Journal of Ethnopharmacology 140 (2012) 333–338

Contents lists available at SciVerse ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Aqueous extract of *Centella asiatica* promotes corneal epithelium wound healing *in vitro*

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ARTICLE INFO

Article history:
Received 6 September 2011
Received in revised form 6 January 2012
Accepted 13 January 2012
Available online 24 January 2012

Keywords:
Centella asiatica
Corneal epithelial cell
Corneal wound healing
CK12
Connexin 43

ABSTRACT

Ethnopharmacological relevance: Centella asiatica is a traditional herbal medicine that has been shown to have pharmacological effect on skin wound healing, and could be potential therapeutic agent for corneal epithelial wound healing.
Aim of the study: This study was done to evaluate the effects of *Centella asiatica* on the proliferation and migration of rabbit corneal epithelial (RCE) cells in the *in vitro* wound healing model.
Materials and methods: RCE cells were cultured with or without supplementation of *Centella asiatica* aqueous extract. Viability and proliferation of the RCE cells was determined by MTT assay and cell cycle was analyzed by flow cytometry. *In vitro* re-epithelization was studied by scratch assay and migration rate was evaluated quantitatively by image analyzer. Expression of corneal specific differentiation markers, CK12 and connexin 43, were studied via RT-PCR.
Results: It was found that supplementation of *Centella asiatica* did not show any significant effect on the RCE cells proliferation at the concentration up to 500 ppm, while at the concentration of 1000 ppm significantly inhibited RCE cells proliferation ($p < 0.05$). However, at the concentration up to 62.5 ppm, RCE cells shows significant enhancement of migration rate compared to the control group ($p < 0.05$). It was also found that the supplementation of *Centella asiatica* aqueous extract did not alter the expression of differentiation markers and cell cycle.
Conclusion: In conclusion, supplementation of *Centella asiatica* aqueous extract at low concentrations could be useful to promote corneal epithelium wound healing.

1. Introduction

Centella asiatica (L.) Urban is a medicinal plant. The therapeutic use of this herbal remedy with its wide range of applications has been well documented in South East Asia and India. The pharmacological effects of *Centella asiatica* has been reported on wounds healing of skin, oxidative stress, bronchitis, dysentery, leucorrhoea, kidney diseases, urethritis, atherosclerosis, venous hypertension, and cardio-protective function (Suguna et al., 1996; Jaganath and Ng, 1999; Shukla et al., 1999a; Jayashree et al., 2003; Gnanapragasam et al., 2004). It was also claimed to be useful in the treatment of inflammation, asthma, tuberculosis, leprosy, psoriasis, keloid and gastric ulcer (Cheng et al., 2004; Zheng and Qin, 2007).

The effectiveness of *Centella asiatica* in promoting wound healing of skin was studied extensively both *in vitro* and *in vivo*. Application of *Centella asiatica* extract shown to promote in incision type wounds and open wounds as represent by a greater collagen content and thickness of epithelium (Rosen et al., 1967; Rao et al., 1996). The extract of *Centella asiatica* especially from roots and leaves contain a high anti-oxidative activity, which was as good as α-tocopherol, a natural anti-oxidant, have been reported to play

Abbreviations: RCE, rabbit corneal epithelial; MTT, 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide; CK12, cytokeratin 12; RT-PCR, reverse transcriptase-polymerase chain reaction; ppm, parts per million; NZW rabbit, New Zealand White rabbit; PBS, phosphate buffer saline; TE, trypsin-EDTA; EDTA, ethylene diaminetetra acetic acid; CM, corneal medium; FRIM, Forest Research Institute of Malaysia; P1, passage 1; ELISA, Enzyme Linked Immuno Sorvent Assay; HCl, hydrochloric acid; RNA, ribonucleic acid; RNase, ribonuclease; DNase, deoxyribonuclease; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PI, propidium iodide.

