Potential of Topical Microemulsion Serum Formulations to Enhance *In Vitro* and Clinical Anti-Skin Wrinkle Benefits of *Cordyceps Militaris* Extracts

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Abstract

This research is the first to investigate the anti-skin wrinkle properties of *Cordyceps mili-taris* extracts both *in vitro* and *in vivo*. Anti-skin wrinkle activities of *C. militaris* were investigated by means of matrix metalloproteinase-1 (MMP-1), elastase, and hyaluronidase inhibitions. Microemulsions and topical serum formulation containing *C. militaris* extract were developed. The anti-skin wrinkle efficacy and irritation properties of the topical serum formulations were clinically investigated in human volunteers. Cordycepin was identified as a major component of *C. miliaris* extract that was responsible for MMP-1, elastase, and hyaluronidase inhibition. The *C. miliaris* water extract possessed the most potent inhibition on MMP-1 (77.9 ± 5.3%) and elastase (84.4 ± 4.0%). Interestingly, CW was as a potent MMP-1 and elastase inhibitor as oleanolic acid and EGCG. CW was incorporated into the microemulsion with the smallest internal droplet size (146.1 ± 1.5 nm) and further developed as a topical serum formulation. The resulting serum formulation effectively enhanced skin moisture (42.2 ± 14.2%), increased the skin elasticity (39.9 ± 7.3%), and induced no skin irritation in 30 human volunteers. The effectiveness on the skin was detected after 1 week of the applications. Therefore, it was suggested as an effective anti-skin wrinkle formulation.

Introduction

Human skin is an organ directly contact with the external environments, which are primary reasons for skin damage and leading to skin-winkles [1]. Skin ageing is naturally occurred by various factors, such as genetics, metabolic process, environments, mechanical stress, ultraviolet (UV) irritation, pollution, etc. [2]. UV irradiation or photo ageing is the one of the important factors which promote the production of matrix metalloproteinase (MMP), which leads to the decrease or degradation of collagen fibers [3]. Recently, plenty of cosmetic products claim to improve skin problems, especially anti-ageing and reduce wrinkles. The active ingredients for anti-ageing can be obtained from both natural and synthetic process. However, some chemicals could cause serious side effects, such as retinoic acid which has been reported to cause serious skin irritations [3]. Therefore, tretinoin (all-trans retinoic acid) is currently not allowed in Europe (cosmetic regulation 1223/09) and strictly regulated by Food and Drug Administration (FDA) [4]. Consequently, natural active ingredients are getting more attentions.

Cordyceps is the fungi that belongs to Clavicipitaceae family. Some cordyceps have been well-known since they have been used as traditional Chinese medicine for over hundred years, such as *C. sinensis* and *C. militaris*. However, cultivation of *C. militaris* is easier and more popular, whereas, *C. sinensis* are commonly collected from natural source. Although *C. militaris* can be cultivated in the laboratory, the price of *C. militaris* is still high. *C. militaris* is commonly available as drug materials and health-promoting supplements in several Asian countries, such as China, Japan, Korea, Taiwan, Thailand, etc. Various biological activities of *C. militaris* have been reported, including antioxidant, antitumor, immunomodulatory, and hypolipidemic activities [5, 6]. Nevertheless, there are few studies investigated the anti-ageing and anti-wrinkle activities of *C. militaris* extracts.

Our previous study developed nanoemulsion for dermal delivery of *C. militaris* extracts by high pressure homogenization [6]. However, there were some limitations since an expensive high-pressure homogenizer is required for the production of nanoemulsions. Additionally, there were some limitations for the scaling up process. Microemulsion, which is an isotropic spontaneously formed colloidal system, requires no energy nor expensive device [7]. Microemulsion requires only cosurfactant to reduce the interfacial tension between oil and aqueous phase. The internal droplet size of microemulsion is smaller comparing to nanoemulsion. Therefore, microemulsion is always transparent, whereas, nanoemulsion is sometimes translucent [8]. Microemulsion has superior advantages over nanoemulsion since microemulsion exhibits excellent thermodynamically stability, required no energy in the preparation process, and easy to be scaled up [9].

Therefore, the present study aimed to develop microemulsion and cosmetic formulation containing *C. militaris* extracts. This research reported on the anti-wrinkle activities of *C. militaris* extracts in vitro and the clinical anti-wrinkle effects of topical formulation in human volunteers.

Materials And Methods

Fungi materials

C. militaris dried powder was obtained from Mushroom Research and Development Center (MRDC), Chiang Mai, Thailand.

Chemical materials

Cordycepin (purity ≥ 98.0%), L-ascorbic acid (purity ≥ 99.0%), epigallocatechin gallate (EGCG; purity ≥ 95.0%), 3β-hydroxyolean-12-en-28-oic acid (oleanolic acid; purity ≥ 97.0%), sodium chloride (NaCl), sodium phosphate (Na_3PO_4), sodium dihydrogen phosphate (NaH_2PO_4), disodium phosphate (Na_2HPO_4), sodium carbonate (Na_2CO_3), tricine, tris base, N-Succinyl-Ala-Ala-Ala-p-nitroanilide (AAAPVN), N-[3-(2-Furyl)acryloyl]-Leu-Gly-Pro-Ala (FALGPA; purity ≥ 99.0%), hyaluronic acid, bovine serum albumin (BSA), sodium lauryl sulfate (SLS), Tween® 85, Tween® 80, Tween® 20, PEG 40-castor oil, Plantacare® 1200 UP, and Triton™ X-114 were analytical grade purchased from Sigma-Aldrich (St. Louis, MO, USA). All enzymes, including elastase from porcine pancreas lyophilized powder (E–E.C.3.4.21.36), metalloproteinase-1 (MMP-1) from Clostridium histolyticum (ChC–EC.3.4.23.3), and hyaluronidase from bovine testes (E.C.3.2.1.3.5), were also purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol, hexane, ethyl acetate, acetic acid, and dimethyl sulfoxide (DMSO), were analytical grade purchased from Labscan (Dublin, Ireland). Apricot kernel oil, argan oil, avocado oil, canola oil, corn oil, jojoba oil, perilla oil, and sugar squalane, butylene glycol, propylene glycol and glycerin were purchased from Acros Organics (Morris Plains, New Jersey, USA). Carbomer U21, hydroxyethyl cellulose, glycerin, ethylenediaminetetraacetic acid, caprylhydroxamic acid (and) 1,2-hexanediol (and) butylene glycol, and triethanolamine were cosmetic grade purchased from Namsiang Co., Ltd. (Chiang Mai, Thailand). Dulbecco modified eagle medium (DMEM), amphotericin B, l-glutamine, penicillin/streptomycin, and

trypan blue were supplied from Invitrogen™ (Grand Island, NY, USA). GlutaMAX™-I supplement was supplied from Thermo Fisher Scientific, Inc. (Grand Island, NY, USA). HPLC-grade methanol was supplied from Labscan (Dublin, Ireland).

Extraction of C. militaris

Crude ethanolic extract (CC), fractionated hexane extracts (CH), fractionated ethyl acetate extracts (CA), fractionated ethanolic extracts (CE), and water extract (CW) of *C. militaris* were prepared by according to the method previously described by Marsup et al. [6]. All organic solvents were completely eliminated by rotary evaporator (N-1001S-W, Eyela, Tokyo, Japan), whereas, the DI water was eliminated by a freeze dryer (Beta 2–8 LD plus, Christ, Osterode, Germany). All extracts were kept in a well-closed container in a refrigerator until further experiments.

In vitro **determination of anti-wrinkle activities of** C. militaris **extracts**

Determination of matrix metalloproteinase-1 (MMP-1) inhibition

C. militaris extracts and cordycepin were investigated for their inhibitory activity against MMP-1 by using spectrophotometric method according to the previously described method of Chaiyana et al. (2019) which was slightly modified from Thring et al. (2009) [10, 11]. The final concentration of cordycepin and *C. militaris* extracts in the tested system was 0.1 mg/ml. Blank of sample, which was a sample solution, was performed to reduce an interference from colored samples. Blank of control, which was the native solvent, was performed to reduce the interference from each solvent. The MMP-1 inhibition was calculated using the following equation; MMP-1 inhibition (%) = [1-(A/B)] ×100, where A is a different absorbance between sample solution with MMP-1 and the sample solution without MMP-1 and B is a different absorbance between MMP-1 solution and its native solvent. Oleanolic acid was used as a positive control. The experiments were analyzed in triplicate.

