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Porcine amniotic fluid as possible antiwrinkle cosmetic agent

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Abstract−Porcine amniotic fluid was investigated for use as a functional cosmetic ingredient. From safety tests by MTT (5-diphenyltetrazolium bromide) assay, cell viability was above 90% for 50-1,000 μg/mL concentration and porcine amniotic fluid was safe for cosmetic ingredient. From stability tests, cream containing 1% porcine amniotic fluid maintained constant physical properties for color, pH and viscosity during 28 days, and porcine amniotic fluid was stable for a cosmetic agent. Efficacy tests were done for antiwrinkle (elastase inhibition and collagenase synthesis inhibition), whitening (tyrosinase inhibition and DOPA (3,4-Dihydroxy-L-phenyl-alanine) oxidation inhibition) and antioxidation. At 500 μg/mL concentration, elastase inhibition of porcine placenta amniotic fluid was 33%, whereas that of adenosine as reference was 14%. However, porcine amniotic fluid showed relatively insignificant effect on collagenase synthesis inhibition, whitening and antioxidation activity. From this study, porcine amniotic fluid showed potential for a future antiwrinkle cosmetic agent.

Key words: Porcine Amniotic Fluid, Antiwrinkle, Whitening, Safety, Stability

INTRODUCTION

In Korea, cosmeceuticals (functional cosmetics) are acknowledged for whitening, antiwrinkle and UV protection area [1]. In 2008, the Korean cosmeceutical market exceeded 1 trillion Won for the first time and occupied the second largest portion among cosmetics after basic cosmetics [2].

Melanin is a heterogeneous polyphenol-like biopolmer with a complex structure and color varying yellow to black. In mammalian melanocytes, melanin is synthesized in melanosomes to play an important role in protecting skin from the harmful effects of solar UV radiation [3]. Tyrosinase is an enzyme that catalyzes the oxidation of phenols (such as tyrosine) and is widespread in plants and animals. The first step of the biosynthetic pathway for both eumelanins and pheomelanins is catalyzed by tyrosinase [4].

The dermis of young people is woven together of collagen fiber and elastic fibers. Because of its net shape, skin elasticity and flexibility are granted. Skin wrinkles typically appear as a result of aging processes such as glycation or, temporarily, as the result of prolonged immersion in water. Wrinkling in the skin is caused by habitual facial expressions, aging, sun damage, smoking, poor hydration, and various other factors. Collagenase and elastase are enzymes that break the peptide bonds in collagen and elastin at the dermis [5].

According to Korea Food and Drug Administration, whitening agents registered include arbutin, niacin amide, *Broussonetia* extract, ascobyl glucoside, ascorbyl tetra isopalmitate, ethyl ascorbyl ether, alpha-bisabolol and *Licorice* extract [1]. Antiwrinkle agents registered contain retinol, retinyl palmitate, adenosine and polyethoxylated retinamide [1].

Recent trend in cosmetic ingredients is to use natural products as long as materials are available. Many organic chemicals are replaced by natural resources, especially plant extracts and microbial products. Whitening agents researched included Scotch pine, citrus fruits, *Angelica* and antiwrinkle agents studied contained Oat kernel, *Geranium* and *Cirsiumsetidens* [6-8].

Porcine placenta has been reported to have skin care effects, improve basic metabolic function and strengthen immune function [9]. Although there are several types of placenta, human and bovine placenta are prohibited in cosmetics. In contrast, the porcine placenta is relatively safe and its immune effect is similar to that of humans so that it is used widely as cosmetic agent. Major components of the porcine placenta are proteins, amino acids, vitamins, minerals, and growth factors.

Amniotic fluid is essential that fluid be breathed into the lungs in order for them to develop normally. As well, amniotic fluid protects the developing baby by cushioning against blows to the mother's abdomen, allows for easier fetal movement, promotes muscular/skeletal development, and helps protect the fetus from heat loss. Recent studies have shown that amniotic fluid contains a considerable quantity of stem cells [10]. These amniotic stem cells are multipotent and able to differentiate into various tissues, which may be useful for future human application.

In literature, porcine placenta extracts are reported to have good antiwrinkle effect [11]. There is also research indicating that pure concentrations of human amniotic fluid had some benefit for wound healing [12]. However, there is no research showing that amniotic fluid is effective for wrinkles or other skin-care needs in cosmetic formulation.

In this study, porcine amniotic fluid was tested to see the possibility for a functional cosmetic agent. Cosmetic tests were done for safety, stability and efficacy (antiwrinkle, whitening and antioxida-

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tion) according to standard methods.

