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Research Article

Physical stability and subjective efficacy study of liposome loaded with *Clitoria ternatea* (butterfly pea) flower extract and *Eugenia caryophyllus* (clove) oil

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ABSTRACT

Clitoria ternatea (butterfly pea) flower and *Eugenia caryophyllus* (clove) oil have been traditionally used as hair growth stimulating herbal and exhibited hair growth effects *in vitro*. This work prepared liposome loading with butterfly pea flower extract and clove oil and studied the hair growth promoting effects on eyebrows in volunteers. The liposome of butterfly pea flower extract and clove oil was prepared by loading the extract and oil into a pro-liposome. The formation of liposome was examined using the microscopy and its physical stability was studied at 4°C, 45°C and ambient temperature (30-35°C) for 1 month. The liposome of butterfly pea flower extract and clove oil was macroscopically stable but microscopic examination showed that the liposome vesicles were larger overtime. The loaded liposome was used for eyebrow growth efficacy test in 15 volunteers. The product was applied on both sides of eyebrows twice daily for 60 days. The evaluation was based on the taken photos and the satisfaction assessment scoring from 1 to 5 (very poor-excellent). The eyebrows appeared darker and thicker after 30 day and progressively more obvious at 2 month use. Participants felt that the enhancement of eyebrow thickness and darkness was good (33.3%) to excellent (66.7%). In addition, there was no irritation present which ensure the product safety. Conclusively, this work reported a stable liposome loaded with butterfly pea flower extract and clove oil. The product was subjectively proven to intensify the thickness and darkness of eyebrows.

Keywords:
Butterfly pea flower, Clove oil, Liposome, Hair growth

1. INTRODUCTION

Clitoria ternatea, commonly known as butterfly pea or blue pea, is an edible plant species belonging to the *Fabaceae* family. The plant has several therapeutic activities like a memory enhancer, antidepressant, anti-stress, sedative agent, anxiolytic and tranquilizing agent[1-2]. The *C. ternatea* blue flower contains anthocyanins, which are mainly delphinidin-glucosides[3-4]. The blue flowers of butterfly peas have good free radical scavenging activity and have potential as antioxidants[4-7]. The flower is commonly used as a source of natural coloring agent for food and cosmetics and as a natural pH indicator in pharmaceutical industry[8]. It has been reported that the butterfly pea flower fermentation solution can be added to cosmetic formula as a natural raw material of skin care products to improve moisture retention and had whitening effects[9]. The application of crushed fresh butterfly pea flower (also known as Aunchan) directly on the eyebrows and scalp for the benefits of coloring and promoting hair growth is traditionally and commonly found in Thailand[10]. The ability to promote hair growth may be related with its inhibition of the 5α-reductase enzyme activity[11-12].

Clove (*Eugenia caryophyllus*) is an important medicinal plant that has been employed for centuries as food preservative and pain reliever. It is also claimed to be the rich source of phenolic antioxidants[13]. Eugenol and eugenyl acetate, which are poorly water soluble

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molecules, are the main compounds of clove associated with its medicinal and nutritional benefits[14]. Clove oil has been recognized as a 'Generally Regarded As Safe' substance by the United States Food and Drug Administration when administered at levels not exceeding 1500 ppm in all food categories[15]. Clove oil was found to be an effective antioxidant in different *in vitro* assays including reducing power, DPPH radical, ABTS radical and superoxide anion radical scavenging, hydrogen peroxide scavenging and metal chelating activities[15]. Additionally, clove oil has been recommended for treatment of hair loss as it boosts blood circulation in the scalp, thereby stopping unnecessary hair loss[16]. Liposomes are enclosed spherical vesicles, organized in one or several concentric phospholipidic bilayers with an internal aqueous phase. Liposomes are biodegradable, nontoxic, non-immunogenic and biocompatible[17]. They represent an efficient approach for incorporating active compounds, both oil and water soluble components, by improving chemical stability and enhancing skin penetration[18-19]. Incorporating the *C. ternatea* flower extract and *E. caryophyllus* oil into liposome would create an effective delivery system for hair growth treatment. Although pharmacological activities of *C. ternatea* and *E. caryophyllus* were well separately investigated, study regarding the effects of the combination of both active compounds in liposome system on promoting human hair growth has not been undertaken. The present study is an effort to simply prepare the liposome loaded with *C. ternatea* flower extract and *E. caryophyllus* oil and subjectively investigate the efficacy on eyebrow growth of volunteers.

