zones. The diameter of the inhibition zone corresponds to the clear area surrounding the paper disc, which indicates the inhibition of bacterial growth. On the other hand, turbidity observed in the media indicates bacterial growth. According to Dafale et al. (2016), the criteria for antibacterial potency are as follows: an inhibition zone area of 78.5 mm² or less is categorized as weak, 78.5 - 314 mm² is categorized as moderate, 314 - 1256 mm² is categorized as strong, and 1256 mm² or more is categorized as very strong. The results of inhibition against S. aureus indicate that both the chloroform and ethanolic extracts have moderate antibacterial activity (Table 2 & Figure 1).

Previous studies have reported that D. trifasciata extract has similar activity in inhibiting Gram-negative bacteria such as E. coli and P. aeruginosa. However, the inhibition zones of the extract against Gram-negative bacteria were larger, ranging from 248 to 314

mm² (Dewatisari et al., 2021). In contrast to previous studies, in this research, the inhibition zones formed by the treatment of chloroform and ethanolic extracts of D. trifasciata against S. aureus were smaller. S. aureus, as a Grampositive bacterium, possesses a thicker cell wall compared to Gram-negative bacteria such as E. coli and P. aeruginosa. Gramnegative bacteria are characterized by a cell wall with a thin layer of peptidoglycan and are encompassed by lipoproteins, lipopolysaccharides, phospholipids, and certain proteins. The cell wall of Gramnegative bacteria is more susceptible to physical disruption, such as the administration of antibiotics or other antibacterial agents, due to their limited peptidoglycan layer and the absence of teichoic acid (Daniels et al., 2021). Gram-positive bacterial cell walls have a characteristic structure consisting of a lipid bilayer cytoplasmic membrane surrounded by a rigid cell wall containing peptidoglycan and teichoic acid or teichuronic acid. This structural composition makes S. aureus more resistant to the exposure of foreign substances (Afroz et al., 2015).

 Table 2. Antibacterial activity of D. trifasciata extract against S. aureus.

Sample	Clear zone area (mm) ±SD
Chloroform extract	73.3±2.8
Ethanolic extract	110±10

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Figure 1. Antibacterial activity of *D. trifasciata* extract against *S. aureus*. a) control, b) chloroform extract, c) ethanolic extract

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Following the disc diffusion antibacterial test, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays were performed. The MIC represents the lowest concentration of an antibacterial compound that inhibits bacterial growth, while the MBC represents the lowest concentration that kills the bacteria. It is crucial to establish this because the disc diffusion antibacterial test lacks the capability to assess the antibacterial effectiveness at various concentrations. The antibacterial activity of a substance is influenced by factors such as the concentration of the antibacterial agent (Frazier & Westhoff, 1983).

Table 3. The MIC and MBC values of D. trifasciata extract against S. aureus

Sample	MIC (mg/mL)	MBC (mg/mL)
Chloroform extract	500	1000
Ethanolic extract	31,25	62,50

The results of the MIC test for the ethanolic extract and chloroform extract on S. aureus vielded distinct outcomes. The MIC value recorded for the ethanolic extract was 31.25 mg/mL, whereas for the chloroform extract, it was 500 mg/mL. This suggests that the ethanolic extract exhibits a greater capacity to hinder the growth of S. aureus, as its minimum inhibitory concentration is lower in comparison to the chloroform extract (Table 3). It is hypothesized that the compounds present in the ethanol extract can disrupt the structure of bacterial cells. Antimicrobial agents can damage the cell wall by inhibiting its formation or altering its structure. The cell wall of gram-positive bacteria is rich

in teichoic acids, teichuronic acids, and polysaccharide molecules. These components serve to protect the cells from enzymatic lysis, while others influence the cells' reactions during gram staining and may attract and bind bacteriophages. This structural composition makes the cell wall of gram-positive bacteria particularly susceptible to the antibacterial effects of the ethanol extract (Daniels et al., 2021).

