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Research Article

Cytotoxicity Profiling and IC_{50} Determination of Madecassoside-Loaded Ultra-Small Unilamellar Nanocarrier System on Keloid-derived fibroblast cell lines: an MTT Assay and Cell Viability Analysis

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Abstract

Objective: To evaluate the in vitro cytotoxicity of a madecassoside-loaded ultra-small unilamellar nanocarrier system (MU) against keloid-derived fibroblasts and to compare it with that of the empty carrier and pure madecassoside. **Design:** In vitro experimental study using keloid-derived fibroblasts and the MTT colorimetric assay; each treatment was performed in triplicate. **Intervention:** MU, prepared from virgin coconut oil (VCO), soy lecithin and Tween 80 by sonication-aided emulsification, its empty carrier and pure madecassoside were applied to the cells at 10, 20, 30 and 40 µg/mL for 72 h. **Main outcome measures:** Cell viability (percentage of untreated control), the half-maximal inhibitory concentration (IC_{50}) calculated by semilogarithmic linear regression, and cell morphology under an inverted microscope. Groups were compared by two-way ANOVA with Bonferroni post hoc test. **Results:** MU reduced fibroblast viability in a concentration-dependent manner, with an IC_{50} of 29.77 µg/mL compared with 58.21 µg/mL for the empty carrier and 78.80 µg/mL for pure madecassoside (the latter two extrapolated beyond the highest tested concentration). At 30 µg/mL, viability after MU treatment was significantly lower than after pure madecassoside (65.1% vs 104.4%; $p < 0.05$). MU was the only sample that reduced viability below 50% within the tested range. **Conclusion:** Loading madecassoside into the ultra-small unilamellar nanocarrier increased its cytotoxic potential against keloid fibroblasts, as reflected by an approximately 2.6-fold lower IC_{50} than that of pure madecassoside, with the carrier itself contributing partly to this effect.

Keywords: madecassoside; ultra-small unilamellar nanocarrier; keloid fibroblast; MTT assay; IC_{50}; cytotoxicity

INTRODUCTION

Facial surgical procedures, acne, injuries, or burns can lead to atypical wound healing and hypertrophic scars and keloids. These lesions are characterized by persistent fibroblast proliferation, excessive extracellular matrix (ECM) deposition, and abundant collagen. Keloids proliferate beyond the confines of the original lesion and fail to resolve spontaneously, leading to discomfort, itching, redness, and functional impairment. Periocular scars, particularly those that occur after xanthelasma excision, have a significant psychosocial burden due to their visible location.[1] The patients may experience psychological and physical distress, resulting in an inferiority complex and thus decreasing their quality of life.[2]

Corticosteroid injections, surgery, laser treatment, pressure therapy, and cryotherapy are all treatments for keloids and hypertrophic scars.[3] Surgical treatment can be fast to relieve symptoms, but the chances of recurrence are greatly increased.[4] Laser therapy is effective, but the treatment of scar tissue is imprecise, as the surrounding healthy tissue as well as the scar tissue is damaged, and it involves high treatment costs.[5,6]

In terms of molecular biology, it is believed that keloids are caused by an imbalance in the TGF-β pathway in wound healing.[7–9] Fibroblasts produce excess TGF-β1 during the proliferative phase, which leads to keloid formation. In our previous study, we performed an in silico screening of 12 natural compounds that have possible therapeutic effects on keloids, based on the

binding affinity to the TGF-β1 receptor. Madecassoside exhibited a more negative docking score (−101.24 kcal/mol) and was the most promising anti-keloid candidate.[10] Madecassoside, a pentacyclic triterpenoid glycoside and a marker compound of *Centella asiatica*, aids in skin repair by stimulating fibroblast proliferation, collagen synthesis, and angiogenesis; it also possesses anti-inflammatory, immunomodulatory, and antibacterial activities[11]. It is vital to encourage epithelialization for the repair of cell damage by collagen synthesis, increased fibroblast proliferation and increased hydroxyproline levels.[12–14] In another study, we have developed an optimal MU formulation as a serum for the prevention and treatment of facial keloids.[15]

The cytotoxic effects of MU, pure madecassoside and the empty carrier on keloid fibroblasts were evaluated in this study. Cytotoxic effects were evaluated by the Microculture Tetrazolium Test (MTT) assay to establish the half-maximal inhibitory concentration (IC_{50}). This study aimed to determine whether pure madecassoside, empty carrier, and MU formulas could serve as a novel approach to enhance the anti-keloid effect of madecassoside.

