

THE SCREENING OF LOCAL HERBS IN TREATING NON HEALING WOUNDS AND DIABETIC FOOT ULCERS COMPLICATIONS USING NIH 3T3 MOUSE FIBROBLAST AND RAW 264.7 MOUSE MACROPHAGE CELLS.

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Abstract

Lee Indica and *Stachytarpetta* is the common plant in Malaysia and people are using as a traditional wound healing agent since an ancient era. The aim of this current study was to determine the diabetic wound healing activity. The NIH 3T3 mouse fibroblast cells and RAW 264.7 mouse macrophage cells cultured to confluence in a six-well culture plate, and the culture medium drained away. The area of cell is scraped gently with a rubber stick to create a wound with 0.5mm width. The herbal extract added at 100 mg/mL to the culture while the control culture received only PBS. The migration of cells were observed after 24 h. The *leea indica* and *stachytarpetta indica* has shown a positive results when tested on RAW macrophage cells compared to its result when tested on NIH 3T3 mouse fibroblast cells which shows a less positive results. It can be concluded that both *leea indica* and *stachytarpetta indica* can be used to treat wound as it enhance the mitochondrial activities of cells. The extract from *leea indica* is more effective on tested cells compare than *stachytarpetta indica*. It is suitable to be used on macrophage cells rather than on fibroblast cells. It will support to the use of *leea indica* for the treatment of wound healing.

Key words: diabetic wound, fibroblast, *lee indica*, *stachytarpetta*

Introduction

A discontinuity in the epithelial cells of the skin is a sign of wound. The function and structure of the tissues were also disrupted. [1-2] Through wound healing, the homeostatic mechanisms of the cells are re-established, infection can be prevented and excessive fluid loss are avoided. [3]

Diabetics are more prone to have wound complications compared to non-diabetics patient and usually they developed an open and non-healing wound of the extremities. [4-5] Majority of Diabetes Mellitus patients are facing with impaired wound healing and usually have chronic non-healing localized at the sites of pressure on the foot. [6] Two National Health and Morbidity Survey were conducted in Malaysia in two different years. The prevalence of Diabetes Mellitus of 6.3% and 8.2% are reported, respectively. The prevalence is reported to be higher in Kelantan at 10.5%. [7]

Many efforts and treatments have been made to lessen the Diabetes Mellitus problems among Malaysians. Some of the treatments that are usually given to patients are medicines that are taken orally and by injecting insulin directly into the system.

In this research, Malaysian medicinal herbs are chosen to cure the non-healing wound of diabetics. There are certain types of natural herbs that have been used by practitioners and doctors in treating wounds. A better results of wound healing can be seen when the aqueous extracts of *Trifolium canescens* and *Trifolium pretense* were applied on the wound. [8] Wound healing involves a complex process at cellular level.

Some of the processes are the integration of inflammation, mitosis, angiogenesis, synthesis and remaking of the extracellular matrix. [9] Large populations of cell are involved in the process of wound healing. Extracellular matrix and the action from some of the mediators such as growth factors and cytokines are also involved. Acute and chronic wounds are categorized based on the time taken for their healing. [10] Acute wounds have the ability to repairs themselves when there is injury and succeeded in following a timely and orderly healing process pathway. Some of the characteristics of acute wounds are the function and the anatomic structure of the cells is restored at the end of the process. The process of wound healing usually occurs within 30 days. Acute wound can be acquired from a traumatic loss of tissue or can be acquired from a surgical procedure [11]. Compared to acute wounds, chronic wounds did not complete the healing process and the cells cannot be repaired to what it supposed to be, which is the normal cells.

