Original Article

Evaluation of the Antioxidant Activity, Collagen Synthesis and Stability of Bright and Firm hybrid emulsion I

Sitthiphon Bunman, MSc[1] ; Pannee Chinjarernpan, PhD[2] ; Sirawit Stitsmith, MD[3] ; Apicha Jabsuwan, MSc[4] ; Sanghyun Kim, MSc[5]

Sitthiphon Bunman, MSc

Abstract

OBJECTIVES: The aim of this study was to evaluate antioxidant activity, collagen synthesis, and stability of bright and firm hybrid emulsion I.

MATERIAL AND METHODS: The stability was evaluated by pH test, heating and cooling test, and microbial growth test. The antioxidant activity was evaluated by 2,2′-Diphenyl-1-picrylhydrazyl (DPPH) method, 2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method, and ferric reducing antioxidant power (FRAP) method. Histological analysis was stained with Masson's trichrome and evaluated for collagen synthesis on days 28.

RESULT: Bright and firm hybrid emulsion I had antioxidant activity and was stable under room temperature. Histological analysis was performed on day 28. Bright and firm hybrid emulsion I increased collagen synthesis when compared with the control group.

CONCLUSION: This study demonstrated that bright and firm hybrid emulsion I has antioxidant effect, increases collagen synthesis and stabilizes under room temperature.

Keywords: bright and firm hybrid emulsion I, antioxidant activity, collagen synthesis, stability

[1] Department of Community and Family Medicine, Faculty of Medicine, Thammasat University, Pathumthani , Thailand
[2] Faculty of Medical Technology, Nakhonratchasima College, Nakhonratchasima, Thailand
[3] Faculty of Medicine, Burapha University, Chon Buri, Thailand
[4] Kochicure Healthcare Co., Ltd, Bangkok, Thailand
[5] Department of Plant Physiology, Umeå Plant Science Centre, Umeå University, Umeå, Sweden

** Address Correspondence to author:*
Sitthiphon Bunman, MSc
Department of Community and Family Medicine, Faculty of Medicine, Thammasat University, 99 Moo 18 Paholyothin Road, Klong Luang Pathumthani 12120, Thailand
email: Acetaminophen55@gmail.com

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In 2011, Bunman et al., extracted proteoglycan from the head of fish cartilage and developed cream extraction from the head of fish cartilage. Proteoglycan is a hybrid molecule composed of a central core protein by bonding it with polysaccharides (glycosaminoglycans or GAGs) with a covalent bond, previously called mucopolysaccharide due to its physical property (jelly-like, sticky and viscosity). GAGs can be divided into 5 groups: chrondroitin/chorndroitan sulfate, keratin/keratin sulfate, dermatin/dermatan sulfate, heparin/heparan and hyaluronan. Proteoglycan in cartilage plays an important role in wound healing because the molecular structure is similar to an epidermal growth factor (EGF)-like domain. The bioactive mechanism of proteoglycan is also similar to that of EGF with regard to wound healing through promotion of epidermal growth and regeneration of tissues and blood vessels.[1] In 2015, this material was added to silver sulfadiazine to accelerate and facilitate wound healing and to promote collagen synthesis.[1] After that, they studied liver and kidney toxicity in rats receiving cream containing proteoglycan extraction from fish cartilage in 2016.

This result demonstrated that the cream containing proteoglycan extracted from fish cartilage accelerated and facilitated wound healing without causing to toxic effects to the liver and kidney in rats in long term use.[2] In 2018, Bunman et al., formulated a new serum for facial use, which when applied promotes collagen synthesis and wrinkle relief. The study demonstrated that this serum has accelerated collagen synthesis and low potential skin sensitization in rat models.[3] In 2019, Bunman developed and formulated a new cream for facial use, called "bright and firm hybrid emulsion I". Bright and firm hybrid emulsion I is composed of many active ingredients such as *pseudoalteromonas* ferment extract, *chlamydomonas* extract, *hamamelis virginiana* (witch hazel) extract, and astaxanthin etc. to reduce wrinkles, to increase facial whitening and to promote collagen synthesis. However, this product does not have scientific findings of antioxidant activity, collagen synthesis, and stability.

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The aim of this study is to evaluate antioxidant activity, collagen synthesis, and stability of bright and firm hybrid emulsion I.

