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Research Article

PRELIMINARY EVALUATION ON THE ANTIBACTERIAL ACTIVITIES OF *CITRUS HYSTRIX* OIL EMULSIONS STABILIZED BY TWEEN 80 AND SPAN 80

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ABSTRACT

A study was conducted to evaluate the antibacterial activities of the emulsions containing essential oil of Kaffir lime, *Citrus hystrix*. The emulsions were formulated according to the information obtained from the constructed phase equilibriums consisting of water, Tween 80, Span 80 and hexane. Stability tests were carried out within the following three weeks to determine the emulsions' stability index, followed by antibacterial tests. Emulsion with surfactant mixture of Tween 80 and Span 80 at the ratio of 90:10 with 2% essential oil had the most effective antibacterial properties.

Keywords: Kaffir lime oil, Emulsion, Antibacterial activity

INTRODUCTION

Citrus hystrix or commonly known as Kaffir lime is a common edible herb species of family Rutaceae that can be found everywhere within South East Asia. Besides playing an important role in the South East Asian cuisine, the oil from the leaves and the fruits are used commercially in Malaysia as flavour and fragrance agents, as well as in perfumery and medicinal preparation. The chemical constituents of the Kaffir lime peel oil are mostly monoterpene hydrocarbons, with limonene (30.73%) and β-pinene (18.76%) as the major components, whereas the minor components are terpinene-4-ol (10.63%), α-terpineol (8.35%), γ-terpinene (6.18%), α-terpinene (5.09%) and terpinolene (4.33%) [1].

In general, essential oils are a rich source of biological active compounds. This is because essential oils have very complex natural mixtures which can contain 20 to 60 components. Therefore, they have been used widely as bactericides, virucides, fungicides, anti-parasites, and insecticides in various applications, especially in the pharmaceutical, sanitary, cosmetic, food and agricultural industries[2]. Whilst the applications of Kaffir lime are expanding in several industries, studies have been conducted to examine the biological activities exhibited by its essential oil. The studies showed that Kaffir lime oil has effective bactericidal effect on bacterial strains such as *Propionibacterium acnes*[3], 20 serotypes of *Salmonella*[4] as well as other bacteria that cause skin diseases such as *Bacillus subtilis, Staphylococcus epidermis, Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*[5].

Due to the Kaffir lime's pleasant aroma and vast biological properties, it has become a great source of economic potential. It is a common component in perfumery and toiletries like shampoo, body wash and lotion. These products fall in a colloidal category known as the emulsion[6]. An emulsion is a dispersion of two immiscible liquids, generally referred to oil and water. These two phases may form some sort of dispersion under intense agitation, whereby fine droplets of one phase were dispersed into the other phase. However, without any stabilizing agent, such dispersion is not stable. The droplets will coalesce and eventually the two phases will be separated, and such action normally starts immediately once the agitation stopped[7]. This is due to the differences in the polarity between the aqueous and non-aqueous solution as well as the high interfacial energy between the two phases. In order to enhance the dispersion process and to stabilize the emulsion, emulsifying agents are added, where the role of emulsifiers in the system is to rectify this instability[8].

With much attention focusing on the essential oil of Kaffir lime, it is thought that a study on formulating emulsions containing the essential oil of Kaffir lime with antibacterial properties will be interesting. In a preliminary investigation, stability of oil-in-water (O/W) emulsions containing the essential oil of Kaffir lime prepared from systems containing water, hexane, Tween 80 and Span 80, was examined. After the stability test, the emulsions were tested for their antibacterial properties against three bacterial strains namely *E. coli*, *B. subtilis* and *S. aureus*.

MATERIALS AND METHOD

Materials

For the non-ionic surfactants, polyoxyethylene (20) sorbitan monooleate (Tween 80) (95%) were purchased from Sigma; Sorbitan monooleate (Span 80) (>95%) was purchased from Sigma-Aldrich; n-hexane was purchased from PC Laboratory; beeswax was purchased from Aldrich and Mueller-Hinton agar was purchased from Merck. All components were directly used without further purification. The essential oil of Kaffir lime was obtained as reported in previous studies[9,10]. Double distilled-water was used throughout the study.

Preparation and stability tests of emulsions

For the preparation of emulsions, inversion technique was applied. The surfactant and the beeswax were first heated to 70°C and mixed. The mixture of hexane and essential oil was added as the solution's temperature has dropped to 50°C. Based on the report that 1% essential oil effectively inhibited bacterial test strains[5], higher percentage of *C. hystrix* oil (2% by weight) was chosen. When the mixture has reached homogeneity, water previously heated to 50°C was added. It was then homogenized and the mixture was stirred slowly until the temperature dropped to 35°C. The emulsion samples were then placed in a cool dark place for storage purpose. For the stability test, the samples were observed daily in order to detect any changes or separation of phases for a period of three weeks.

