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Evaluation of Physicochemical and Antifungal Properties of Microemulsions of Lemongrass Oil (*Cymbopogon citratus*) and Clove Oil (*Syzygium aromaticum*)

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ABSTRACT

Onychomycosis is a nail infection caused by the fungus *Trychophyton rubrum*. Current treatments are sometimes ineffective due to poor patient compliance during the long duration therapy, besides their undesired side effects. Owing to the potency of some natural ingredients as antifungals, topical herbal formulations may be an alternative treatment as they can be applied directly to the infected nails providing an intra-dermal action with minimum side effects. This study aims to develop microemulsion formulations of lemongrass oil (*Cymbopogon citratus*) and clove oil (*Syzygium aromaticum*) at a concentration of 3%, each followed by an *in-vitro* antifungal activity evaluation against *T. rubrum*. The formula consists of Virgin Coconut Oil (oil phase), tween 80 – polyethylene glycol 400 (surfactant-cosurfactant), and water (aqueous phase) at various compositions plotted on a pseudo-ternary phase diagram as a platform for screening of the best microemulsion compositions. Only six from fifty-four selected compositions in the diagram (MA1, MB1, MC1, MD1, ME1 and MF1) produced transparent and homogenous solutions as an indication of microemulsion. They were subjected to evaluation procedures, including determination of interfacial tension, physical stability, transmittance, relative density, droplet size, pH, and viscosity. *In vitro* antifungal activity test of the six formulations against *T. rubrum* was conducted by disc diffusion method using Potato-Dextrose Agar. The inhibition zone of the antifungal test of the six formulations was in the range 7.05±0.07 to 8.85±0.21 mm, compared to itraconazole (positive control) with inhibition zone of 7.70±0.14 mm. The MD1, ME1 and MF1 formulations showed a better inhibition diameter than the positive control.

Keywords: Clove oil, Lemongrass oil, Microemulsion, Onychomycosis, Pseudo-ternary phase diagram, *Trychophyton rubrum*

Introduction

Fungal infections often occur in tropical countries like Indonesia. A tropical climate with high humidity supports fungal growth. One of the most common diseases is a fungal infection of the nails known as onychomycosis, which accounts for approximately 50% of all nail diseases.[1] *Trychophyton rubrum* (*T. rubrum*) is the dermatophyte that causes the majority of superficial fungal infections worldwide.[2]

Currently, available antifungal drugs for oral use include itraconazole, terbinafine, fluconazole, griseofulvin, and ketoconazole.[3] The nails are in the third compartment in the pharmacokinetic model where drug distribution is slower with low bioavailability, making oral drug administration less effective for this disease.[4] Further, prolonged oral therapy is more likely to cause side effects than topical use. Therefore, the use of herbal-based transdermal preparations can be an alternative because they can be applied directly to the infected nails to provide rapid therapeutic effects with minimal side effects.

A literature search shows that the chemical compounds in lemongrass essential oil have antifungal activity.[5,6] Lemongrass oil at a concentration of 100 µg/mL inhibits the growth of *C. albicans* mycelia. In addition, pre-clinical studies of ointments containing lemongrass oil show an inhibition effect on dermatophyte fungal infections caused by *T. rubrum* and *M. gypseum*.[5] While clove oil contains phenolic compounds such as carvacrol and eugenol which show strong fungistatic and fungicidal activities against *M. canis*, *M. gypseum*, *T. rubrum*, *T. mentagrophytes*, and *E. floccosum* isolated from nails and skin.[6]

The objectives of this study are to develop a microemulsion (ME) formulation of the lemongrass and clove essential oils by using an optimization technique and to evaluate the antifungal activity of the formulas against the growth of *T. rubrum*. Topical use of MEs for intradermal or transdermal purposes has gained increasing interest. [7] Drug-loaded MEs are suitable for antifungal compounds due to the advantage of the system that might eliminate the unfavorable physicochemical properties of most antifungal agents such as low solubility that lead to poor skin absorption or bioavailability.[4] In the treatment of onychomycosis, the oil phase in ME has double advantages as it does not only dissolves the nonpolar drug but also hydrates the skin, therefore increase penetration.