Elastase enzyme inhibition

C. militaris extracts and cordycepin were investigated for their inhibitory activity against elastase by using spectrophotometric method according to the previously described method of Chaiyana et al. (2019) which was slightly modified from Thring et al. (2009) [10, 11]. The final concentration of cordycepin and *C. militaris* extracts in the tested system was 0.1 mg/ml. Blank of sample, which was a sample solution, was performed to reduce an interference from colored samples. Blank of control, which was the native solvent, was performed to reduce the interference from each solvent. The inhibition of elastase was calculated using the following equation; Elastase inhibition (%) = [1-(A/B)] ×100, where A is a different absorbance between sample solution with elastase and the sample solution without elastase and B is a different absorbance between elastase solution and its native solvent. EGCG was used as a positive control. The experiments were analyzed in triplicate.

Hyaluronidase inhibition

C. militaris extracts and cordycepin were investigated for their hyaluronidase inhibitory activity by using spectrophotometric method according to the previously described method of Nema et al. (2011) [12] with slight modifications. The final concentration of cordycepin and *C. militaris* extracts in the tested system was 0.1 mg/ml. Blank of sample, which was a sample solution, was performed to reduce an interference from colored samples. Blank of control, which was the native solvent, was performed to reduce the interference from each solvent. The inhibition of elastase was calculated using the following equation; Hyaluronidase inhibition (%) = [1-(B/A)] ×100; where A is a different absorbance between a sample solution with hyaluronidase, hyaluronic acid, and BSA and a sample solution without these reagents, whereas, B is a different absorbance between hyaluronidase solution and its native solvent. EGCG was used as a positive

Chemical characterization of CW

The chemical characterization of CW, which was the most potent anti-wrinkle extract, were performed using high performance liquid chromatography (HPLC) regarding to our previous study [6]. Cordycepin was used as a standard compound. The experiments were analyzed in triplicate.

Development of microemulsion

Pseudoternary phase diagram construction

Pseudoternary phase diagrams were prepared by using water titration method [13]. The samples were defined as microemulsions when they were transparent liquid with low viscosity. Various factors affecting microemulsion formation were investigated, including oil types, surfactant types, co-surfactant types, and ratio of surfactant to co-surfactant (Smix).

To investigate the effect of oil type, various types of oils, including jojoba oil, argan oil, apricot kernel oil, perilla oil, corn oil, canola oil, and sugar squalane were used as the oil phase in microemulsion system when other components are fixed Tween® 85 was used as a surfactant, butylene glycol was use as a co-surfactant, and the Smix ratio was 1:1. To investigate the effect of surfactant type, various types of non-surfactants, including Tween® 20, Tween® 80, Tween® 85, PEG 40-caster castor oil, Plantacare® 1200 UP, and Triton™ X-114 were used as the surfactant in microemulsion system when sugar squalane was used as an oil, butylene glycol was use as a co-surfactant, and the Smix ratio was 1:1. To investigate the effect of co-surfactant type, various co-surfactant types, including glycerin, propylene glycol, and butylene glycol were used as the co-surfactant in microemulsion system when sugar squalane was used as an oil, Tween® 85 was use as a surfactant, and the Smix ratio was1:1. To investigate the effect of surfactant to co-surfactant ratio, various surfactant to co-surfactant ratio, including 1:2, 2:1. 3:1, 4:1, and 5:1 were investigated when sugar squalane was used as an oil, Tween® 85 was used as a surfactant, and butylene glycol was used as a co-surfactant.

Microemulsions from the pseudoternary phase diagram which give the largest area of microemulsion region, were prepared.

Characterizations of microemulsions

Organoleptic inspections were used to assess the external presence of microemulsions. The zeta sizer (Zetasizer®, Malvern Instruments Ltd., Malvern, UK) was used to investigate the internal droplet size, polydispersity index (PDI), and zeta potential. A pH meter was used to measure the pH of the formulation.

Stability of microemulsions

The stability of microemulsions was investigated after 8 heating-cooling cycles in which the microemulsions were stored at 45°C for 24 h and then at 4°C for 24 h. The physical appearance, particle size, PDI, and zeta potential were then investigated as previously stated.

Preparation of microemulsion containing C. militaris extract

C. militaris extract with the most potent anti-wrinkle activities was selected for incorporating into the best microemulsion system. Isotropic appearance, small internal droplet size, narrow size distribution, stable after storage, and appropriate concentration of surfactant used in the formulation were among the selection criteria for the best microemulsion. To incorporate the *C. militaris* extract, it was first dissolved in DI water and then combined with the other ingredients until homogeneous. The microemulsions containing *C. militaris* extract were characterized and investigated for their stability in heating-cooling conditions as described above.

In vitro **cytotoxicity effect determination on HaCaT cells**

Before the preparation of topical serum formulations for evaluation in human volunteers, the cytotoxicity effects of on HaCaT cells of microemulsion containing *C. militaris* extract were investigated in a comparison with their blank formulations. Additionally, aqueous solution of CW was also evaluated for the cytotoxicity effect. In brief, the HaCaT cells (Cell Lines Service, Eppelheim, Germany) were grown and the cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay regarding to our previous study [6].

Preparation of topical serum formulation from microemulsion containing C. militaris **extract**

The microemulsions containing *C. militaris* extract were incorporated into the serum formulation, the ingredients of which are mentioned in Table 1. The topical serum formulation from microemulsion containing *C. militaris* extract were prepared regarding good manufacturing practices (GMP), characterized and investigated for the stability in heating-cooling conditions as described above.

Table 1
Composition of serum formulation

Ingredients	Amount (% w/w)
Carbomer U21	0.15
Hydroxyethyl cellulose	0.15
Glycerin	7.5
Ethylenediaminetetraacetic acid	0.75
Caprylhydroxamic acid (and) 1,2-hexanediol (and) butylene glycol	0.75
Triethanolamine	0.11
Water	q.s. 100

Microbial enumeration test of non-sterile serum formulation

Methods for microbial limit numbers and specified microorganisms in non-sterile cosmetic products were assessed in accordance with the procedure and criteria laid down in standard requirements of the United States Pharmacopeia 32–National Formulary 27 [14] and the European Pharmacopoeia (EP) [15]. Total viable aerobic microbial count (TAMC) and total yeasts and molds count (TYMC) were performed by plating serum sample on soybean casein digest agar (SCDA) and Sabouraud dextrose agar (SDA), respectively. The specified harmful pathogens, such as *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Clostridium* sp. and *Candida albicans* were determined dependent on the method validation under the guidelines. Culture plates were incubated and the results are displayed as colony forming units per mL of serum (CFU/mL).

In vivo **irritation test in human using human patch test**

Before the *in vivo* skin irritation study, the ethic for human research was approved by the Human research ethics committees, Faculty of Pharmacy, Chiang Mai University. The ethics approval number was 04/2563. The skin irritation was investigated in 30 healthy volunteers by using human patch test with some modifications [16, 17]. The samples were applied on the inner lower arm skin for 4 h. During the study, the skin irritation was observed at 15, 30, 60, 120, 180, and 240 min. After that the patch was taken off and the skin irritation was further assessed at 24, 48, and 72 h.

Efficiency evaluation in human volunteers

Before the efficiency evaluation, the ethic for human research was approved by the Human research ethics committees, Faculty of Pharmacy, Chiang Mai University. The ethics approval number was 04/2563. The efficacy of microemulsion containing *C. militaris* extract were investigated in 30 healthy volunteers. Two products, including topical serum formulation from microemulsion containing *C. militaris* extract (CW serum) and blank serum formulation (Blank) were applied on the inner forearm skin of the left and right arm, respectively. The blank serum formulation was served as placebo. During the

experimental period of 14 days, the volunteers can use all other products in normal daily life. Before and after applying the products on the skin twice daily for 14 days, the skin was evaluated for moisture and elasticity by using portable skin test equipment (M-6602, Shenzhen Fuhengtong Technology, Shenzhen, China). The exact area of the skin, where skin elasticity and moisturization were evaluated, was marked by using a plastic sheet and punching a hole with an area of 3×3 cm placed on the inner forearm area, setting the edge on the volunteers' wrist. Skin moisturization, which was recorded as a function of electrical conductance, and skin elasticity, which was recorded as a function of skin stretching, were recorded and the changes in these parameters were calculated using the equations; Skin elasticity change (%) = A/B×100, where A is a different skin elasticity between before and after application of the formulations and B is a skin elasticity before the formulation application. Additionally, Skin moisture change (%) = A/B×100, where A is a different skin moisture between before and after application of the formulations and B is a skin moisture before the formulation application.