Comparably, 62.50 mg/mL of MBC was found for the ethanolic extract and 1000 mg/mL for the chloroform extract. When the ethanolic extract was used for treatment, the growth of S. aureus was not observed in tube 6. Conversely, in the case of the chloroform

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extract, *S. aureus* growth was already absent in tube 1 (Figure 2 and Figure 3). This also indicates that the ethanolic extract requires a lower concentration to kill *S. aureus* compared to the chloroform extract. The negative controls for both extracts showed no growth of *S. aureus*, while the positive control exhibited a white membrane (bacterial growth) (Figure 2 and Figure 3).

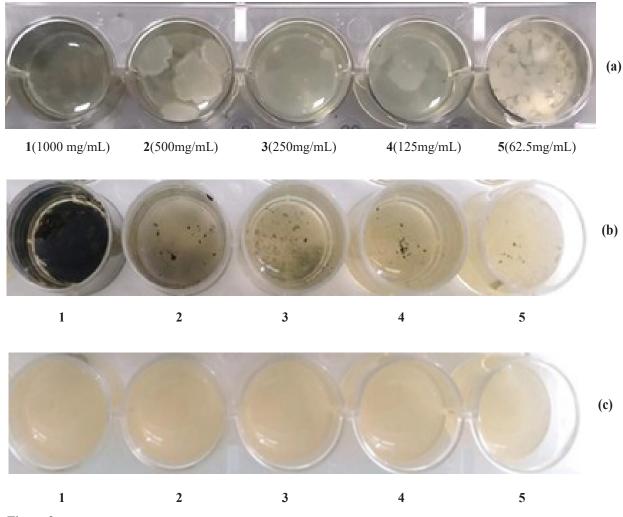
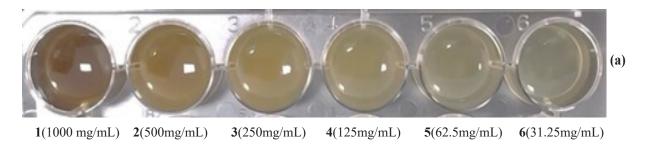


Figure 2. The results of the MIC-MBC test for the chloroform extract of *D. trifasciata* against *S. aureus* are as follows: a) Chloroform extract with various concentrations, b) Negative control, c) Positive control



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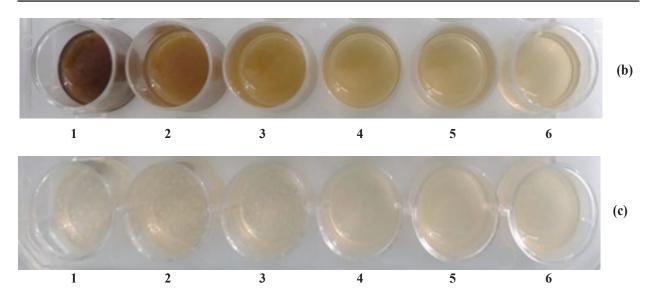


Figure 3. The results of the MIC-MBC test for the ethanolic extract of *D. trifasciata* against *S. aureus* are as follows: a) Ethanolic extract with various concentrations, b) Negative control, c) Positive control

Antioxidant Activity

Table 4 showcases the antioxidant activity of the chloroform and ethanolic extracts obtained from *D. trifasciata* leaves, as determined by the DPPH assay. The data collected indicates that both the ethanol and

chloroform extracts of Dracaena leaves do not demonstrate notable antioxidant activity when compared to ascorbic acid. This disparity in antioxidant activity is distinct from the findings of previous studies on *D. trifasciata*.

Table 4. Antioxidant activity of D. trifasciata

Sample	IC50 (µg/mL)±SD	Category
Chloroform extract	370,8±0,07	Not potential
Ethanolic extract	647,4±0,12	Not potential
Ascorbic acid	4,17±0,02	Very potential

Note: One-way ANOVA followed by post hoc Tukey HSD test resulted in the mean (\bar{x}) values from three replicates. Subscripts with different letters represent significance (p<0.05) between IC50 (µg/ml) samples. SD = Standard Deviation. Classification of IC50 values: <10 µg/ml (highly potent), 10-50 µg/ml (strong), 50-100 µg/ml (weak), 100-250 µg/ml (very weak), and >250 µg/ml (inactive) (Reviana et al., 2021)

The ethanolic extract of D. trifasciata leaves has been previously reported to exhibit highly potent antioxidant activity, with an IC50 value greater than 10 μ g/mL (Lontoc et al., 2018; Sarjani et al., 2021). The difference in antioxidant activity can be affected by the extraction method utilized. In the studies by Sarjani et al. (2021) and Lontoc et al.

