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Antibacterial Properties of Essential Oil Extracted from Kaffir Lime (*Citrus Hystrix*) Peel

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Citrus hystrix (C. hystrix), locally known as 'limau purut,' is Malaysia's major commercial fruit harvest. Besides being a common ingredient in Asian cuisines, the extracted essential oil has diverse applications. The essential oil can be extracted from peels or leaves, which could give strong aromatic properties. The main purpose of this study is to evaluate the antibacterial activity of C. hystrix essential oil by using disc diffusion method. C. hystrix essential oil was extracted from the peels using the Clevenger apparatus of hydro-distillation method. The antibacterial activity of essential oil was evaluated by agar disc diffusion method against four strains of bacteria: two Gram-positive bacteria (Staphylococcus aureus ATCC 25923 and Staphylococcus epidermidis ATCC 12228) and two Gram-negative bacteria (Escherichia coli ATCC 25922 and Shigella dysenteriae ATCC 13313). Each assay was done in triplicates. In this research, positive controls gentamicin and streptomycin were used as indicators to prove the research's validity. The Gram-positive bacteria were more susceptible to the essential oil (average zone of inhibition diameter; S. aureus = 19.3 ± 1.5 mm and S. epidermidis = 19.3.0 ± 0.6 mm) as compared to the Gram-negative bacteria (average zone of inhibition diameter; E. coli = 8.3 ± 0.6 mm and S. dysenteriae = 11.7 ± 0.6 mm). Gentamicin was recorded to be most effective against all tested bacteria (more than 15 mm zone of inhibition diameter). However, only S. epidermidis showed resistance to streptomycin. C. hystrix essential oil was found to possess antibacterial activity. Thus, these findings indicated that C. hystrix essential oil could be developed as an antibacterial agent in various applications.

Keywords: Antibacterial; Citrus Hystrix Peel; Disc Diffusion, Essential Oil, Hydro-Distillation.

Antibacterial activity of any substance can be defined as its ability to either inhibit (bacteriostatic) or kill (bactericidal) the bacteria. It is highly beneficial to the human body in counteracting skin infection and related diseases (Chaudhari, 2016). Recently, microbial infections have become one of the most severe health problems at the international level, as the established conventional antibiotics have been reported to lose their effectiveness against pathogens (Das *et al*., 2013). Arise with the situation, plants have attracted great interest as part of pharmaceutical development and medication since they display wide therapeutic actions (Dulay & De Castro,

2016). The plant components, especially fruits and leaves, have antibacterial components that reduce bacterial growth (Agudo et al., 2007). In addition, the presence of active secondary metabolites from plants has been beneficial as antibacterial and antioxidant agents, which can contribute significantly to the pharmaceutical industry (Dulay & De Castro, 2016).

Kaffir lime (*Citrus hystrix)*, which belongs to the family Rutaceae, is a tropical plant with reported antibacterial activity. Locally in Malaysia, *C. hystrix* is known as 'limau purut'. Several parts of *C. hystrix,* such as peel, leaf, and fruit, may contain medicinal properties (Srisukh *et al*., 2012). The essential oil extracted from *C. hystrix* was a concentrated aromatic colourless, and strong odour (Giacoma & Giacoma 2002). According to Sreepian (2019), chemical composition from *C. hystrix'*s fruit was reported to possess antibacterial activity after being screened for various pharmacological activities. Due to its great potential in commercial applications is also commonly used as an essential ingredient in aromatherapy (Anuja *et al*., 2020). Furthermore, the essential oil obtained from the plant's fruits and leaves are widely used in cosmetics and pharmaceutical industries due to is numerous bioactivities, such as antibacterial, antioxidant, and anti-inflammatory (Tsai *et al*., 2011).

