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AQUEOUS EXTRACT OF *CENTELLA ASIATICA* AS A POTENTIAL ANTI-KELOID AGENT

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ABSTRACT: *Centella asiatica* (CA) is a reputed medicinal plant that has been shown to have pharmacological effect on skin wound healing. The aim of the study was done to assess the effects of aqueous extract of CA on human dermal fibroblasts in an in vitro model of wound healing. Dermal fibroblasts were cultured with or without supplementation of aqueous extract of CA. Viability and proliferation of the fibroblasts cells were determined by MTT assay and cell cycle were analyzed by flowcytometry. *In-vitro* re-epithelization was assessed by scratch assay and migration rate was evaluated quantitatively by image analyzer. Gene expression of type I collagen, type III collagen, fibronectin and SMAA2 were studied *via* real-time RT-PCR. Supplementation with the aqueous extract of CA inhibited the fibroblasts' proliferation significantly ($p<0.05$) at high concentration. Moreover, it also inhibited fibroblasts' migration across the denuded area significantly ($p<0.05$) at a concentration of 194.15 µg/mL (IC_{50}) when it was compared to the control. The gene expression of type I collagen, type III collagen, fibronectin and SMAA2 decreased with increasing concentration of aqueous extract of CA. However, supplementation of aqueous extract of CA did not alter the cell cycle even at high concentrations. In conclusion, CA has a potential to be used as an anti-keloid agent as it down regulates the gene expression of type I collagen, type III collagen, fibronectin and SMAA2. These genes are found to be up regulated in keloid scars.

INTRODUCTION: *Centella asiatica* (CA) is a medicinal plant from the family of Umbelliferae [1]. Locally known as Pegaga in Malaysia, *Centella asiatica* has been documented to exhibit various medical beneficial effects such as wound healing, memory improvement, treating mental fatigue, bronchitis, asthma, dysentery, kidney trouble, urethritis, antiallergic and anticancer purposes, curing leukorrhea and toxic fever [1].

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It has been used in traditional medicine throughout Asia, India and China. Traditionally, it is used as remedies for the treatment of burn lesions, wounds and scars [2, 3]. *Centella asiatica* have been reported to induce wound healing by stimulating the synthesis of collagen type I and type III, both *in-vitro* and *in-vivo* [4, 5] inducing angiogenesis [5, 6] and escalating the proliferation of fibroblast [7].

Centella asiatica also displayed antioxidant properties which is very important in the process of wound healing. Recent studies further reinforced the promising potential of *Centella asiatica* extracts as an agent of wound healing *via* its antioxidant, antimicrobial, and stimulation of collagen synthesis properties [8, 9]. The ethanol extract of *Centella asiatica* stimulates normal and delayed wound

healing by increasing the wound breaking strength and rate of re-epithelialization [10]. In addition, *Centella asiatica* reported to be effective in treatment of wounds, burns and hypertrophic scar by promoting fibroblast proliferation, increasing the collagen synthesis and inhibiting the inflammatory phase of hypertrophic scars and keloid [11]. *Centella asiatica* given topically promote wound healing and relieve the associated pain [12, 13]. Centiderm ointment which contained *Centella asiatica* improved the re-epithelialization and resulted in complete healing in a clinical trial [3].

The principle active compound in *Centella asiatica* extracts has been reported to be made up of four major triterpenoid: asiatic acid, madecassic acid, asiaticoside and madecassoside. In terms of its active compound, evaluation study on the effects of asiaticoside topical application on the wound of Sprague Dawley rats was done and successfully demonstrated enhanced wound healing activity [8].

Moreover, the effects of asiaticoside treatment on the *in-vitro* model of fibroblasts showed asiaticoside increased gene expressions of cell proliferations and extracellular matrix formation such as collagen type 1A2 (COL1A2), collagen type 3A1 (COL3 A1) and Lysyl like-oxidase 3 (LOXL3) [14]. Various studies have also confirmed that *Centella asiatica* compound such as titrated extract from *Centella asiatica* (TECA) and asiaticoside can stimulate the accumulation of extracellular matrix macro-molecule in the wound chamber and increase the rate of wound healing in the Sprague Dawley male rats [7] and showed significantly stimulates the synthesis of collagen type I [4].

In this study, the effects of *Centella asiatica* on human dermal fibroblasts was investigated by analyzing the *in-vitro* migration rate, wound healing gene expression analysis using real-time RT-PCR and analyzing cell cycle analysis using flow cytometry.