Additionally, Likert-scale questionnaires were also used to evaluate the efficiency of the products in terms of moisturizing, elasticity, and winkle reducing effects.

Investigation of satisfaction in human volunteers

Before the satisfaction evaluation, the ethic for human research was approved by the Human research ethics committees, Faculty of Pharmacy, Chiang Mai University. The ethics approval number was 04/2563. The satisfaction on microemulsion containing *C. militaris* extract was investigated in 30 healthy volunteers after 14 days of application. Likert-scale questionnaires were used to evaluate the volunteer's satisfaction in terms of physical appearance (viscosity, color, and odor) and topical application (non-sticky, emollient, skin absorption, non-skin irritation, moisturizing, skin texture, and skin slippery).

Statistical analysis

Data was analyzed and reported as mean and standard division (S.D.). The statistical analysis was processed by t-test and ANOVA using SPSS program (SPSS Statistics 17.0, IBM Corporations, New York, USA). Statistically significant different was denoted when $P < 0.05$.

Results And Discussion

C. militaris **extracts**

The external appearance of each *C. militaris* extracts were different. CC, CA, and CE were brown-yellowish semisolid masses with a distinct odor on the outside, while CH was a lighter brown-yellowish semisolid mass. In contrast, CW was a light-brown dry powder.

Anti-wrinkle activities of C. militaris **extracts**

Skin ageing is naturally occurred by a decline of the extracellular matrix (ECM), including collagen fibers, elastin fibers, and hyaluronan by the favor of MMP-1, elastase, and hyaluronidase, respectively [18].

These enzymes mostly presented in the dermis layer and played important roles in the degradation of ECM and resulting in the skin winkles [19]. Inhibitory activities against MMP-1, elastase, and hyaluronidase of *C. militaris* extracts are shown in Table 2. Among different *C. militaris* extracts, CW possessed the significantly highest MMP-1 and elastase inhibitory activities with the inhibition of 77.9 ± 5.3% and 84.4 ± 4.0%, respectively. Interestingly, the MMP-1 and elastase inhibition of CW was as potent as oleanolic acid and EGCG, a naturally occurring triterpenoid widely known for MMP-1 and elastase cascade [11]. Therefore, CW was suggested as a potent natural extract which exerted anti-wrinkle activities. On the other hand, cordycepin was noted as a major component of CW which responsible for MMP-1 and elastase inhibition since it exhibited potent MMP-1 and elastase inhibitory activities with the inhibition of 58.5 ± 4.9% and 76.5 ± 7.1%, respectively. Although, there are some previous reports revealed about the MMP-1 inhibitory activity of *C. militaris*, they suggested cordycepin as the key compound that inhibited the activator protein-1 and resulting in the suppression of MMP-1 expression [20, 21]. However, the present study was the first to remarked that both cordycepin and *C. militaris* extracts exerted a direct effect on the MMP-1 enzyme. Additionally, the present study was the first to reveal anti-elastase of *C. militaris* extracts and suggested CW as the most potent anti-wrinkle ingredient for cosmetic formulations. However, the IC_{50} value was preferable to comparing the strength of the inhibition of CW with the standard compound. Therefore, it was suggested for further study to investigate the IC_{50} value of CW in a comparison with oleanolic acid and EGCG on the inhibition of collagenase and elastase, respectively.

Table 2
Anti-ageing activities of *C. militaris* extracts

Samples	Collagenase inhibition (%)	Elastase inhibition (%)	Hyaluronidase inhibition (%)
OA	71.7 ± 0.2^{a}	N.D.	N.D.
EGCG	N.D.	89.6 ± 4.1^{a}	74.8 ± 2.3^{a}
COR	58.5 ± 4.9^{b}	$76.5 \pm 7.1^{a,b}$	65.5 ± 2.1^{a}
CH	0.0 ± 0.0^{c}	32.9 ± 0.2^{d}	30.7 ± 8.5^{b}
CA	0.0 ± 0.0^{c}	$55.8 \pm 8.5^{b,c}$	69.0 ± 3.9^{a}
CE	56.6 ± 3.0^{b}	$42.4 \pm 5.0^{c,d}$	33.2 ± 7.6^{b}
CC	10.8 ± 0.9^{c}	26.5 ± 7.3^{d}	41.5 ± 7.1^{b}
CW	77.9 ± 5.3^{a}	84.4 ± 4.0^{a}	14.3 ± 5.6^{c}

Note: OA = oleanolic acid; EGCG = epigallocatechin gallate; COR = cordycepin; CH = hexane extract; CA = ethyl acetate extract; CE = ethanolic extract; CC = crude ethanolic extract; CW = water extract; N.D. = not determined. The final concentration of all tested compounds and extracts in the tested system was 0.1 mg/ml. Data are shown in mean value ± S.D. (n = 3). There letters a, b, c, and d denoted significant difference between samples analyzed using post-hock Tukey ANOVA ($p < 0.05$).

In contrast to MMP-1 and elastase inhibition, CW possessed the least inhibitory activity against hyaluronidase. CA was noted as the most potent hyaluronidase inhibitor with the inhibition of 69.0 ± 3.9%. Interestingly, CA was as potent as EGCG which was well-known as anti-ageing compound. Although cordycepin possessed a potent inhibitory activity of 65.5 ± 2.1% against hyaluronidase, the cordycepin content was not related to the hyaluronidase inhibition. Therefore, cordycepin was not the only compound responsible for the hyaluronidase inhibitory activity. Previous studies suggested that *C. militaris* contained large quantity of polysaccharides [22, 23], which has been reported for the hyaluronidase inhibition [24]. Since the retardation in hyaluronan degradation and increasing hyaluronan level in the skin layer by both putting hyaluronan in topical skin care products or using as a temporary dermal filling agent were suggested for plumping and youthful skin [25]. CA would be another natural extract potentially retard the skin ageing.

Although CA possessed the significantly highest hyaluronidase inhibition ($p < 0.05$), it had no effect on MMP-1 inhibition. Therefore, CW was selected for further formulations development since CW exerted the most potent MMP-1 and elastase inhibition ($p < 0.05$), as well as a moderate hyaluronidase inhibitory activity (14.3 ± 5.6%). The effective concentration of CW was suggested at 0.1 mg/ml because this concentration led CW to possess approximately 80% inhibitory activity against both MMP-1 and elastase. However, only some active compounds could release and deeply penetrate into the target site of the skin. Therefore, approximately 100 times higher than the effective concentration, which was 10 mg/ml or 1% w/v, was suggested for further product development [6].

Chemical characteristic of CW

CW, which was the most potent anti-wrinkle extract, was characterized for its chemical constituent. The HPLC chromatograms of cordycepin and CW are shown in Fig. 1. Cordycepin was detected at the retention time of 6.347 min, whereas, there was a major peak detected around 6.3 min in the HPLC chromatograms of CW. Therefore, it could be noted that cordycepin was a major chemical constituent of CW, which could be further used as a marker for the quantitative determination of CW in the further study.

Microemulsions development

Effect of oil type

Various oil types generated different microemulsion region in the pseudoternary phase diagrams as shown in Fig. S1. Sugar squalane gave the largest area of microemulsion (9.5%), followed by jojoba oil, argan oil, apricot kernel oil, canola oil, corn oil, avocado oil, and perilla oil, respectively. Since sugar squalane is a unique cosmetic ingredient which is a mobile, colorless, odorless, and has good physical and chemical stability, it was selected for the further studies. Additionally, squalene has been known as a natural triterpene hydrocarbon which is one of the most important lipids in human skin cell and accounted for up to 13% of total lipids synthesized from the sebaceous glands [26].

Effect of surfactant type

Pseudoternary phase diagrams constructed using various types of surfactant. Tween® 85 was the only one surfactant which could generate microemulsion with the region of 9.5% in the pseudoternary phase diagram. The results were well accordance with previous studies, which suggested Tween® 85 as the most suitable surfactant for microemulsion development [27, 28]. Therefore, Tween® 85 was selected for the further studies.

Effect of co-surfactant type

The single surfactant is not enough for reducing interfacial tension between water and oil and generate microemulsion, of which the internal droplet size is in the range of 10–200 nm [29]. Therefore, co-surfactant is required in the development of microemulsion. Pseudoternary phase diagrams constructed using various types of co-surfactant are shown in Fig. S2. The results showed that propylene glycol gave the largest microemulsion area in pseudoternary phase diagram (11.1%), followed by butylene glycol. On the other hand, glycerin could not generate microemulsion. The likely explanation might be due to the presence of alkane triol which composed of three hydroxyl groups in the glycerin molecule and led to high hydrophilic property but lower efficacy in the interfacial tension reduction [30]. Therefore, propylene glycol was selected for the further studies.