Materials and Methods

Lemongrass and clove essential oils were purchased from PT. Lansida (Indonesia); tween 80, polyethylene glycol (PEG) 400, Virgin Coconut Oil (VCO), Potato Dextrose Agar (PDA) media were from Bratachem® (Indonesia). Fungi *Trychophyton rubrum* (ATCC 28288) was obtained from the Faculty of Medicine, University of Indonesia (Indonesia). Sodium chloride (NaCl), dimethyl sulfoxide (DMSO), and ethanol were purchased from Merck® (Indonesia). *Itraconazole*

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capsules 100 mg were purchased from Bernofarm® (Indonesia). The chemicals used were of pharmaceutical or analytical grade.

Pseudo-ternary phase diagram

The proportion of the oil phase (VCO), surfactant (S) - cosurfactant (CoS) mixture (tween 80 - PEG 400), and the aqueous phase was determined by using a pseudo ternary phase diagram. The diagram was created by using CHEMIX School version 3.60. The ratio of tween 80-PEG 400 was predetermined using variations of (1: 1); (2: 1) and (3: 1), at various VCO : a mixture of tween 80 and PEG 400 proportions as in Table 1. The microemulsions were prepared by using the water titration method at a stirring rate of 700 rpm with a magnetic stirrer (IKA, Germany) for 30 minutes at a temperature of 70ºC. The occurrence of a ME system was characterized by the formation of a clear and transparent liquid. Furthermore, the ME formulations of lemongrass and clove oils were prepared by using tween 80-PEG 400 ratios of 2:1, which were mixed with VCO at the various ratios of 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1 (Table 2). Transparent ME formulations were subjected to evaluation procedures.

Table 1: Optimization of the ratio of S-CoS (tween 80 – PEG 400)

Formula	A	B	C
VCO + (S + CoS) (%)	20	20	20
Ratio S:CoS	1:1	2:1	3:1
	1:9	1:9	1:9
	2:8	2:8	2:8
	3:7	3:7	3:7
	4:6	4:6	4:6
Ratio VCO:(S + CoS)	5:5	5:5	5:5
	6:4	6:4	6:4
	7:3	7:3	7:3
	8:2	8:2	8:2
	9:1	9:1	9:1
Water (%)	80	80	80
Total	100	100	100

Table 2: Composition of ME bases at S-CoS ratio of 2:1

Formula	A	B	C	D	E	F
VCO + (S + CoS) (%)	20	30	40	50	60	70
	1:9	1:9	1:9	1:9	1:9	1:9
	2:8	2:8	2:8	2:8	2:8	2:8
	3:7	3:7	3:7	3:7	3:7	3:7
	4:6	4:6	4:6	4:6	4:6	4:6
Ratio VCO:(S + CoS)	5:5	5:5	5:5	5:5	5:5	5:5
	6:4	6:4	6:4	6:4	6:4	6:4
	7:3	7:3	7:3	7:3	7:3	7:3
	8:2	8:2	8:2	8:2	8:2	8:2
	9:1	9:1	9:1	9:1	9:1	9:1
Water (%)	80	70	60	50	40	30
Total	100	100	100	100	100	100

Evaluation of the ME bases

Determination of interfacial tension

The interfacial tension of the ME bases was measured using Du-Nouy Tensiometer.[8] The interfacial tension was calculated using the equation:

$$\sigma = \frac{\text{The force required to remove the ring in the dyne}}{2 \times \text{circumference of the ring}} \times \text{correction factor} \quad (1)$$

Measurement of turbidity

The degree of turbidity was measured by using Spectrophotometer UV-Visible (Genesys 10S) at a wavelength of 502 nm. Turbidity was calculated using the equation:

$$\text{Turbidity (\%)} = \frac{2.303 \times \text{Absorbance}}{\text{Cuvet width (cm)}} \quad (2)$$

Turbidity of lower than 1% indicated that the system is a transparent liquid.[9]

Relative density measurement [9]

The relative density of ME bases was determined using a pycnometer at 25°C. Relative density was calculated using the equation:

$$\rho = \frac{(W_2 - W_0)}{(W_1 - W_0)} \quad (3)$$

Where: W_2 = the weight of pycnometer filled with ME bases
W_1 = the weight of pycnometer filled with water
W_0 = the weight of an empty pycnometer

Physical stability test [10]

The physical stability tests performed are the mechanical test by centrifugation (Centrifuge PLC series) at 3000 RPM for 30 minutes and the freeze and thaw cycling test. For the latter, the ME bases were kept on storage at temperature -5°C for 24 hours and then at 25 °C for another 24 hours. The test was repeated for three cycles. The physical stability of the ME bases was observed.

