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Identification of Antibacterial Compounds in Methanol Extract of Clove Stems (*Syzygium aromaticum*) using Liquid Chromatography Mass Spectrometry

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ABSTRACT

Introduction: *Clove is well-known as an herbal plant that is also included in the spice group which is known to have an antibacterial activity spread across the stems, leaves, and flowers.*
Objectives: *The purpose of this study was to identify the antibacterial compounds that are found in the methanol extract of clove stems and the antibacterial activity of clove stem extract against Bacillus cereus.*
Methods: *Liquid Chromatography Mass Spectrometry (LC-MS) method to identify the antibacterial compounds, and the paper disc method (Kirby Bauer) for antibacterial test with clove stem extract concentrations of 30%, 50%, and 70%, positive control of Amoxicillin at a dose of 0.5 mg/ml, also a negative control of sterile aquadest.*
Results: *The results showed four antibacterial compounds in the extract, namely (+)-Licarin A, 4-O-Caffeoylquinic acid_1, Eckol, and Leucodelphinidin. Methanol extract of clove stems at the concentrations of 30%, 50%, and 70% inhibited the growth of Bacillus cereus significantly ($p < 0.05$). The concentration of 30% was categorized as quite sensitive, meanwhile, both 50% and 70% were categorized as sensitive.*
Conclusions: *Four compounds with antibacterial properties were found in the methanol extract of clove stems based on LCMS analysis, and supported by the results of antibacterial tests which showed the formation of an inhibition zone in the clove stem extract against the growth of Bacillus cereus.*

Introduction

Indonesia is a tropical country that has high biodiversity, herbal plant is one of the biggest biodiversity that spread across Indonesia (Novianti, 2017). Most Indonesian people often use several types of herbal plants as medicine, one of which is the clove plant (Safitri & Purnamawati, 2021). The clove plant is one of the herbal plants in the spice group which is known to have antioxidant and antimicrobial activity (Gülçin et al., 2004; Nzeako et al., 2006).

The antioxidant and antimicrobial content of the clove plant is spread throughout all parts of the plant, both stems, leaves, and flowers. The use of cloves in the industrial world is often found as a mixture of cigarette ingredients, toothpaste, perfumes, and medicinal ingredients such as cough medicine. In daily life, cloves are also used as a mixture of herbal drinks and as a mixture of some cookies (Safitri et al., 2024; Triana, 2023). The main antibacterial compound in clove plants is clove oil. The clove oil content in clove stems is not much different from the flower part, but the utilization of clove stems is still relatively low compared to the flowers (Widayat et al., 2012).

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Identification of antibacterial compound content is often carried out using the Gas Chromatography Mass Spectrometry (GCMS) and Liquid Chromatography Mass Spectrometry (LCMS) methods. Previous research stated that clove stem is known to have antibacterial compounds including alpha-pinene, hexylene glycol, and mycrene based on identification using GCMS (Safitri et al., 2022). This study aims to identify antibacterial compounds in methanol extract of clove stems using LCMS along with its antibacterial activity against *Bacillus cereus* bacteria.

Methods

Extract Preparation

Extraction was showed in Figure 1, the process carried out by mixing dry clove stem powder with 80% methanol in a ratio of 2:15 (g/ml) and left at room temperature for 24 hours in dark conditions. We covered the bottle of homogenate with aluminium foil to prevent light-catalyzed reactions or color changes. We stirred the homogenate twice in 24 hours, so that the extract mixed perfectly (Safitri & Fatimah, 2023).

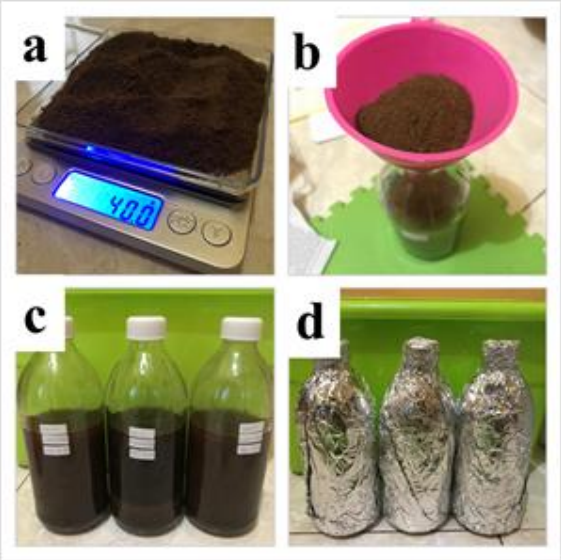

Figure 1. Clove stem extraction: a) measured clove stem powder; b) poured into a transparent glass bottle; c) the homogenate from extraction; d) covered the bottle with aluminium foil

