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Asiaticoside induces cell proliferation and collagen synthesis in human dermal fibroblasts

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ABSTRACT

BACKGROUND
Asiatiocoside, a saponin component isolated from *Centella asiatica* can improve wound healing by promoting the proliferation of human dermal fibroblasts (HDF) and synthesis of collagen. The skin-renewing cells and type I and III collagen synthesis decrease with aging, resulting in the reduction of skin elasticity and delayed wound healing. Usage of natural active compounds from plants in wound healing should be evaluated and compared to retinoic acid as an active agent that regulates wound healing. The aim of this study was to compare and evaluate the effect of asiaticoside and retinoic acid to induce greater cell proliferation and type I and III collagen synthesis in human dermal fibroblast.

METHODS
Laboratory experiments were conducted using human dermal fibroblasts (HDF) isolated from human foreskin explants. Seven passages of HDF were treated with asiaticoside and retinoic acid at several doses and incubated for 24 and 48 hours. Cell viability in all groups was tested with the MTT assay to assess HDF proliferation. Type I and III collagen synthesis was examined using the respective ELISA kits. Analysis of variance was performed to compare the treatment groups.

RESULTS
Asiaticoside had significantly stronger effects on HDF proliferation than retinoic acid ($p<0.05$). The type III collagen production was significantly greater induction with asiaticoside compared to retinoic acid ($p<0.05$).

CONCLUSION
Asiaticoside induces HDF proliferation and type I and III collagen synthesis in a time- and dose-dependent pattern. Asiaticoside has a similar effect as retinoic acid on type I and type III collagen synthesis.

Keywords: Asiaticoside, type I collagen, type III collagen, fibroblast, proliferation, retinoic acid

Asiaticoside menginduksi proliferasi sel dan sintesis kolagen pada fibroblas dermis kulit manusia

ABSTRAK

LATAR BELAKANG

Asiatiocosida merupakan komponen saponin yang diisolasi dari Centella asiatica yang bermanfaat untuk mempercepat penyembuhan luka dengan meningkatkan proliferasi dan sintesis kolagen dalam fibroblas dermis kulit manusia. Proses pembaharuan sel kulit dan sintesis kolagen tipe I dan III menurun dengan proses penuaan, elastisitas kulit berkurang dan penyembuhan luka melambat. Penggunaan senyawa aktif alami dari herbal dalam penyembuhan luka dievaluasi dan dibandingkan dengan asam retinoat sebagai bahan aktif yang mengatur penyembuhan luka. Tujuan penelitian ini adalah untuk membandingkan dan mengevaluasi pengaruh asiaticosida dan asam retinoat terhadap proliferasi fibroblas dan sintesis kolagen tipe I dan III pada dermis kulit manusia.

METODE

Sebuah penelitian eksperimental laboratorik menggunakan sel dermis fibroblas kulit manusia yang diisolasi dari eksplan kulit. Generasi ke tujuh fibroblas dermis kulit manusia dipajankan dengan beberapa dosis asiaticosida dan asam retinoat, diinkubasi selama 24 dan 48 jam. Viabilitas sel diuji dengan uji MTT untuk menilai proliferasi fibroblas kulit manusia. Sintesis kolagen Tipe I dan III diperiksa menggunakan ELISA kit, masing-masing dievaluasi setelah 24 dan 48 jam. Analisis uji ANOVA digunakan untuk menguji perbedaan antara kelompok perlakuan.

HASIL

Penelitian ini menunjukkan, asiaticosida menginduksi proliferasi fibroblas dermis kulit manusia lebih tinggi secara bermakna dibandingkan asam retinoat ($p<0,05$). Asiaticosida menginduksi sintesis kolagen tipe III lebih tinggi secara bermakna dibandingkan asam retinoat ($p<0,05$).