2. MATERIALS AND METHODS

2.1. Materials

Clove (*E. caryophyllus)* bud oil was obtained from TCFF, Thailand. Pro-liposome (Lipobelle® ST) was of Mibelle-Biochemistry, Switzerland. All other chemicals are of cosmetic grade.

2.2. Methods

2.2.1. Preparation of C. ternatea flower extract

The fresh *C. ternatea* flowers were collected from a local market in Thailand and its identity was confirmed by comparing with herbarium specimens and database from the Concise Encyclopedia of Plants in Thailand. The flower petals were macerated in 70% ethanol at the plant to solvent of 1:2 mass ratio for seven days. The extract was then filtered using double layered cheesecloth. The extract was concentrated by partially removing the solvent using a rotary evaporator until the final volume was 10% of the starting volume. The concentrated *C. ternatea* flower extract was then preserved by addition of phenoxyethanol (1%, w/w) and stored in an amber bottle at 4°C until further use.

2.2.2. Preparation of liposome loaded with C. ternatea flower extract and E. caryophyllus oil

C. ternatea flower extract and *E. caryophyllus* oil (TCFF, Thailand) were loaded in a Pro-liposome according to the previous report[20-21]. Briefly, a mixture of Pro-liposome and 2% w/w of clove oil solution (50% w/w clove oil in ethanol 95%) was stirred at 200 rpm, 60°C for 5 min. In another container, a mixture of Pro-liposome and of *C. ternatea* flower concentrated extract (45%, w/w) was stirred using the same condition. Then, without heating, the two mixtures were mixed and stirred at 200 rpm for 60 min and was cooled to room temperature. The size of the prepared liposome was further reduced using sonication bath (Crest Ultrasonics P360, USA) for 10 min. The formation of liposome was examined under microscopy. Loading efficacy was determined according to the previous report with modification[21]. Free extract or clove oil was removed from liposome dispersions by centrifugation at 10,000 rpm for 30 min at 4°C in a Thermo IEC centrifuge (Thermo Scientific, USA). The amount of butterfly pea flower extract or clove oil in supernatant was defined spectrophotometrically (Biochroms/Libra S22, UK) at 546 and 239 nm, respectively. Entrapment efficiency (EE%) was calculated as the amount of encapsulated extract/oil in liposomes divided by the amount of extract/oil used for the preparation as shown in the following equation:

$$EE\% = \frac{Mi - Msup}{Mi} * 100$$

where Mi is the initial amount of the extract/oil used for the preparation of liposomes and Msup is the amount of the extract/oil determined in supernatant.

The stability of the prepared liposome was evaluated under various storage conditions which are ambient temperature (30-35°C), 4°C, and 45°C for 1 month according to the Colipa guidelines on stability testing of cosmetic products[22]. The physiochemical properties, i.e., appearance, color, odor, and pH were collected every week.

2.2.3. Subjective efficacy studies

All of the study in human volunteers was depicted in accordance with the Declaration of Helsinki[23-24]. Thai healthy volunteers both male and female aged between 15 and 60 years old were enrolled in the study. The inclusion criteria is the volunteer whose has thin or uneven eyebrows which was subjectively graded by participant and approved by the principal investigator. The study was approved by the ethical committee of Mae Fah Luang University prior to enrollment (REH-

61070). All participants were informed about the study and signed an informed consent form and were free to discontinue their participation at any time during the study without any consequences.

