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The effect of the *swietenia mahagoni* seed extracts on the production of collagen in human fibroblast cell (HSF1184)

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Abstract. We have used the in vitro sircol collagen test to investigate the effects of *Swietenia mahagoni* seed extract on collagen production in human fibroblast cells (HSF1184). The results showed that *Swietenia mahagoni* seed extract with concentrations of 0.001, 0.01 and 0.1 mg / mL caused stimulation in the synthesis of type I collagen in HSF1184 cells. Although, three concentration of SC-CO₂ and soxhlet that showed no statistically significant of collagen on the HSF1184 when compared with the negative control, while LA (fibroblast cells with pure Linoleic acid as supplement in culture medium) gave significant effect on the collagen production.

1. Introduction

Swietenia mahagoni is commonly used as traditional medicine in several countries including, Indonesia, Malaysia, India and China. The *S.mahagoni* seeds studied have several uses as herbal medicines and are proven to have antifungal and antibacterial properties [1] and can inhibit platelet aggregation induced by platelet activating factor [2] and has anti-human immunodeficiency virus activity [3]. Ethanol extract from *S.mahagoni* seeds has antioxidant activity in vitro [4]. Linoleic acid as an important fatty acid that must be consumed for good health care. Linoleic acid has become popular in the beauty products industry in skin care. Research shows that linoleic acid is anti-inflammatory, reductive and can moisturize when applied topically to the skin [5]. *Swietenia mahagoni* has higher linoleic acid content of 30.1% [6].

Fibroblasts are found in inflammatory lesions due to tissue injury by various different agents. They influence the repair of damaged tissue by synthesizing and placing extracellular components that form scars. The mechanism by which special effector cells are attracted to the site of tissue injury and which is inflamed. Collagen is an important component in the wound healing stage. Collagen is produced from fibroblast cells to give strength to all tissues and play an important role for tissue growth and recovery. Fibroblast are responsible for the synthesis of collagen and other protein regenerated during the repair process [7].



2. Method

2.1. Fibroblast Cell Culture

Human Skin Fibroblast 1184 (HSF 1184) was used in this study. HSF 118 was cultured by seeding the cells using 3×10^5 cells/cm² cells density with MEM medium. Firstly, the MEM media was mixed with 1% penicillin streptomycin and 10% Fetal Bovine Serum (FBS) and then cells cultured in a 75 cm² T-flask and maintained at 37°C, 5% humidified CO₂ incubator until cells were 90-95% confluent. The medium in the flask was changed every two or three days until cells become 90-95% confluent.

The subculture procedure was conducted immediately when cells achieved 90-95% confluent monolayer. Firstly, the medium in 75 cm² T-flask was aspirated and rinsed three times with 5 ml phosphate buffer saline (PBS) to get rid of cellular debris or serum which could interfere the action of trypsin. Then, remove PBS and add 3 ml of trypsin-EDTA. The flask was incubated at temperature 37°C for three minutes. Then, a flask was observed under microscope to ensure the cells detach from the flask's surface. 3 ml of MEM was added to neutralize the trypsin. Next, the cell mixture in the flask was poured into a centrifuge tube and was centrifuged at 3300 rpm for 10 minutes. Then, the supernatant is removed and the pellet is diluted MEM medium. Lastly, Fibroblasts cells (HSF 1184) were incubated in 5% CO₂ air atmosphere at 37°C.

2.2. Cell Cryopreservation

Cells in the log phase are stored to be frozen. Cells are mixed and centrifuged. Clotting media containing 90% fetal bovine serum and 10% Dimethyl Sulfoxide slowly added to cell suspension. After that, the cells were aliquoted into 1.2 ml of the cryogenic vial that had been labeled before and the vials were put into the Nalge Nunc freezing container (for adaptation process) with temperature of -70°C for 24 hours. Then, a vial containing cells is stored in liquid nitrogen storage [8].

2.3. Cell Viability

Method of determining the number of living cells, these cells need to be calculated using the dye exclusion test. The principle of this test is that living cells have intact cell membranes that will exclude certain dyes while dead cells do not. Trypan blue and Neubauer exclusion tests increase bright line hemocytometers (Fortuna®, Germany) used to apply cell counts. The dead cells will accumulate trypan blue whereas live cells will exclude it. Firstly, a clean coverslip was placed on the top of haemocytometer slide. Then, 50µL suspension of fibroblast cells mixed with dye 50µL trypan blue. After that, the mixture is transferred to the edge of the coverslip. Then, the amount that is not stained (living cell) and stained (dead cell) were counted under a light microscope (Zeiss, axiovert 40CFL, Germany). The concentration of viable cells was calculated using the following equation:

$$C = NA \times D \times 10^4 \quad (1)$$

C = Concentration of cells (Cells/mL)

NA = The average number of living cells

D = Dilution factor

Whereas, percentage of cell viability was calculated using the following formula:

$$\text{Percent of viability (\%)} = (\text{Number of cells unstained}) / (\text{Total number of cells}) \times 100 \quad (2)$$

2.4. Growth Profile

A growth profile or growth curve can be useful in assessing the growth characteristic of fibroblast. From the growth curve, information of lag phase, exponential phase, saturation density and stationary phase can be easily determined.

