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Preliminary Study on Antioxidant and Antibacterial Activity of Kaffir Lime (*Citrus hystrix* DC) Leaf Essential Oil

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Abstract: This preliminary study aimed to examine the antioxidant and antibacterial activity of kaffir lime leaf essential oils (KLLEOs) in response to different growing locations and post-harvest handling. This study tested 9 samples, i.e., 8 KLLEOs with variation in growth location and post-harvest handling, and 1 standard citronellal solution. Thin layer chromatography was used to test the antioxidant and antibacterial activity of samples. Eluent in a mixture of hexane: ethyl acetate (5: 1) was chosen because it could provide a clear separation effect under visible light and after being sprayed with vanillin sulfate. KLLEOs originated from brown and green leaf powder had a stronger antioxidant activity than other samples. The sensitivity of KLLEOs to *E. coli* was lower than *S. aereus*, as indicated by fewer stains observed in *E. coli* glass plates rather than *S. aereus* ones. Standard citronellal compound at a concentration of 5 l ml^{-1} showed no antioxidant activity and at a concentration of 10 l ml^{-1} showed no antibacterial activity against both *E. coli* and *S. aereus.*

Keywords: Citronellal; DPPH; *Escherichia coli*; Post-harvest; *Staphylococcus aureus*.

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INTRODUCTION

The kaffir lime (*Citrus hystrix* DC.) is a functional citrus plant with a high potential to develop as essential oil, aside from its main role as a condiment. Condiments from the citrus family vary, e.g., the leaf of kaffir lime (Budiarto *et al.*, 2021a), the fruit of limau (Budiarto *et*

al., 2017), lemon and lime. Kaffir lime can be easily distinguished from another citrus because it has *bifoliate* leaves consisting of the main blade and a winged petiole that resembles an imaginary lower leaf blade (Budiarto *et al.,* 2021b; Budiarto *et al.,* 2021c). The development of kaffir lime as essential oil has been initiated by local communities in Tulungagung regency, Indonesia (Budiarto *et al.,* 2019). The commercial value of kaffir lime leaf essential oil lies in its various uses, including fragrances, cosmetics, and pharmaceuticals (Murni *et al.,* 2017). In the pharmaceutical field, kaffir lime essential oil can be used as an antioxidant and antibacterial agent.

Antioxidant compounds play an important role in counteracting free radicals that enter the body and further endanger human health. Consumption of foods rich in natural antioxidants needs to be increased to maintain the health of the human body (Katalinic *et al.,* 2006). In addition to natural antioxidants, there are also synthetic antioxidants. However, natural antioxidants are more in demand than synthetic products because of the higher trust in natural ingredients, which have generally been used by previous generations (Maestri *et al*., 2006).

Antibacterial compounds can be used to inhibit bacterial growth (bacteriostatic) and even kill bacteria (bactericide) (Jawetz *et al.,* 1996). Antibacterial compounds can be obtained from natural ingredients (natural products) or from the synthesis of the pharmaceutical industry, which is often referred to as antibiotics. The use of antibiotics as antibacterial substances also negatively affects the emergence of bacterial resistance to drug activity. Therefore, the use of natural ingredients that are useful as an antibacterial is strongly recommended.

The antioxidant and antibacterial activity of kaffir lime essential oil is influenced by the composition of the oil. Differences in plant parts used as raw materials indicate differences in antioxidant and antibacterial activity. Kaffir lime oil from fresh fruit peel has the strongest antioxidant and antibacterial activity compared to twig oil and leaf oil of kaffir lime. This is due to the higher content of monoterpene hydrocarbons (pinene, sabinene, myrcene, limonene) in fruit peel oil compared to leaf and twig oils (Warsito *et al.,* 2017). The citronellal content which is much lower in fruit peel oil compared to leaf oil actually has the strongest DPPH antioxidant and antibacterial activity against *Escherichia coli* (Warsito *et al.,* 2017). Variability of environmental conditions and post-harvest handling has been shown to affect the composition and levels of chemical compounds that make up kaffir lime leaf essential oil (Efendi *et al.,* 2021; Wulandari *et al.,* 2019). Therefore, this study aimed to test the antioxidant and antibacterial activity of kaffir lime leaf essential oils with different growing locations and post-harvest methods.