Until recently, there has been limited research on the antibacterial activity of *C. hystrix* extracts on a broad range of pathogenic bacteria. Previous research from Srisukh (2012) found that *C. hystrix* peel essential oil (CHPEO) has shown great antibacterial activity against multi-resistant bacteria, including the respiratory pathogen. Therefore, this research focused on investigating the antibacterial activity of *C. hystrix* peel extract's essential oil on Gram-positive and Gram-negative bacteria strains. According to Sabulal (2016), the *in vitro* antibacterial activity can be performed using the modified Kirby-Bauer disc diffusion method as it is the easiest standard reference method.

MATERIALS AND METHODS

Plant Materials

The fresh fruits of *C. hystrix* were purchased from a market located in Ipoh, Perak, Malaysia. The fruits were washed under running tap water. The peel was separated and cut into 4-5 mm size dice, dried in an oven for at least 5 days at 50 °C, grinded to a fine powder, and then processed through an extraction step using the hydro-distillation method.

Extraction of Essential Oil

About 100 g of the *C. hystrix* peels were soaked in the distilling flask containing 600 ml of distilled water. A Clevenger-type apparatus was used for the hydro-distillation method. The hydro-distillation process ran for 3 hours, and temperature was controlled below 100 °C to avoid excess evaporation. After the distillation process, the oily layer was separated using dried anhydrous sodium sulphate from the distillate. In this study, the percentage yield of extracted CHPEO was 8.34% (w/v). Extracted CHPEO was stored at 4 °C and protected from light until used (Abdallah, 2016). A stock solution of CHPEO was prepared at a 400 mg/mL (v/v) concentration in DMSO before being used.

Proximate Analysis

The parameters used for proximate analysis included total ash value, acid-insoluble ash, water-soluble extractives, alcohol-soluble extractives, and loss on drying (Bhargava *et al*., 2013).

Total Ash

Two grams (2 g) of *C. hystrix* peel powder was cremated at 450 °C. The ash was left in a desiccator for 20 minutes to let it cool. The total ash had been calculated by measuring the differences between crucible containing ash and empty crucible.

Acid-Insoluble Ash

The ash is mixed with 25 ml of dilute HCl, covered with a watch-glass, and boiled for 5 minutes. The insoluble matter was collected using an ashless filter paper and washed with hot water until the filtrate was neutral to collect all residue. The solution was ignited until it reached a constant weight.

Water-soluble Extractive Value

Four grams (4 g) of *C. hystrix* peel powder was mixed with 100 ml of distilled water. The mixture was regularly shaken for 24 hours. After 24 hours, the solution was filtered. About 25 ml of the collected filtrate was heated in the evaporating dish until a damp mass formed. Continuously, the evaporating dish was directly cooled and weighed.

The upper part, which was the solid content, was discarded.

Alcohol-soluble Extractive

Four grams (4 g) of *C. hystrix* peel powder was mixed with 100 ml of methanol. The mixture was regularly shaken for 24 hours intervals. After 24 hours, the solution was filtered. About 25 ml of the collected filtrate was heated in the evaporating dish until a damp mass formed. Continuously, the evaporating dish was cooled and weighed. The upper part, which was the solid content, was discarded.

Loss on Drying

Two grams (2 g) of *C. hystrix* peel powder was dried in the oven at 105 °C for 3 hours. The weight was obtained every 1 hour until it reached a constant weight. After 3 hours, the sample was cooled in a desiccator for 15 minutes.

Phytochemical Screening

Qualitative Analysis

The essential oils of the *C. hystrix* peel were subjected to qualitative phytochemical analysis for flavonoids, alkaloids, tannins, phenols, and terpenoids by following standard procedures.

Tannin Test

In a small centrifuge tube, about 0.5 ml of essential oil of *C. hystrix* peel was mixed with 0.1 ml 5% $FeCl_3$. The appearance of dark blue colour indicated the presence of tannins.

Flavonoid Test

0.1 ml essential oil was mixed with NaOH in a small centrifuge tube. The appearance of yellow fluorescence showed the presence of flavonoids.