Measurement of pH

The pH of microemulsions was measured by using a pH meter (Hanna Instrument, Germany) which was previously calibrated with standard buffer solution of pH 7 and 4, respectively. The electrode was immersed into the microemulsion and the pH value was observed. The measurement was done once a week during a six-week storage at room temperature.

Formulation of lemongrass and clove oils ME

Preparation of ME containing a combination of lemongrass and clove oils were made by adding the oil to the VCO and then mixing it with the S-CoS, water was added by titration as a method of preparation a ME base. The concentration of lemongrass and clove oils used were 3%, respectively, which is the concentration that showed antifungal activity against T. rubrum in a preliminary test. The transparent MEs were chosen and subjected to the same evaluation procedures applied for the bases, as well as the measurement of droplet size distribution, viscosity, and the antifungal activity by disc diffusion method.

Measurement of the droplet size distribution

The mean droplet size distribution was determined using dynamic light scattering Particle Size Analyzer *(Shimadzu SALD 2300,* Japan). The samples were diluted with distilled water before measurement.

Viscosity measurement

The viscosity of microemulsions was determined using Brookfield Viscometer (DV2T, USA)) attached with spindle number 3 at a speed of 100 rpm.

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Determination of antifungal activity

The antifungal activity of ME containing lemongrass and clove oils against *T rubrum* was determined using the disc diffusion method.[11] A suspension of *T rubrum* colony was prepared in 5 mL saline solution to obtain the turbidity level of equivalent to 0.5 McFarland standard units (10^6 CFU/ml), then homogenized using a vortex.[11] The suspension (0.1 mL) was inoculated into 3.9% PDA media in Petri dishes. Sterile disks were impregnated with 10 µL test compound in DMSO and placed over the media. Petri dishes were incubated at 20-25^{o}C for 5-14 days. Itraconazole was used as a positive control and DMSO as a negative control. The diameter of inhibition was measured in millimeters.

Statistical analysis

Data were expressed as mean ± standard deviation. The diameter of inhibition was analyzed by one-way ANOVA ($\alpha = 0.01$) to determine the significant differences between means followed by the Duncan Multiple Range Test at 5% significance level.

Results and Discussion

The ME formulations consist of three components, first is the essential oils in VCO as the oil phase, the second is a mixture of Tween 80 as the main surfactant strengthens with PEG 400 as co-surfactant and the third is water as the aqueous phase. The composition of the three components was plotted on a pseudo ternary phase diagram to screen the formulation composition that might produce ME. The development and optimization of ME formulation were carried out in two stages, first to determine the optimal proportion of S-CoS and second to obtain the proportion of oil: S-CoS: water. The results showed that from the variations of S-CoS ratio of 1:1, 2:1 and 3:1 in the oil to water ratio of 20: 80, the S-CoS 2:1 ratio produced clear and transparent ME bases compared to other ratios. Using this ratio, 54 formulas were generated based on the pseudo ternary phase diagram (Figure 1). Transparent solutions were obtained from the green zone in the diagram showing that ME had formed. The six microemulsion based formulas (A1, B1, C1, D1, E1, and F1) obtained contain (VCO + S-CoS) in the range of 20% - 70% and an aqueous phase between 80% - 30%, with the VCO : S-CoS ratio of 1: 9 (Figure 2). This means that it takes nine parts of S-CoS for 1 part of the oil, the high concentration of S-CoS at the interface solubilized the globules in the continuous phase resulting in a transparent and stable microemulsion.[7] ME bases were evaluated for interfacial surface tension, turbidity, relative density, pH value, and physical stability. The determination of the surface tension of ME bases showed a lower value than the surface tension of water (Table 3). This is due to the presence of surfactant and cosurfactant in the formulation solubilizing the globules, reducing the interfacial tension which results in a thermodynamically stable ME. All formulas showed a turbidity value <1% which indicates the transparent solution obtained.[9] ME base D1 showed higher interfacial tension and turbidity level than other bases, which was consistent with the appearance of a cloudy solution (Figure 2).

