Analysis of Open Skin Wound Tissue Treated by *Alocasia denudata* Enggler Stem Extracts.

Analisa luka terbuka tisu kulit yang dirawat dengan ekstrak batang *Alocasia denudata* Enggler

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ABSTRACT

Malaysia has abundance of herbs and the majority of them have medicinal value. Keladi candik or its scientific name, *Alocasia denudata* Enggler (*A. denudata*) is an herbal plant that has been used traditionally for skin wound treatment. The objective of the study was to observe a healed open wound tissue sample of Wistar rats treated with aqueous extract of *A. denudata* stem for 14 days. The method uses Hematoxylin & Eosin (H&E) and Masson's Trichrome (MT) staining to analyze tissue healing and total protein determination by Bradford method. 20 tissue samples (n=20) were used to investigate tissue healing with 15 mg/ml of the aqueous extract of *A. denudata* stem. The negative control wound tissue samples received 0.1M sterile PBS solution and the positive control wound tissue samples received 15 mg/ml of pure curcumin extract solutions. As a result, the *A. denudata* treatment of histological observation showed the epithelialisation process begins on the 6th day after induced of wound with the differentiation of epithelium occurs on the 14th day. Collagen deposition begun on the 6th day and becomes denser until the 14th day. where collagen fibres dominated the wound area. The total protein concentration shows a pattern of decreasing of the protein concentration from day 1 (one) of induced wound until the 6th day but later increases at day 10th and finally decreases at day 14th. Statistical analyses showed that there are no significant differences with total protein concentration among the negative control compared to the positive control group with curcumin and the A. *denudata* treatment group. In conclusion, the treatment of *A. denudata* stem aqueous extract show healing effect on open-wound and has a great potential to be developed as wound healing agent.

Keywords: *Alocasia denudata* Enggler, Skin, Histology, Bradford test, Wound healing

ABSTRAK

Malaysia adalah negara yang kaya dengan tumbuhan herba dan sebahagian besarnya mempunyai khasiat dan manfaat kesihatan. Keladi candik atau nama saintifiknya *Alocasia denudata* Enggler (*A. denudata*) adalah tumbuhan herba yang digunakan dalam perubatan tradisional bagi penyembuhan luka kulit. Kajian ini dilakukan untuk menganalisis sampel tisu penyembuhan luka terbuka pada tikus Wistar yang telah dirawat dengan ekstrak akueus *A. denudata* selama 14 hari. Kaedah yang digunakan untuk analisa ialah ujian histologi dengan pewarnaan Hematoksilin & Eosin (H&E) dan Masson's Trichrome (MT) serta ujian penentuan kandungan protein melalui kaedah Bradford. Sampel tisu luka yang dirawat dengan larutan PBS dijadikan sebagai kawalan negatif dan sampel tisu luka yang dirawat kurkumin tulen berkepekatan 15 mg/ml pula dijadikan sebagai kawalan positif manakala sampel tisu luka yang dirawat dengan ekstrak akueus batang *A. denudata* berkepekatan 15 mg/ml dijadikan sebagai kumpulan rawatan. Sebanyak 20 sampel tisu (n=20) dianalisa bagi setiap kumpulan. Pemerhatian histologi menunjukkan epitelialisasi luka rawatan *A. denudata* pada tisu luka hari ke-6 dan pembezaan epitelium pada tisu luka hari ke-14. Penimbunan kolagen mula berlaku pada tisu luka hari keenam namun semakin padat pada tisu luka hari ke-10 dan tisu luka hari ke-14 menunjukkan serat kolagen mendominasi kawasan tisu luka. Penentuan aras protein jumlah menunjukkan pola penurunan aras protein dari hari pertama hingga hari ke-6 dan aras protein meningkat semula pada hari ke-10 dan menurun semula pada hari ke-14. Analisis statistik menunjukkan tiada perbezaan signifikan antara purata kepekatan protein antara kumpulan kawalan PBS dan kurkumin dengan kumpulan rawatan *A. denudata*. Kesimpulannya, rawatan ekstrak akueus batang *A. denudata* mempunyai kesan penyembuhan pada luka kulit yang terbuka dan berpotensi dibangunkan sebagai agen penyembuhan luka.

