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

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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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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,