MATERIALS AND METHODS

1. Reagents and Cells

Porcine amniotic fluid was collected from a pig farm in Hamyang-gun Gyeongnam, Korea. Porcine amniotic fluid was centrifuged at 5,000 rpm for 10 min and supernatant was collected. Porcine amniotic fluid was dried with freeze-dryer (Supra 30 K, Hanil science industrial, Korea) to keep the sample for longer period and to measure porcine amniotic fluid concentration accurately. Dried porcine amniotic fluid was used as testing material in the experiment. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), mushroom-tyrosinase, L-tyrosine, 3,4-dihydroxy-L-phenyl-alanine (DOPA), elastase(pancreatic solution), N-succinyl-$(Ala)_3$-p-nitroanilide,adenosine, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (USA). Matrix metalloproteinase-1 (MMP-1) human biotrak ELISA system kit (collagenase synthesis inhibition assay) was purchased from GE Healthcare (USA). Human fibroblast cells (CCD-986sk) were purchased from Korea Cell Line Bank. Cells were cultured at 37 °C under 5% CO_2 in incubator (MCO-15AC, Sanyo, Japan) using Dulbecco's modified eagle's medium (DMEM, Lonza, USA), which contained 10% (v/v) FBS (fetal bovine serum) and 1% (v/v) penicillin/ streptomycin.

2. Safety Test (MTT Assay)

Cell toxicity test was done using MTT assay with modified Mosmann method [13]. After seeding human fibroblast cell (CCD 986sk) on 24-well plate in 1×10^5 cell/mL concentration, cells were cultured for 24 hours in CO_2 incubator. New cell medium including porcine amniotic fluid was exchanged and cultured for another 24 hours. MTT solution (5 mg/mL) was added and left for 2 hours in 37 °C, 5% CO_2 incubator. Formazan was dissolved in DMSO (dimethyl sulfoxide) and transferred to 96 well plate. Absorbance was measured at 595 nm by Microplate Spectrophotometer (Power Wave XS2, BIOTEK, USA) and cell viability was calculated as follows:

$$\text{Cell viability (\%)}=[(\text{Exp.}-\text{Blank})/\text{Control}]\times100 \quad (1)$$

Table 1. Formulation of cream containing 1% porcine amniotic fluid

	Components	g
Water phase	D.I water	54.05
	Methylparaben	0.2
	Ethylparaben	0.1
	1,3 Butylene glycol	4.00
Oil phase	Macadamia nut oil	2.00
	Olivem1000	1.50
	Tween 60	2.00
	Sweet almond oil	7.00
	Jojoba oil	10.00
	Wax	10.00
	Propylparaben	0.10
	Buthylparaben	0.05
Thickening agent	Carbopol 941 (1%)	8.00
Active ingredient	Porcine amniotic fluid	1.00
Total		100

3. Stability Test

Cream including 1% porcine amniotic fluid was prepared as shown in Table 1. As stability test, color, viscosity and pH of the cream were observed at 4 °C, 25 °C and 45 °C for 28 days.

4. Antiwrinkle Effect Test

Antiwrinkle effect was measured by elastase inhibition assay and collagenase synthesis inhibition assay [14]. Elastase inhibition assay was done as follows: N-succinyl-$(Ala)_3$-ρ-nitroanilide (as substrate) was dissolved in 0.2 M tris-Cl buffer (pH 8.0) to make 1.0 mM concentration. 20 μL porcine amniotic fluid and 20 μL 2.5 U/mL porcine pancreas elastase were added to 200 μL tris-Cl buffer including substrate, and reacted for 10 min at 25 °C. Absorbance was measured at 410 nm by microplate spectrophotometer.

Collagenase synthesis inhibition assay was done as follows [1]: Human fibroblast cells were seeded in 48-well plate including 10% FBS/DMEM media for 24 hours to 5×10^5 cell/well. The porcine amniotic fluid was added in serum free media and left for 48 hours under CO_2 incubator. Collagenase concentration was measured by Lowry assay (Matrix metalloproteinase-1 human biotrak ELISA system kit, GE Healthcare, USA). Absorbance was measured at 450 nm by microplate spectrophotometer.

