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Original Research Paper

Stability and solubility improvement of Sompoi (*Acacia concinna* Linn.) pod extract by topical microemulsion

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ABSTRACT

The aim of this study was to enhance the solubility and stability of *Acacia concinna* extract by loading in a microemulsion for topical application. Both physical appearance and biological activities of the extract-loaded microemulsion were determined in comparison with the extract solution. Pseudoternary phase diagrams of three oil types including tea seed oil, grape seed oil, and sesame oil, together with polysorbate 85 or the mixture of polysorbate 85 and sorbitan oleate as surfactants, and absolute ethanol as a co-surfactant were constructed to optimize the microemulsion area. The selected microemulsion was then characterized for droplet size, polydispersity index, and viscosity. Tea seed oil exhibited the highest microemulsion area in the phase diagram because it had the highest unsaturated fatty acid content. The microemulsion composed of tea seed oil (5%), polysorbate 85 (40%), ethanol (20%), and water (35%) exhibited Newtonian flow behavior with the droplet size and polydispersity index of 68.03 ± 1.09 nm and 0.44 ± 0.04, respectively. After 4% w/w of the extract was incorporated into the microemulsion, larger droplets size was observed (239.77 ± 12.69 nm) with a lower polydispersity index (0.37 ± 0.02). After storage in various conditions, both physical appearances and the stability of biological activity of the extract-loaded microemulsion were improved compared to the solution. Therefore, the *A. concinna* loaded microemulsion may be a promising carrier for further development into a topical formulation and clinical trials for pharmaceutical and cosmeceutical applications are also suggested.

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1. Introduction

Acacia concinna Linn. or Sompoi, belonging to the family *Fabaceae*, is widely grown in Southern and Southeast Asia for medicinal purposes [1,2]. Its pod has been recognized as a component in holy water used for paying respect to elderly people in many special festivals in Thailand, especially the Songkran festival [3]. The Indian Ayurvedic pharmacopeia stated that *A. concinna* pod has been used as an active ingredient in anti-dandruff shampoos [1]. The main chemical constituent of the pod is saponin (20.8%) which was responsible for antidermatophyte and antimicrobial properties [2]. An ointment containing *A. concinna* pod extract has been widely used as an antimicrobial for skin disorders. The ethanolic, ethyl acetate, and hexane extracts of the pod showed fungicidal activity against several dermatophytes including *Trichophyton rubrum*, *Trichophyton mentagrophyte*, *Microsporum nanum*, and *Epidermophyton floccosum* [2,4].

Our previous study indicated that *A. concinna* pod extracted by hydroethanolic maceration exhibited the highest antioxidant and antityrosinase activities among all the extracts due to its highest phenolic content [3]. Moreover, the safety profile on human peripheral blood mononuclear cells (PBMCs) of the extract was comparable to ascorbic acid [3]. Thus, the *A. concinna* extract is suggested to be a promising compound for topical treatment of microbial infections and UV-induced skin disorders as well as melasma [3,5]. However, our preliminary study found that the antioxidant and antityrosinase activities of the extract declined at ambient temperature and especially at high temperature with sedimentation occurring in various solvents after a period of storage. Therefore, microemulsions which possess several advantages such as increasing solubility of both lipophilic and hydrophilic compounds, higher stability, and efficacy enhancement over conventional formulations were investigated for loading the *A. concinna* extract for topical application [6–8].

Microemulsions can be defined as optically isotropic and transparent oil or water dispersions with diameters a bit greater than swollen micelles. Their particles sizes are in the range of 20–200 nm. Dinielsson and Lindman (1981) gave the definition of microemulsions as the systems of water, oil and amphiphile that are thermodynamically stable with low viscosity and Newtonian behavior [9,10]. Microemulsion components are usually optimized by using pseudoternary phase diagrams which present the suitable amount of oil, water and surfactant mixtures. The microemulsion region can be identified in the phase diagram [10]. Microemulsions are categorized into 3 types: oil in water (O/W), water in oil (W/O) and bicontinuous microemulsions. Microemulsions have been reported to enhance the solubility and oxidation stability of Silymarin and ascorbyl palmitate [11,12]. Therefore, microemulsion systems are attractive topical formulations to investigate for not only improving the stability of *A. concinna* extract but also delivering its active compounds through the skin barrier for better biological results [13].

The purposes of this study were to develop and characterize the microemulsions loaded with the *A. concinna* extract. Both physical stability and biological activities of the extract-loaded microemulsion (MES) were also determined in comparison with the extract solution (HES solution).

