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Anti-Inflammatory Activity of Topical Anthocyanins by Complexation and Niosomal Encapsulation

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***Abstract*—**Anthocyanins are natural pigments with effective UV protection but their topical use could be limited due to their physicochemical characteristics. An attempt to overcome such limitations by complexation of 2 major anthocyanin-rich sources, C. *ternatea* and *Z. mays*, has potentiated its use as topical anti-inflammatory. Cell studies indicate no cytotoxicity of the anthocyanin complex (AC) up to 1 mg/ml tested in HaCaT and human fore head fibroblasts by MTT. Croton oil-induced ear edema in Wistar rats suggests an effective dose of 5 mg/cm^2 of AC as a topical anti-inflammatory in comparison to 0.5 mg/cm^2 of fluocinolone acetonide. Niosomal encapsulation of the AC significantly prolonged the anti-inflammatory activity particularly at 8 h after topical application (p = 0.0001). The AC was not cytotoxic and its anti-inflammatory and activity was dose-dependent and prolonged by niosomal encapsulation. It has also shown to promote collagen type 1 production in cell culture. Thus, AC could be a potential candidate for topical anti-inflammatory agent from natural resources.

***Keywords*—**Anthocyanin complex, ear edema, inflammation, niosomes, skin.

I. Introduction

ANTHOCYANINS are natural pigments daily intake from coloured fruits, flowers, and vegetables [1]. Anthocyanins possess anti-inflammatory activity [2], [3], anti-oxidant [4], [5], anti-carcinogenic [6] and anti-microbial including anti-candida activities [7], [8]. Topical application of anthocyanins has been reported for UV protection on the skin [9], however, the use of anthocyanins can be restricted due to physicochemical charateristics including pH, colour staining and poor skin permeation. Complex formation of natural anthocyanins is common in plants so as to physicochemically stabilize the molecular conformation of anthocyanins [10], [11]. Complex formation of natural anthocyanins is common in plants so as to physicochemically stabilize the molecular conformation of anthocyanins [10], [11]. Niosomes are self-assembly vesicles which mimic bilayer structure of cell membrane, and could be used to enhance skin permeation of the encapsulated compounds [12]. An anthocyanin complex (AC) formed with 2 major anthocyanins, i.e. delphinidin- and cyanidin-rich sources, was able to be taken up by one of the human fibroblast cells [13]. Since fibroblasts play a vital role in the viable skin, including responses to inflammatory stimuli [14]. Forming a complex might retard skin permeation, thus an in vivo test on the effectiveness of topical applications of the anthocyanin complex is required. In this light, niosomal encapsulation was introduced for comparison on the permeation of anthocyanin complex.

Topical steroids are commonly prescribed as effective anti-inflammatory property, however, there are many common side effects and complications occurred such as steroid allergy, steroid-induced skin atrophy, including secondary infection from bacteria, virus and fungus, if long-term or high dose uses [15]. If the anthocyanin complex has been shown to be effective as a topical anti-inflammatory, and also the niosomes could encapsulate the anthocyanin complex, a potential use of the anthocyanins from abundant natural resources is made possible.

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II. Materials and Methods

A. Anthocyanin Complex (AC) Preparation

Dried blue flowers of *Clitoria ternatea* L. (Sisaket, Thailand), identified [16] and herbarium collected no.KKUAP_03 at the institute plant collection, were extracted by water and freeze-dried to obtain its crude extract (CT). Dried cobs of purple waxy corn (*Zea mays* L. *ceritina* Kulesh.) from an open-pollinated variety (Kao Kum) harvested in the university campus were grinded and extracted by water followed by freeze-dried to obtain its crude extract (CC).

An anthocyanin complex (AC) was formed using the previously described protocol [13]. In brief, an aqueous mix of CC, CT and turmeric rhizome (ratio = 5:5:1) was heated to 80ºC and trace amount of Zn^{2+}(Ajax Finechem, Australia) and caffeic acid (Sigma-Aldrich, China) were mixed and then cooled to -20°C to precipitate. AC powder was obtained from the oven-dried precipitate.

B. Preparations of AC

AC solution was prepared by dissolving AC powder with 0.1% sodium polyacrylate (GMP, Bangkok, Thailand) in isotonic phosphate buffer saline at pH 7.4.

Span 60 (Sigma, St. Louis, MO, U.S.A.) and cholesterol (Sigma, St. Louis, MO, U.S.A.) were dissolved in 1:1 chloroform and methanol and rotary evaporated under vacuum to form a thin film. The thin film was rehydrated with the AC solution to form AC niosomes. Blank niosomes were prepared

by the same method without the AC. These were freshly prepared for use.

