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ANTIBACTERIAL ACTIVITY OF KAFFIR LIME LEAVES (*Citrus hystrix*) ON SELECTED FOODBORNE PATHOGENS

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One of the major problems that cause harm to humans and food spoilages is the microorganism contaminating the food. The survival of the microbes in food is a crucial issue which can lead to the spoilage and reduce the quality of food products and also cause harm to human if they are being ingested (Celiktas *et al*., 2007). Food poisoning is usually caused by ingesting food containing microbes that can be harmful to humans. These microbes would usually produce toxins and also gas in which will give a bad side effect on humans. It was reported that in Malaysia the cases of food poisoning increase by the rate of 62.47 cases in 100,000 people (Soon *et al*., 2017). Centers for Disease Control and Prevention (CDC), 2017, had stated that foodborne diseases are caused mainly by bacteria, parasites, and viruses in which *Salmonella* and *S. aureus* are on the top list for bacteria that can cause illness via food. Kaffir lime leaves or in Bahasa Melayu known as "daun limau purut", is a type of common aromatic plant in Malaysia that people used in cooking as an ingredient and have been reported to contain many benefits (Wongpornchai, 2012). It belongs to the citrus family and has the potential to inhibit biofilm produced by *Streptococcus mutans* (Kooltheat *et al*., 2016). Besides, according to Srifuengfung *et al*. (2020), antibacterial oral sprays which are derived from kaffir lime leaves oil and kaffir lime fruit oil was proven to be effective against respiratory tract pathogens. Thus, the initial work presented here focused on the antibacterial activity of kaffir lime leaves extracts on selected foodborne pathogens namely *Salmonella typhi* ATCC 14028, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 11778, and *Escherichia coli* ATCC 25922.

Kaffir lime leaves were washed first before being left dried in an oven at 30°C until a constant weight was obtained. Then, the dried leaves were grounded by using a blender into powder form. As for the preparation of extract, 200 g of powdered leaves were soaked with 500 mL of distilled water for 5 days and were kept inside a refrigerator to preserve the samples. Next, the soaked leaves were boiled for 1 hr and then filtered by using a filter paper (Whatman no 4.) before placing them in a freezer at -20°C. Later, the samples were placed in a freeze dryer, and extracts obtained were prepared into concentrations of 150 mg/mL, 200 mg/mL, 250 mg/mL by mixing the powdered extract with distilled water. Next, the agar disc diffusion method and broth microdilution method were performed according to the Clinical and Laboratory Standard Institute (CLSI, 2019). The 24 hr bacterial cultures were placed inside culture bottles that contained saline and were compared with 0.5 McFarland standard ($\pm$ 1.0 $\times$ 10^8 cfu) (Hardy Diagnostics) and 0.5 mL bacteria suspensions were spread on MHA (Mueller Hinton agar, Oxoid CM0337). Next, 6 mm blank paper discs (Oxoid antimicrobial susceptibility test disc) were dipped into the kaffir lime leaves extract and were placed on the agar and incubated overnight at 37°C. Then, the agar plates were observed for clear inhibition zones and the diameter of inhibition zones was measured (Yildrim *et al*., 2017). The antibiotic Gentamicin disc (CN,10, Oxoid) was used as a positive control and distilled water was used as a negative control. This was repeated three times to obtain mean $\pm$ SEM and data was analyzed by one-way ANOVA (Sample size, n=48) (IBM SPSS Statistic 22, 2013). The broth microdilution method was used to determine the minimum inhibitory concentrations (MICs). An amount of 0.5 mL of sterile MHB (Mueller Hinton broth, SRL 49550(MM0210)) was added into the wells of the microtiter plate (HmbG PO458 Microplate 96 wells Flat Bottom) and 0.5 mL of the kaffir lime leaves extract was added into the first well. Serial dilution was performed from the first well to the eighth well. The ninth well was used as a control. Then, 0.2 mL of the bacteria suspension was added to all of the wells and then incubated overnight.