2. Materials and methods

2.1. Plant material

A. concinna pods were collected at maturity between May and September 2014 in Chiang Mai Province, Thailand. The seeds were removed, then the remaining pods were dried in a hot-air oven at 45 °C for 24 h and ground into fine powder.

2.2. Chemical materials

Polysorbate 85 (Tween 85®), sorbitan oleate (Span 80®), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) were purchased from Sigma-Aldrich Inc., USA. Absolute ethanol was purchased from Labscan Asia Co., Ltd., Thailand. Linoleic acid was purchased from Fluka Buchs, Switzerland. Tea seed oil (TO) was purchased from Naturel Organic Inc. Thailand. Grape seed (GO) was purchased from United Chemical & Trading CO., Ltd., Thailand. Sesame oil (SO) was purchased from Kuanw brand, Thailand.

2.3. Extraction

Pod powder was extracted according to the method of Poomanee et al. using 50% ethanol in water by a maceration technique for 48 h each time [3]. The extractant was filtered through Whatman® Qualitative filter paper No.1 and the marc was then re-extracted by the same process twice. The solvent in collected filtrate was then evaporated and dried to powder by spray drier. The obtained extract was named as HES.

2.4. Pseudoternary phase diagram construction

Three different oil phases, TO, SO, and GO, which have the same required hydrophilic–lipophilic balance (rHLB) value of 7.0, were used for the construction of pseudoternary phase diagrams. Polysorbate 85 and sorbitan oleate were used as surfactants, and absolute ethanol was used as a co-surfactant. The pseudoternary phase diagram of each oil was constructed using various surfactants including polysorbate 85 and the mixture of polysorbate 85 and sorbitan oleate (2:3 and 1:1). The ratios of surfactant to co-surfactant were 1:1, 2:1, or 4:1. The effect of pH of the aqueous phase on microemulsion area was also studied. The pH values were adjusted using 1% citric acid solution to pH 3.0, 5.0, and 7.0.

The Origin 8.0 program was used for the construction of the pseudoternary phase diagrams. Percentage microemulsion area was calculated by Image J version 1.45.

2.5. Preparation of microemulsions

The composition of the microemulsion was selected from the pseudoternary phase diagram that gave the largest microemulsion area. The selected microemulsion (ME) was com-

posed of 5% TO, 40% polysorbate 85, 20% ethanol, and 35% water. The microemulsion was prepared by mixing all the components.

The HES-loaded microemulsion (MES) was prepared by adding the HES into the aqueous phase before mixing with other components. The final concentration of the HES in the microemulsion was 4% w/w.

2.6. Characterization of microemulsions

Physical appearances of ME and MES were observed in terms of color, isotropic, and transparency. The internal droplet size and polydispersity index (PDI) of each formulation were measured using photon correlation spectroscopy (Zetasizer®) at 25 ± 1 °C. Rheology and viscosity of each formulation were determined using a Brookfield® rheometer (Model: R/S-CPS plate & plate). The rheogram of each microemulsion systems was constructed by graph of shear stress (y) and shear rate (x) using Microsoft Excel 2013.

2.7. Stability study of microemulsions

2.7.1. Storage conditions

ME, MES and 4% w/w HES solution in 20% ethanol were kept in air-tight containers under various accelerated conditions including 6 heating–cooling cycles (HC) (4 ± 1 °C for 48 h followed by 45 ± 1 °C for 48 h as 1 cycle), 4 °C, room temperature (RT), and 40 °C/75% humidity for 90 d.

2.7.2. Physical stability investigation

Physical appearances including phase separation, transparency, and the sedimentation of MES were observed and compared with the HES solution. Droplet size, PDI, viscosity and pH value of the ME and MES were also determined after 90 d and at the end of 6 heating–cooling cycles.

2.7.3. Biological activities and stability study

Antioxidant and anti-tyrosinase activities of MES and HES solution were determined after 90 d and at the end of 6 heating–cooling cycles. The stability of MES was compared with that of HES solution and expressed as percentage reduction of the activities between the starting point and at the end of the test.

2.7.3.1. *Antioxidant activity*.

2.7.3.1.1. *DPPH assay*. Each formulation was dissolved in 50% ethanol and agitated by vortex for 1 min. Then, the mixture was sonicated twice for 30 min and centrifuged at 10,000 rpm, 10 °C for 30 min. The supernatant was collected and tested for antioxidant activity by the DPPH assay following the method of Poomanee et al. with some modifications [3]. Each sample (20 μl) was mixed with 120 mM DPPH in ethanol (180 μl) and left in the dark for 30 min. Then the absorbance of the solution was measured at 520 nm using a Beckman Coulter®, DTX 880 multimode detector, Beckman Coulter GmbH, Austria. The percentage inhibition was calculated using the following equation: %inhibition = [(Ac – As)/Ac] × 100%, where Ac was the absorbance of control and As was the absorbance of the sample.

