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Identification of Bioactive Compounds and Antibacterial Activity of Limeberry (*Triphasia trifolia*) Fruit Ethanol Extract against *Staphylococcus aureus*

Novita Herdiana*, Sri Hidayati, Dewi Sartika, Subeki, Suharyono, Puspita Yuliandari, Ahmad S. Zuidar

Department of Agricultural Product Technology, Faculty of Agriculture, Lampung University, Bandar Lampung 35141, Lampung, Indonesia

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ABSTRACT

Bacteria are microorganisms that are widely distributed in various habitats and environments, including food. Food poisoning can be caused by enterotoxin contamination from *Staphylococcus aureus*. Soft tissue and skin infections are associated with staphylococci or streptococci. The use of synthetic drugs, including antibiotics, is generally done to prevent bacterial infections, but this can result in resistance. Therefore, natural compounds are a potential source used as an antibacterial. Limeberry (*Triphasia trifolia*) fruit is a potential source of bioactive compounds. Thus, this study aimed to investigate the bioactive compounds and antibacterial activity of limeberry (*T. trifolia*) fruit ethanol extract against *S. aureus*. The bioactive compound was extracted using an ethanol solvent. The functional groups of the compounds were analyzed using *gas chromatography-mass spectroscopy* and Fourier transform infrared. The antibacterial activity was analyzed using disk diffusion by the Kirby-Bauer method. The result showed that the extraction yield was about 22.5%. The ethanol extract of limeberry fruit contains alkaloids such as piperidine alkaloids and polyphenol compounds such as flavonoids and tannins. The hydroxyl (OH) group was detected in the ethanol extract of limeberry and indicated the presence of polyphenol compounds. This extract exhibits antibacterial activity against *S. aureus*, with an inhibition zone of about 11.94±0.18 mm at a 25% extract concentration. Therefore, this ethanol extract of limeberry fruit can be used as an alternative source of antibacterial agents.

Keywords: Antibacterial activity, bioactive compound, limeberry fruit, *Staphylococcus aureus*.

Introduction

Bacteria are microorganisms that are widely distributed in various habitats and environments, including food. Pathogenic bacteria in food can cause various diseases if consumed by humans and are also known as foodborne diseases.[1] The cases of food poisoning in Indonesia have occurred in several areas, such as an increase in diarrhea cases from 2017-2019 in Tikala Baru Village, Manado City and Pelabuhan Ratu in 2019 due to a bacterial infection that causes fever, stomach cramps, and bloody diarrhea.[2, 3] Food poisoning can be caused by enterotoxin contamination from *Staphylococcus aureus* which is a gram-positive bacterium.[4, 5] Additionally, soft tissue and skin infections are associated with staphylococci or streptococci. These infections can range in severity from self-limiting superficial infections to life-threatening conditions.[6]
The use of synthetic drugs, including antibiotics, is generally done to prevent bacterial infections, but this can result in resistance.[7] Therefore, natural extracts from various plants can be a potential alternative to antibiotics. Plants have bioactive compounds that can inhibit and kill microorganisms by influencing host cellular processes (immune response, mitosis, apoptosis, and signal transduction). Natural antibacterial agents are also not causing a resistance.[8] The use of natural extracts is not only used in the treatment phase, but is also used in the prevention phase. These extracts generally have bioactive components that can act as antibacterial, such as alkaloid and polyphenolic compounds.[7, 9]
Polyphenolic compounds are secondary metabolite compounds that can commonly be extracted from plants. Polyphenolic compounds from plants have various biological activities, such as antibacterial, anti-inflammatory, antiviral, anti-allergic, and antioxidant.[10] Polyphenolic compounds from the plant *Stryphnodendron adstringens* were reported to have inhibitory activity against *S. aureus* bacteria.[11] A polifenol compound from the *Filipendula ulmaria* plant also exhibits antioxidant activity against *S. aureus*.[6] Additionally, alkaloids also show antibacterial activity against some pathogenic bacteria.[12] A previous study reported that the leaf and steam of limeberry (*Triphasia trifolia*) contain bioactive compounds including terpenoids, alkaloids, and polyphenol compounds such as flavonoids and tannins.[13] Limeberry (*T. trifolia*) is a species in the Rutaceae (Citrus) family, is native to tropical southeastern Asia, including Indonesia and Philippines. Additionally, an essential oil from *Triphasia trifolia* exhibits antioxidant activity.[14] However, there is yet no study reporting the antibacterial activity of limeberry fruit ethanol extract against *S. aureus*. Therefore, this study aimed to identify the bioactive compounds and antibacterial activity of limeberry (*T. trifolia*) fruit ethanol extract against *S. aureus*.

