



Effects Of *Eclipta prostrata* And *Eclipta alba* On Survival, Proliferation, Migration Of Periodontal Ligament Cells

Thuy Thu Nguyen^{1,2}, Sirinthip Choonate¹, Duangchewan Puengsurin¹, Ratchaporn Srichan³, Suparporn Mala³, Rudee Surarit²

¹ Department of Oral Biology, Faculty of Dentistry, Mahidol University

² Department of Periodontology, Faculty of Odonto-Stomatology, University of Medicine and Pharmacy – Ho Chi Minh City

³ Research Service Center, Faculty of Dentistry, Mahidol University

Abstract

Objective: The study aimed at investigating the effects of juice extract of *Eclipta prostrata* (EP) and *Eclipta alba* (EA) on the viability, migration and proliferation of periodontal ligament cells (PDLs).

Materials and methods: The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay) was used to assess the cytotoxicity and the proliferation. The cell migration test was performed by scratched assay, estimated by counting the number of cells in the scratched area.

Results: The IC₅₀ of EA on PDLs was approximately 0.12 mg/ml after 48 hours. The IC₅₀ of EP on PDLs was approximately 0.23 mg/ml after 48 hours. For the proliferation, the absorbance of living cells of EP and EA began to increase at day 3. Both EP and EA could inhibit the migration of PDLs in a dose dependent manner.

Keywords: *Eclipta alba*, *Eclipta prostrata*, migration, periodontal ligament cells, proliferation, viability.

How to cite: Nguyen Thu T, Choonate S, Puengsurin D, srichan R, Mala S, Surarit R. Effects of *Eclipta prostrate* and *Eclipta alba* on survival, Proliferation, Migration of periodontal ligament cells. M Dent J 2016; 36: 165-173.

Corresponding author:

Rudee Surarit
Department of Oral Biology,
Faculty of Dentistry, Mahidol University
6 Yothi Street, Ratchathewi, Bangkok,
10400 Thailand
Office phone: 02-200-784-950
Mobile phone: 08-156-30-361
Email address: rudee.sur@mahidol.ac.th

Received: 20 April 2016

Accepted: 12 May 2016

Introduction

In Thailand, the percentage of subjects classified as mild, moderate, and severe periodontitis was 30.5, 53.6, and 15.9, respectively¹. In Vietnam, oral hygiene status was poor with high accumulation of plaque and calculus in subjects aged 35-44 years; almost all subjects presented with at least one site with loss of attachment ≥ 2 mm². In both countries, traditional herbal medicine came to be recruited as an important component of national efforts to promote the public health of urban and rural populations, which covered the oral health including periodontal status^{3,4}.

Eclipta prostrata (EP) (Illustration 1A) juice has been showed to inhibit cancer and endothelial cell migration *in vitro* and to have anti-angiogenic activity *in vivo*⁵. The echinocystic acid extracted from EP concentration-dependently inhibited not only lipopolysaccharide-induced inducible nitric oxide synthase (iNOS) expression at the protein level but also iNOS, tumor necrosis factor α (TNF α), and interleukin-6 expression at the messenger ribonucleic acid (mRNA) level. It also inhibited lipopolysaccharide-induced iNOS promoter binding activity. In addition, echinocystic acid suppressed the lipopolysaccharide-induced transcriptional activity of nuclear factor- κ B by blocking the nuclear translocation of p65⁶. Furthermore, an

anti-inflammatory compound, isolated from EP, was identified to be wedelolactone, an inhibitor of a crucial kinase enzyme in the activation of inflammation response, I κ B kinase⁵. However, this plant has not been used in oral cavity, both for research and treatment purposes.