2.7.3.1.2. *Linoleic acid peroxidation assay*. The protective effect of the supernatant mentioned above on linoleic acid peroxidation was determined following the method of Poomanee et al. [3]. Phosphate buffer (PBS) pH 7.0 (1.40 ml), 1.3% linoleic acid in methanol (1.40 ml), and deionized water (0.70 ml) were mixed with the sample (0.30 ml) in test tubes. AAPH solution at 46.35 mM (0.20 ml) was added to start the lipid peroxidation process and the tubes were incubated in the dark at 50.0 °C for 4 h or until the absorbance of the control was 0.55 ± 0.02 at 500 nm. The degree of lipid peroxidation was determined by the ferric thiocyanate method. The reaction mixture (0.10 ml) was mixed with 20 mM $FeCl_2$ solution in 3.5% HCl (0.10 ml), 10% NH_4SCN solution (0.10 ml), and 75% methanol (9.70 ml) for 3 min. The absorbance was measured at 500 nm using UV–VIS spectrophotometer (Shimadzu, UV-2450). The experiments were done in triplicate. The percentage inhibition was determined using the following equation: %inhibition = [(Ac – As)/Ac] × 100%, where Ac was the absorbance of control and As was the absorbance of sample.

2.7.3.2. *Anti-tyrosinase activity*. The inhibitory effect of the supernatant above on mushroom tyrosinase was determined following the method of Manosroi et al. with some modifications [14]. The sample (70 μl) was added to 1.66 mM mushroom tyrosinase solution (70 μl) in 0.1 M PBS (pH 6.8) and left at room temperature for 10 min. The reaction was started by adding 0.85 mM L-tyrosine solution (70 μl) in PBS. The mixture was then incubated at room temperature for another 20 min. The absorbance was measured at 450 nm using a Beckman Coulter®, DTX 880 multimode detector, Beckman GmbH Austria. The percentage inhibition on mushroom tyrosinase was calculated by the following equation: %inhibition = [(Ac – As)/Ac] × 100%, where Ac was the absorbance of control and As was the absorbance of sample.

2.8. Statistical analysis

The tests were done in triplicate and the results were expressed as mean ± SD. Statistical analysis was performed by independent t-test using SPSS 17.0 (IBM SPSS Statistics Inc.). *P*-values of less than 0.05 ($P < 0.05$) were recognized as statistically significant.

3. Results and discussion

3.1. Optimization of microemulsions

3.1.1. Effects of oil type

The solubility of HES in DI water, absolute ethanol, TO, GO, SO and polysorbate 85 were 0.44 g/mL, 0.19 mg/mL, 0.17 mg/mL, 0.16 mg/mL, 0.14 mg/mL and 0.035 g/100 mL, respectively indicating that the extract was freely soluble in water. However, extract sedimentation occurred in aqueous solution after 3 months of storage. ME was developed to address this problem.

Pseudoternary phase diagrams constructed using different oil phases with the surfactant mixture of polysorbate 85:ethanol (2:1) are shown in Fig. 1. The systems using TO presented the largest microemulsion area due to their higher unsaturated fatty acid (UFA) content. These three oil types contain UFA including oleic acid and linoleic acid. Saturated fatty acids such as stearic acid and palmitic acid are also found in these oils [15–17]. Previous reports showed that TO