Primary skin irritation patch test

The potential of skin irritation of the prepared liposome was investigated by closed patch test method for 24 h using Finn chambers® (8mm, Smart Practice, USA). Sodium lauryl sulfate (SLS) 0.1% was used as a positive control and DI water as a negative control. Observation was undertaken 30 min, 24, 48 and 72h following patch removal. Mean Irritation Index (M.I.I) was then calculated from the sum of irritation grade per total number of subject[25-27].

Eyebrow growth efficacy

The prepared liposome was filled in a tube with applicator brush. Efficacy test on eyebrow growth was performed by the enrolled 15 volunteers for 2 months. The test was designed to mimic the real life application. The participants were instructed to use the product twice a day, in the morning and evening. For each application, the product was applied 2 times on both sides of the eyebrows. The evaluation of hair growth was based on the photo taken on Day 0, 15, 30, 45 and 60. The photos were taken under fluorescent lamp (cool light , 36 watt).

Figure 1. Appearance of (a) *C. ternatea* flower and (b) its concentrated extract.

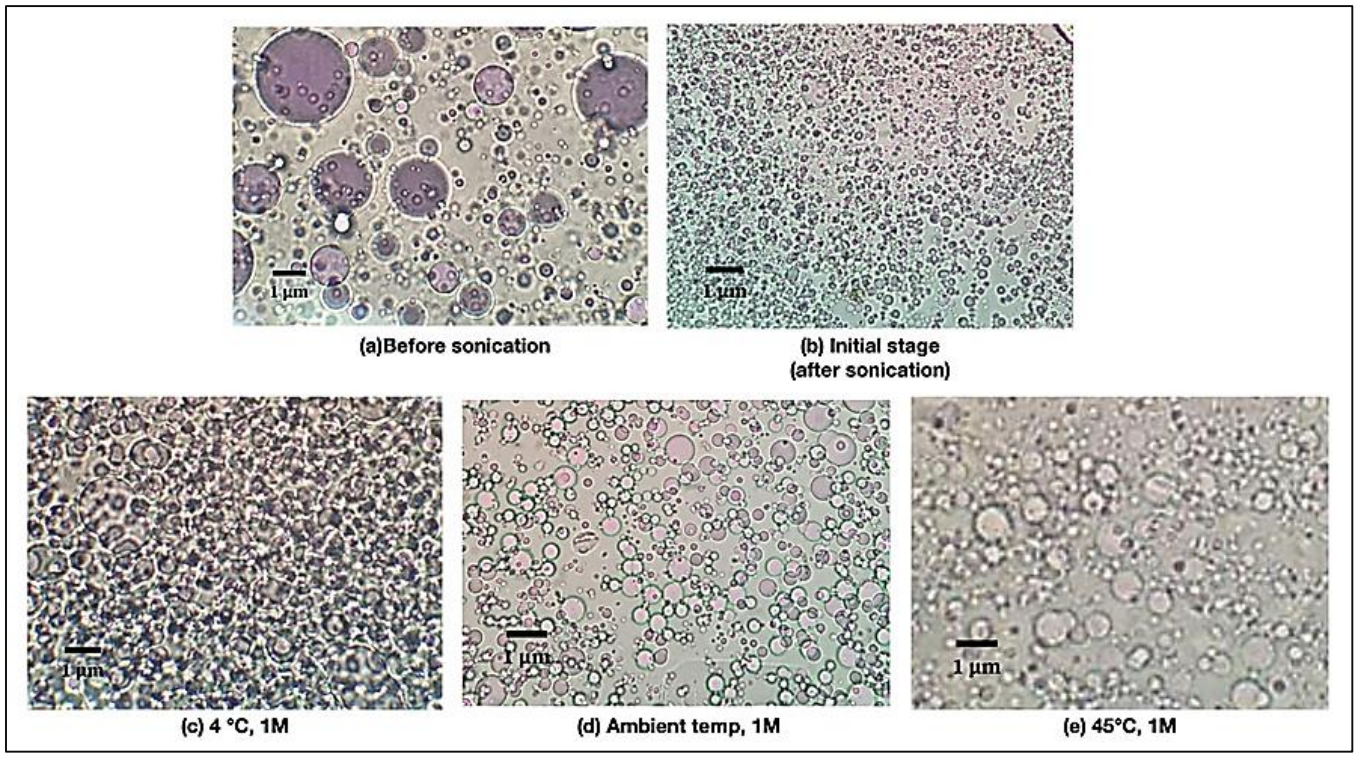

Figure 2. Microscopic examination of *C. ternatea* flower extract and *E. caryophyllus* oil loaded liposome at 100x magnification.