Effect of surfactant to co-surfactant ratio

Various ratio of surfactant to co-surfactant showed different microemulsion region in the pseudoternary phase diagrams as shown in Fig. S3. The results represented that microemulsion region increased when the surfactant proportion in Smix increased from 1:2 to 2:1. Therefore, it could be concluded that there should be higher content of surfactant than co-surfactant in the Smix. However, microemulsion region tended to decrease after increasing of the surfactant from 1:2 to 5:1. The likely explanation might be due to the lower co-surfactant content which was not enough to reduce the interfacial tension. Therefore, Smix ratio of 2:1 was selected for further studies.

Microemulsion preparation

Since all parameters, including oil type, surfactant type, co-surfactant type, and Smix ratio affected the microemulsion region in the pseudoternary phase diagrams, the system that generated the largest region of microemulsion was selected for further microemulsion development. In brief, the selected system composed of sugar sqaulane, Tween® 85, propylene glycol, and DI water. The Smix ratio was 2:1. Four formulations (ME1 – ME4) along the line of 30% w/w water content as shown in Fig. 2 were developed and characterized to investigate the effects of different Smix and oil content.

All formulations (ME1 – ME4) were homogeneous transparent liquid with low viscosity which could be defined as microemulsion [31]. Higher content of Smix led to darker yellow color and more viscosity. The internal droplet size of each microemulsions were ranging from 107.2 ± 2.9 nm to 366.3 ± 16.0 nm as shown in Fig. 3. Since all microemulsion contained 30% w/w of water phase, the increasing of Smix but decreasing of oil phase were used in ME1 to ME4, respectively. ME1, which contained the highest oil content (40% w/w), tended to be W/O microemulsion since the amount of oil phase was higher than the

water phase. ME2, which contained an equal amount of oil and water phase (30% w/w), tended to be O/W microemulsion since Tween® 85 is hydrophilic surfactant with high HLB value (11.0). The results noted that ME2 had the smallest internal droplet size (107.2 ± 2.9 nm). However, the size of O/W microemulsion increased with the increasing amount of Smix although the oil content decreased. The likely explanation might be due to their higher viscosity since size of internal droplets has been reported to increase with an increase of the viscosity [32]. PDI of these microemulsions were ranging from 0.7 ± 0.1 to 1.0 ± 0.0, which were very high. The likely explanation might be due to the measurement of undiluted sample of which the internal droplets were not well dispersed and led to the detection of larger size than usual [33]. However, microemulsion could not be diluted before the size and PDI measurement because the microemulsion system would be changed. On the other hand, all microemulsion had the same pH value around 6.9 ± 0.1 to 7.1 ± 0.2. Additionally, all microemulsions were stable since no changing in external appearance, e.g., separation, sedimentation, or coagulation, were not detected after 8 cycles of heating-cooling condition. The internal droplet size was still remained the same. Therefore, ME1 was selected as a representative of W/O microemulsion and ME2 was selected as a representative of O/W microemulsion for further incorporation of *C. militaris* extract (CW).

Microemulsion containing C. militaris extract

ME1 and ME2 were selected for the incorporation of CW. The external appearance of microemulsion containing *C. militaris* extract (ME1-CW and ME2-CW) were homogeneously transparent and brown-yellow color with low viscosity. The internal droplet size of ME2-CW (146.1 ± 1.5 nm) was significantly smaller than that of ME1-CW (212.4 ± 1.5 nm) ($p < 0.05$). On the other hand, the PDI of ME2-CW (0.5 ± 0.0) was significantly narrower than that of ME1-CW (0.9 ± 0.1) ($p < 0.05$). Therefore, ME2-CW tended to be more stable than ME1-CW due to its narrower size distribution of the internal droplets. Interestingly, the internal droplet size of ME1-CW and ME2-CW were larger than their own blank microemulsions as shown Fig. 4. Hence, it could be assumed that CW was entrapped inside the droplet of microemulsion and led to larger internal droplet size after the *C. militaris* extract incorporation.

A basic analysis of the stability under heating-cooling conditions could be used as a fast tool to predict the stability of emulsions, such as cosmetic formulations [34, 35]. After 8 cycles of heating-cooling condition, both ME1-CW and ME2-CW were physically stable since no changing in external appearance were detected. However, the internal droplet size of ME1-CW was significantly increased ($p < 0.05$). The results were well accordance with the previous PDI data noted that the internal droplet size distribution of ME1-CW was board. On the other hand, ME2-CW was stable since its internal droplet size was still remained unchanged. Although the internal droplet size of ME1-CW increased, the size was still in the nano scale (212.4 ± 1.5 nm). Therefore, ME2-CW were selected for further preparation of topical serum formulation.

Cytotoxicity effects on HaCaT cells of microemulsion containing C. militaris extract

The cytotoxicity effects on HaCaT cells of microemulsions with and without *C. militaris* extract (CW) are shown in Fig. 5. All formulations were found to be safe since the HaCaT cell viability was approximately

higher than 80%. No significant difference was detected among these formulations. The HaCaT cells viable after being exposed to the aqueous solution of *C. militaris* water extract (CW), ME1, ME2, ME1-CW, and ME2-CW were 78.5 ± 1.3%, 86.26 ± 1.0%, 81.6 ± 0.0%, 80.2 ± 1.9%, and 79.4 ± 4.6%, respectively. The results were well comparable with our previous study which reported that nanoemulsions had no cytotoxicity effect on the HaCat cells and biocompatible [6]. Therefore, both ME1-CW and ME2-CW could be used for the preparation of topical serum formulations for evaluation in human volunteers. ME2-CW, on the other hand, with a smaller internal droplet scale and a narrower PDI, was chosen for further production of topical serum formulations.

Topical serum formulation from microemulsion containing C. militaris extract

The external appearance of topical serum formulation from microemulsion containing *C. militaris* extract was homogeneously transparent yellow liquid. The viscosity was 0.8 ± 0.0 mPas. The formulation was stable since no changing in external appearance, e.g., separation, sedimentation, or coagulation, were not detected after 8 cycles of heating-cooling condition. Additionally, the viscosity was still remained the same. Therefore, it was a good formulation suggested for further applied in the human volunteers.

Microbial enumeration test of non-sterile serum formulation

Microbial contaminants may grow by using ingredients in the products causing physicochemical changes or spoilage and finally loss in product safety, quality, activity, and stability. In tested serum, the TAMC was ≤ 1 x 102 CFU/mL and the TYMC was not detected. The specified pathogens, *E. coli, P. aeruginosa, S. aureus, Clostridium* sp. and *C. albicans* were not detected in tested serum formulation. The tropical serum products were compliant with the accepted criteria according to guidelines of non-sterile and cosmetic products due to non-excessive microbial count and the absences of specified pathogens. The compliant serum formulation was applied use in human volunteers.

Irritation properties of topical formulation from microemulsion containing C. militaris extract

Thirty healthy human volunteers (77% of female and 23% of male), ranged in age from 21 to 60, were tested on the serum formulation. The results noted that the topical formulation from microemulsion containing *C. militaris* extract induced no irritation sign on human skin. Therefore, it was suggested as safe for using topically on the skin.

Efficacy of topical formulation from microemulsion containing C. militaris extract

After two weeks of the application, the skin appearance improved as shown in Fig. 6. The results showed that the skin elasticity and skin moisture significantly improved after 1 week of serum products application and continuously increased after 2 weeks as shown Fig. 7. Interestingly, the topical formulation from microemulsion containing *C. militaris* extract could enhance the skin elasticity and skin moisture when compared to its own base formulations. The topical formulation from microemulsion containing *C. militaris* extract could improve the skin elasticity by 39.9 ± 7.3% from the baseline after 1 week and maintained at 38.5 ± 9.0% after 2 weeks of application. The potent inhibitory activities of CW

on MMP-1 and elastase may be the likely explanation of the effective results on the skin since it could reduce the damage of collagen and elastin fiber. Apart from the enhancement of skin elasticity, the skin moisture was also improved after the application of serum formulation. The topical formulation from microemulsion containing *C. militaris* extract could increase the skin moisture by 42.2 ± 14.2% from its baseline after 1 week and 38.3 ± 15.2% after 2 weeks of applications. The likely explanation might be due to the composition of various polysaccharide in the *C. militaris* extracts, which could reduce transdermal water loss (TEWL) and protect the skin barrier [36]. Although the skin moisturization measure in the present study was based on the electrical conductance, the reduction in TEWL would result in increased skin moisturization since water can be retained in the skin layer. Apart from *C. militaris* extracts, sugar squalane, which was used as an oil phase in the microemulsion, would be another component that would help to moisturize the skin. Sugar squalene is commonly used as an occlusive ingredient, so it could reduce TEWL and thereby in-creases the skin moisture. Therefore, the serum formulation not only effectively enhanced the skin moisture, but also improved the skin elasticity. Although the clinical studies regarding cosmetic formulations efficacy are usually performed for two or three months because the epidermal transit time is around one month, the skin improvement could be detected since the first week of a serum formulation application. The likely explanations were not only from the inhibitory activity on extracellular matrix degradation in the dermis layer but also attributed to the enhancement of the skin moisturization.