MATERIALS AND METHODS:

Isolation of Epidermal and Dermal Tissue from Full Thickness Skin: Skin samples were obtained from consented patients (**Table 1**) and approved by Universiti Kebangsaan Malaysia Research and Ethical Committee. The skin was rinsed briefly in 70% isopropanol and then placed into Dulbecco's Phosphate Buffered Saline (DPBS) (Gibco/BRL, USA) containing 20 µg/ml Gentamycin (Gibco/ BRL, USA) for 1 hour. The skin was cut into small pieces (1-2 cm^2) and soaked in a 25 caseinolytic units/ml solution of Dispase (Sigma-Aldrich Co., USA) in Defined Keratinocyte-Serum Free Medium (DKSFM) (Gibco/BRL, USA) with 5 µg/ml Gentamycin for 8-12 hours at 2 to 8 °C to separate the epidermis and dermis.

TABLE 1: TISSUE DONORS, AGE AND REGION OF HARVESTED BIOPSIES

Sample	Age (years)	Region of explants
1	33	Abdomen
2	35	Abdomen
3	31	Abdomen
4	36	Abdomen
5	40	Abdomen
6	38	Abdomen

Isolation and *In-vitro* Culturing of Human Dermal Fibroblasts Cells: Human dermis layer was digested with 0.6% collagenase type I enzyme (Gibco/BRL, USA) for 12-18 hours in an incubator-shaker. The cell suspension obtained was centrifuged at 600 x g for 5 minutes at room temperature. The resulting pellet was washed with DPBS buffer. Dermal fibroblasts will be cultured in either Ham's F12:DMEM (1:1) + 10% FBS or Ham's F12:DMEM (1:1) + 10% HS.

Cells were plated in 6-well plate (Becton Dickinson, USA) at the density of 1 x 10^5 cells per well in either Ham's F12:DMEM (1:1) + 10% FBS or Ham's F12:DMEM (1:1) + 10% HS. Cells were cultured at 37 °C in 5% CO_2 (Jouan, France) with medium changed every 2-3 days. The fibroblasts monolayer culture was subcultured to passage 1 when the culture reached confluency about 90 %.

Preparation of *Centella Asiatica* Aqueous Extracts: Dry CA powder was obtained from Forestry Research Institute of Malaysia (FRIM). Three grams of dry CA powder were dissolved in 100 mL of PBS (1X) and stirred with a magnetic stirrer for 12 hours to produce a stock solution with the concentration of 30,000 µg/mL. The stock solution was then filtered through Whatman filter paper. The filtered stock solution was then sterile-filtered using 0.2 micron syringe filter (Sartorius, USA). For this study, 0 µg/mL, 7.81 µg/mL, 15.63 µg/mL, 31.25 µg/mL, 62.5 µg/mL, 125 µg/mL, 250 µg/mL, 500 µg/mL and 1000 µg/mL concentration

of CA in F12 Ham's: DMEM 10 % + 10 % FBS were studied. The culture mediums containing CA aqueous extracts was kept at 4 °C and in the dark to prevent oxidation of CA components.

Dermal Fibroblasts Proliferations Assay: Six samples of human dermal fibroblasts at passage 1 was cultured in 96 well plate at seeding density of 2000 cells/well in culture medium without CA aqueous extract for 24 hours. After 24 hours, the cells were exposed with culture medium containing CA aqueous extracts at different dose. Medium without CA aqueous extract was used as a control. Every treatment was performed as triplicate for each skin samples.

The proliferation assay was performed on day 1, day 4, and day 8 of treatment by using MTT with modifications described by Mosmann, 1983 [15]. The plate was then read using a microplate spectrophotometer PowerWave™ XS Universal Microplate Spectrophotometer (Bio-Tek® Instrument, Inc., USA) with absorbance measured at 570 nm and correction wavelengths measured at 60 nm.

***In-vitro* Wound Healing Assay (Scratch Assay):** Scratch assay was performed according to methods described by Phan *et al*., 2001 [16]. Dermal fibroblasts were cultured at seeding density of 5000 cell/cm^2 in 6-well plate in culture medium without CA aqueous extract. After 100% confluence, a scratch was made using sterile disposable micropipette crystal tip to the cell culture. Culture mediums with CA supplementations at 0 µg/mL (control), 7.81 µg/mL and 194.15 µg/mL (IC_{50} value) were added to each individual well (duplicate for each dosage).

Three criteria were taken into considerations during data observations: low number of floating cells, a straight scratch and the end of the scratch must be able to be observed under phase contrast microscope at 40X magnification. Photograph for the scratch area was taken every 6 hours for 24 hours period using a digital camera Evolution MP 5.0 Cooled, RTV (Media Cybernetics, Canada) connected to phase contrast microscope. The initial scratch area and dermal fibroblasts cell migrations was calculated to determine the average rate of dermal fibroblasts migrations for all 6 dermal fibroblasts samples by using Image Analyzer software QCapture Pro (QImaging Corp., USA).