Eclipta alba (EA) (Illustration 1B) is a small branched perennial herb, which has been used as a traditional medicine in different countries mainly in tropical and subtropical regions of the world. The herb has been claimed for its medicinal value and has been traditionally used as an analgesic, antimutagenic, antihepatotoxic, antibacterial, antioxidant, antihemorrhagic, antihyperglycemic, immunomodulatory therapy and also recognized as a reincarnated plant⁷. Main active principles consist of coumestans like wedelolactone, desmethylwedelolactone, furanocoumarins, oleanane & taraxastane glycosides⁸. In Vietnam, this herb is usually used in treatment of periodontal diseases in rural provinces. A clinical research in 2005 stated that when being used as a mouthwash, EA juice had anti-inflammatory effect similar to Chlorhexidine in patients with gingivitis, based on clinical assessment⁹. However, the study mentioned did not evaluate the antibacterial effect of EA juice, whereas Chlorhexidine was also used in treatment and prevention of oral diseases for its antiseptic properties¹⁰.



A



B

Figure 1 *Eclipta prostrata* and *Eclipta alba* plants.

These medicinal herb extracts may have potential effects in periodontal diseases treatment⁸, possibly owing to their biochemical components and activities^{11, 12}. However, their *in vitro* effects, especially on cells of periodontal tissues have never been assessed up to now. The aim of this study was to investigate the effects of EP and EA on the viability and the migration of periodontal ligament cells (PDLs) by using basic techniques.

Materials and methods

PDLs were purchased from American Type Culture Collection (ATCC). Dried EP was kindly supplied by the Faculty of Pharmacy, Chulalongkorn University, Thailand. Dried EA was kindly supplied by the Institute of Traditional Medicine - Ho Chi Minh City, Vietnam.

Maintenance of the cell line

The primary PDLs were maintained in Dulbecco's Modified Eagle Medium (DMEM) (90% DMEM + 10% Fetal Bovine Serum (FBS) + 1% antibiotics) and sub-cultured when cells reached confluence.

Preparation of test sample

The fresh herb was dried and cut out into small pieces. Then they were dried and grinded into powder. The stock was made by diluting the herb powder with sterile water at the concentration of 50 mg/ml, filtering through 0.45 µm filter membrane before diluting the stock with media at lower concentrations.

Cytotoxicity EP and EA on PDLs

The PDLs at passage 6 from ATCC were seeded into microtiter plates at 2×10^4 cells/well. The cell counter was realized by using hemacytometer (Improve Neubauer). The plates were then incubated in 24 hours in CO₂ incubator (37° C, 5% CO₂, 100% humidity) for cells to reach confluence in each well. The EP and EA juices were diluted into solutions at different concentrations: 5mg/ml, 1 mg/ml, 0.1 mg/ml, 0.01 mg/ml. These solutions

were then added into wells (4 wells for each concentration). Chlorhexidine 0.12% was used as positive control and media as negative control. Phosphate Buffered Saline (PBS) 1X was put in blank wells. The plates were incubated in carbon dioxide (CO₂) incubator in 48 hours, before being tested for cytotoxicity with MTT test. The results were read as absorbance (OD540) by spectrophotometer (Epoch™ microplate reader spectrophotometer, Biotek®, USA) at 540 nm. The experiment was repeated two times. The results with coefficient of variation less than 15% were analyzed and reported. The IC50 or concentration which 50% of cells are still alive was determined by graph between concentration and cell viability.

Effects of EP and EA on the proliferation of PDLs

The PDLs at passage 8 were seeded into microtiter plates at 2×10^4 cells/well. The EP and EA were diluted into different concentrations which had been proved to have ≥85% of cells being alive after 48 hours of treatment in the previous experiments: 1 mg/ml and 0.05 mg/ml for EP, 0.05 mg/ml and 0.001 mg/ml for EA, then added to cells. Chlorhexidine 0.12% was used as negative control and Human Basic Fibroblast Growth Factor (hFGF basic/FGF2, Cell Signaling Technology®, United States) at the concentration of 0.2 µg/ml as positive control. The plates were then incubated in CO₂ incubator for 7 days. The cell viability was determined at day 1, 3, 6 and 7. The absorbance was read by spectrophotometer at 540 nm. The experiment was repeated two times. The results with coefficient of variation less than 15% were analyzed and reported.