Materials and Methods

Material and chemical

Hydrochloric acid, 2,2′-Diphenyl-1-picrylhydrazyl (DPPH), Distilled water, Ethanol, Methanol, Ascorbic acid, 2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic Acid (ABTS), Iron (II) sulfate heptahydrate, Iron (III) chloride hexahydrate, 2,4,6-tri (2-pyridyl)-1, 3, 5-triazine (TPTZ), Acetic acid, Sodium acetate, Manganese dioxide, Potassium persulfate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 96-well microtiter plate, microplate reader, UV-Vis spectrophotometer

Stability evaluation

Determination of pH

The pH meter was calibrated using standard buffer solution. A 0.5 g of the bright and firm hybrid emulsion I was weighed and dissolved in 50 ml of distilled water and its pH was measured.[4]

Heating and cooling test

The heating and cooling test was used for the stability determination of bright and firm hybrid emulsion I. The heating and cooling method was investigated using the modified method from Biskup et al.[5] (2013). Bright and firm hybrid emulsion I was kept at a temperature of 4ºC (48 hours) and a temperature of 45ºC (48 hours) for 7 cycles. The physical appearances of the bright and firm hybrid emulsion I such as color, smoothness, and pH were observed before and after testing.

Microbial growth test

The bright and firm hybrid emulsion I formulation was inoculated on the plate of Muller Hilton agar media by streak plate method. The plates were incubated at 37 ºC for 24, 48, 72, 96, 120, 144, and 168 hours. After incubation time, plates were taken out and checked for microbial growth by comparing it with the control.[4]

Antioxidant methods

2,2′-Diphenyl-1-picrylhydrazyl (DPPH) method

DPPH (2,2-diphenyl-1-picryl-hydrazyl) method is a rapid way to evaluate antioxidants by spectrophotometry. This assay measures the ability of antioxidants which will donate hydrogen (H) on DPPH. It is based on changing the DPPH free radicals which are purple to pale yellow. In brief, 0.22 mg/ml DPPH stock solution was prepared by accurately weighing 22 mg of DPPH in 100 ml of absolute methanol and allowed to settle overnight at 4 °C. To obtain DPPH working solution, the DPPH stock solution was diluted at 1:5 by adding 20 ml stock solution to 80 ml of methanol. To 1.9 ml of DPPH working solution, 100 µl of the final concentration of the extract and standard solution were added separately. Each solution was incubated in the dark at 37 ºC for 30 minutes. After incubation absorbance of the solution, it was measured at 517 nm by using UV-Vis spectrophotometer. Ascorbic acid was used as the reference standard compound. All determinations were carried out in triplicate.[6] Antioxidant capacity is expressed in mg Ascorbic acid equivalent /100g extract.

The percentage inhibition was calculated by using the following equation:

$$\% \text{ Inhibition} = \left[\frac{1 - (\text{A sample})}{\text{A control}} \right] \times 100$$

Asample = Corrected absorbance value for sample
A control = Corrected absorbance value for control

2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic Acid (ABTS) method

ABTS method was evaluated for antioxidant activity. The experiment was performed according to Censi et al.(2018).[7] In brief, ABTS•+ stock solution was prepared by 1:1 chemical reaction of 15 mM ABTS and 5mM potassium persulfate in dark condition, overnight. To obtain ABTS•+ working solution, the ABTS•+ stock solution was diluted 1:50 with 5 mM phosphate buffer saline, pH 7.4. In the assay, either final concentration of the extract (10 µl), Trolox standard solution (10 µl) or ethanol (10 µl) for controls were mixed with ABTS•+ working solution (290 µl) in 96-well microplate. The microplate was incubated in the dark at 37°C for 6 minutes. All determinations were carried out in triplicate. After incubation, the absorbance of each well was determined at 734 nm by using a microplate reader. Background absorbance was corrected by subtracting the absorbance value of blank. The antioxidant activities are expressed in percentage inhibition and mg Trolox equivalent/100 g extract. The ABTS percentage inhibition was calculated by using the equation already described for the DPPH method. Antioxidant capacity is expressed in mg Trolox equivalent /100g extract.