Total RNA Extraction for Quantitative Gene Expression Analysis: Dermal fibroblasts at passage 1 were cultured in culture medium at seeding density of 5000 cells/cm^2 with supplementations of CA aqueous extract at the concentrations of 0 µg/mL, 7.81 µg/mL and 194.15 µg/mL (IC_{50} value). Total RNA extractions was done using TRI reagent (Molecular Research Center, Cincinnati, OH). Polyacryl Carrier (Molecular Research Center) was added in each extraction to precipitate the total RNA.

Extracted RNA pellet was then washed with 75 % ethanol and dried before dissolved in RNAse and DNAse free distilled water (Invitrogen, Carlsbad, CA). RNA purification was done using RNA Clean-Up Kit-5™ (Zymo Research Corp., USA) according to manufacturer instructions. Yield and purity of the extracted RNA was determined by spectro-photometer (Bio-Rad, Hercules, CA) and 1.5 % agarose gel electrophoresis.

Quantitative Gene Expression Analysis by Real-Time PCR: Expression of type I collagen, type II collagen, fibronectin and alpha smooth muscle actin-2 was quantitatively analyzed using real-time PCR. GAPDH was used to normalize the expression of targeted genes. All primers (**Table 2**) were designed with Primer 3 software and blasted with GeneBank database sequences in order to obtain primers with high specificity.

The efficiency and specificity of each primer set was confirmed with the standard curve (Ct value versus serial dilution of total RNA) and melting profile evaluation. Real-time PCR reaction was performed with 100 ng of total RNA, 400 nM of each primer and iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad) according to the manufacturer's instruction. Reactions were run using Bio-Rad iCycler with reaction profile of; cDNA synthesis for 30 min at 50 °C; pre-denaturation for 2 min at 94 °C; PCR amplification for 38 cycles with 30 sec at 94 °C, 30 sec at 60 °C, and 30 sec at 72 °C. This series of cycles was followed by a melt curve analysis to check the reaction specificity. Real-time PCR products were confirmed using 1.5 % agarose gel electrophoresis.

TABLE 2: PRIMER SEQUENCES USED IN REAL-TIME PCR FOR QUANTITATIVE GENE EXPRESSION ANALYSIS

Genes	Access no.	Primer 5' - 3'	PCR product (bp)
GAPDH	BC020308	F: 5'-tcc ctg agc tga acg gga ag-3' R: 5'-gga gga gtg ggt gtc gct gt-3'	217
Collagen type I	NM 000088	F: 5'-agg gct cca acg aga tcg aga tcc g-3' R: 5'-tac agg aag cag aca ggg cca acg-3'	222
Collagen type III	NM 000090	F: 5'-gtt gac cct aac caa gga tgc a-3' R: 5'-gga agt tca gga ttg ccg tag-3'	203
Fibronectin	M10905	F: 5'-aag acc agc aga ggc ata agg-3' R: 5'-cca ctt cca aag cct aag cac-3'	196
α-SMAA2	NM 001613	F: 5'-tgg ccg aga tct cac tga cta-3' R: 5'-ctt ctc aag gga gga tga gga-3'	172

Cell Cycle Analysis Using Flow Cytometry: Dermal fibroblasts at passage 1 were cultured in culture mediums of Ham's F12: DMEM (1:1) + 10 % FBS supplemented with CA aqueous extract at concentrations of 0 µg/ml, 7.8125 µg/ml and 194.1492 µg/mL (IC_{50} value) in T-25 flasks with seeding density of 5000 cells/cm^2. A total number of one million cells per sample were used for cell cycle analysis using flow cytometry (Becton Dickinson, Rutherford, NJ).

Dermal fibroblasts were processed using CycleTEST^{TM} PLUS DNA Reagent Kit (Becton Dickinson, Rutherford, NJ) following the manufactures instructions. The propidium iodide (PI) stained single nuclei suspensions were analyzed using FACScan^{TM} flow cytometers (Becton Dickinson, Rutherford, NJ) and raw data was collected using CELLQuest software (Becton Dickinson, Rutherford, NJ). Data analysis was performed using Modfit Cell Cycle Analysis Software (Verity House Software, Topsham, ME). A total of 6 samples of dermal fibroblasts were used for this study.

Statistical Analysis: The comparisons between CA treated group of dermal fibroblasts and untreated group of dermal fibroblast were done using variants analysis (ANOVA) and student t-test. Values were presented as average ± standard error of mean (SEM). Statistical analysis was performed using Statistic Package for Social Science 12.0 (SPSS 12.0) for Windows software. Dunnett test was used to determine the significance of sample when compared to control during the one-way ANOVA test. The result is significance when the significance level is less than 0.05 ($P<0.05$).