Ten 25cm² t-flasks were prepared and labeled for the cell culture. Each of the flasks was seeded with similar concentration of fibroblast which is 5×10^5 cells/ml. Other than that, this procedure also involved basic handling of cells such as washing, trypsinization and centrifugation so that the pellet can be obtained for cell counting. After 24 hours, one of the flasks were taken out and the number of fibroblast cells were counted using the method described. All steps were repeated until day 10.

2.5. Sircol Collagen Assay

Sircol Collagen Assay that can be used to evaluate and monitor the amount of collagen produced in cell culture in vitro Cultured human skin fibroblast cell (HSF1184), standard reagent blank (100 ml) mixed with Sircol Dye reagent (1 ml) into a microcentrifuge tube and left for 30 minutes. The collagen-dye complex forms precipitation from the solution. The complex was packaged firmly to the bottom of the tube by centrifugation at 12,000 rpm for 10 minutes. Unbound dyes are removed by turning and drying the tube. Washing cold acid salts (750 μ l) was added gently to remove unbound dyes from the surface of the pellets and microcentrifuge tubes. The tube was centrifuged at 12,000 rpm for 10 minutes and the washing was drained. Bound dye is released and dissolved through the addition of alkaline reagents (250 μ l or 1000 μ l depending on the size of the pellet) and mixing the vortex for 10 minutes. The dyes released are measured spectrophotometry at 555 nm or colorimetry with blue-green filters using multi-wall plate readers [9].

3. Result and Discussion

3.1 The Growth Curve of Human Skin Fibroblast (HSF1184) Cells Line

Fibroblasts are a type of primary cell located within the dermis of the skin. They present an important role in wound healing through collagen synthesis and extracellular matrix (ECM) in connective tissue. Furthermore, they also capable to produce growth factor such as PDGF, TGF-B and KGF-1 which plays a role in proliferation, migration, and differentiation of other cells that help the wound healing process [10].

Thus, the growth cycle of fibroblast was investigated as its provide the necessary information of cell that is handle such as seeding concentrations, the duration of growth before subculture and the duration of the experiments [11]. The growth curve of fibroblast was implemented using ten 25cm² T-flask without medium replenishment. The density used was 5×10^4 Cells/mL and the number of fibroblasts cells was calculated daily by haemocytometer. Figure 1 presented the growth curve of fibroblasts cell line.

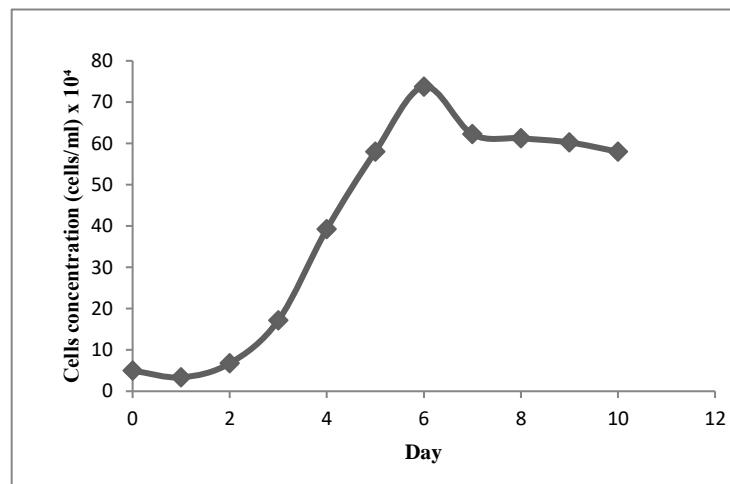


Figure 1. The normal curve growth of human skin fibroblast cells (HSF 1184)

Normally, the growth cycle of fibroblast consist three phases which are lag phase, log phase and stationary phase [11]. The figure 1 show that the lag phase occurs for 2 days. The lag phase from fibroblasts were determined because of extrapolated the intercept between the straight line from the log phase and cell density at day 0 (5×10^4 cells/ml). During this phase, the cells tried to adapt with the new growth environment such as Fetal bovine serum which needed for the cells growth. Furthermore, at day 1 and 2, the cells were unable to duplicate themselves and the number of cell

constant due to incapable to survive with the new environment. However, the remaining cells that survived started to duplicate themselves and increased the cell number.