Kata kunci: *Alocasia denudata Enggler*, Kulit, Histologi, Ujian bradford, Penyembuhan luka

INTRODUCTION

A wound occurs when the integrity of any tissue is compromised for example skin breaks, muscle tears, burns, or a bone fracture. A wound can be caused by harmful physical forced which result from a fall, bad knock, cuts and puncture or by an infection. An open wound is a contusion in which

the skin is broken and capillary or blood vessel leaks blood into the surrounding area. (Mallefet & Dweck 2010). Acute wounds normally heal in a very orderly and efficient manner characterized by four distinct, but overlapping phases: haemostasis, inflammation, proliferation and remodeling. Haemostasis is the normal response of vessel to injury by formation of clot to establish a barrier against the invasion of microorganisms, formation of fibrin network for cell migration thus restoring skin function as a protective barrier for skin integrity. Inflammation phase involves migration of cells such as neutrophils and macrophages which activated by growth factors signal which contribute to the continuance of inflammatory response, clearing of the tissue and destroying invading agents. Proliferative phase is represented by process such as angiogenesis, extracellular matrix formation and epithelization of the wound site. Remodeling phase involves repair process at the wound site which is replacement of rich-collagen type I granulation tissue with collagen type III and migration and proliferation of new cells at the wound site triggered by the presence of growth factors (Gonzalez AC et al. 2016; Luis Cañedo-Dorantes & Mara Cañedo-Ayala 2019; Mehmet Evren Okur et al. 2020; Melanie Rodrigues et al. 2019

Traditionally, when antibiotic was not known to exist, few species of plant were used to treat disease, infection and even injury. These plants have some richness in medicinal values. Malaysia is a tropical country that has vast diversity of tropical plant. There are about t 1200 plant species that are identified as medicinal plant in Malaysia. One of it is Keladi candik or *Alocasia denudata* Enggler (*A. denudata*) which belongs to the Araceae family. The herbal plant grows wildly in the forest and it requires dark and a humid condition to grow (Musa et al. 2009). The plant was used to treat open wound or injury. In traditional usage, the plant was torn and strip of its stem. The thin layer stem was placed on top of the wound site. The plant was secure on the wound by using a piece of cloth to cover on top and tied firmly. It is useful to treat dry wound for a quick healing (Noraida 2005). Previous studies showed that *A. denudata* extracts induce skin wound healing in animal studies (Zulasyraf et al. 2011; Mazlyzam et al. 2015). The main objective of this study was to determine the healing effect of aqueous extract of *A. denudata* stem in terms skin tissue remodelling, reconstruction and reorganization of skin cells and extracellular matrixs due to the open wound healing processes.

MATERIALS AND METHODS

Wound tissue sample

Wound tissue samples of 1.0 cm x 0.2 cm were immersed in 10% formalin and later used for histology observation. While some similar size tissue sample were frozen at 4˚C was used for total protein determination. The wound tissue samples were obtained from the previous in vivo experiment that was conducted according to the studies by Zulasyraf et al. (2011). Fifty Wistar rats (250-300 g) were divided into two main groups. The *A. denudata* aqueous stem extract treated group (15 mg/ml) and pure curcumin treated group (15mg/ml). Both groups were further divided again into five groups, which consists of five rats in each group. Four circular (6 mm in diameter) full-thickness wounds (2 treatments & 2 controls) were induced bilaterally on the dorsal of each rat skin. Each group of rats has their own control wound, which was treated with PBS solution. The study took 14 days to complete with five rats from each group sacrificed on days 1, 3, 6, 10 and 14 and the wound tissue were harvested for histological analysis and total protein determinations. For histology, Haematoxylin & Eosin (H&E) and Masson's Trichrome stains were used. Wound tissue samples were treated with the aqueous extract from *A. denudata* and observed its healing processes. The negative control received uses 0.1M Phosphate buffered saline (PBS) pH7.4 as negative control. The curcumin extract was used as positive control. All tissue samples from each group were continuously exposed for a period of time, which was consist of 1st, 3rd, 6th, 10th and 14th days.