The incomplete healing process might be disturbed by various factors. Those factors may have caused one or more of the healing stages to be longer or shorter than usual. Some examples for the factors are infection, tissue hypoxia, necrosis, exudate and high levels of inflammatory cytokines. Unlike in acute wound healing process, the end products of chronic wound healing process are upsetting. The functional and the anatomical outcomes are poor and the wounds are frequently relapsing. Chronic wounds may be resulted from naturopathic, pressure, arterial and venous insufficiency, burns and also vasculitis. [12] Many types of treatments have been introduced in order to treat Diabetes Mellitus wounds. One of the treatments is antibiotic therapy. This treatment functions by using antimicrobial therapy to lessen the number of microorganisms contaminating the wound surface. [13] There are also hyperbaric oxygen treatments which help in fighting infections and also improving the wound healing by increasing the oxygen delivery to the ischemic tissue. Other than that, revascularization treatments are also introduced. It improved the blood flow to the ischemic foot to control infections. Besides, larval (maggot) therapy is also used in chronic wound treatment. It acts in a way that treats the infections on the soft tissue and bone, debriding the wound and also lessens the wound odor. [14]

Herbal medicine has also become one of the choices of treatment for medical practitioners nowadays. Some of the plants used in treating diabetes are *Antigonon leptopus*, *Bidens alba* and also *Bidens pilosa*. [15] Natural products are believed to be safer because they can easily adapt to the biological systems. [15]

***Leea indica* (Vitaceae)**

Leea indica (Burm. F.) Merrill is one of the many kinds of Chinese medicine that belongs to the Leeaceae family. This plant usually can be found in tropical and subtropical country such as Malaysia, China, India and also Thailand. Its leaves and roots are used to treat various kind of diseases including diabetes. [16] Its flowers also have been studied for various activities such as anti-oxidants, anti-microbial and many other activities. The root of *L. indica* is used as a sudorific, anti-diarrhoeal, anti-dysentric, anti-spasmodic and to treat cardiac and skin problems. The methanolic extract of *L. indica* was reported to have a strong antioxidant activity mainly because the existence of gallic acid, a type of antioxidant compound. [4]

***Stachytarpheta Indica* (Verbenaceae)**

Stachytarpheta indica Vahl (Verbenaceae) is commonly known as snake weed. It is an annual herb that can reach to about 2-3 feet high with its stems erected. *S. indica* has been used traditionally to treat various kinds of diseases such as asthma and headache. The plant has also been used in the traditional system for diabetes and liver components treatments. [16] The plant contains Ipolamide, C29-C35 hydrocarbons, α -spinasterol, saturated aliphatic commonly known as snake weed. It is an annual herb that can reach to about 2-3 feet high with its stems erected. *S. indica* has been used traditionally to treat various kinds of diseases such as asthma and headache. The plant has also been used in the traditional system for diabetes and liver components treatments. [16] The plant contains Ipolamide, C29-C35 hydrocarbons, α -spinasterol, saturated aliphatic ketone and aliphatic carboxylic acid and also an unsaturated hydroxycarboxylic acid. The stem and leaves of this plant have a lot of chemical properties that may contribute to the research such as iridoid glycoside, tarphetalin, choline and many more. [17-18]

Methods

Chemicals

Trypsin, Analytical grade ethanol (70%) was used for extraction purposes, antibiotic streptozocin (STZ), sulforhimine b sodium salt (SRB), rosiglitazone (RS), ethanol, Phosphate Buffer Solution (PBS), Dulbecco's Modified Eagle Medium (DMEM).

Materials

Serological pipette, tips (yellow, white and blue tips), T flask, petri dish, 96 well plate (flat bottom), syringe, filter (0.02 μ m), Appendorf tube.

Collection of plant material

The ripen fruits (500 g) of EEPM was collected in September 2013 from local area of Kuantan, in the state of Pahang, Malaysia. Taxonomic identification was done by Dr. Norazian Mohd Hassan (Voucher specimen no: PIIUM 0233) and deposited in the Herbarium, Kulliyah of Pharmacy, IIUM, Malaysia. The mesocarp & pericarp of the fruits were sliced and dried in a normal room temperature at 25°C for 10 days, then pulverized to powdered form (378 g) using Fritsch Universal Cutting Mill-PULVERISETTE 19, Germany, and kept at 4°C until further use.