Ferric reducing antioxidant power (FRAP) method

The FRAP method measures the antioxidant potential in a sample based on the ability of a compound to reduce the complex of ferric ion (Fe^{3+}) and TPTZ to the ferrous form (Fe^{2+}) in acidic condition. The reaction was monitored by determining the change of absorbance at 593 nm. This method was investigated using the modified method from Biskup et al. (2013).[5] FRAP working solution was freshly prepared each time by adding 0.3 M acetate buffer (pH 3.6) to 0.01 M TPTZ (2,4,6-tripyridyl-s-triazine) and 0.02 M $FeCl_3 \bullet 6H_2O$ (Iron (III) chloride hexahydrate) in ratio 10:1:1

(v/v/v). This working solution was protected from light and prewarmed to 37°C before used. In the assay, the final concentration of the extract and standard solution were added to 2.25 mL FRAP working solution and 0.225 mL of deionized water. $FeSO_4 \bullet 7H_2O$ (Iron (II) sulfate heptahydrate) was dissolved in deionized water for using as standard solution. The reaction mixture was shaken and incubated at 37°C for 5 min. Absorbance was measured at 593 nm by using the UV-Vis spectrophotometer. FRAP working solution with deionized water instead of a sample and was used as a blank. All determinations were determined in triplicate. Results are expressed in mg Iron (II) sulfate heptahydrate equivalent /100g extract.

Histological analysis

Animals

In this study the histological analysis was investigated using the method of Bunman et al. (2015)1, which was modified from Chuenwattana et al. (2018).2 Male Wistar rats (age 8 weeks, weighing 250-300 grams) were purchased from the national Laboratory Animal Centre, Mahidol University, Salaya, Thailand. Rats were housed in the Laboratory Animal Unit under standard conditions of temperature 25 ± 2 °C, 50 - 60 % humidity, and a 12 hours/12 hours light/dark cycle. The rats were kept under laboratory conditions for one week prior to the start of the experiments and allowed food and water ad libitum. At the end of each experiment, the rats were sacrificed with carbon dioxide asphyxiation. Animal experiments in this study were carried out in accordance with the Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes of the National Research Council of Thailand.

Animal preparation

Male Wistar rats were randomly divided into 2 groups of 6 animals, and assigned to receive 0.5 gram of cream base (control), and bright and firm hybrid emulsion I applied to the skin. Histological analysis evaluated collagen synthesis on day 28 post applied.

The Masson's trichrome examination

Collagen synthesis was proved by skin stained with masson's trichrome. This study was investigated using the method of Chuenwattana et al. (2018).[3] Skin tissue sections were deparaffinized in xylene, hydrated, and stained in Weigert's iron hematoxylin solution for 10 minutes. They were washed and stained in acid fuchsin solution for 3 minutes, rinsed in distilled water, treated with phosphomolybdic-phosphotugstic acid solution for 15 minutes, immediately submerged into aniline blue solution for 10 minutes, rinsed in distilled water, treated with acetic solution for 10 minutes, dehydrated in 95% alcohol, cleared twice in xylene, mounted with a cover slip, and observed under a light microscope.

Statistical analysis

The results are expressed as means ± standard deviation (SD). Stability studies and histological analysis were analyzed using descriptive statistics.

Results

Heating and cooling test of bright and firm hybrid emulsion I evaluated color, smoothness, and pH. The color of bright and firm hybrid emulsion I appeared as pearl like color. On 1st to 7th cycle, the color and pH of bright and firm hybrid emulsion I was unchanged when compared with the control group (Table 1). On 1st to 7th cycle, the physical of bright and firm hybrid emulsion I appeared smooth, the physical appearance was unchanged or no separation phase (Figure 2).

The microbial growth test was observed post incubate 24, 48, 72, 96, 120, 144, and 168 hours, respectively. Bright and firm hybrid emulsion I was not found to have a bacterial colony or no bacterial growth on media plates when compared with the control (Table 2).

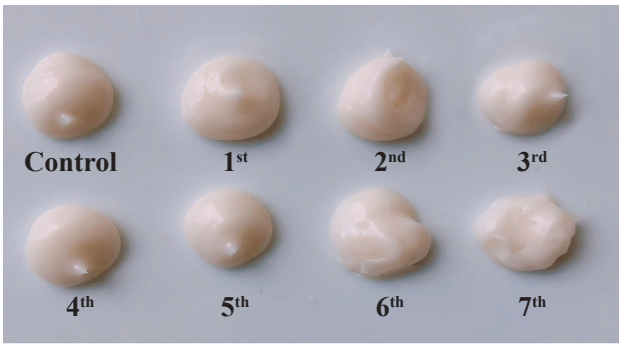

Figure 1: Color of bright and firm hybrid emulsion I on 1st to 7th cycles.