METHOD

Testing of antioxidant and antibacterial activity was carried out at the Laboratory of Natural Product Chemistry, Research Center for Biology, Indonesian Institute of Sciences (LIPI) Cibinong in April 2019. This test was carried out on 9 types of samples consisting of 8 types of kaffir lime leaf essential oils and 1 standard solution of citronellal compounds. The eight types of kaffir lime essential oils (KLLEOs) were KLLEOs originated from fresh leaves from Bogor (i), Pasuruan (ii), Tulungagung (iii), West Bandung (iv), shaded fresh leaves from Bogor (v), green leaf powder, (vi) brown leaf powder (vii), and brown dried leaf (viii).

Essential oil samples were prepared for thin layer chromatography (TLC) analysis. The essential oil sample was dissolved using hexane to a concentration of 5 µl ml^{-1}. The TLC plate used was a silica gel G60F254 aluminium plate from Merck. The sample was spotted on a TLC plate and then eluted using hexane: ethyl acetate (5: 1) as eluent in a glass vessel. After that, the TLC plate was observed under UV light with wavelengths of 254 nm and 366 nm to check whether any stains appeared. The TLC plate was then sprayed with a staining solution, namely vanillin sulfate and photographed.

The antioxidant test procedure initiated by preparing KLLEOs a concentration of 5 µl ml^{-1}. The used TLC plate for antioxidant testing was an aluminium plate of the type silica gel G60F254 from Merck. The sample was spotted on a TLC plate and then eluted using hexane: ethyl acetate (5 : 1) as eluent in a glass vessel. After that, the TLC plate was observed under UV light with wavelengths of 254 and 366 nm to check whether any stains appeared. After that, the plates were sprayed with 0.2% DPPH solution evenly. The plate became purple while the active point of the sample showing antioxidant activity against DPPH radicals became yellowish white.

Antibacterial testing began with the sterilization of tools and materials. Heating-resistant glassware was sterilized in an oven at 160 ^oC for 2 hours. Bacterial culture media, cotton and aquadest were sterilized by autoclaving at 121 ^oC for 20 minutes. The test was carried out in an aseptic laminar flow that had previously been irradiated with a UV lamp for 5 minutes. The bacterial culture medium used was Mueller Hinton Broth (MHB). MHB was weighed as much as 0.63 g and then dissolved in 30 ml of tap water. The media was stirred to make it homogeneous and then sterilized by autoclave at a temperature of 121 ^oC and a pressure of 1 atm for 20 minutes. Two bacteria were planted in the culture, namely *Staphylococcus aureus* (*S. aereus*) and *Escherichia coli* (*E. coli*). Each type of bacteria was inoculated into the MHB culture medium by using inoculating needle. Inoculation was carried out in an aseptic laminar flow. The media was then incubated in a shaker incubator for 18 hours at 37 ^oC. Bacteria that grew on the media were counted using the Mac Farland solution absorption approach ($BaCl_2$ 1% + H_2SO_4 1%) and the same number of *S. aureus* and *E. coli* was obtained, i.e., 10^9 CFU ml^{-1}. The bacterial suspension was re-diluted with MHB until the number of bacteria was about 10^5 CFU ml^{-1}. To formulate a new bacterial suspension with a volume of 100 ml, 0.1 ml of previous bacterial suspension solution was needed.

Previously, the TLC plate had been prepared by spotting the sample on the TLC plate and then eluting it using a mixed eluent (hexane : ethyl acetate = 5 : 1) in a glass vessel. The TLC plate prepared for antibacterial testing differed from the TLC plate for antioxidants, which was the glass base. A bacterial suspension (10^5 CFU ml^{-1}) was poured into a stainless tray that had been pre-sterilized with 75% alcohol. Next, the TLC plate was dipped in the bacterial suspension and placed into another sterile stainless tray. The TLC plates were arranged in a manner not to touch each other and each side of the TLC plate was given moist cotton to maintain relative humidity during incubation. The incubation process was carried out in an incubator for 18 hours at 37 ^oC. After that, the cotton was removed and the plate was sprayed by using Iodonitrotetrazolium (INT) indicator solution. The *S. aureus* antibacterial test sample immediately showed an active stain, while the *E. coli* antibacterial test sample need to re-incubated for about 90 minutes. Samples that have antibacterial ability were

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identified from the white stain on the purplish red background of TLC plate (Das et al., 2010).