MATERIALS AND METHODS

Materials and Instruments

The MU formulation (particle size 10.33 nm) and the MU base (empty carrier) were prepared as described previously. [15] Madecassoside (purity ≥90%) was sourced from MarkHerb (EBM Scitech, Indonesia).. Food-grade soy lecithin (Shankar Soya Products, Indonesia), Tween 80 (Bratachem, Indonesia) and virgin coconut oil (VCO; Wahana, Indonesia) were used as carrier components. The MU formulation and its empty carrier (MU base without madecassoside) were prepared by sonication-aided emulsification as described previously. The optimized MU had a mean particle size of particle size 10.48±0.25 nm, PDI 0.186±0.007, peak intensity 96.9±0.3%, and zeta potential −33.3±1.65 mV and EE 80.33% with spherical nano-vesicular morphology[15]. Keloid-derived fibroblasts were supplied by the Biomedical Laboratory, Faculty of Medicine, Universitas Andalas, Indonesia. MTT reagent and cell culture supplies were obtained from Sigma-Aldrich. All solvents and reagents were of analytical grade. The main apparatus comprised an autoclave (All American, USA), CO_2 incubator (NU-5100E; Nuaire, USA), analytical balance (Precisa, China), ELISA microplate reader (Dynatech MR5000, USA), inverted microscope (Meiji Techno TC5400, Japan), laminar airflow cabinet (Thermo Fisher Scientific, USA), refrigerated centrifuge (Universal 32R, USA), water bath (Memmert, Germany) and standard cell culture equipment.

In Vitro Cytotoxicity Assay (MTT Assay)

The viability of fibroblast keloid cells was evaluated using the MTT colorimetric test, which is based on the reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to insoluble formazan by mitochondrial reductase. Cells were inoculated onto 96-well plates at a concentration of 1 × 10^4 cells per well in media supplemented with 10% fetal bovine serum (FBS) and incubated for 72 hours at 37 °C and 5% CO_2 in a humidified atmosphere to facilitate cell adhesion. Serial dilutions (10 µg/mL, 20 µg/mL, 30 µg/mL, and 40 µg/mL) of the test samples were prepared in a medium with 0.1% DMSO and administered to the cells for 72 hours. Untreated cells and those subjected to vehicle treatment (0.1% DMSO) served as controls, with each treatment condition executed in triplicate. Following the exposure duration, 10 µL of MTT solution (5 mg/mL in PBS) was introduced to each well and incubated for a further 4 hours to generate formazan. The culture media was subsequently removed, and the resulting crystals were dissolved in 100 µL of DMSO. The absorbance was measured at 595 nm with an ELISA microplate reader. Cell viability was shown as a percentage relative to the untreated control. Dose-response correlations were examined, and IC_{50} values were ascertained by linear regression analysis. The proportion of cell viability was determined in comparison to the untreated control, and IC_{50} values were derived using the semilogarithmic concentration–viability graphs. The experimental approach was derived from an earlier published technique with few alterations. Morphological alterations were examined using an inverted microscope at 100× magnification, encompassing cellular contraction, rounding, membrane blebbing, and reduced confluence.

Statistical Analysis

Data are presented as mean ± standard deviation (SD), n = 3. Statistical analyzes were conducted utilizing R (version 4.6.1; R Foundation for Statistical Computing, Vienna, Austria) within R Studio, employing the emmeans and car packages. The half-maximal inhibitory concentration (IC_{50}) was established by linear regression analysis of the dose-response curve, which plotted cell viability percentage against the logarithm of sample concentration. The calibrated curve was utilized to determine the concentration associated with 50% cellular viability. Comparative evaluations among treatment groups, assessing MU, the unoccupied carrier, and pure madecassoside at varying concentrations, were conducted utilizing Two-way ANOVA followed by a Bonferroni post hoc multiple comparison test. The Shapiro-Wilk test was employed to evaluate the normality of residuals, whereas Levene's test was utilized to examine the homogeneity of variance. Statistical significance was established at $p < 0.05$.