Satisfaction on the topical formulation from microemulsion containing C. militaris **extract**

The topical formulation from microemulsion containing *C. militaris* extract gained high level of the volunteers' satisfaction after two weeks of application in the term of physical appearance and efficacy as shown in Fig. 8. It obtained a satisfactory score in terms of viscosity (4.4 ± 0.6/5.0), odor (4.5 ± 0.8/5.0), moisturizing effect (4.6 ± 0.7/5.0), emollient (4.7 ± 0.5/5.0), skin absorption (4.6 ± 0.6/5.0), skin texture (4.6 ± 0.7/5.0), skin slippery (4.6 ± 0.8/5.0), no skin irritation (4.9 ± 0.3/5.0), skin elasticity enhancement (4.3 ± 0.8/5.0), winkle reduction (4.2 ± 0.9/5.0), and non- sticky (4.3 ± 1.1/5.0).

Conclusions

CW was the most potent natural anti-wrinkle extract with potent inhibitory activities against MMP-1 (77.9 ± 5.3%) and elastase (84.4 ± 4.0%). Interestingly, CW exhibited comparable MMP-1 and elastase inhibition to that of oleanolic acid and EGCG. Cordycepin, which was a major chemical constituent of CW, was the compound responsible for its anti-skin ageing activities. Therefore, CW has a great potential to be used as active cosmetic ingredient for anti-wrinkle. CW was incorporated in the microemulsion and topical serum formulation. No cytotoxicity effect of the microemulsions was observed in HaCaT cells. The topical serum formulation from microemulsion containing *C. militaris* extract induced no skin irritation and significantly improved the skin moisture (42.2 ± 14.2%), as well as skin elasticity (39.9 ± 7.3%) from its baseline after 1 week of the applications. Therefore, this formulation was recommended for further use as anti-wrinkle cosmetic/cosmeceutical products.

Abbreviations

CA: fractionated ethyl acetate extract; CC: crude ethanolic extract; CE: fractionated ethanolic extract; CH: fractionated hexane extract, CW: water extract; ECM: extracellular matrix; HaCaT: human keratinocyte; IC_{50}: The half maximal inhibitory concentration; MMP-1: Matrix metalloproteinase-1.

Declarations

Completing Interests: The authors declare no completing interests.

Author Contributions: P.M. and W.C.: experiment design and methodology. P.M., Sriyab S., Sirilun S. and W.N.: formal analysis. P.M., Sirilun S. and W.N.: investigation. W.C., J.S., S.A. and C.T.: resources. W.C. and P.M.: writing-original draft preparation. Sirilun S., A.P., W.C. and P.M.: writing-review and editing. W.C.: supervision and project administration. W.C., P.M., and Sriyab S: funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials: The used datasheets and materials are available from the corresponding authors on reasonable request.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Faculty of Pharmacy, Chiang Mai University (protocol code: 04/2563; approved in 2020).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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Figures

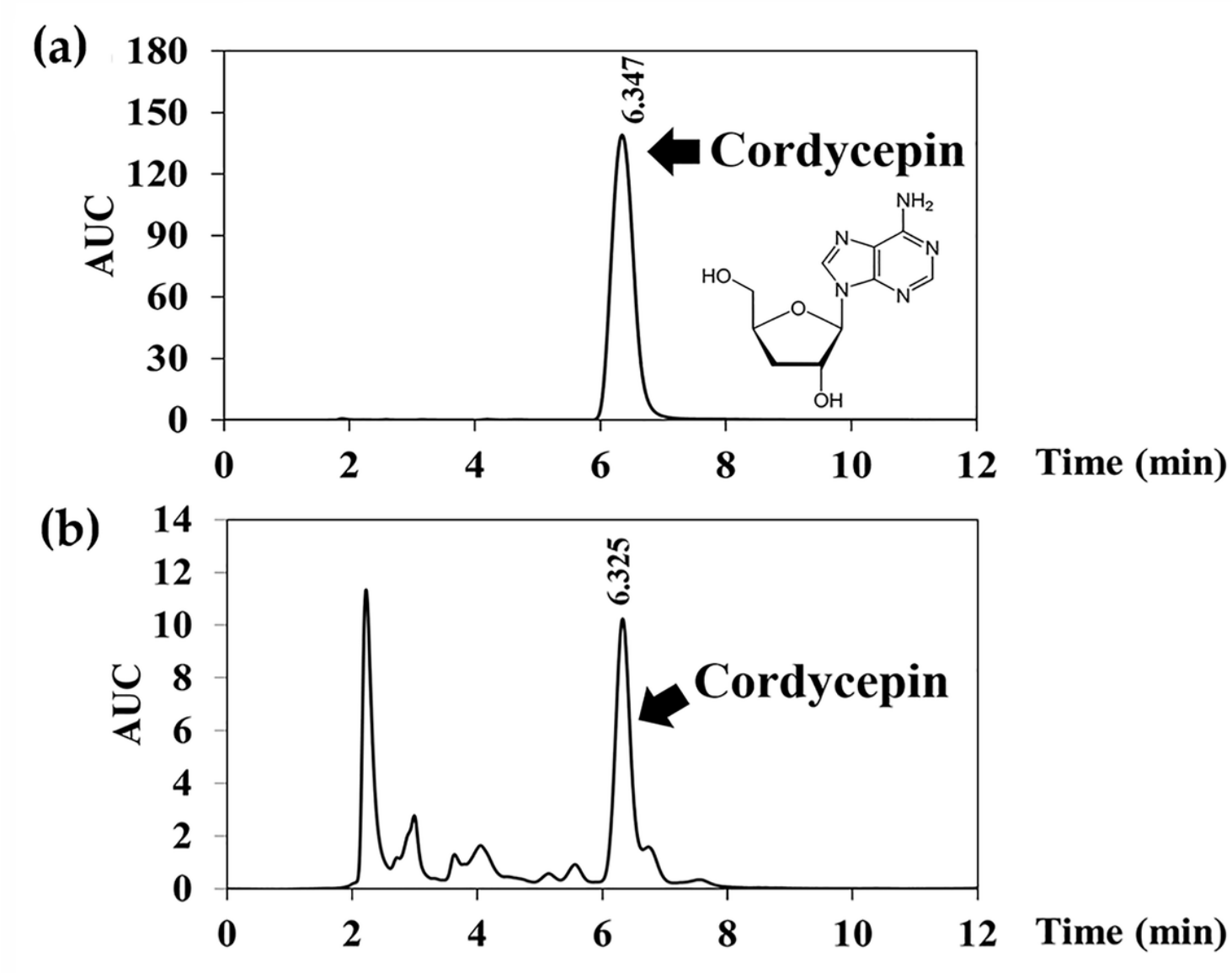

Figure 1

HPLC chromatograms of cordycepin (a) and CW: C. militaris water extract (b).