The antifungal compounds, lemongrass oil, and clove oil were formulated as MEs using A1 – F1 bases, each at a concentration of 3%. The essential oils were dissolved in VCO and mixed with other components in the same way as for the preparation of the ME base. Formulated MEs (Figure 3) were evaluated in terms of droplet size distribution, viscosity, % transmittance, relative density, pH value, and in vitro antifungal activity tests. The physicochemical properties of the essential oil ME formulations (Table 4) showed that the droplet sizes were in the range of 134.76-280 µm with an average diameter of 199.70 µm, not in the range of ideal ME globule sizes of 100-200 nm.[7] These results suggest that the transparent and stable dispersed system is likely the result of solubilization of oil in water or vice versa by the presence of S-CoS rather than ME formation. This fact may differ from one expected, but a transparent and physically stable preparation such as this formulation will be preferred in the treatment of onychomycosis. The transmittance of formula MB1, MC1, MD1, ME1, and MF1 was more than 95 %. The highest was 100.2% (MF1) (Table 4). An increase in the transmittance value indicates an increase in the clarity and homogeneity of the formulation. Data in Table 4 also showed an increase in the relative density with an increase in the amount of oil and S-CoS. The pH value is in the neutral to the weak alkaline range. The pH increases with the increase in the proportion of the oil phase. There was an increase in pH of all formulations during storage, indicating a decrease in H^+ concentration in the solution which might arise due to degradation reaction. The addition of a buffer solution can stabilize the pH of the formula during storage. The viscosity of formulations was in the range of 16 to 661 cP. It increased with the increase in the oil phase concentration. Thick solutions such as ME1 and MF1 (viscosity 527 and 661 cP) are preferred for topical application because they are easily applied to the surface of the skin and nails. Furthermore, viscous solutions will stay longer on the skin and nails' surface, which provides good penetration of the essential oils. Essential oils are good candidates for the treatment of nail infections because their main content, terpenes, has a low molecular weight that easily penetrates the nail surface and under the skin where the fungus grows.[6] The previous report shows that lemongrass oil or clove oil inhibits the growth of fungus that cause onychomycosis.[5] The in-vitro antifungal test showed that the ME formulations had shown antifungal activity against *T. rubrum* ATCC 28288 (Figure 4). The zone of inhibition of the MEs is in the range of 7.05±.07 to 8.85±0.21 mm, compared with 7.70±0.14 mm (itraconazole) as a positive control (Table 5). The one-way ANOVA test results show a significance of 0.002 ($P<0.01$) which means that the inhibition zone of each formulation is significantly different. The Duncan Multiple Range Test indicates that the inhibition zone diameter of MB1 and MC1 formulations are not significantly different from the positive control ($P > 0.05$). The MD1, ME1, and MF1 formulations show a better inhibition zone than the positive control ($P < 0.05$).

Table 3: The physicochemical properties of ME bases A1 – F1

No	Solution	Interfacial tension (dyne/cm)	Turbidity level (%)	Relative density (g/cm^3)	pH value	Physical stability*
1	Water	72.79 ± 1.19	n.d	n.d	n.d	n.d
2	A1	46.46 ± 0.87	0.39	0.955	6.72 ± 0.11	Stable
3	B1	46.46 ± 0.20	0.41	0.984	6.80 ± 0.07	Stable
4	C1	46.46 ± 0.69	0.47	0.988	6.84 ± 0.17	Stable
5	D1	49.29 ± 0.57	0.81	1.006	7.16 ± 0.30	Stable
6	E1	42.44 ± 1.36	0.45	1.012	7.50 ± 0.19	Stable
7	F1	41.05 ± 0.70	0.44	1.021	7.70 ± 0.46	Stable

Note: n.d = not determined; *by centrifugation and freeze and thaw method after 6 week storage

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Table 4: The physicochemical properties of the formulations containing lemongrass and clove oils 3% each

No	Formulation	Range of droplet size (µm)	Transmit-tance (%)	Relative density (g/cm^3)	pH value	Viscosity (cP)
1	MA1	134.76 – 280.00	59.4	1.0188	6.07 ± 0.28	16
2	MB1	147.66 – 255.54	98.7	1.0341	6.30 ± 0.18	25
3	MC1	134.76 – 280.00	98.8	1.0404	6.62 ± 0.40	52
4	MD1	134.76 – 280.00	99.8	1.0437	7.13 ± 0.41	100
5	ME1	134.76 – 280.00	99.5	1.0586	7.40 ± 0.32	527
6	MF1	134.76 – 280.00	100.2	1.0712	7.65 ± 0.17	661