Wound tissue samples preparation

The tissue samples were dehydrated by immersing in a series of different alcohol concentration which has 50%, 70%, 90% and absolute alcohol of I & II. The total time for immersing each wound tissue in each alcohol concentration (50%, 90% and absolute alcohol I) was 3 hours treatment with 50% alcohol, 90% alcohol and absolute alcohol II. The tissues samples were continued to be treated with 70% alcohol and absolute alcohol I overnight. For the cleansing process, the samples were immersed in

xylene I & II for 2 hours respectively. This process was further continued with tissue infiltration where the samples were immersed in paraffin I & II for 2 hours respectively and with overnight immersion in paraffin III. The embedding and sectioning process were carried out with tissue thickness of about 5-6 µm. This is followed by the de-waxing process of the tissue slides by dipping in a series of immersion with xylene I & II for 5 minutes respectively and later continued with 100% alcohol, 95%, 90%, 80%, 70 % and 50% alcohol for 3 minutes each respectively. Finally, the tissue slide was rinsed in distilled water for 5 minutes. All tissues samples were divided into two groups for further staining with either Haematoxylin & Eosin (H&E) or the Masson's Trichrome stain.

Staining

Haematoxylin & Eosin (H&E)

De-waxed tissue slides were immersed in Haematoxylin solution for 10-15 minutes and rinsed with flowing tap water. The slides were then immersed in 1% acid alcohol for 3 second and rinsed again with tap water. Slides were again rinsed in distilled water for 5 minutes and dipped in Eosin solution for 5 minutes followed by final rinsed with tap water.

Masson's Trichrome (Modification of Luna 1968)

The de-waxed tissue slides were immersed in Iron-Weigert Haematoxylin solution for 10 minutes, followed by rinsing with flowing tap water for 5 minutes. Next the slides were rinsed in distilled water 3 times and dipped in Biebrich Scarlet-Acid fuchsin for 5 minutes. The slides were then rinsed in distilled water for 3 times and immersed in phosphomolybdic acid for 10 minutes. The process continues to immersed the slides in Light Green SF-Yellowish solution for 5 minutes and rinsed with distilled water for 3 times. Slides decolourization was carried out with 1% acetic acid for 1 minute and finally rinsing with distilled water for 3 times.

Tissue slide dehydration

All of the stained tissue slides were immersed in 95% alcohol I & II and absolute alcohol I & II for 3 minutes respectively. The slides were then immersed in xylene I & II for 5 minutes respectively and dried at room temperature. The slides were then mounted with Di-N-butyl Phthalate in Xylene (DPX).

Total Protein determination (Bradford (1976)

Cold tissue samples were warmed at room temperature and washed with 0.15 M sodium chloride. The tissue was weighted and mixed with 0.1 M Phosphate buffer pH 7.4 solution at ratio of 1 g tissue: 4 ml phosphate buffer. The mixture was homogenized at 16000 rpm for 1-2 minutes. The homogenized tissue was centrifuged at 3000 rpm, 4˚C for 5 minutes and the supernatant was obtained. A total of 0.1 ml of supernatant was added to 9.9 ml 0.1 M Phosphate buffer pH 7.4 solution (100 times dilution). Next, 0.1 ml of the solution mixture was added to 5 ml of Bradford solution. The reading was done with a spectrophotometer with wavelength of 595 nm. The total protein concentration was determined through standard curve.

Statistical analysis

Statistical analyses used were independent t-test and one-way ANOVA for normal distributed data. For the not normal distributed data, Mann-Whitney test and Kruskal-Wallis test were used. All test was based on significance level, $p<0.05$. The value was stated in mean ± Standard deviation (SD).

