

# Anti-Wrinkle Activity of *Clausena harmandiana* Essential Oil and Development of a Bioactive Nano-Drug Delivery System for Cosmetic Applications

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## ABSTRACT

**Background:** *Clausena harmandiana* (Song Fa) leaves are a source of essential oil, in which the dominant compound is *trans*-anethole and reported the high antioxidant activity but the inhibition of biological enzymes related to anti-wrinkle activity is limit. **Objectives:** The objectives of this study are to investigate the ability of Song Fa leaf essential oil (SFEO) to inhibit enzymes that cause skin wrinkles and then to develop a bioactive ingredient in a nano-drug delivery system for anti-ageing cosmetic products. **Methods:** Fresh leaves of Song Fa were distilled and their essential oil obtained. Then, the anti-collagenase, elastase, and hyaluronidase activities were investigated and compared with those of an oleanolic acid standard. Then, SFEO was developed into a microemulsion by using Tween 80 as a surfactant and ethanol as a co-surfactant in a ratio of 1:1. **Results:** SFEO presented the highest inhibitory activities against hyaluronidase and collagenase (IC<sub>50</sub> 10.94±1.06 and 19.06±0.06 µg/ml, respectively), which were close to those of oleanolic acid (IC<sub>50</sub> 7.43±0.58 and 16.75±0.14 µg/ml), followed by elastase (IC<sub>50</sub> 121.47±2.80 µg/ml). Two microemulsion formulas with different amounts of essential oil, 1% and 5% w/w, were formulated and evaluated for their enzyme inhibitory activities. The results showed that 5% Song Fa microemulsion inhibited collagenase and elastase (35.78% and 99.35%, respectively) by more than 1% Song Fa microemulsion (34.22% and 92.67%, respectively) and only the 5% Song Fa microemulsion formula exhibited anti-hyaluronidase activity. **Conclusion:** This information will benefit the development of essential-oil-based products and increase the utilization of medicinal plants in the cosmetic industry.

**Key words:** *Anti-collagenase, Anti-elastase, Anti-hyaluronidase, Anti-wrinkle, Clausena harmandiana, Microemulsions.*

## INTRODUCTION

*Clausena harmandiana* (Pierre) Guillaumin is an aromatic plant in the Rutaceae family and is known locally as Song Fa in northern Thailand. The characteristics of its leaves include pellucid dots containing essential oils. The young leaves are consumed as part of the local cuisine in Northern Thailand.<sup>1</sup> The root part is used in Thai traditional medicine for the treatment of headaches, colic, and bronchitis, and the leaf is used as a carminative.<sup>2</sup> Essential oil extracted from dried leaves of Song Fa was re-reported to have an anethol content of 46.09%, along with lower proportions of camphene (9.61%), β-terpinene (7.87%), and D-limonene (7.07%), and also presented antioxidant activity in DPPH, ABTS, and FRAP assays.<sup>3</sup> Furthermore, essential oil distilled from fresh leaves of Song Fa was investigated and revealed to have 91.44% *trans*-anethole as the principal chemical compound; inhibitory activity against DPPH and ABTS radicals; and the ability to reduce the Fe<sup>3+</sup>-TPTZ complex, as well as anti-collagenase activity.<sup>1</sup> From the above information, *trans*-anethole is the major component contained in Song Fa essential oil that might affect the enzymes that cause skin wrinkles. One medicinal herb, *Illicium verum*, rich in *trans*-anethole, was extracted and evaluated for its enzyme-inhibitory ability. The results showed that the crude extract presented anti-collagenase and -elastase activities.<sup>4</sup> Moreover, *I. verum* is used in the perfumery and cosmetic industries.<sup>5</sup> Therefore, the essential oil

extracted from Song Fa, which notably contains *trans*-anethole, would be beneficial for cosmetic applications.

