

Full Length Research Paper

Anti-proliferative activities of *Centella asiatica* extracts on human respiratory epithelial cells *in vitro*

Mohd Heikal, M. Y.^{1,2}, Siti Mariam, H¹, Mohd Ilham, A.^{3,4}, Mee Fong, C.¹, Aminuddin, B. S.^{5,1} and Ruszymah BHI^{1,2*}

¹Tissue Engineering Centre, UKM Medical Centre, Jalan Yaacob Latiff, Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Malaysia.

²Department of Physiology, Medical Faculty UKM, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia.

³Malaysian Institute of Pharmaceuticals and Nutraceuticals (IPHARM) Persiaran Bukit Jambul, 11900 Bayan Lepas, Pulau Pinang, Malaysia.

⁴Forest Research Institute of Malaysia (FRIM), Kepong, 52109 Selangor, Malaysia.

⁵Ear, Nose & Throat Consultant Clinic, Ampang Puteri Specialist Hospital, Taman Dato Ahmad Razali, 68000 Ampang, Selangor, Malaysia.

Received 17 April, 2012; Accepted 19 June, 2014

Centella asiatica or “pegaga” is well known for its ability in promoting wound healing. This study focused on the effect of *C. asiatica* on the proliferation of human respiratory epithelial (RE) cells. RE cells were cultured using co-culture techniques until first passage (P1). Viability cell test by trypan blue dye exclusion assay showed that there was high percentage of cell viability at both P0 (74%) and P1 (91.61%). Triplicate tetrazolium dye (MTT assays) were carried out with different concentrations of *C. asiatica* from 15.6, 31.3, 62.5, 125, 250, 500, 1000, until 2000 ppm. The higher the concentration of *C. asiatica*, the more inhibitory effect was seen. *C. asiatica* aqueous extract at concentration 1000 and 2000 ppm demonstrated a significant ($p < 0.05$) inhibitory effect on human RE cells proliferation on day 4 and 7 after treatment. This provides potential use of *C. asiatica* extract for the treatment of conditions with respiratory epithelial cells overgrowth.

Key words: *Centella asiatica*, respiratory epithelial cells, anti-proliferative.

INTRODUCTION

Centella asiatica (family: Umbelliferae) is also known as Gotu kola in Indian or pennywort in English. It originates from Asia (India, Sri Lanka) and East Africa widespread

to South America, West Indies and South East Asia like Malaysia, Pakistan, Japan, China and Australia. It is commonly found in swampy areas of India, as a weed

*Corresponding author. E-mail: ruszy@medic.ukm.my, ruszyidrus@gmail.com. Tel: +603 91457670. Fax: +603 91457678.

Author(s) agree that this article remain permanently open access under the terms of the [Creative Commons Attribution License 4.0 International License](http://creativecommons.org/licenses/by/4.0/)

crop fields and other waste places throughout India up to an altitude of 600 m (Dastur et al., 1962). The extract of *C. asiatica* consists of bioactive terpene acids such as asiatic acids, madecassic acid and their respective glycoside, asiaticoside and madecassoside (Inamdar et al., 1996). *C. asiatica* has been reported to possess antiulcer (Yoshinori et al., 1982; Gohil et al., 2010), anti-inflammatory (Guo et al., 2004; George et al., 2009; Huang et al., 2011), immunomodulating (Punuree et al., 2005), antitumor (Babu et al., 1995), antiproliferative (Yoshida et al., 2005), antibacterial (Zaidan et al., 2005), antioxidant (Gupta et al., 2006), and antigenotoxic (Siddique et al., 2007) properties. It is believed that the antioxidation activity of the phenolic compound from *C. asiatica* can prevent certain diseases like arteriosclerosis, cancer, diabetic and arthritis (Zainol et al., 2003). Besides, *C. asiatica* could act as cardio protective agent that can enhance myocardial antioxidants and thus prevents the extent of cardiac damage (Gnanapragasam et al., 2004).

A number of studies have demonstrated the effectiveness of *C. asiatica* triterpenes, in particular the glycoside asiaticoside, in promoting wound healing (Maquart et al., 1999; Shukla et al., 1999a, b). Wound and ulcer healing are enhanced by the promotion of fibroblast proliferation and collagen synthesis in response to topical treatment with extracts of *C. asiatica* herb (Maquart et al., 1990, 1999). *C. asiatica* increase the production of basic fibroblast growth factor (bFGF) induce angiogenesis and cell proliferation, therefore it promotes wound healing activity (Shukla et al., 1999a, b; Chuen et al., 2004; Gohil et al., 2010). Asiaticoside enhance the burn wound healing at low doses by promoting angiogenesis during skin wound repair (Kimura et al., 2008).