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doi:10.1016/j.jep.2012.01.023

a significant role in wound healing (Zainol et al., 2003). *Centella asiatica* extract contains four principle bioactive compounds – asiatic acids, madecassic acid, asiaticoside and madecassoside (Inamdar et al., 1996; Zheng and Qin, 2007), in which asiaticoside was identified as the main active constituent responsible for wound healing (Shukla et al., 1999a). Topical application of asiaticoside in normal as well as diabetic rats significantly enhanced the rate of skin wound healing, which was attributed to increase collagen synthesis and angiogenesis (Shukla et al., 1999a). Moreover, topical application of asiaticoside to excision-type cutaneous wounds in rats led to increase of enzymatic and non-enzymatic tissue antioxidants levels at the initial stage of healing (Shukla et al., 1999b). Considering the potential effect of *Centella asiatica* extracts on skin re-epithelialization, this extract could be a possible candidate for treating corneal epithelial wound.

Corneal epithelial wound healing is an important process for maintaining the homeostasis of the cornea. Corneal transparency, which provides appropriate optical refraction are dependent on the ability of the epithelial layer to undergo continuous renewal and on the endothelial fluid transport activity to maintain stromal thinness. Due to the cornea's unique location at the outermost surface of the eye globe, it can be damaged from ultraviolet light exposure, by physical wounding, and bacterial or fungal infections. The important steps of corneal epithelial wound healing are cell proliferation from the healthy area and cell migration to cover the damaged area, and form epithelial intercellular junctions to restore corneal epithelial integrity (Imanishi et al., 2000; Zelenka and Arpitha, 2008). Herbal components from *Pothomorphe umbellata* ethanolic crude extract and Chinese herbal medicine component, emodin have been reported to possess therapeutic value for corneal wound healing (Barros et al., 2007; Kitano et al., 2007). In addition, exogenous application of natural anti-oxidant such as ascorbic acid and vitamin E analog (Trolox) shown to improve the corneal wound healing activity in animal model by re-epithelialization in the wounded area (Hallberg et al., 1996). However, almost no scientific information is available for the effect of *Centella asiatica* extract on corneal epithelial cells. Therefore, the present study was designed to evaluate the effect of an aqueous extract of *Centella asiatica* on proliferation and migration of rabbit corneal epithelial (RCE) cells which corresponded to *in vitro* re-epithelization model. Moreover, RCE cells were characterized for expression of differentiation markers and cell cycle to evaluate any adverse effect on growth and differentiation.

2. Materials and methods

2.1. Isolation and culture of rabbit corneal epithelial (RCE) cells from rabbit corneal tissue

Isolation of corneal epithelial cells was performed according to the protocol described by Wei et al. (1996) with some modification. Corneas from sacrificed New Zealand White (NZW) rabbits were excised and washed three times with phosphate buffer saline (PBS; Gibco, Grand Island, NY, USA). Connective tissues, extra-ocular muscle, sclera and iris, and endothelium side of the cornea were removed from the corneal tissues. The remaining corneal tissue was rinsed with PBS and then digested in dispase solution (1.2 μU/ml; Sigma–Aldrich, USA) at 20 °C for 18 h to separate epithelial and keratocytes layers. The corneas were placed upside down (with the concave surface touching the dispase solution).

The epithelial cells were separated from stromal keratocytes on a petri dish containing 5 ml of trypsin-EDTA (TE; Gibco/BRL) using dissecting microscope. Suspended epithelial cells then transferred into a centrifuge tube and placed in incubator shaker for 5 min, followed by addition of 5 ml trypsin inhibitor (Gibco/BRL) to stop the activity of TE. The cell suspension was then centrifuged at 7000 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in Corneal Medium, CM (Epilife basal medium supplemented with Ca^{2+}, human corneal growth supplement and antibiotic–antimycotic Cascade Biologics, Gibco). The numbers of viable cells were counted using trypan blue dye (Gibco/BRL) in hemocytometer. Suspended cells were then plated in 6-well culture plate (Becton Dickinson, NJ, USA) at the density of 2×10^5 viable cells per well. Corneal epithelial cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C with medium changed every two days. The culture was continued until reaching 80% confluency. RCE cells were detached from the culture surface using TE, and seeded at a density of 5×10^3 cells/cm^2 and incubated in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2. Preparation of Centella asiatica (CA) aqueous extract

Fresh leaves of *Centella asiatica* were purchased from the wet market in Kepong, Selangor, Malaysia. The plant material was identified and deposited at the Medicinal Plant Division, Forest Research Institute of Malaysia (FRIM; voucher specimen no. FRI50032). The aqueous extract of *Centella asiatica* was prepared by method described by Flora and Gupta (2007). The leaves were sun-dried and grounded into a powder. Powdered samples (250 g) were refluxed with 1.5 l of distilled water at ratio 1:6 for 3 h at temperature approximately 40 °C. The extracts were left to cool at room temperature before it was filtered using Whatman filter paper. The extract was concentrated on magnetic stirrer hotplate until it became half of the initial volume. The extract was freeze dried to remove the solvent and the dried extract was stored at 4 °C until further use.