RESULTS AND DISCUSSION

Thin layer chromatography (TLC) is one of the chromatographic analysis techniques to determine the separation of compounds in a complex mixture, using a stationary phase of TLC plate and a mobile phase of eluent. Determination of the best eluent for KLLEOs was obtained by trial and error. The eluent in the form of a mixture of hexane and ethyl acetate in a ratio of 5:1 showed a clearer separation and the separation was evenly distributed on the plate rather than the ratio of 1:1 (Figure 1). If the stationary phase and the mobile phase are correctly selected, the separation of sample components will be clear enough to observe. A mixture of hexane and ethyl acetate in a ratio of 5:1 was then used for TLC in antioxidant and antibacterial study

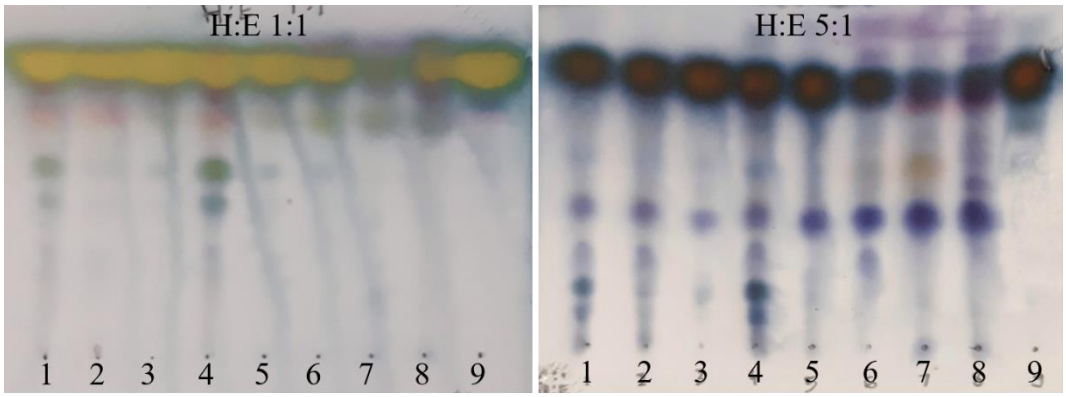

Figure 1. The results of thin layer chromatographic elution of kaffir lime leaf essential oil using an eluent in the form of a mixture of hexane and ethyl acetate in a ratio of 1:1 (A) and a ratio of 5:1 (B), under visible light after spraying with vanillin sulfate.

Note: 1-KLLEOs originated from fresh leaves from Bogor, 2-KLLEOs originated from fresh leaves from West Bandung, 3- KLLEOs originated from fresh leaves from Pasuruan, 4-KLLEOs originated from fresh leaves from shaded fresh leaves from Bogor, 5-KLLEOs originated from fresh leaves from Tulungagung, 6-KLLEOs originated from green leaf powder, 7-KLLEOs originated from brown leaf powder, 8-KLLEOs originated from brown dried leaf, 9-Standard citronellal solution

The results of antioxidant bioautography showed some yellowish white spots between the purple background on the aluminium foil TLC plate. The chromatogram pattern of KLLEOs sample could only be seen under visible light after spraying with DPPH (Figure 2A), however, it was not visible under UV light at 366 nm (Figure 2B), implied that there was no fluorescent compound in samples. Yellowish white stains seen after spraying DPPH indicated antioxidant activity. The stain was most clearly seen in the KLLEOs originated from brown leaf powder and KLLEOs originated from green leaf. This finding implied that the milling process could increase the antioxidant activity of the kaffir lime leaf. The stain was also observed in KLLEOs originated from brown

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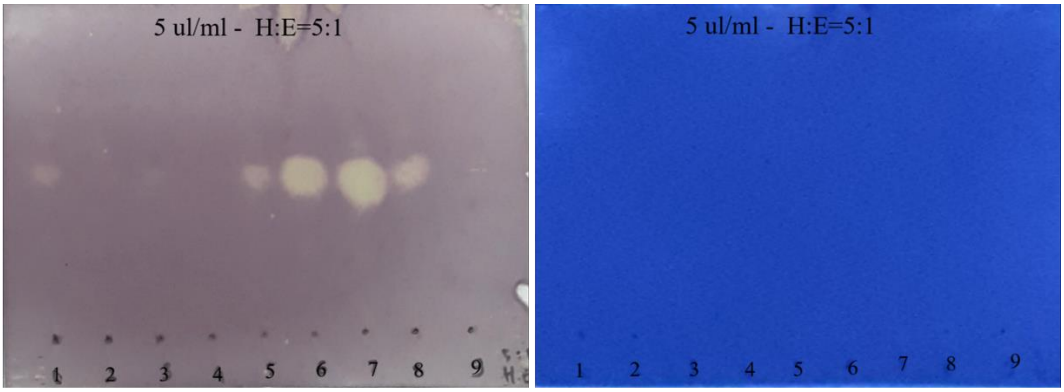
dried leaf, KLLEOs originated from fresh leaves from Tulungagung and Bogor. There were no stains on standard citronellal solution, implied that no antioxidant activity recorded. This could be due to the low concentration of the standard citronellal compound used, i.e., 5 l ml^{-1}. The IC_{50} of citronellal for the inhibition of DPPH free radicals is 10.1 l ml^{-1} (Warsito *et al.*, 2017).