Besides the aforementioned activities, previous studies reported that *C. asiatica* extract possess anti-psoriatic effect due to an inhibition of keratinocyte proliferation by its constituent triterpenoid glycosides (Sampson et al., 2001). Recent study by Babykutty et al. (2009) show that *C. asiatica* extracts induced apoptosis on human breast cancer cells. To date, almost no scientific information is available for the study of effect of *C. asiatica* aqueous extract on human respiratory epithelial cells. Therefore, the present study was designed to study the effect of an aqueous extract of *C. asiatica* on primary human respiratory epithelial cells.

MATERIALS AND METHODS

Respiratory epithelial tissues isolation and cultivation

The University of Kebangsaan Malaysia Research and Ethical Committee have approved the usage of redundant human nasal turbinate specimens from consented patient. Human nasal turbinate were obtained from six consented patients undergoing turbinectomy. The tissues were rinsed in phosphate buffer saline (PBS) (Gibco,

Grand Island, NY) supplemented with 1% antibiotic-antimycotic (Invitrogen, Carlsbad, CA) and cut into small pieces (2 mm³) and digested with 0.3% collagenase type I (Gibco/BRL) for 6 to 7 h. After complete digestion, the cell suspension was centrifuged at 5000 rpm for 5 min at room temperature. The resulting cell pellet was washed with PBS and centrifuged again to obtain a cell pellet. The cell pellet consisted of respiratory epithelial cells and fibroblasts were then cultured using co-culture technique (Noruddin et al., 2007; Mohd et al., 2010) in the combination medium of defined keratinocyte serum free medium, Ham's F12 and Dulbecco's modified eagle medium (defined keratinocytes serum free medium (DKSFM): F12: DMEM; 2:1:1) (Gibco/BRL) + 5% fetal bovine serum (FBS) in six-well plate culture dish. All cultures were maintained at 37°C in a humidified 5% CO₂ incubator (Jouan). When the cells reached 80 to 90% confluency, fibroblast were trypsinized with 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco/BRL) leaving the colonies of respiratory epithelial cells in DKSFM only as respiratory epithelial cells culture passage zero (P0). The respiratory epithelial cells were trypsinized again and sub cultured into passage one (P1) once reached 80 to 90% confluency.

Determination of cell number and viability

Morphological features of the cells were examined every day using the inverted light microscope (Olympus, Shinjuku-ku, Tokyo) and photomicrographs were recorded. Growth rates (cell/d/cm²) of cultured respiratory epithelial cells were calculated at every passage.

Preparation of *C. asiatica* (CA) aqueous extract

Fresh leaves of *C. asiatica* were identified and voucher specimen no. FRI50032 was deposited at the Medicinal Plant Division, Forest Research Institute of Malaysia (FRIM). The aqueous extract of *C. 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 until it became half the initial volume. The extract was freeze dried to remove the solvent and the dried extract was stored at 4°C until further use.

MTT assay

MTT assay to measure cell viability and proliferation was used to quantitatively determine the number of viable cells after treatment with *C. asiatica* aqueous extract. Respiratory epithelial cells at P1 were cultured triplicate in 96-well micro titer plate at a density of 1 × 10⁴ cells/well in DKSFM culture medium for 1 to 2 days. After the cells reached confluence, medium was changed to F12: DMEM (FD; 1:1) supplemented with indicated amounts of *C. asiatica* (15.6, 31.3, 62.5, 125, 250, 500, 1000 and 2000 ppm). Culture plate was then incubated for 24 h at 37°C in 5% CO₂. MTT solution was added and incubated with the cells for 4 h. The cells were then lysed to release and solubilize purple formazan. The test was carried out in 3 different days, day-1, day-4 and day-7. The absorbance value of culture in each well was measured at wavelength 570 nm with background subtraction at 690 nm by spectrophotometer.