2.3. MTT assay

For quantitative evaluation of cell viability and proliferation, MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide; Sigma-Aldrich) assay was used, in which only viable cells can reduce MTT to insoluble purple formazan. Thus, the intensity of purple color in turns represents the number of viable cells. The MTT assay was performed according to the manufacturer instructions. For MTT assay, passage 1 (P1) RCE cells were used. The cells were cultured in 96-well micro titer plate at a density of 5×10^3 cells per 100 μl CM for 48 h. Then the medium was changed to fresh CM (control culture) and CM supplemented with different concentrations of *Centella asiatica* that includes 7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1000 parts per million (ppm). RCE cells were then incubated in a humidified atmosphere of 5% CO_2 at 37 °C, and medium was change every two days (where applicable). MTT assay was performed on 1st, 4th and 7th day post-treatment. To evaluate the number of viable cells, 100 μl of MTT solution was added into each well and incubated for 4 h at 37 °C in dark. The formazan crystals that formed by living cells were solubilized with 100 μl MTT solvent (0.1 N HCl in anhydrous isopropanol) and the absorbance was measured at 570 nm with background subtraction at 690 nm by ELISA reader.

2.4. Wound healing assay

To evaluate the migration of RCE cells, wound healing assay was performed by scratching the confluent culture, according to the method described elsewhere (Liang et al., 2007). RCE cells of P1 were plated into 6-well plate at the concentration of 5×10^3 cells/cm^2 in CM with medium changed every 2 days, and culture was continued until reaching approximately 80% confluency. The medium was discarded and a scratch was made using micropipette tip, followed by washing with PBS to remove cell debris resulted from the scratching. The cultures were then feed with CM supplemented with indicated amounts of *Centella asiatica* (7.8, 15.6, 31.2,

Table 1
The sequence of forward and reverse primers used for evaluating gene expression.

Gene	GenBank accession no.	Primers (5′–3′)	PCR product size (bp)
GAPDH	AB128158	F: atcactgccacccagaagac R: gtgagtttcccgttcagctc	146
CK12	X77665	F: accacgaggaggagctacaaa R: ggtgctgatctccttcctgag	203
Connexin 43	AY382590	F: gcctttcgttgtaacactcagc R: ggcaactttgagttcctcctct	198

62.5 and 125 ppm). The cells with CM without supplement were used as control. The images of the wound area were captured on day 0 (day of scratching), 1 and 2 using Olympus SC 35 camera connected to inverted microscope. The migration rate of RCE cells was calculated using Image Analyzer software by measuring the distance traveled by cells, and represents as average migration rate in μm/h. For each concentration of *Centella asiatica* and each time frame, two scratched areas were captured to get an average cell migration rate.

2.5. Gene expression analysis

For gene expression analysis, total RNA from cultured cells (P1 RCE cells cultured with or without supplementation of *Centella asiatica*) was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instruction. Polyacryl Carrier (Molecular Research Center) was added in each extraction to precipitate the total RNA, which was then washed with 70% ethanol and air-dried before dissolved in RNase and DNase free distilled water (Gibco). The yield and purity of the isolated RNA was determined by spectrophotometer. Purity value in between 1.8 and 2.0 was accepted for gene expression analysis. Total RNA was stored at −80 °C immediately after extraction. The expression of corneal epithelial cells markers cytokeratin 12 (CK12) and connexin 43 were studied with semi-quantitative RT-PCR as described elsewhere (Ruszymah et al., 2005). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as control. The specific sense and antisense primers (as shown in Table 1) were designed using the primer 3 software (http://frodo.wi.mit.edu/). RT-PCR reaction was performed with 200 ng of total RNA using one step iScriptTM RT-PCR kit (Bio-Rad, Hercules, CA, USA). Reactions profile for RT-PCR were follows: 30 min at 50 °C for reverse transcription; pre-denaturation for 2 min at 94 °C; PCR amplification for 38 cycles with 30 s at 94 °C, 20 s at 60 °C and 1 min at 72 °C. Subsequently PCR products were separated by electrophoresis on a 1.5% agarose gel (Gibco), and visualized by UV transillumination (Vilber Lourmat, Marne La Vallee, France).