Table 5: The diameter of inhibition of ME formulations against *T. Rubrum*

No.	Formulations	n	Diameter of inhibition (mm)
1	MA_1	2	7.05 ± 0.07 [a]
2	MB_1	2	7.50 ± 0.14 [a,b]
3	Positive control	2	7.70 ± 0.14 [b]
4	MC_1	2	7.80 ± 0.28 [b]
5	MD_1	2	8.45 ± 0.49 [c]
6	ME_1	2	8.55 ± 0.21 [c]
7	MF_1	2	8.85 ± 0.21 [c]

[a,b,c]: data with different superscripts showed significant differences ($P < 0.05$)

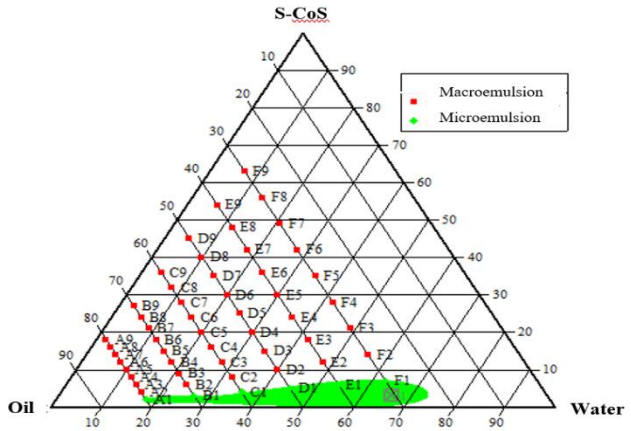

Figure 1: A pseudo ternary phase diagram of ME bases at various weight ratio of VCO : tween 80-PEG 400 : Water

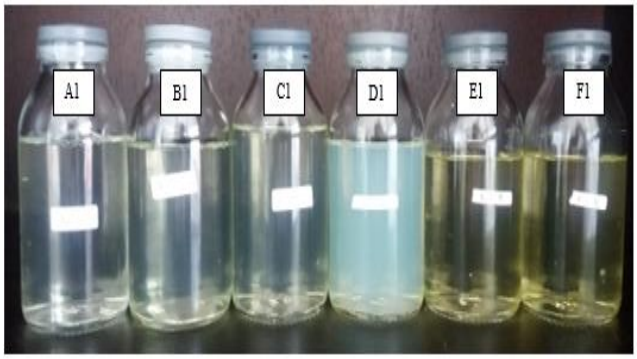

Figure 2: ME bases obtained from the composition of VCO : tween80-PEG400 : Water at the ME are of the pseudo ternary phase diagram

Figure 3: The formulations containing lemon grass (3%) and clove oil (3%), VCO as the oil phase, tween 80-PEG 400 as surfactant-cosurfactant and water

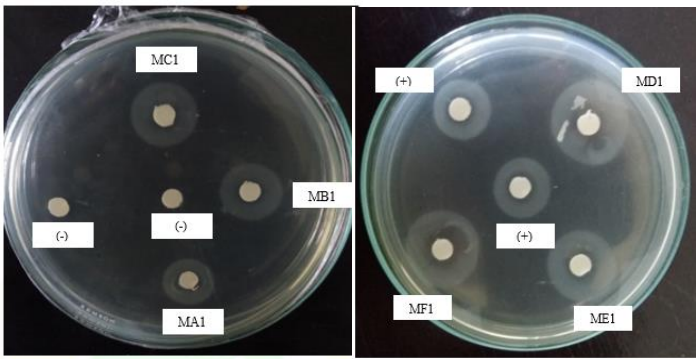

Figure 4: The *in vitro* antifungal activity of MA1, MB1, MC1 and negative control (left), MD1, ME1, MF1 and positive control (right) against *Trychophyton rubrum* ATCC 28288 using media PDA

Conclusion

Formulation of 3% lemongrass oil and 3% clove oil in microemulsion containing VCO : (tween 80-PEG 400): water with the proportion determined based on a pseudo ternary phase diagram, produces six formulas, namely A1 (2:18:80), B1 (3:27:70), C1 (4:36:60), D1 (5:45:50), E1 (6:54:40), and F1 (7:63:30) as transparent and physically stable solutions. The formulations have shown antifungal activity against *T. rubrum* ATCC 28288.

Conflict of interest

The authors declare no conflict of interest.

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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.

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