RESULTS

Histology

Haematoxylin & Eosin

Observation of 1st day wound tissue showed necrotic layer and inflammation cells at the wound area. Necrotic layer consists of fibrin, blood cells, cell debris and damaged collagen fibres. Observation on 3rd day wound tissue doesn't show any difference except increase of inflammation cells that migrated to wound area. The observation of 6th day wound tissue showed the formation of the epithelial layer in all group, control and treatment tissue. The necrotic layer was separated from wound area and replaced with new epidermal layer. Keratin layer was also observed over the new epidermal layer. Fibroblast cells were dominating the wound area. Observation on the 10th day did not show any difference except fibroblast cells were arranged better compared to the 6th day. Further observation until 14th day wound tissue showed the epithelial cells were differentiated into stratified squamous cells. (Figure 1)

Masson's Trichrome

The observation of 1st day wound tissue showed the formation of necrotic layer inflammation cells and damaged collagen fibres at the wound area. The following 3rd day, the wound tissue samples didn't show any difference except there are increase of inflammation cells that migrated to wound area and a complete degrading collagen fibre. Observation of the 6th day wound tissue, all samples showed the deposition of collagen fibre at the wound area. Observation for the 10th day, the wound tissues didn't show any difference except denser collagen fibres deposited at wound area. Observation of the 14th day wound tissue showed that collagen fibres are dense and arranged better than 10th day wound. (Figure 2)

Total protein determination

The total protein concentration determination shows a pattern of decreasing of protein concentration levels from 1st day wound tissue until the 6th day wound tissues. The protein concentration increases on the 10th day wound tissues and decreases back on the 14th day. Statistical analysis shows that there are no significant differences of total protein concentration among the negative control group of (PBS solution), curcumin (positive control) and treatment group of *A. denudata*. The mean of wound tissue total protein concentrations is showed in table 1.

DISCUSSION

Histological observation with Hematoxylin & Eosin (H & E) staining is focused on the migration of cells and organizations, including the formation of epithelial tissue injury, formation, and changes occur in blood vessels and the density and morphology of cells and tissues of the wound. Observations for all wound tissues on the 1st and 3rd day did not show much difference in morphology. It showed the dominance presence of necrotic and inflammatory cells. This indicates that the wound is in the inflammatory phase. The presence of necrotic layer is due to the accumulation of dead cells of the skin with induced wound. Necrotic layer is composed of fibrin, blood cells, debris of cells and damaged collagen fibers. Observations on all samples in the 3rd day wound samples tissues showed an increase of infiltration and migration of inflammatory cells. Epidermis and dermis layer were not visible because the injuries have caused damage to both layers of the epidermis and dermis. According Diegelmann & Evans (2004), inflammatory cells such as neutrophils would dominate the wound area within 24 hours after an injury has occurred. Its role is to remove impurities, bacteria, dead cells and damaged cell matrixes. Observations to all samples on the 6th day of wound tissue showed there were morphological changes observed in injured tissue significantly compared to the 1st and 3rd day. The formation of keratin was also observed at the epithelium layer. This process is called re-epithelialization and the formation of epithelial wound is visible to the curcumin group compared to PBS negative control wounds and *A. denudata* wound treatment. Sidhu et al (1998) reported that curcumin treatment can expedite the process of reconstruction of the epithelium. No epithelial hyperplasia observed on all of the wounds on 6th day of treatment. Formation of granulation tissue showed proliferative phase was taking place. Observations on 10th day for all wound tissues showed there were no morphological differences compared to the 6th day. Squamous cell differentiation of epithelial layer can only be seen on the 14th day of the injury. According Mallefet & Dweck (2010), epithelial cells that are not damaged during the wound formation on the epithelium will beg the reconstruction process through the process of mitosis. Bandaged wound with blood supply will induce migration of new cells into the wound area. These migrating cells remain attached to the stem cells and cause pressure on the normal cells around the wound. This pressure causes the wound appeared to shrink. According Rigal et al. (1991), the rapid reconstruction of the epithelium can avoid disruption from the environment that can interferes with wound healing processes.