KESIMPULAN

Asiaticosida mampu menginduksi proliferasi fibroblas dermis kulit manusia dan sintesis kolagen tipe I dan III yang dipengaruhi oleh dosis dan waktu inkubasi. Asiaticosida mempunyai efek yang sama seperti asam retinoat pada sintesis kolagen tipe I dan III

Kata kunci: *Asiaticosida, kolagen I, kolagen III, fibroblas, proliferasi, asam retinoat*

INTRODUCTION

Skin wound healing is a complex process carried out by cells that are involved in inflammation, re-epithelialization, angiogenesis, granulation, tissue formation and differentiation, and deposition of interstitial matrix. In general, wound healing can be divided into three stages: inflammation, proliferation and remodeling.[1] *Centella asiatica* (L.)Urb. (CA) has been widely used as traditional herbal medicine in Malaysia, India and Nepal as part of the traditional Ayurvedic Medicine for hundreds of years.[2,3] It is commonly known as pegaga in Malaysia, pegagan in Indonesia and as penny wort or gotu kola in America.[2-4] This tropical plant can be used as "longevity herb" or herbal anti-aging cosmetic [5,6] and traditionally serves various medicinal purposes such as wound healing, treatment of asthma, ulcers, leprosy, lupus erythematosus, psoriasis, venous disorders, for memory improvement, and as antidepressant, antibacterial, antifungal, and anti-cancer agent.[7-9]

Centella asiatica has been used traditionally to improve wound healing.[3-5] *Centella asiatica* ethanolic extract treated wounds can epithelialize faster and have greater collagen content.[9] The biologically active ingredients in CA are triterpenes, namely asiatic acid, madecassic acid, asiaticoside and madecassoside.[8-10] Asiaticoside isolated from CA promotes fibroblast proliferation and extracellular matrix synthesis in wound healing [11,12] by enhancing collagen formation and angiogenesis.[12,13] In particular, asiaticoside and madecassoside stimulate type I and type III collagen, respectively.[12,14] A study by Hadi et al.[13] indicated that allogenic human dermal fibroblasts (HDF) are viable in peripheral blood. Monolayer co-culture *in vitro* can induce synthesis of type I and III collagen, the major structural and functional components of the skin. Asiaticoside and retinoic acid can regulate (i.e. stimulate or inhibit) the production of these collagens.[11,14]

In skin, retinoids are involved in keratinocyte cell differentiation, epidermal cell adhesion and corneocyte exfoliation from the surface of epidermis. More specifically to the dermis, retinoids regulate fibroblast proliferation, induce angiogenesis and play an essential role in the synthesis of collagen and elastin fibrils.[14] Natural products contain a wealth of interesting pharmaceutically active compounds and various active agents are expected to be beneficial in wound healing and as preventive anti-aging cosmetics.[1] The stimulation of collagen synthesis is an important criterion for plant extracts to be considered as a potential ingredient in skin care anti-aging products.[11,12] Due to its ability to stimulate collagen synthesis, CA has been used in skin care products for anti-aging, restoring skin firmness and elasticity and improving skin appearance.[11,12]

Lu et al.[10] reported that asiaticoside isolated from *Centella asiatica* promotes fibroblast proliferation and extracellular matrix synthesis in wound healing, the precise mechanism, however, being still only partially understood. The aim of this study was to evaluate fibroblast proliferation and type I and III collagen synthesis by asiaticoside as an active compound from CA in comparison to retinoic acid, a well-known active ingredient in the prevention of skin aging.

METHODS

Research design

In the present study, experiments on fibroblast proliferation were conducted at the Laboratory of the Centre for Pharmaceutical and Medical Technology, Agency for the Assessment and Application of Technology, from January 2013 to April 2013, and on collagen synthesis at the Integrated Laboratory of Medicine, Yarsi University, from April 2014 to June 2014.

Preparation of *Centella asiatica* extract

Centella asiatica (CA) aqueous and ethanolic extracts (Tawangmangu Strain) and *Centella asiatica* chitosan nanoparticles were prepared in the laboratory of the Agency for the Assessment and Application of Technology. *Centella asiatica* was encapsulated in the chitosan nanoparticles by ionotropic gelation. Then, proliferative and cytotoxic effects of nanoencapsulated *Centella asiatica* extracts was evaluated *in vitro* on human dermal fibroblasts (HDF). The MTT proliferation assay was performed with the encapsulated CA extracts in comparison with the unencapsulated CA extracts and with empty chitosan nanoparticles (CNP) as controls.