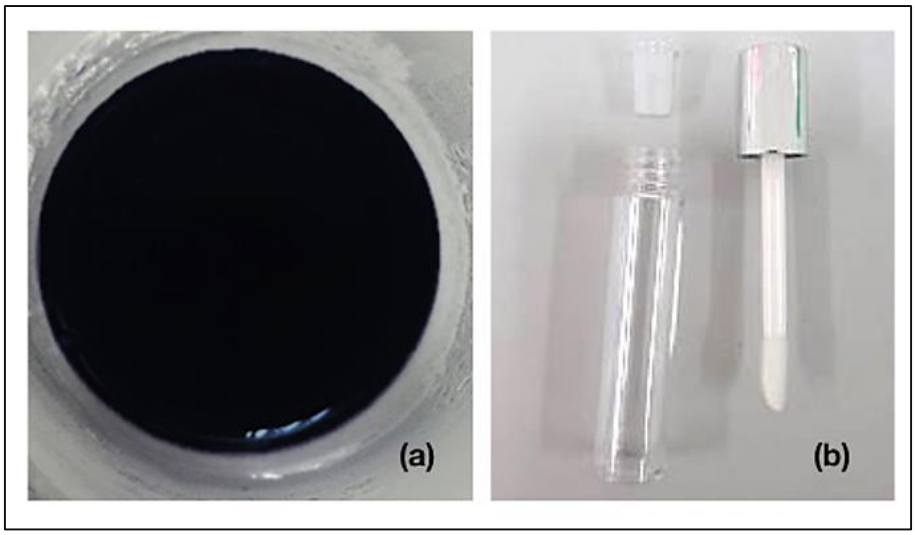

Figure 3. The physical appearance of (a) the *C. ternatea* flower extract and *E. caryophyllus* oil loaded liposome and (b) the packaging for filling the liposome product.

Table 1. pH values of the *C. ternatea* flower extract and *E. caryophyllus* oil loaded liposome at different storage condition.

Storage time	Storage temperature		
	4°C	Ambient temperature	45°C
W0	5.22±0.20	5.20±0.20	5.22±0.20
W2	5.15±0.10	5.28±0.10	5.13±0.10
W4	5.18±0.10	4.31±0.00	4.24±0.00

Table 2. Grading scale to score irritation test performed on subjects using patches.

Score	Skin reaction
0	No visible erythema
0.5	Doubtful reaction, pinkish, mottled
1	Slight diffuse redness
2	Moderate uniform redness
3	Intense redness
4	Very intense redness with oedema or epidermal lesions

Table 3. Interpretation of the Mean Irritation Index (M.I.I) obtained from irritation test performed on subjects using patches.

MII value	Interpretation
M.I.I < 0.2	Non-irritant
0.2 ≤ M.I.I < 0.5	Slight irritant
0.5 ≤ M.I.I < 1.0	Moderate irritant
M.I.I ≥ 1	Irritant

Subjective sensory test

Subjective sensory assessment was evaluated by using hedonic scale method scoring from 1 to 5 (very poor-excellent). The evaluation was done by volunteers after being finished the testing period on Day 60[25].