Table 1: Heating and cooling test of bright and firm hybrid emulsion I

Parameters	Control	Cycles						
		1st	2nd	3rd	4th	5th	6th	7th
Color	Like pearl	Unchanged	Unchanged	Unchanged	Unchanged	Unchanged	Unchanged	Unchanged
pH	5.5-6	Unchanged	Unchanged	Unchanged	Unchanged	Unchanged	Unchanged	Unchanged
Smoothness	No separation phase	No separation phase	No separation phase	No separation phase	No separation phase	No separation phase	No separation phase	No separation phase

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Table 2: The result of microbial growth test

Time (Hours)	Control	The bright and firm hybrid emulsion I
24	No growth	No growth
48	No growth	No growth
72	No growth	No growth
96	No growth	No growth
120	No growth	No growth
144	No growth	No growth
168	No growth	No growth

Table 3: The antioxidant activity of bright and firm hybrid emulsion I.

Methods	Results
DPPH method	% inhibition 14.93 ± 0.39 Ascorbic acid equivalent 0.53 ± 0.01 mg/100 g extract
ABTS method	% inhibition 59.60 ± 6.21 Trolox equivalent 14.91 ± 1.50 mg/100 g extract
FRAP method	Iron (II) Sulfate Heptahydrate equivalent 8.32 ± 0.44 mg/100 g extract

The antioxidant activity of bright and firm hybrid emulsion I was evaluated by 3 methods as DPPH, ABTS, and FRAP methods respectively. The result of antioxidant activity of bright and firm hybrid emulsion I had a percentage of inhibition 14.93 ± 0.39 ascorbic acid equivalent 0.53 ± 0.01 mg/100 g extract by DPPH method, percentage of inhibition 59.60 ± 6.21 Trolox equivalent 14.91 ± 1.50 mg/100 g extract by ABTS method, and Iron (II) Sulfate Heptahydrate equivalent 8.32 ± 0.44 mg/100 g extract by FRAP method, respectively (Table 3).

The results from Masson's trichrome of rat skins staining can clearly differentiate important morphological keys for collagen synthesis assessment. Muscle, Hemoglobin, keratin and fiber are stained red color. Adipose tissue and cytoplasm are stained light red or pink. Cell nuclei show dark brown to black and collagen fiber is stained aniline blue. On day 28 post applied with bright and firm hybrid emulsion I showed higher levels of collagen fiber when compared to the control group (Figure 2).