Preparation of ethanolic extract

The sample (378 g) was extracted using cold

ethanolic maceration for 72h at room temperature, then filtered into a sterile round bottom flask using adsorbent cotton wool and filter paper (Whatman No. A-1). [17] The earlier stated extraction procedure was repeated for eleven times to ensure the highest percentage yield of *ethanol* soluble compounds from the EEPM powder. The *ethanol* extract was concentrated in vacuo (temperature at 45°C, 175mbar and rotation 80-85rEPM) using a rotary vacuum evaporator (BUCHI R-205) to a final corrected volume of 500 ml. This was further frozen at -70 °C and shifted instantly to three weeks successive freeze drying at -50 °C using bench top freeze dryer (ALPHA 1-4LD-2), to give a ultimate yield of 83 g.

Methods

Cell Culture (The method follows IVIS Imaging Protocol). Frozen cell vial (NIH/3T3) thawed by putting it in 37°C water bath. After it thawed, the cells transferred into 15 mL conical tube. 5 mL of growth medium (DMEM) added into the tube. Later, the tube is spinned at 1000rpm at 4°C for 5 min. The medium aspirated and the cell pellet resuspended with 5 mL of growth medium. The cell suspension put in T25 flask, and is grown at 37°C, 5% CO₂, 100% humidity. The cells washed with PBS 3 times after it is 75% confluent. 1 mL trypsin is added into the flask to dissociate the cells from the bottom of the flask. The flask incubated for a while and examined under microscope. Then, 5 mL of DMEM was added into the flask, and the cells resuspended by pipetting up and down. 0.5 – 1.0 mL of the cells were transferred into another T25 flask, and DMEM added to both flasks. Flasks were incubated to promote cell growth.

MTT Assay

The cells were cultured and transferred into 96-well plate and cells were incubated. After 24 hours, 10 μ L of MTT reagent was added to the plate. The plate was then be incubated about 2 to 4 hours until purple precipitate is visible. Then, 100 μ L of detergent was added. The plate will be left at room temperature in the dark for 2 hours. The absorbance at 570 nm will be recorded.

Scratch Assay

The normal human fibroblasts; NIH/3T3 cells cultured to confluence in a six-well culture plate, and the culture medium drained away. The area of cell is scraped gently with a rubber stick to create a wound with 0.5mm width. The rubber stick moved back and forth against the top of the culture. The well is washed three times with phosphate buffered saline

(PBS) to remove remaining cellular debris. The cultures maintained with a medium supplemented with 5% (FBS). The herbal extract added at 100 mg/mL to the culture while the control culture received only PBS. The migration of cells was observing and measured how much of the gap had been closed after 24 hours. Different concentration of extract was used to determine the most suitable concentration that can promote cell migration.

Results

MTT Assay

MTT assay test on RAW cells by using *leea indica* and *stachytarpetta indica* extract. Percentage of cell viability with different extract concentrations of *leea indica* (P9) and *stachytarpetta indica* (P10) on RAW 264.7 mouse macrophage cells. Based on the graph shown, the plant extract has no cytotoxic effect on the tested cells. (P10) on NIH 3T3 mouse fibroblast cells. From the given graph, it shows that the plant extract has no cytotoxic effect on the cells tested.

Scratch assay

The bar chart below shows the average distance of the migration of cells using different type of treatment on RAW 264.7 mouse macrophage cells. Based on the chart, both extracts give effects on the migration of the cells compared to untreated cells.

Discussion

Scratch Assay

Scratch assay is a process where cells are cultured to confluent and once it has confluent, a segment from the cells will be destroyed by scratching a line through it. The scratched line will creates a gap between these cells. The gap imitates the characteristics of wound and observed by using microscope from time to time to record the movements of the cells towards those gaps and thus closing it. The time taken for the gap to close is depend on characteristics such as the width of the gap, the movement of the cells are being induced by some drugs and many other examples. Scratch assay was conducted after the MTT assay test to observe the migration of cells in this study.