The homogenate from the extraction was filtered using filter paper and processed using a rotary evaporator to remove the solvent to obtain a concentrated clove stem extract. The methanol-free concentrate extract stored at a temperature of 4 °C to prevent the contamination.

Identification of antibacterial compounds using LCMS

Identification of antibacterial compounds using LCMS was carried out at the Integrated Laboratory of Bioproduct, National Research and Innovation Agency (BRIN), Bogor, West Java. The principal of LCMS is the separation of analytes based on its polarity and time to reach the detector which known as retention time (RT). The results of LCMS analysis will be shown as a chromatogram picture with some RT peaks, and its mass of each molecule (Mangurana et al., 2019).

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Antibacterial activity test of clove stem extract

Antibacterial tests were carried out using the paper disc method with variations in extract concentrations of 30%, 50%, and 70%, equipped with a positive control of amoxicillin antibiotic 0.5 mg/ml, and a negative control of sterile aquadest. *Bacillus cereus* were spread on agar media, then we inserted paper discs which contained both extracts and controls on the surface of it, and leave it for 24 hours in temperature of 37 °C (Safitri et al., 2022). The test results were carried out by measuring the clear inhibition zone formed in the paper disc area (Figure 2).

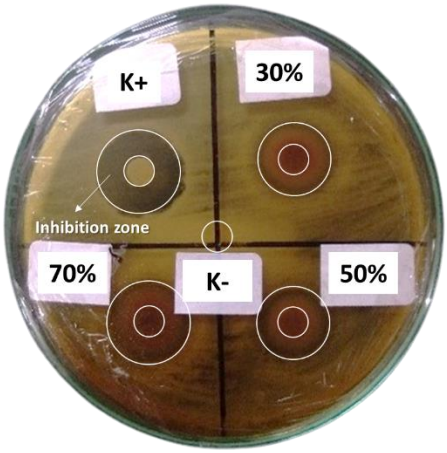

Figure 2. Antibacterial activity test that showed inhibition zones around the paper discs

The observation results were categorized based on the classification of antibacterial substance strength, namely insensitive with the inhibition zone less than 8 mm; quite sensitive with the inhibition zone between 8 mm and 14.0 mm; sensitive with the inhibition zone between 14 and 20 mm; and very sensitive with the inhibition zone more than 20.0 mm (Xiao et al., 2019). The research data were analyzed statistically using SPSS software version 26 using Kruskal Wallis and the Mann Whitney as a Post Hoc test.

Results

Antibacterial Compounds of Clover Stem Extract Analysed using LCMS

The results of the compound profile test on the clove stem extract based on LCMS analysis obtained a total of 5 highest peaks in the chromatogram analysis. These compounds include (+)-Licarin A with a molecular weight of 326.1 g/mol, 4-O-Caffeoylquinic acid_1 with a molecular weight of 354.0 g/mol, Eckol with a molecular weight of 372.0 g/mol, Leucodelphinidin with a molecular weight of 322 g/mol, and Candidate Mass C26H48O14 with a molecular weight of 584.3 g/mol (Table 1).

Table 1. Antibacterial Compounds of Clover Stem Extract Analyzed using LCMS

Compounds name	Retention time (RT)	Chemical formula	Molecular mass (g/mol)
(+)-Licarin A	9.30	C20H22O4	326.1
4-O-Caffeoylquinic acid_1	3.42	C16H18O9	354.0
Eckol	8.36	C18H12O9	372.0
Leucodelphinidin	7.48	C15H14O8	322.0
Candidate Mass C26H48O14	9.56	C26H48O14	584.3

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Based on the chromatogram analysis in Figure 3, many compounds were detected in the LCMS test of clove stem methanol extract. The highest peak seen from the LCMS chromatogram results was Candidate Mass C26H48O14 with a retention time of 9.56. In second place was (+)-Licarin A with a retention time of 9.3, followed by Eckol, Leucodelphinidin, and 4-O-Caffeoylquinic acid_1 in sequence with retention times of 8.36, 7.48, and 3.42 respectively.