Antibacterial tests of *C. hystrix* oil and oil emulsions

In the antibacterial test, disc diffusion method or also known as the Kirby-Bauer method was applied using Mueller-Hinton agars as the nutrient agar. The agars were swabbed separately with approximately 100µL of each test strain bacteria: *E. coli*, *B. subtilis* and *S. aureus*, and left to dry. Antibacterial tests for *C. hystrix* essential oil and oil emulsions were carried out separately, where discs with diameter of 6.0 mm were soaked in essential oil of 0.5, 1.0, 1.5 and 2.0% (by weight), and in the oil emulsions, respectively. The discs were then placed gently onto the agars containing the cultured bacteria. For positive control, ampicilin was used as standard. The Petri dishes were then sealed and incubated at 37°C for 24 hours. After incubation, the inhibition zones produced by different discs

were measured, where it represented the sample's ability in inhibiting the growth of the targeted bacteria. By comparing the diameters of their inhibition zones with that of ampicillin, the strength of the antibacterial activity of the samples can be categorised into four levels namely strong (>16mm), good (11-16mm), weak (7-10mm) and none (<7mm).

RESULTS AND DISCUSSION

Formulations and stability of *C. hystrix* oil emulsions

During the preparation of emulsions, the compositions of water, hexane, Tween 80 and Span 80 were based on the phase equilibriums constructed previously[9,10]. The percentage of beeswax was maintained at 10% where it acted as an effective thickening agent and 2% (by weight) essential oil was used to obtain optimum antibacterial performance. From the phase equilibriums, composition points at 60% of water, 4% of hexane and 36% of surfactants were selected to formulate the O/W emulsion since it has the lowest possible content of surfactant and hexane. Furthermore, it was within the region that is in equilibrium with the liquid crystalline phase; consequently this would increase the stability of the emulsions since liquid crystalline phase is a very stable association structure[11]. The formulations of the emulsion system are shown in Table 1.

Table 1: The formulations of *C. hystrix* emulsion systems

Ingredient	Percentage by weight (%)			
	A3	B3	C3	D3
Beeswax	10.0	10.0	10.0	10.0
Essential oil	2.0	2.0	2.0	2.0
Tween 80	31.7	-	28.5	23.8
Span 80	-	31.7	3.2	7.9
Hexane	3.5	3.5	3.5	3.5
Water	52.8	52.8	52.8	52.8

By substituting the surfactant from Tween 80 to Span 80 (A3 and B3), and with the mixtures of both surfactants at the ratio of 90:10 and 75:25 (C3 and D3, respectively), it was found that the emulsions' viscosity and texture had changed after three weeks of storage (Figure 1). As the content of Span 80 increased (C3 and D3), the viscosity of the emulsion increased as well. Furthermore, when Span 80 alone was used (B3), the emulsion appeared to be cream-like while the rest appeared to be lotion-like.

In terms of stability, all emulsions did not undergo separation after three weeks of storage regardless of which surfactants or at which ratios of surfactants were used (Table 2).

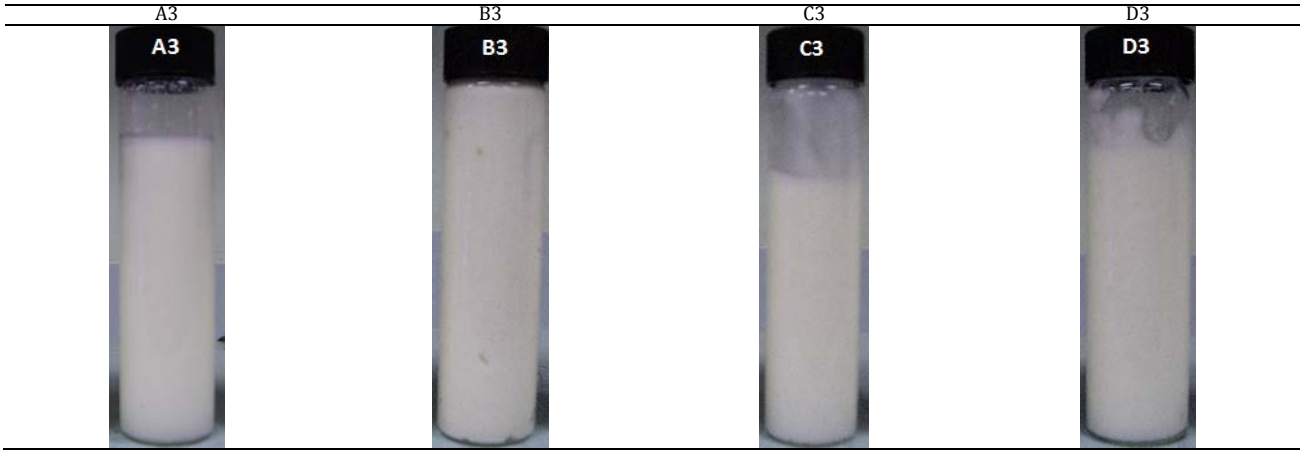

Fig. 1: The appearance of the *C. hystrix* emulsions after three weeks of storage