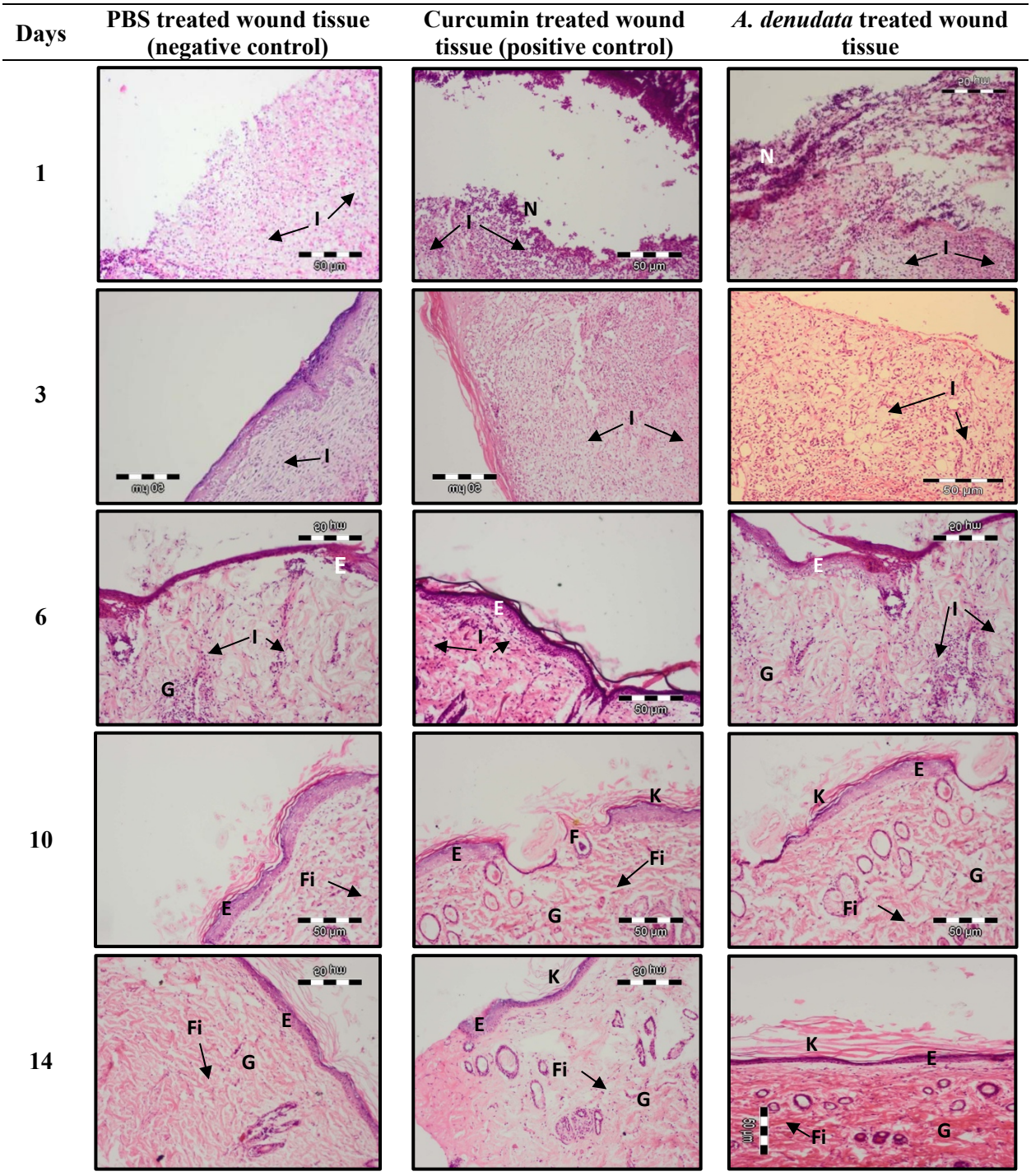

FIGURE 1. Photomicrograph of wound tissue treated with PBS (negative control), Curcumin (positive control) and *A. denudata* with H&E staining. On the 1st day, all wound displayed necrotic layer and presence of inflammation cells. All the wounds appeared to be healing gradually according to time, with the formation of epidermis layer within day 6 of treatment and complete healing within day 14 of treatment. Legend: I=inflammation cells, E=epithelium layer, G=granulation tissues, Fi=fibroblasts, F=hair follicle, K=keratin layer. 100x magnification.