Effects of EP and EA on the migration of PDLs

PDLs at passage 7 were seeded into 24 well plates at the concentration of 5×10^3 cells/well and incubated (37° C, 5% CO₂, 100% humidity) for 48 hours. After taking out the

media, 1,000 µl micropipette tip was used to draw a horizontal line across the center of the surface of the well. The wells were checked under inverted microscope to verify that there was no cell left in the scratched area. 500 µl of extract juice at different non-toxic concentrations was then added to each well (EP at 1 mg/ml and 0.05 mg/ml, EA at 0.05 mg/ml and 0.001 mg/ml). Media served as negative control. For the positive control, we used 500 µl of Human Basic Fibroblast Growth Factor (hFGF basic/FGF2, Cell Signaling Technology®, United States) at the concentration of 0.2 µg/ml. The plate was then incubated for 24 hours and the migration of cells was checked after 3 hours, 6 hours and 24 hours. After 24 hours, the cells were washed with PBS 1X, fixed with methanol in 2 minutes, washed with PBS 1X two times before being stained with toluidine blue 0.25%. Photographs were taken under inverted microscope (Nikon HMC 0.4/0.044, Japan). The number of cells in the scratched area was estimated by counting.

Data analysis

The inhibitory concentration at 50% (IC_{50}) was calculated by Microsoft Excel program. For the effects of solutions on the migration

of PDLCs, all values were expressed as mean \pm standard error of the mean (SEM). The distribution of the data was verified by using test of normality - Kolmogorov Smirnov test. Statistical analysis of number of PDLCs at migrated area was determined with one-way ANOVA test. The Kruskal-Wallis one-way analysis of variance on ranks was used to analyze the proliferation of PDLCs. All pairwise multiple comparison procedures were realized by using Tukey test. The P values ≤ 0.05 were considered statistically significant. The SigmaStat version 3.5 logistics was used for statistical analysis.

Results

Cytotoxicity of EP and EA on PDLCs:

The cytotoxicity is generally showed as the IC_{50} value derived from the concepts of the concentration of an agent that cell viability is reduced by half. The MTT assay showed the percentage of viable cells in the presence of test substances when compare to the negative control (media only) (Table 1). The positive control showed 5% viable cells when compare to the negative control. The IC_{50} of EA and EP on PDLCs were approximately 0.12 mg/ml

Table 1 MTT results after 48 hours of incubation with EA and EP.

Solution	OD 540nm Mean \pm SD*	Living cells (%)
Blank	0.04 \pm 0.00	-
Negative control	0.46 \pm 0.04	100.00
Positive control	0.07 \pm 0.00	6.05
EP 5 mg/ml	0.05 \pm 0.00	1.94
EP 1 mg/ml	0.46 \pm 0.03	89.85
EP 0.1 mg/ml	0.45 \pm 0.05	88.55
EP 0.01 mg/ml	0.50 \pm 0.07	98.92
EA 5 mg/ml	0.05 \pm 0.00	2.10
EA 1 mg/ml	0.33 \pm 0.03	65.65
EA 0.1 mg/ml	0.40 \pm 0.02	83.41
EA 0.01 mg/ml	0.42 \pm 0.01	87.15

* SD: Standard deviation

and 0.23 mg/ml after 48 hours, respectively as shown in Table 2. It was found that more than 89% of cells are still viable when treated with EP at the concentration of less than 1 mg/ml while for EA cell viability were higher than 87% at the concentration of 0.001 mg/ml. Therefore, EA at the concentrations of 1 mg/ml and 0.05 mg/ml and EP at the concentrations of 0.005 mg/ml and 0.001 mg/ml where cells should be alive more than 85% were selected for further study.

Effect of EP and EA on the proliferation of PDLCs:

It was found that both EP and EA showed no toxicity to PDLCs and cells could proliferate at similar rate as shown in Figure 2.

Table 2 Estimated IC₅₀* of EP and EA on PDLCs.

Substance	IC ₅₀ after 48 hours (mg/ml)
EP	0.23
EA	0.12

* IC₅₀: inhibitory concentration at 50%

The number of cells began to increase at day 3 with no significant difference among groups.