Cell culture and treatment

Human dermal fibroblast cultures were established individually from preputial skin of an 8 year-old child and prepared at Yarsi laboratorium by Dr. Indra Kusuma. The seventh passages of cultured cells were used for all experiments. Normal human dermal fibroblasts (NHDF) were grown in Roswell Park Memorial Institute (RPMI) medium containing 10% fetal

bovine serum (FBS) and 1% penicillin-streptomycin. Cells were reseeded at a ratio of 1:3 and density of 1 x 10^6 cell/mL and subjected to treatment with 0.3125 mg/mL, 0.625 mg/mL, 1.25 mg/mL, 2.5 mg/mL, 5 mg/mL and 10 mg/mL of asiaticoside or retinoic acid. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was repeated three times using MTT as substrate, which is actively absorbed by fibroblasts and then reduced by mitochondrial succinate dehydrogenase under the addition of nicotinamide adenine dinucleotide-H (NADH) from water-soluble MTT to insoluble purple formazan. The number of viable cells was then evaluated at 24 and 48 hours after treatment, as an indicator of fibroblast proliferation.[17] Obtained values were then normalized against the control group, which was taken as 100%.

Type I and III collagen synthetic activity by human dermal fibroblasts

Collagen synthesis was analyzed using ELISA kit for human collagen type I and III, alpha 1 from Cusabio. Fibroblast collagen synthetic activity was evaluated using ELISA, 24 and 48 hours after treatment and compared with untreated HDF as negative control. Results for collagen synthesis were reported in µg/mL.

Statistical analysis

For statistical evaluation of HDF proliferation and collagen type I and III synthesis, one-way ANOVA as well as Tukey's post hoc analysis was performed when one-way ANOVA results were significant. Significance was set to $p<0.05$, and for highly significant differences to $p<0.001$.

RESULTS

Table 1 shows that after 24 hours, asiaticoside at all concentrations had stronger effects on HDF proliferation than retinoic acid. The optimal stimulatory effect was observed at 0.625 mg/mL. In statistical analysis with

Table 1. Mean distribution of HDF proliferation and collagen synthesis by asiaticoside and retinoic acid

Fibroblast proliferation (%)	Treatment												
	Asiaticoside							Retinoic acid					
	I	II	III	IV	V	VI	p	I	II	III	IV	V	VI	p
24 hours	149.45	150.00	71.73	163.04	137.50	127.72	0.237	144.02	30.43	93.48	132.06	154.34	135.32	0.027
48 hours	139.37	150.00	71.74	108.69	137.50	127.72	0.285	144.02	30.43	93.48	132.06	102.89	135.32	0.025
Type I collagen synthesis (µg/mL)														
24 hours	0.935	0.930	0.930				0.465	0.915	0.940	0.940				0.465
48 hours	0.940	0.940	0.935				0.465	0.940	0.940	0.935				0.465
Type III collagen synthesis (µg/mL)														
24 hours	0.367	0.443	0.406				0.465	0.389	0.341	0.376				0.465
48 hours	0.527	0.517	0.497				0.465	0.622	0.479	0.418				0.465

Test compound concentration for HDF proliferation (mg/L) : I : 0.3125;II : 0.625;III : 1.25;IV : 2.50;V : 5.0;VI : 10.0

Test compound concentration for collagen synthesis (µg/mL): I : 0.025;II : 0.5;III : 0.1

ANOVA test the difference between asiaticoside and retinoic acid was significant ($p<0.05$). After 48 hours, asiaticoside has its strongest effect at 0.625 mg/mL *vs.* retinoic acid ($p<0.025$).