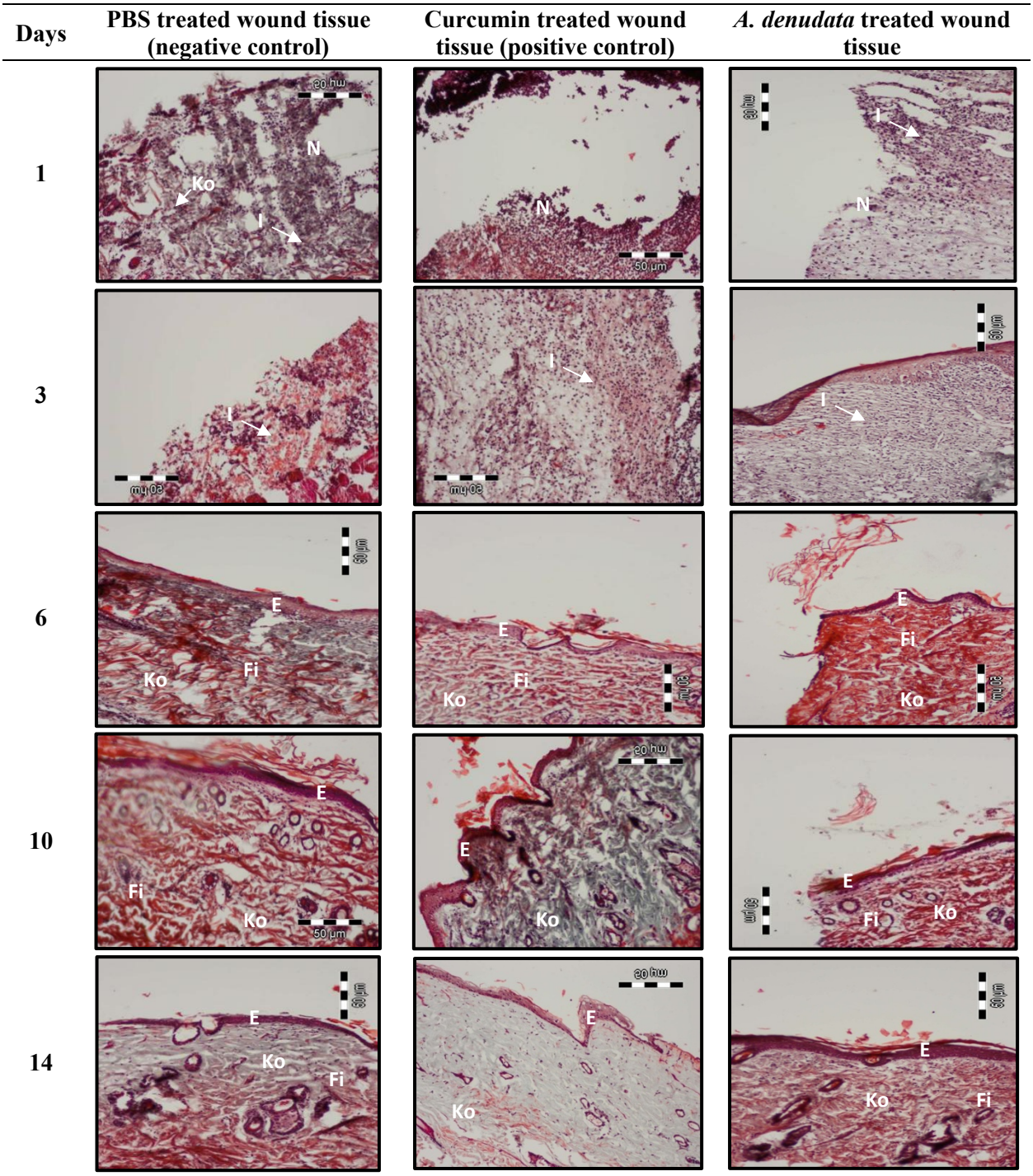

FIGURE 2. Photomicrograph of wound tissue treated with PBS (negative control), Curcumin (positive control) and *A. denudata* with Masson's Trichrome staining. On the 1st day, all wound displayed necrotic layer, presence of inflammation cells and damaged collagen fibers due to wound. All the wounds appeared to be healing gradually according to time, with accumulation and rearrangement of collagen fibers with increasing number of fibroblasts cells within 6 days of treatment by wound tissue treated by curcumin and *A. denudata* extract. All wounds show packed collagen formation and deposition within 14 days of treatment. Legend: I=inflammation cells, E=epithelium layer, Ko=collagen, Fi=fibroblasts. 100x magnification.

Day of wound	Mean of total protein concentrations (µg/ml)		
	PBS–Negative control (n=3)	**Curcumin–Positive control (n=3)**	***A. denudata*-Treatment (n=3)**
1st day	34.21 ± 15.01	20.18 ± 8.39	32.11 ± 2.41
3rd day	28.07 ± 7.01	16.67 ± 4.38	20.40 ± 8.36
6th day	24.21 ± 9.25	9.12 ± 2.37	17.37 ± 5.69
10th day	32.46 ± 7.37	20.70 ± 3.42	28.76 ± 2.63
14th day	15.44 ± 4.38	7.89 ± 1.39	20.88 ± 9.48

TABLE 1. Mean of wound tissue total protein concentrations comparison.

Histological observation with Masson's Trichrome staining was more focused on the accumulation of collagen and fibroblasts. Masson's Trichrome staining was specific to observe the distribution and accumulation of collagen. Histological observation on all wound tissue from 1st and 3rd day displayed no significant differences since only damaged collagen fibers, necrotic layers and inflammation cells can be detected. Observation of all 1st day wound showed degradation of damaged collagen fibers caused by wound, however less collagen fibers degradation was observed from 3rd day wound along with the increase of inflammation cells. According to Mallefet & Dweck (2010), damaged collagen will be degraded by collagenase enzyme. Observation of all 6th day wound tissues showed significant morphological changes. Fibroblasts cells dominate the wound area with accumulation of collagen fibers. Accumulation of collagen in wound tissue treated with curcumin (positive control) was much better compared to negative control wound (PBS only) and wound treated with *A. denudata* (treatment). Study by Sidhu