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Geraniin supplementation increases human keratinocyte proliferation in serum-free culture

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ABSTRACT

BACKGROUND
Various products used in cellular therapy utilize tissue culture techniques requiring keratinocyte culture. An efficient and clinically acceptable keratinocyte culture system requires supplements with mitogenic activity. Geraniin is a phytochemical with the potential as a supplement for expansion culture of keratinocytes. The objective of the present study was to verify the mitogenic activity of geraniin on human keratinocytes.

METHODS
This was an experimental study using two samples of human foreskin obtained by circumcision of a male child. Epidermal keratinocytes were isolated from the foreskin samples and were divided into paired groups, comprising intervention and control groups. The intervention groups were cultured with geraniin supplementation, whereas the control groups with standard supplements, without the addition of geraniin. Mitochondrial activity of the cells was evaluated by means of the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium-bromide (MTT) proliferation assay. Absorbance values in each of the groups was measured at 450 nm. Data analysis was performed with the paired t-test.

RESULTS
Geraniin supplementation significantly increased the keratinocyte proliferation rates at dosages of 0.8 to 3.1 µM. An increase of 57% in the proliferation rate was obtained at a dosage of 1.6 µM, while at a dosage of 12.5 µM toxic effects were starting to appear. Geraniin presumably causes increased cellular energy status, resulting in increased proliferation rates.

CONCLUSION
The findings in this study provide evidence in support of the utilization of geraniin as a supplement for expansion culture of keratinocytes. Further studies may presumably identify the molecules acting as geraniin receptors and the intracellular mechanisms underlying the increase in proliferation rates.

Key words: Keratinocyte, geraniin, foreskin, proliferation

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Suplementasi geraniin meningkatkan proliferasi kultur keratinosit manusia bebas serum

ABSTRAK

LATAR BELAKANG

Beberapa produk yang digunakan dalam terapi sel menggunakan tehnik rekayasa jaringan yang membutuhkan kultur keratinosit. Sistem kultur keratinosit yang efisien dan dapat diterima oleh klinisi membutuhkan suplemen yang bersifat mitogenik. Geraniin adalah suatu zat fitokimia yang memiliki potensi sebagai suplemen kultur ekspansi keratinosit. Penelitian ini bertujuan untuk melakukan verifikasi aktivitas mitogenik geraniin terhadap keratinosit manusia.

METODE

Penelitian ini menggunakan desain eksperimental. Dua sampel kulit preputium manusia diperoleh dari prosedur sirkumsisi pada anak laki-laki. Keratinosit epidermal diisolasi dari kulit preputium dan dikultur dengan suplemen berupa epidermal growth factor *dan* bovine pitutary extract*. Keratinosit dibagi dalam kelompok berpasangan. Kelompok perlakuan dikultur dengan tambahan suplemen geraniin, sedangkan kelompok kontrol dikultur dengan suplemen standar tanpa penambahan geraniin. Aktivitas mitokondria sel dievaluasi dengan uji proliferasi* 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium-bromide *(MTT). Nilai absorbansi dari setiap kelompok diukur pada panjang gelombang 450 nm. Analisis dilakukan menggunakan uji t pasangan.*

HASIL

Suplementasi geraniin menunjukkan peningkatan proliferasi keratinosit secara signifikan pada dosis antara 0,8 sampai 3,1 µM. Peningkatan proliferasi sebesar 57% dicapai pada dosis 1,6 µM sementara efek toksik mulai terlihat pada dosis 12,5 µM. Geraniin menyebabkan peningkatan status energi yang mengakibatkan laju proliferasi sel yang lebih tinggi.

KESIMPULAN

Temuan ini mendukung penggunaan geraniin sebagai suplemen kultur keratinosit untuk tujuan ekspansi. Penelitian selanjutnya diharapkan dapat mengidentifikasi molekul yang berperan sebagai reseptor untuk geraniin dan mekanisme intraseluler yang mendasari peningkatan laju proliferasi sel.