Table 2: Stability of *C. hystrix* emulsions within three weeks of storage

	A3	B3	C3	D3
Day 1	√	√	√	√
Day 3	√	√	√	√
Day 5	√	√	√	√
Day 7	√	√	√	√
Week 2	√	√	√	√
Week 3	√	√	√	√
Appearance	Milky white	Milky white cream	Milky white	Milky white
Stability Index	1.00	1.00	1.00	1.00

Antibacterial activity of *C. hystrix*

The results on the inhibition zone caused by the essential oil were tabulated in Table 3. In general, the Kaffir lime oil was effective in inhibiting the growth of these bacterial strains, which is consistent with the finding by another study[5]. The effective concentration obtained in this study however differs; Kongtun and Sarcherdkaiti[5] showed that the effective concentration of Kaffir lime oil that inhibited these bacterial strains was at 1%, whereas the effective percentage of essential oil obtained in this study is at 2.0% against

all three bacteria. Apart from higher concentration, this is also probably due to the variability of the essential oil constituents, as it may differ according to their geographical origin[2].

Antibacterial susceptibility tests against *E. coli*, *B. subtilis* and *S. aureus* were conducted after the emulsions had undergone stability test. In order to ensure that the emulsions produced had sufficient activity to inhibit bacterial growth, the percentage of the essential oil usage was maintained at 2% by weight in the formulation of the emulsions. Table 4 shows that all emulsions inhibited the growth of all three bacterial strains.

Table 3: The inhibition zones produced by the pure essential oil of *C. hystrix* at different concentration

[Essential oil] (% by weight)	Diameter of the inhibition zone (± 1.0 mm)		
	E. coli[a]	*B. subtilis*[b]	*S. aureus*[c]
0.5	-	-	-
1.0	7.0	11.0	-
1.5	7.0	12.0	6.0
2.0	16.0	15.0	9.0

[a] inhibition zone of ampicilin = 11.0 mm, [b] inhibition zone of ampicilin = 10.0 mm, [c] inhibition zone of ampicilin = 8.0 mm

Table 4: The inhibition zones produced by *C. hystrix* oil emulsions

Emulsions	Diameter of the inhibition zone (± 1.0 mm)		
	E. coli[a]	*B. substilis*[b]	*S. aureus*[c]
A3	17.0	8.0	9.0
B3	11.0	9.0	9.0
C3	18.0	8.0	11.0
D3	14.0	7.0	10.0

[a] inhibition zone of ampicilin = 13.0 mm, [b] inhibition zone of ampicilin = 8.0 mm, [c] inhibition zone of ampicilin = 10.0 mm

In the case of *E. coli*, formulations A3, C3 and D3 were able to strongly inhibit the bacterial growth as the zones produced are larger than ampicillin where their diameters are 17.0 mm, 18.0 mm and 14.0 mm, respectively, compared to only 13.0 mm by ampicillin. Meanwhile, formulations A3, B3 and C3 effectively inhibited the growth of *B. subtilis* with inhibition zones of 8.0 mm, 9.0 mm and 8.0 mm, respectively. Only emulsions C3 and D3 were successful in preventing the growth of *S. aureus* effectively with inhibition zones of 11.0 mm and 10.0 mm, respectively. Overall, formulation C3 had the most effective antibacterial property as the inhibition zones produced by the formulation are bigger or similar to that of ampicillin against all three bacterial strains. On the other hand, the results also showed that antibacterial activities of the emulsions were not influenced by the concentration of the surfactant. This is consistent with a similar study conducted to examine the effect of surfactant type on the antibacterial activity of *Ocimum gratissimum Linn*. emulsion where non-ionic surfactant had no significant effect on the antibacterial activities[12].

CONCLUSION

From this study, it may be concluded that composition point at 60% of water, 4% of hexane and 36% of Tween 80 and/or Span 80 is suitable to formulate a stable O/W emulsion. The emulsions with at least 2% of the essential oil of Kaffir lime proved to be able to inhibit the growth of *E.coli*, *B. subtilis* and *S. aureus* at a similar effect as ampicillin. Emulsion with surfactant mixture of Tween 80 and Span 80 at the ratio of 90:10 with 2% essential oil had the most effective antibacterial properties.

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