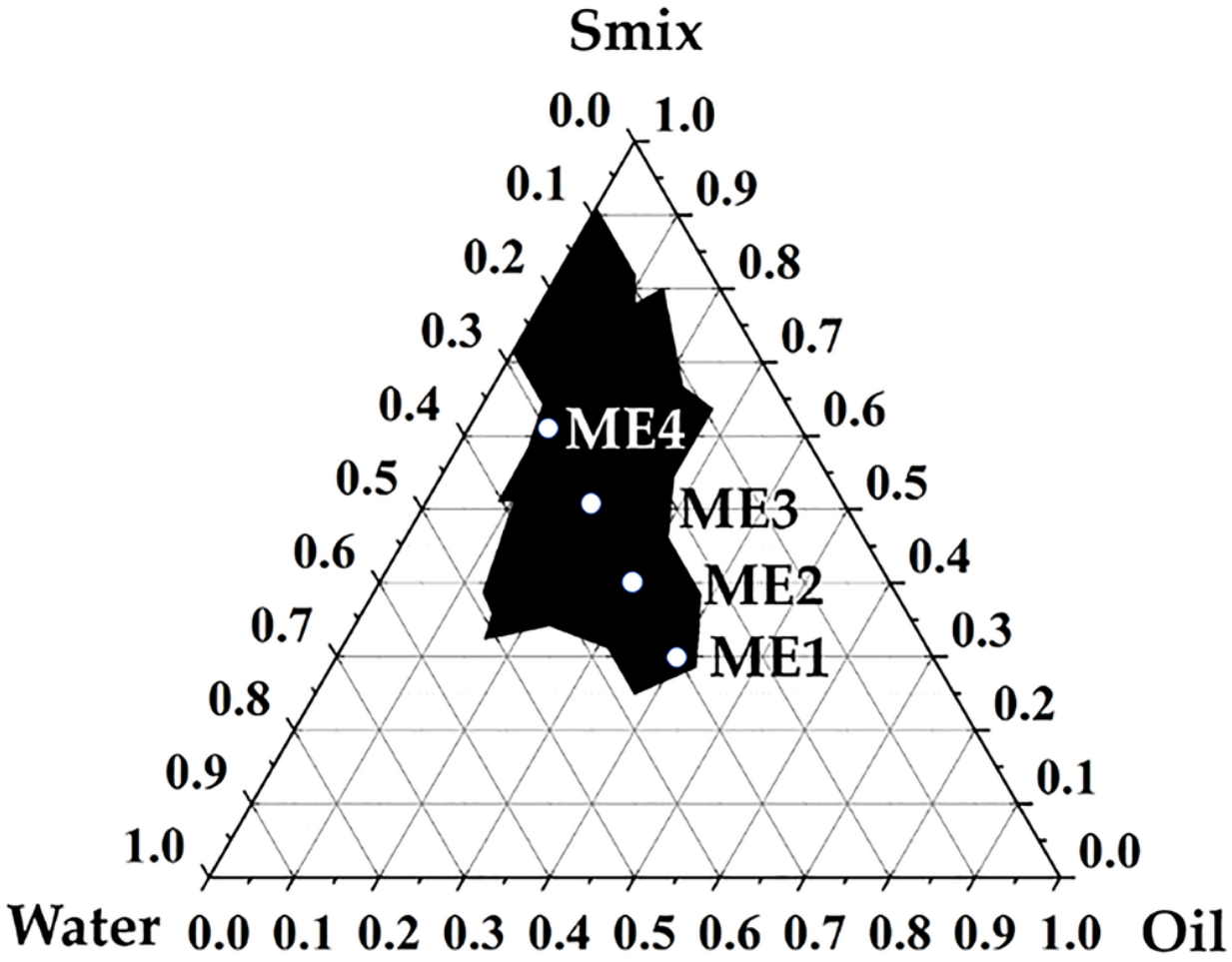

Figure 2

Pseudoternary phase diagram of sugar squalane/Tween® 85/propylene glycol/DI water. The Smix ratio was 2:1.

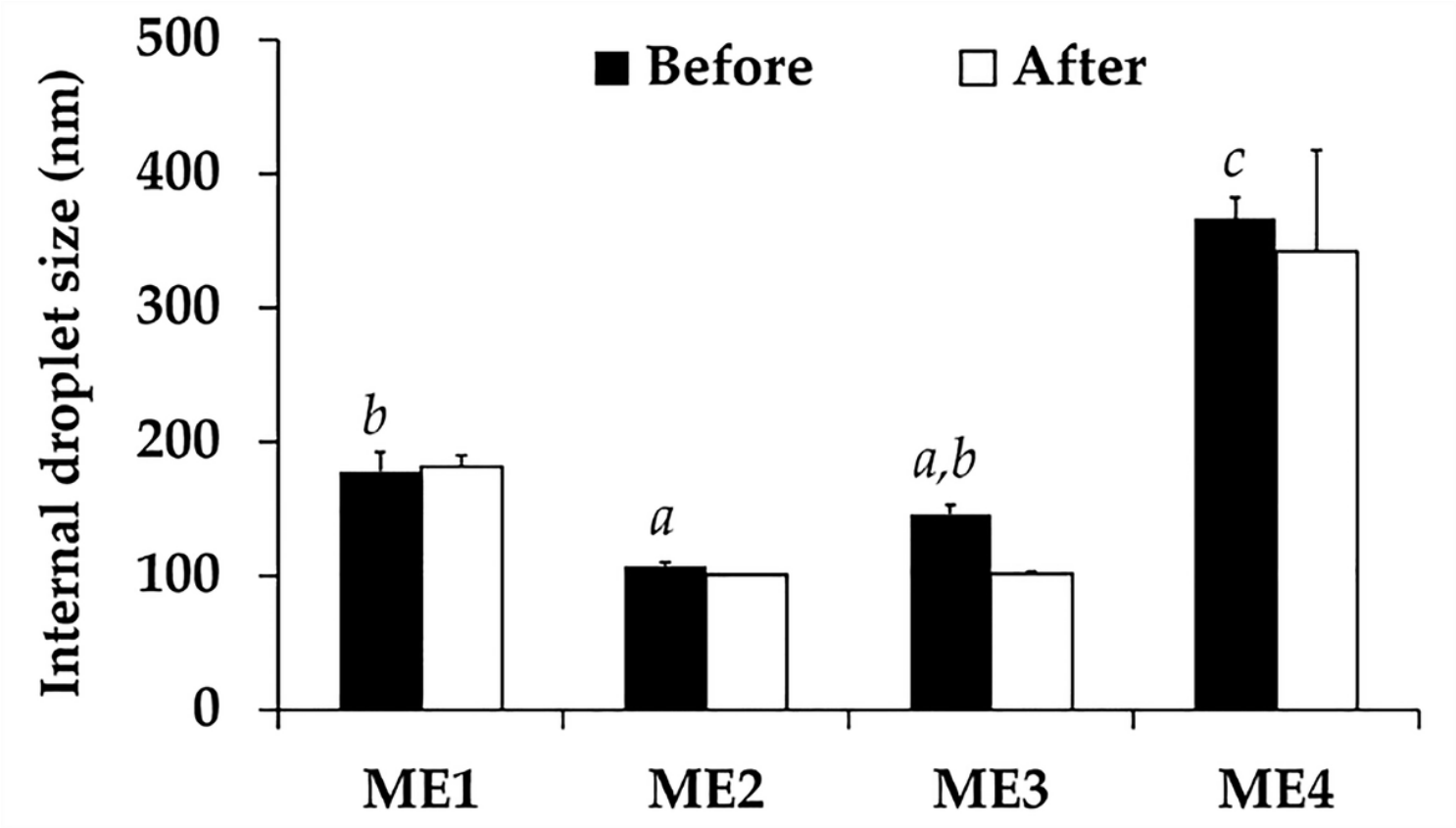

Figure 3

Internal droplet size of microemulsions before and after 8 cycles of heating-cooling condition. Different letters (a, b, and c) denoted significant difference between the internal droplet size of each microemulsion formulations ($p<0.05$).