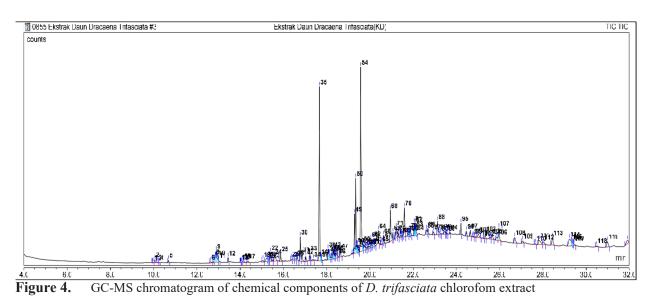
(2018), the maceration method was employed to obtain phytochemical compounds. Maceration extraction is performed without high-temperature intervention, allowing phytochemical compounds with temperaturesensitive antioxidant activity to remain undamaged.

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Flavonoids are phytochemical compounds known for their antioxidant activity. However, flavonoids are sensitive to high temperatures (Chaaban et al., 2017). Therefore, the selection of an appropriate extraction method is crucial to ensure that phytochemical compounds responsible for antioxidant activity are not damaged. The results of compound identification using GC-MS showed that the chloroform extract contained a total of 47 different compounds. The highest peak detected corresponded to Heptadecanoic acid, 16-methyl-, methyl ester (17.12%) with a retention time of 19.58. This was followed by Hexadecanoic acid, methyl ester (13.88%) (Figure 4 & Supplementary file). Both compounds belong to the group of fatty acids.

GC-MS Analysis Results



Through compound identification of the ethanolic extract, it was determined that the extract contained a total of 49 different compounds. This identification was supported by the chromatogram, which displayed distinct peaks corresponding to specific retention times (RT), phytoconstituents, chemical structures, and the percentage of peak area (Figure 5 and Supplementary file). Methyl stearate was identified as the compound with the highest peak, accounting for 8.77% with a retention time of 19.57 minutes. Methyl stearate belongs to the group of fatty acids. This compound is known to possess antimicrobial, anticancer, anti-arthritis, anti-inflammatory, and antiviral activities (Olajuvigbe et al., 2018; Painuli et al., 2015). The compound n-Hexadecanoic

acid is the compound with the highest percentage, accounting for 12.40% of the total composition.

The ethanolic extract also contains dominant fatty acid compounds, including cis-13-Eicosenoic acid (3.28%) and Ethyl 9.cis.,11.trans.-octadecadienoate (2.19%). Another prominent compound is Phytol, which is a diterpene alcohol known for its anti-inflammatory properties. Phytol, found in the methanolic extract of P. nigrum, exhibits biological activity as an antioxidant. Phytol is an alcohol diterpene that has been found to inhibit inflammation. Fitol, found in the 95% ethanolic extract of Clitoria ternatea, possesses antimicrobial and anti-asthma activities (Dewatisari, 2023.). Phytol is one of

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the compounds that can contribute significantly to drug discovery (Mapanawang & Elim, 2019).

The ethanolic extract reveals a greater number of detected compounds in comparison to the chloroform extract. Therefore, it is plausible that the higher number of identified compounds corresponds to the extract's potential as an antibacterial and antioxidant agent. Both extracts are predominantly composed of compounds belonging to the fatty acid group. Several fatty acids have been studied and found to be partially responsible for the antimicrobial activities of plants (Daniels et al., 2021). Several studies have reported that hexadecanoic acid, methyl ester exhibits antimicrobial activity with antifungal and antibacterial properties (McGaw et al., 2002; Seidel & Taylor, 2004). Hexadecanoic acid exhibits properties that enable it to function as an anticancer, antioxidant, and antimicrobial agent (Daniels et al., 2021)