Alkaloid Test

In Mayer's test, 0.1 ml of essential oil was mixed with 0.1 ml HCl. Then, two drops of Mayer's reagents were added to the solution. A creamy white precipitate confirmed the presence of alkaloids.

Quinone Test

In a small centrifuge tube, 0.1 ml of essential oil was mixed with 0.1 ml H_2SO_4. The appearance of a reddish colour indicated the presence of quinone.

Phenol Test

The test was performed by adding a few drops of 10% $FeCl_3$ in a tube containing 0.5 ml distilled water. Then, 0.1 ml of essential oil was mixed into the solution. The formation of blue or green colour showed the presence of phenolic compounds.

Saponins Test

In a small centrifuge tube, 0.3 ml of essential oil was mixed with 0.6 ml of distilled water. The mixture was shaken for at least 15 minutes. A formation of foam at 1 cm layer indicated the presence of saponins.

Terpenoids Test

In this test, 0.1 ml essential oil was mixed with 0.5 ml H_2SO_4. A few drops of acetic anhydride were added to the mixture. An appearance of a reddish-brown colour indicated the presence of terpenoids.

Quantitative Analysis

The essential oils of the *C. hystrix* peel were subjected to the determination of Total Phenolic Contents and Total Flavonoid Contents by following standard procedures (Biswash, 2020).

Total Phenolic Content

Total Phenolic Content (TPC) was determined using the Folin-Ciocalteu (FC) method. About 0.5 ml of dilute Folin-Cioca reagent (FCR) solution was added to 1 ml CHPEO. After five minutes of incubation, 1.5 ml of prepared Na_2CO_3 solution was added and incubated for one and half hours in the dark at room temperature. The absorbance was measured at 725 nm using a UV spectrophotometer. TPC was expressed as µg of Gallic acid equivalent per mg (GAE/mg) of plant extract. Each assay has been performed in triplicates.

Total Flavonoid Content

$AlCl_3$ colourimetric method was used to determine Total Flavonoid Content (TFC). 1 ml of CHPEO was added to a test tube containing 0.3 ml of 5% $NaNO_3$ solution. The mixture was allowed to remain for five minutes. After that, about 0.5 ml of 10% of $AlCl_3$ solution was mixed with the solution, followed by the addition of 0.5 ml of NaOH solution. The absorbance was taken at 510 nm using the UV-Visible spectrophotometer. TFC was expressed as µg of Quercetin equivalent per mg (QE/mg) of plant extract. Each assay was performed in triplicates.

Screening of Antibacterial Properties

Bacteria

The bacterial organisms determined in this study consisted of four bacterial strains from American Type Culture Collection (ATCC). The

bacterial strains were composed of Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* ATCC 12228) and two Gram-negative bacteria (*Escherichia coli* ATCC 25922 and *Shigella dysenteriae* ATCC 13313). These bacteria were obtained from the laboratory of UniKL-RCMP. The bacteria were cultivated in a solid medium of Nutrient Agar (NA) plates by streaking method and stored at 4 °C.

Preparation of Culture Media (Agar)

Mueller Hinton (MH) agar was used for the antibacterial test. 13.3 g of MH powder was dissolved in 350 ml of distilled water and autoclaved at 121 °C for 20 minutes. Once the agar cooled to 45 °C, the medium was poured into a sterile disposable petri dish. The pouring plate process was operated in the laminar airflow and left at room temperature until solidified. The prepared plates were put in upside-down positions and kept in the refrigerator.

Preparation of Bacterial Suspension

A single colony of each bacteria culture in the plate for Gram-positive and Gram-negative were transferred in 10 ml of MH using a sterile loop. The prepared bacterial suspension was incubated for 3 hours at 37 °C to achieve the exponential phase of 0.5 McFarland Standard (Abdallah, 2016). Each bacterial suspension was subjected to turbidity check using a single-beam spectrophotometer at 600 nm. A 0.5 McFarland Standard has an absorbance reading of 0.08 to 0.1. All bacterial suspensions were directly used in the antibacterial activity assay.