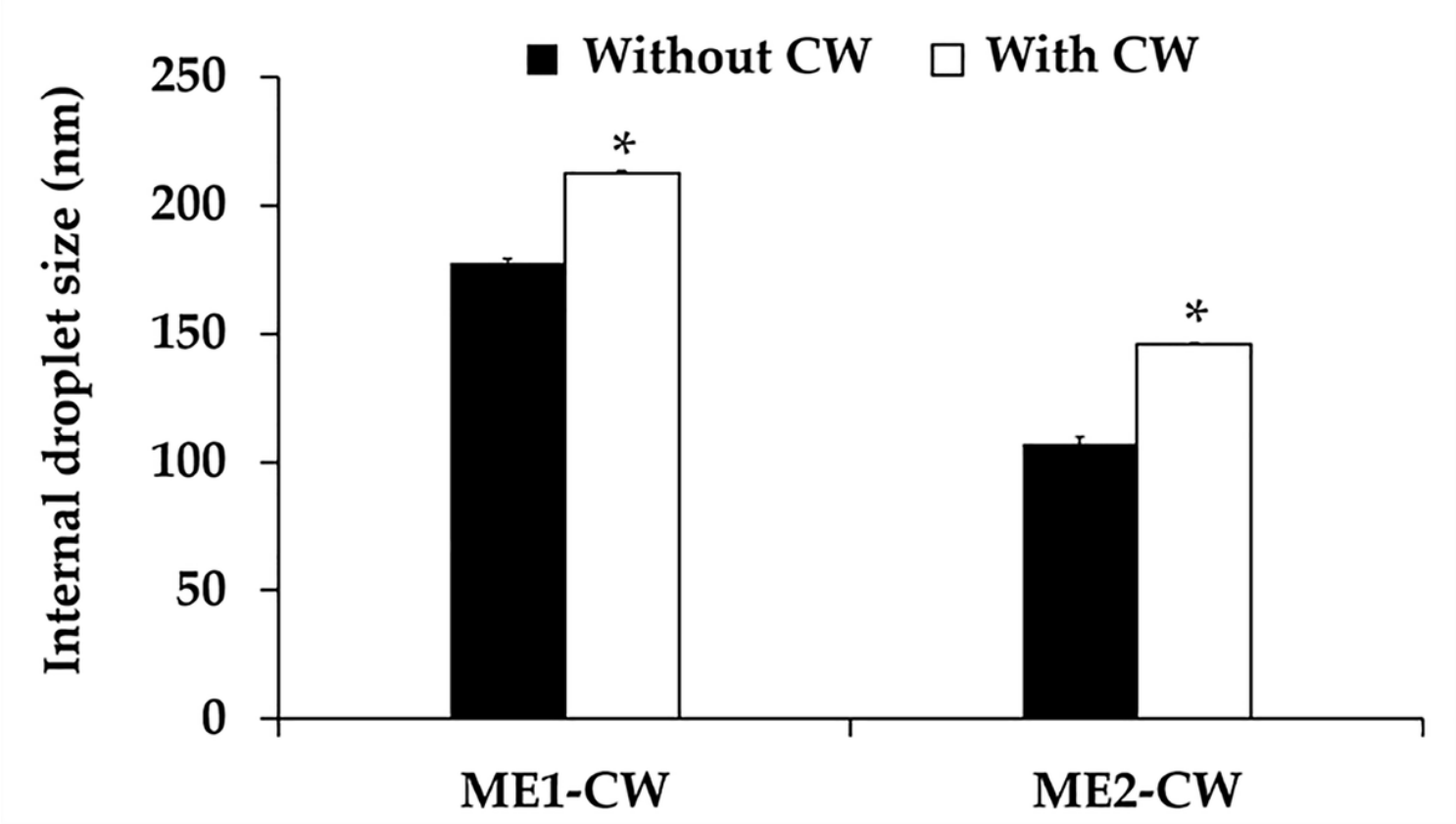

Figure 4

Internal droplet size of microemulsions with and without C. militaris water extract (CW). Asterisk (*) denoted significant difference between the microemulsions with and without CW analyzed using t-test ($p < 0.05$).

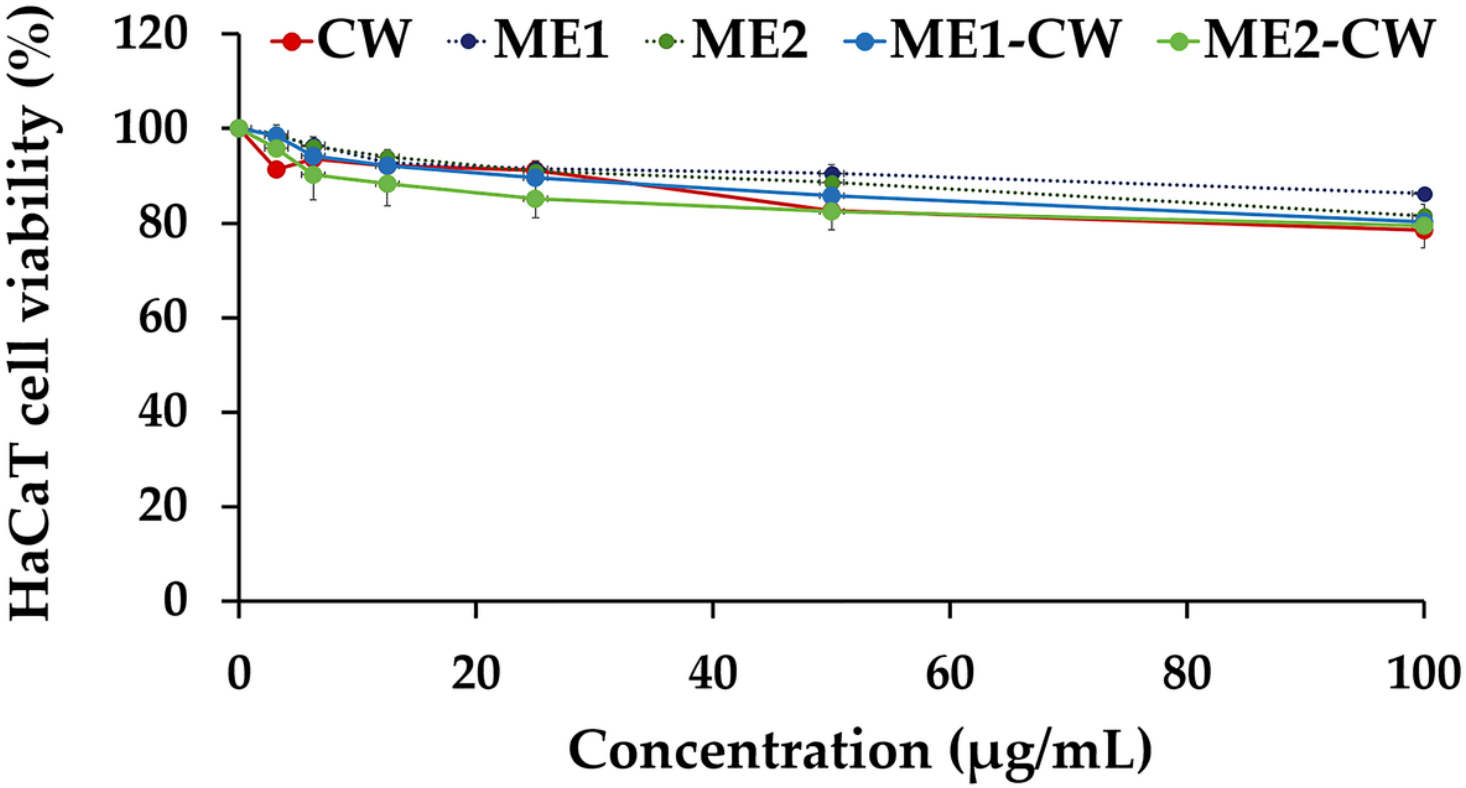

Figure 5

The viability of HaCat cells after exposed to the aqueous solution of C. militaris water extract (CW), blank microemulsion (ME1 and ME2), and microemulsion containing C. militaris water extract (ME1-CW and ME2-CW).

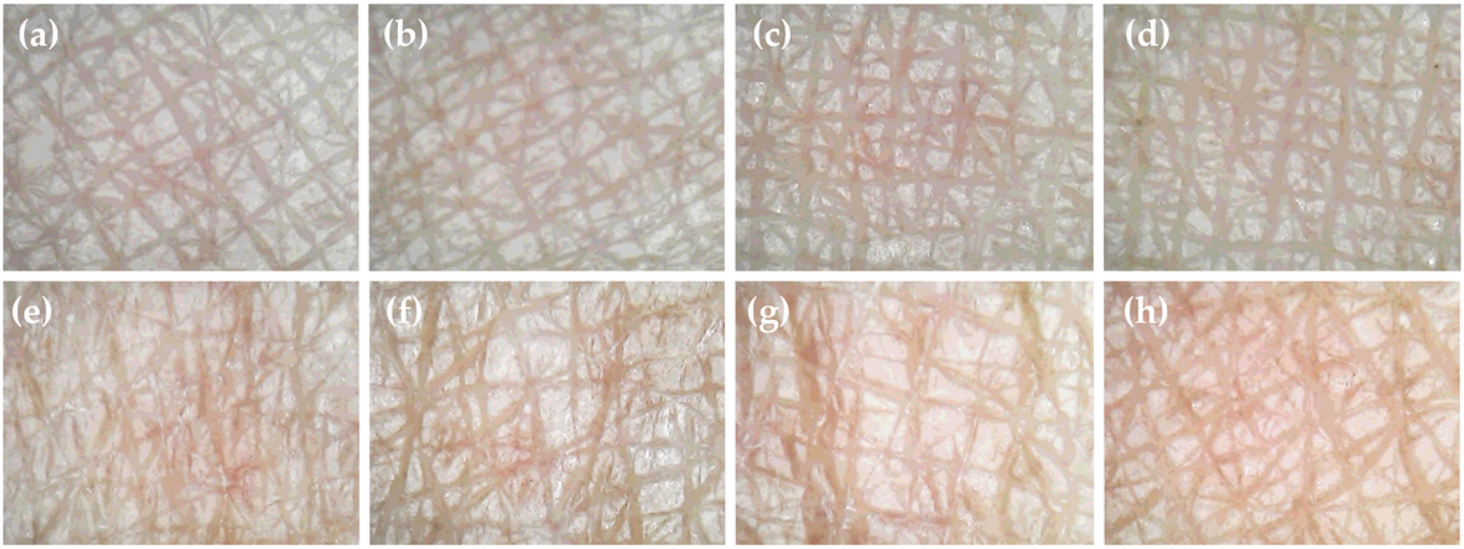

Figure 6

Skin photographs before (a) and after (b) the application of blank serum formulation (Blank) for 14 days (volunteer age: 36 years). Skin photographs before (c) and after (d) the application of topical formulation