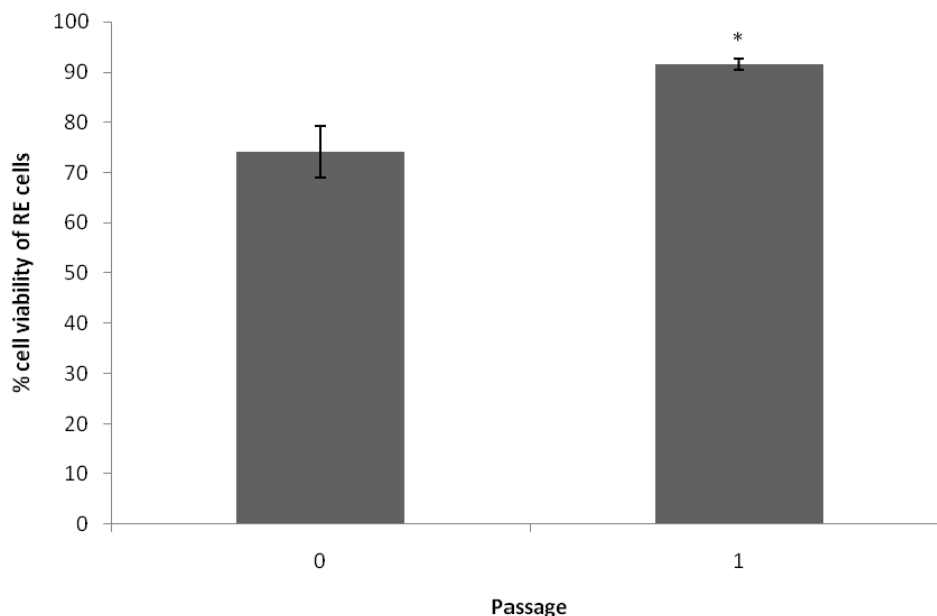


Figure 1. Percentage of respiratory epithelial cell viability at passage zero (P0) and passage one (P1). * $p < 0.05$ showed the significant differences in percentage of cell viability in passage one when compared to the passage zero. The mean values of cell viability for each passage are 74.2 ± 5.6 (P0) and 91.6 ± 5.6 (P1).

Statistical analysis

Statistical calculation was done with the SPSS 13.0 software. Data for respiratory epithelial cells viability and absorbance values were performed as mean \pm standard error of mean (SEM). Statistical differences were determined by Student's t-test and one-way analysis of variance (ANOVA), and the differences among means were considered significant at $p < 0.05$.

RESULTS

Morphological analysis and cell viability of human respiratory epithelial cells

Observation under inverted microscope of the co-culture system showed that fibroblast and respiratory epithelial cells have different morphology. Respiratory epithelial cells appeared polygonal while fibroblasts were flat and elongated. The respiratory epithelial cells maintained polygonal morphology from primary culture (P0) to passage one (P1). Both P0 and P1 showed high percentage of cell viability which was 74.2% (P0) and 91.6% (P1), respectively. The number of respiratory epithelial cells at P1 was slightly higher as compared to the number of cells in P0 (Figure 1). Statistical analysis through Student's t-test showed that the percentage of cell viability in P1 was significantly higher at $p < 0.05$ when

compared to percentage of cell viability in P0. The results are supported by the findings that respiratory epithelial cells from the nasal turbinates cultured using a co-culture system produced superior quality of respiratory epithelial cells (Noruddin et al., 2007).

C. asiatica extract effect on proliferation of respiratory epithelial cells

The proliferation of respiratory epithelial cells culture was evaluated by MTT assay after 1, 4 and 7 days treated with *C. asiatica* aqueous extract. On day-1, *C. asiatica* aqueous extract at all concentration (15.6, 31.3, 62.5, 125, 250, 500 and 1000 ppm) except 2000 ppm demonstrated increases in total number of human nasal respiratory epithelial cells when compared to DKFSM control but these increments were not significant. On day-4, all culture treated with *C. asiatica* aqueous extract showed an inhibitory pattern towards the respiratory epithelial cells compared to DKFSM control but only extract at concentration 125, 1000 and 2000 $\mu\text{g}/\text{ml}$ showed significant inhibitory effect when compared to the control (Table 1). On day-7, culture treated with *C. asiatica* aqueous extract at concentration of 62.5, 1000 and 2000 $\mu\text{g}/\text{ml}$ showed significant inhibitory effect when compared to the DKFSM control (Table 1). The numbers of viable