Agar Disc Diffusion

Agar disk diffusion was performed to screen for *in vitro* antibacterial activity of CHPEO as previously described (Sabulal *et al*., 2016) with some modifications. The bacterial suspension was spread homogeneously onto the surface of MH agar using a sterile cotton swab. Sterilized disks (6 mm) impregnated with 20 µl of CHPEO were placed on the surface of each plate. In addition, gentamycin and streptomycin disks (10 µg and 120 µg respectively) were included as positive controls, and 20 µl of 1% DMSO impregnated disc was used as a negative control. The plates were incubated for 24 hours at 37 °C. The inhibition zone diameter (IZD) of CHPEO was measured and interpreted using the following criteria: no activity, IZD = 6 mm; weak activity, 6 mm < IZD < 12 mm; moderate activity, 12 mm < IZD < 20 mm; and strong activity, IZD > 20 mm (Lv *et al*., 2011).

Table 1. Proximate Analysis

No.	Experimental studies	Experimental value (%)
1	Total ash value	8%
2	Acid insoluble ash value	6.25%
3	Water soluble extractive value	32%
4	Alcohol soluble extractive value	21%
5	Loss on drying	14.5%

RESULTS AND DISCUSSION

Proximate Analysis

The proximate analysis provided valuable information on the plant composition that helped to assess the CHPEO sample (Table 1).

Ash value designates the presence of remaining inorganic residues after heating. The value provides the minerals' amount as they cannot be destroyed by heating (Vidita *et al*., 2013). Less extractive values indicate that there is an addition of exhaustive materials due to incorrect processing during the drying process. Alcohol soluble extractive value is also indicated for the same purpose as the water-soluble extractive value. This study showed that the constituents in *C. hystrix* peel were more extracted and soluble in water than alcohol with 32% and 21%, respectively.

Phytochemical Analysis

Qualitative and quantitative analyses are essential to identify phytochemical constituents present in the herbal plant. Therefore, phytochemical

Table 2. Phytochemical Analysis of CHPEO

No	Test	Extracted Sample		
		1	2	3
1	Tannin	-	-	-
2	Flavonoid	-	-	-
3	Alkaloid	-	-	-
4	Quinone	+	+	+
5	Phenols	-	-	-
6	Saponins	+	+	+
7	Terpenoid	+	+	+

(+) indicates positive results, (-) indicates negative results

screening was conducted to evaluate the active phytoconstituents in CHPEO.

Qualitative Analysis

Phytochemicals are biologically active, where the secondary metabolites may exert antimicrobial properties (Aziman *et al*., 2012). The overall results of the phytochemical analysis are shown in Table 2.

The presence or absence of phytochemicals was confirmed by observing colour intensities when reacting with different reagents. According to Barile *et al.* (2007), saponins are active constituents which are known for their antimicrobial activity, a trait which is desirable for disease resistance in plants.

Quantitative Analysis

TPC and TFC are listed in Tables 3 and 4, respectively. All determinations were carried out in triplicates, and the values were expressed as mean (Biswash, 2020).

TPC was quantified for essential oil extraction by Folin-Ciocalteu (FC) method using Gallic acid as standard. TPC for essential oil was equivalent to 23.904 ug/mg Gallic acid equivalent. The reaction between the Folin-Ciocalteu reagent and the CHPEO resulted in blue colour formation, which absorbed the radiation and allowed quantification.

TFC was quantified for essential oil extraction by the $AlCl_3$ colourimetric method using Quercetin as standard. TFC content for essential oil was equivalent to 145.767 ug/mg Quercetin equivalent. The colour formation was light blue with a small layer of separation. According to a study by Francisco (1995), it was emphasized that the conversion from yellow colour to orange colour was regarded as a marker for flavonoid content. The current results indicated that CHPEO extraction did not contain flavonoid contents.