et. Al (1998) reported that wound treatment with curcumin can increase collagen expression. However, the accumulation of collagen was still low on the 6th day on all treated wounds. Histological observations on 10th day wound tissue showed an increase in accumulation of collagen deposition on the wound treated with curcumin (positive control) and *A. denudata* while no changes in collagen accumulation on the PBS treated wound (negative control). This indicates good healing process on the *A. denudata* treatment which was comparable to the treatment of curcumin (positive control). Distribution of collagen fibers on the positive control wound treated with curcumin is more compact compared to other wounds indicating curcumin can increase collagen expression as reported previously by Sidhu et al (1998). Histological observation on skin wound tissues showed collagen fibers have dominated wound area of *A. denudata* treated wound compared to control negative wound (PBS treatment). Distributions of collagen fibers on wound treated by curcumin (positive control) is more organized compared to wound tissue treated with *A. denudata*. There is also an increased of accumulation of collagen in wound treated with PBS (negative control) showing wound healing is progressing. Mallefet & Dweck (2010) states that the formation of collagen is important in wound healing. Fibroblasts migrate to the wound area producing and secreting collagen. Collagen is produced at high rates up to a stage where new collagen production and degradation of old collagen reach a balance rate. This process will continue until the end phase of remodeling. According to Fossum et al. (1997) when the content of collagen in the wound increases, the number of fibroblasts and collagen synthesis rates will also decrease along with increased accumulation of collagen. Based on histological observations, it can be concluded that *A. denudata* treatment provided healing effect on open skin wound. The epithelium reconstruction process was much faster in wounds treated with aqueous juice of *A. denudata* stem compared to wounds treated with PBS solution (negative control) on the 6th day after treatment. The accumulation of collagen is also more compact in the wound that was treated with *A. denudata* stem extracts from wounds treated with PBS solution.

