Time- and Dose-Dependent Effects of Centella Asiatica Ethanolic Extract Encapsulated Into Chitosan Nanoparticles on Collagen III Synthesis and Proliferation in Human Dermal Fibroblasts

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Abstract
Natural aging process comes with the decline of skin cells renewal and collagen III production. *Centella asiatica* L. (CA) extracts are natural products which were shown to promote fibroblast proliferation during wound healing. In
In this study, we evaluated therapeutic potential of ethanolic CA extract (CAEE) when encapsulated into chitosan nanoparticles (CNP) by ionotropic gelation. Effects of CAEE-loaded CNP (CAEE+CNP) on the proliferation rate and collagen III synthesis of human dermal fibroblasts (HDF) were evaluated and compared against un-encapsulated CAEE. Proliferation rate of HDF was assessed with 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT assay), while ELISA was performed to evaluate Collagen III synthesis after 24, 48, or 72 hours incubation. For 24h incubation period, CAEE+CNP stimulates significantly greater HDF proliferation than CAEE alone. Moreover, in general we observed higher proliferation rate and collagen III synthesis on fibroblasts stimulated with CAEE+CNP as compared to CAEE alone. Thus, nano-encapsulation is important to maximize therapeutic potential of CAEE. In the future, the proposed formulation of CAEE-loaded CNP may be explored further as effective herbal agents in dermacosmetics for wound healing and anti-aging.

**Keywords:** *Centella asiatica* extract, chitosan nanoparticles, fibroblast, proliferation, MTT assay, Collagen III

**Introduction**

For hundreds of years, *Centella asiatica* (L.)Urb. (CA) has been widely used in Malaysia, India and Nepal as traditional herbal medicine [1,2]. CA which is more commonly known as *pegaga* in Malaysia, *pegagan* in Indonesia or *pennywort / gotu kola* in America [1,3,4], can be used as “longevity herb” and herbal anti-aging cosmetic [2-6]. Additionally, it has served various other medicinal purposes including wound healing, treatment of asthma, ulcers, leprosy, lupus erythematosus, psoriasis, vein diseases and even cancer treatment [7-11]. Although CA extracts (CAE) possess high potential of biological activities, their clinical usage is limited due to poor physical stability and high hygroscopy; powder extracts are promptly liquefied within a few minutes when exposed to normal environment. Therefore, the development of nanoparticles, which entrap the extract and protect it from external moisture, could lead to stabilization [12]. The biologically active ingredients in CA are triterpenes, namely asiatic acid, madecassic acid, asiaticoside and madecassoside[8,9]. Asiaticoside isolated from CA promotes fibroblast proliferation and extracellular matrix synthesis in wound healing [11] by increasing collagen formation and angiogenesis [13,14].

Chitosan is derived from the shells of crustaceans, including shrimps. It is produced by deacetylation of chitin, the structural element of the crustacean exoskeleton and the cell walls of fungi [15]. Chitosan is a polysaccharide, which consists of acetylated and deacetylated units. Its acetylated units are formed by N-acetyl-D-glucosamine while its deacetylated units are composed of β-(1,4)-D-glucosamine. Its hypoallergenic and natural antibacterial properties makes it useful as wound healing agent [16,17]. Furthermore its biocompatibility,
Time- and dose-dependent effects

Biodegradability and low costs suggest versatile biomedical applications extending to anti-aging skin care (both as carrier and active compound) [18-20].

Skin aging is a complex process, controlled by both, genetic determination (intrinsic, chronologic aging) and the influence from external factors (extrinsic aging). Skin aging leads to various modifications in cells and tissue structure. A number of studies have shown reduced proliferation capacity of keratinocytes and fibroblasts as a result of aging process. Aside from the cell renewal rate, the synthesis of collagen III in skin fibroblasts also decreases with aging process [21].

Cosmeceutical anti-aging formulations contain active ingredients to achieve local biological effects for cell renewal and synthesis of extra cellular matrix without adverse side effects [21,22]. Thus far, retinoic acid (RA) is widely used in cosmetics for skin aging treatment, but causes regular side effects such as skin irritation [23]. An innovative concept for cosmetic anti-aging is to prevent skin aging or delay aging process with minimal side effects. [21,23].