C. Cytotoxicity of Anthocyanin Complex

Human keratinocyte cell line (HaCaT) (CLS, Germany) and human forehead fibroblasts (HFF, a gift from Assist. Prof. Dr. Wilairat Leeanansaksiri) were sub-cultured in DMEM medium (high glucose) supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS) (Gibco, U.S.A.), 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 25 µg/ml of amphotericin B (Invitrogen Cooperation, Carlsbad, CA, U.S.A.) in a humidified incubator (Shel Lab, U.S.A.), 5% CO_2 and 37°C. Reaching its growth plateau, the cells were treated with 0.25% trypsin/EDTA solution (Gibco, U.S.A.) and transferred to obtain about 20,000 cells/well (HaCaT) or 10,000 cells/well (HFF) prior to use.

The prepared cells were incubated with samples or controls (positive or negative) for 24 h and then subjected to 3-(4,5-dimethylthiazol-2-yl-2-yl)-2,5-diphenyltetrazolium (MTT, Biobasic, Canada) as previously described [17]. In brief, cells were treated with samples or controls for 24 h. After that, the medium was removed and the viable cells were stained with MTT (0.5 mg) for absorbance reading at 550 nm using a microplate reader 680 (Bio Rad, Japan).

AC samples for cytotoxicity were prepared by dissolving in 1% DMSO solution and further diluting with culture medium solution to the final concentrations, ranging from 0.01-20 mg/ml of AC and 0.1% of DMSO. The negative control was the culture medium and the positive control was 10% hydrogen peroxide (H_2O_2). Cell viability was estimated in comparison to the negative control of the same setting, as the following equation:

$$\%\ \text{cell viability} = \frac{\text{absorbance of sample}}{\text{absorbance of negative control}} \times 100 \qquad (1)$$

D. Analysis of Collagen Type I

Prepared HFF were incubated with AC at a concentration range of 1-1000 µg/ml in free FBS medium solution. L-ascorbic acid (Sigma U.S.A.) at a concentration of 2 mM was the positive control as it is reported to be a collagen inducer [18]. After exposure to the AC or the controls, the cells were kept in humidified CO_2 incubator containing 5% CO_2 at 37ºC for 24 h, then the medium solution from each treatment was sampled for analysis of collagen by picro-sirius red method.

This method is based on the observation that Sirius red in saturated picric acid selectively binds to fibrillar collagens (types I to V), specifically to the $(Gly\text{-}X\text{-}Y)_n$ helical structure. For the test samples, each sample was acidified 5 M acetic acid for 30 min, followed by dyeing with serious red (Sigma-Aldrich, USA) for 30 min and centrifuging at 1350 rpm for 15 min for collection of the collagen pellet. The unbound dye was dissolved and removed from the pellet by 0.1M HCl. The pellet was then collected after centrifugation at 1350 rpm for 15 min and then 1 ml of 0.5M NaOH was added with vigorously mix to release the bound dye. The released dye was subjected to be analyzed by UV spectrophotometry at 540 nm using a microplate reader.

Standard human collagen type I (Sigma-Aldrich, USA) was pretreated in accordance to the instruction, diluted to a concentration range of 0-0.8 mg/ml in 0.5 M acetic acid and subjected to dyeing with acid and alkali treatments as the test samples. A linear plot between concentrations of the standard collagen type 1 and the absorbance at 540 nm was used as the standard of analysis of the test samples.

E. Anti-Inflammatory by Croton Oil-Induced Ear Edema

Adult male Wistar rats (weighing 250 ± 6 g, 60 in total) were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakhonpathom, Thailand, and the animal handling were under supervision of the certified veterinarian of the Northeast Laboratory Animal Center, Khon Kaen University, Thailand. The study protocol has been reviewed and approved by the Animal Ethics Committee of Khon Kaen University, based on the Ethic of Animal Experimentation of the National Research Council of Thailand (NELAC 23/2557). The animals were housed under natural conditions (22±3°C, 50±5%RH, 12h/12h light-dark cycle) for 1 week prior to experimentation. They had free access to a rodent diet and clean water. Experiments were carried out on groups of 6 animals with free access to food and water in a Heating Ventilating and Air Conditioning (HVAC) system (Northeast Laboratory Animal Center, 2010).

Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (Nembutal™, Ceva Corporate, France). Inflammation of the posterior edge of the left ear of each rat was induced by applying 0.5 mg/cm^2 of croton oil (diluted in acetone). Right ears of the rats were treated with the same volume of acetone for blank comparisons. Immediately after the inducement, predetermined topical preparations, i.e. AC, AC niosomes, fluocinolone acetonide (Sigma, U.S.A.) in the same formula of polyacrylate solution and the relevant blanks, were topically applied to the assigned ears of the rats. Thicknesses of both ears of each rat were measured at predetermined time intervals using a digital vernier caliper (Mitutoya, Japan). Anti-inflammatory activity was calculated from ear edema inhibition based on thicknesses of the right ear of each rat, compared to an equation, as follows:

$$\%\ \text{ear edema inhibition} = \frac{\Delta T_{\text{blank}} - \Delta T_{sample}}{T_{\text{pretreated}}} \times 100 \qquad (2)$$

where ΔT_{blank} = difference of ear thickness between after and before treated with blank, $T_{pretreated}$ = ear thickness of the same rat at the same site before the treatment (mm) and ΔT_{sample}= difference of ear thickness between after and before treated with samples

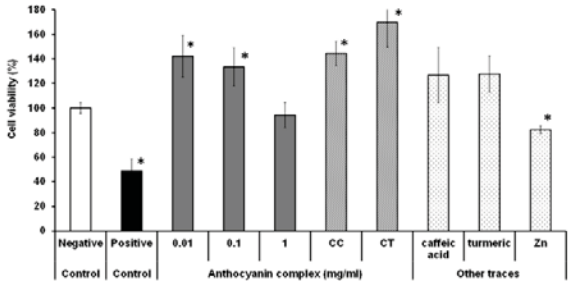
F. Statistical Analysis

Cell viabilities were expressed as mean ± standard deviation (SD) and statistically compared by one-way analysis of variance (ANOVA). Paired t-test was used to monitor the measurement of untreated ear thicknesses in all rats. ANOVA and paired t-test were used to analyze ear edema inhibition. The significant level was considered at $p< 0.05$.

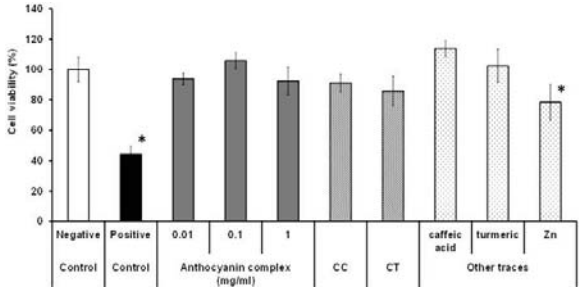
III. Results and Discussion

The anthocyanin complex was dark odorless fine powder. Its color was darker than CT and CC, used to produce the AC. AC solution was prepared in the presence of a bioadhesive and biocompatible polymer so as to adhere the product onto the rat skin. The AC solution was clear viscous liquid which could be dropped onto the rat ears without any spillage throughout the study. Niosomal preparation of AC gave light cloudy liquid which readily mix with polyacrylate solution to form the AC niosomal dispersion which giving opaque teal liquid solution.

HaCaT, representing cells derived from human keratinocytes, while HFF, representing cells from the skin fibroblasts, have shown to well tolerated the AC up to 1 mg/ml. The concentration of AC at 1 mg/ml was almost the limit of this cytotoxicity test since high concentration of the AC could produce similar color to interfere MTT staining to the viable cells.



(a)



(b)

Fig. 1 Mean viability (%) of HaCaT cells (a) and HFF cells (b) treated with 0.01-1 mg/ml of AC and other traces for 24 h. Negative control using medium solution for its relevant blanks, positive control using 10%H_2O_2. The error bars represent %CV (n = 8), asterisks (*) indicate significant differences at $p< 0.05$ comparing to negative control

AC at 0.01 to 0.1 mg/ml significantly enhanced the viability of HaCaT cells ($p< 0.001$) as shown in Fig. 1 (a). CC- and CT-treated HaCaT cells also gave an increase trend in cell viabilities ($p< 0.001$). At the highest concentration of AC of 1 mg/ml, viabilities of HaCaT cells did not significantly differ from the control. HFF showed similar effect as HaCaT when exposed to the AC.