RESULTS:

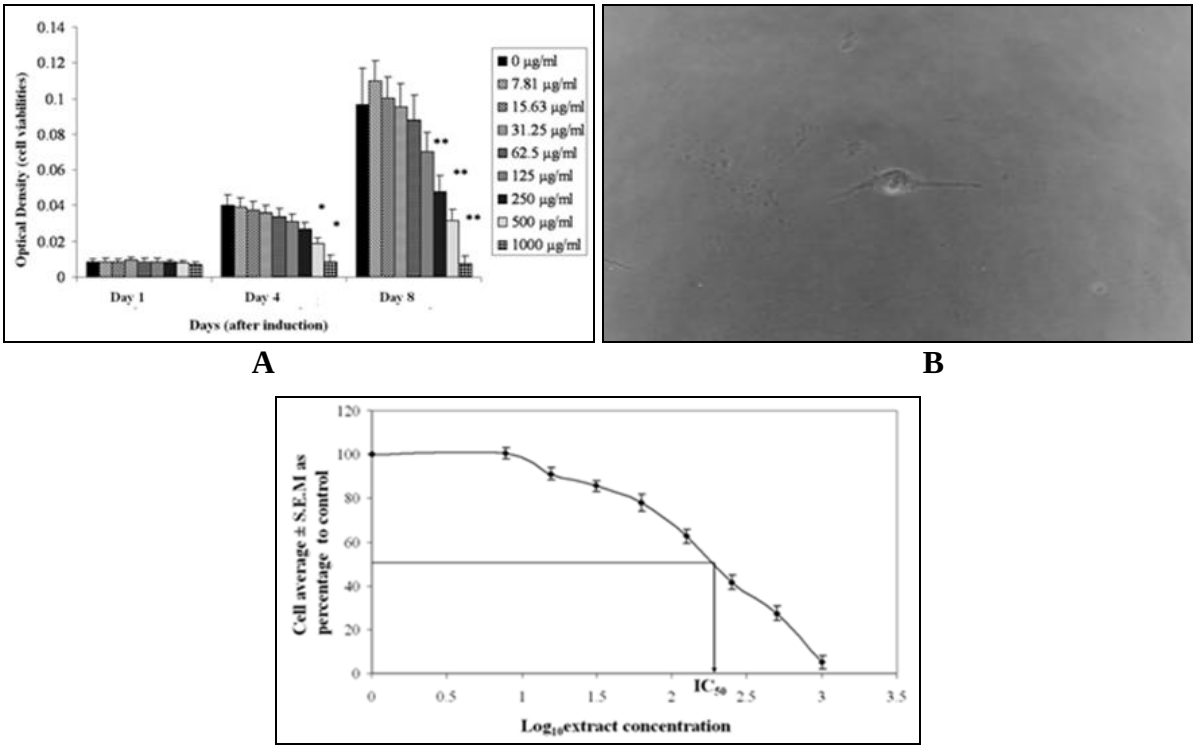
Effects of CA Aqueous Extract on the Human Dermal Fibroblast Proliferations: The result displayed an anti-proliferative effect by the CA on the growth of dermal fibroblasts (n=6) on day 4 and 8. The average viability significantly reduced when compared to control (0 µg/mL) at high concentrations of CA on day 4 (500 µg/mL and 1000 µg/mL) and day 8 (250 µg/mL, 500 µg/mL and 1000 µg/mL) (as shown in **Fig. 1A**).

Under visual observation using inverted microscope, morphological changes were detected where dermal fibroblasts with normal spindle shape changes to round shape in the center with pointed end (as shown in **Fig. 1B**).

After treatment with CA extract, it was also noticed that dermal fibroblast does not form formazan crystal after MTT salt was added. This show possibility of toxicity effects on dermal fibroblast at day 4 and day 8 at high level of CA concentration. The value of IC_{50} (194.15 µg/mL) was determined from dose-response curve on day 8. IC_{50} is an inhibiting concentration for 50% populations of dermal fibroblast exposed to the CA aqueous extract (**Fig 1C**).

Effects of CA Aqueous Extract on the Dermal Fibroblast Migration Rate: CA aqueous extract inhibits the migration of dermal fibroblasts. At the concentration of 194.15 µg/mL, average value of migration reduced significantly when compared to the control (0 µg/mL) (as shown in **Fig. 2A**). This is also proven through observation by comparing 0 µg/mL (control) and 194.15 µg/mL between 0 hour and 12 hours (**Fig. 2B**).