3. RESULTS

3.1. Preparation of *C. ternatea* flower extract

The fresh petal of butterfly pea flower (Figure 1(a)) was extracted using ethanol (70%). The concentrated extract was obtained by removing 90% of the solvent using rotary evaporator. A viscous liquid with deep blue color as shown in Figure 1(b) was obtained. Phenoxyethanol (1%, w/w) was added to preserve the extract. The concentrated extract has pH value of 4.31.

3.2. Preparation of liposome loaded with *C. ternatea* flower extract and *E. caryophyllus* oil

The liposome of *C. ternatea* flower extract and *E. caryophyllus* oil (TCFF, Thailand) was simply prepared by loading into a ready-made, commercial Pro-liposome (Lipobelle® ST, Mibelle-Biochemistry) according to the registered Thai petty patent[20]. The formation of the liposome loaded with *C. ternatea* flower extract and *E. caryophyllus* oil before and after size reduction by sonication technique was examined

under microscopy (Motic, BA300 Compound Microscope, USA) as shown in Figure 2(a) and Figure 2(b), respectively. The particle size of the prepared liposomes was gauged to be in the usual micron range (0.1-0.5 micrometers) as this can be examined under microscopy. The butterfly pea flower extract or clove oil in liposome was quantified spectrophotometrically at 546 and 239 nm which represent the anthocyanin and eugenol, respectively[28-30]. The loading efficacy of *C. ternatea* flower extract and *E. caryophyllus* oil was 99.6% and 99.8%, respectively.

The physical stability of *C. ternatea* flower extract and *E. caryophyllus* oil loaded liposome was investigated at different storage temperatures, i.e., 4°C, ambient temperature (30-35°C) and 45°C for 1 month. It was found that there was no change regarding appearance, color and odor. However, the pH values of the liposome tend to decrease from 5.20±0.20 to 4.31±0.00 and 4.24±0.00 when being stored at ambient temperature (30-35°C) and 45°C, respectively (Table 1). The difference of pH values were statistically significant (p<0.05) evaluated by independent pair t-test. The change of the pH to lower values may be contributed to the leakage of the *C. ternatea* flower extract from the vesicles as at higher temperature the size of liposome increased (see Figure 2(b-e)).

3.3. Subjective efficacy studies

The potential skin irritation of the prepared liposome was investigated by closed patch test method. The patch test was removed at the end of the 24 h test period. The score of skin irritation was graded according to Table 2[27]. The results showed that there was no irritation, redness and itching on the skin of volunteers. The calculated M.I.I. value was 0, which indicated that the *C. ternatea* flower extract and *E. caryophyllus* oil loaded liposome is potentially non-irritating product, Table 3[27]. Then, the eyebrow growth efficacy was consequently studied. The loaded liposome, which is a viscous liquid with dark blue color (Figure 3a) with characteristic clove oil odor, was filled into a tube with applicator wand (Figure 3b). The product was applied on both sides of eyebrows 2 times. After application, the product can instantly darken the eyebrows (Figure 4) as the butterfly pea flower possessed dark blue color. The participants were asked to continually use the product twice daily (in the morning and evening) for two month period. The evaluation was based on the taken photos. As the product can color the hair and skin, thus the eyebrows were thoroughly cleaned using micelle cleansing solution before the photo was taken. The photos of eyebrows of the selected 2 participants depicting the area of the change of eyebrow thickness and darkness in the circle were presented in Figure 5. It showed that the eyebrows appeared darker, thicker and the hair fibre was longer after using the liposome loaded with *C. ternatea* flower extract and *E. caryophyllus* oil. The effects were apparently seen after 1 month application and became increasingly obvious at 2 months.