*Corresponding author. E mail: novita.herdiana@fp.unila.ac.id
Tel: (+62) 721 781036

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Materials and Methods

Sample preparation and extraction

Two kilograms of limeberry (*T. trifolia*) fruits were collected from Pasar Mulya Village, Krui, West Lampung in October 2022. Then, it was dried by sun-drying for 8 hours and then oven-drying at 50 °C for 72 hours. After the drying process, it was ground to powder and kept for further used. The extraction process used a maceration method, according to the previous study.[13, 15] Briefly, 450 g of fruit powder was mixed with 750 mL of a 96% ethanol solvent. The extraction was performed at room temperature and stirred with a magnetic stirrer

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(Thermo Scientific Cimarec Stirring hot plate, Massachusetts, USA) at 120 rpm for 3 days. The filtrate and residue were separated using filter paper (Whatman No. 42). The filtrate's solvent was evaporated using a rotary vacuum evaporator (Eyela N-1110 rotary evaporator, Tokyo, Japan) at 30 °C. The limeberry ethanol extract was put in the new sample bottle and kept at a cold temperature for further analysis.

Qualitative phytochemicals analysis

The qualitative phytochemical compounds including alkaloids, flavonoids, and tannin, were analyzed according to the previous methods.[16, 17] For the alkaloid test, each extract was dissolved in an ethanol solvent and then filtrated to separate the residue from the filtrate. The filtrate was divided into 3 tubes (5 mL/tube) and then added to 3 reagents (Mayer's, Wagner's, and Dragendorff's). Alkaloid was detected in white or yellow precipitates in Mayer's reagent, brown precipitates in Wagner's reagent, and orange precipitates in Dragendorff's reagent. In the flavonoid test, 5 mL of extract was dissolved in ethanol, then Mg powder was added, and 5 drops of concentrated HCl were added. The presence of flavonoids was detected by red, yellow, or orange color formation. For the tannin test, 5 mL of extract dissolved in ethanol was added to the $FeCl_3$ reagent. The presence of tannin was indicated by blue, blackish, or green color formations.

Gas chromatography-mass spectroscopy and Fourier-transform infrared analysis

The identification of bioactive compounds in limeberry fruit ethanol extract were also analyzed using a gas chromatography-mass Spectroscopy (GC-MS, Agilent 8890) according to previous study.[18] The HP5-MS column (30 m × 250 µm × 0.25 µm) was used and the injection temperature was at 280 °C, the initial oven temperature was at 60 °C and the initial time was 2.0 minutes. The rate 1 was 20.0 °C/min and the final temperature was 120 °C. The rate 2 was 4.0 °C/min and the final temperature was 290 °C. The MS quad temperature was 150 °C and MS source temperature was 230 °C. Whereas, the Fourier-transform infrared (FT-IR) analysis was performed according to the previous methods to analyze the functional group of the limeberry ethanol extract. A Fourier transform infrared (Agilent Cary 630 FTIR Spectrometer) equipped with an infrared source and potassium bromide beam splitter according to the previous method.[19]

Antibacterial activity analysis

Staphylococcus aureus bacteria were used to evaluate the antibacterial activity of the limeberry fruit ethanol extracts, which performed according to the previous method.[20] Whereas, the 96% ethanol extract was tested as a positive control for inhibition, while distilled water was used as a negative control. The paper discs that had been dipped in extracts with different concentrations (5%, 10%, 15%, 20%, and 25%) were placed on the agar medium, which contained *S. aureus* cultures, and then incubated at 37°C for 24 hours. Antibacterial activity is carried out by measuring the diameter of the clear zone, which indicates the inhibition of the growth of bacteria.

Data analysis

The qualitative phytochemicals, GC-MS, and FT-IR data were used for descriptive analysis. The antibacterial activity was assessed using a one-way ANOVA and a Duncan post-hoc test ($p<0.05$) using SPSS software.

