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Wound contraction and epithelisation effects of *Acrostichum aureum* L. in rabbits

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ABSTRACT

Introduction: Rhizomes paste from *Acrostichum aureum* Linné was used traditionally by Malays ethnic groups in Malaysia for wound healing treatment. To evaluate wound healing properties of aqueous and ethanolic extract of *A. aureum* on rabbits.

Method: There were four treatments namely aqueous extracts of rhizomes, leaves and stems *A. aureum* with low and high dose (5 % and 10 %). There were four rabbits in three treatment groups with each rabbit were inflicted with excisional wounds on their back near the neck with 6 mm in diameter. Topical treatment was applied once daily until complete healing with Solcoseryl jelly served as positive control group and blank aqua cream served as negative control group. The percentage period of epithelisation and wound contraction were measured every 3 days interval. At day 15, all healed wound specimens were biopsied and stained with Masson's trichrome staining for histopathological study.

Results and Discussion: From the results, all extracts from *A. aureum* possessed tannins and total tannin content showed that ethanolic extracts had higher total tannin content compared to aqueous extracts. Based on percentage wound contraction and epithelisation period, the treatment with 5 % aqueous extracts of leaves *A. aureum* was the most effective wound healing agent in enhancing higher percentage wound contraction, rapid epithelisation period, producing more collagens and fibroblasts proliferation.

Conclusion: It was suggested that wound healing properties of rhizomes and leaves *A. aureum* was contributed by its high total tannin content. This finding would be able to justify its traditional claim as wound healing treatment by Malays communities in Malaysia.

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Introduction

Malaysia is one of the twelve megadiversity countries in the world, with 1100 species of ferns and fern allies found in its rainforests and mangroves (Ministry of Science, Environment, and Technology, 1998). From these large families, Pteridaceae is among prominent family found worldwide with 50 genera and 950 species (Smith et al., 2008). Example of ferns in Pteridaceae family that used ethnomedicinally is *Acrostichum aureum* (Bandaranayake, 1998). Acrostichum spp. is a large ferns that can grow up to 4 m tall with not more than 30 leaflets. Giesen et al., (2006) described that *A. aureum* and A. speciosum sharing the same habitat, one vital characteristic that could differentiate them is blunt leaflet and elongate-pointed leaflet, respectively. These ferns are found in Malaysia, Indonesia, Thailand, Singapore, and India (Kathiresan & Rajendran, 2005). The methanol extract of *A. aureum* was reported to have phenolic sulfates which are active against SK-LU-1, HepG2, and MCF7 cell lines (Mint et al., 2021).

Wound occurs when the continuity of tissues is interrupted. Goss (1992) described that wound healing process primarily involves in three stages, namely proliferation, migration, and differentiation of cells. Since people in rural area still depend on medicinal plants for wound healing treatment, it is a great advantage to further analyse its properties scientifically in order to justify its traditional claim. Malays communities, which are the majority ethnic group in multi-racial Malaysia, have been reported to use the rhizomes of *A. aureum* for wound healing treatment (Bandaranayake, 1999; Mannan & Maridass, 2008; Hossan et al., 2010). Other traditional uses of this plant in this region are treatment cloudy urination and sexual stimulant in Bangladesh (Rahmatullah et al., 2010), anti-inflammation, malaria treatment, and antidote to poison victims in Vietnam (Hong & San, 1993; Hout et al., 2006). The water extract of *A. aureum* was reported to have gastroprotective effect of ethanol-induced gastric ulcer in rats (Wu et al., 2019). The phenolic compound, (+)-pinoresinol-4-O-sulfate showed a moderate activity against SK-LU-1, HepG2, and MCF7 cell lines (Thi Minh et al., 2022).

Various medicinal plants found in Malaysian virgin rainforests and mangroves offer huge natural resources for a novel medicinal product. In this study, the wound healing property of *A. aureum* as was evaluated by in vivo model and it serves as a basis for development of the plant-based products in Malaysia. Based on previous preliminary study of wound scratch assay on NIH/3T3 fibroblasts cell line (Herman et al., 2013), there were Three types of crude extracts to be tested in vivo which were aqueous extracts of rhizomes, leaves, and stems of *A. aureum*.

Materials and methods

Plant material

The whole samples (rhizomes, stems, and leaves) of *A. aureum* were obtained from Matang mangroves, Perak in February 2011. The plant was authenticated by Dr. Shahbudin Saad from the Department of Biotechnology, Kulliyyah of Science with herbarium specimen (MT 1011-9) was kept in the Herbarium Kulliyyah of Pharmacy, International Islamic University Malaysia.

Ethanolic extracts

The leaves, rhizomes and stems of the plants were dried in the oven at 30 °C for 7 days. Then, the samples were powdered using a mechanical grinder and 200 g of samples were for extraction purposes. Each sample was extracted with ethanol (90%) using a Soxhlet extractor and dried under reduced pressure to give dark brown colour to yield rhizomes (5.27 %), stems (6.90 %) and leaves (4.05 %) ethanol extracts. The extracts were stored in a 4 °C chiller until further use.