Additionally, the density of the eyebrows in the depicted area (Figure 5) was calculated as mean gray value using ImageJ software (Rasband 1997-2018)[31]. The measurement was set from 0 (dark) to 255 (bright). It can be seen that the calculated mean gray value decreased (Table 4) indicating the darkness increased after using the product for 2 months. The calculation helps support the appearance of the eyebrows in Figure 5.

Subjective sensory assessment was finalized by volunteers after using the product for 60 days. The hedonic scores from 1 to 5 (very poor-excellent) were collected and the percentage distribution of each score was reported in Figure 6. The volunteers felt that the *C. ternatea* flower extract and *E. caryophyllus* oil loaded liposome has high skin and hair adhesion after apply and it enhanced the thickness and darkness of eyebrows. The overall satisfaction was also quite high as the product can provide both instant coloring ability and long-term treatment effect on the eyebrows. The likeness on the product's texture, color and odor was also high. However, the volunteers felt the stickiness after apply the product as 53.3% of volunteers gave score of 3 (fair) on the light feeling property (Figure 6). Additionally, there was no irritation reported after the product being used by volunteers twice daily for two months which ensure that the product is topically safe.

4. DISCUSSION

The liposome was loaded with 45% butterfly pea flower extract and 2% clove oil. The loading efficacy of butterfly pea flower extract and clove oil was 99.6-99.8% which was very high. The high loading efficacy of liposome was also previously reported in the previous research[21]. The size of the loaded liposome was approximately in the micron range (0.1-0.5 micrometers) as it can be examined under microscopy. The loaded liposome was macroscopically stable but the vesicles were microscopically larger but still in usual micron range (0.1-1.0 micrometers) after 1 month stability study. The changes of particle size and size distribution may be due to the aggregation and fusion of vesicles; and leakage of entrapped active ingredient. Such destabilizations have been reported to happen at a faster rate when the surface charges of liposomes are decreased by pH or the presence of strong ions[32]. In our study the pH of the liposome significantly decreased (p<0.05) overtime from 5.20±0.20 about 17-19% especially for those stored at higher temperatures and value may be contributed to the leakage of the *C. ternatea* flower extract which has pH value of 4.31.

The results of increasing in thickness and the

Table 4. The brightness-darkness of eyebrows in the depicted area.

Subject		Area	Mean gray value*
Participant #1	Before use	1	122.6±34.0
	After 2 months	1A	102.3±34.5
	Before use	2	186.2±35.6
	After 2 months	2A	154.6±41.7
Participant #2	Before use	3	181.4±30.4
	After 2 months	3A	163.5±45.8
	Before use	4	199.1±25.9
	After 2 months	4A	175.9±46.9

*Note: The scale was set in arbitrary unit from 0 (dark) to 255 (bright)

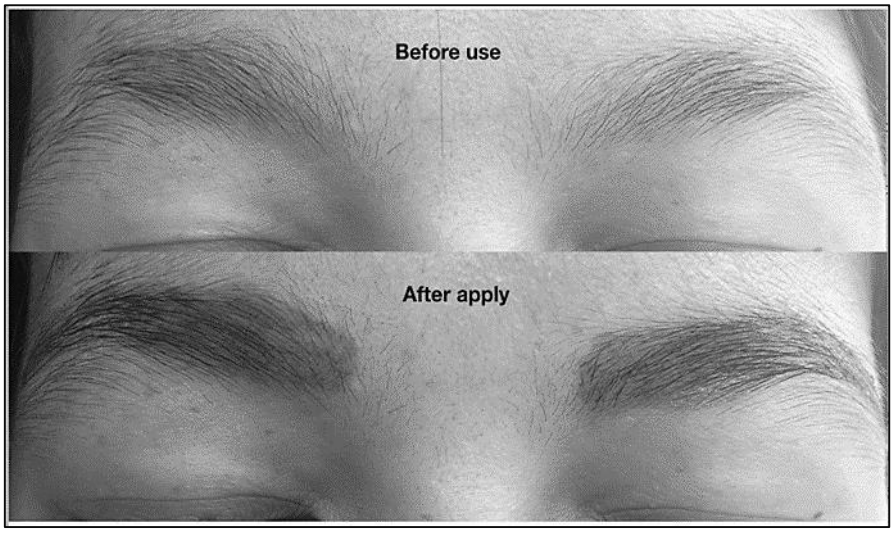

Figure 4. The photos of eyebrows before and after apply the liposome loaded with *C. ternatea* flower extract and *E. caryophyllus* oil.