Antibacterial Activity of CHPEO by Agar Disk Diffusion

Antibacterial activity of CHPEO against the bacterial strains, clinically isolated Gram-positive bacteria and Gram-negative bacteria are shown in Table 5. The results demonstrated various antibacterial activities of CHPEO against four selected bacterial strains. Overall, moderate to the strong antibacterial activity of CHPEO was observed in *S. aureus* and *S. epidermidis*, while weak antibacterial activity was observed in *E. coli* and *S. dysenteriae.*

The results demonstrated that CHPEO displayed moderate to strong antibacterial activities against Gram-positive bacteria [*S. epidermidis* (Figure 1) and *S. aureus* (Figure 2)]. This is an interesting finding as compared to the Gram-

Table 3. Total Phenolic Content Determination

Absorbance	Total Phenolic Content (ug GAE/mg)	Observation
0.211	23.904	Blue

Table 4. Total Flavonoid Content Determination

Absorbance	Total Flavonoid Content (ug QAE/mg)	Observation
2.332	145.767	Light blue

Table 5. Antibacterial activity of CHPEO against bacterial strains

Bacteria	Agar disk diffusion; IZD (mm)			
	CHPEO[a]	Gentamicin	Streptomycin	1% DMSO
Staphylococcus aureus	19.3 ± 1.5	15 ± 0.0	13 ± 1.0	ND[b]
Staphylococcus epidermidis	19.3 ± 0.6	50.7 ± 0.6	ND[b]	ND[b]
Escherichia coli	8.3 ± 0.6	23.7 ± 0.6	17.7 ± 0.6	ND[b]
Shigella dysenteriae	11.7 ± 0.6	27.3 ± 0.6	24.7 ± 0.6	ND[b]

Values are expressed as mean±SD of triplicate experiments.

[a]Interpreted criteria of antibacterial activities: IZD = 6 mm is no activity (N), 6 mm < IZD < 12 mm is weak activity (W), 12 mm < IZD < 20 mm is moderate activity (M), and IZD > 20 mm is strong activity (S) (Lv et al., 2011)

[b]ND – not determined. Negative control (1% DMSO) indexes were not determined when the inhibition zone was not presented (IZD = 6 mm).