5. Whitening Effect Test

Whitening effect was measured by tyrosinase inhibition assay and DOPA oxidation inhibition assay [15]. Tyrosinase inhibition assay was done as follows: 220 μL of 0.1 M potassium phosphate buffer (pH 6.8), 40 μL of 1.5 mM L-tyrosine and 20 μL of mushroom tyrosinase (2,000 U/mL, Sigma, USA) were reacted with 20 μL of porcine amniotic fluid for 10 min at 37 °C. Absorbance was measured at 490 nm by microplate spectrophotometer and inhibition ratio was calculated as follows:

$$\text{Inhibition ratio (\%)}=[1-(\text{Exp.}-\text{Blank})/\text{Control}]\times100 \quad (2)$$

DOPA oxidation inhibition assay was done as follows: 200 μL of 0.1 M potassium phosphate buffer (pH 7.0) and 20 μL of mushroom tyrosinase (2,000 U/mL) were mixed with 40 μL of porcine amniotic fluid for 6 min at 37 °C. 40 μL of 2 mM DOPA was added and reacted for 1 min at 37 °C. The absorbance was measured at 475 nm by microplate spectrophotometer.

6. Antioxidation Effect (DPPH Free Radical Scavenging Assay)

Antioxidation effect was measured by DPPH free radical scavenging assay [16]. DPPH free radical scavenging assay was done as follows: 100 μL of DPPH (α,α-diphenyl-β-picrylhydrazyl, Sigma, USA) was reacted with 200 μL of porcine amniotic fluid for 20 min and absorbance was measured at 517 nm by microplate spectrophotometer.

7. Statistical Analysis

Each experiment was repeated three times and analyzed by 95%-confidence interval using Student's t-test.

RESULTS AND DISCUSSION

1. Safety

As safety test, MTT assay was performed in 50-1,000 μg/mL concentration using human fibroblast cell (CCD-986sk) as shown in Fig. 1. Cell viability of porcine amniotic fluid was 98% at 50 μg/mL and decreased slightly to 89% at 1,000 μg/mL, whereas that for vitamin C (reference material) was 92% at 50 μg/mL and decreased

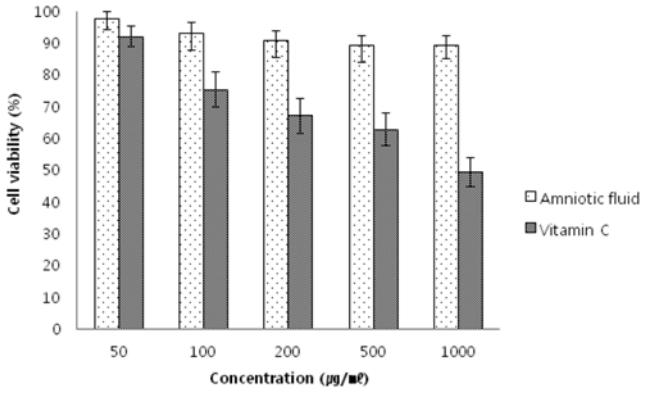

Fig. 1. Cell viability for porcine amniotic fluid on human fibroblast cells (MTT assay).

significantly to 50% at 1,000 μg/mL. This indicated that porcine amniotic fluid had minimum cell toxicity. Cell viability for porcine amniotic fluid was similar to that for porcine placenta extract [17]. Cell toxicity of porcine amniotic fluid was negligible compared with typical plant extracts such as *Nelumbo nucifera* [18]. Therefore, porcine amniotic fluid was stable enough to use as cosmetic agent.

2. Stability Test

Cream containing 1% amniotic fluid was prepared to examine stability of the material. Color, viscosity and pH of the cream were measured at 4 ℃, 25 ℃ and 40 ℃ for 28 days. Color, viscosity and pH of cream containing 1% amniotic fluid were not changed significantly for experimental condition tested in Table 2. Porcine amniotic fluid could be regarded as stable for cosmetic ingredient.

3. Efficacy

As an efficacy test, antiwrinkle, whitening and antioxidation effect were measured to evaluate porcine amniotic fluid as a possible functional cosmetic agent. Even though antioxidation was not explicitly mentioned in functional cosmetic effect, it was indirectly related to UV protection and whitening effect.

Collagen and elastin are primary proteins which compose the dermis and they maintain the skin's structure and elasticity. Collagen decreases as people grow older, but its catabolic rate increases. When exposed to UV rays, tertial structure of elastin is twisted by elastase, which increases activation. Those symptoms go on to dents of the dermis; therefore, skin wrinkles are formed.

