

RESEARCH ARTICLE



Wound healing activity of *Pluchea indica* leaf extract in oral mucosal cell line and oral spray formulation containing nanoparticles of the extract

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ABSTRACT

Context: *Pluchea indica* (L.) Less (Asteraceae) is an herb used as a traditional medicine for wound healing. The chemical compounds found in *Pluchea indica* leaves are phenolic acids, flavonoids, anthocyanins and carotenoids.

Objective: This study investigates the effect of *Pluchea indica* leaf ethanol extract and its nanoparticles (NPs) on cytotoxicity, cell survival and migration of human oral squamous carcinoma cell line.

Materials and methods: Cell viability was measured using MTT assay to assess the effect of *Pluchea indica* leaf extract and NPs (1–500 µg/mL) on cytotoxicity and cell survival. The effect of *Pluchea indica* leaf extract and NPs on cell migration was determined by scratch assay. The % relative migration was calculated after 24, 48 and 72 h of treatment.

Results: The sizes of *Pluchea indica* leaf extract NPs were in a range of nanometers. NPs possessed negative charge with the polydispersity index (PDI) smaller than 0.3. After the treatment for 24, 48 and 72 h, *Pluchea indica* leaf extract had IC₅₀ value of 443.2, 350.9 and 580.5 µg/mL, respectively, whereas the IC₅₀ value of NPs after the treatment for 24, 48 and 72 h were 177.4, 149.2 and 185.1 µg/mL, respectively. The % relative migration of cells was significantly increased when the cells were treated with 62.5 and 125 µg/mL of the extract and 62.5 µg/mL of NPs.

Discussion and Conclusions: NPs increased cytotoxicity of the *Pluchea indica* leaf extract, increased the migration of cells at low concentration and increased colloidal stability of the extract in an oral spray formulation.

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Introduction

Oral mucositis refers to an inflammation of the oral mucosa; it is observed in patients with cancer being treated with chemotherapy, and/or with radiotherapy involving the oropharynx (Naidu et al. 2004; Scully et al. 2004; Lionel et al. 2006; Lalla et al. 2008). This toxicity has been shown in patients receiving chemotherapy with both hematologic malignancies and solid tumours such as non-Hodgkin lymphoma, breast, lung, or colorectal cancer (Rosen et al. 2006; Niscola et al. 2007; Blijlevens et al. 2008; Seiler et al. 2014). Chemotherapeutic agents such as 5-fluorouracil (5-FU), anthracyclines and taxanes are associated with a high incidence of oral mucositis (Keefe et al. 2007). The symptoms of oral mucositis include mucosal erythema, oral ulceration, oral discomfort, severe pain and dysphagia. The severe pain and inflammation reduce the ability to intake food or drinks. Therefore, patients have to receive nutrients intravenously or by tubing, which may cause a subsequent infection. The wound is characterized as a necrotic patch that appears in the oral cavity. The healing process usually lasts from 12 to 16 days depending on epithelial proliferation rate, hematopoietic recovery, half-life of the drug and other factors affecting wound healing such as microbial infection (Naidu et al. 2004). Currently, there is no drug or agent used as a standard therapy for chemotherapy- and radiotherapy-induced oral mucositis (Plevova 1999; Saadeh 2005). The hygiene of oral cavity and teeth, colour

and moisture of oral mucosa, pain, wound and frequency of salivation are monitored, recorded and evaluated by physicians or nurses (Cheng et al. 2001; Velez et al. 2004). The patients are advised to keep their teeth and oral cavity clean, avoid spicy foods and take fluidic foods to ease swallowing. Cytokines such as growth factor G-/GMCSF, TGF-β and IL-11 available for use in oral mucositis has high cost limiting the access of most patients in developing countries (Sonis et al. 2000; Foncuberta et al. 2001; Stokman et al. 2006).

Pluchea indica (L.) Less (Asteraceae) is found in mangrove forests in several countries in Asia including Thailand. *Pluchea indica* has been used as a traditional medicine. The leaf of *Pluchea indica* tastes salty and astringent. In Thai traditional medicines, the leaf was used to treat diabetes, gallstone, haemorrhoids, fever and to heal wounds. According to the studies, *Pluchea indica* leaf extract exhibited anti-tuberculosis activity and potent anti-inflammatory activity (Mohamad et al. 2011; Buapool et al. 2013).

Regarding the application of herbal ethanol extract in the formulation, a problem lies in their tendency to precipitate due to the low solubility of the extract. To solve this problem, this study is designed to develop NPs of ethanol leaf extract of *Pluchea indica* with desirable colloidal stability in oral spray formulation. The NPs are attractive for medical purposes because it has unique features such as their surface area to mass ratio which

is much larger than that of extract particles, and surface chemistry modification. Therefore, they have ability to bind, adsorb and interact with the target cells. This study investigates wound healing activity of *Pluchea indica* leaf extract *in vitro*. The oral spray product containing *Pluchea indica* leaf extract NPs for relieving oral mucositis was formulated and its stability was tested by an accelerated stability test of heating and cooling cycles and storage for 30 days.

Materials and methods

Materials

The leaves of *Pluchea indica* were purchased from Thai medicinal herb store, Jao-Kom-Per Co. Ltd. in Bangkok, on May 2015. The plant was identified by Associate Professor Nijisiri Ruangrunsi. A voucher specimen of this plant (SWU09) was deposited at Faculty of Pharmacy, Srinakharinwirot University, Nakhonnayok, Thailand. Human oral squamous carcinoma cell line (HO-1-N-1) was purchased from Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan. Dulbecco's modified eagle's medium (DMEM) – F12 medium (1:1 mix), foetal bovine serum and penicillin-streptomycin were purchased from Gibco, MA. MTT [3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] was purchased from Bio-world, OH. Pluronic® F-127 was purchased from BASF, Ludwigshafen, Germany.