Effect of EP and EA on the migration of PDLCs:

Both EP and EA could inhibit the migration of PDLCs in a dose dependent manner (Table 3). The number of cells migrated into the wounded area showed in Table 3. Usually after scratching and removing of cells, cells started to move slowly into the scratched area and covered about 50% of the area after 24 hours as shown in Figure 3. Therefore we select 24 hours incubation before determination of cell migration. It was found that both EA and EP showed inhibitory effect to the cell migration. EP at the concentration of 0.05 mg/ml and 1.0 mg/ml and EA at the concentration of 0.05 mg/ml could significantly inhibit cell migration while EA at the concentration of 0.001 mg/ml showed no significant different to negative control (Figure 4)

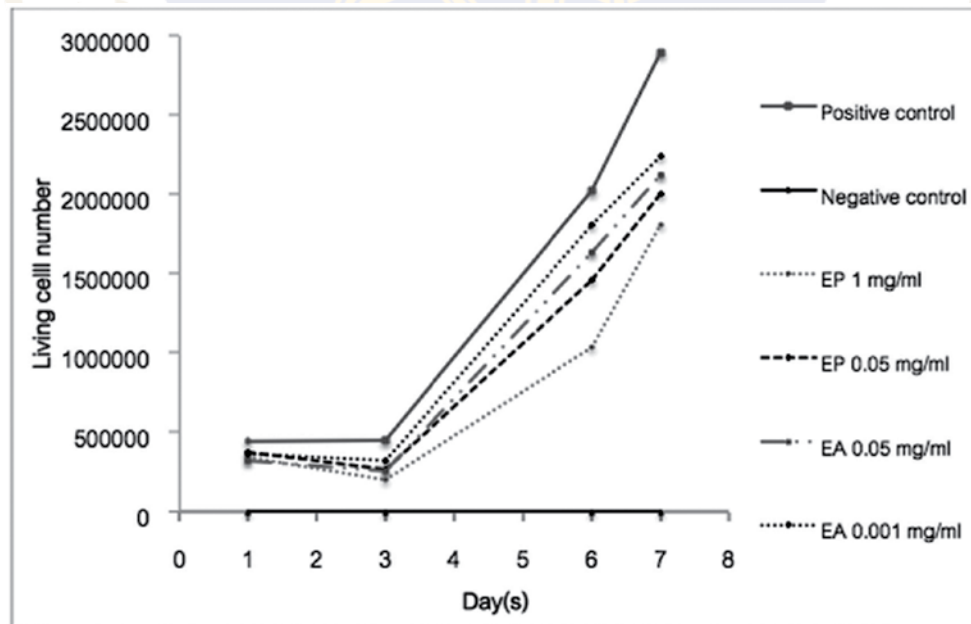


Figure 2 Number of living cells after 1, 3, 6, 7 day(s) of incubation with solutions at different concentrations.

Table 3 Number of PDLCs in the scratched area after 24 hours of incubation.

Solution	Concentration (mg/ml)	Number of PDLCs Mean \pm SEM* (cells)
Negative control	-	259.67 \pm 17.68
Positive control	0.0002	334.44 \pm 22.11 ²
EP	1.00	174.67 \pm 13.62 [§]
	0.05	192.67 \pm 11.34 [§]
EA	0.05	175.33 \pm 12.63 [§]
	0.001	253.33 \pm 13.82 ^{**}

* SEM: standard error of the mean

§ Different when comparing to negative control ($p < 0.05$)