Table 2. Stability test of cream including 1% porcine amniotic fluid

		4 ℃	25 ℃	40 ℃
0 Days	Color	white	white	white
	Viscosity (cP)	35,000	35,000	35,000
	pH	4.83	4.83	4.83
7 Days	Color	white	white	white
	Viscosity (cP)	36,000	34,000	33,000
	pH	4.75	4.63	4.73
14 Days	Color	white	white	white
	Viscosity (cP)	35,600	37,000	36,500
	pH	4.83	4.69	4.75
21 Days	Color	white	white	white
	Viscosity (cP)	35,000	36,200	35,800
	pH	4.80	4.78	4.90
28 Days	Color	white	white	white
	Viscosity (cP)	34,900	35,400	36,000
	pH	4.75	4.80	4.85

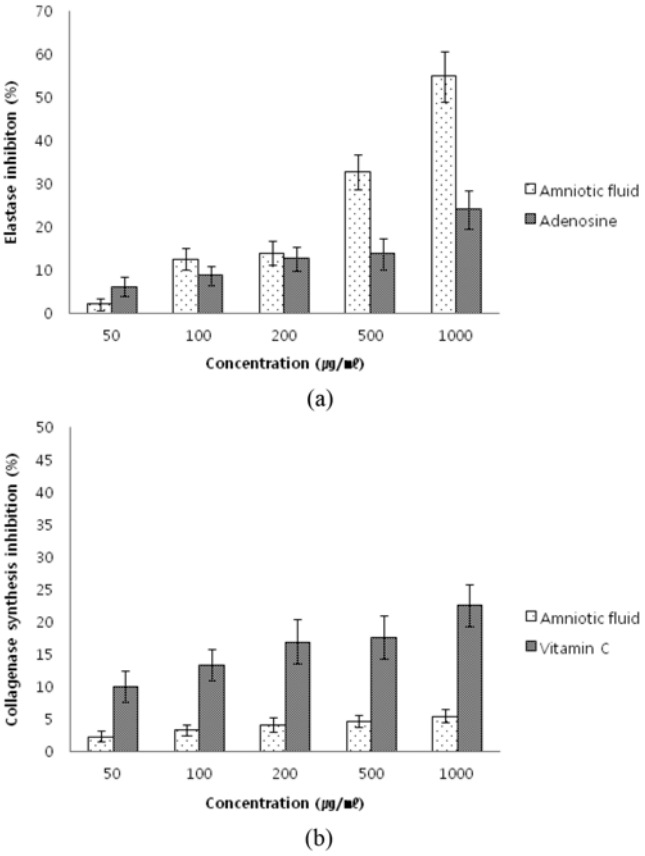

Fig. 2. Antiwrinkle effect of porcine amniotic fluid. (a) Elastase inhibition effect; (b) collagenase synthesis inhibition effect.

When exposed to UV rays, tertial structure of elastin is twisted by elastase, which increases activation. Those symptoms go on to dents of the dermis; therefore, skin wrinkles are formed.

Antiwrinkle effect of porcine amniotic fluid was measured by elastase inhibition and collagenase synthesis inhibition as shown in Fig. 2. Elastase inhibition effect of porcine amniotic fluid was increased monotonically as concentration of porcine amniotic fluid increased from 50 to 1,000 μg/mL. At 500 μg/mL concentration, elastase inhibition of porcine placenta amniotic fluid was 33%, whereas that of adenosine (reference material) was 14%. However, collagenase synthesis inhibition effect of porcine amniotic fluid was significantly lower compared with vitamin C (reference material) in 50 to 1,000 μg/mL. From the experiment, porcine amniotic fluid contained strong elastase inhibition effect, whereas it did not have meaningful collagenase inhibition.

IC_{50} (50% elastase inhibition concentration) of porcine amniotic fluid was 800 μg/mL, whereas IC_{50} of porcine placenta extracts by enzyme treatment was 1,100 μg/mL in literature [17]. Porcine amniotic fluid showed better elastase inhibition capacity than porcine placenta extract. Elastase inhibition effect of porcine amniotic fluid was as good as other natural products such as *Geranium nepalense* [19] and *Astragalus membranaceus* [20]. From this result, porcine amniotic fluid has potential for antiwrinkle agent as elastase inhibitor.