Preparation of *Pluchea indica* leaf crude extract

Pluchea indica leaves were dried in a hot air oven at 50 °C, then powdered. The powdered plant material (100 g) was extracted in Soxhlet apparatus for 7 h with 95% ethanol. The plant extract was concentrated and dried under reduced pressure using a rotary vacuum evaporator. The dried extract was weighed and the yield was calculated to be 15.6% w/w.

Preparation of *Pluchea indica* leaf extract NPs

The NPs containing *Pluchea indica* leaf extract were prepared with the solvent displacement method (Chittasupho et al. 2009). Pluronic® F127 (0.1% w/v) was used as a stabilizing agent. *Pluchea indica* leaf extract was dissolved in 2 mL of 95% ethanol and was infused into 15 mL of 0.1% Pluronic® F127 under magnetic stirring (540 rpm). The NPs were stirred to remove organic solvent overnight and kept in water until they were characterized.

Characterization of *Pluchea indica* leaf extract NPs

The size, size distribution and zeta potential of prepared NPs were evaluated with the dynamic light scattering (DLS) technique. The NPs of *Pluchea indica* leaf extract were dispersed in deionized water at a concentration of 6.7 mg/mL for size, PDI and zeta potential measurements. The effective diameter and PDI were recorded at 173° scattering angle under 25 °C (Zetasizer Nanoseries, Malvern instruments, Malvern, UK) (Kaszuba et al. 2008).

Morphology of *Pluchea indica* leaf extract NPs

The morphology of *Pluchea indica* leaf extract NPs was observed using a transmission electron microscope (TEM) (Tecnai™

S/TEM Family, FEI Company, OR). A drop of nanoparticle suspension was deposited on a copper grid with carbon film and dried at room temperature. After another drop was repeatedly deposited on the grid, samples were allowed to dry at room temperature before investigation.

Colloidal stability study of *Pluchea indica* leaf extract NPs in deionized water and cell culture medium

The colloidal stability of *Pluchea indica* leaf extract NPs was determined by measuring size, size distribution and zeta potential. The NPs were prepared by using the above procedure. The NP dispersion (6.7 mg/mL) was sealed and stored in deionized water at 4 °C. After 1, 2, 3 and 4 weeks, samples were analyzed using DLS. The NPs dispersed in cell culture medium were characterized for size, and PDI to determine the stability of NPs when exposed to cell culture.

Formulation of oral spray solution containing *Pluchea indica* leaf extract or *Pluchea indica* leaf extract NPs

The oral spray solution base was prepared by mixing 10 mL of glycerine, 20 mL of sorbitol 70 in 55 mL of deionized water. Then, 0.1 g of sodium benzoate was weighed and added into the above solution. The 6.25 mg of *Pluchea indica* leaf extract or *Pluchea indica* leaf extract NPs were mixed with the oral base, followed by the addition of peppermint as a flavouring agent. The volume of the total solution was adjusted to 100 mL. The size, PDI and pH values of *Pluchea indica* leaf extract particles or *Pluchea indica* leaf extract NPs in the oral solution were measured. The obtained oral solutions containing *Pluchea indica* leaf extract and *Pluchea indica* leaf extract NPs were further subjected to the accelerated stability test of heating-cooling cycles for six cycles and stored isothermally at room temperature, 4 °C and 45 °C for 30 days.

In vitro cell viability study

HO-1-N-1 cells were cultured in DMEM-F12 (1:1) medium supplemented with 10% FBS and 1% penicillin-streptomycin, and maintained at 37 °C in a humidified incubator containing an atmosphere of 5% CO₂. HO-1-N-1 cells were detached by trypsinization and transferred from the culture flask to a 96-well plate. The initial concentration of cells added into the 96-well plate (8000 cells/well) was counted by using a haemocytometer. The cells were allowed to grow in the 96-well plate until 80% confluent. *Pluchea indica* leaf extract or *Pluchea indica* leaf extract NPs was added to the wells at defined concentrations (1–500 µg/mL), and incubated for 2 h at 37 °C. The extract or NPs were removed and cells were further incubated with a culture medium for 24, 48 and 72 h. For a negative control experiment, cells were not treated and incubated with the culture medium. The cells were washed three times with phosphate buffered saline (PBS), and then incubated in the culture medium containing MTT (0.5 mg/mL) for 2 h at 37 °C. After incubation, the media were removed, and 100 µL of DMSO was added to solubilize the water insoluble formazan product which was a metabolite of a tetrazolium salt (Twentyman & Luscombe 1987). The absorbance was measured at 550 nm and at 650 nm for reference. Percentage of cell viability was calculated as the ratio of mean absorbance of

triplicate readings with respect to mean absorbance of control wells. Dose-response curves were plotted to determine half maximal inhibitory concentrations (IC₅₀) for *Pluchea indica* leaf extract and nanoparticles using the GraphPad Prism5 (GraphPad Software, San Diego, CA).