Liposomes, solid lipid nanoparticles of 100-300 nm in diameter are nanocarrier systems which have been widely studied for drug and cosmetic delivery [25,26]. Nanoparticles have unique physical properties for skin penetration and cellular internalization, making them ideal for usage in various skin care products [27]. Therefore, this study incorporate nanoparticle technology to maximize therapeutic benefits of CA, based on chitin-chitosan copolymer nanoparticles [28,29].

The major aim of this study is to evaluate the in vitro activity of CA ethanolic extract (CAEE) compared to CAEE-encapsulated chitosan nanoparticles (CNP) at various concentrations. More specifically, activity of asiaticoside as an active compound of CA is evaluated on inducing human dermal fibroblast (HDF) proliferation, as compared to retinoic acid. Collagen III synthesis in HDF after incubation with CAEE and CAEE+CNP at various times is another parameter evaluated.

Methods

Cell isolation and culture

Human dermal fibroblast cultures were established from the foreskin (preputium) of an eight year-old circumcised boy. NHDFs were cultured using RPMI medium supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin, and kept in humidified incubators (5% CO2, 37°C). For all experiments, cells were used between the second and seventh passages.

Nanoparticle fabrication

Firstly, ethanolic extracts from Centella asiatica, Tawangmangu strain were separated (CAEE). Then CAEE encapsulation into CNPs was done by
ionotropic gelation, as previously studied [12]. Here, chitosan with a deacetylation degree of more than 75% (Sigma-Aldrich) was used. For all experiments, CAEE+CNP formulation is prepared fresh to obtain true therapeutic benefits.

**CAEE+CNP effect on fibroblast proliferation**

One day after re-seeding, NHDFs were treated with various concentrations of *Centella asiatica* ethanolic extract (CAEE), CAEE+CNP, Asiaticoside, and Retinoic acid. Untreated cells were used as negative control. In previous study, effects from CAEE, CNP, and CAEE + CNP had been compared.

At various timepoint after treatment introduction (i.e. 24, 48, and 72hr), fibroblast proliferation (and viability) was evaluated through MTT assay, with previously published protocol [17]. Briefly, water-soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was used as substrate. As it enters into the fibroblast cells, mitochondrial succinate dehydrogenase under the presence of cellular NADH forms insoluble purple formazan. At varous timepoint, absorbance measurement at 570nm was taken to correlate NADH concentration present with cell density. MTT experiment was repeated three times for each treatment conditions.

**CAEE+CNP effect on Type III Collagen Synthesis**

One day after re-seeding, NHDFs were treated with various concentrations of *Centella asiatica* ethanolic extract (CAEE) and CAEE+CNP. At various timepoints (i.e. 24, 48, and 72hr), collagen III synthesis of treated cells was evaluated using ELISA and compared against untreated control. For statistical evaluation, one-way ANOVA was used with Tukey's post hoc analysis. Significance was set to p<0.05 and for highly significant p<0.001.

**Results and Discussion**

![Figure 1. Transmission electron micrograph (TEM) of CAEE+CNP, magnification set at x100,000; scale bar = 50.0 nm.](image-url)
TEM imaging showed that the proposed encapsulation of ethanolic extract of *Centella asiatica* into chitosan yielded nanoparticles with diameters in the range of 50 nm (Fig.1).

Figure.2. Proliferative activity of fibroblasts following 24hr incubation of unencapsulated *Centella asiatica* ethanolic extract (CAEE), blank chitosan nanoparticles (CNP), and proposed formulation (CAEE+CNP), as compared to un-treated control. The concentrations of 6.25 mg/mL and 3.125 mg/mL were accomplished by dilution steps from 12.5 mg/mL.

In our preliminary experiments shown in Fig.2, we compared the activities of CAEE, CNP, and CAEE+CNP at three different concentrations on the proliferation of HDF within 24 hours. Surprisingly, we observed an interesting pattern whereby the middle concentration (6.25 mg/mL) exerts better proliferative effects than both 3.125 and 12.5 mg/mL. Interestingly, blank chitosan nanoparticles exerted greater proliferative effect than those with CAEE.