RESULT AND DISCUSSION

In Vitro Cytotoxic Activity Against Keloid Fibroblast Cells

MTT assay exhibited the inhibitory effect of all three formulations on the viability of scar fibroblasts in a concentration-dependent manner (Table I, Figure 1). At lower concentrations of 10 and 20 (µg/mL), cell viability was found to be over 100% in all groups, which is consistent with the reported physiological function of madecassoside in promoting fibroblast proliferation and

tissue repair under sub-inhibitory conditions. At higher concentrations (30 and 40 µg/mL), a significant reduction in viability was noticed, particularly in the MU group, with cell viability decreasing to 65.1% and 35.7% at 30 and 40 µg/mL, respectively. Two-way ANOVA indicated significant main effects of treatment ($F(2,24) = 7.32$, $p<0.05$) and concentration ($F(3,24) = 78.02$, $p < 0.05$), but no significant interaction of treatment x concentration ($F(6,24) = 1.26$, $p = 0.314$). Post hoc analysis with Bonferroni correction indicated a significant reduction in fibroblast viability by MU relative to pure madecassoside at 30 µg/mL. However, the difference between MU and the empty carrier at 30 µg/mL, as well as between MU and the other two groups at 40 µg/mL ($p<0.05$) was not statistically significant after correction.

Table 1. Cytotoxic activity of MU, empty carrier, and pure madecassoside against scar fibroblast cells

Conc. (µg/mL)	MU		Empty carrier		Pure Madecassoside	
	Abs.	Viability (%)	Abs.	Viability (%)	Abs.	Viability (%)
10	0.386±0.046	144.805	0.412±0.034	157.468	0.405±0.049	153.734
20	0.333±0.024	118.994	0.357±0.029	130.357	0.330±0.041	117.208
30	0.223±0.028	65.097**	0.285±0.028	95.292	0.303±0.003	104.383
40	0.162±0.006	35.714	0.217±0.012	62.500	0.220±0.005	63.636
IC_{50}	**29.770 µg/mL**		**58.210 µg/mL**		**78.80 µg/mL**	

**Absorbance of DMSO (negative control) = 0.089. Values expressed as mean ± SD (n = 3); **p < 0.05 vs pure madecassoside at the same concentration (two-way ANOVA test)*

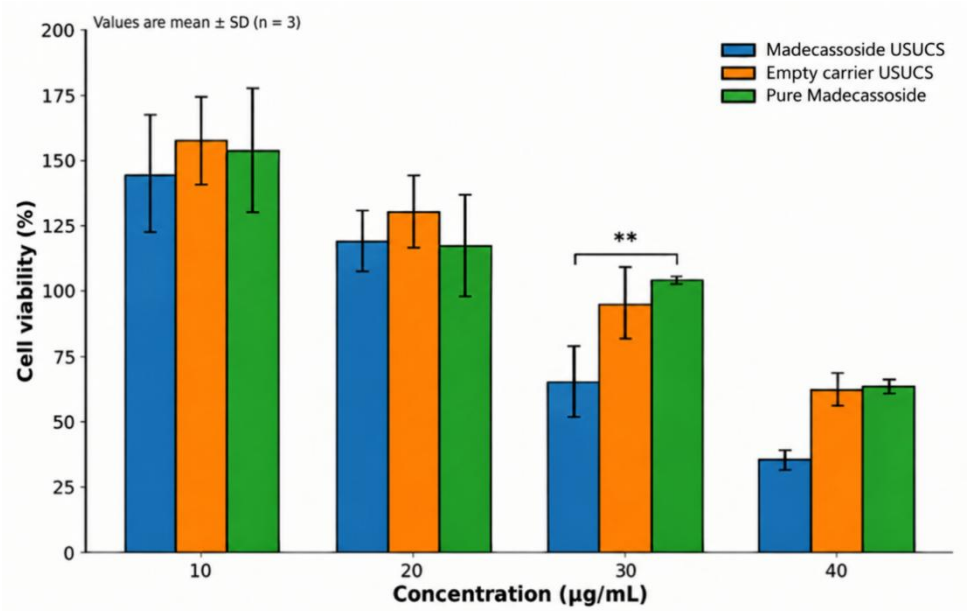


Figure 1. Viability percentage of fibroblast cell lines following 72-hour exposure to (a) MU, (b) empty carrier, and (c) pure madecassoside. All data are reported as means (n = 3) ± SD. **p < 0.05, MU versus pure madecassoside at 30 µg/mL (two-way ANOVA with Bonferroni post hoc evaluation).