The one-way ANOVA test results indicated that between the asiaticoside dose groups there were significant differences in fibroblast proliferation after 24 and 48 hours of treatment ($p<0.001$). The post hoc Tukey test found significant differences in the asiaticoside groups at 24 hours, between dose I and II, II and III, II and IV, II and V, II and VI, III and IV, III and VI (Table 2).

According to the one-way ANOVA test, between the retinoic acid dose groups there were significant differences in fibroblast proliferation after 24 hours and 48 hours of treatment ($p<0.001$). The post hoc Tukey test found significant differences in the retinoic acid groups at 24 hours, between dose I and II, I and III, I and V, I and VI, II and III, II and IV, II and V, II and VI, III and V, III and VI (Table 3).

Type I collagen synthesis by HDF after 24 and 48 hours of treatment at all three doses of asiaticoside and retinoic acid, was observed to actually be lower than controls. However, the differences were statistically not significant. For type III collagen production, we observed the greatest induction at 24 hours with asiaticoside treatment at 2.50 ìg/mL. On the other hand, at 48 hours the maximum effect was achieved by retinoic acid at 0.50 ìg/mL.

Table 2. Post hoc Tukey multiple comparisons on HDF proliferationby asiaticoside

		Fibroblast proliferation (%) Mean differences	p
24 hours			
I	II	-0.54	1.000
	III	77.71	0.333
	IV	-13.58	0.999
	V	11.95	0.999
	VI	21.74	0.989
II	III	78.26	0.327
	IV	-13.04	0.999
	V	12.50	0.999
	VI	22.28	0.988
III	IV	-91.30	0.197
	V	-65.76	0.978
	VI	-55.97	0.919
IV	V	25.354	0.978
	VI	35.32	0.978
V	VI	9.78	1.000
48 hours			
I	II	-10.62	0.999
	III	67.63	0.395
	IV	30.68	0.937
	V	1.87	1.000
	VI	11.65	0.999
II	III	78.26	0.259
	IV	41.30	0.818
	V	12.5	0.999
	VI	22.28	0.983
III	IV	-36.95	0.875
	V	-65.76	0.422
	VI	-55.97	0.581
IV	V	-28.80	0.951
	VI	-19.02	0.992
V	VI	9.79	1.000

Asiaticoside concentration (mg/L) : I: 0.3125; II: 0.625; III: 1.25; 28.80 IV: 2.50; V: 5.0; VI: 10.0

Table 3. Post hoc Tukey multiple comparisons on HDF proliferation by retinoic acid

		Fibroblast proliferation (%) Mean differences	
24 hours			
I	II	113.58	0.049
	III	50.54	0.670
	IV	11.95	0.999
	V	-10.32	1.000
	VI	0.69	1.000
II	III	-63.04	0.461
	IV	-101.63	0.088
	V	-123.91	0.029
	VI	-104.89	0.075
III	IV	38.58	0.856
	V	-22.28	0.496
	VI	-3.26	0.000
IV	V	-22.28	0.983
	VI	-3.26	1.000
V	VI	19.02	0.992
48 hours			
I	II	113.58	0.025
	III	50.54	0.570
	IV	11.95	0.998
	V	41.12	0.748
	VI	8.69	1.000
II	III	-63.04	0.353
	IV	-101.62	0.049
	V	-72.46	0.229
	VI	-104.88	0.041
III	IV	-38.58	0.790
	V	-9.41	0.999
	VI	-41.84	0.734
IV	V	29.16	0.920
	VI	-3.26	1.000
V	VI	-32.42	0.882

Retinoic acid concentration (mg/L) : I : 0.3125; II : 0.625; III : 1.25; IV : 2.50; V : 5.0; VI : 10.0

DISCUSSION

Our results indicate that 24 hours after treatment, asiaticoside has a stronger effect on fibroblast proliferation at all concentrations compared to retinoic acid. This is in accordance to the previous study by Lu et al.,[10] in which they reported alterations in COL18A1, COL1A2, and COL3A1 gene profile, as well as in the fibroblast cell division cycle, in response to asiaticoside stimulation.