Tyrosinase is a prime enzyme that engages in melanin formation that decides skin color. Tyrosine is oxidized to DOPA and DOPA

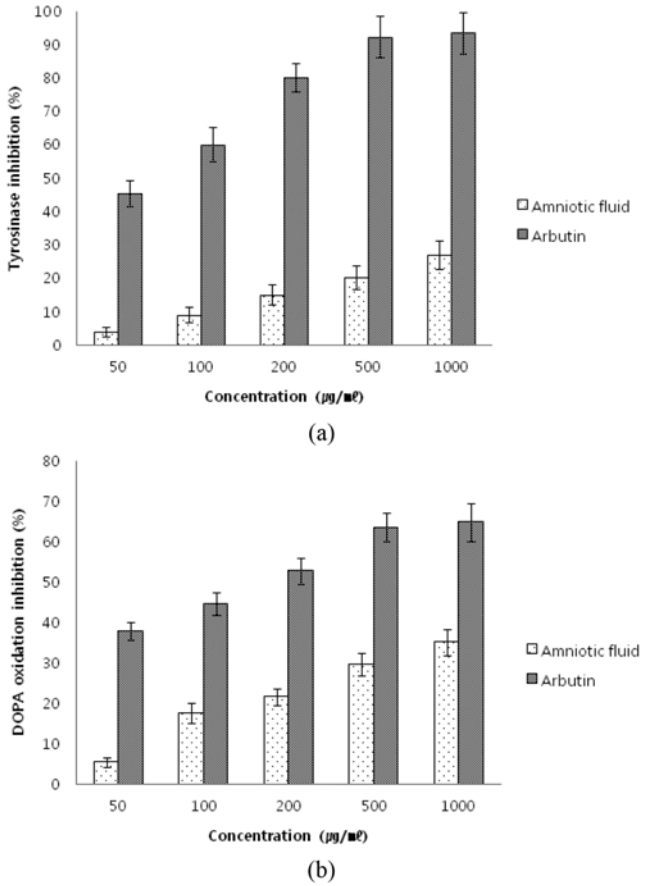

Fig. 3. Whitening effect of porcine amniotic fluid. (a) Tyrosinase inhibition effect; (b) DOPA oxidation inhibition effect.

quinone by tyrosinase. After being oxidized, DOPA quinone changes to DOPA chrome, 5,6-quinone and produces melanin because of polymerization to indole-5,6-quinone; therefore, we can expect skin whitening effect by examining the inhibition degree of tyrosinase enzymatic reaction.

Whitening effect of porcine amniotic fluid was measured by tyrosinase inhibition assay and DOPA oxidation inhibition assay as shown in Fig. 3. Tyrosinase inhibition effect and DOPA oxidation inhibition effect were increased as concentration of porcine amniotic fluid increased from 50 to 1,000 μg/mL. However, tyrosinase and DOPA oxidation inhibition effects were relatively lower compared with arbutin (reference material). At 200 μg/mL concentration, tyrosinase inhibition and DOPA oxidation inhibition of porcine amniotic fluid were 15% and 22%, respectively, whereas those of porcine placenta extract were 19% and 15%, respectively, in the literature [17]. Tyrosinase inhibition and DOPA oxidation inhibition were similar for both porcine placenta extract and amniotic fluid. Tyrosinase inhibition for native plants extracts showing strong whitening effect was generally 40-70% at 200 μg/mL [21]. From the experiment, porcine amniotic fluid did not possess strong whitening activity.

Antioxidation effect of porcine amniotic fluid was measured by DPPH free radical scavenging assay as shown in Fig. 4. Antioxidation effect was increased as concentration of porcine amniotic fluid increased from 50 to 1,000 μg/mL. However, antioxidation effect of porcine amniotic fluid was considerably lower than vitamin C (reference material). FSC_{50} (50% free radical scavenging capacity) for natural plants with high capacity was generally 5-100 μg/mL [21]. Porcine amniotic fluid did not show 50% inhibition even at 1,000 μg/mL. Free radical scavenging activity for porcine amniotic fluid was not strong enough for other potent natural agents.

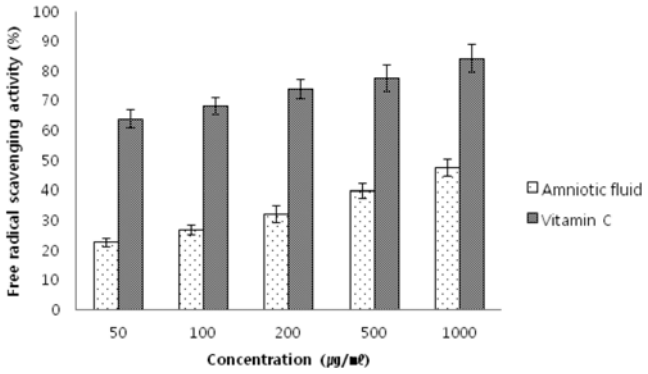

Fig. 4. Antioxidation effect of porcine amniotic fluid by DPPH free radical scavenging assay.