Kata kunci: *Keratinosit, geraniin, preputium, proliferasi*

INTRODUCTION

Human keratinocyte culture systems for the production of various epithelial cells initially required mouse fibroblast feeder layers and serum-containing media. Serum-free culture without feeder layer was later described as using a collagen coating and mitogen-rich supplementation such as epidermal growth factor (EGF) and bovine pituitary extract (BPE).[1,2] The use of basic fibroblast growth factor (bFGF), embryonic stem cell-conditioned medium, bone morphogenetic factor-4 (BMP-4) and transcription factor Oct-4 was proved to be able to increase the total number of cells harvested and induce de-diferentiation, and was specifically directed to neuronal lineages.[3-5] Recently, keratinocytes were succesfully reprogrammed to become induced pluripotent stem cells (iPSCs).[6,7]

Keratinocytes were expanded to produce products such as cultured epithelial autografts (CEA) and bilayered human skin equivalents (HSE). Bioreactors were developed to increase culture efficiency.[8] However, the use of serum and animal products in the culture system would cause the products to become unqualified for human therapy. Therefore, the need to develop chemically-defined or plant-based mitogenic agents is increasing. Geraniin is a phytochemical isolated from *Phyllanthus* sp., also known as *meniran* in Indonesia. *Phyllanthus urinaria L*., locally known as *rumput pacar* by the Dayak Tunjung tribe of Kalimantan, was traditionally used as medication for wounds and skin rashes.[9] Studies on traditional herbs recognized that *Phyllanthus niruri* was used to alleviate symptoms of ascites, urinary tract infection, enteritis, conjunctivitis, hepatitis, jaundice, and aphtae.[10,11] The isolated geraniin demonstrated inhibition of tumor necrosis factor (TNF)-á release and stimulation of celullar activities, such as proliferation, collagen biosynthesis and formation of extracelullar matrix proteins.[12,13] These properties of geraniin suggest that it may be a potential mitogenic agent for keratinocytes. A recent study by Agyare et al.[12] shows that geraniin isolated from *Phyllanthus muellerianus* was able to increase proliferation of HaCaT cells, a human keratinocyte cell line. Agyare et al.[12] did not use a normal human epidermal keratinocyte culture in their study. However, they revealed an increase in collagen production in dermal fibroblast culture.

The present study was designed to evaluate the ability of geraniin to increase human keratinocyte proliferation rates in culture. This study focused on keratinocytes that are readily used for cellular therapy or as a component to construct the epidermal layer of HSE.

METHODS

Research design

The present study using an experimental design was conducted from February to August 2012.

Cell culture

Cell culture and proliferation assays were conducted at the Kimia Farma Cell Culture Laboratory, Jakarta. Two human foreskin samples were obtained after a circumcision procedure conducted at Indonesia House of Circumcision (IHC) in accordance with the manual provided by WHO.[14] Keratinocytes, unless otherwise stated, were cultured in Epilife medium supplemented with human keratinocyte growth supplement (HKGS, Gibco; Invitrogen) which contains BPE, human epidermal growth factor, hydrocortisone, insulin and transferrin. Antibiotics and antimycotics used were a combination of penicillin-streptomycin (Sigma) and fungizone (Sigma). This supplementation will be referred to in this paper as standard supplementation.

Sample preparation

The keratinocytes used in this study were derived from foreskins collected after circumcision procedures conducted on two children (6 and 8 years old). To obtain the proper number of cells, we performed keratinocyte expansion culture (Figure 1). Keratinocytes were subcultured 7 times, with 5 times population doubling at the third passage.

Collected samples were transferred to 10 ml Epilife medium (Gibco; Invitrogen) to which 20 ug/ml gentamycin (Sigma) and standard antibiotic-antimycotic combination[1] were added, and then transported at 2-8^{o}C to the laboratory. Samples were refrigerated for 60 minutes before decontamination using povidone iodine and 70% ethyl alcohol (EtOH).[12,15]

Samples were then rinsed using phosphate buffered saline (PBS, Sigma). Subcutaneous fat and blood clots were dissected away leaving the dermis and epidermis. The skin was then cut into strips and placed in a 60 mm dish containing 1 U/ml dispase II (Stem Cell Technologies) for overnight incubation at 2-8^{o}C.[1,6] Digested skin samples were placed in a dry 100 mm sterile glass petri dish cover. The epidermis was then peeled off and folded using a pair of fine