Microemulsions are thermodynamically stable liquid systems that form spontaneously and are simple to prepare because their formation consumes no energy. The main components of microemulsions are oil and water, and they are stabilized by an interfacial film of surfactant, which is frequently combined with a co-surfactant.<sup>6</sup> Microemulsions are colloidal dispersions with small droplets (usually up to 150 nm) that appear clear or translucent, while emulsions are milky, coarse dispersions with droplet sizes in the micrometer range and slightly below.<sup>7</sup> Microemulsions can incorporate both hydrophilic and lipophilic drugs and can enhance the skin-penetrating ability of the active ingredients.<sup>7-11</sup> Many research studies have used essential oils, such as eucalyptus oil, citrus peel and leaf oil, and cinnamon oil, as the oil phase in microemulsions for transdermal drug delivery,<sup>12-14</sup> resulting in a high penetration rate of the active drug. This study aims to investigate the anti-wrinkle activity of SFEO against enzymes that cause skin ageing and then to develop this essential oil as a nano-bioactive compound delivery system for anti-wrinkle cosmetic product applications.

## MATERIALS AND METHODS

### Plant material

Fresh leaves of *Clausena harmandiana* were collected, and voucher specimen No. Nichakan-005

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was deposited at the Herbarium of Faculty of Pharmacy Chiang Mai University. Leaves were then distilled by hydro-distillation for 2 hours. The essential oil was obtained and the moisture absorbed by using anhydrous sodium sulphate. The essential oil was stored in a brown glass container in a refrigerator until investigation.

### Determination of anti-wrinkle activity

The anti-ageing activity assay evaluated the ability of essential oil to inhibit the enzymes that cause skin wrinkles. The evaluation procedures are as follows.

#### Anti-collagenase activity

The anti-collagenase activity assay was performed according to Thring,<sup>4</sup> with some modifications. N-[3-(2-furyl) acryloyl]-Leu-Gly-Pro-Ala (FALGPA) 1 mM was prepared in 50 mM tricine buffer, pH 7.4. The stock solution 1 U/ml of collagenase from *Clostridium histolyticum* was prepared fresh and dissolved with ultra-pure water (18.2 MΩ) at 2-8°C. The sample was dissolved in a small volume of ethanol and diluted with tricine buffer. Fifty microliters of sample were transferred to a 96-well microplate, and then collagenase enzyme solution (50 μL) was added and incubated at 37°C for 20 minutes. Subsequently, 50 μL of substrate was added and the mixture again incubated at the identical temperature for 30 min. The absorbance at 340 nm was measured by using a UV-Vis spectrophotometer. The result was presented as the percentage of collagenase inhibition according to the equation (1) below.

$$\% \text{Inhibition} = \frac{[A_{\text{con}} - (A_s - A_{\text{sb}})]}{A_{\text{con}}} \times 100 \quad (1)$$

$A_{\text{con}}$ : the absorbance of control

$A_s$ : the absorbance of sample with enzyme

$A_{\text{sb}}$ : the absorbance of control without enzyme

#### Anti-elastase activity

The elastase-inhibition assay was performed according to Chattuwatthana<sup>15</sup> and Azmi.<sup>16</sup> A stock solution (1 U/ml) of elastase was prepared and diluted to 0.03 U/mL by using 0.2 M Tris-HCl buffer, pH 8.0. The substrate (0.8 M N-Succinyl-Ala-Ala-Ala-p-nitroanilide [AAPVN]), was dissolved in the same buffer before use. The sample solution was dissolved with ethanol and diluted with buffer. Then, 150 μL buffer, elastase enzyme, and 20 μL sample solutions were mixed in a 96-well plate and incubated at room temperature for 20 min. After that, AAPVN substrate was added and incubated at room temperature for 20 min. A UV-Vis spectrophotometer was used to measure the absorbance at 410 nm. The percentage inhibition was calculated using the same equation (1) as above.

#### Anti-hyaluronidase activity

The assay to evaluate the anti-hyaluronidase effect of SFEO employed the procedure of Jitrachayamaethasakul<sup>17</sup> and Kolayli,<sup>18</sup> with some modifications. Three units of Bovine hyaluronidase was prepared in 20 mM sodium phosphate buffer, pH 7.0, 77 mM sodium chloride (NaCl), and 0.01% of bovine serum albumin (BSA). Hyaluronic acid (HA) substrate 0.03% was dissolved in 300 mM sodium phosphate buffer, pH 5.35. Acid albumin solution was prepared by using 0.1% BSA in 24 mM sodium acetate buffer and 79 mM acetic acid, pH 3.75. The sample was dissolved in ethanol and diluted with phosphate buffer. Five microliters of sample solution were transferred to a microcentrifuge tube. Then, hyaluronidase enzyme solution 100 μL was added and the mixture incubated for 10 min at 37°C. Next, HA substrate 100 μL was mixed

and incubated at the identical temperature for 45 min. Subsequently, the reaction was stopped by addition of 1 mL of acid albumin solution and allowed to stand at room temperature for 10 min. The absorbances at 600 nm were detected, and the percentage inhibition was calculated according to the equation (2) below.