2.6. Cell cycle analysis

RCE cells were processed using CycleTEST PLUS DNA Reagent Kit (Becton Dickinson) according to the manufacturer instruction. In brief, P1 RCE cells were cultured separately in CM with or without supplementation of CA (7.8, 15.6, 31.2 and 62.5 ppm). After reaching 100% confluency, cells were detached from the culture surface by trypsinization followed by centrifugation at 4500 rpm for 5 min. The pellet was then resuspended in 1 ml of the buffer solution. A total number of 5×10^5 cells per sample were used. Cells were then stained with propidium iodide (PI). PI-stained single nuclei suspensions were analyzed using FACS Calibur flowcytometers (Becton Dickinson) and raw data were collected using CELLQuest software (Becton Dickinson). A minimum of 5×10^4 cells was analyzed for one cycle of test for one sample and was repeated six times for each sample. Data analysis was performed using Modfit Cell Cycle Analysis Software (Verity House Software, Topsham, ME).

2.7. Statistical analysis

Values were presented as mean ± standard error of mean (SEM). The data were analyzed with paired *t*-test using SPSS version 12.0 and the difference was considered significant if $p < 0.05$.

3. Result

3.1. Effect of Centella asiatica on the proliferation of RCE cells

The proliferation of RCE cells was evaluated by MTT assay at 1, 4 and 7 days of post-treatment with different concentrations of *Centella asiatica* aqueous extract. As shown in Fig. 1, the number of viable RCE cells on day 1 were almost similar in all concentration of *Centella asiatica* extract (7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1000 ppm), which is comparable with control culture and not significantly different. It was also found that the increment of viable RCE cells with increasing culture time was almost similar in all culture conditions except for the culture supplemented with 1000 ppm of *Centella asiatica*. In the culture supplemented with 1000 ppm of *Centella asiatica* extract, the number of viable cells increases on day 4 (but comparatively lower than other culture conditions) and decreases significantly on day 7, giving the absorbance value of 0.113 ± 0.051, which is 78% lower than that in case of control culture. These results suggested that aqueous extract of *Centella asiatica* at a concentration of ≤500 ppm has no adverse effect on the viability and proliferative ability of RCE cells during *in vitro* culture. But at higher concentration of 1000 ppm of *Centella asiatica* shows anti-proliferative effect on cultured RCE cells. Considering the effect of *Centella asiatica* extract on RCE cells proliferation, the

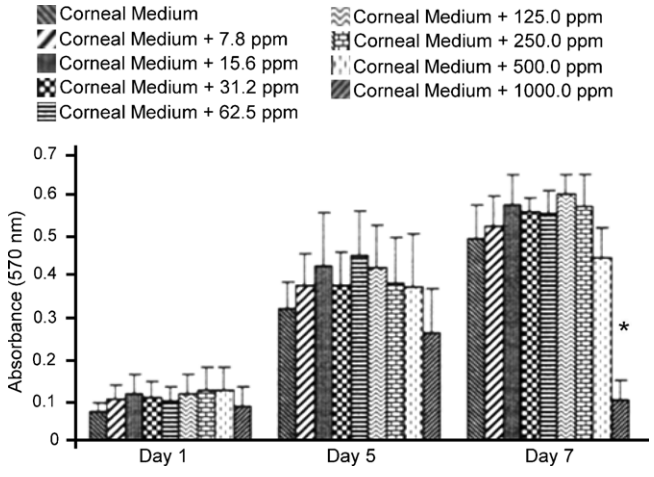

Fig. 1. Effect of *Centella asiatica* aqueous extract on RCE cells proliferation. RCE cells were cultured with various concentration of *Centella asiatica*, and number of viable cells was quantified by MTT assay for 1, 5 and 7 days. The results indicated that aqueous extract of *Centella asiatica* at a concentration of ≤500 ppm has no adverse effect on the viability and proliferative ability of RCE cells, while 1000 ppm of *Centella asiatica* shows anti-proliferative effect. All data were reported in mean ± SEM ($n = 6$). The asterisk (*) indicates significant difference ($p < 0.05$) in absorbance between RCE cells cultured at 1000 ppm of *Centella asiatica* and control culture.