(A) (B)

Figure 2. The stain on aluminium foil plate with hexane and ethyl acetate 5:1 mobile phase under visible light after spraying with 0.2% DPPH (A) and under UV light at 366 nm (B)

Note: 1-KLLEOs originated from fresh leaves from Bogor, 2-KLLEOs originated from fresh leaves from West Bandung, 3- KLLEOs originated from fresh leaves from Pasuruan, 4-KLLEOs originated from fresh leaves from shaded fresh leaves from Bogor, 5-KLLEOs originated from fresh leaves from Tulungagung, 6-KLLEOs originated from green leaf powder, 7-KLLEOs originated from brown leaf powder, 8-KLLEOs originated from brown dried leaf, 9-Standard citronellal solution

The results of antibacterial bioautography were varied in response to different growing location and post-harvest treatment. All samples showed antibacterial activity, as evidenced by white stains (zone of bacterial inhibition), except for the standard citronellal (Table 1). The low concentration of citronellal compound, i.e., 10 l ml^{-1}, was thought to be the cause of the absence of white stains on the TLC plate. The minimum inhibitory concentration (MIC) against *E. coli* of the citronellal was 25 l ml^{-1} (Warsito *et al.*, 2017). The antimicrobial activity of kaffir lime leaf essential oil was assumed to be increased by interaction of several compounds. In similar to that argument, the previous study on *Eucalyptus citriodora* oil showed the synergistic effect of citronellal and citronellol behind the presence of antimicrobial activity (Low *et al.*, 1974).

This study also showed that the sensitivity of KLLEOs to *E. coli* was lower than that to *S. aereus*. The number of stains on the glass plate with *E. coli* suspension was 17 stains, lower than those with *S. aereus* suspension, i.e., 23 stains (Table 1). The difference in bacterial sensitivity was thought to be related to differences in the structure and bacterial cell wall composition. *S. aereus* which is a Gram-positive bacteria has a cell wall with an outer layer of peptidoglycan which makes it easier for antibacterial compounds to pass. In opposite, *E. coli* is a Gram-negative bacteria that has a cell wall

with an outer layer of lipopolysaccharide that does not easily pass antibacterial compounds (Denis *et al.*, 2010).

Table 1. The number of stains on glass plate with hexane and ethyl acetate 5:1 mobile phase under visible light after spraying with Iodonitrotetrazolium (INT) for testing the antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*

No	Sample	The number of stains	
		E. coli	*S. aereus*
1	KLLEOs originated from fresh leaves from Bogor	3	1
2	KLLEOs originated from fresh leaves from West Bandung	3	1
3	KLLEOs originated from fresh leaves from Pasuruan	2	1
4	KLLEOs originated from shaded fresh leaves from Bogor	3	1
5	KLLEOs originated from fresh leaves from Tulungagung	1	3
6	KLLEOs originated from green leaf powder	2	3
7	KLLEOs originated from brown leaf powder	2	7
8	KLLEOs originated from brown dried leaf	1	6
9	Standard citronellal solution	0	0
Total stains		17	23

The finding of present preliminary study need to be followed up with an analysis of the metabolite profile of each KLLEOs sample, and strengthened by in-depth antioxidant and antibacterial analysis in the future.

CONCLUSION

The differences in growing locations and post-harvest handling caused a variation in term of antioxidant and antibacterial activity of KLLEOs. KLLEOs originated from brown and green leaf powder had a stronger antioxidant activity than others. The sensitivity of KLLEOs to *E. coli* was lower than *S. aereus*, as indicated by less stains observed in *E. coli* glass plate rather than *S. aereus* ones. Standard citronellal compound at a concentration of 5 l ml^{-1} showed no antioxidant activity and at a concentration of 10 l ml^{-1} showed no antibacterial activity against both *E. coli* and *S. aereus*.

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