The IC_{50} values calculated from the semilogarithmic plot of concentration vs cell viability (Figure 2) were 29.77 µg/mL, 58.21 µg/mL and 78.80 µg/mL for MU, empty carrier and pure madecassoside, respectively. The MU activity with IC_{50} between 21 and 50 µg/mL showed moderate to strong cytotoxicity, while the empty carrier and pure madecassoside were weakly cytotoxic (IC_{50} > 50 µg/mL) on fibroblast cell proliferation according to the criteria of the National Cancer Institute (NCI).[16,17] Previous studies have shown that encapsulation of herbal ingredients in liposomes may improve their bioavailability and cytotoxic activity.[18] For instance, liposomes carrying silibinin showed a tenfold increase in in vitro cytotoxicity against liver cancer when compared to silibinin alone (IC_{50} of 48.680 µg/mL versus 485.450 µg/mL for silibinin alone).[16]

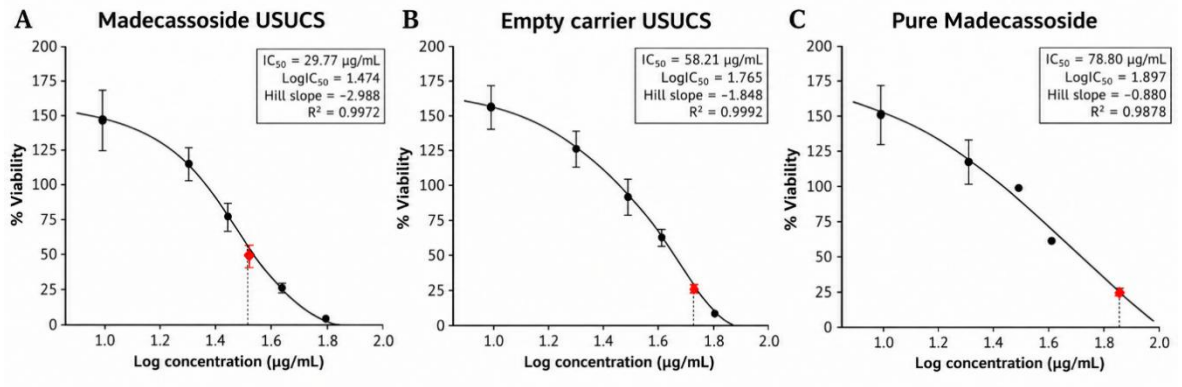


Figure 2. Semilogarithmic concentration–viability curves from the MTT cytotoxicity assay. (A) MU, (B) empty carrier, and (C) pure madecassoside against keloid fibroblast cells; (n = 3). Solid line, linear regression of mean % viability against log concentration; dashed line, extrapolation beyond the highest tested concentration (40 µg/mL); red diamond, IC_{50}.

The curves in Figure 2 show that cell viability decreased with increasing concentrations for all three samples, albeit at different rates. A more rapid decline in viability at low concentrations corresponds to a lower IC_{50} value and greater cytotoxic potential. The MU formula exhibited the sharpest decline, dropping from 145.04 ± 22.22% at 10 µg/mL to 35.75 ± 3.14% at 40 µg/mL. This formula was the only one capable of inhibiting cells (reducing viability below 50%) within the tested concentration range. In contrast, viability for the formula base (62.60 ± 6.02%) and pure madecassoside (66.66 ± 0.28%) remained above 60% at the highest concentration; consequently, the IC_{50} for both exceeded 40 µg/mL. The order of cytotoxic potential was MU formula > formula base > pure madecassoside. This indicates that the USUCS system enhanced the effect of madecassoside on the cells—likely because the nanocarrier delivery system effectively inhibited keloid fibroblast growth—while the base alone also reduced viability (though less potently than the MU formulation), suggesting that the carrier itself contributed to the observed effect.

The results indicate that nanoencapsulation of madecassoside in Ultra Small Unilamellar Carrier System (MU) enhances the biological efficacy of the active compound as compared to its free form, probably by improving cellular penetration and bioavailability.[17] The Ultra Small Unilamellar Carrier for nano-delivery system plays an important role in modulating the response of keloid fibroblast cells, as the IC_{50} values are different.[189] Nanoencapsulation enables a more controlled release of madecassoside and more effective interaction with cell signaling pathways, particularly the TGF-β1 pathway, which plays a dominant role in fibroblast proliferation and keloid formation.[19]

The present study was performed following the in silico screening of madecassoside as the most potent anti-keloid among the 12 plant-derived bioactive compounds. MTT assay results showed a 1.6-fold decrease in the IC50 value compared with pure madecassoside, suggesting the enhanced antiproliferative potential of the MU formulation on keloid fibroblasts. This result is due to the higher uptake of the natural active ingredient by the nano-vesicular liposome delivery system, sustained drug release and increased membrane permeability.[14]