At the same amounts used to form AC, cell viabilities of are not significantly affected by caffeic acid and turmeric (all $p > 0.05$), but significantly by Zn^{2+} ($p = 0.01$) in both cells. However, it is noticed that the presence of Zn^{2+} in the AC did not induce cytotoxicity to both cells. HaCaT and HFF cells did not show toxicity signs after exposure to CT and CC anthocyanins as well as an anthocyanin complex (AC) composed of both, even at milligram level. Caffeic acid and turmeric used in the complexation did not affect both cells either. Zinc, at the concentration used to form the AC, significantly affected cell viabilities of HaCaT ($p = 0.002$) and HFF ($p = 0.001$). This implies that cytotoxic property of zinc was diminished when complex with anthocyanins.

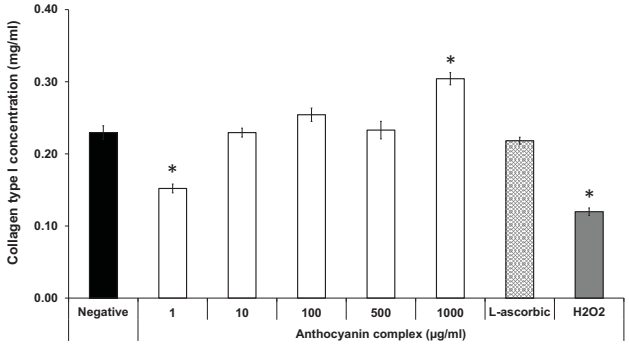


Fig. 2 Collagen type I synthesis from HFF cells after 24 h treatment of anthocyanin complex comparing to L-ascorbic acid (Vitamin-C)

Fig. 2 illustrates a positive trend of collagen type 1 production increases with the concentrations of the AC exposed to the cultured HFF for 24 h. Collagen type I produced when the cells exposed to L-ascorbic acid was not different from blanks used as negative control. The results demonstrate that AC induced collagen synthesis in a dose dependent manner. At 1 mg/ml of AC, cells significant increased the collagen type I production comparing to blanks and L-ascorbic acid ($p< 0.0001$ both). This concentration of AC did not affect cell viability of HFF, as shown in Fig. 1 (b), thus, it leads to conclude that AC showed capability to induce collagen type 1 production provided that it reached the cells.

Croton oil contains 12-o-tetracanoilphorbol-13-acetate and other phorbol esters as the main irritant agents [19]. It was diluted with acetone which rapidly vaporizes and lefts croton oil to activate a cascade pathway of arachidonic acid production. Irritants stimulate the release of inflammatory mediators such as histamine, serotonin, prostaglandins and leukotrienes by cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) enzymes. COX and 5-LOX can be inhibited by topical corticosteroids used as anti-inflammatory [20]. Applying croton oil to rat ears resulted in signs of inflammation, i.e. redness, swelling, within 2 h.

Fig. 3 demonstrates a dose dependent manner of anti-inflammatory activity, as ear edema inhibition, of AC. Fluocinolone acetonide in the same base formula as the AC gave the peak of anti-inflammatory activity (about 10% inhibition) at 2 h after application, then the activity began to decline. Topical AC at a low dose of 0.5mg/cm^2 began to exhibit some anti-inflammatory activity but to a non-significantly lower extent than fluocinolone. At a dose of 5mg/cm^2 of AC, ear edema was inhibited and prolonged till 8

h at which the extent of activity was significantly higher than fluocinolone (p = 0.01). This suggests that a single topical application of AC to the skin could inhibit inflammation caused by an irritant used in croton oil-induced ear edema model. It was anticipated that an effective dose of AC should be higher than corticosteroids. This study showed that it was about 10 times less potent than fluocinolone acetonide. Anthocyanins inhibit formation of inflammatory cytokines, including interleukin-1 beta and tumor necrosis factor alpha, as well as prevent damage due to free radicals scavenging activity [21]. The mechanisms of actions of anthocyanins are not likely to be the same as fluocinolone, thus, the dose and duration of action were found to be different.