In vitro scratch assay

In vitro cell migration can be measured by scratch assay which is convenient, inexpensive and widely used to mimic cell migration during wound healing *in vivo* (Liang et al. 2007). HO-1-N-1 cells were plated in a 24-well plate (15,000 cells/well) and cultured at 37°C, 5% CO₂ for 48 h. The 100% cell confluent was observed before the scratch assay was performed. A sterile 200 µL pipette tip was used to make a straight scratch on the monolayer of cells, simulating a wound. *Pluchea indica* leaf extract dissolved in serum-free DMEM-F12 at concentrations of 62.5 and 125 µg/mL were added into the wells and incubated for 2 h at 37°C, 5% CO₂. For a negative control, cells were not treated and incubated in serum free-DMEM-F12. After incubation, the cells were washed with serum free-DMEM-F12 three times, incubated with serum free-DMEM-F12, and the images of cells were captured under an inverted microscope equipped with a camera (TS100 Nikon, Japan) after 24, 48 and 72 h. The % relative migration of HO-1-N-1 cells treated with the extract, NPs and control were calculated according to the following equation.

% Relative migration

$$= \frac{\text{Area between cells}_{0\text{h}} - \text{Area between cells}_{24\text{ or }48\text{ or }72\text{ h}}}{\text{Area between cells}_{0\text{h}}} \times 100$$

Evaluation of microbiological stability of oral spray containing *Pluchea indica* leaf extract NPs

Counts for *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans* and *Aspergillus niger* were determined according to manufacturer's instruction of 3MTM PetrifilmTM *E.coli*/Coliform count plates, 3MTM PetrifilmTM Staph Express count plates, 3MTM PetrifilmTM Aerobic count plates and 3MTM PetrifilmTM yeast and mold count plates. The diluted oral spray (1 mL) containing *Pluchea indica* leaf extract NPs (1:1000), which were freshly prepared, stored at 4°C and 45°C for 30 and 120 days, was dispensed onto the centre of PetrifilmTM plates. The 3MTM PetrifilmTM Aerobic Count Plate, 3MTM PetrifilmTM *E. coli*/Coliform Count Plate, and 3MTM PetrifilmTM Staph Express Count Plate were sealed and incubated at 37°C for 24 h. The 3MTM PetrifilmTM Yeast and Mold count plate was sealed and incubated at 37°C for 96 h. After an incubation, the 3MTM PetrifilmTM count plates were counted and compared with the plates containing sterile buffered sodium chloride-peptone solution pH 7.0 used as a negative control and *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans* and *Aspergillus niger* used as a positive control.

Statistical analysis

Statistical evaluation of data was performed using an analysis of variance (one-way ANOVA). A value of $p < 0.05$ was accepted.

Results

Characterization and colloidal stability of *Pluchea indica* leaf extract NPs

The mean particle size, PDI and zeta potential values of *Pluchea indica* leaf extract NPs after fresh preparation and after storage periods of 1, 2, 3 and 4 weeks are presented in Table 1. The sizes of NPs after fresh preparation and after storage for 1, 2, 3 and 4 weeks were in a range of 259–306 nm. The PDI of NPs after fresh preparation and after storage for 1, 2, 3 and 4 weeks were in a range of 0.125–0.173. The zeta potential values of NPs after fresh preparation and after storage for 1, 2, 3 and 4 weeks were in a range of –15.8 to –21.0 mV. The size and PDI of *Pluchea indica* leaf extract NPs after incubation with a cell culture medium containing serum for 24 h were 277.2 ± 8.0 nm and 0.260 ± 0.032, respectively.

Morphology of *Pluchea indica* leaf extract NPs

Figure 1 showed the TEM image of *Pluchea indica* leaf extract NPs prepared by solvent displacement method. According to TEM image, it was clear that NP size was consistent with the results obtained from DLS technique. TEM image of *Pluchea indica* leaf extract NPs revealed that these particles were spherical in shape and well-separated from each other.

Table 1. Size, PDI and zeta potential values of *Pluchea indica* leaf extract NPs after storage in deionized water at 4°C for 4 weeks.

	Size (nm)	PDI	Zeta potential values (mV)
Freshly prepared	259.3 ± 3.3	0.125 ± 0.023	–15.8 ± 0.8
1 week	272.5 ± 6.9	0.132 ± 0.020	–20.0 ± 1.6
2 weeks	284.7 ± 1.1*	0.160 ± 0.010	–18.0 ± 0.3
3 weeks	297.5 ± 13.9*	0.173 ± 0.038	–21.0 ± 0.9
4 weeks	306.3 ± 8.8*	0.163 ± 0.029	–18.8 ± 3.9

*Indicates $p < 0.05$ compared with freshly prepared NPs.

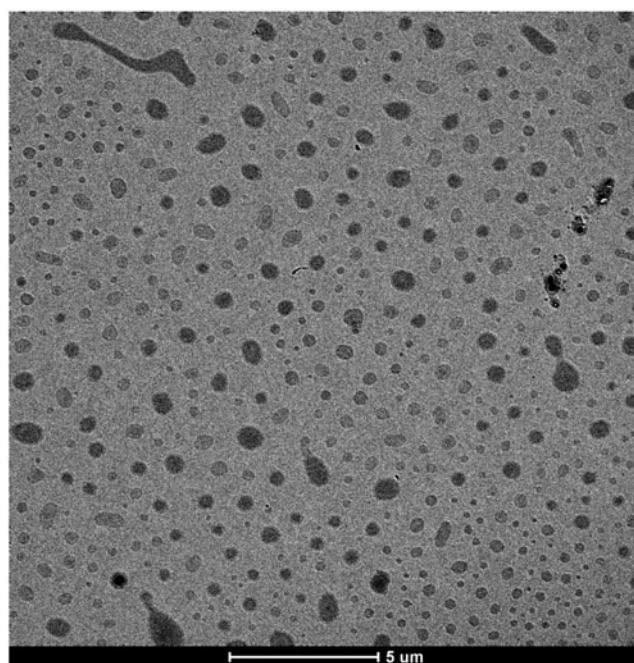


Figure 1. TEM image of *Pluchea indica* leaf extract nanoparticles.