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Table 1. Mean ± SEM of the diameter of inhibition zone (mm) of KLLE on foodborne pathogens

Sample	Concentration (mg/mL)	Zones of inhibition (mm)			
		S. typhi	*S. aureus*	*B. cereus*	*E. coli*
KLLE	150	7.33 ± 0.33	7.33 ± 0.33	NIZ	6.83 ± 0.17
	200	7.67 ± 0.33	7.83 ± 0.44	NIZ	7.00 ± 0
	250	7.67 ± 0.33	8.17 ± 0.17	NIZ	7.67 ± 0.33
	300	8.67 ± 0.67	8.17 ± 0.17	NIZ	8.33 ±0.88
Gentamicin (+)	–	16	20	21	17
dH_2O (–)	–	NIZ	NIZ	NIZ	NIZ

KLLE: Kaffir lime leaves extract, NIZ: No inhibition zone.

Table 2. MIC (mg/mL) and MBC (mg/mL) of KLLE on foodborne pathogens

	S. typhi	*S. aureus*	*B. cereus*	*E. coli*
MIC KLLE (mg/mL)	150	100	–	100
MBC KLLE (mg/mL)	150	100	–	100
MBC/MIC ratio	1	1	–	1

–: Not available.

Next, resazurin dye was added into all of the wells and incubated for 24 hr. The growth of bacteria was observed by the color changed from dark blue to pink. The lowest concentration of extract without growth was taken as MIC (Hastey *et al*., 2017). Minimum bactericidal concentration (MBC) was determined by sub-culturing the identified MIC content onto the MHA (Mueller Hinton Agar, Oxoid CM 0337) and was incubated for 24 hr. The agar plates with no bacterial growth were taken as the MBCs.

Results (Table 1) showed that at concentration 150 mg/mL clear inhibition zones were observed against *S. typhi, S. aureus* and *E. coli* are 7.33 ± 0.33 mm, 7.33 ± 0.33 mm, and 6.88 ± 0.17 mm of mean values of the diameter of inhibition zone respectively while at a concentration of 200 mg/mL, the mean values of the diameter of inhibition zone obtained are 7.67 mm ± 0.33 mm against *S. typhi,* 7.83 ± 0.44 mm against *S. aureus* and 7.00 ± 0 mm against *E. coli*. At a concentration of 250 mg/mL, clear inhibition zones were observed against *S. typhi, E. coli,* and *S. aureus* with mean values of the diameter of inhibition zone were 7.67 ± 0.33 mm, 7.67 ± 0.33 mm, and 8.17 ± 0.17 mm in diameter respectively. At a concentration of 300 mg/mL, the highest mean value of diameter was obtained against *S. typhi* with the value of 8.67 ± 0.33 mm followed by *S. aureus* and *E. coli* in which the values were at 8.33 ± 0.33 mm and 8.33 ± 0.88 mm in diameter. Unfortunately, there were no inhibition zones observed against *B. cereus.* As for the MICs and MBCs (Table 2), 150 mg/mL were obtained against *S. typhi,* 100 mg/mL against *S. aureus* and *E. coli.* If the MBC/MIC ratio ≤ 4, the effect of the extract will be taken as bactericidal, but if the MBC/MIC ratio is ≥ 4, the effect of the extract will be taken as bacteriostatic (Levison, 2004; Thomas *et al*., 2012; Mogana *et al*., 2020). Thus, this extract is bactericidal to *S. typhi, E. coli,* and *S. aureus*. According to Yasurin (2015), compounds identified in kaffir lime leaves were terpenoids, monoterpenes, oxygenated monoterpenes, sesquiterpenes, citronellic acid, nerolidol, δ-cadinene, citronellal, and citronellol. These compounds were responsible for kaffir lime leaves exhibiting antimicrobial properties. This was supported by Siripongvutikorn *et al*. (2015) which found that the extract of kaffir lime leaves showed antibacterial activity against *S. aureus* as well as on *E. coli* in which the diameter of the inhibition zone was less than 9 mm. As a conclusion, kaffir lime leaves extract exhibited antibacterial activity on *S. aureus, S. typhi,* and *E. coli* except on *B. cereus* with the most effective was demonstrated against *S. typhi* at 300 mg/mL concentration with 8.67 ± 0.67 mm diameter of inhibition zone. The increase in extracts concentration can also increase the antibacterial activity of the extracts.

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