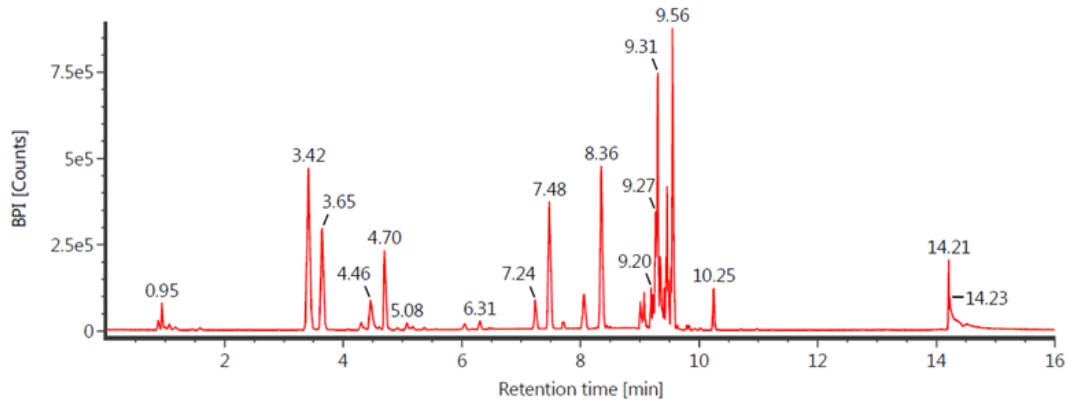

Figure 3. Liquid Chromatography Mass Spectrometry chromatogram of clove stem extract

Antibacterial Activity Results of Clove Stem Methanol Extract on the Growth of *Bacillus cereus*

The results of the antibacterial test of clove stem methanol extract on the growth of *Bacillus cereus* bacteria with various concentration variations can be seen in Table 2. Observations of the inhibition zone showed that the most effective concentration to inhibit the growth of *Bacillus cereus* was a concentration of 70% with an inhibition zone diameter of 15.3 mm with a sensitive category. At concentrations of 30% and 50%, respectively, the inhibition zone was 13.6 mm with a quite sensitive category and 14.5 mm with a sensitive category.

Table 2. Methanol Extract of Clove Stem on the Growth of *Bacillus cereus*

Treatments	Inhibition zone (mm)	Claassification (Xiao et al., 2019)
C + (Amoxicilin 0.5 mg/ml)	19.8	Sensiitive
C – (Sterile aquadest)	0	Resistant
E-30%	13.6	Quite sensitive
E-50%	14.5	Sensitive
E-70%	15.3	Sensitive

Antibacterial test on positive control of amoxicillin dose of 0.5 mg/ml showed the inhibition zone formed by 19.8 mm with sensitive category. A negative control that used sterile aquadest showed no inhibition zone in the antibacterial test.

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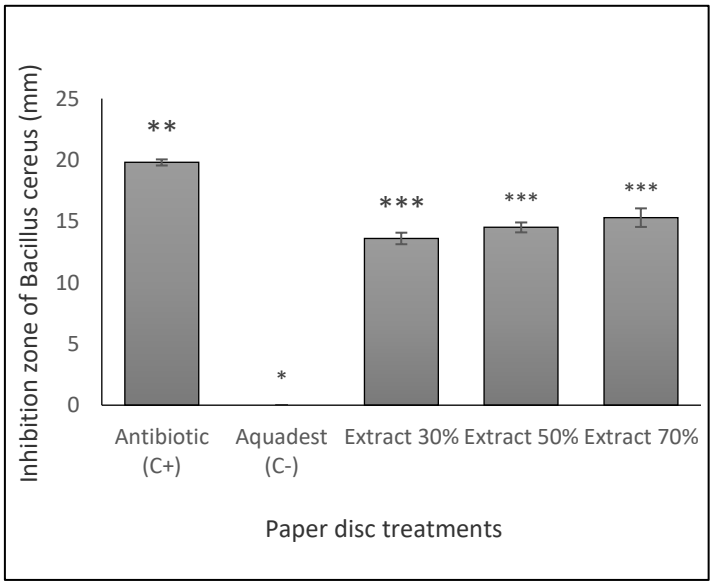