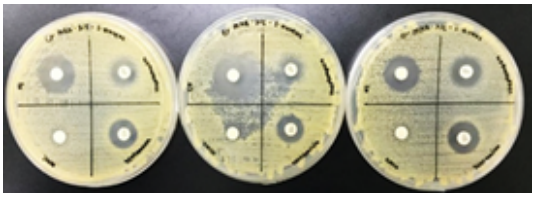

Fig. 1. Zone of inhibition against *S. aureus*

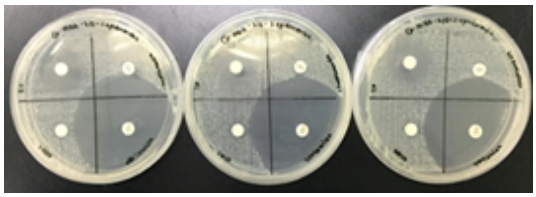

Fig. 2. Zone of inhibition against *S. epidermidis*

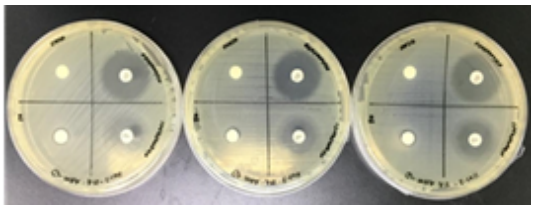

Fig. 3. Zone of inhibition against *E. coli*

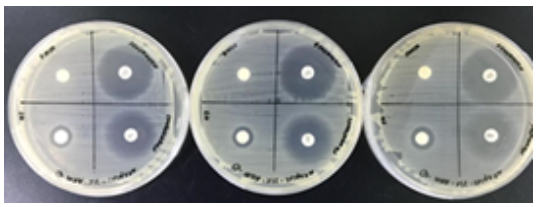

Fig. 4. Zone of inhibition against *S. dysenteriae*

negative bacteria, these Gram-positive bacteria have a thicker peptidoglycan layer in their cell walls which usually makes them more resistant towards antibacterial agents (Katas *et* al., 2019; Venkatpurwar and Pokharkar, 2011). In addition, the highest potency of CHPEO could be observed for S. aureus compared to the positive controls (gentamicin and streptomycin). The susceptibility of the bacteria towards CHPEO could be attributed to the presence of several phytochemical constituents such as terpenoid, quinone and saponins. The results obtained were also in line with the previous study, which stated that the essential oil containing terpenoids and having phenol and alcohol functionalities showed higher bactericidal activity against Gram-positive and Gram-negative bacteria (Lo Cantore *et* al., 2009). Both gentamicin and streptomycin, which are positive controls, generated moderate inhibition for bacterial growth. For *S. epidermidis,* the bacteria were more susceptible to only one positive control, which was gentamicin. However, the bacteria showed resistance against streptomycin antibiotics. Thus, this proved that CHPEO has antibacterial activity against the genus *Staphylococcus*.

For Gram-negative bacteria, CHPEO displayed weak antibacterial activity against *E. coli* (Figure 3) and moderate antibacterial activity against *S. dysenteriae* (Figure 4). The results obtained were probably due to the presence of a cell wall in Gram-negative bacteria, which prevented passage of hydrophobic molecules, thus increasing resistance to biological activities imposed by the essential oils and their components (Nazzaro *et* al., 2013). Furthermore, it could be observed that the bacteria were more susceptible to the positive controls than the plant extracts as gentamicin and streptomycin generated potent inhibition by having greater diameter sizes.

Data obtained in this study were similar to the previous study by Sreepian *et al.* (2019). In their study, CHPEO displayed moderate antibacterial activity against *S. aureus* and weak antibacterial activity against *E. coli.* In the present study, Gram-positive bacteria showed higher sensitivity to the plant extracts compared to Gram-negative bacteria. This could be explained as Gram-negative bacteria contain complex components in their cell wall compared to Gram-positive bacteria (Nazzaro *et al.,* 2013). The presence of the lipopolysaccharide layer in Gram-negative bacteria could limit the permeability of essential oil in penetrating the bacterial cell (Chimmnoi *et al.,* 2018).

Apart from that, all bacteria tested were affected by the positive control, gentamicin (*S. aureus*; IZD 15 ± 0.0, *S. epidemidis*; IZD 50.6 ± 0.6, *E. coli*; IZD 23.7 ± 0.6, and *S. dysenteriae*; IZD 27.3 ± 0.6). A standard susceptible zone diameter of inhibition for gentamicin is e”15 mm, and this study reported equal and higher values for all bacterial strains (Table 5). Meanwhile, only *S. epidermidis* were resistant against streptomycin antibiotic, in which no antibacterial activity was observed in all three replicates. Collectively, CHPEO displayed

promising results as a potent antibacterial agent, with its ability to inhibit both Gram-positive and Gram-negative bacteria.

CONCLUSION

Our findings demonstrated that CHPEO exerted antibacterial activities against a broad range of bacterial organisms, thus implying that CHPEO has the potential to be developed as an effective antibacterial agent. Therefore, CHPEO, which is less toxic to humans than other existing antibiotics, would be able to reduce unnecessary antibiotic use and subsequently decrease the probability of antibiotic resistance development. Further evaluations on the mode of action of CHPEO, potential synergistic effects with essential oil from other herbal plants, as well as *in vivo* adverse effects, are needed further to develop CHPEO as a new alternative antibacterial agent.

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Conflict of Interest

We declare that we have no conflict of interest.

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