$$\% \text{Inhibition} = 100 - \frac{[(A_{\text{test}} - A_{\text{con}}) \times 100]}{A_{\text{con}}} \quad (2)$$

$A_{\text{con}}$ : the absorbance of control

$A_{\text{test}}$ : the absorbance of sample

### Development of microemulsions

#### Construction of pseudo-ternary phase diagrams

The pseudo-ternary phase diagrams of microemulsions (ME) were constructed using the water titration method.<sup>14</sup> Four components of ME were composed of SFEO: the oil phase, the water phase, Tween 80 as a surfactant, and ethanol as a co-surfactant. The surfactant-co-surfactant mixture (Smix) was prepared in different weight ratios, 1:1, 2:1, and 3:1, of surfactant and co-surfactant. Then, the Smix was dissolved in the oil phase in the vial at weight ratios of 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1 (oil: Smix). Each vial of mixture was titrated drop-wise with purified water from a burette and stirred with a magnetic stirrer until the mixture became turbid. The quantity of water required was recorded, and then the percentage of each component was calculated. A pseudoternary phase diagram was constructed to delineate the area of ME and plotted by using Prosim<sup>®</sup> ternary diagram software.

#### Preparation of SFEO microemulsions

Two ME formulations of SFEO were prepared with different amounts of the three compositions shown in Table 1. The Smix was prepared by using Tween 80 and ethanol in a suitable ratio and then mixed with SFEO by using a magnetic stirrer. ME1 and ME2 were the microemulsions prepared with 1% and 5% w/w of SFEO, respectively. When the first two compositions reached homogeneity, purified water was added to the mixture and stirred for 15 minutes to assess the equilibrium.<sup>19</sup> These two ME formulations were characterized by their physicochemical properties and evaluated for their anti-collagenase, anti-elastase, and anti-hyaluronidase activities.

#### Characterization of SFEO microemulsions

The external appearance, internal droplet size, size distribution, electrical conductivity, and pH of MEs were characterized. The dynamic light scattering technique (Zetasizer Nano ZS, Malvern Instruments Worcestershire, UK) was used to evaluate the droplet size and size distribution. The results were recorded as the mean droplet size and polydispersity index (PDI). Electrical conductivity was measured using a conductivity meter (S230 SevenCompact<sup>™</sup>, Mettler Toledo, Switzerland). The pH of MEs was determined using a pH meter (S220 SevenCompact<sup>™</sup>, Mettler Toledo, Switzerland). Each measurement was determined in triplicate at 25°C, and then the mean and standard deviation were calculated.

**Table 1: The composition of SFEO microemulsion formulas from the position of microemulsion ratios A, B, and C.**

Formulations	Composition (%w/w)		
	SFEO	Smix	Water
A	10	70	20
B	10	85	5
C	25	70	5

## Statistical analysis

The results were presented as the mean  $\pm$  SD. In each test, at least three iterations were performed, and one-way ANOVA followed by Tukey's was used to assess statistically significant differences in Minitab version 16.

## RESULTS AND DISCUSSIONS

### Enzymatic inhibitory activities

The evaluation of enzymatic inhibitory effects on collagenase, elastase, and hyaluronidase of SFEO for application as a bioactive ingredient in microemulsion formulas found that SFEO inhibited all enzymes tested. The results are shown in Figure 1. Collagenase is an enzyme that breaks down collagen, which is a key component of skin. This destruction of collagen is a major cause of wrinkles and aging skin. Inhibition of collagenase activity is one path to reducing wrinkle formation. The results showed that SFEO inhibited collagenases. The  $IC_{50}$  value obtained here ( $19.06 \pm 0.06 \mu\text{g/ml}$ ) approximated that of oleanolic acid ( $16.75 \pm 0.14 \mu\text{g/ml}$ ).