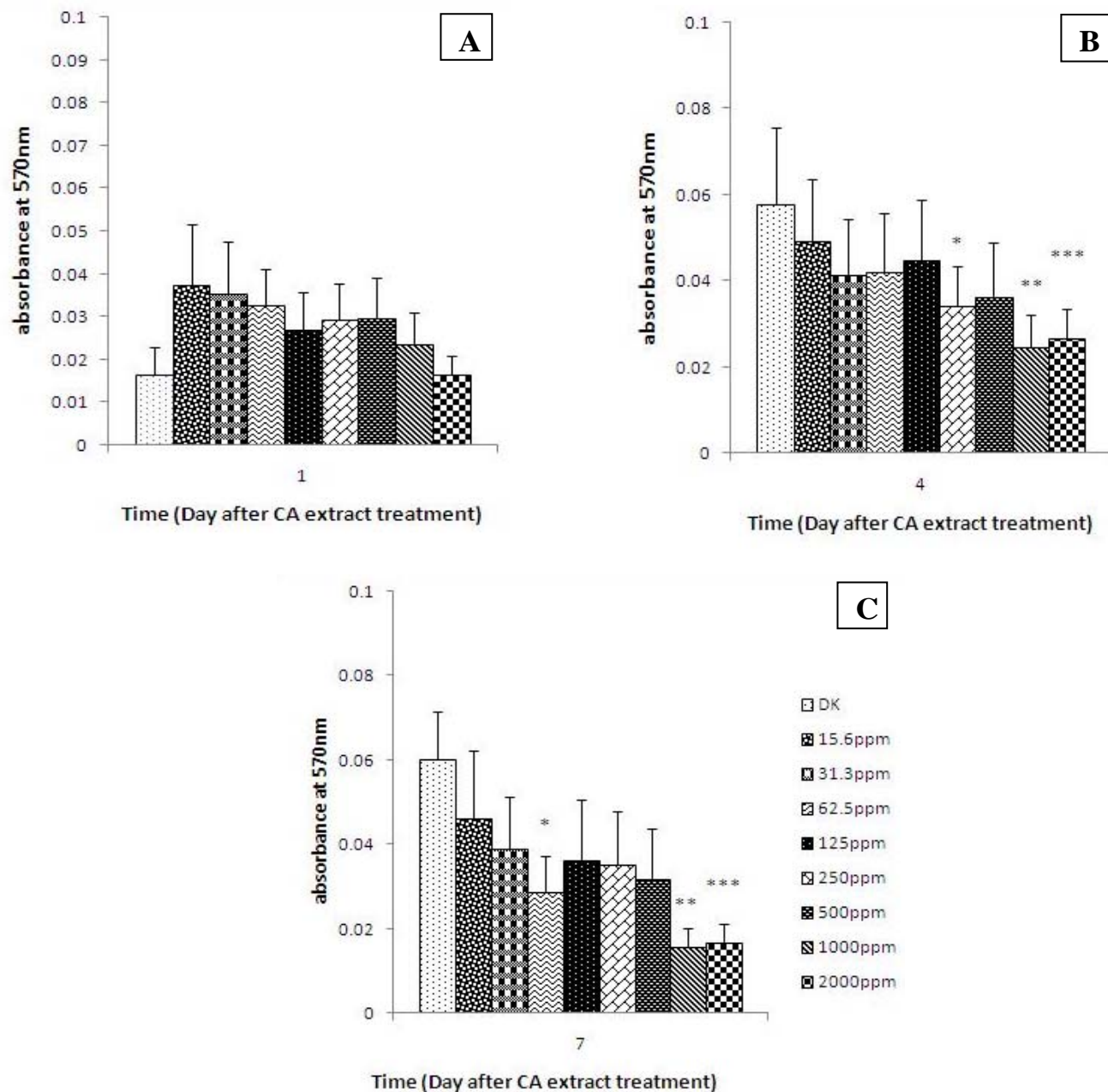


Figure 2. Concentration-dependent effects of *C. asiatica* extract on human Respiratory epithelial cells. Human RE cells were incubated with *C. asiatica* aqueous extracts for the indicated time: day-1(A), day-4 (B) and day-7 (C). Cell viability was measured by MTT assay. The absorbance value at 570nm are expressed as the mean \pm SEM. * $p < 0.05$, ** $p < 0.05$ and *** $p < 0.05$ are significant compared with the DK control. DK: Respiratory epithelial cells in DKSFM without *C. asiatica* treatment.

of viable cells in culture treated with all concentration on day-7 were lower when compared to day-4. Overall, among the various concentrations of *C. asiatica* extract, 2000 and 1000 ppm were found to have anti-proliferative effect on cultured human respiratory epithelial cells. As

illustrated in Figure 2, the effect of *C. asiatica* aqueous extract on human respiratory epithelial cells seems to be concentration-dependent, where the higher the *C. asiatica* extract concentration, the lower the number of viable human respiratory epithelial cells.