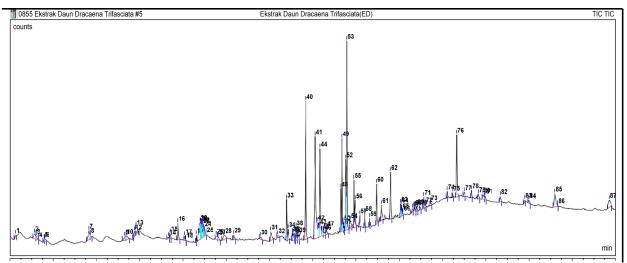


Figure 5. GC-MS chromatogram of chemical components of *D. trifasciata* ethanolic extract

Both extracts of D. trifasciata contain numerous active compounds. However, based on the yield and the compounds detected by GC-MS analysis, the ethanolic extract shows higher potential compared to the chloroform extract. The yield value is directly linked to the quantity of chemical compounds present in the plant material. A higher extract yield indicates a greater amount of substances extracted from the source material (Senduk et al., 2020). Therefore, with the highest yield, the ethanolic extract of D. trifasciata potentially has the highest phytochemical content compared to the chloroform extract.

The potential as an antibacterial agent is more promising than as an antioxidant. The antibacterial activity of both tested extracts of *D. trifasciata* falls within the moderate category. However, the ethanolic extract demonstrates particularly promising values for the MIC and MBC. The MIC value of the ethanolic extract in this study is superior to previous research on the ethanolic extract fraction of *D. trifasciata* obtained through maceration extraction. In that study, the MIC value of the ethanolic extract fraction obtained through maceration was found to be 32 mg/mL, slightly higher in concentration compared to the findings of this study (Dewatisari et al., 2021).

D. trifasciata's antibacterial activities are due to the presence of fatty acid molecules such as methyl stearate, hexadecanoic acid, and heptadecenoic acid. It is essential to take

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take into account the potential synergistic effects of other fatty acids and additional components. Numerous studies have illustrated the significance of synergy among phytochemicals in enhancing the antibacterial efficacy of plants. Octadecenoic acid acts as an antiandrogenic, antiarthritic, antimicrobial, and anti-coronary agent. Research conducted by Hema et al. (2011) also reported that octadecenoic acid acts as an inhibitor of 5-alpha reductase and finds applications in cosmetics, flavorings, hypokolesterolemia treatment, lubricants, fragrances, propecies, and suppositories. Palmitic acid, Dodecanoic acid, n-hexadecenoic acid, 9-octadecanoic acid, pentadecanoic acid, tetradecanoic acid, and heptadecanoic acid have potential antioxidant, anticancer, anti-eczema, anti-inflammatory, anti-enzymatic, and antimicrobial properties (Bodoprost & Rosemeyer, 2007; Preethi et al., 2010; Xie et al., 2018).

The leaves of D. trifasciata exhibit a more dominant antibacterial action. Therefore, additional research utilizing the ethanolic extract of D. trifasciata is required to advance the development of natural remedies. Enhancing the antibacterial activity of the ethanolic extract can potentially be achieved by implementing fractionation techniques to isolate the active compounds and identify the dominant compounds responsible for its antibacterial effects.

To determine the precise bioactive compounds accountable for the observed antibacterial effects of the ethanolic extract derived from D. trifasciata leaves, additional investigations are required. It is important to note that the composition and effectiveness of bioactive compounds found in plant extracts can be influenced by various factors, such as extraction techniques, plant species, geographical location, and environmental conditions. Therefore, more research is needed to completely comprehend the antibacterial activity of the ethanolic extract of D. trifasciata leaves, including phytochemical investigation and in vivo evaluations.

CONCLUSION

Ethanol extract of D. trifasciata leaves showed higher yield values and antibacterial activity compared to chloroform extract. Both extracts contain compounds that have the potential to be active substances. This is supported by the results of the Ethanol extract of D. trifasciata containing more compounds than the chloroform extract. The potential of D. trifasciata extract as an antibacterial agent shows greater promise than its antioxidant activity.

AUTHOR CONTRIBUTION

WFD designed the research, wrote the manuscript, and supervised all the processes. The data was collected and analyzed by NT, who also authored the manuscript.

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CONFLICT OF INTEREST

Authors have no conflict of interest.

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