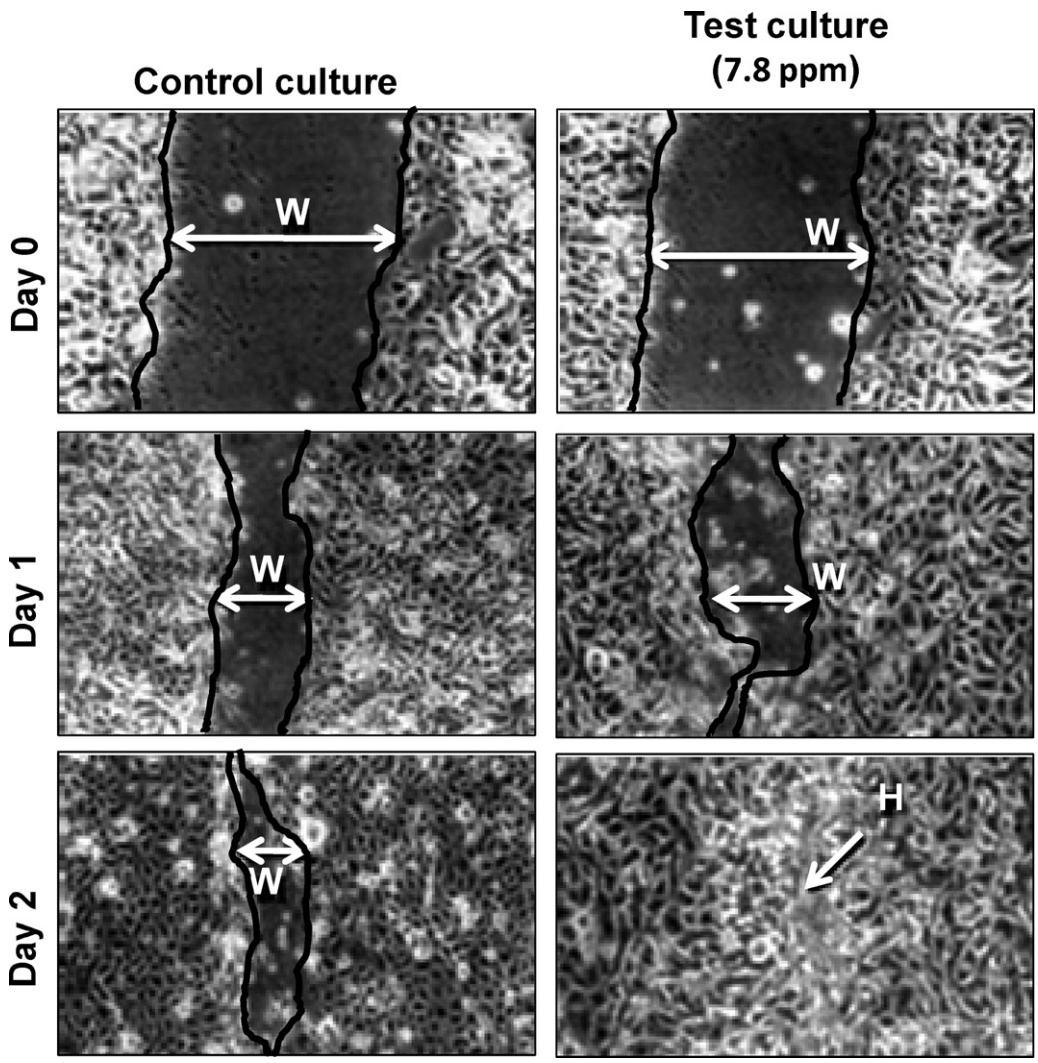

Fig. 2. Representative images of *in vitro* wound healing of RCE cells cultured in control and test (supplemented with 7.8 ppm of *Centella asiatica* aqueous extract) conditions. RCE cells migration was observed for 2 days after scratching confluent culture. The black lines show the margin of scratched area in which double headed arrow indicating scratch width (W) and white arrow was indicating complete healing of scratch wound (H). These images clearly shows that the migration was faster in test culture compared to control culture.

concentrations of 7.8–125 ppm of *Centella asiatica* were used for further experiments.