Table 1. Antiproliferative effect of *C. asiatica* aqueous extract on human respiratory epithelial cells.

| Concentration of <i>C. asiatica</i> (ppm) | Day-4 | | Day-7 | |
|---|----------------------------|---------------------------|----------------------------|----------------------------|
| | Absorbance value at 570 nm | Sig (1-tailed) *P<0.05 | Absorbance value at 570 nm | Sig (1-tailed) **P<0.05 |
| 15.6 | 0.0493±0.0143 | 0.079 | 0.0460±0.0163 | 0.066 |
| 31.3 | 0.0415±0.0127 | 0.065 | 0.0388±0.0124 | 0.054 |
| 62.5 | 0.0422±0.0135 | 0.056 | 0.0287±0.0084** | 0.049 |
| 125 | 0.0447±0.0142 | 0.059 | 0.0362±0.0145 | 0.051 |
| 250 | 0.0343±0.009* | 0.045 | 0.0352±0.0127 | 0.050 |
| 500 | 0.0363±0.0125 | 0.055 | 0.0318±0.0118 | 0.052 |
| 1000 | 0.0245±0.0078* | 0.039 | 0.0157±0.0045* | 0.042 |
| 2000 | 0.0267±0.0068* | 0.042 | 0.0167±0.0044* | 0.044 |

Results are mean ± SEM of culture treated with *C. asiatica* after day-4 and day-7; *Significant compared to the DKFSM control at p<0.05.

DISCUSSION

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay applied the principal of formazan crystal formation through dehydrogenase mitochondria activities in living cells. The rate of formation of MTT crystal formazan is directly proportional to the number of living cells. According to Sampson et al. (2001), *C. asiatica* aqueous extract suppressed the proliferation of keratinocytes cell line (SVK-14). However, Lu et al. (2004) found *C. asiatica* aqueous extract induced proliferation of human dermal fibroblast cells. Figure 2 showed that there was inhibitory effect of *C. asiatica* on human RE cells proliferation activities with the decrease in absorbance values at all concentration. According to Sampson et al. (2001), the active compounds in *C. asiatica* extract which are asiaticoside and madecassoside have anti-proliferative effect on keratinocyte cells.

In this study, an inhibitory trend was detected as a response to *C. asiatica* extract. The higher the concentration of *C. asiatica*, the more inhibitory effect was seen. This indicated that the inhibitory effect of *C. asiatica* aqueous extract on respiratory epithelial cells is concentration-dependent. Observation under inverted microscope showed that nearly all cells contained purple crystal. However, statistical analysis showed no significant mean difference to the formation of crystal formazan when compared to DKFSM control. This might be caused by the condition of cell physiology and variation in dehydrogenase activities in the mitochondria of respiratory epithelial cells. Babykutty et al. (2009) had found that *C. asiatica* extracts induced apoptosis on human breast cancer cells by induction of nuclear condensation and loss of mitochondrial membrane potential. This study also showed that the exposure of human respiratory epithelial cell cultures to high

concentration of *C. asiatica* aqueous extract (500 to 2000 ppm) may cause cytotoxicity effect on cells.

Studies on the effect of *C. asiatica* on human nasal epithelial cells could help to investigate the potential of this herb for the treatment of nasal polyposis. Nasal polyposis is a chronic inflammatory disease of the mucous membranes in the nose and paranasal sinuses. Increased proliferation of epithelial cells has been well documented in nasal polyp (Coste et al., 1996). The fibroblasts are the main cells of the polyp architecture and play an important role in nasal polyposis (Xing et al., 1993). A number of differentiation factors and inflammatory mediators secreted by fibroblast cells are involved in the growth of nasal polyps (Saji et al., 2000). Activated epithelial cells may be the major source of mediators inducing influx of inflammatory cells that further cause proliferation and activation of fibroblasts leading to nasal polyp formation (Pawliczak et al., 2005). The innate immune mechanism, the growth and homeostasis of epithelial cells may also play a role in the formation of nasal polyps (Fundova et al., 2008). Since the present study revealed that *C. asiatica* aqueous extract exhibited some inhibitory effect on the proliferation of human respiratory epithelial cells, the identification and characterization of the compounds present in *C. asiatica* extract to determine their particular functions will be part of our future study.