Measurements of total protein concentration levels patterns showed a decrease and increase the protein levels in all group of wound tissues. On the 1st day, all wound tissues displayed high protein levels and protein levels progressively decreased until day 6th, but protein level was increased again at the 10th and dropped again on the

14th day. Murray (2003) states that a group of proteins known as acute phase protein was produced during inflammation phase. Diegelman & Evans (2004) states that collagen is a protein produced during wound healing. Acute phase protein and collagen contributes to the fluctuation's levels of total protein. The protein levels were high on the first day is due to the process of hemostasis and the inflammatory phase which occurs immediately after inducing injury (Leaper & Enoch 2007). During the process of hemostasis and inflammation, chemical mediators such as enzymes, cytokines, hormones, C-reactive protein and coagulation protein components, cell debris, immunoglobulins and components of the complement system are involved during the inflammatory phase. This is supported by Walter (1975) which states that all components involved in blood clotting factors consists of 13 types of protein and one-part of calcium ion. On the third day, decreased in protein levels suggest inflammation process began to subside in addition to the degradation of old and damaged collagen which damaged by wound. This process happens through action of collagenase, a leucocytic enzyme involved during the recovery of injured tissue. The process simultaneously occurred together with the degradation of protein by infiltrating macrophage and neutrophils during previous inflammatory phase. On the 6th day, protein levels on all wound samples were decreased due to the end of inflammatory process and marked the beginning of proliferation phase which was characterized by collagen synthesis from fibroblasts and macrophages. This is supported by previous study by Mallefet & Dweck (2010) which stated inflammatory phase ended during fibroplasia process. Reconstruction of epithelium, wound contraction and collagen productions all happened during fibroplasia process. Protein levels increase in all wound samples during 10th day of treatment suggest increased accumulation of collagen in wound tissues. This finding is supported by Irvin (1981) which stated that collagen is the major structural protein and contributes to 70% of tissue weight. On the 14th day, there is decrease in protein levels in all tissues due to maturation and remodeling of collagen. Pilcher et al. (1999) and Park (1999) stated that during the process of collagen remodeling, there was degradation of collagen through specific enzyme from macrophages, neutrophils and fibroblasts which cuts collagen structures, and the excised parts will undergo denaturation by other protease enzymes. This process was associated with the conversion of collagen type III to collagen type I in the skin tissues. Low protein level by curcumin-treated wound tissue (positive control) were associated with anti-inflammatory effects of curcumin as described in the study by Srimal & Dhawan (1973) and Satoskar et al. (1986). Decrease in protein levels although not significant on day 14 for tissue treated with *A. denudata* may be due to late degradation of collagen.

Based on the results of total protein concentration from wound tissues, it can be concluded that the treatment of *A. denudata* enhanced the open wound healing in Wistar rats. The pattern of increase and decrease in total protein levels was due to an acute phase protein produced during inflammation phase and the production of collagen during the process of fibroplasia demonstrating healing process occurs. Overall, the quality of healed wound tissue treated with *A. denudata* extract is better than wounds treated with PBS and is parallel with the wound tissue healed with curcumin extract.

CONCLUSION

In conclusion, *Alocasia denudata* Enggler extract treatment on open skin wound can promote wound healing trough promoting migration of inflammation cells, re-epithelialization, epithelial differentiation and collagen disposition.

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