The morphological changes of cell lines treated with MU, empty carrier and pure madecassoside, respectively, are shown in Fig. 3. The morphology of cells treated with MU at 40 µg/mL (Figure 3A) shows a predominance of rounded and shrunken cells, some forming clusters (aggregates) and accompanied by cellular debris; few cells remain elongated, and the monolayer is discontinuous with numerous empty spaces, indicating strong cytotoxic activity (+++). Figure 3B shows the morphology of cells treated with empty carrier at 40 µg/mL, where almost all cells are rounded, refractile, evenly dispersed and detached from the substrate; spindle-shaped cells are virtually absent and no monolayer formation, indicating strong cytotoxic activity (+++). Fig. 3C Morphology of cells treated with pure madecassoside at 40 µg/mL; cells are adherent, spindle-shaped or fibroblast-like morphology, arranged in a tight organized pattern with only occasional rounded cells observed; monolayer is intact and nearly confluent, suggesting non-cytotoxic or minimal cytotoxic activity (–/+). The observed cytotoxicity of the empty carrier suggests that the components of the carrier (e.g., surfactants or co-surfactants at high concentrations) may be responsible for cell death. This is in agreement with the findings in Table 1 and Figure 2, where the IC_{50} value of the empty carrier is lower than that of pure madecassoside. Non-ionic surfactants like Tween 80 at high concentration may interfere with the lipid bilayers of cell membranes to lead to the membrane being fluidized, permeabilized, or solubilized within cell monolayer preparations. This is the reason for the reported cellular rounding and detachment seen in Figure 3B. But as the MU nanocarrier system as an entire formulation, the cellular uptake and antiproliferative effect of madecassoside become improved

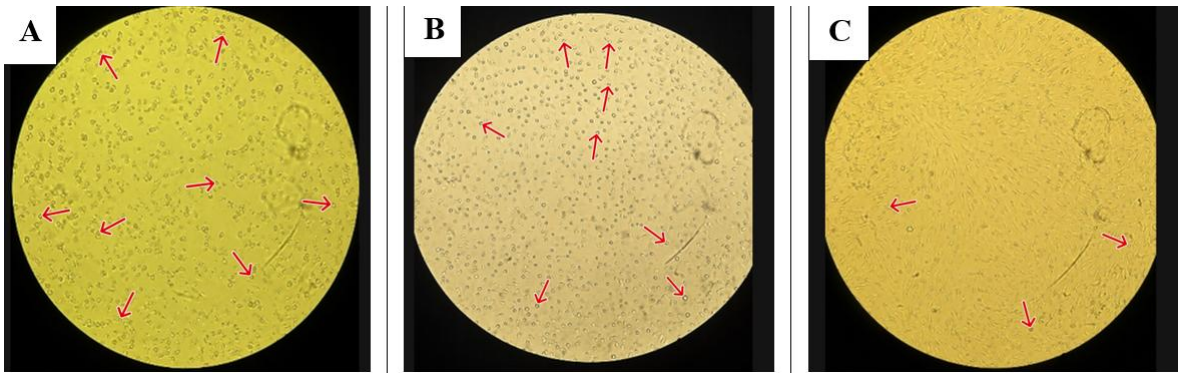


Figure 3: Morphological changes of fibroblast cell lines after 72-hour treatment with (A) MU, (B) empty carrier, and (C) pure madecassoside observed under an inverted microscope. Cells that are non-viable are denoted by the red arrow, whereas those displaying morphological changes are highlighted. Cellular morphology was analyzed utilizing an inverted microscope at a magnification of 100x.

Keloid fibroblasts are characterized by resistance to apoptosis, allowing the cells to persist and continue producing collagen[20]. Inducing apoptosis leads to fibroblast death and a reduction in the number of hyperproliferating fibroblasts, which in turn limits excessive extracellular matrix (ECM) deposition[21]. On a mechanistic level, madecassoside inhibited the TGF-β1 pathway, leading to increased anti-keloid action of MU[12]. The TGF-β1 protein is the primary activator of fibroblasts, causing an excess deposition of ECM and collagen in keloids[21]. Madecassoside has been found to inhibit migration of fibroblasts and induce apoptosis in keloid fibroblasts through inhibition of the p38 MAPK and PI3K pathways[20]. USUCS is likely to potentiate these molecular effects by increasing the efficiency of drug delivery at the cellular level, enhancing therapeutic suppression of pathological scar remodeling.

CONCLUSION

Nanoencapsulation of madecassoside in an ultra-small unilamellar carrier system resulted in the improvement of its biological performance in regulating fibroblast activity in abnormal wound healing. The MU formulation showed enhanced cytotoxic potential against keloid fibroblast cells compared with the empty carrier and pure madecassoside, with lower IC_{50} values, which were related to the improved delivery efficiency and cellular interactions. These results collectively show the potential of MU formulation as a promising transdermal nanotherapeutic system for targeting TGF-β1 mediated fibroblast proliferation.

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