Conclusion

The *C. asiatica* aqueous extract demonstrated an inhibitory effect towards the proliferation activities of human respiratory epithelial cells. The effect of inhibition is dose-dependent; with the higher the dose of *C. asiatica* extract the more inhibition will be observed. Further

studies are necessary to understand the compounds of *C. asiatica* that is responsible for the anti-proliferative effect on human respiratory epithelial cells. This finding provides the possibility of using *C. asiatica* extract as an anti-polyps therapy.

ACKNOWLEDGEMENT

This study would not be possible without the research grants for Ministry of Science, Technology and Innovation Malaysia, 06-02-02-0003-BTK/ER/022 and also grants from Ministry of Higher Education Malaysia UKM-OUP-TKP-21-97/ 2008 and UKM-OUP-TKP-20-94/ 2009.

Conflict of interests

The author(s) have not declared any conflict of interests.

REFERENCES

- Babu TD, Kuttan G, Padikkala J (1995). Cytotoxic and anti-tumor properties of certain taxa of Umbelliferae with special reference to *Centella asiatica* (L.) Urban. *J. Ethnopharmacol.* 48:53-57.
- Babykutty S, Padikkala J, Sathiadevan PP, Vijayakurup V, Abdul Aziz TK, Srinivas P, Gopala S (2009). Apoptosis induction of *Centella asiatica* on human breast cancer cells. *Afr. J. Tradit. CAM* 6(1):9-16.
- Chuen LC, Jin SG, Luk J, Koo MWL (2004). The healing effects of *Centella extract* and asiaticoside on acetic acid induced gastric ulcers in rats. *Life Sci.* 74:2237-2249.
- Coste A, Rateau JG, Bernaudin JF, Peynègre R, Escudier E (1996). Nasal polyposis pathogenesis: a flow cytometric and immunohistochemical study of epithelial cell proliferation. *Acta Otolaryngol.* 116(5):755-761
- Dastur JF (1962). *Medicinal Plants of India and Pakistan*. D.B. Taraporevala Sons & Co., Bombay.
- Flora SJ, Gupta R (2007). Beneficial effects of *Centella asiatica* aqueous extract against arsenic-induced oxidative stress and essential metal status in rats. *Phytother. Res.* 21(10):980-988.
- Fundova P, Filipovsky T, Funda DP, Hovorka O, Holy R, Navara M, Tlaskalova-Hogenova H (2008). Expression of IGF-1R and iNOS in nasal polyps; epithelial cell homeostasis and innate immune mechanisms in pathogenesis of nasal polyposis. *Foliar Microbiol.* 53:558-562.
- George M, Linchy J, Ramaswamy (2009). Anti-allergic, anti-pruritic and anti-inflammatory activities of *Centella asiatica* extracts. *Afr. J. Tradit. CAM* 6(4):554-559.
- Gnanapragasam A, Ebenezer KK, Sathish V, Govindaraju P, Devaki T (2004). Protective effect of *Centella asiatica* on antioxidant tissue defense system against adriamycin induced cardiomyopathy in rats. *Life Sci.* 76:585-597.
- Gohil KJ, Patel JA, Gajjar AK (2010). Pharmacological review on *Centella asiatica*: A potential Herbal Cure-all. *Indian J. Pharmaceut. Sci.* 72(5):546-556.
- Guo JS, Cheng SL, Koo MW (2004). Inhibitory effect of *Centella asiatica* water extract and asiaticoside on inducible nitric oxide synthase during gastric ulcer healing in rats. *Planta Med.* 70:1150-1154.
- Gupta R, Flora SJ (2006). Effect of *Centella asiatica* on arsenic induced oxidative stress and metal distribution in rats. *J. Appl. Toxicol.* 26:213-222.
- Huang SS, Chiu CS, Chen HJ, Hou WC, Sheu MJ, Lin YC, Shie PH, Huang GJ (2011). Antinociceptive activities and the mechanisms of anti-inflammation of Asiatic Acid in Mice. *Evidence-Based Complement. Altern. Med.* 10 p.
- Inamdar PK, Yeole KD, Cibogare AB, Desoza NJ (1996). Determination of Biologically Active Constituents in *Centella asiatica*. *J. Chromatogr.* 742:127-130.