from microemulsion containing C. militaris extract (CW serum) for 14 days (volunteer age: 36 years). Skin photographs before (f) and after (f) the application of blank serum formulation (Blank) for 14 days (volunteer age: 60 years). Skin photographs before (g) and after (h) the application of topical formulation from microemulsion containing C. militaris extract (CW serum) for 14 days (volunteer age: 60 years). The photographs were taken using INSHERE 50x-1600x USB Microscope, Handheld Magnifier with the magnitude of 1000x.

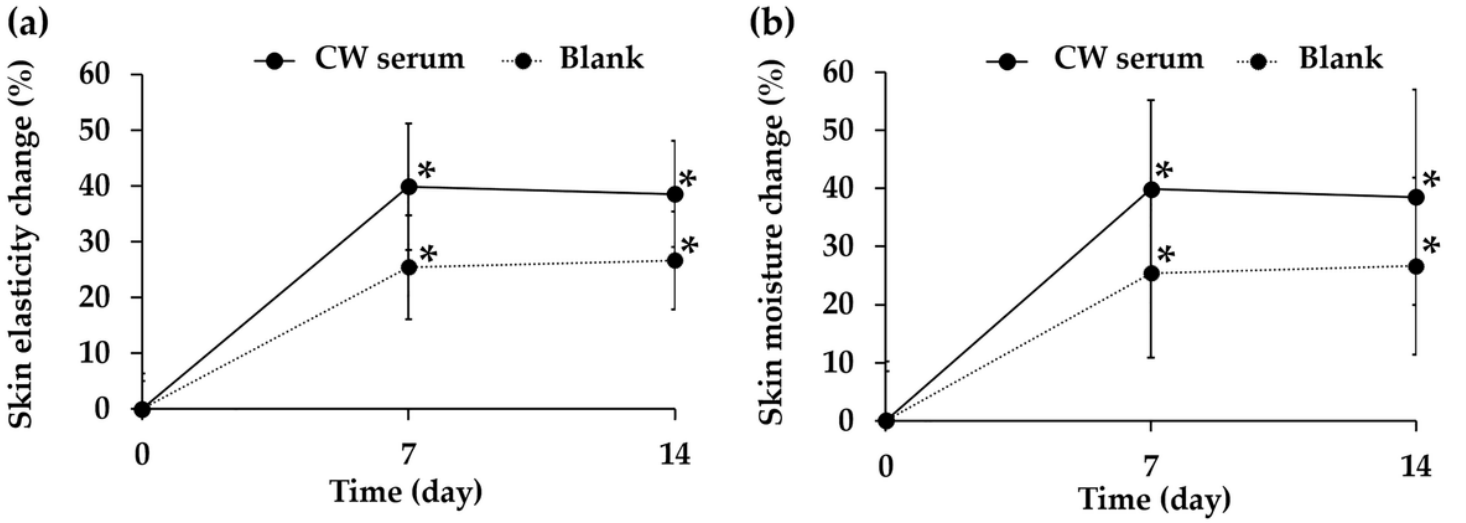

Figure 7

Skin elasticity change (a) and skin moisture change (b) after application of topical formulation from microemulsion containing C. militaris extract (CW serum) and blank serum (Blank). Asterisk (*) denoted significant difference between before and after application of the formulations, analyzed using t-test ($p < 0.05$).

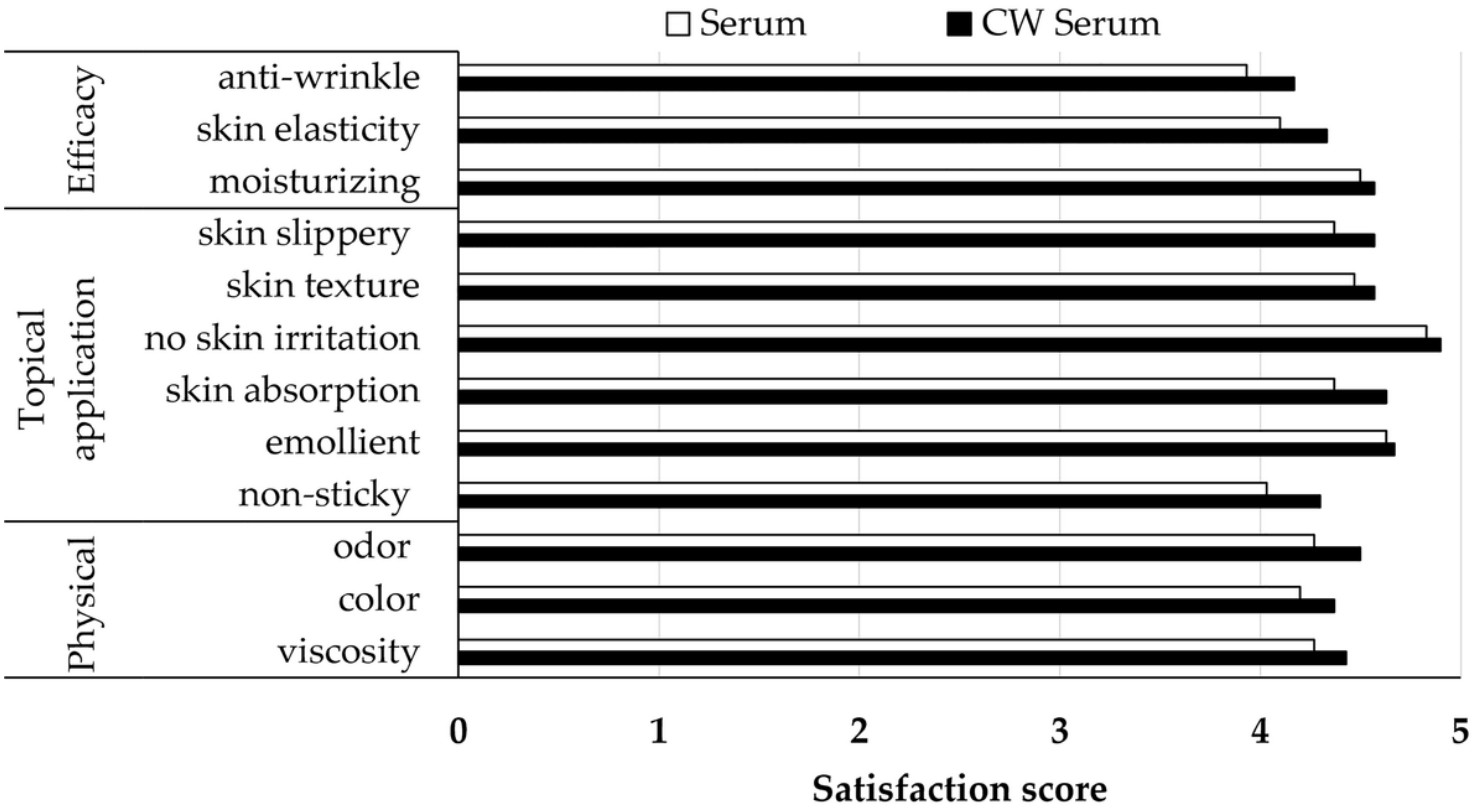

Figure 8

Satisfactory score on the physical appearance, topical application, and efficacy of serum containing CW (Serum; □) and topical formulation from microemulsion containing C. militaris extract (CW Serum; ■).

Supplementary Files

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