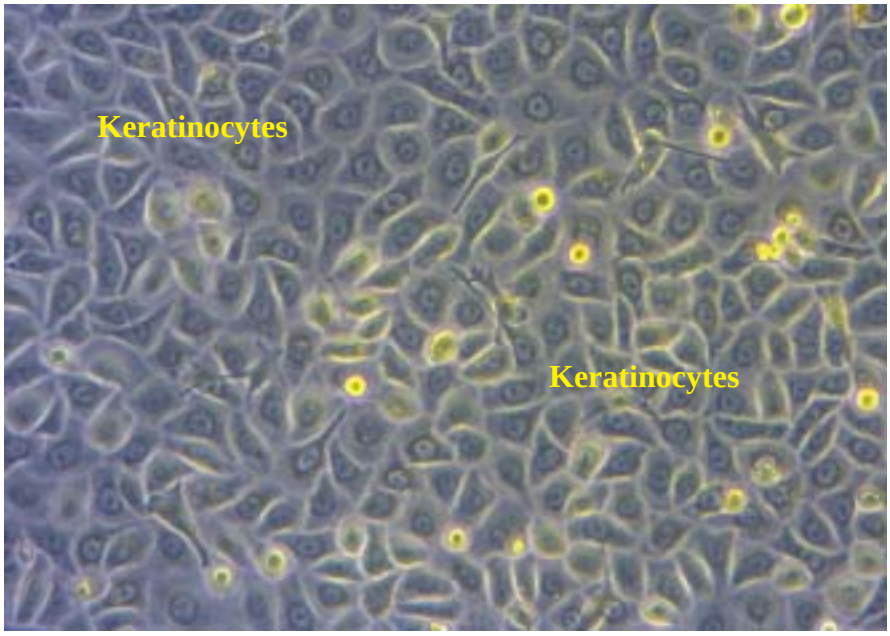

Figure 1. Primary keratinocytes for expansion culture
(Cobblestone morphology, 80-90% confluent, DIV 1, 20x Axiocam; DIV: day in vitro)

tweezers. The recovered epidermis was cut into smaller pieces and placed in a 15 ml tube containing 5 ml of 0.1% trypsin/EDTA (Sigma) for a 30-minute incubation at 37° C.[15]

Trypsin/EDTA was deactivated using equal an amount of fetal bovine serum (FBS, Sigma). The cell suspension was then triturated using a 10-ml serological pipette and passed through a 70–µm cell strainer (Cascade Biologix) into a 50-ml tube.[6,15] The collected cell suspension was then centrifuged at 1500 rpm for 7 minutes. The pellet was resuspended using pre-warmed Epilife medium supplemented with HKGS (Gibco; Invitrogen). Resuspended cells were then seeded (at $1x10^5$ viable cells/sqcm) on 60-mm culture dishes precoated with 10 ug/sqcm collagen type IV.[16] The primary culture (passage 0;P0) was incubated at 37° C with 5% CO_2 and the medium was changed after 24 hours and then every other day. The culture reached 80-90% confluency in 7 days and was subcultured in a 1:3 split ratio.

Geraniin isolated from *Phyllanthus urinaria* to 98% purity by HPLC was obtained from Biopurify for use in this study. The agent was dissolved in 98% EtOH prior to usage. The presence of EtOH corresponding to the amount used in high dose geraniin experiments was evaluated. The experiment showed no conclusive evidence of toxicity. Therefore, the use of EtOH presumably does not affect keratinocyte viability. Other studies using agents dissolved in 98% EtOH also showed no significant functional interference.[17] 20 mg of geraniin was dissolved in 98% EtOH and kept at -20° C before use. The keratinocyte culture groups for experimentation were seeded on a 96-well plate overnight. Geraniin solutions titrated for dose ranging were mixed with pre-warmed Epilife medium to replace the spent culture medium. Keratinocytes were divided into 6 distinct groups with various dosages of geraniin supplementation. These groups were the positive control group, negative control group, and the four geraniin groups (at dosages of 0.8 ìM, 1.6 ì M, 3.12 ì M and 6.25 ì M, respectively). The negative control group was supplemented with 25 µM geraniin which is well above the geraniin toxic dose. The positive control group comprised keratinocytes cultured without geraniin.

Cell viability

Keratinocytes seeded on 60-mm culture dishes were subcultured by trypsinization and enumerated using a hemocytometer. Subcultures were performed when the cultures reached 80% confluence. Viable and non-viable cells from each subculture were identified using the trypan blue exclusion method. Population doublings were calculated using:

$$n=3.32 (\log UCY-\log I) + X$$

(n= population doubling, UCY= cell yield, I= inoculum numbers, X=population doubling rate of inoculum)

Proliferation assay

Keratinocyte groups were incubated for 48 hours, then mitochondrial activity was assesed in triplicate by colorimetric methods using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium-bromide (MTT) assay (Biovision) on a 96-well culture plate. A microplate reader with 450 nm filter was used to read the absorbance. Cellular dehydrogenase activity (MTT assay) was used to quantify cell proliferation.