Pluchea indica leaf extract and NPs increased cell viability

Pluchea indica leaf extract and *Pluchea indica* leaf extract NPs showed no cytotoxic effects at concentrations up to 125 and 62.5 µg/mL, respectively. After incubation of cells with *Pluchea indica* leaf extract at concentrations of 1–125 µg/mL for 24, 48 and 72 h, the viability of the treated cells was in the range of 100–110%, 100–112% and 114–129%, respectively (Figure 2). The viability of cells treated with 250 and 500 µg/mL of *Pluchea indica* leaf extract were significantly decreased to 57–69% and 4–15%, respectively. The viability of cells treated with 1–62.5 µg/mL of *Pluchea indica* leaf extract NPs were in the range of 101–120%, 109–118% and 107–129% after incubation for 24, 48 and 72 h, respectively (Figure 2). The viability of cells treated with 125, 250 and 500 µg/mL of *Pluchea indica* leaf extract NPs were significantly decreased to 35–54%, 3–12% and 3–8%,

respectively. The IC₅₀ value of *Pluchea indica* leaf extract after incubation with cells for 24, 48 and 72 h were 443.2, 350.9 and 580.5 µg/mL, whereas the IC₅₀ of *Pluchea indica* leaf extract NPs incubation for 24, 48 and 72 h were 177.4, 149.2 and 185.1 µg/mL, respectively. These results suggested that *Pluchea indica* leaf extract NPs increased cytotoxicity of the extract.

Pluchea indica leaf extract and NPs enhanced oral mucosal cell migration

Cell proliferation and migration play an important role in the wound healing process. The scratch assay has been widely used to study the *in vitro* wound closure effect of plant extract. In this study, *Pluchea indica* leaf extract and *Pluchea indica* leaf extract NPs at 62.5 and 125 µg/mL were selected to study the effect of the extract and NPs on HO-1-N-1 cell migration. Although the NPs showed cytotoxicity to the cells at a concentration of 125 µg/mL, NPs at this concentration was chosen to compare the activity on cell migration with the unprocessed extract. The results showed that % relative migration of cells treated with *Pluchea indica* leaf extract, *Pluchea indica* leaf extract NPs and untreated

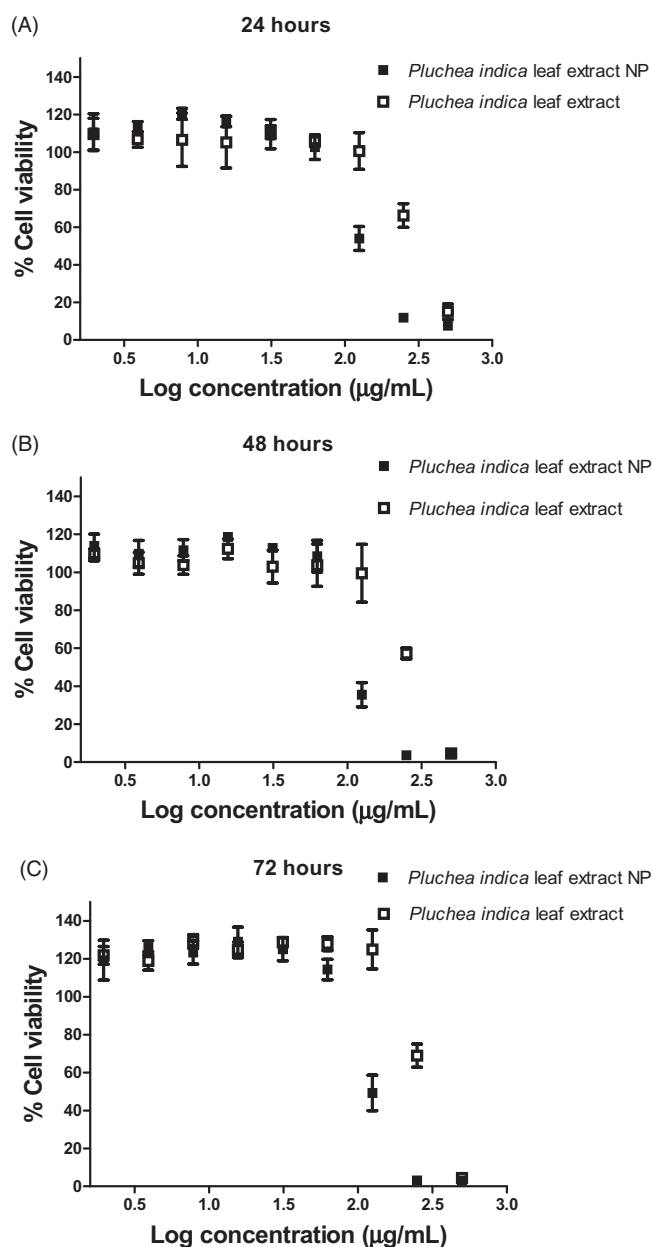


Figure 2. Effect of *Pluchea indica* leaf extract and *P. indica* leaf extract NPs on viability and proliferation of HO-1-N-1 cells. The cells were treated with the indicated concentration of *Pluchea indica* leaf extract or *Pluchea indica* leaf extract NPs for 2 h, followed by incubation in a serum free medium for (A) 24 h, (B) 48 h and (C) 72 h.