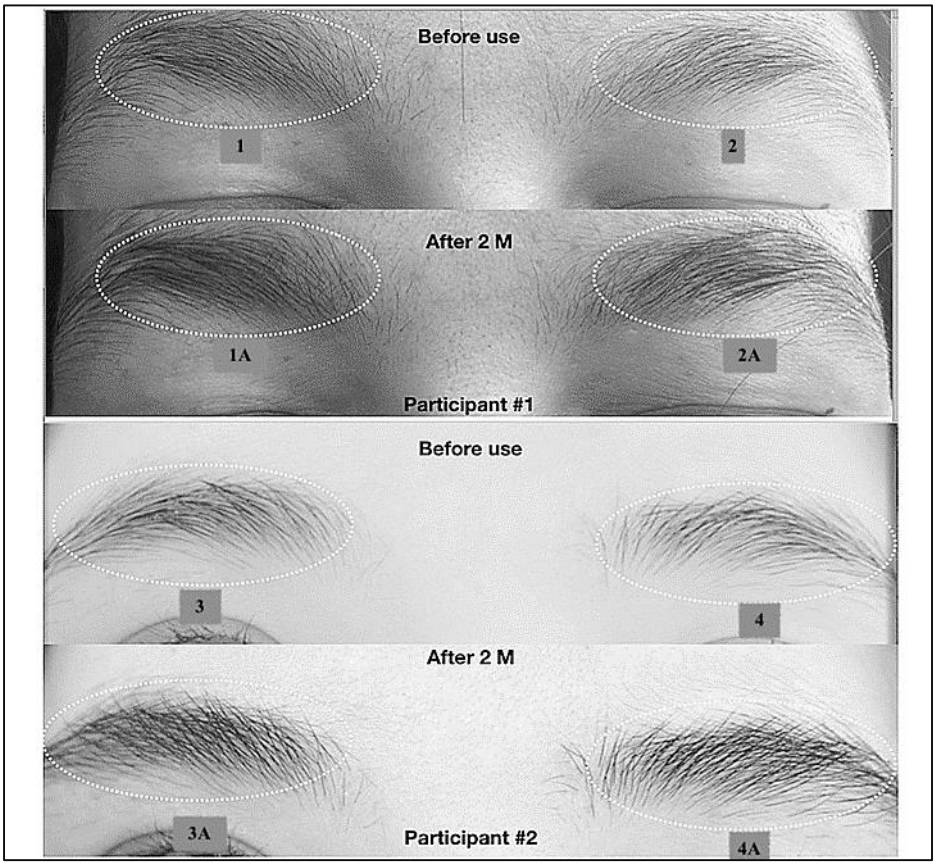

Figure 5. The photos of eyebrows before and after using the *C. ternatea* flower extract and *E. caryophyllus* oil loaded liposome for 2 months.