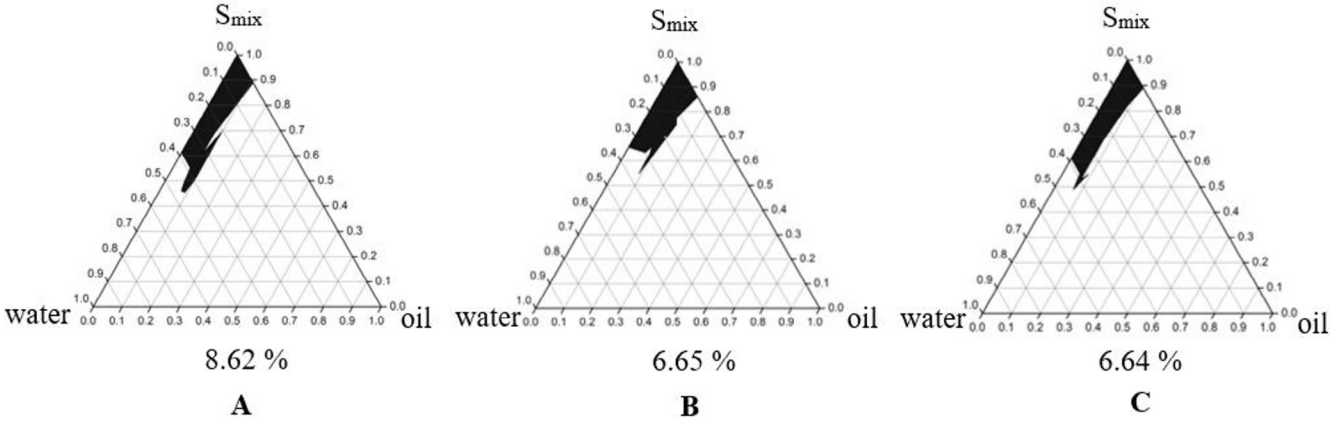

Fig. 1 – Pseudoternary phase diagrams of (A) TO, (B) GO, and (C) SO with polysorbate 85/ethanol (2:1) as a surfactant mixture. Microemulsion area in black.

contained 91.2% UFA which was 82.3% oleic acid [15] while the amounts of UFA in SO and GO were 75.5%, and 85.4%, respectively [16,17]. Moreover, SO contained the highest saturated fatty acid including stearic acid (6%) when compared with TO (1.1%) and GO (4%).

Furthermore, TO contains the highest amount of oleic acid, which is known as skin penetration enhancer [18]. Oleic acid can reduce the diffusional resistance of the skin by reacting with the lipid matrix in stratum corneum increasing lipid fluidity [18,19]. The extract was also found to have the highest solubility in TO. Therefore, TO was then selected for further study.

3.1.2. *Effects of surfactant to co-surfactant ratios*

The effects of surfactant to co-surfactant ratio are shown in Fig. 2. The system composed of polysorbate 85/ethanol at the ratio of 2:1 showed the highest microemulsion area. In addition, the amount of surfactant mixture of this system could be minimized as it can incorporate more water content. The microemulsion area was smaller at the ratio of 4:1 due to the lower amount of ethanol since ethanol was a co-surfactant which can decrease the interfacial tension between oil and water in microemulsion [6]. The lower ethanol content also leads to the decrease of hydrophilicity of the surfactant mixture and resulted in a smaller microemulsion area, whereas at the ratio of 1:1, no microemulsion area was obtained because of the low proportion of surfactant [20]. In conclusion, the ratio of 2:1 was the optimum ratio of surfactant to co-surfactant for the TO system due to its highest microemulsion area.

3.1.3. *Effects of surfactant type*

The effects of the surfactant type are shown in Fig. 3. The surfactant system composed of polysorbate 85 and ethanol showed the highest microemulsion area (Fig. 3A). The ratio of the surfactant mixture in Fig. 3C which was calculated from rHLB exhibited smaller microemulsion area than Fig. 3B. A previous study reported that the emulsification was the best when rHLB of the system were equal to the HLB of surfactant [6]. However, the results of this study demonstrated that rHLB value was not the main factor affecting microemulsion formation. The optimum microemulsion system was TO with polysorbate 85/ethanol (2:1) as a surfactant mixture. So, it was selected for loading with HES.