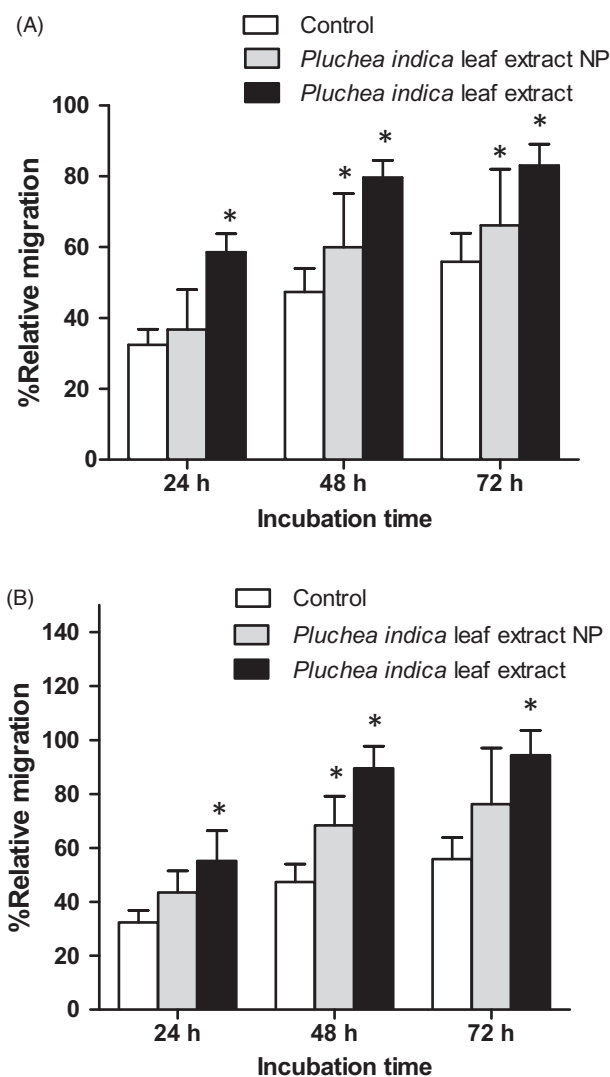


Figure 3. Migration of cells in serum free medium in the absence or presence of (A) 62.5 µg/mL and (B) 125 µg/mL *Pluchea indica* leaf extract and *Pluchea indica* leaf extract NPs. **p* < 0.05 indicates a statistically significant difference between untreated and extract or NP treated cells.

cells increased when increasing incubation time, e.g., 24, 48 and 72 h. The % relative migration of cells treated with *Pluchea indica* leaf extract at 62.5 and 125 µg/mL for 24, 48 and 72 h were significantly higher than that of untreated cells in the control (Figure 3). NPs containing *Pluchea indica* leaf extract significantly increased migration of HO-1-N-1 cells at a concentration of 62.5 µg/mL after incubation with cells for 48 and 72 h, whereas at 125 µg/mL, NPs enhanced migration of cells only at 48 h. The results suggested that *Pluchea indica* leaf extract at 62.5 and 125 µg/mL can increase the migration of HO-1-N-1 cells in dose and time dependent manner, while the optimal concentration of NPs to induce migration was 62.5 µg/mL. Micrographs showing the coverage of scratched wounds by HO-1-N-1 in the absence or presence of *Pluchea indica* leaf extract and *Pluchea indica* leaf extract NPs at 62.5 µg/mL were shown in Figure 4.

***Pluchea indica* leaf extract NPs increased colloidal stability of particles in oral spray formulation**

Characterization of NPs in oral spray solution by measuring the size of *Pluchea indica* extract and *Pluchea indica* extract NPs showed that the extracts and NPs had diameters of 2.5 µm and 680 nm, respectively. The size of *Pluchea indica* leaf extract particles were significantly larger than that of *Pluchea indica* extract NPs prepared by the solvent displacement method at every heating-cooling cycle (Figure 5(A)). Figure 5(B) exhibited measured

size distribution of *Pluchea indica* leaf extract and *Pluchea indica* leaf extract NPs. The results showed that particle size distribution of the extract was quite wide compared to the particle size distribution of NPs. The pH of the oral spray formulation containing *Pluchea indica* leaf extract and *Pluchea indica* leaf extract NPs were in a range of 5.7–5.9 and 5.5–5.8, respectively (Figure 5(C)).

Pluchea indica leaf extract NPs were stored in oral spray formulation at room temperature, 4 °C and 45 °C for 30 days. The size of NPs when stored at room temperature were not changed from that of freshly prepared NPs, whereas the size of NPs stored at 4 °C and 45 °C were significantly larger than the freshly prepared NPs indicating instability of NPs (Table 2). Similarly, the PDI of NPs stored at room temperature was close to the freshly prepared NPs and was in an acceptable range. The PDI of NPs stored at 4 °C and 45 °C were significantly increased and were not acceptable. Thus, the oral spray formulation containing NPs should be stored at room temperature to ensure the stability of the NPs.