This result was concordant with previous research detailing the anti-collagenase activity of essential oil extracted from the leaf of this plant, which could be an indicator of potential future applications in cosmetics and other products.<sup>1</sup> Regarding elastase, SFEO showed the capacity to inhibit this enzyme. When compared with the standard, the  $IC_{50}$  of SFEO ( $121.47 \pm 2.80 \mu\text{g/ml}$ ) was lower than that of oleanolic acid ( $3.24 \pm 0.08 \mu\text{g/ml}$ ). However, the degree of elastase inhibition by this essential oil is still relevant to cosmetic applications. When used as an ingredient in cosmetic products, SFEO may also slow down the appearance altering effects of skin wrinkles. Furthermore, SFEO showed the ability to inhibit hyaluronidase, presenting an  $IC_{50}$  for this enzyme of  $10.94 \pm 1.06 \mu\text{g/ml}$ . The data revealed that SFEO possesses a mechanism for arresting hyaluronidase before it reacts with

hyaluronic acid. This function can help to slow down the formation of skin wrinkles. The information from this examination supports the idea of applying SFEO as the anti-skin-wrinkle bioactive ingredient in cosmetic products.

### Development of a microemulsion of Song Fa essential oil

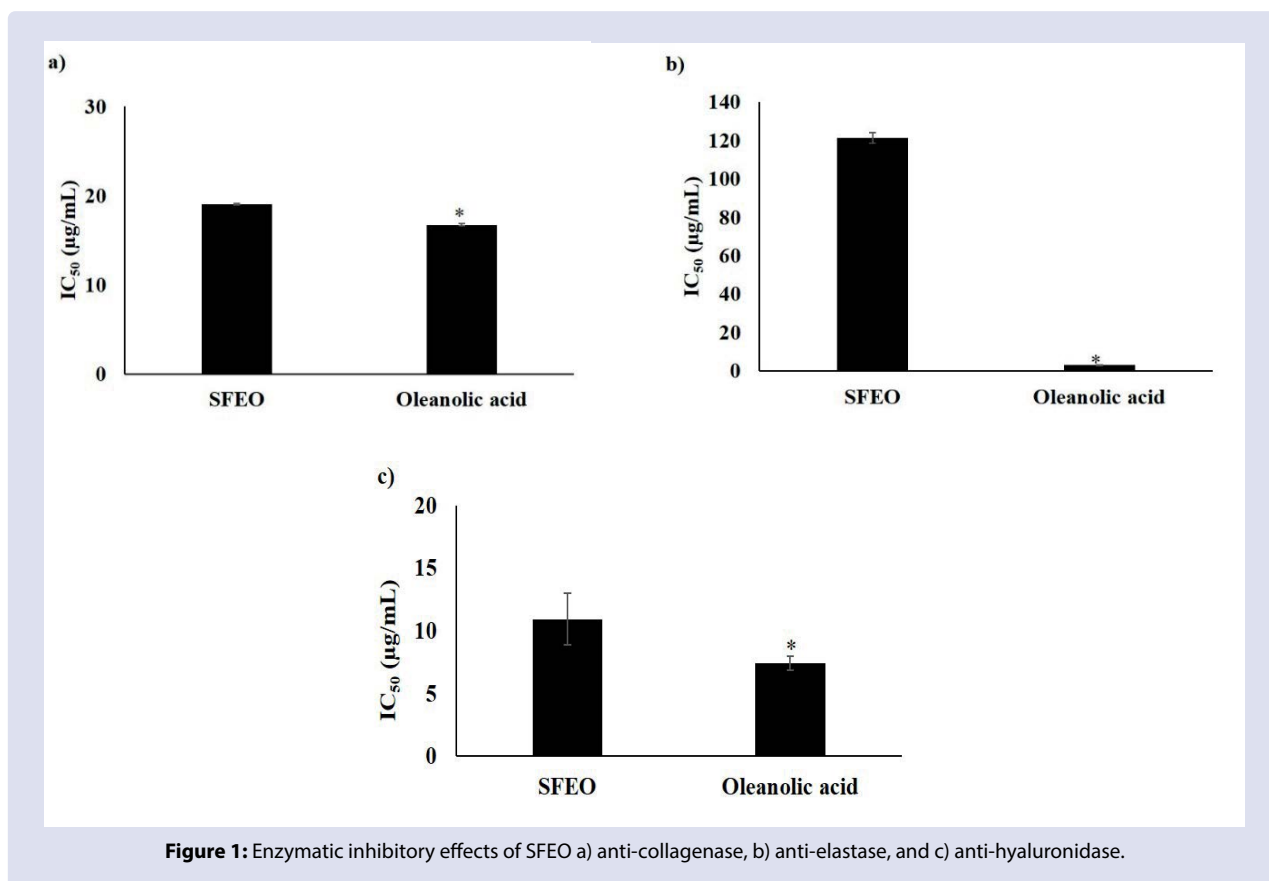
The SFEO was developed as a microemulsion for use in cosmetic products in the future by following the procedure below.