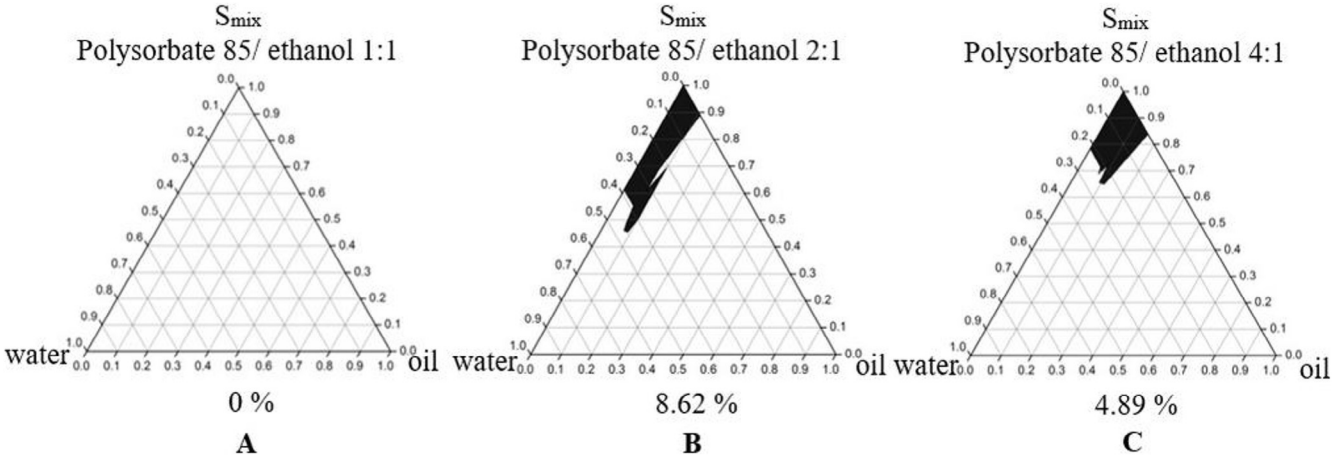

Fig. 2 – Pseudoternary phase diagrams of TO with various ratios of surfactant and co-surfactant; (A) 1:1; (B) 2:1; (C) 4:1. Microemulsion area in black.

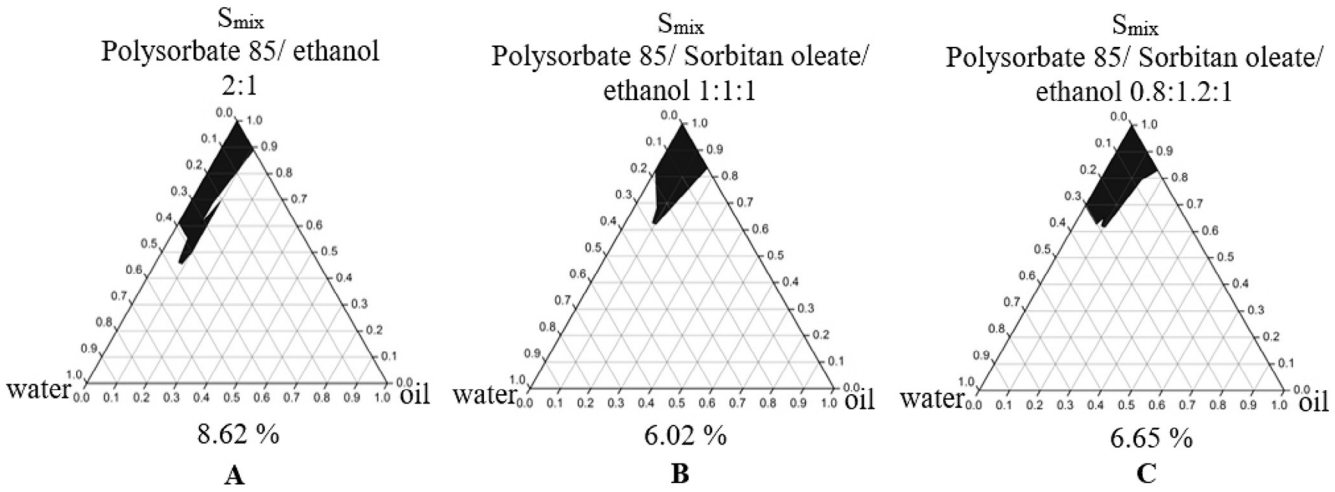

Fig. 3 – Pseudoternary phase diagrams of TO in the surfactant mixture ratio of 2:1; (A) Polysorbate 85/ethanol (2:1), (B) Polysorbate 85/sorbitan oleate/ethanol (1:1:1), (C) Polysorbate 85/sorbitan oleate/ethanol (0.8:1.2:1). Microemulsion area in black