Effects of CA Aqueous Extract on the Dermal Fibroblast Gene Expressions: Expressions of collagen type I, collagen type III, fibronectin and α-SMAA2 relative to GAPDH was reduced with the increase of the concentration of CA aqueous extract supplementations. However, the reduction at the concentration of 7.81 µg/mL and 194.15 µg/mL was not significant when compared to control (0 µg/mL) (as shown in **Fig 3**).

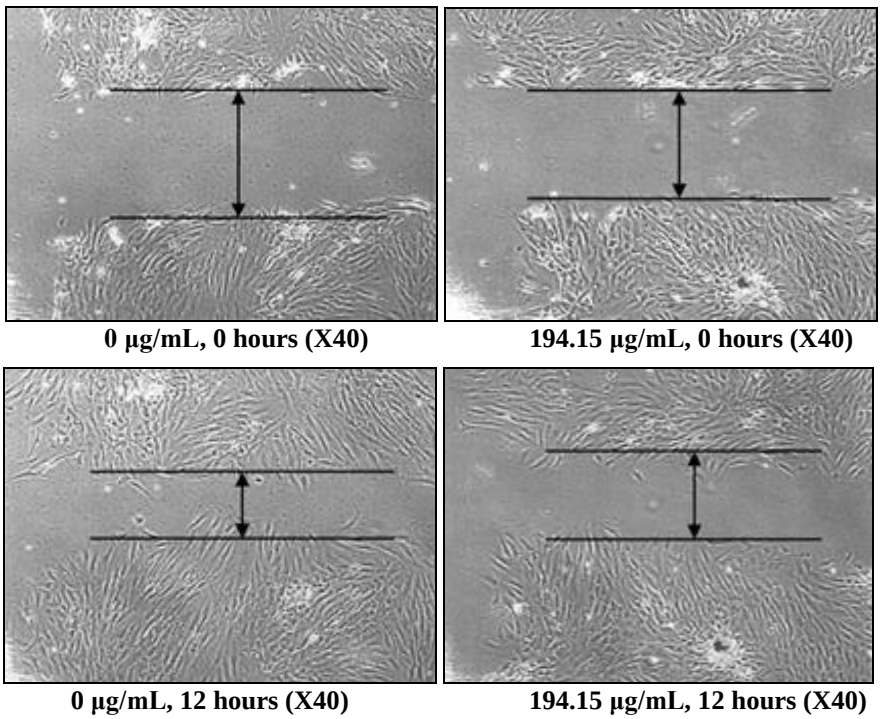


A **B**

C

FIG. 1: EFFECT OF *CENTELLA ASIATICA* AQUEOUS EXTRACT EFFECT ON THE PROLIFERATION

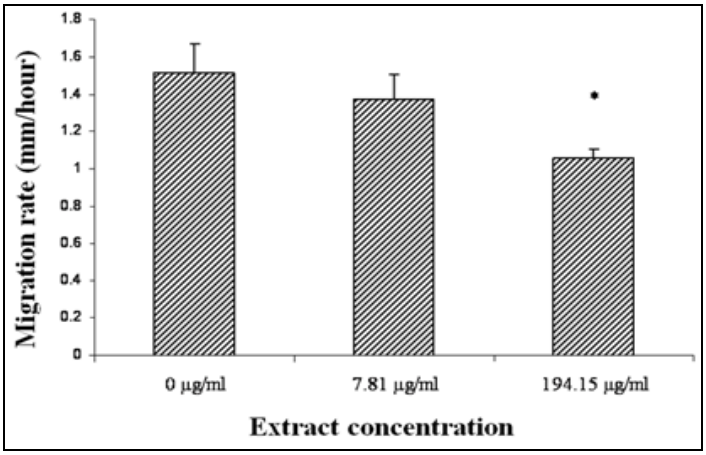
(A), Morphology changes at concentration 1000 µg/ml (Day 4) (B) and dose response curve (C) of dermal fibroblasts (N=6) (Average ± S.E.M). The proliferation rate was significant (**P*<0.05 and ***P*<0.05) compared to optical density at concentration of 0 µg/ml at day 4 and 8.