In this study, *Stachytarpetta indica* and *leea indica* has been choose as the cells movement inducer. Both of the plant has been discovered to have the properties of flavanoids, terpenoids, tannins and also reducing sugar. Flavonoids came from a large class of pigments that gave the plants its colour and have a potent antioxidant property. There has never been a report about these two plants having

characteristics such as toxicities and side effects up until today. Tannin has an angiogenic properties that can cause the up-regulation of VEGF-A expression during inflammatory phase. Due to this up-regulation, the maturation of wound was accelerated. VEGF is one of the long-term signals for angiogenesis stimulation in wounds. Thus, with the properties that tannins had the process of wound healing could be speed up and a faster recovery could be achieved. Referring to the chart as attached in the result section, both *leea indica* and *stachytarpetta indica* gave positive effects on RAW 264.7 mouse macrophage cells in enhancing the migration of the cells towards the closure of the gap. But the result is not the same when tested with NIH 3T3 mouse fibroblast cells. In this chart, only *leea indica* gave a positive effect on the migration of the cells. While *stachytarpetta indica* does not give any effects on the fibroblast cells. This is because the concentration of the *stachytarpetta indica* is too high and toxic for the cells which then caused the cells to die. From the results obtained from the scratch assay of the plant extract on both types of cells, it can be concluded that *leea indica* has a better wound healing properties than *stachytarpetta indica*.

MTT Assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay applied the principal conversion of MTT into formazan crystals and the determination of mitochondrial activity and cell proliferation by living cells. [15] This method is used to know the in vitro cytotoxic effects of any chosen drugs applied usually on cells lines or the primary patient cells. From the results obtained, *leea indica* and *stachytarpetta indica* has shown a positive results when tested on RAW macrophage cells compared to its result when tested on NIH 3T3 mouse fibroblast cells which shows a less positive results. The changes of yellow colour to purple colour of tetrazole to formazan indicate that there are cells activities such as cells proliferation, mitochondrial activities and other activities. The cells viability is also measured using spectrometer light absorbance. And from the graph obtained, it shows that the plant extracts that has been used has no cytotoxicity effects on the cells. By using MTT assay method, it can be concluded that both *leea indica* and *stachytarpetta indica* can be used to treat wound as it enhance the mitochondrial activities of cells. This study has partially achieved the objective because only the extract from *leea indica* gave a positive effect on all tests while *stachytarpetta indica* does not give the expected outcomes. Based on the results, both plant extract

are more suitable to be used on macrophage cells rather than on fibroblast cells. In conclusion, the results from this study gave the scientific support to the use of *leea indica* for the treatment of wound healing.

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Figure 1.1 A) *Leea indica* (Vitaceae) B) *Stachytarpheta Indica* (Verbenaceae)

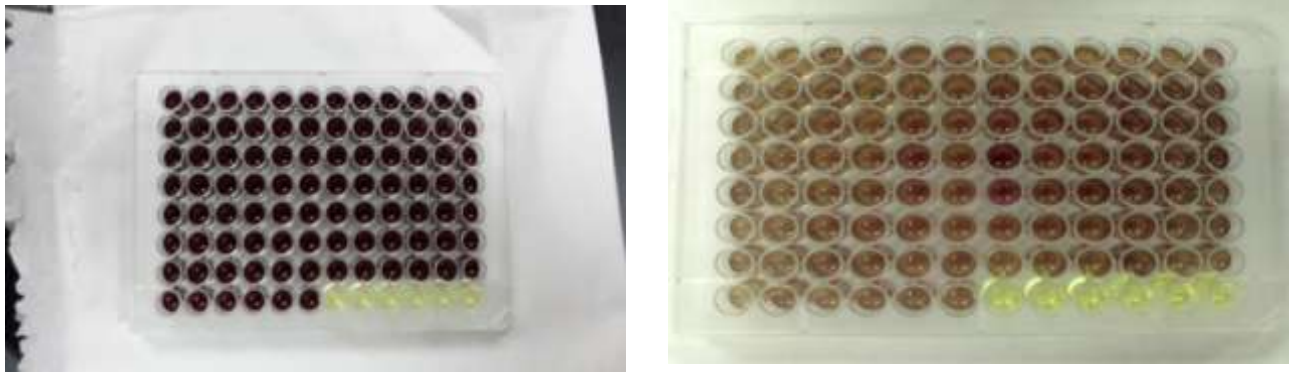


Figure 2.1 MTT assay test on NIH 3T3 cells by using *lea indica* and *stachytarpheta indica* plant extracts.