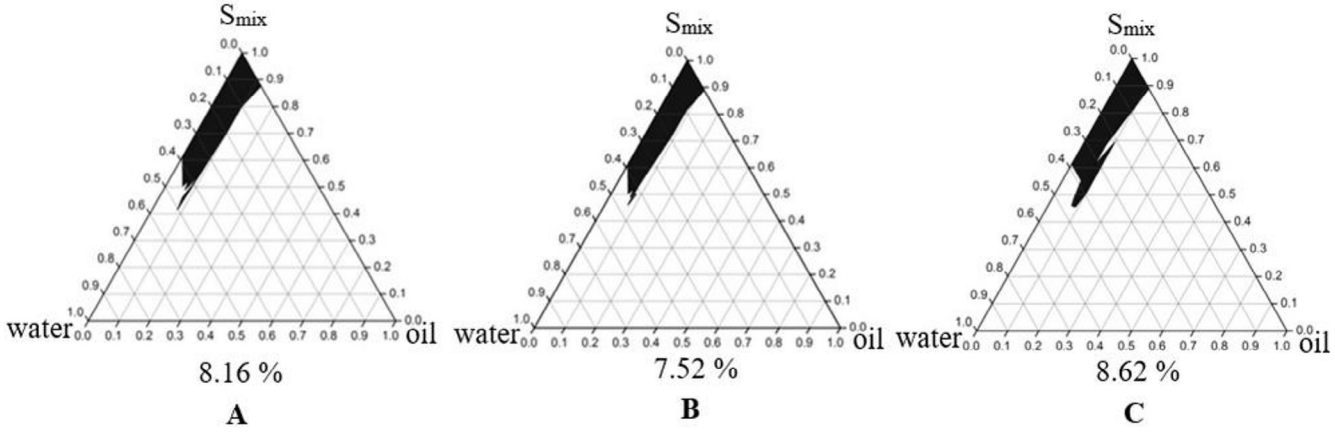

Fig. 4 – Pseudoternary phase diagrams of TO system (Polysorbate 85/Ethanol 2:1) with variation of pH; (A) pH 3.0, (B) pH 5.0 and (C) pH 7.0. Microemulsion area in black.

3.1.4. *Effect of pH values*

The interfacial tension in microemulsion systems containing fatty acids may depend on pH due to changes in ionization [21]. Therefore, the microemulsion might be affected by the acidity of HES [3]. The effects of pH on microemulsion area are shown in Fig. 4. The pH ranged from 3.0 to 7.0. These results indicate that pH does not significantly affect this microemulsion system.

3.2. *Characterization of microemulsions*

The physical appearance of ME (Fig. 5) was yellowish transparent liquid. Our previous results demonstrated that IC_{50} of the HES on DPPH, linoleic acid peroxidation and tyrosinase inhibition assays were 0.87 ± 0.01 mg/ml, 1.88 ± 0.03 mg/ml and 1.10 ± 0.01 mg/ml, respectively [3]. Consequently, the HES was dissolved in the aqueous phase of ME in the final concentration of 4% w/w which is greater than 20-fold of its IC_{50} value in these assays of potential skin benefits. MES was consequently a dark brown transparent liquid.

The droplet size of ME was 68.03 ± 1.09 nm. When HES was loaded into the microemulsion, larger particle sizes were observed with a lower PDI value (Table 1). Based on the solubility of the HES, it might mainly be localized in the external phase and surfactant layer between internal phase and external phase of the microemulsion. This might have resulted in larger droplet size.

The viscosity of each formulation is given in Table 1. Both ME and MES exhibited Newtonian flow behavior (data not shown) with constant viscosity under shear stress corresponding to the general property of microemulsions [9].

Table 1 – Droplet size, PDI, viscosity, pH and conductivities of microemulsions.

Formulations	Droplet size (nm)	PDI	Viscosity (Pa·s)	pH
ME	68.03 ± 1.09	0.44 ± 0.04	0.38 ± 0.01	7.0
MES	239.77 ± 12.69	0.37 ± 0.02	0.43 ± 0.01	4.0

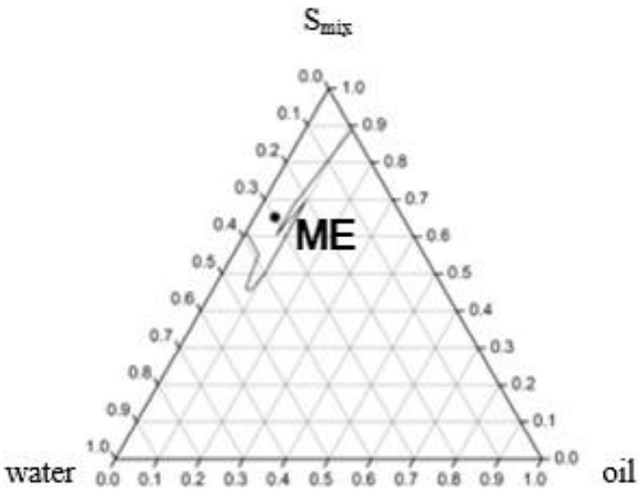

Fig. 5 – Pseudoternary phase diagram showing the components of TO microemulsion (ME).

The pH values of the microemulsions are shown in Table 1. ME has pH value of 7.0 which is suitable for topical application. However, the acidity of HES might affect the pH of the microemulsion.

3.3. *Physical stability study of microemulsions*

The physical appearances of ME and MES did not change after stability testing. Droplet size, PDI, and viscosity of both microemulsions also did not significantly change under test conditions (Figs. 6 and 7). These results indicate that both ME and MES were physically stable after 90 d of storage. After stability testing, the HES solution showed sediment under all conditions while sedimentation of MES did not occur. A unique property of microemulsions is their ability to improve the solubility and physical stability of loaded active compounds [8,20,22]. Due to the high amount of surfactant used in microemulsion, the solubility of the HES in the microemulsion was better than that of HES in 20% ethanol. Physical stability of the HES was consequently improved by loading into microemulsion since sedimentation did not occur. Therefore, HES solubility and physical stability were successfully improved via the microemulsion system.