Gimemo et al.[15] investigated the time-dependency effect of four different retinoids in the μM dose range on human dermal fibroblasts cultivated in vitro. This study showed that retinol and retinal at concentrations greater than 20 μM, can damage cells as evaluated by lactate dehydrogenase activity released into the culture medium, and induce oxidative stress and apoptosis in human dermal fibroblasts.

Type I and III collagens are major structural and functional components of skin connective tissue. Therefore, we investigated the intracellular and extracellular modifications of type I and III collagen, as in the cross-linked step by asiaticoside stimulation.[11] Hadi et al.[13] investigated allogeneic HDF viability in peripheral blood mononuclear co-culture and reported that allogeneic HDF were able to secrete type I and III collagen. Our study showed that

asiaticoside and retinoic acid induced the synthesis of type I collagen in HDF after 24 and 48 hours of incubation, but after 24 hours 0.05 µg/mL of asiaticoside induced in HDF significantly higher type III collagen synthesis compared to retinoic acid.

Our experiment indicated that after 24 hours, type III collagen synthesis in HDF was significantly higher after treatment with asiaticoside compared to retinoic acid and controls (without treatment). Maximal effect was achieved at 2.50 µg/mL of asiaticoside, but after 48 hours retinoic acid had a greater effect than asiaticoside at the same dose.[7]

A study in Malaysia showed that CA extract has a dose-dependent stimulatory effect on collagen synthesis, which was enhanced three-fold over controls at a dose of 50 mg/mL.[5] Compared to our experiment, another study in Malaysia by Hashim et al.[4] evaluated the effect of *Centella asiatica* on collagen synthesis in HDF, compared to vitamin C. The effect of CA extract was stronger than that of vitamin C, which was used as a positive control. The CA extract at 50 mg/mL showed three-fold collagen enhancement, whereas vitamin C had a two-fold collagen enhancing response.[4]

Lu et al.[10] showed that asiaticoside isolated from CA induced collagen III synthesis with maximum effect at 48 hours. In our study, both asiaticoside and RA showed greatest effects at 48 hours. This is supported by the previous study of Wu et al.,[16] which reported significant elevation of procollagen III level following stimulation with low concentrations of asiaticoside and madecassoside. Moreover, research by Pareda et al.[17] indicated a dose-dependent in vitro stimulation on collagen synthesis by green *Coffea arabica* (GCO). It was found that collagen synthesis was enhanced 1.8 fold in human dermal fibroblasts than in negative controls after 48 hours of treatment with 3.125 mg/mL and 6.25 mg/mL GCO. These results demonstrated a more pronounced stimulatory effect of biosynthetic compounds on collagen production. Nevertheless, with further improvement, we expect a similar stimulatory performance of asiaticoside.

Song et al.[18] conducted a study of madecasoside (one of the active compounds of *Centella asiatica*) and found that this compound induces apoptosis of keloid fibroblasts in a mitochondrial-dependent pathway, in addition to inhibiting type I collagen synthesis. Our study with asiaticoside matches this observation, with greater type III collagen and lower type 1 collagen synthesis in human dermal fibroblast cultures of preputium explants of young children (4-8 years). Ju-lin et al.[19] had previously reported that asiaticoside induces the expression and activation of the TGF-â signaling pathway in hyperthropic scars, while inhibiting Smad7. This is the underlying mechanism behind the decreased type I collagen production. In the present study, however, we isolated human dermal fibroblast from one subject only. Therefore, this may not fully represent some genetic variations related with collagen synthesis. A clinical study by Kaji et al.[20] concluded that topical retinol treatment for 24 weeks improved fine wrinkles associated with natural aging, and that increased collagen production by retinol is the most likely reason behind it. Clinical application of the present study will be done to investigate the effect of asiaticoside in accelerating wound healing. Another possible clinical study is to investigate the anti-aging effect of asiaticoside after topical treatment, in comparison with retinoic acid.