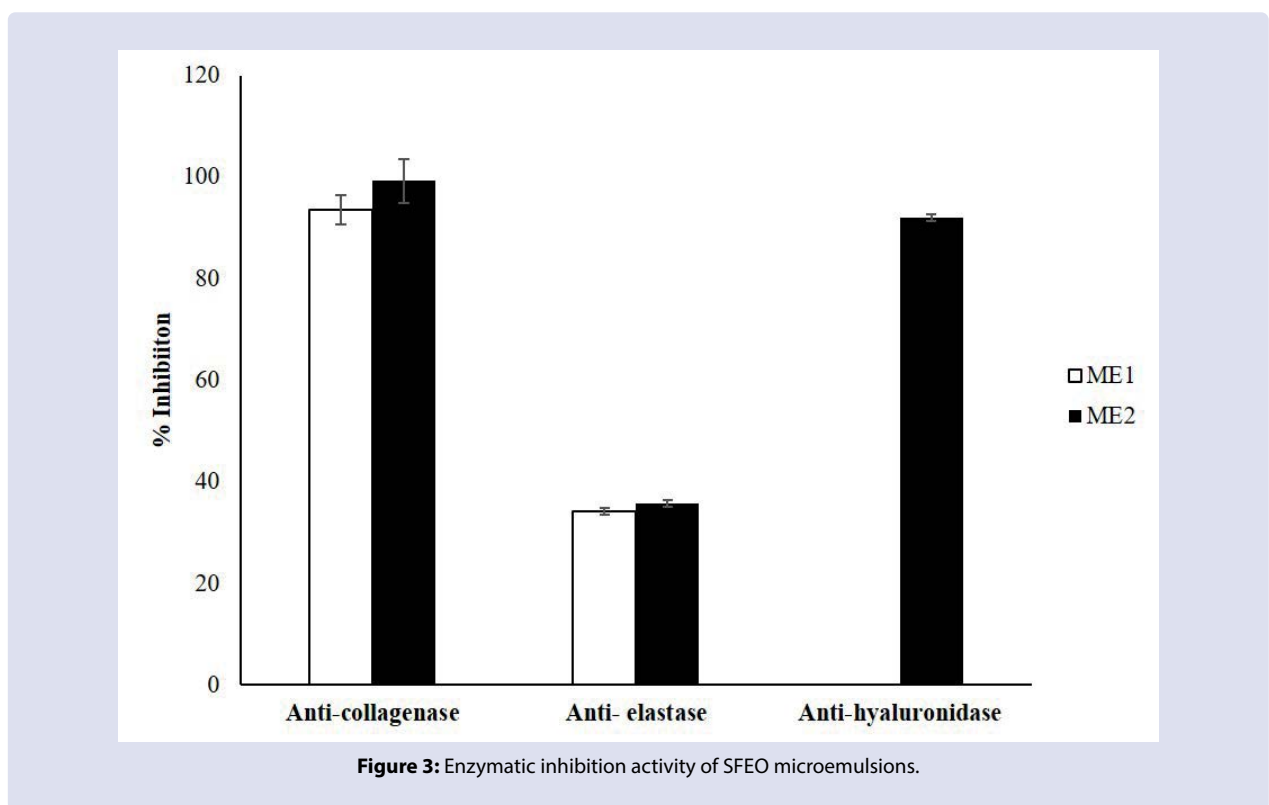
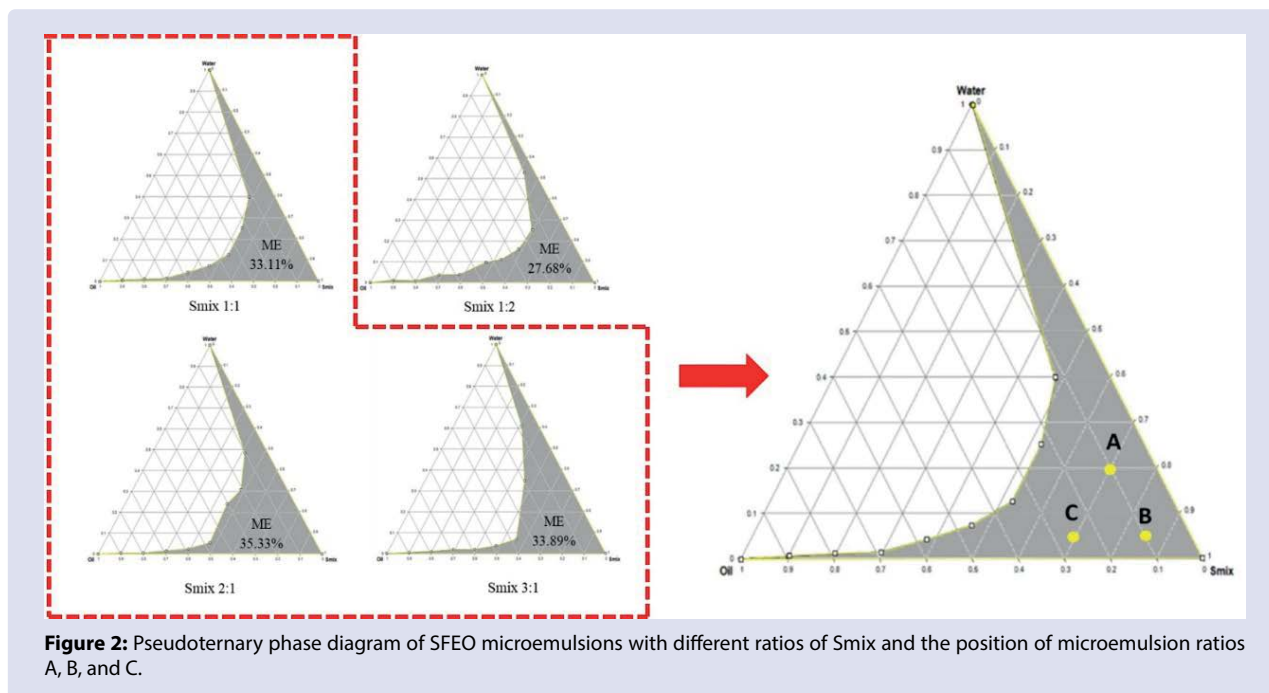
### Construction of a pseudo-ternary phase diagram