3.4. *Stability study of biological activities of microemulsions*

HES solution was kept in the same conditions as MES. Biological activities of ME were also determined to determine the influence of the excipients in the microemulsion system. Initially, HES solution and MES showed high antioxidant activity in the DPPH assay with the percentage inhibition of 90.99 ± 0.01% and 61.20 ± 0.11%, respectively. The results from the linoleic acid peroxidation assay were similar to those with the DPPH assay with percentage inhibition of 51.69 ± 0.81% and 42.59 ± 1.34%, respectively. The percentage inhibition of ME on DPPH and linoleic acid peroxidation assays was 14.99 ± 2.14% and 3.42 ± 2.64%, respectively, significantly less than with MES. Therefore, the data indicate that the antioxidant effect of MES resulted primarily from HES. The relatively small antioxidant effect of ME might have resulted from tea seed oil which was in the oil phase of the system. Tea seed oil, consisting of polyphenols, carotenoids and vitamin E, has shown inhibitory effects on intercellular reactive oxygen species and low density lipoprotein (LDL) oxidation [23,24].

Heating–cooling (HC) was performed for accelerated stability testing and predicting the stability results. The percentage reduction of activity of HES solution on both DPPH and linoleic acid peroxidation assays was higher than that of MES as shown in Fig. 8A perhaps because of extract sedimentation in HES. The effect of temperature on antioxidant activity is shown in Fig. 8. The results indicated that reduction of activity of HES solution was clearly observed in all storage condition including 4 °C, RT, and 40 °C/75% humidity which were significantly different from that of MES. Reduction of activity in this test may have also resulted from the sedimentation of HES solution. Furthermore, at 40 °C/75% humidity, the percentage reduction of activity of HES solution was the highest compared with other storage temperatures as seen in both Fig. 8A

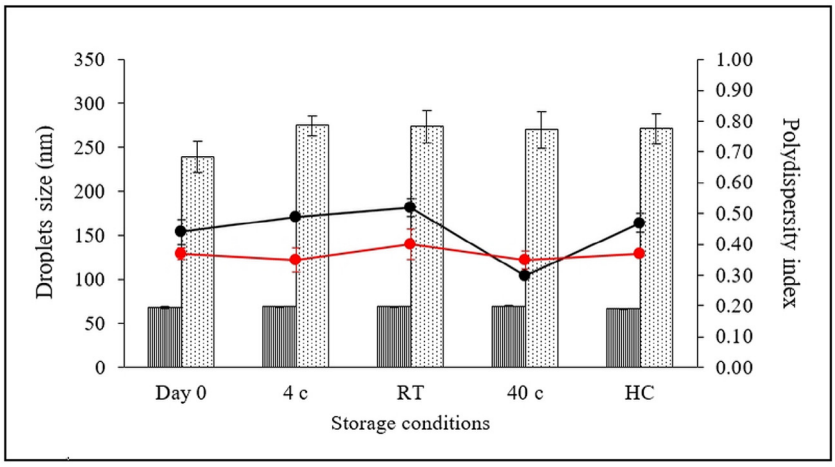

Fig. 6 – Droplet size and polydispersity index of ME and MES after storage in various conditions; droplet size: (▮) ME, (▨) MES; Polydispersity index: (•) ME, (•) MES.

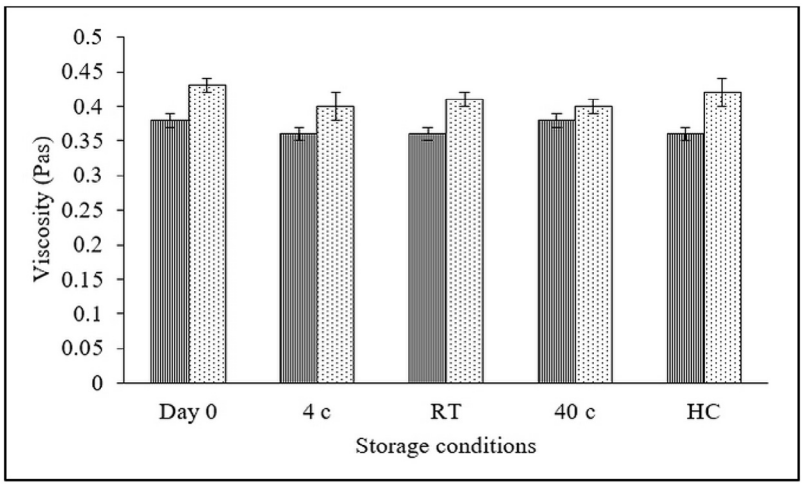

Fig. 7 – Viscosity of ME and MES after storage in various conditions; (▩) ME, (▨) MES.

and 8B. These results indicate that some active compounds of HES might be degraded by heat. Therefore, the stability of HES was successfully improved by loading into microemulsion.