0 µg/mL, 0 hours (X40) **194.15 µg/mL, 0 hours (X40)**

0 µg/mL, 12 hours (X40) **194.15 µg/mL, 12 hours (X40)**

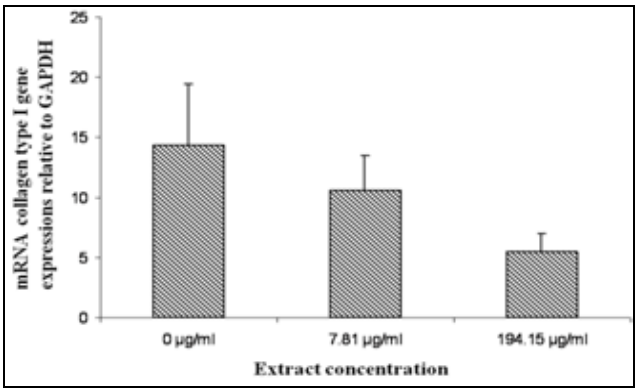
A



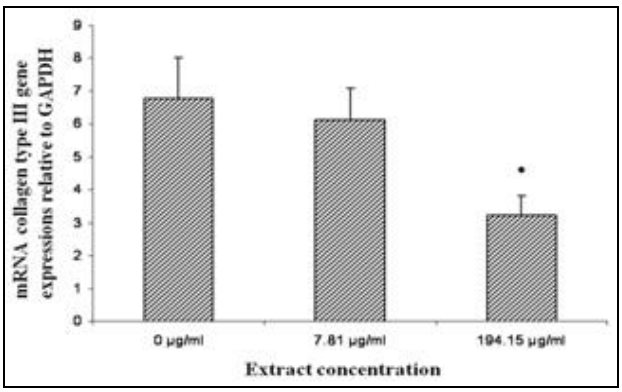
B

FIG. 2: EFFECTS OF *CENTELLA ASIATICA* AQUEOUS EXTRACT EFFECTS ON THE MIGRATION OF DERMAL FIBROBLASTS

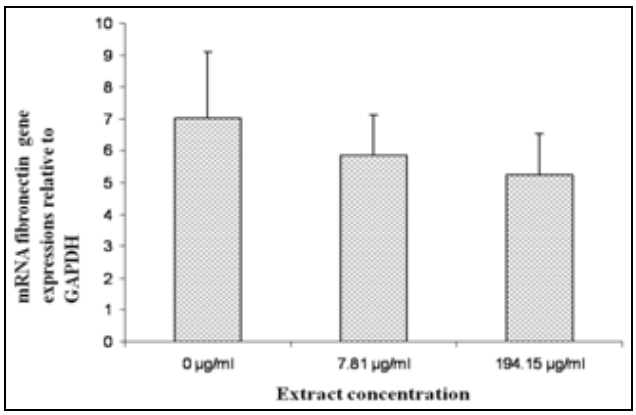
Microscopic observation showed inhibition of dermal fibroblasts migration at concentration 194.15 µg/mL compared to 0 µg/mL (Control) (A). Graph showed significant (*P< 0.05) in dermal fibroblasts migration at concentration 194.15 µg/mL compared to 0 µg/mL (Control) in 24 hours (n=6) and (Average ± S.E.M) (B)



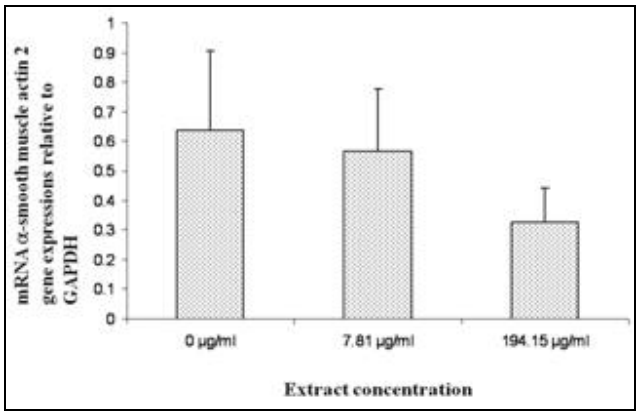
A



B



C



D

FIG. 3: EFFECTS OF *CENTELLA ASIATICA* AQUEOUS EXTRACT SUPPLEMENTATIONS AT GENE EXPRESSION LEVEL Graph showed mrna expression of collagen type I (A), collagen type iii (B), fibronectin (C) and A-SMAA2 (D) reelative to Gapdh (N=6) (Average ± S.E.M).

Effects of CA Aqueous Extract Supplementations on the Cell Cycle of Cultured Dermal Fibroblasts: Most of the dermal fibroblasts were in the G_0/G_1 phase compared to S phase and G_2/M phase. From the result, statistical test revealed the average value is not significant at G_0/G_1 and S phase at concentrations of 7.81 µg/mL and 194.15 µg/mL when compared to control (0 µg/mL).