Previously, Leonida *et al.* demonstrated application of chitosan as an antimicrobial, wound-healing, and anti-aging compound. The authors reported that “nano-sizing” enhanced effectiveness [25]. This supports the notion that materials may undergo major changes in their properties following size reduction to nanometer scale. In view of this, nanotechnology could re-invent the field of cosmetic dermatology, to greatly enhance therapeutic efficacy of substances currently used [30].

Nanoparticles delivery of active molecules also carries additional benefit to protect leakage of molecules, thereby reducing non-specific, undesirable side effects. A study utilizing idebenone antioxidant showed that chitosan and N-carboxymethyl chitosan incorporation significantly decrease topical irritation which is important for their nasal application [31]. Moreover, Okonogi *et al.* observed that *Centella asiatica* extracts loaded in chitosan-alginate nanoparticles retains their functionality better. In other words, the nanocarriers helped to stabilize the active CA extract [12].
In this study we used chitosan with a degree of deacetylation of more than 75%. A study by Howling et al. demonstrated that chitosan with a relatively high degree of deacetylation strongly stimulated fibroblast proliferation while chitosan with lower levels of deacetylation showed less activity [17].

After this preliminary study, we were interested in the time-dependent effect of CAEE+CNP on fibroblast proliferation. In this case, proliferation activity was traced for longer incubation period (24, 48, and 72 hours). In addition, wider range of treatment concentrations was used in order to find conditions which optimize therapeutic efficacy.

![Figure 3](image_url)

**Figure 3.** Proliferative activity of fibroblasts after treatment with *Centella asiatica* ethanolic extract (CAEE), *Centella asiatica* ethanolic extract encapsulated into chitosan nanoparticles (CAEE+CNP), asiaticoside, and retinoic acid for 24, 48, and 72hr.

As presented in Fig.3, CAEE and CAEE+CNP exerted similar proliferative effects in almost all of the conditions tested. Only at the concentration of 6.25 mg/mL, does CAEE+CNP induce greater proliferative activity than CAEE (160% to 120% of untreated control; respectively). At the same time, highest proliferative activity was observed at this concentration. This result is consistent with our preliminary study (Fig.2), where the same concentration of 6.25 mg/mL also showed the greatest effect towards cell proliferation.

Importantly, we once again observed that the proliferative effect of CAEE+CNP is not strictly dose-dependent. Furthermore we noted that asiaticoside showed a similar concentration profile as CAEE+CNP with its highest effect achieved at 6.25mg/mL. Meanwhile, RA demonstrated a distinct concentration profile with greatest effects at 50 and 6.25 mg/mL. This is quite surprising, as previously Song et al. showed that madecassoside, another bioactive
compound of *Centella asiatica* inhibited proliferation of keloid fibroblasts in a time and concentration-dependent manner (34).

In general, the proliferative effect vs concentration trend differs between 24 and 48hr stimulation. For 48 hours timepoint, asiaticoside exhibits the strongest effect among all the compounds on fibroblast proliferation at 6.25 mg/mL (about 150% of control and p<0.001 against all other compounds). The strongest effect of CAEE+CNP is seen at the lowest concentration (3.125 mg/mL; about 140% of control), CAEE alone is at 25 mg/mL, and retinoic acid at the lowest concentration of 3.125 mg/mL. At this timepoint, all the other values are in very similar from control (insignificant difference). Crucially, it should be noted that retinoic acid generally exerts higher proliferative activity at 48 hours than at 24 hours.

Asiaticoside had the highest stimulating effect on HDF proliferation after 48 hours, compared to all the other preparations. The differences were highly significant for concentration of 6.25 mg/mL (p <0.001). A study by Lu *et al.* [13] found that asiaticoside strongly induced cell-cycle progression, proliferation and collagen synthesis in dermal fibroblasts [13]. Another study by Wu *et al.*[33] also investigated the effect of four triterpenes from *Centella asiatica* on the proliferation of HDF. At low concentrations (1, 3, 10 µM) all compounds tested did not enhance proliferation compared with the control group under the applied experimental conditions [33].

Our experiment indicates that after 48 hours the proliferative activity of the various compounds exerts a concentration profile which differs from that of 24 h which needs further investigation. This result mirrors a study by Pereda *et al.*[32] on the effect of green *Coffea arabica* L. seed oil in HDF [32]. Interestingly Shigematsu *et al.* has reported that retinoids (all-trans retinoic acid) and etretinate decreased HDF proliferation after 48 hour of treatment [35].