** Not different when comparing to negative control ($p > 0.05$)

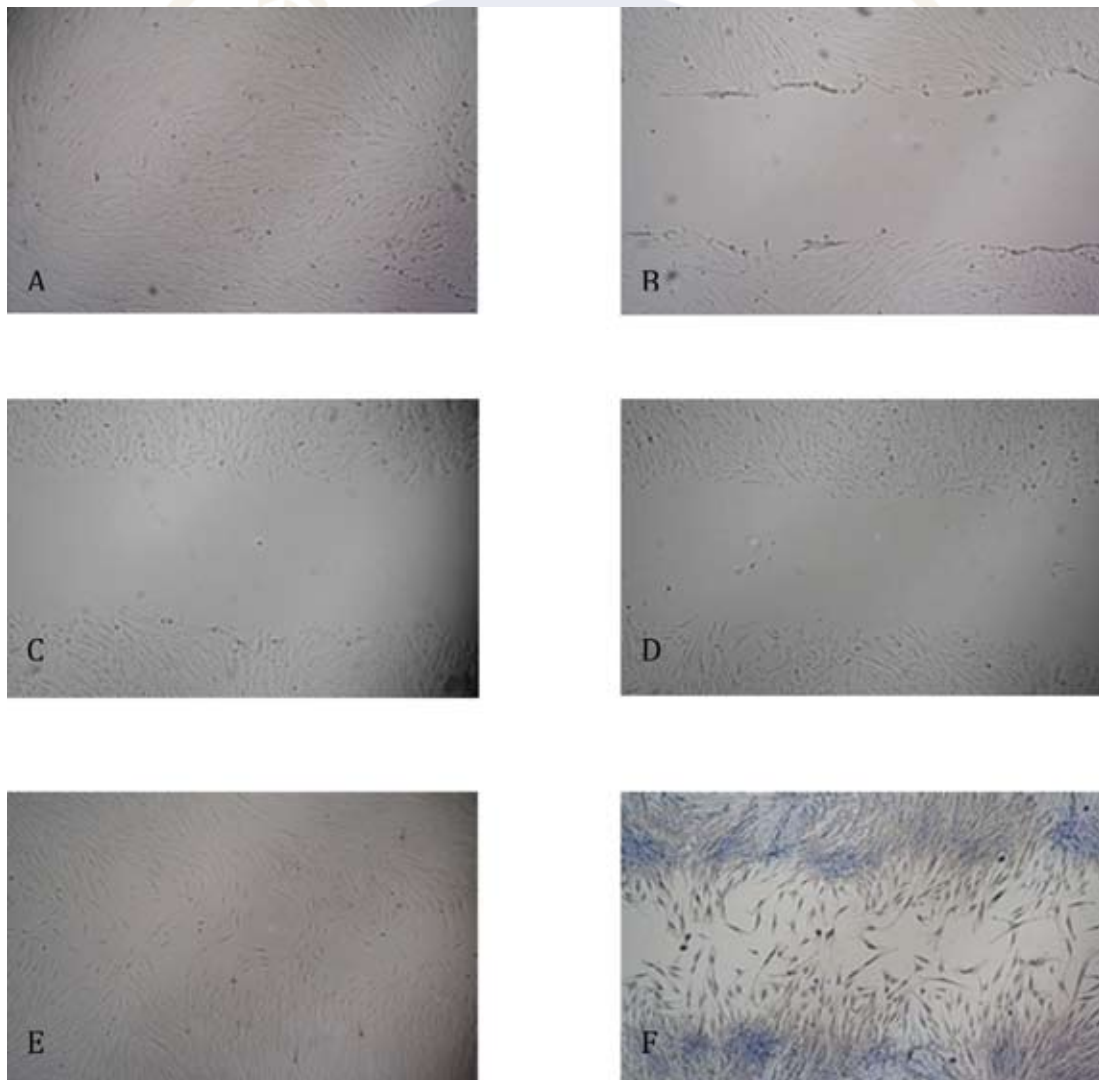


Figure 3 PDLCs migration (negative control well): (A) before scratching, (B) with scratch line, (C) after 3 hours of incubation, (D) after 6 hours of incubation, (E) after 24 hours of incubation (before staining with Toluidine blue 0.25%), (F) after 24 hours of incubation (after staining).