Results and Discussion

Extraction yield and phytochemical compositions

The bioactive compounds from limeberry fruit were successfully extracted using a 96% ethanol solvent. The extraction yield was about 22.5%. Previous studies also reported that bioactive compounds from *Pistia stratiotes* leaf were successfully extracted using an ethanol solvent, with a yield extraction of about 16.80%.[21] Additionally, a 96% ethanol solvent was also used to extract bioactive compounds from *Cnidoscolus aconitifolius* leaf.[22] According to the qualitative phytochemical analysis, this extract contains alkaloids, flavonoids, and tannins (Table 1). A previous study reported that 96% ethanol was used to extract various bioactive compounds, including alkaloids and polyphenol compounds, such as flavonoids and tannin.[22] A previous study also reported that different ethanol concentrations have been widely used in bioactive compound extraction.[23] Ethanol, or mixture of ethanol and water, is widely used as a solvent due to its safety and it is also categorized as a food-grade solvent.[24]

Bioactive compounds composition of limeberry fruit ethanol extract using GC-MS

The gas chromatography-mass spectroscopy chromatograms of limeberry fruit ethanol extract are shown in Figure 1 and the bioactive compounds are shown in Table 2. The presence of some alkaloid compounds, such as piperidine alkaloids ([1,3,4]Thiadiazol, 2-amino-5-(2-piperidin-1-ylethyl) and 1,1'-Methylenebis(3-methylpiperidine)) was observed in the limeberry fruit ethanol extract. A previous study reported that piperidine belongs to the alkaloids group and the important feature of true piperidine alkaloids is monocycle compounds with the C5N nucleus.[25] Additionally, 1,6-dehydropinidine (a piperidine alkaloid) is present in *Picea abies* extract, as identified by using GC-MS.[26] This limeberry fruit ethanol extract also contains some polyphenol compounds, such as flavonoids (4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl and 7H-Furo[3,2-g][1]benzopyran-7-one,4,9-dimethoxy). Previous studies reported that 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl (DDMP) and 7H-Furo[3,2-g][1]benzopyran-7-one,4,9-dimethoxy also known as isopimpinellin (furano-coumarin), belong to flavonoid group.[27, 28]

Functional group of limeberry fruit ethanol extract using FT-IR

The FT-IR spectra of limeberry fruit ethanol extract are shown in Figure 2 and the functional groups are shown in Table 3. The absorption of limeberry fruit ethanol extract at 3265.1 cm^{-1} indicates the presence of a hydroxyl (O-H) group and also indicates the presence of polyphenol compounds.[29, 30] The wavenumbers 1760 to 1600 cm^{-1} and 1230 to 1140 cm^{-1} was recognized as the C-O stretching vibration absorption bands and it indicate the presence of phenol compounds. Additionally, that C-O stretching at ~ 1200 cm^{-1} due to the C–O of pyran is typical of flavonoid C-rings.[31, 32]

Table 1: Phytochemicals composition of limeberry (*T. trifolia*) fruit ethanol extract

Phytochemicals	Results
Alkaloids	+
Flavonoids	+
Tannin	+

Note: +, presence in the extract.

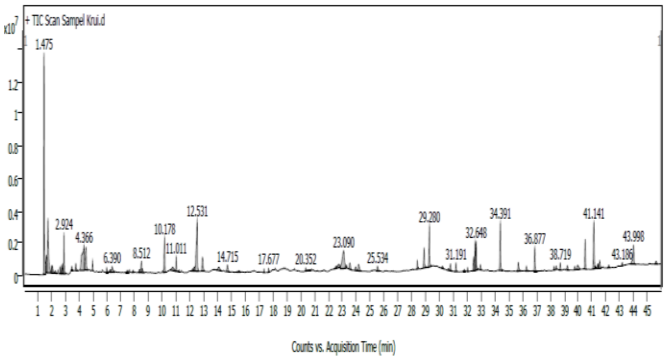

Figure 1: Chromatogram of GC-MS of limeberry (*T. trifolia*) fruit ethanol extract.

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Table 2: The bioactive compounds of limeberry (*T. trifolia*) fruit ethanol extract based on the GC-MS chromatograms

Compounds	Formula	Retention time (min)
Acetic acid	$C_2H_4O_2$	1.755
[1,3,4]Thiadiazol, 2-amino-5-(2-piperidin-1-ylethyl)	$C_9H_{16}N_4S$	4.983
4H-Pyran-4-one, 2,3-dihydro-3,5 dihydroxy-6-methyl	$C_6H_8O_4$	5.998
1,1'-Methylenebis(3-methylpiperidine)	$C_{13}H_{26}N_2$	8.358
terpinen-4-ol	$C_{10}H_{18}O$	11.011
Caryophyllene	$C_{15}H_{24}$	17.348
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	29.280
cis—Vaccenic acid	$C_{18}H_{32}O_2$	32.648
7H-Furo[3,2-g][1]benzopyran-7-one,4,9-dimethoxy	$C_{11}H_{10}O_5$	34.391