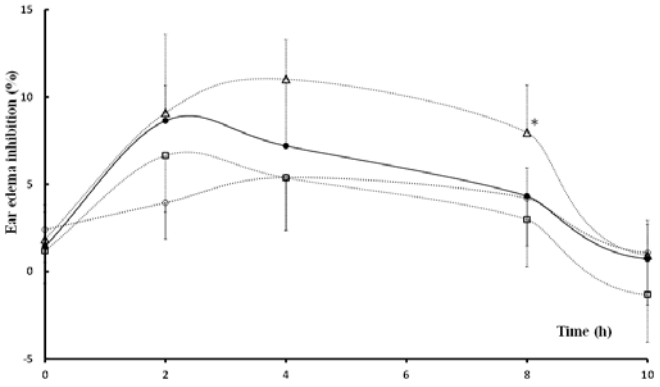


Fig. 3 Croton oil-induced ear edema tested in rats after topical applications of 0.5 mg/cm^2 of AC in solution(○), 2.5mg/cm^{2}of AC in solution (□), 5 mg/cm^{2}of AC in solution(Δ) and 0.5 mg/cm^2 of fluocinolone acetonide in solution (●), n = 6 each. * = significant differences compared to fluocinolone acetonide (p< 0.05)

Niosomal encapsulation of the AC at doses of 0.5 and 5 mg/cm^2 was topically applied on the inflamed ears of the rats for comparison to fluocinolone in the same solution base. Fig. 4 illustrates a higher extent of ear edema inhibition was obtained from topical applications of AC niosomes at 5 mg/cm^2 than of fluocinolone. At 4 and 8 h, the anti-inflammatory activity of AC niosomes was significantly higher than fluocinolone (p = 0.03 and 0.0001, respectively). AC niosomes at 0.5 mg/cm^2 showed some but lesser activity than fluocinolone and AC niosomes at 5 mg/cm^2.

In comparison, at 8 h niosomal encapsulated AC gave a significant higher activity than non-encapsulated AC (p = 0.03). AC and AC niosomes at 0.5 mg/cm^2 were not significantly different (p> 0.05). This indicates niosomes promote the permeation of AC into the inflamed sites of the rat skin due to encapsulation of the AC which might have also been deposit in the skin. However, the dose of AC is also vital to exert its action. Thus, a therapeutic dose of AC of 5 mg/cm^2 is defined. It is worth notified that niosomes prolonged the action of the AC but this requires further investigation for a concrete support.

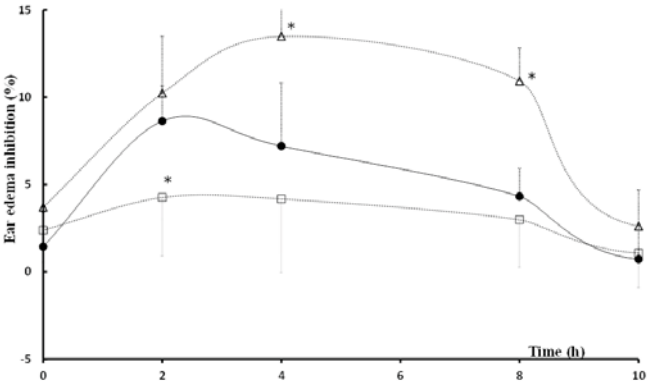


Fig. 4 Croton oil-induced ear edema tested in rats after topical applications of 0.5mg/cm^{2}of AC encapsulated in niosomes (□),5mg/cm^{2}of AC encapsulated in niosomes (Δ), and 0.5 mg/cm^2 of fluocinolone acetonide in solution (●), n = 6 each. * = significant differences compared to fluocinolone acetonide (p< 0.05)

IV. Conclusion

A complex formed from anthocyanins from 2 natural resources, blue petals of *C. ternatea* and purple cobs of *Z. mays*, which was previously shown to overcome the physicochemical burdens of anthocyanins in product development, has shown to be a potential as topical anti-inflammatory agent with no cytotoxic effect to HaCaT (representing human keratinocytes) and human forehead fibroblasts (HFF). AC showed a potential capability to induce collagen type 1 production in human fibroblasts. Using a croton oil-induced ear edema models in Wistar rats, an effective topical dose of the AC was determined to be 5 mg/cm^2 when applied in the presence of polyacrylate in aqueous solution. This dose is comparatively as efficient as 0.5 mg/cm^2 of fluocinolone acetonide in the same formula. Niosomal encapsulation of the AC significantly prolonged the anti-inflammatory activity particularly at 8 h after topical application (p = 0.0001). Thus, AC was a dose-dependent anti-inflammatory agent in which its action could be prolonged by niosomal encapsulation. It is a potential candidate for topical anti-inflammatory agent. Moreover, its production could provide the use of an agricultural waste abundantly available from natural resources.