To construct a pseudo-ternary diagram of SFEO microemulsion by using the water titration method, SFEO was used as the oil phase, Tween 80 as the surfactant, and ethanol as the co-surfactant. A mixture of surfactant and co-surfactant (Smix) was blended at different ratios, 1:1, 1:2, 2:1, and 3:1, and the area of the microemulsion was compared using the Image J program. The results of the experiment are shown in Figure 2.

The microemulsion with a Smix ratio of 2:1 had the largest microemulsion area, 35.33% of the total area, followed by microemulsions with Smix ratios of 3:1, 1:2, and 1:1, which had microemulsion areas of 33.89%, 33.11%, and 27.68% of the total area, respectively. Since the Smix ratios of 1:1, 2:1, and 3:1 was similar, we were unable to select the appropriate Smix ratio. However, the three ratios of Smix microemulsion agents were selected for the study of physicochemical characteristics, inner droplet size, and size distribution, which will be used as the selection criteria for the appropriate Smix ratio.

From the construction of a pseudo-ternary phase diagram of ME formulations of SFEO, three ME formulations were formulated from the ME area with Smix ratios of 1:1, 2:1, and 3:1, each containing the same amounts of components (A, B, and C), as shown in Figure 3 and Table 1.







**Table 2:** Droplet size and size distribution of each microemulsion formulation according to the Smix ratio.

Formulation	Size (nm)			PDI		
	Smix 1:1	Smix 2:1	Smix 3:1	Smix 1:1	Smix 2:1	Smix 3:1
A	81.25±16.62 <sup>cd</sup>	148.43±29.90 <sup>b</sup>	210.00±16.01 <sup>a</sup>	0.42±0.17 <sup>b</sup>	0.40±0.04 <sup>b</sup>	0.38±0.03 <sup>b</sup>
B	86.49±19.59 <sup>cd</sup>	63.00±7.79 <sup>d</sup>	109.55±18.85 <sup>c</sup>	0.43±0.13 <sup>b</sup>	0.65±0.19 <sup>a</sup>	0.73±0.20 <sup>a</sup>
C	54.15±7.68 <sup>d</sup>	77.12±11.70 <sup>cd</sup>	150.23±26.14 <sup>b</sup>	0.23±0.02 <sup>b</sup>	0.27±0.04 <sup>b</sup>	0.71±0.01 <sup>a</sup>

Results are presented as mean ± SD of three determinations. <sup>a, b, c, d</sup> within the same characteristic indicates a significant difference ( $p < 0.05$ ).



**Table 3: Characterizations of SFEO microemulsion formulas.**

Formulations	Appearance	Size (nm)	PDI	Conductivity (mS/cm)	pH
ME1		63.22±21.65	0.39±0.11	0.273±0.009	4.5
ME2		86.53±25.68	0.30±0.12	0.384±0.018	4.5

### Characterization of SFEO microemulsions (droplet size and size distribution)

#### Droplet size

Table 2. shows the findings of internal droplet size measurement for each microemulsion formulation. The internal droplet sizes of points A, B, and C did not exceed 100 nm at Smix ratio of 1:1, and point C had the smallest droplet size of  $54.15 \pm 7.68$  nm, which was significantly different from those of the other formulations. Point A had a particle size of more than 100 nm at the 2:1 Smix ratio, which was larger than the other locations. All of points A, B, and C had particle sizes surpassing 100 nm, which were significantly different from those of the other formulations at Smix ratio of 3:1.

#### Size distribution

The results of size distribution are presented in terms of the polydispersity index (PDI). The formulation with low PDI ( $< 0.2$ ) resulted in a narrow size distribution. In Table 2, the microemulsion formulation with Smix ratio of 1:1 showed the narrow size distribution, especially at point C, where it showed the lowest PDI ( $0.23 \pm 0.02$ ).

#### Preparation and characterization of SFEO microemulsions

In general, the addition of the active ingredient in cosmetic formulations was 5-6 times the  $IC_{50}$  value to ensure high effectiveness of the active ingredient and to prevent its degradation during storage. In this study, we investigated the ability of SFEO to inhibit enzymes related to skin aging. The results of enzymatic inhibition, expressed as the  $IC_{50}$  value, which differed in  $\mu\text{g/ml}$  and was considered a very low dose, made the formulation difficult to determine. Therefore, the amount of SFEO was selected following the guidelines in the book Aromatherapy Science.<sup>20</sup> The requirements of essential oils that contain *trans*-anethole (80%-95%) as the major component, such as anise oil and star anise oil, presented dermal toxicity with  $LD_{50} > 5$  g/kg (rabbit).<sup>20</sup> Therefore, the volume of SFEO (1% and 5%) used in the two microemulsion models (Table 1) would not be toxic to human skin. Each microemulsion was prepared three times, and the physicochemical characteristics of each microemulsion were assessed upon completion.

Particle size and size distribution are important factors in transdermal drug delivery. The approximate size range of drugs delivered transdermally is 10-600 nm. The term PDI is used to describe the degree of non-uniformity of the size distribution. A PDI value is smaller than 0.05 indicates a high degree of monodispersity, and a PDI value larger than 0.7 indicates a very broad particle size distribution.<sup>21</sup> The results of the preparation and characterization of SFEO microemulsions are presented in Table 3.

ME1 and ME2 contained 1% and 5% w/w SFEO, respectively, and gave the appearance of clear, yellow liquids with low viscosities. The inner droplet size of ME1 and ME2 did not exceed 100 nm ( $63.22 \pm 21.65$  nm and  $86.53 \pm 25.68$  nm, respectively), and the size distribution was narrow ( $PDI < 0.7$ ). The conductivity of two MEs ( $> 0.01$  mS/cm) indicated that ME1 and ME2 were oil-in-water MEs.<sup>22-24</sup> However, the classification of microemulsion types by using conductivity may be

unsuitable for non-ionic surfactants such as Tween 80, so it should be confirmed by other procedures, for example, the dye test method. The pH value was 4.5, which was near normal skin pH and indicated that the microemulsions would not cause skin irritation.<sup>25</sup>