Data Analysis

Data analysis was performed with Excel (version 2007; Microsoft). Statistical significance was determined using the paired Student t-test.

Ethical Clearance

Ethical clearance was obtained from the Ethical Commission of YARSI University. Informed consent was obtained from the patients' parents

RESULTS

This study showed an increase in cell proliferation in a dose-dependent manner (Figure 2). At a dosage of 1.6 µM there was a 57% increase in proliferation, compared to standard keratinocyte culture supplementation. Optimal stimulation was achieved at dosages of 0.8 µM to 3.1 µM. Geraniin supplementation clearly increased the keratinocyte proliferation rate. Lower doses of geraniin did not increase the keratinocyte proliferation rate significantly.

This study also revealed that geraniin supplementation started to reduce proliferation at 12.5 µM. To clarify the effect of high dose

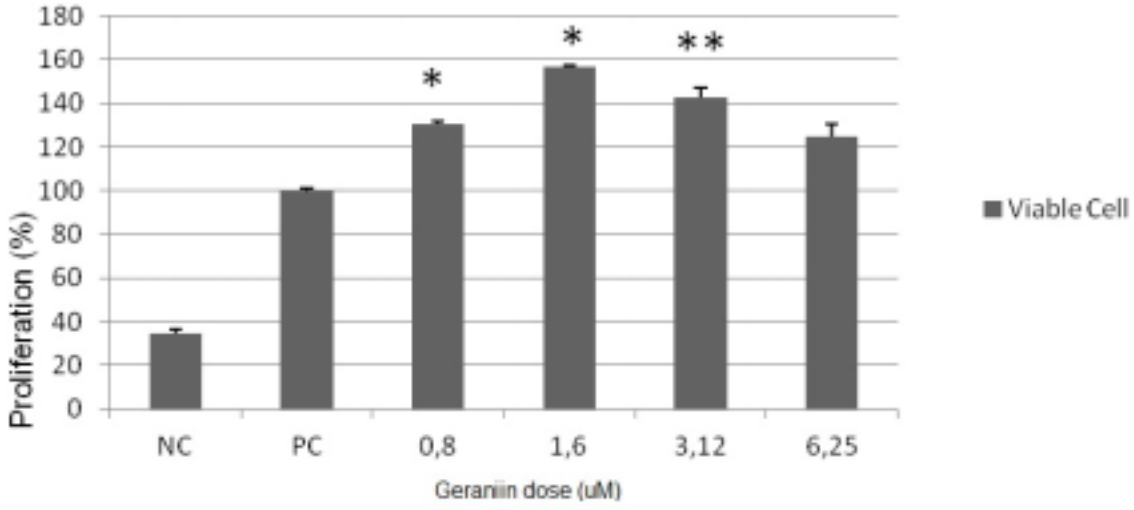

Figure 2. Geraniin supplemented keratinocyte culture

NC=negative controls; PC= positive controls; N=2 keratinocyte cultures for each data set. Error bars represent SD. Statistical significance *p<0.01, **p<0.05