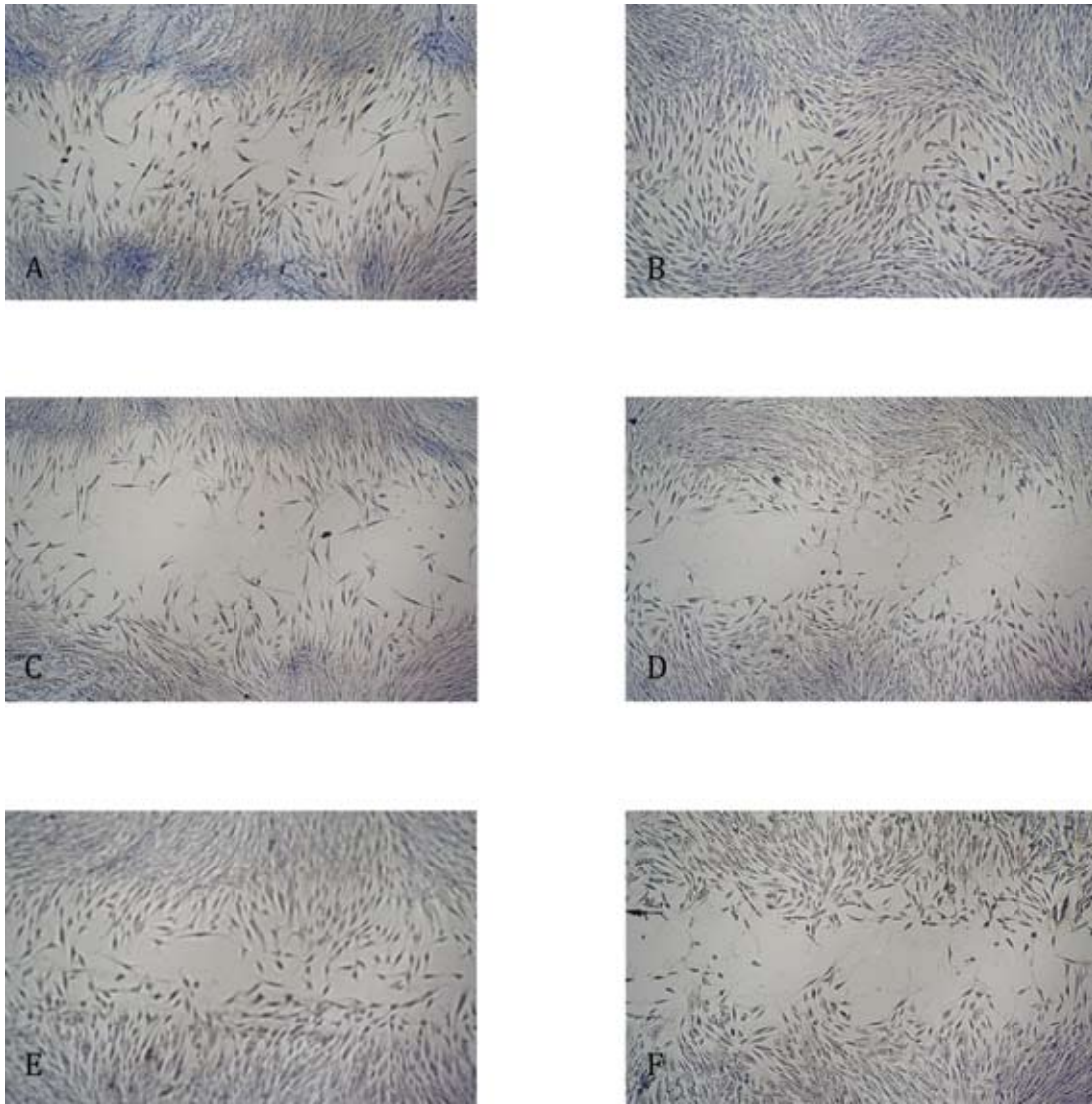


Figure 4 PDLCs in migration assay after 24 hours of incubation with: (A) negative control, (B) positive control, (C) EP 0.05 mg/ml, (D) EP 1 mg/ml, (E) EA 0.001 mg/ml, (F) EA 0.05 mg/ml.

Discussion

From the cytotoxicity test suggested us to use the test substances at different concentrations (EP at 1 mg/ml and 0.05 mg/ml, EA at 0.05 mg/ml and 0.001 mg/ml) in the next steps since the effect of plant extracts on PDLCs cell proliferation and migration could only be performed when cells are still alive. The different concentrations were used to confirm the effect of each extract. In this cytotoxicity assay, we used Chlorhexidine at the concentration of 0.12% for positive control.