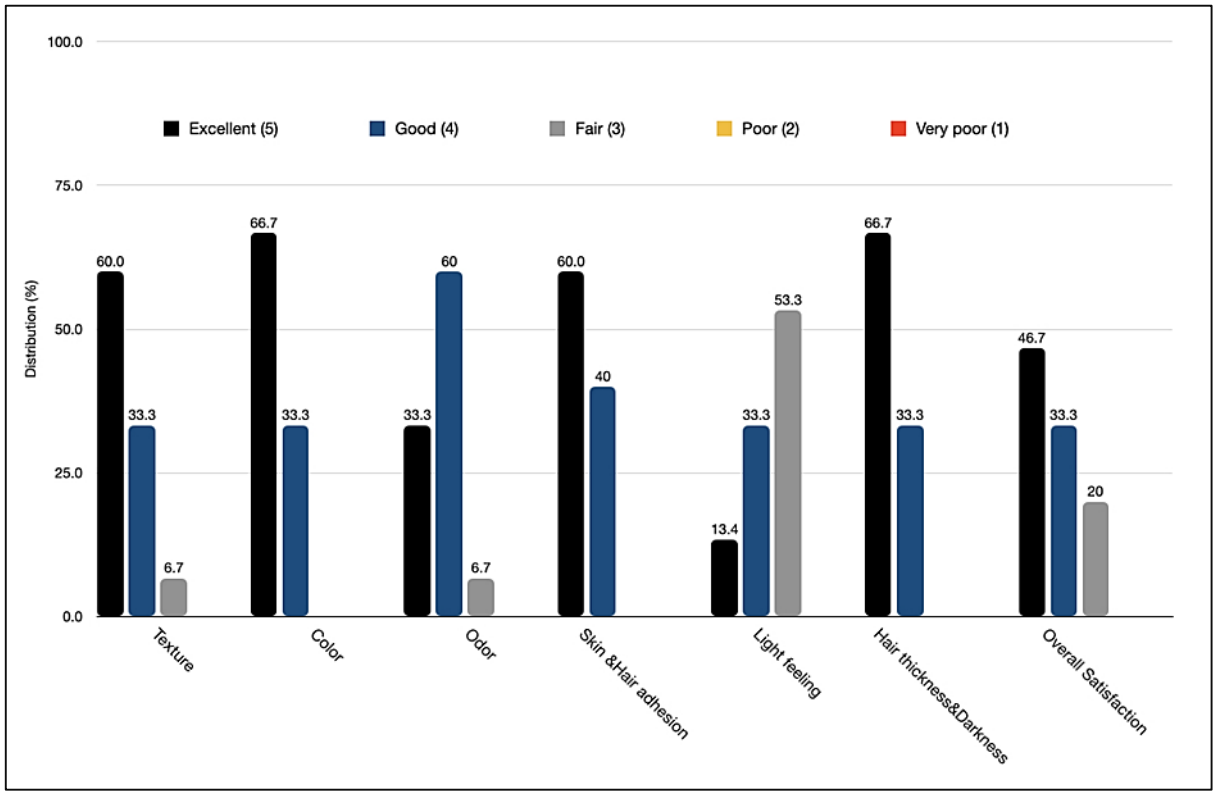

Figure 6. Subjective sensory evaluation of the *C. ternatea* flower extract and *E. caryophyllus* oil loaded liposome.

darker color of eyebrows obtained in this work may confirm the *in vitro* pharmacological activities of *C. ternatea* and *E. caryophyllus* previously reported[10-12,16]. The data obtained in this study is, for the first time, the report on efficacy proof of the butterfly pea flower extract and clove oil loaded liposome on promoting growth of eyebrows. Nonetheless, the results are largely subjective, it is therefore propounding that objectively designed study would conclusively strengthen their hair growth effects. Additionally, the patch test results also indicated that the product is potentially safe for consumer use.

5. CONCLUSIONS

Clitoria ternatea(butterfly pea) flower extract and *Eugenia caryophyllus*(clove) oil were loaded into pro-liposome. The liposome was macroscopically stable but the vesicles were microscopically larger overtime. Subjectively, the eyebrows appeared darker and thicker and the participants feel the enhancement of thickness and darkness of eyebrows with high score of satisfaction. The loaded liposome was topically safe as no irritation reported after daily use for 2 months.

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Conflict of interest

The authors declare that they have no conflict of interest.

Funding

None to declare.

Ethics approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the ethical committee of Mae Fah Luang University and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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