- Kimura Y, Sumiyoshi M, Samukawa K, Satake N, Sakanaka M (2008). Facilitating action of asiaticoside at low doses on burn wound repair and its mechanism. *Eur. J. Pharmacol.* 584:415-423.
- Maquart FX, Bellon G, Gillery P, Wegrowski Y, Borel JP (1990). Stimulation of collagen synthesis in fibroblast cultures by a triterpene extracted from *Centella asiatica*. *Connect Tissue Res.* 24, 107-120.
- Maquart FX, Chastang F, Simeon A, Birembaut P, Gillery P, Wigrowski Y (1999). Triterpenes from *Centella asiatica* stimulate extracellular matrix accumulation in rat experimental wounds. *Eur. J. Dermatol.* 9:289-296.
- Mohd MYH, Aminuddin BS, Jeevanan J, Chen HC, Sharifah SH, Ruszymah BHI (2010). Cells Tissues Organs, Autologous Implantation of Bilayered Tissue-Engineered Respiratory Epithelium for Tracheal Mucosal Regeneration in a Sheep Model. *Cells Tis. Organs.* 192(5):292-302.
- Noruddin NA, Saim AB, Chua KH, Idrus R (2007). Human Nasal turbinate as a viable source of respiratory epithelial cells using a co-culture system versus dispase dissociation technique. *Laryngoscope* 117:2139-2145.
- Pawliczak R, Lewandowska-Polak A, Kowalski ML (2005). Pathogenesis of nasal polyps: an update. *Curr. Allergy Asthma Rep.* 5:463-471.
- Punuree K, Wild CP, Kasinrerker W, VinitKetkumnuen U (2005). Immunomodulatory activities of *Centella asiatica* and *Rhimachantus nasutus* extracts. *Asian Pac. J. Cancer Prev.* 6:396-400.
- Saji F, Nonaka M, Pawankar R (2000). Expression of RANTES by IL-1 beta and TNF-alpha stimulated nasal polyp fibroblasts. *Auris Nasus Larynx* 27:247-252.
- Sampson JH, Raman A, Karlsen G, Navsaria H, Leigh IM (2001). *In vitro* keratinocyte antiproliferant effect of *Centella asiatica* extract and triterpenoid saponins. *Phytomedicine* 8:230-235.
- Shukla A, Rasik AM, Dhawan BN (1999a). Asiaticoside-induced elevation of antioxidant levels in healing wounds. *Phytother. Res.* 13:50-54.
- Shukla A, Rasik AM, Jain GK, Shankar R, Kulshrestha DK, Dhawan BN (1999b). *In vitro* and *in vivo* wound healing activity of asiaticoside isolated from *Centella asiatica*. *J. Ethnopharmacol.* 65:1-11.
- Siddique YH, Ara G, Beg T, Faizal M, Ahmad M, Afzal M (2007). Protective role of *Centella asiatica* L. extract against methyl methanesulphonate and cyclophosphamide induced genotoxic effect in cultured human lymphocytes. *Recent Progress Med. Plants* 19:369-381.
- Xing Z, Jordana M, Braciak T, Ohtoshi T, Gauldie J (1993). Lipopolysaccharide induces expression of granulocyte/macrophage colony-stimulating factor, interleukin-8, and interleukin-6 in human nasal, but not lung, fibroblasts: evidence for heterogeneity within the respiratory tract. *Am. J. Respir. Cell Mol. Biol.* 9:255-263.
- Yoshida M, Fuchigami M, Nagao T, Okabe H, Matsunaga K, Takata J, Karube Y, Tsuchihashi R, Kinjo J, Mihashi K, Fujioka T (2005). Antiproliferative constituents from Umbelliferae plants VII. Active triterpenes and rosmarinic acid from *Centella asiatica*. *Biol. Pharm. Bull.* 28:173-175.
- Yoshinori A, Reiko M, Tsumematsu T (1982). Mono and sesquiterpenoids from hydrocotyle and *Centella* species. *Phytochemistry* 21:2590-2592.
- Zaidan MR, Noor RA, Badrul AR, Adlin A, Norazah A, Zakiah I (2005). *In vitro* screening of five local medicinal plants for antibacterial activity using disc diffusion method. *Trop. Biomed.* 22:165-170.
- Zainol MK, Abd Hamid A, Yusof S, Muse R (2003). Antioxidative activity and total phenolic compounds of leaf, root and petiole of four accessions of *Centella asiatica* (L.) urban. *Food Chem.* 81:575-581.