Less than 7% of PDLCs still survived after 48 hours of incubation at this concentration of Chlorhexidine which is usually recommended to be applied in mouthwashes for treatment of periodontal diseases and after oral surgery¹³. This result completed the conclusion of another study that diluted Chlorhexidine reduced both cell migration and long-term survival¹⁴.

For the effect of EP and EA on the proliferation of PDLCs after 7 days, the data from day 1, day 3, day 6 and day 7 were significantly different ($p < 0.01$). At day 1, all the data are

not significantly different except between the positive and negative controls ($p < 0.01$). The number of living cells in wells containing EP, EA and positive control began to increase at day 3. No significant difference was found between these groups; which may suggest that EP and EA at the tested concentrations did not have negative effect on the proliferation of PDLCs. This result could be comparable to that of a study in 2010; in which both volatile components and ethanolic extract from EP significantly stimulated the proliferation of primary osteoblasts¹⁵. The EP has been widely investigated for its constitutive components and pharmacological effects on snakebite, tumor etc.^{16,17,18} but not in the oral treatment. Also, the antibacterial activity, cerebro-protective effect and anti-cancer potential of EA^{19,20,21} have been recently studied, but its possible application in cure for oral diseases except oral cancer²³ has not occupied the attention.

The estimated numbers of cells in the migration area were significantly different between negative and positive control ($p = 0.02$). Both EP and EA with concentrations of higher than 0.001 mg/ml could inhibit the migration of PDLCs in a dose dependent manner, which could be supported by the conclusion of Lirdprapamongkol *et al* in 2008 that EP juice inhibited cancer and endothelial cell migration *in vitro*⁵. Therefore these herbs should not be applied directly into the periodontal pocket in the periodontal patients since it may inhibit cell movement leading to tissue regeneration. We suggest that the effects of these medicinal herbs should be investigated more in the context that they may have some values for clinical application.

To the best of our knowledge, this is the first study on the effects of EP and EA on the cytotoxicity, migration and proliferation of PDLCs while these medicinal herbs have been silently used in treatment for oral diseases in

some populations, especially in rural regions in Vietnam. However, further researches concerning these herbs both *in vitro* and *in vivo* are in needs to clarify their activities in the field.

In conclusion, EP and EA have effects on the viability, the proliferation and the migration of PDLCs. These herbs may give new orientation in research for treatment of periodontal diseases based on non-expensive natural products. Further studies both *in vitro* and *in vivo* are in needs to investigate their useful properties and components.

Acknowledgement

This study was supported by the International Dental Collaboration of the Mekong River Region (IDCMR) scholarship from the Faculty of Dentistry, Mahidol University, Bangkok, Thailand.

Funding: IDCMR scholarship

Competing interests: None

Ethical approval: None

References

1. Torrungruang K, Tamsailom S, Rojanasomsith K, Sutdhibhisal S, Nisapakultorn K, Vanichjakvong O, et al. Risk indicators of periodontal disease in older Thai adults. *J Periodontol* 2005; 76: 558-565.
2. Do LG, Spencer JA, Roberts-Thomson K, Ha DH, Tran TV, Trinh HD. Periodontal disease among the middle-aged Vietnamese population. *J Int Acad Periodontol* 2003; 5: 77-84.
3. Wahlberg A. Bio-politics and the promotion of traditional herbal medicine in Vietnam. *Health* 2006; 10: 123-147.
4. Sastravaha G, Yotnuengnit P, Booncong P, Sangtherapitikul P. Adjunctive periodontal treatment with *Centella asiatica* and *Punica granatum* extracts. A preliminary study. *J Int Acad Periodontol* 2003; 5: 106-115.
5. Lirdprapamongkol K, Krumb JP, Chokchaichamnankit D, Srisomsap C, Surarit R, Sila-Asna M, et al. Juice of *Eclipta prostrata* inhibits cell migration *in vitro* and exhibits anti-angiogenic activity *in vivo*.