The oral spray containing Pluchea indica leaf extract NP showed no microbial contamination

There were no colonies observed on the 3M™ Petrifilm™ *E. coli*/Coliform count plates, 3M™ Petrifilm™ *Staph Express* count plates, 3M™ Petrifilm™ Aerobic count plates and 3M™ Petrifilm™ Yeast and Mold count plates containing the negative

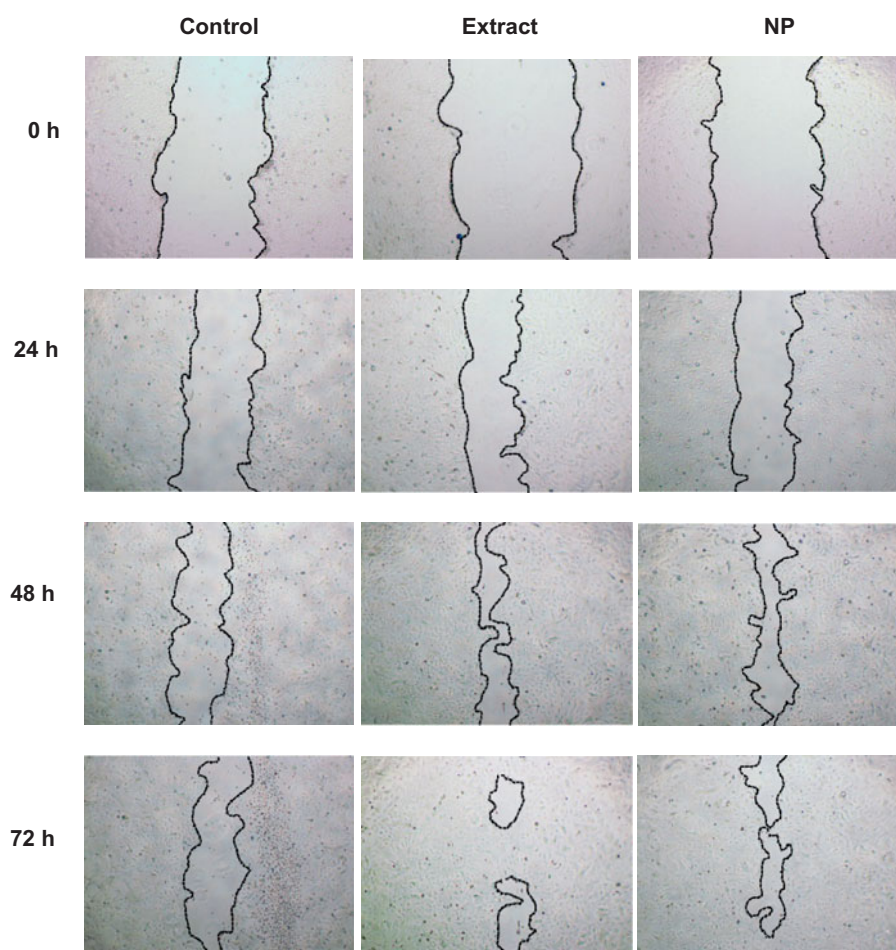


Figure 4. Micrographs showing the coverage of scratched wounds by HO-1-N-1 in the absence or presence of *Pluchea indica* leaf extract and *Pluchea indica* leaf extract NPs. (A) Negative control (serum free medium); (B) *Pluchea indica* leaf extract at 62.5 µg/mL; and (C) *Pluchea indica* leaf extract NPs at 62.5 µg/mL. The dash lines marked the boundaries of the scratched wounds.