At 72 hours, retinoic acid dominates the picture with the highest fibroblast proliferation at all concentrations tested (Fig.3). Both the lowest and highest concentrations of retinoic acid showed greatest proliferative effects of about 185% of control. CAEE+CNP formula exerts its highest effect at the lowest concentration of 3.125 mg/mL (155% of control). All other values do not differ considerably and do not contribute much to the interpretation of the results of this study.

Comparing between retinoic acid and all other compounds, highly significant difference is observed at the highest concentration of 100 mg/mL (p<0.001). At the lowest concentration of 3.125 mg/mL, RA differs significantly against asiaticoside and CAEE, but not against CAEE+CNP. Moreover, significant differences are observed for RA (p<0.05), relative to other compounds at 12.5 and 25 mg/mL.
Retinoic acid exerts clinical activity on dermal tissue, but all-trans-retinoic acid (t-retinoid) reduced proliferation of HDF cultures [36]. In the dermis, retinoic acid was found to regulate fibroblast proliferation and aging process, through its inhibitory effects on the expression of metallo-proteases, which are responsible for skin aging [37]. A study by Varani et al. demonstrated that the ability of retinoic acid to stimulate HDF proliferation depends on the concentration of Ca\(^{2+}\) in the extracellular environment [38].

Figure 4. Collagen III synthesis in HDF after 24, 48, and 72 hours of treatment with Centella asiatica ethanolic extract (CAEE) or Centella asiatica ethanolic extract encapsulated into chitosan nanoparticles (CAEE+CNP); control (collagen III synthesis without treatment)

After 24 hours HDF collagen III synthesis was significantly higher with CAEE+CNP than with CAEE at all concentrations tested (p<0.05; Fig.4). Here, treatment with CAEE alone was found to actually decrease collagen III synthesis than control. Wu et al. investigated the effect of 4 triterpene compound of collagen III synthesis in human skin fibroblast and found both asiaticoside and madecassoside (dose of 3µM and 10 µM) significantly elevated the levels off procollagent type III as detected by ELISA. In contrast, neither Asiatic acid nor madecasic acid could influence collagen synthesis in fibroblast as compare with untreated cell. They also suggested that asiaticoside and madecassoside elevate collagen synthesis by activating the TGF-β/Smad signaling pathway (33). Maquart et al. also showed dose-dependent stimulation of collagen synthesis in fibroblast cultures by a triterpene extracted from Centella asiatica [39].

At 48 hours of treatment, trend in collagen III synthesis is generally the same as that at 24 hours. However, a more pronounced difference was observed between CAEE+CNP and CAEE alone. Moreover, at this timepoint CAEE+CNP significantly elevates collagen III synthesis relative to untreated control as well, at all concentration (p<0.05). This matches previous observation by Lu et al., in which they saw maximal Asiaticoside-induced collagen III synthesis at 48hr treatment [13].
At 72 hours, similar trend is still retained as compared to 24 and 48 hours. At all concentration tested, CAEE+CNP stimulation induces higher collagen III synthesis than CAEE, with an optimal concentration of 6.25 mg/mL. Again, CAEE alone exerts inhibitory effects on collagen III synthesis than control, at all concentrations tested. Differences between CAEE+CNP and CAEE are significant for 6.25 and 12.5 mg/mL.

**Conclusions**

Our results suggest that chitosan nano-encapsulation of CAEE enhances collagen III synthesis significantly for all the conditions tested. Furthermore, CAEE+CNP exert positive effect on fibroblast proliferation in a time and dose-dependent manner. Best stimulation was observed with short term incubation for 24hr at lower concentration between 3.125-6.25 mg/mL. At longer incubation period, asiaticoside and retinoic acid exert greater effects.

**Suggestion**

Our experiments suggest that under defined conditions *Centella asiatica* ethanolic extracts encapsulated in chitosan nanoparticles can be generally adapted to stimulate collagen III synthesis and fibroblast proliferation. Surprisingly however, chitosan nanoparticles itself showed even higher proliferative activity. Thus, the procedures involved in the proposed formulation still require further optimization, in order to achieve maximum therapeutic potential.

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**References**


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