Figure 4. Comparison of the inhibition zone activity of clove stem methanol extract against *Bacillus cereus*

Based on the results of statistical analysis, show that the clove stem extract in each treatment has a significantly different inhibition zone activity ($p < 0.05$) compared to the negative control (aquadest) and positive control (antibiotic dose 0.5 mg/ml). This is indicated by the different notations in the post hoc diagram of the treatment in Figure 4. In the comparison of each treatment, both extracts with concentrations of 30%, 50%, and 70% did not have a significant difference between each other. However, the three concentrations of clove stem methanol extract have quite high inhibition zone activity and are close to the diameter of the inhibition zone produced by antibiotics at a dose of 0.5 mg/ml.

Discussion

Clove plants are one of the spice plants that are often used as herbal plants that have many benefits. The stems, leaves, and flowers contain clove oil which can be used as an ingredient for making herbal medicines and drinks (Triana, 2023). Clove plants are known to have many uses in the world of health, namely having antibacterial, antiviral, and fungicide activities (Chee & Lee, 2017; Kiki, 2023). Clove stems have the second highest content of clove oil after the flower part, which is 5 - 10% with an oil content of 94.73% (Nahrowi et al., 2023).

Antibacterial activity tests showed that the methanol extract of clove stems had inhibition activity with a quite sensitive category at a concentration of 30% and a sensitive category at concentrations of 50% and 70%. These results indicate that the methanol extract of clove stems at concentrations of 50% and 70% has antibacterial activity that is almost equivalent to the inhibition zone produced by antibiotics at a dose of 0.5 mg/ml with the same category (sensitive). This antibacterial activity is supported by the results of the LCMS test which showed the presence of antibacterial compounds in the methanol extract of clove stems, including (+)-Licarin A, 4-O-Caffeoylquinic acid_1, Eckol, and Leucodelphinidin.

Bacillus cereus is gram-positive spore-forming bacterium commonly found in soil and vegetables, infecting the human digestive tract through contamination in the food that is consumed (Bottone, 2010). The results of this study indicate the presence of antibacterial activity that is thought to be able to significantly inhibit the growth of *Bacillus cereus*. One of the compounds thought to have antibacterial activity is 4-O-Caffeoylquinic acid_1 which has been

reported that able to inhibit the growth of Bacillus bacteria (Han et al., 2014). Flavonoids, such as Leucodelphinidin, are known to be able to reduce bacterial growth by inhibiting the process of cell wall biosynthesis (Donadio et al., 2021). Licarin A, which is found in abundance in spice plants, and the Eckol compound are known to be able to inhibit the growth of gram-positive and gram-negative bacteria (Alvarenga et al., 2020). Based on that, this research showed that the methanol extract of clove stem has the potential to be an antibacterial agent against bacteria both gram-positive and negative.

Based on the statistics analyzed, there is no significant difference between each dose of clove stem extract to inhibit the growth of *Bacillus cereus.* All doses of the extract have a high potential to inhibit bacterial growth effectively compared to aquadest as a negative control treatment. Amoxicillin as a positive control showed the highest antibacterial activity compared to all treatments. A broad-spectrum antibiotic, such as amoxicillin has a high ability to kill bacteria through the inhibition of cell wall synthesis during cell division (Resnik & Cillo, 2018). As shown in the results of this study, clove stem extract has the potential to be developed more widely and become an herbal medicine with antibiotic properties. It can be used as an alternative treatment for bacterial infection with minimal side effects.

Conclusion

In conclusion, the antibacterial compounds of clove stem extract identified using LCMS obtained four compounds that have antibacterial properties, namely (+)-Licarin A, 4-O-Caffeoylquinic acid_1, Eckol, Leucodelphinidin. The clove stem extract also has antibacterial activity in inhibiting the growth of *Bacillus cereus* effectively.

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