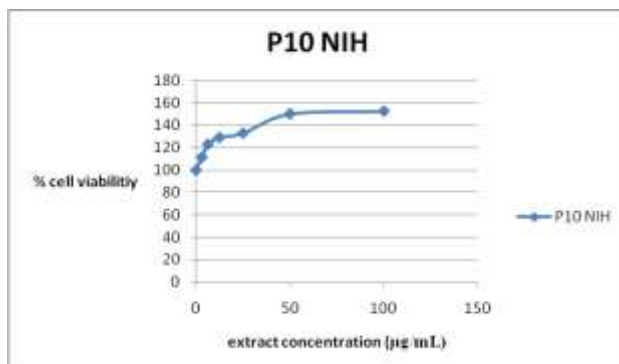
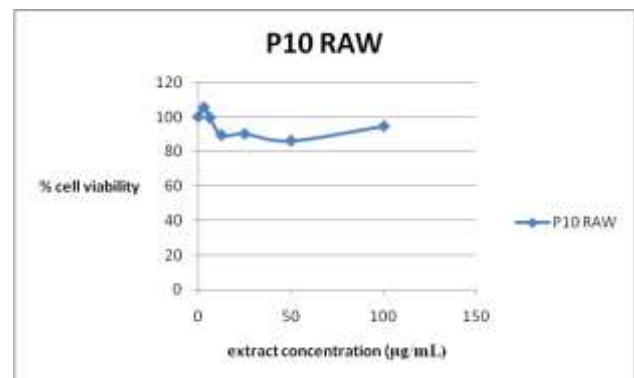
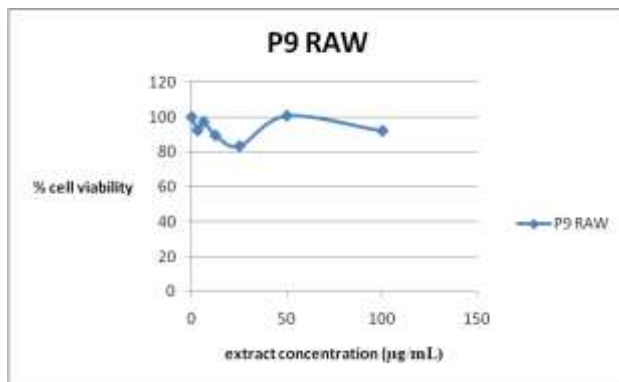


Figure 2.2 Percentage of cell viability with different extract concentrations of *lea indica* (P9) and *stachytarpheta indica*

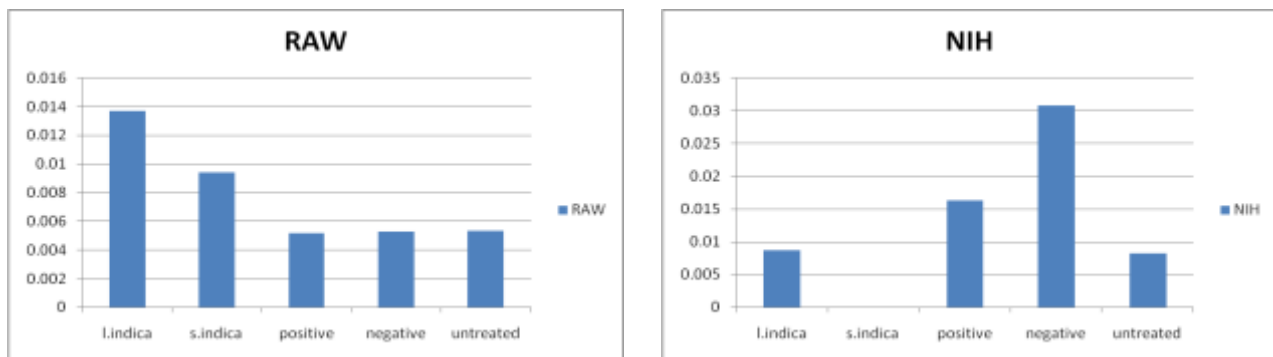


Figure 2.3 Bar chart shows the average distance of the migration of cells by using different type of treatment on NIH 3T3 mouse fibroblast cells. Based on the chart, *leea indica* give effects on the migration of the cells while *stachytarpeta indica* does not give any effects on the tested.