Acknowledgments

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REFERENCES

[1] E. Pojer, F. Mattivi, D. Johnson, C. S. Stockley, "The Case for Anthocyanin Consumption to Promote Human Health: A Review" *Compr. Rev. Food Sci. F.* vol. 12, pp. 483, 2013.
[2] M. Shiono, N. Matsugaki, K. Takeda, "Phytochemistry: structure of the blue cornflower pigment" *Nature* vol. 436, pp. 791, 2005.
[3] Y. Zhu *et al.*, "Anti-inflammatory effect of purified dietary anthocyanin in adults with hypercholesterolemia: A randomized controlled trial" *Nutr. Metab. Cardiovasc. Dis*. vol. 23, pp. 843, 2013.
[4] J. Y. Choi *et al.*, "Analysis and tentative structure elucidation of new anthocyanins in fruit peel of Vitis coignetiae Pulliat (meoru) using LC-MS/MS: Contribution to the overall antioxidant activity" *J. Sep. Sci.* vol. 33, pp. 1192, 2010.
[5] C. Ubeda *et al.*, "Employment of different processes for the production of strawberry vinegars: Effects on antioxidant activity, total phenols and monomeric anthocyanins" *LWT - Food Sci. Technol.* vol. 52, pp. 139, 2013.
[6] S. C. Thomasset, University of Leicester (2008).
[7] L. C. Vasconcelos, M. C. Sampaio, F. C. Sampaio, J. S. Higino, "Use of Punica granatum as an antifungal agent against candidosis associated with denture stomatitis" *Mycoses* vol. 46, pp. 192, 2003.
[8] A. Cisowska, D. Wojnicz, A. Hendrich, "Anthocyanins as antimicrobial agents of natural plant origin." *Nat. Prod. Commun.* vol. 6, pp. 149, 2011.
[9] R. J. Mankovitz. (United States, 2004).
[10] C. H. Brenes, D. D. Pozo-Insfran, S. T. Talcott, "Stability of copigmented anthocyanins and ascorbic acid in a grape juice model system" *J. Agri.Food Chem.* vol. 53, pp. 49, 2005.
[11] C. Zhang, Y. Ma, X. Zhao, J. Mu, "Influence of copigmentation on stability of anthocyanins from purple potato peel in both liquid state and solid state" *J. Agri.Food Chem.* vol. 57, pp. 9503, 2009.
[12] I. F. Uchegbu, S. Vyas, "Non-ionic surfactant based vesicles (niosomes) in drug delivery" *Int. J. Pharm*. vol. 172, pp. 33, 1998.
[13] B. Khampaenjiraroch, A. Priprem, K. Lertrat, T. Damrongrungruang, "Rapid HPLC of cyanidin and delphinidin of an anthocyanin complex exposed to human gingival epithelial cells" *Appl. Mech. Mater.* vol. 563, pp. 403, 2014.
[14] H. Ikeda *et al.*, "EM703, the new derivative of erythromycin, inhibits transcription of type I collagen in normal and scleroderma fibroblasts." *J. Dermatol. Sci.* vol. 49, pp. 195, 2008.
[15] W. Muincharern, P. Louwakul, P. Pavasant, V. Lertchirakarn, "Effect of fluocinolone acetonide on human dental pulp cells: cytotoxicity, proliferation, and extracellular matrix formation" *J. Endod.* vol. 37, pp. 181, 2011.
[16] S. Singhabutta, *200 types of Thai herbal activities by Sountaree Singhabutta*. (Koon, Bangkok, 1993).
[17] M. B. M. Huveneers-Oorsprong, L. A. P. Hoogenboom, H. A. Kuiper, "The use of MTT test for determining the cytotoxicity of veterinary drugs in pig hepatocytes. " *Toxicol. in Vitro* vol. 11, pp. 385, 1997.
[18] J. Tiedtke, O. Marks, J. Morel, "Stimulation of collagen Production in Human Fibroblasts" *Cosmet. Sci. Technol.*vol., pp. 15, 2007.
[19] N. Garbacki *et al.*, "Inhibition of croton oil-induced oedema in mice ear skin by capsular polysaccharides from cyanobacteria" *Naunyn Schmiedebergs Arch. Pharmacol.* vol. 361, pp. 460, 2000.
[20] C. Charlier, C. Michaux, "Dual inhibition of cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) as a new strategy to provide safer non-steroidal anti-inflammatory drugs" *Eur. J. Med. Chem.* vol. 38, pp. 645, 2003.
[21] W. G. Li, X. Y. Zhang, Y. J. Wu, X. T. X, "Anti-inflammatory effect and mechanism of action of proanthocyanidins from grape seeds." *Acta Pharmacol. Sin.* vol. 22, pp. 1117, 2001.