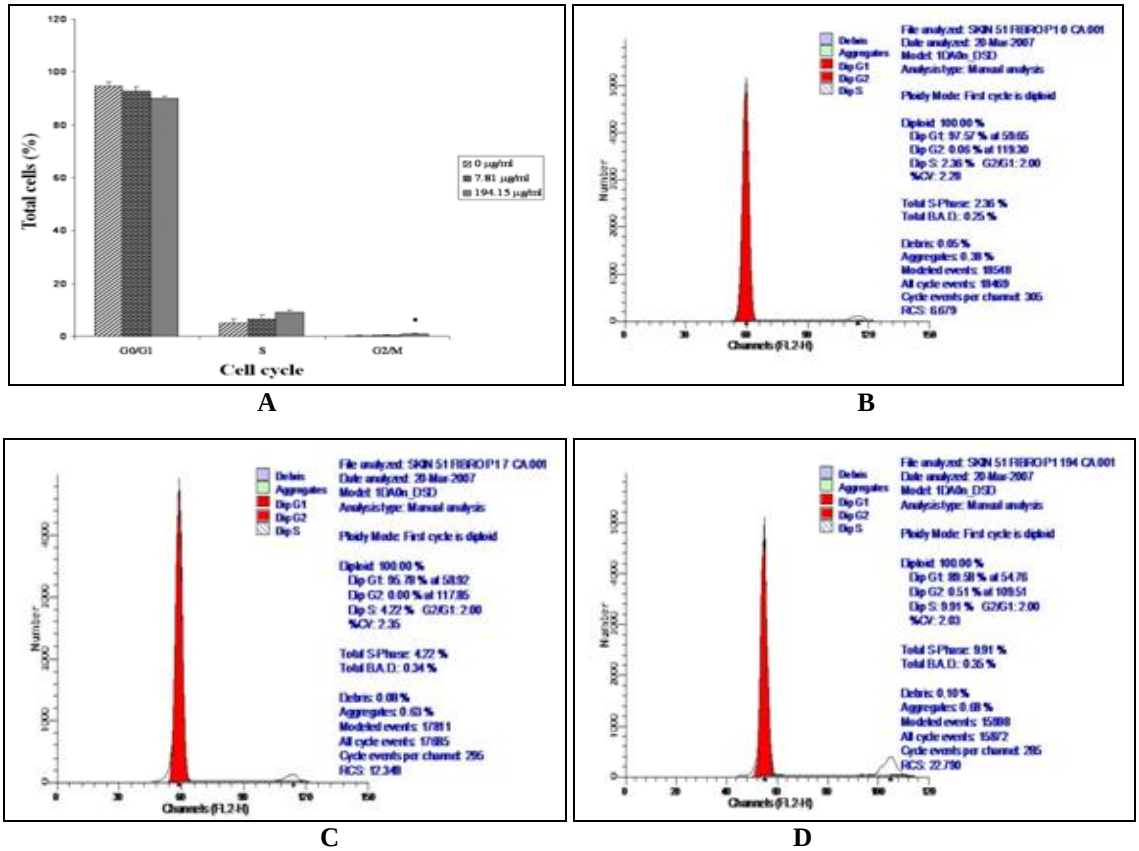


FIG. 4: EFFECTS OF *CENTELLA ASIATICA* AQUEOUS EXTRACT ON CELL CYCLE OF DERMAL FIBROBLASTS
Graph showed significant value of dermal fibroblasts cell cycle versus total cells (%) (A) at G2/M phase (n=6) (Average ± S.E.M). Histogram showed all the cells are normal ploidy diploid (G2/G1 IS 2.00) at control (B), 7.81 µg/mL *Centella asiatica* aqueous extract (C) and 194.15 µg/mL *Centella asiatica* aqueous extract (D)

However, G2/M phase displayed significant average value at concentrations of 194.15 µg/mL when compared to control (0 µg/mL) (as shown in **Fig. 4A**). Cell ploidy is normal (100% diploid) after treatment with 0 µg/mL (control) (as shown in **Fig. 4B**), 7.81 µg/mL (as shown in **Fig. 4C**) and 194.15 µg/mL (as shown in **Fig. 4D**) CA aqueous extract supplementations.

DISCUSSION: Human skin wound healing is a complex, dynamic and organized process. Dermal fibroblast involves actively in the process of wound healing; its proliferation and migration to the wound site, resulted in the synthesis of extracellular matrix, creating and producing granulation tissue that start the process of wound shrinkage and closure [17, 18]. In this study, CA aqueous extract significantly inhibit the growth of *in-vitro* dermal fibroblasts culture at high concentration of supplementations. Antiproliferative effects might be due to the cytostatic or cytotoxic effects of CA aqueous extract. The absence of formazan crystal formation observed under phase contrast microscope at the concentration of 1000 µg/ml on day 4 and 8 suggested the possibility of toxic effects of CA extract on dermal fibroblast culture at this concentration. Antiproliferative effects of CA triterpenoid on the *in-vitro* dermal fibroblasts culture have also been reported in previous studies [19]. In this study, CA aqueous extract inhibit the in vitro migration of dermal fibroblasts which coincides with the antiproliferative effect of the extract observed in the MTT study. The IC_{50} value for CA aqueous extract in this study is at 194.15 µg/mL and was determined from dose-response curve study.