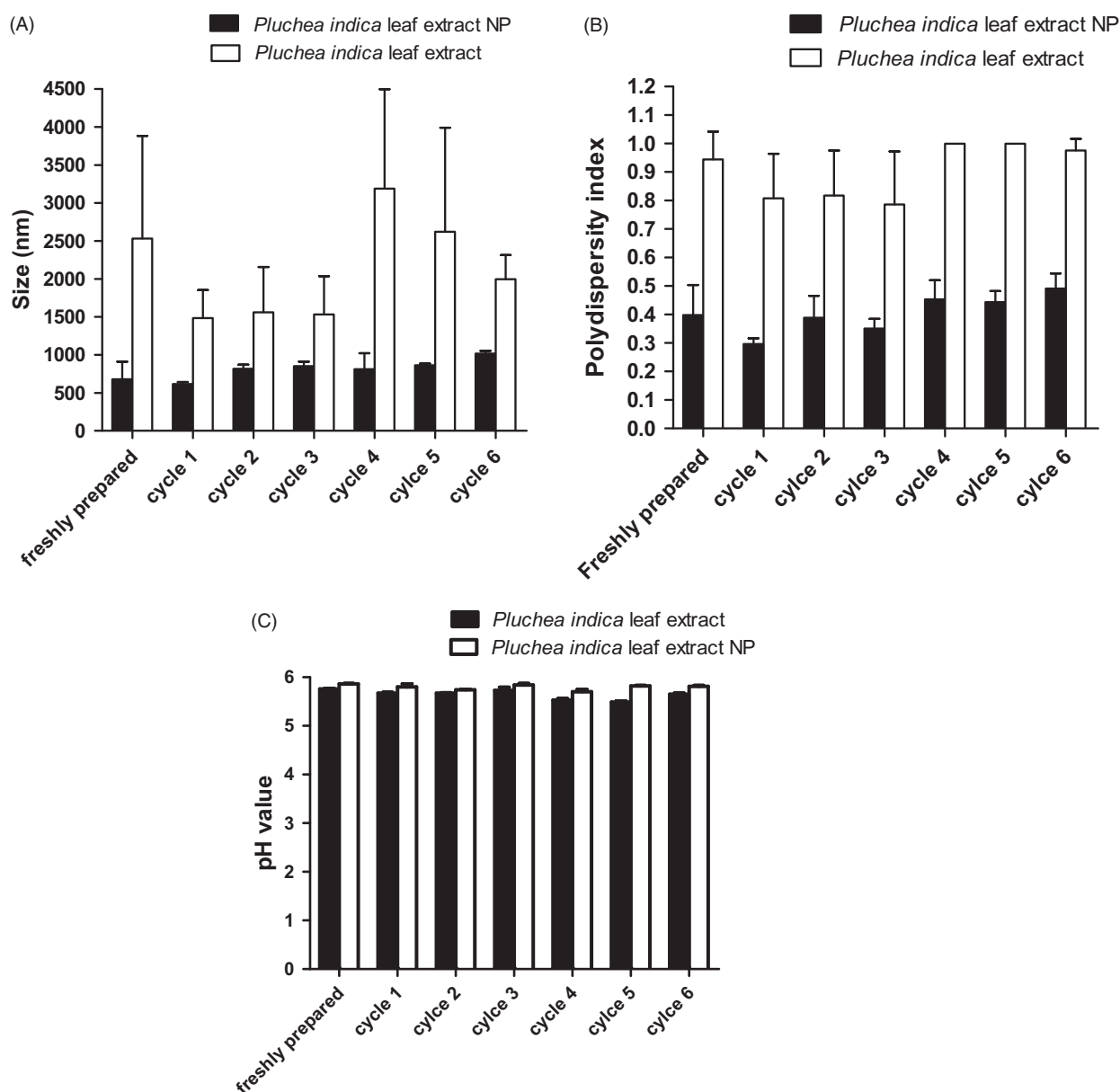


Figure 5. Characterization of unprocessed particles in *Plucheia indica* leaf extract and *Plucheia indica* leaf extract NPs in oral spray formulation after tested accelerated stability test of heating-cooling cycles for six cycles. (A) Hydrodynamic diameter, (B) PDI, (C) pH of oral spray formulation.

Table 2. Size, PDI and pH of *Plucheia indica* leaf extract NPs before and after storage in oral spray formulation at room temperature, 4 °C and 45 °C for 30 days.

	Size (nm)	PDI	pH
Freshly prepared	680.3 ± 230.6	0.398 ± 0.106	5.55 ± 0.02
Room temperature	627.8 ± 72.9	0.307 ± 0.021	5.69 ± 0.02
4 °C	1612.5 ± 122.3	0.753 ± 0.147	5.79 ± 0.01
45 °C	1253.4 ± 391.6	0.647 ± 0.253	5.53 ± 0.04

control (sterile buffered sodium chloride-peptone solution) and the oral spray containing *Plucheia indica* leaf extract NPs which were freshly prepared or stored at 4 °C and 45 °C for up to 30 and 120 days, indicating that there was no microbial contamination with negative control and samples. In contrast, the blue, red-violet, red colonies and blue-green colonies were present on the entire growth area of 3MTM PetrifilmTM *E. coli*/Coliform count plate, 3MTM PetrifilmTM *Staph* Express count plate, 3MTM PetrifilmTM Aerobic count plate and 3MTM PetrifilmTM Yeast and Mold count plate containing positive control (*Staphylococcus*

aureus, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans* and *Aspergillus niger*), respectively, indicating that there was microbial contamination with the positive control.

Discussion

The effect of ethanol leaf extract of *Plucheia indica* and NPs containing *Plucheia indica* leaf extract on promoting oral wound healing *in vitro* was evaluated in this study. In the proliferation phase of the wound healing process, angiogenesis, collagen deposition, granulation tissue formation, re-epithelialization and wound contraction occur (Midwood et al. 2004; Safferling et al. 2013). Re-epithelialization of the epithelium occurs by proliferation and migration of epithelial cells atop of the wound bed providing cover for the new tissue (Safferling et al. 2013). In this study, the proliferation and migration of oral mucosal cells treated with *Plucheia indica* extract and NPs containing *Plucheia indica* leaf extract were assessed to investigate the *in vitro* wound

healing properties. The anti-inflammatory, anti-nociceptive, anti-tuberculosis and antioxidant activities of *Pluchea indica* leaf ethanol extract were reported (Sen et al. 1993; Mohamad et al. 2011; Buapool et al. 2013; Suriyaphan 2014). In Asian traditional medicines, a poultice of leaves is applied externally on top of the wound to treat ulcers and sooth sores (Wiat 2006). In Thai folk medicine, the leaf of *Pluchea indica* is used as astringent to heal ulcers. Phytochemical compounds found in *Pluchea indica* leaf are phenolic acids, flavonoids, e.g., quercetin, kaemferol, myricetin, anthocyanins and carotenoids (Suriyaphan 2014).

The wound healing process consists of haemostasis, inflammation, proliferation and tissue remodelling. The proliferative phase is characterized by epithelial proliferation and migration over the matrix within the wound (Guo & Dipietro 2010). *Pluchea indica* leaf extract and its NPs were allowed to expose to the oral mucosal cells for only 2 h, and the cell viability or cell survival were measured for 24, 48 and 72 h after the incubation. This condition mimics the short contact time of the oral spray in the mouth and allows the extract or NPs exposed to the cells gradually transport into the cells. The incubation time of 2 h and the dose tested were considered optimal because the results showed the % cell viability was in the range of less than 50% to more than 100%, which the IC_{50} value can be calculated. In this study, the results of the scratch assay and MTT assay indicated that the ethanol extract of *Pluchea indica* leaf possesses wound healing activity, based on the ability of *Pluchea indica* leaf extract and NPs containing *Pluchea indica* leaf extract to enhance the cell survival of oral mucosal cell line and accelerated wound closure *in vitro*. Based on these properties, *Pluchea indica* leaf extract has a potential for wound healing intended for use in the oral cavity. However, the isolation and the mechanism of wound healing have to be further investigated.