The cells started to enter log phase after 2 days and continued increasing exponentially until day 6. At this phase, the growth of the cells is high (usually 90-100%) and cells were in the most reproducible form. Thus, time optimum for sampling is at this phase due to it showed the most consistent and high viability of the cells. At the end of this phase, the cells become confluent and all the available surface of the growth was occupied.

After that, at day 6 until day 10 the cells started to decrease and get into the stationary stage. The saturation density of the cells was estimated to be 62.25×10^4 cells/mL. This saturation density showed the maximal population growth achieved using particular growth condition. At this phase the cells proliferation was decelerated due to lack of medium for essential nutrient and also the division of the cells was balanced with the death of the cells [12]. However, regular media changes will make the cell continue to proliferate (although at reduce rate) well beyond confluence which resulted in multilayer of cells [11].

3.2. The Effect of *Swietenia mahagoni* seed on collagen production in human fibroblast cells (HSF1184)

Collagen is an important component in all phases of wound healing. Collagen is synthesized by fibroblast cells, they provide integrity and strength for all tissues and play an important role in increasing the proliferation and remodeling phase. Collagen is the basis for intracellular matrix formation in wounds.

In this study, the Sircol™ Collagen Assay was used to evaluate the collagen synthesis in the human fibroblast cell (HSF 1184).

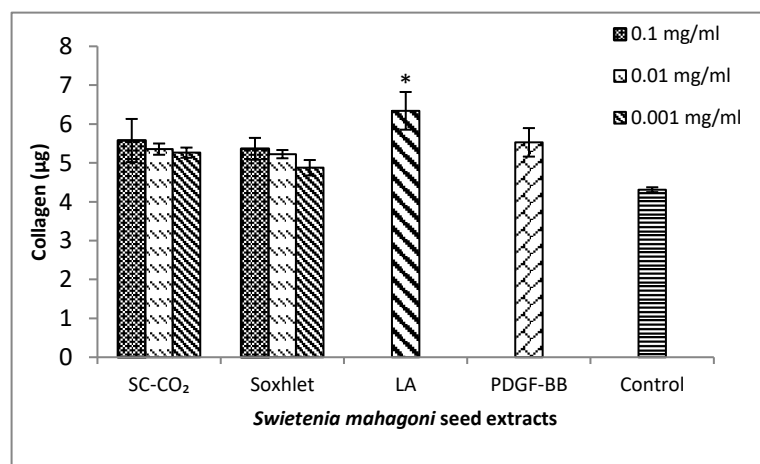


Figure 2. The effect of *Swietenia mahagoni* seed extracts on the collagen synthesis in human skin fibroblast cell (HSF 1184). LA (linoleic acid) and PDGF-BB (positive control) and MEM (negative control).

Increasing fibroblast cells are accompanied by new capillary growth in the wound to provide for metabolic processes. The strength and vascular integrity of the new capillary layer are caused by collagen production and cross-linking. Cross-linking of one of the collagen fibers has been assessed for postoperative bleeding in patients with normal coagulation parameters. In the initial phase of proliferation, fibroblast cell activity occurs in cellular replication and migration. On the third day after the wound, the mass of growing fibroblast cells begins to synthesize and release collagen production. Collagen levels rise continuously for about three weeks. The amount of collagen released during this

period determines the tensile strength of the wound. Increased collagen production is an important factor for wound healing. Type-I collagen is the main collagen of bones, tendons, skin, and wounds that have just healed [13].

Figure 2 shows the effect of extract from *S. mahagoni* seeds on collagen synthesis from fibroblast cells (HSF1184). In this study only three concentrations were chosen by reason of these three concentrations proved to be consistent in stimulating the growth of fibroblast cells (HSF1184) based on the results of previous studies. The graph in Figure 2 the three concentrations cause stimulation in the synthesis of type I collagen in HSF1184 cells. Although, three concentration of SC-CO₂ and soxhlet that showed no statistically significant of collagen on the HSF1184 when compared with the negative control, while LA (fibroblast cells with pure Linoleic acid as supplement in culture medium) gave significant effect on the collagen production ($p < 0.05$).

4. Conclusion

Swietenia mahagoni seed extract with concentrations of 0.001, 0.01 and 0.1 mg / mL caused stimulation in the synthesis of type I collagen in HSF1184 cells. Although, three concentrations of SC-CO₂ and soxhlet are showed no statistically significant of collagen on the HSF1184 when compared with the negative control, while linoleic acid (fibroblast cells with pure Linoleic acid as supplement in culture medium) gave significant effect on the collagen production.

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