Tyrosinase enzyme activity has been recognized as the rate limiting step of melanin synthesis. HES exhibited tyrosinase inhibition indicating potential for treatment for melasma [5]. MES and HES solution showed good anti-tyrosinase activity with percentage inhibition of 55.75 ± 0.17% and 81.49 ± 4.64%, respectively while the percentage inhibition of the ME on mushroom tyrosinase enzyme was 1.54 ± 1.69% indicating that the excipients in ME had no effect on tyrosinase activity. The effect of temperature on tyrosinase inhibition is shown in Fig. 9. After heating–cooling for 6 cycles and 40 °C, the percentage reduction of activity by HES solution was comparable to MES whereas it was higher at 4 °C and RT conditions. It might be assumed that tyrosinase inhibition by HES was not affected by temperature but was reduced by sedimentation in HES solution. Therefore, not only antioxidant activity but also anti-tyrosinase activity of the HES was enhanced by loading in microemulsion due to improvement in its solubility.

Panapisal et al. reported that the phenol group of silymarin could be protected from oxidative degradation by hydrogen bonding with oxyethylene group of surfactants [11]. Since the active compound of HES was a phenolic compound, it might also be protected by h-bonding to oxyethylene of polysorbate 85.

Physical appearance and biological activities of MES were stable after storage in various conditions for 90 further indicating that the microemulsion has potential to improve stability and solubility of HES.

4. Conclusions

A thermodynamically stable microemulsion of the *A. concinna* extract was successfully developed to improve the solubility and stability compared to the extract solution. Both physical appearances and biological activities of the extract were maintained after loading into the microemulsion. Therefore, this system is interesting for further investigation of skin penetration and skin irritation along with performing clinical trials for topical pharmaceutical and cosmeceutical application.

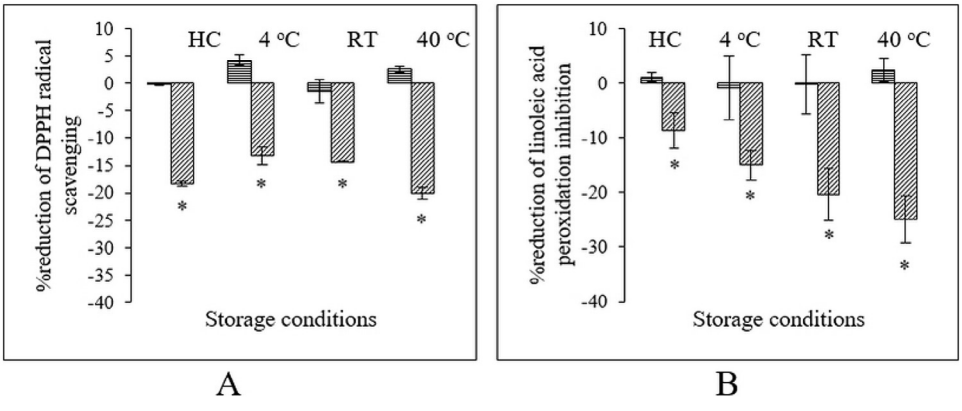

Fig. 8 – Percentage reduction of antioxidant activity of MES and HES solution after storage in various temperatures (90 d) and 6 heating–cooling cycles; (A) DPPH assay, (B) Linoleic acid peroxidation assay; (▩) MES, (▨) HES solution (*$P < 0.05$).

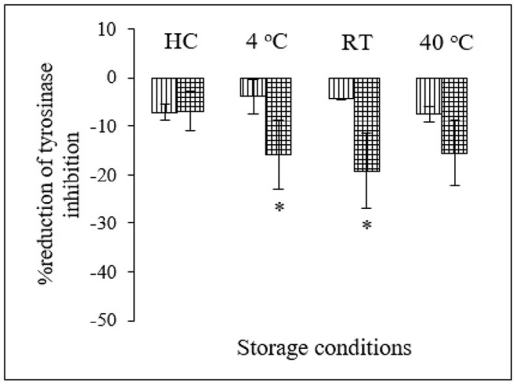

Fig. 9 – Percentage reduction of anti-tyrosinase activity of MES and HES solution after storage in various temperature (90 d) and 6 heating–cooling cycles; (▥) MES, (▦) HES solution (*$P < 0.05$).

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