The development of NPs for herbal drug delivery has a number of advantages including enhancement of solubility and pharmacological activity, improving stability, sustaining the release of the drug and protecting the drug from physical and chemical degradation (Ajazuddin 2010). In this study, NPs containing *Pluchea indica* leaf extract were fabricated with the solvent displacement method for the first time. The particle size is an important characteristic to confirm the production of nanometre sized particles. The statistical analysis revealed that size of NPs was significantly increased after storage in deionized water for 2, 3 and 4 weeks at 4 °C. However, the sizes of NPs were in a nanometre range and were acceptable. The PDI and zeta potential were not changed during storage for 1, 2, 3 and 4 weeks at 4 °C. The PDI of NPs was less than 0.5 indicating that the size distribution of NPs is acceptable (Kaur et al. 2008). Zeta potential is an electrostatic potential caused by the presence of a charge on the NP surface and prevents particle aggregation during storage. The result shows that zeta potential of *Pluchea indica* leaf extract NPs is negative which creates a repulsive electrostatic double layer between particles which may stabilize the NPs in the oral spray formulation (Moore et al. 2015). The effects of components of the cell culture medium on the size and PDI of NPs were evaluated. The results showed that the size and PDI of NPs were increased but were in nanometre-range, which might be due the interaction of NPs with the components of the cell culture medium such as protein supplemented and electrolytes (Moore et al. 2015). Overall, the nanometer particle size, size distribution and surface charge of *Pluchea indica* leaf extract NPs can be controlled with a coating of poloxamer and solvent displacement method is suitable to produce NPs for delivering *Pluchea indica* leaf extract.

The results of the colloidal stability study of *Pluchea indica* leaf extract and NPs containing *Pluchea indica* leaf extract in oral spray formulation revealed that particles of *Pluchea indica* leaf extract were polydisperse at all heating-cooling cycles, whereas *Pluchea indica* leaf extract NPs prepared with a coating of poloxamer had significantly lower PDI suggesting narrow NP size distribution. The NPs containing *Pluchea indica* leaf extract increased the colloidal stability of the extract particles, compared with unprocessed extract particles when tested under accelerated stability test of heating-cooling cycles of six cycles and stored at room temperature for 30 days. These results indicated that *Pluchea indica* leaf extract contained in an oral spray solution formed agglomerates with large hydrodynamic diameters and wide size distribution, and NPs of *Pluchea indica* leaf extract increased the colloidal stability of *Pluchea indica* leaf extract in an oral spray formulation.

In this study, we aimed to compare the effect of *Pluchea indica* leaf extract with NPs at the same concentrations tested. NPs containing *Pluchea indica* leaf extract, at a concentration of 62.5 µg/mL, significantly enhanced oral mucosal cell migration after incubation for 48 and 72 h when compared to untreated cells. The acceleration of cell migration, when treated with *Pluchea indica* leaf extract NPs at a concentration of 125 µg/mL, was observed only at 48 h after incubation, whereas at 72 h, the % relative migration of the cells was not statistically higher than that of the control. The reduced migration rate of cells treated with NPs compared to the extract might be due to the cytotoxicity of NPs at high concentration. The process of cell migration is complex which involves hundreds of molecules. The factors affecting the promotion of directional migration include microtubule dynamics, cellular polarity machinery, receptor signalling, integrin trafficking, integrin-coreceptors and actomyosin contraction and lamellipodial protrusions (Horwitz & Webb 2003; Petrie et al. 2009). Therefore, deregulation and fail-safe may occur. The activity of NPs was less potent compared to the extract itself, which might be due to the sustained release of the extract from NPs. The release rate of the extract might be retarded by the presence of poloxamer 407, acting as a stabilizer, on the surface of NPs. The release of the extract into the medium might be consecutive to either drug diffusion into the coated poloxamer or poloxamer dissolution. The effect of poloxamer 407 on the sustained release of the drug from NPs has been previously reported (Redhead et al. 2001; Dumortier et al. 2006). These results suggested that NPs at 62.5 µg/mL has better potential for wound healing than a concentration of 125 µg/mL.

The NPs, in this case, was aimed to be administered at the oral mucosa of the buccal. The buccal oral mucosa is comprised of stratified epithelium over the lamina propria, which consists of fibroblasts and connective tissue, blood vessels, macrophages and extracellular matrix. After the administration of chemotherapy for 10 days or at cumulative radiation doses of about 30 Gy, the epithelium disintegrates and ulceration occurs. At this phase, there is an infiltration of inflammatory cells such as macrophage, plasma and mast cells. Therefore, the NPs should be applied to the epithelial cells before or right after the administration of chemotherapy and radiation to promote the proliferation and migration of the new cells and to avoid the uptake of the macrophage during the ulcerative stage.

Conclusions

NPs containing *Pluchea indica* leaf extract are appropriate for use as a drug delivery system, which shows the wound healing

activity at 62.5 µg/mL and increase colloidal stability of the extract in oral spray formulation.

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Disclosure statement

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