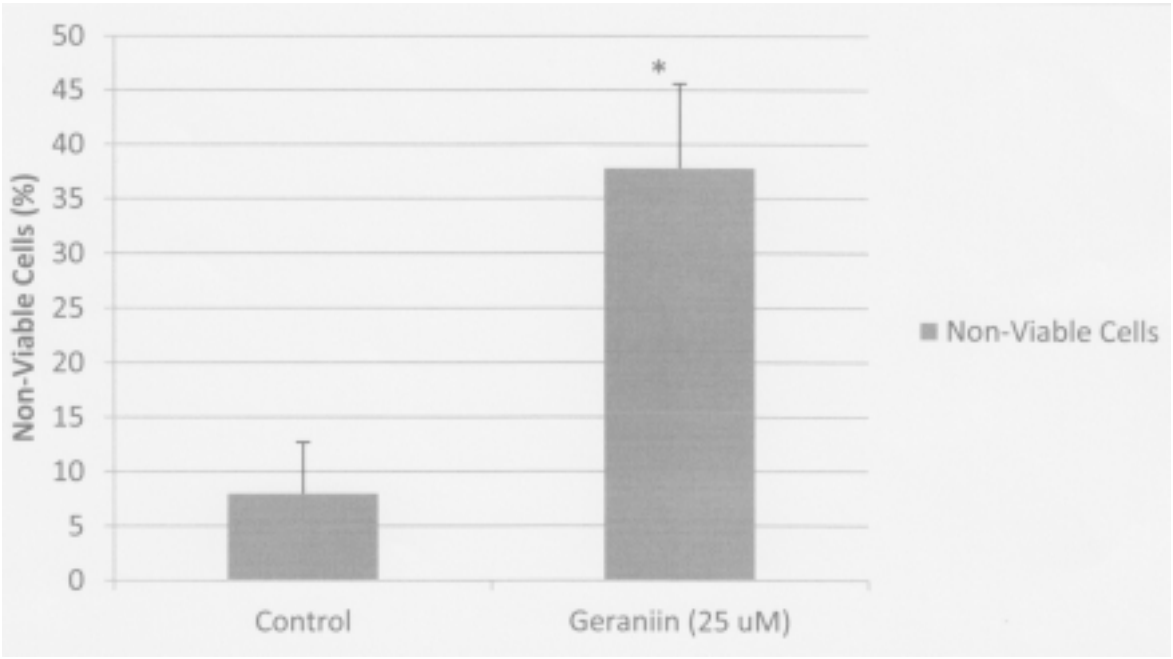

Figure 3. Non-viable cells during routine subculture

Significant increase of non-viable cell number were observed at geraniin-supplemented flasks N=2 keratinocytes culture for each data set. Statistical significance *p<0.01

geraniin supplementation, we evaluated cell viability data from routine keratinocyte subcultures. These data showed a quadruple number (32 ± 8.5; p<0.01) of non-viable cells recorded from subculture of flasks with 25 µM geraniin supplementation, compared to routine subcultures (7.7 ± 4.6) of non-geraniin supplemented flasks. This finding confirms that 25 µM geraniin is toxic to keratinocyte cultures (Figure 3).

DISCUSSION

Our findings reveal that 1.6 µM of geraniin is able to increase the keratinocyte proliferation rate by 57%. A similar study showed that geraniin could increase the proliferation rate of HaCaT at a dose of 20 to 150 µM and induce celullar necrosis at >150 µM. Increase in mitochondrial activity reflects increase of celullar energy status, which triggers the cell toward a higher proliferation rate.[12] These differences in proliferation rate and toxic dose could be attributed to differences in the purity of the geraniim used in these experiments. Agyare et. al. used 5.5% geraniin derived from an aqueous extract of *Phyllanthus muellerianus* leaves.[12] The present study uses geraniin derived from *Phyllanthus urinaria* with a purity of >98% as determined by HPLC.

Okabe et al.[13] showed that geraniin demonstrates inhibition of TNF-á release. TNF-á is a proinflammatory cytokine that is produced by keratinocytes and is increased after UVB irradiation. Recent findings show that TNF-á is able to diminish DNA repair and cause cell cycle arrest.[18] These findings are in support of the ability of geraniin to increase cell viability by maintaining DNA repair during periods of mitotic activity.

The considerably lower population doubling in this study could be attributed to the age of the skin sources. Most experiments use keratinoytes derived from neonatal foreskins, which are able to achieve 12-50 population doublings in 4 to 15 passages.[1,19,20] Older sources of skin samples would be reflected by lower population doublings and earlier senescence. This study was

conducted using high passage keratinocytes (P5-6); therefore, the cells have undergone a certain extent of differentiation. In spite of this, geraniin supplementation was able to significantly increase the proliferation rate.

This study was limited by the number of samples obtained. Genetic differences in the population may cause different responses of keratinocytes toward geraniin supplementation. This study was also limited by the presence of standard keratinocyte supplementation containing EGF and BPE, both of which are potent mitogenic agents and are absolutely required to enable initial plating out of the keratinocytes.

This study demonstrated the use of phyto-based supplementation in cell culture systems, which are important in cell therapy and tissue engineering for the medical industry. These findings would prompt researchers in herbal study to look for other potential candidates that can be used not directly as therapeutical agent, but as a component in regenerative medicine industry.

CONCLUSION

This study concludes that geraniin can be used as supplementation for keratinocyte expansion culture within optimal doses. Further study should be directed to elucidating celullar mechanisms interacting with geraniin. Research could also be directed to verify the effects of geraniin as a substitute for EGF or BPE in keratinocyte supplementation.

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