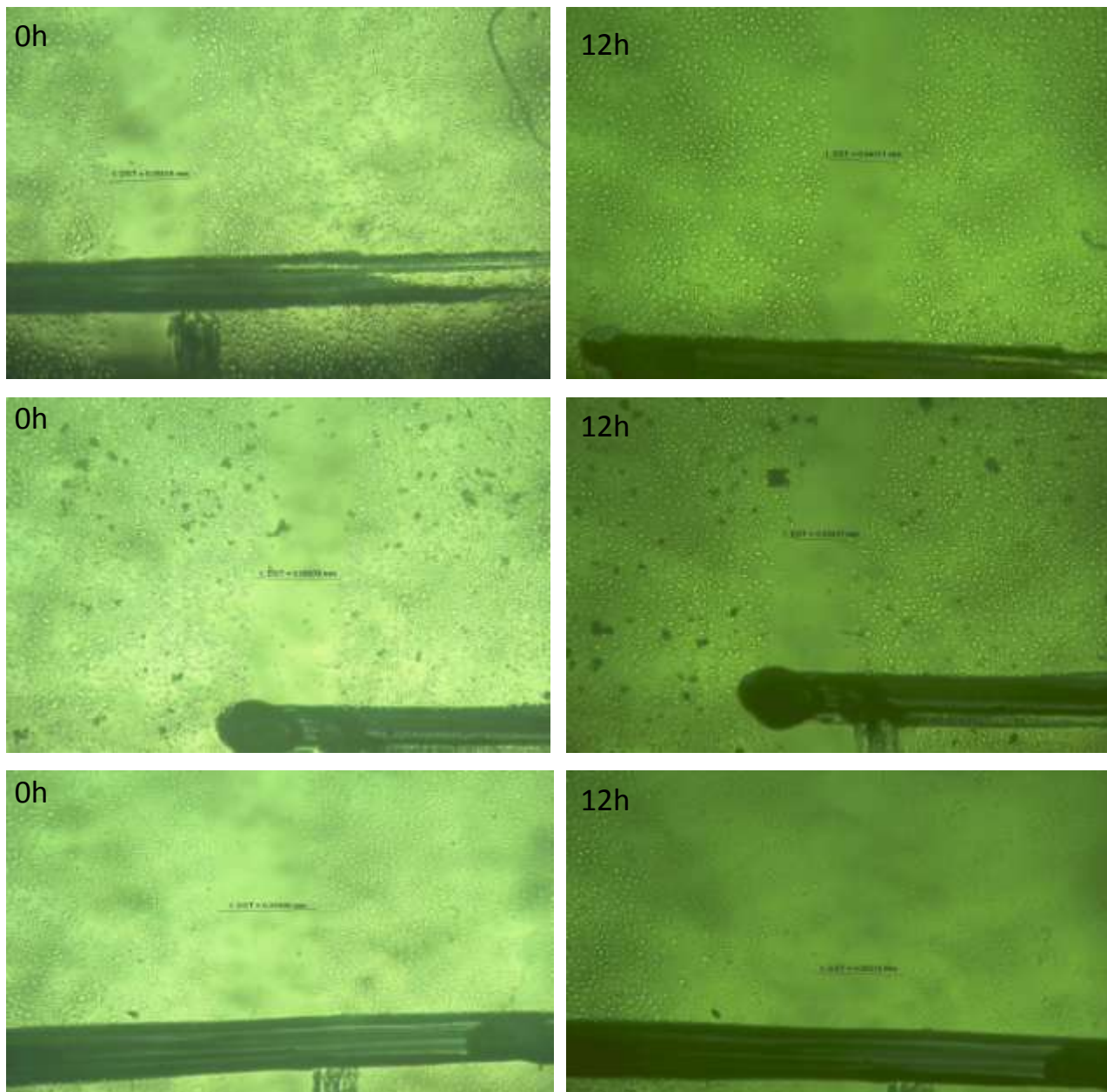


Figure 2.4 Scratch assay of *Leea indica* and *Stachytarpeta indica*