3.2. Corneal epithelial cell migration

In vitro migration of RCE cells was studied by wound healing assay in confluent culture. The migration rate of RCE cells were evaluated for 2 days after making wound in the cultures with supplementation of *Centella asiatica* (concentration of 7.8, 15.6, 31.2, 62.5 and 125 ppm) along with control group. Fig. 2 shows the representative images of the RCE cells migration in the cultures supplemented with 7.8 ppm of *Centella asiatica* extracts and control culture for day 0, 1 and 2 after making wound. It was found that the culture supplemented with *Centella asiatica* extracts, RCE cells migrate faster and recovery of wound area was completed within 2 days, whereas part of wound area was still visible in the control culture. As shown in Fig. 3, the migration rate of RCE cells in the culture supplemented with *Centella asiatica* extract at concentrations of 7.8 ppm (0.180 ± 0.015 μm/h), 15.6 ppm (0.150 ± 0.009 μm/h), 31.2 ppm (0.130 ± 0.019 μm/h) and 62.5 ppm (0.180 ± 0.031 μm/h) were significantly higher compared to control cultures (0.060 ± 0.025 μm/h). However, the migration rate of RCE cells decreased drastically in the culture supplemented with 125 ppm (0.060 ± 0.006 μm/h) of *Centella asiatica* extract, which is almost similar with the migration rate of control culture. These results indicated that *Centella asiatica* at lower concentration has significant effect on the migration rate of RCE cells *in vitro*, which will be advantageous for the re-epithelization during corneal wound healing.

3.3. Effect of C. asiatica extract on the gene expression of RCE cells

The expression of two corneal specific markers of CK12 and connexin 43 was analyzed by RT-PCR. Fig. 4A–C show the expression GAPDH, CK12 and connexin 43, respectively. It was found that expression of CK12 and connexin 43 was almost similar in the control culture of RCE cells and the culture supplemented with *Centella asiatica* extract of 7.8, 15.6, 31.2 and 62.5 ppm. This indicated that the addition of *Centella asiatica* does not affect the expression of corneal specific differentiation gene markers.

3.4. Effect of C. asiatica on the cell cycle of RCE cells

The cell cycle properties of RCE cells were analyzed in the culture with or without supplementation of *Centella asiatica*. DNA histograms shown in Fig. 5 were generated from P1 RCE cells cultured

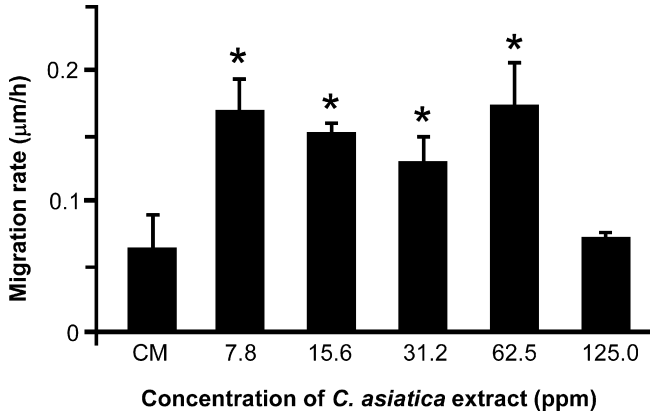

Fig. 3. Migration rate of RCE cells cultured in control and test conditions. Migration rate was evaluated using image analyzer software by measuring the distance traveled by cells, and represents as average migration rate in μm/h. The data indicated that *Centella asiatica* at lower concentration has significant effect on the migration rate of RCE cells. All the data were reported in mean ± SEM ($n = 6$). The asterisk (*) indicates the significantly higher migration rate in test culture compared to control group ($p < 0.05$).