CONCLUSIONS

Asiaticoside induces HDF cell proliferation and collagen synthesis in a time- and dose-dependent manner. Asiaticoside as an active agent of *Centella asiatica* has a similar effect as retinoic acid on type I and III collagen synthesis in human dermal fibroblasts.

CONFLICT OF INTEREST

The authors have no conflict of interest.

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REFERENCES

1. Davis SC, Perez R. Cosmeceutical and natural products: wound healing. Clin Dermatol 2009; 27:502-6.
2. Chanchal D, Swarniata S. Novel approaches in herbal cosmetics. J Cosmet Dermatol 2008;7:89-95.
3. Tiwari S, Geniot S, Gambhir IS. Centella Asiatica: a concise drug review with probable clinical uses. J Stress Physiol Biochem 2011;7: 39-42.
4. Hashim P, Sidek H, Helan MHM, et al. Triterpene composition and bioactivities of *Centella asiatica*. Molecules 2011;16:1310-22.
5. Pitella F, Dutra RC, Junior DD. Antioxidant and cytotoxic activities of *Centella asiatica* (L) Urb. Int J Mol Sci 2009;10:3713–21.
6. Paocharoen V. The efficacy and side effects of oral *Centellla asiatica* extract for wound healing promotion in diabetic wound patients. J Med Assoc Thai 2010;93:S166–9.
7. Zainol NA, Voo SC, Sarmidi MR, et al. Profiling of *Centella asiatica* (L.) Urban extract. Malaysian J Anal Sci 2008;12;322-7.
8. Gohil KJ, Patel JA, Gajjar AK. Pharmacological review on *Centella asiatica*: a potential herbal cure-all. Indian J Pharm Sci 2010;72:546-56.
9. Bhavna D, Jyoti K. *Centella asiatica*: the elixir of life. IJRAP 2011;2:431-8.
10. Lu L, Ying K, Wei S, et al. Asiaticoside induction for cell-cycle progression, proliferation and collagen synthesis in human dermal fibroblasts. Int J Dermatol 2004;43:801-7.
11. Loc NH, Tam An NT. Asiaticoside production from Centella (*Centella asiatica* L. Urban) cell culture. Biotechnol Bioprocess Engineering 2010;15:1065–70.
12. Lintner K, Chamberlin CM, Modun P, et al. Cosmeceuticals and active ingredients. Clin Dermatol 2009;27:461-8.
13. Hadi RS, Kusuma I, Sandra Y. Allogenic human dermal fibroblast are viable in peripheral blood mononuclear co-culture. Univ Med 2014;33:34-42.
14. Jurzak M, Latocha M, Goiniczek K. Influence of retinoids on skin fibroblasts metabolism in vitro. Acta Pol Pharm Drug Res 2008;1:85-95.
15. Gimeno A, Zarogoza R, Vivosese I. Retinol at concentration greater than the physiological limit, induces oxidative stress and apoptosis in human dermal fibroblast. Exp Dermatol 2004; 13:45-54.
16. Wu F, Bian D, Xia Y, et al. Identification of major active ingredients responsible for burn wound healing of *Centella asiatica* herbs. J Evid Based Complement Altern Med 2012. Article ID 848093, 13 pages. doi:10.1155/2012/848093.
17. Pareda MDCV, Dieamant GDC, Eberlin S, et al. Effect of green Coffea Arabica L. seed oil on extracellular matrix components and water-channel expression in *in vitro* and *ex vivo* human skin models. J Cosmetic Dermatol 2009;8:58-62.
18. Song J, Day Y, Bian D, et al. Madecassoside induce apoptosis of keloid fibroblast via a mitochondria–dependent pathway. Drug Dev Res 2011;72:315-22.
19. Ju-lin X, Shao-hai Q, Tian-zeng L, et al. Effect of asiaticoside on hypertrophic scar in the rabbit ear model. J Cutan Pathol 2009;36:234-9.
20. Kaji R, Kwak HSR, Schumacker WE, et al. Improvement of naturally aged skin with vitamin A (retinol). Arch Dermatol 2007;143: 606-12.