Table 3: The functional groups of limeberry (*T. trifolia*) fruit ethanol extract

Functional groups	Wavelength (cm^{-1})
O-H stretching	3265.1
C-H asymmetric and symmetric C-H stretching vibrations of sp3 hydrocarbons	2929.7
C=O Carbonyl group	1632.6
C-O ether	1252.2 – 1013.8

Antibacterial activity

The antibacterial activity of limeberry fruit ethanol extract against *S. aureus* is shown in Figure 3. In this present study, the bacterial inhibition significantly ($p<0.05$) increased when the dose of extract also increased, with a value range from 6.52 mm to 11.94 mm. Whereas, positive and negative control were about 20.92 and 0 mm, respectively. These results indicated that the bioactive compounds in the limeberry fruit ethanol extract exhibit antibacterial activity. According to the data from phytochemical analysis, identification of bioactive compounds, and functional group analysis, the ethanol extract contains piperidine alkaloids. A previous study reported that epidihydropinidine (a piperidine alkaloids) from *Picea abiesi* exhibits antibacterial activity against some bacteria, including *S. aureus*, *Bacillus cereus*, *Salmonella enterica*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis.*[33] The limeberry fruit ethanol extract also contains polyphenol compounds, such as flavonoid and tannin, that are also involved in its antibacterial activity. A previous study reported that a flavonoid from some Rubiaceae plants exhibits antioxidant activity against *S. aureus* and *Escherichia coli.*[34] Flavonoid exerts antibacterial activity by some mechanisms, such as decreasing cytoplasmic membrane function, nucleic acid synthesis, and energy metabolism.[35] Additionally, tannin compounds also reported their ability to destroy cell bacteria by passing through the cell wall of bacteria up to the internal membrane and then interference with cell metabolism.[36] A previous study also reported that tannin extract from *Neolamarckia cadamba* fruit successfully inhibits *S. aureus* bacteria.[37]

Conclusion

In this present study, the bioactive compounds from limeberry (*T. trifolia*) fruit ethanol extract were successfully extracted using a 96% ethanol solvent. The yield of extraction is about 22.5%. This extract contains various bioactive compounds, including piperidine alkaloid and some polyphenol compounds such as flavonoid and tannin. This extract exhibits antibacterial activity against *Staphylococcus aureus* with a diameter of inhibition zone of about 11.94 mm at a 25% extract concentration. Therefore, this extract has potential use as an antibacterial agent.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

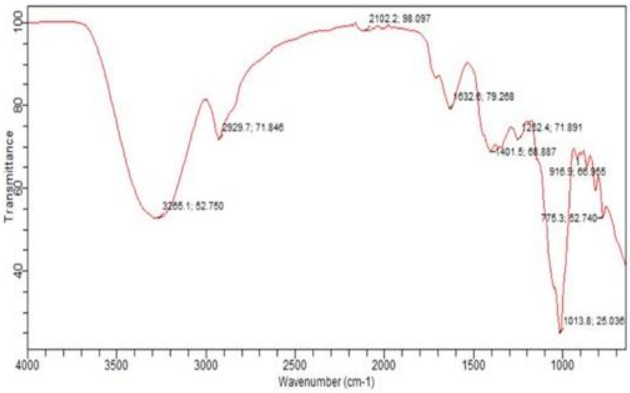

Figure 2: FT-IR spectrum of limeberry (*T. trifolia*) fruit ethanol extract.

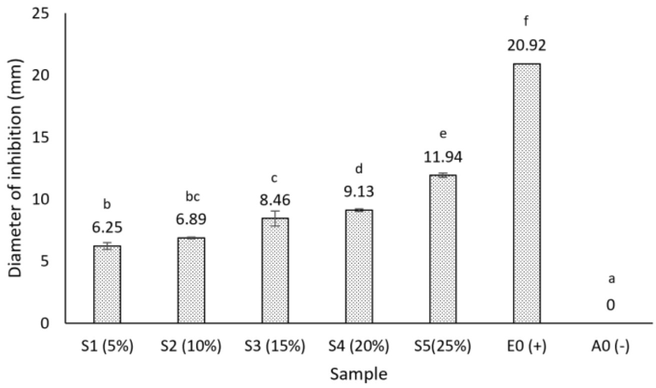

Figure 3: The diameter of inhibition zone of limeberry (*T. trifolia*) fruit ethanol extract against *S. aureus*. E0(+), 96% ethanol as positive control; A0(-), distilled water as negative control.

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