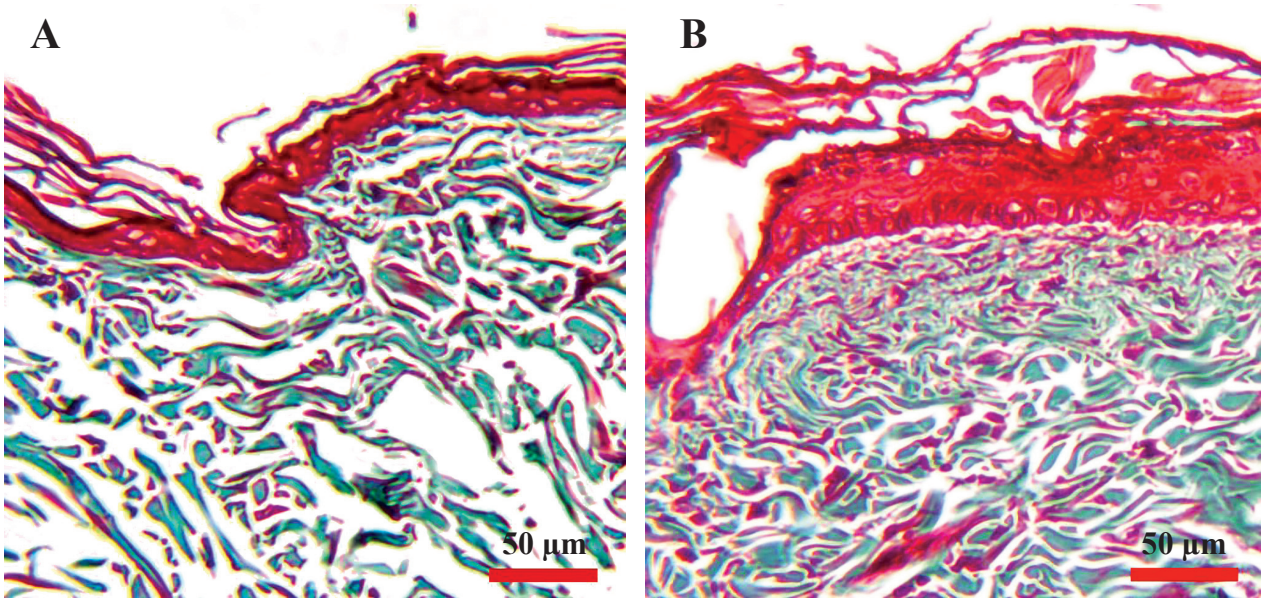

Figure 2: Histological appearance on day 28 post applied. Masson's trichrome stains of rat skins applied with (A) cream base (control), and (B) bright and firm hybrid emulsion I, Bar = 50 µm.

Discussion

Determination of pH, heating and cooling test, and microbial growth test were used for evaluation of the stability of a cosmetic creams.[8] The general properties of the bright and firm hybrid emulsion I had a pH that ranged between 5.5 and 6. This pH range is necessary and appropriate for skin pH and because the preservatives are effective at those levels. Study of the effect of temperature on the stability of the bright and firm hybrid emulsion I showed that the temperatures did not affect the physical appearance of the bright and firm hybrid emulsion I through the period of 7 cycles. The temperature ranged between 4°C and 45ºC and is necessary and appropriate for storage.

The study on the effect of light on the stability of the bright and firm hybrid emulsion I demonstrated that the physical appearances (color and solidity) of the bright and firm hybrid emulsion I were not changed throughout the period of 7 cycles. However, antioxidant ingredients such astaxanthin, ascorbic

acid, niacinamide etc. can degrade in light conditions, because the antioxidant ingredients are oxidized when they are exposed to light.[9] Therefore, bright and firm hybrid emulsion I is recommended to be kept under dark condition.

The microbial growth test was evaluated for preservative property, which control microbial growth.[10-12] Bright and firm hybrid emulsion I formulation added Caprylyl Glycol (1,2-Octanediol), Sodium Benzoate, and Potassium Sorbate as a preservative. The result demonstrated that bright and firm hybrid emulsion I formulation did not have microbial growth. Therefore, this preservative of bright and firm hybrid emulsion I can control microbial growth.

UV radiation from sun light damages the skin by increasing levels of free radicals. Cigarette smoke, pollution, stress, illnesses, and drugs etc. can increase levels of free radicals in the physical body.[7] For their elimination antioxidants contained in antioxidant creams are helpful.[13] The study demonstrated that bright and firm hybrid emulsion I can reduced free radical scavenging activity because it contained high active ingredients such as *pseudoalteromonas* ferment extract, *chlamydomonas* extract, *hamamelis virginiana* (witch hazel) extract, and astaxanthin etc.

Collagen and hyaluronan are synthesized in the fibroblast cells, which is the main component of connective tissue such as skin, tendon, etc., giving it strength and flexibility. The fibroblasts' proliferation is related to collagen content in skin.[14] Skin aging is also associated with loss of skin moisture. The key molecule involved in skin moisture is a glycosaminoglycan (GAG), hyaluronic acid (HA), or hyaluronan with a unique capacity to bind and retain water molecules.[15] From the results, the collagen of skin in the treated group on days 28 was higher than the control group. Therefore, bright and firm hybrid emulsion I may play an important role in fibroblasts proliferation, which increases collagen and HA synthesis in skin.

Conclusion

This study was able to show that bright and firm hybrid emulsion I not only displays cosmetic efficacy as an antioxidant effect and to accelerate collagen synthesis, but also has pleasant sensorial characteristics for cosmetic use.

Conflicts of interest

The authors declare that they have no conflicts of interest

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