in control condition and supplementation with 7.8 ppm of *Centella asiatica* aqueous extract, and same cell cycle pattern was seen for both culture conditions. It was also found that no ratio of aneuploidy or tetraploidy was detected in both histograms, indicating cells are all in diploid state (100% for both cells). The same results

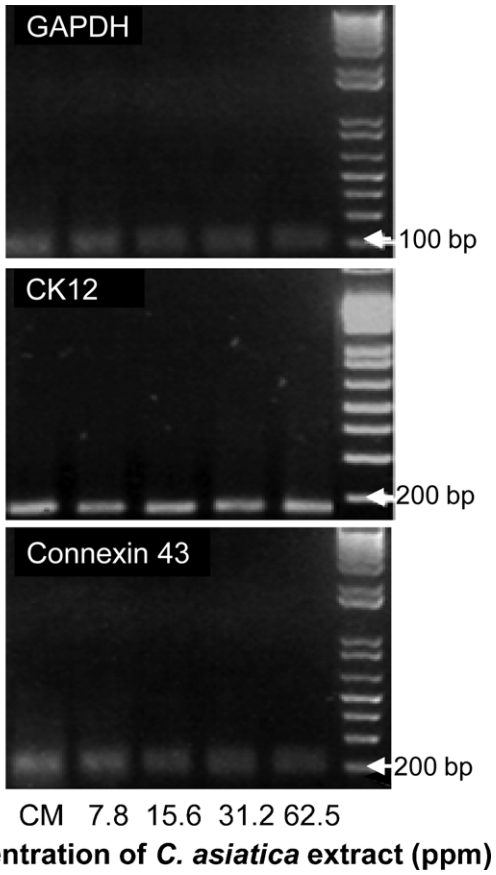

Fig. 4. Gene expression of CK12 and connexin 43 for RCE cells cultured in control and test conditions performed by RT-PCR. Expression of GAPDH was used as control for gene expression. The results indicated that the addition of *Centella asiatica* does not affect the expression of corneal specific differentiation gene markers.

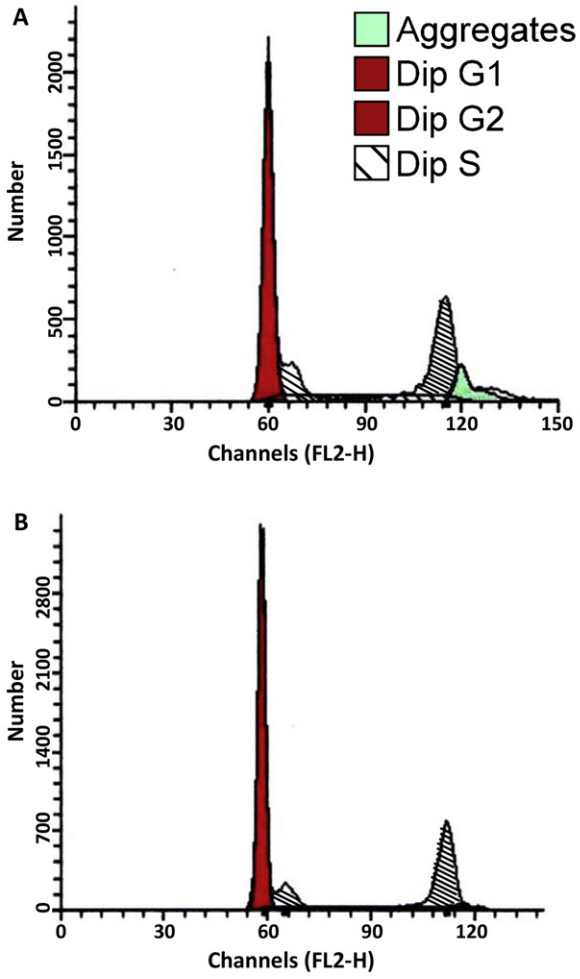

Fig. 5. DNA histogram generated from RCE cells cultured in control (A) and supplemented with 7.8 ppm aqueous extract of *Centella asiatica* (B) showing the percentages of G0–G1, S, G2 and M phases. *X*-axis represents the relative fluorescence intensity proportional to DNA content. The same results were also seen for *Centella asiatica* aqueous extract of 15.6 and 31.2 ppm (not shown), indicating that the addition of *Centella asiatica* does not affect the cell cycle of RCE cells in culture.

were also seen for *Centella asiatica* aqueous extract of 15.6 and 31.2 ppm (data not shown).