- In Vivo* 2008; 22(3): 363-368.
6. Ryu S, Shin JS, Jung JY, Cho YW, Kim SJ, Jang DS, et al. Echinocystic acid isolated from *Eclipta prostrata* suppresses lipopolysaccharide-induced iNOS, TNF- α , and IL-6 expressions via NF-KB inactivation in RAW 264.7 macrophages. *Planta Med* 2013; 79: 1031-1037.
 7. Sidra S, Hussain S, Malik F. Review: Accentuating the prodigious significance of *Eclipta alba* - An inestimable medicinal plant. *Pak J Pharm Sci* 2013; 26: 1259-1266.
 8. Chokotia LS, Vashistha P, Sironiya R, Matoli H. Pharmacological activities of *Eclipta Alba* (L.). *Int J Res Dev in Pharm L Sci* 2013; 2: 499-502.
 9. Pham LA, Nguyen TB, Huynh AL. Effect of *Eclipta alba* solution in treatment of patients with gingivitis. *Scientific researches in Odontostomatology* 2006; 2: 34-38. (Article in Vietnamese).
 10. Watanabe E, Nascimento AP, Guerreiro-Tanomaru JM, Razaboni AM, de Andrade D, Tanomaru-Filho M. Antiseptic mouthwashes: in vitro antibacterial activity. *Acta Odontológica Latinoamericana* 2015; 28: 180-184.
 11. Singh B, Saxena AK, Chandan BK, Agarwal SG, Anand KK. In vivo hepatoprotective activity of active fraction from ethanolic extract of *Eclipta alba* leaves. *Indian J Physiol Pharmacol* 2001; 45: 435-441.
 12. Patel MB, Kadakia VM, Mishra SH. Simultaneous estimation of andrographolide and wedelolactone in herbal formulations. *Indian J Pharm Sci.* 2008; 70: 689-693.
 13. Rath SK, Singh M. Comparative clinical and microbiological efficacy of mouthwashes containing 0.2% and 0.12% chlorhexidine. *Dent Res J* 2013; 10: 364-369.
 14. Tsourounakis I, Palaiologou-Gallis AA, Stoute D, Maney P, Lallier TE. Effect of essential oil and chlorhexidine mouthwashes on gingival fibroblast survival and migration. *J Periodontol* 2013; 84: 1211-1220.
 15. Arumugam R, Ragupathi Raja Kannan R, Jayalakshmi J, Manivannan K, Karthikai Devi G, Anantharaman P. Determination of element contents in herbal drugs: chemometric approach. *Food Chem* 2012; 135: 2372-2377.
 16. Samy RP, Thwin MM, Gopalakrishnakone P, Ignacimuthu S. Ethnobotanical survey of folk plants for the treatment of snakebites in Southern part of Tamilnadu, India. *J Ethnopharmacol* 2008; 115: 302-312.
 17. Liu QM, Zhao HY, Zhong XK, Jiang JG. *Eclipta prostrata* L. phytochemicals: isolation, structure elucidation, and their antitumor activity. *Food Chem Toxicol* 2012; 50: 4016-4022.
 18. Ray A, Bharali P, Konwar BK. Mode of antibacterial activity of Eclalbasaponin isolated from *Eclipta alba*. *Appl Biochem Biotechnol* 2013; 171: 2003-2019.
 19. Mansoorali KP, Prakash T, Kotresha D, Prabhu K, Rama Rao N. Cerebroprotective effect of *Eclipta alba* against global model of cerebral ischemia induced oxidative stress in rats. *Phytomedicine* 2012; 19: 1108-1116.
 20. Chaudhary H, Dhuna V, Singh J, Kamboj SS, Seshadri S. Evaluation of hydro-alcoholic extract of *Eclipta alba* for its anticancer potential: an in vitro study. *J Ethnopharmacol* 2011; 136: 363-367.
 21. Panghal M, Kaushal V, Yadav JP. In vitro antimicrobial activity of ten medicinal plants against clinical isolates of oral cancer cases. *Ann Clin Microbiol Antimicrob* 2011; 20: 10-21.
 22. Lin XH, Wu YB, Lin S, Zeng JW, Zeng PY, Wu JZ. Effects of volatile components and ethanolic extract from *Eclipta prostrata* on proliferation and differentiation of primary osteoblasts. *Molecules* 2010; 15: 241-250.