Previous study has chosen concentration of CA triterpenoids at 160 µg/mL for gene expression analysis using cDNA microarray analysis and real-time RT-PCR [2]. Other study has concluded that the IC_{50} for SVK-14 cells (keratinocytes cell line) is at 209.9 µg/mL [20]. The IC_{50} value for CA aqueous extract used in this study is comparable to other studies. This minimal difference might be due to the different concentration level of active

component in the different extracts used in each study. Influence of the concentration is also cell specific. The difference in active component level might be caused by the different methods of extraction, whether water-based or solvent-based extraction. Genetic or environmental modifications of the plant might also influence the outcome of the extraction.

The investigated genes in this study are collagen type I, collagen type III, fibronectin and α-smooth muscle actin 2 which plays a major role during the skin wound healing process. The mRNA expressions for collagen type I, collagen type III, fibronectin and α-smooth muscle actin 2 relative to GAPDH showed decreasing trends with the increasing supplementations of CA aqueous extract. However, the average value for the gene expressions was not significant after treatment with CA aqueous extract at concentration of 7.81 µg/mL and 194.15 µg/mL (IC_{50}) when compared to control.

Previous findings reported no changes in the levels of collagen gene expressions (COL1A2 and COL3A1), fibronectin and collagen processing enzymes when dermal fibroblasts were treated with 160 µg/ml of CA for 24 hours [2]. On contrary, another study demonstrated that asiaticoside stimulates synthesis of human collagen type I through TGF-β receptor I kinase-independent Smad activation pathway [4]. In addition, previous studies reported by using cDNA microarray analysis, asiaticoside treatment on dermal fibroblast increased expressions of collagen type I and III. The changes of mRNA levels for collagen type I and III was confirmed using Northern blots [14, 21].

The different observation might be due to the difference in reaction mechanism between asiaticoside and combination of active compounds of CA. Another notable component of CA extract, asiatic acid, has been reported to inhibit TGF-β1-induced collagen type I and PAI-1 expression while elevating Smad 7 protein level in keloid fibroblasts via PPAR-γ activation [22].

The reduced expressions of collagen type I caused by CA aqueous extract in this study was believed to be one of the factors that caused the antimigratory effects of dermal fibroblasts [19]. Despite the antiproliferative effect exerted to the dermal fibroblasts, result from this study showed that the cell ploidy is normal even after treatment with high concentrations of CA aqueous extract.

Any changes or abnormality in wound healing process resulted in various complications. One of it is the formation of keloid fibroblast. Keloid fibroblast produced collagen (predominantly collagen type I) [22, 23], elastin [24], fibronectin [25, 26] and proteoglycan [27] at high levels and showed abnormal responses to stimulations [28]. Previous study demonstrated an increasing production of soluble collagen types I and III in the normal fibroblasts when co-cultured with keloid-derived keratinocytes [29]. They also concluded that the overlying epidermis of keloid tissue has profound effects on the production and organization of collagen by fibroblasts in the mesenchyme.

The progression of keloid formation, *via* the TGF-ß1 expression in the inflammation stage of wound healing could positively be influenced by nitric oxide pathway [30]. Increased infiltration of fibrocytes compared to normal tissue was apparent in keloids [31]. Total relative collagen type III produced in normal scar is much higher compared to normal skin. In keloid fibroblasts, the total fibronectin expression has been shown to be higher compared to the control dermal fibroblasts [32].

Activin-A and α-SMA, are a phenotypic marker for myofibroblasts which was found to be upregulated in keloid tissues compared to normal fibroblast [26, 33]. Myofibroblast characterized by alpha-smooth muscle actin 2 (SMAA2) is found in high number in the collagen nodules of hypertrophic scar. CA aqueous extract might probably be able to reduce the productions of high extracellular matrix in keloid by reducing the gene expressions of collagen type I, collagen type III, fibronectin and α-SMAA2. Dermal fibroblasts from keloid have high capacity to proliferate [34].

Since CA aqueous extract showed antiproliferative effects on human dermal fibroblasts, it might also have antiproliferative effects on dermal fibroblasts in keloids. With that, CA aqueous extract have high potentials to be used as antikeloid agent. However, further study on the effect of CA on keloid fibroblasts of the skin is necessary.

CONCLUSION: Aqueous extract of *Centella asiatica* caused antiproliferative and antimigratory effect on *in-vitro* primary human dermal fibroblasts culture. However, it could have huge potential as an antikeloid agent since it can reduce the gene expressions of type I collagen, type III collagen, fibronectin and SMAA2. These genes are highly expressed in keloid scars.

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CONFLICT OF INTERESTS: The authors have declared no conflict of interests.

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