4. Discussion

There are several types of corneal wounds such as superficial scrape, alkaline burn, infection (Dayhaw-Barker, 1995a,b), and anterior keratotomy (Sheardown and Cheng, 1996). Generally, corneal injury initiates the healing process, which is characterized into three phase's namely epithelial cell migration, proliferation, and differentiation (Dua et al., 1994). Immediately after wounding, the neighboring intact epithelial cells begin to migrate actively over the affected area until they cover the defect with a layer of cells. Then the cells begin to proliferate to increase cell number that results restoration of the normal epithelial thickness. A few weeks after wounding, the epithelial cells begin to differentiate, the surface of the healed region becomes smooth, and a well-layered structure is restored (Dua et al., 1994). In this study, effect of *Centella asiatica* aqueous extract was studied on the *in vitro* migration and proliferation of RCE cells to understand the potential therapeutic use of this extract in corneal wound healing.

Even though the migration and proliferation of epithelial cells function independently during re-epithelialization of corneal

wound healing, the two processes complement each other. In this study, it was found that supplementation of *Centella asiatica* aqueous extract at lower concentration, ranging from 7.8 to 62.5 ppm; significantly enhance the migration of RCE cells after wounding (Fig. 3). Previous study in Wistar albino rat models shows that ethanolic extract of *Centella asiatica* promote faster epithelialization of skin wounds by promoting the migration of surrounding cells (Shetty et al., 2006). It was also found that asiaticoside, one of active component in the *Centella asiatica* extract, promote skin epithelialization in normal and diabetic animals (Shukla et al., 1999a).

In contrast to migration, supplementation of *Centella asiatica* aqueous extract does not show any significant improvement in the proliferation of RCE cells *in vitro* at the concentration up to 500 ppm of *Centella asiatica*. Besides, supplementation of *Centella asiatica* aqueous extract at high concentration of 1000 ppm show anti-proliferative effect on the RCE cells at day 7 (Fig. 1). Similar anti-proliferative effect of *Centella asiatica* extract towards *in vitro* skin epithelial cells, keratinocytes, has been reported by Sampson et al. (2001), and suggested that this may be attributed by two terpenoids found in extract namely asiaticoside and madecassoside. Besides, *Centella asiatica* extract has shown cytotoxicity towards Ehrlich and Dalton's lymphoma ascites tumour cells as well as various transformed cell lines (Babu et al., 1995), fibroblasts (Coldren et al., 2003) and SW480 human colon cancer cells (Tang et al., 2009).Cell–cell adhesion is also an important factor in corneal epithelial wound healing. Connexin 43 is one of the gap junction proteins identified in the corneal epithelium. It was reported that in the intact rabbit corneal epithelium, connexin 43 was abundantly expressed in the proliferative basal cell layer but not in the limbal stem cells, suggested that connexin 43 may represent a critical differentiation marker for corneal epithelial cells (Matic et al., 1997). It was found that the supplementation of *Centella asiatica* extract does not affect the expression of connexin 43 in RCE cells, suggesting that the differentiation properties of RCE cells were maintained by the extracts.

The intermediate filament components cytokeratin 12 (CK12) had showed that its expression was corneal epithelial cell specific, differentiation dependent, and developmentally regulated, suggesting the role of CK12 in maintaining the normal functions of corneal epithelium (Lu et al., 2001). In CK12-deficient animals, the corneal epithelial layer becomes thinner and fragile, and corneal epithelial cells are not able to adhere firmly onto the corneal surface (Kao et al., 1996). Supplementation of *Centella asiatica* extract maintains the expression of CK12 in RCE cells, indicating no adverse effect on the differentiation of corneal epithelial cells during wound healing.

5. Conclusion

In conclusion, *Centella asiatica* did not give any significant effect on the RCE cells proliferation and cell cycle at low concentration but significantly enhance the migration, suggesting the potential therapeutic effect on re-epithelialization of corneal epithelium during wound healing whilst maintaining the expression of corneal-specific genes.

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