In summary, porcine amniotic fluid showed good elastase inhibition, safety and stability for possible functional cosmetic agent. However, whitening and antioxidation effect were relatively poor. Therefore, porcine amniotic fluid has good possibility for antiwrinkle cosmetic agent.

CONCLUSIONS

Porcine amniotic fluid was tested for use as a functional cosmetic ingredient. It showed negligible cell toxicity in 50-1,000 μg/ml concentration by MTT assay. Cream containing 1% amniotic fluid maintained stable color, viscosity and pH for 28 days, and amniotic fluid was very stable as a cosmetic agent. At 500 μg/mL concentration, elastase inhibition of porcine placenta amniotic fluid was 33%, and porcine amniotic fluid showed good elastase inhibition effect. However, collagenase synthesis inhibition effect was negligible in concentration range tested. Whitening effect (tyrosinase inhibition and DOPA oxidation inhibition) and antioxidation of porcine amniotic fluid were relatively weak. From the study, porcine amniotic fluid was concluded to have good potential for antiwrinkle functional cosmetic agent.

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REFERENCES

1. Korea Food and Drug Administration, http://www.kfda.go.kr/index.html (2009).
2. Cosmetics Industry Analysis Report 2009, KHIDI, Seoul (2009).
3. P. Elsner and H. I. Mailbach, *Cosmeceuticals and active cosmetics*, 2nd Ed., Taylor & Francis, New York (2005).

4. B. C. Ha, *Cosmeceuticals*, Shin Kwang Publishing, Seoul (2005).
5. KDA Textbook Editiong Board, *Dermatology*, 5th Ed., Ryo Mook-Gak, Seoul (2008).
6. G. Vielhaber, G. Schmaus, K. Jacobs, H. Franke, S. Lange, M. Hermann, H. Joppe and O. Koch, *IFSCC*, **9**, 227 (2006).
7. S. H. Park, B. Y. Lee, C. S. Han, J. G. Kim, K. T. Kim, K. H. Kim and Y. H. Kim, *J. Soc. Cosmet. Scientists Korea*, **33**, 101 (2007).
8. R. Pillai, M. Redmond and J. Roding, *IFSCC*, **8**, 17 (2005).
9. J. C. Park, *Herbal functional food*, Hyoilbooks, Seoul (2007).
10. Y.-M. Zheng, H.-Y. Zhao, X.-E. Zhao, F.-S. Quan, S. Hua, X.-Y. He, J. Liu, X.-N. He and H. Lin, *Reproduction*, **137**, 793 (2009).
11. B. Y. Kim, T. Kim, W. Y. Kang, B. Hyun, H. Y. Cheon and D. Kim, *Korean Chem. Eng. Res*., **48**, 327 (2010).
12. G. Y. Ozqenel, B. Samil and M. Ozcan, *J. Hand Surgery*, **26**, 332 (2001).
13. T. Mosmann, *J. Immun. Method*, **65**, 55 (1983).
14. N. Erdemogu, B. Sener and S. Ide, *J. Mol. Struct*., **559**, 227 (2001).
15. A. Yagi, T. Kanbara and N. Morinobu, *Plantamedica*, **3981**, 517 (1986).
16. Y. Fujita, I. Hehara, Y. Morimoto, M. Nakashima, T. Hatano and T. Okuda, *YakugakuZasshi*, **108**, 129 (1988).
17. T. Kim, Master's Thesis, Inje University, Gimhae, Korea (2011).
18. H. J. Kim, T. Kim, W. Y. Kang, B. Hyun, H. Y. Cheon, B. Y. Kim and D. Kim, *Korean Chem. Eng. Res*., **48**, 413 (2010).
19. K. H. Lee and S. N. Park, *J. Soc. Cosmet. Scientists Korea*, **34**, 25 (2008).
20. M. J. Kim, K. R. Lim, T. K. Jung and K.-S. Yoon, *J. Soc. Cosmet. Scientists Korea*, **33**, 33 (2007).
21. J. Y. Kim, H. J. Yang, K. H. Lee, S. M. Jeon, Y. J. Ahn, B. R. Won and S. N. Park, *J. Soc. Cosmet. Scientists Korea*, **33**, 165 (2007).