### Anti-wrinkle activity related to enzymatic inhibition by SFEO microemulsion formulas

After the successful development of SFEO ME formulations (ME1 and ME2), the anti-wrinkle activity against collagenase, elastase, and hyaluronidase were evaluated. The results are presented in Figure 3. ME1 and ME2 showed high anti-collagenase activity with percentages of inhibition of  $93.67 \pm 2.87\%$  and  $99.35 \pm 4.34\%$ , respectively. Both microemulsions also inhibited elastase, showing  $34.22 \pm 0.63\%$  and  $35.78 \pm 0.56\%$  inhibition, respectively. For anti-hyaluronidase activity, only ME2 inhibited this enzyme ( $92.1 \pm 0.6\%$ ), while the inhibitory activity of ME1 could not be detected. To summarize this information, SFEO microemulsions have potential anti-wrinkle properties, and the preparation of SFEO as a microemulsion did not decrease the enzymatic inhibitory activities of the essential oil.

### CONCLUSION

This study concluded that essential oil extracted from Song Fa leaves inhibits the enzymes collagenase, elastase, and hyaluronidase, which are responsible skin wrinkles. Moreover, the microemulsion formulation showed the ability to inhibit these enzymes as well. Therefore, SFEO has the potential for application as a bioactive anti-wrinkle ingredient in cosmetic products. However, cytotoxicity and skin irritation tests should be conducted to confirm the safety of cosmetic application of SFEO microemulsions before such products become available on the market.

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### ABBREVIATIONS

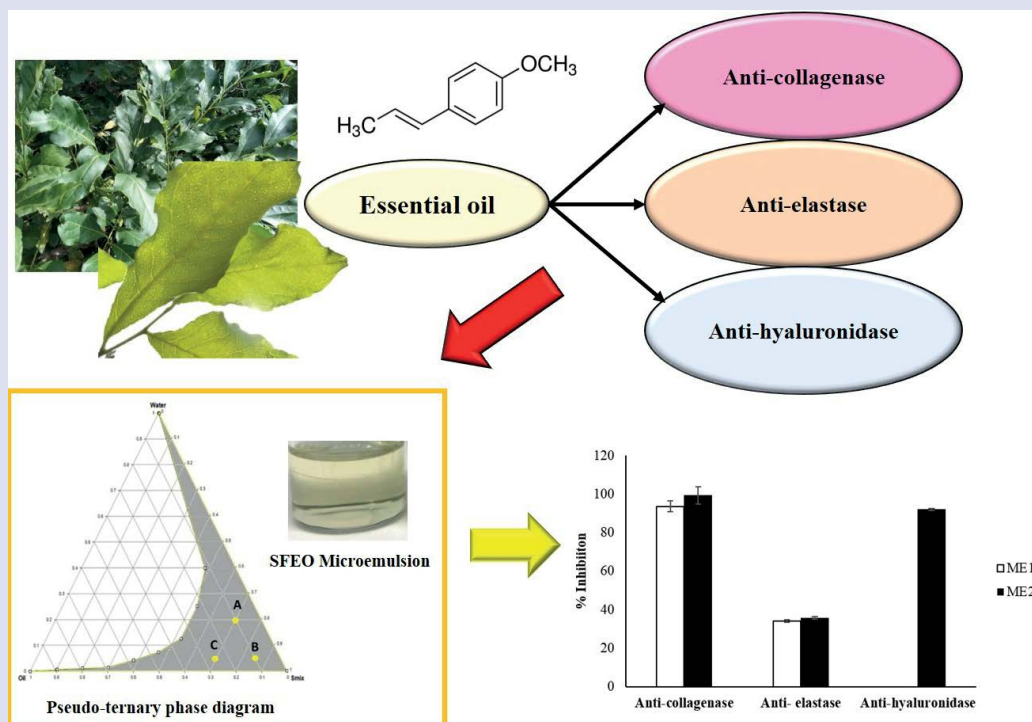
SFEO	Song Fa essential oil
ME	Microemulsion
Smix	Surfactant and co-surfactant mixture
$IC_{50}$	Half-maximum inhibitory concentration

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## GRAPHICAL ABSTRACT



## ABOUT AUTHORS



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