Aqueous extracts

The aqueous extract was prepared by maceration. 50 g of finely ground plants was macerated in 1000 mL of distilled water overnight in a 40 °C water bath. The distilled water was added in ratio 1: 20 (Mahmood & Phipps, 2006). After cooling, the extract was filtered by Whatman No. 1 filter paper and filter tunnel. The whole process was repeated three times to obtain a maximal extraction. Then, it was subject to a freeze drying process to produce yellowish powder extracts. Yield of extract for *A. aureum*'s rhizomes was 6.44 %, stems (9.64 %) and leaves (6.76 %). The extracts were stored in a 4 °C chiller for further testing.

Total Tannin Content

The extract (0.1 mL) was added with 7.5 mL of distilled water. Then 0.5 mL of Folin-ciocalteu phenol reagent, 1 mL of 35 % Na_2CO_3 solution and 10 mL of distilled water were added. The mixture was mixed well and stored for 30 min at room temperature. The absorbance was measured at 725 nm with a UV/Visible spectrophotometer. A set of standard solutions of tannic acid was read against the blank. The results were expressed as tannic acid in mg/g of extract. Total tannin content was measured as mg of tannic acid equivalent per gram using the equation which was obtained from a standard tannic acid calibration curve (Haile and Kang, 2019).

Excisional wound study

12 male, New Zealand rabbits (2.5+/- 0.5 kg)) were

purchased from a supplier in Seri Kembangan, Selangor and were acclimatized for least two weeks in an animal room. They were fed on a standard pellet diet (Bendera®) with fresh carrots and water ad libitum throughout the experiment. For each group, there were four individual rabbits used. This study was approved by IIUM Research Ethics Committee (IREC) Meeting No 3/2011 on 5th December 2011.

Preparation of wound

This animal study was used to monitor periods of epithelisation and wound contraction. The excisional wounds were inflicted according to previous methods (Ravishankar et al., 2018; Xie et al. (2002). Each rabbit was anaesthetized intraperitoneally using a cocktail of ketamine/ xylazine (22 and 2.5 mg/kg, respectively). The drugs prevent any movement of the animals at least for 2 hours after the administration of the anaesthetic solution. First of all, the hair on that wounding area was removed by shaving the dorsal back of the rabbit. Then, the circular wound on the dorsal interscapular region of each animal sized 6 mm biopsy was left open. All wounds were treated once daily until the wound completely healed. The healing progress in the wound area was monitored with transparent graph paper with an accuracy of 1/20 mm. Wound areas were also monitored with a camera on day 0, 3, 6, 9, 12, and 15.

Topical wound application

Aqua cream was purchased from UPHA Pharmaceutical Manufacturing (M) Sdn containing white soft paraffin 12 % w/w, emulsifying wax 8 % w/w, and liquid paraffin 8 % w/w. Each extract was homogenized with 1 g of aqua cream according to the designed concentration. Solcoseryl® jelly was purchased from Legacy Pharmaceuticals Switzerland GmBH.

For treatment 1 group, wounds were treated with positive, negative control, 5 % and 10 % of aqueous extracts of rhizomes *A. aureum*. For treatment 2 group, wounds were treated with 5 % and 10 % of aqueous extracts of stems *A. aureum* and control while for treatment 3 group, wounds were treated with 5 % and 10 % of aqueous extracts of leaves *A. aureum* and control. For negative control, 1 g of blank aqua cream was applied and for positive control, 1 g of Solcoseryl® jelly was applied once daily. The wound contraction was evaluated as follows (Herman et al., 2013):

$$\text{Wound contraction (\%)} = \frac{(Wdo - WDt)}{Wdo} \times 100$$

Where:
Wdo = The wound diameter on day zero
WDt = The wound diameter on day t

Histopathological study

At the last day of the experiment (Day 15), the cross-sectional of full-thickness skin specimens from each group were histopathologically evaluated. Skin samples were fixed in Bouin's solution before being processed and blocked with paraffin and then sectioned into 5 µm sections and stained with modified Masson's trichrome staining (MT). The method of staining was done following from Suvik & Effendy (2012). Firstly, the skin tissue slides was deparaffinised by submerging into three series of absolute xylene followed by 100 %, 95 %, 90 %, 80 %, and 70 % of ethanol for 4 minutes, respectively.

The slides were soaked in warmed Bouin's solution for 45 minutes at 60 °C and the slides were rinsed in running tap water until yellow colour in samples disappeared. Then, in order to differentiate nuclei, slides were immersed in modified Weigert's haematoxylin for 8 minutes, then washed in running tap water for 2 minutes. To stain erythrocytes and cytoplasms, slides were submerged in acid fuchsin for 5 minutes, then again washed with running tap water for 2 minutes. Next, the slides were treated with a solution of phosphomolybidic acid for another 10 mins and immediately submerged into a methyl blue solution for 5 minutes for staining. After that, slides were washed in running tap water for 2 minutes and lastly treated with 1 % acetic acid solution for 1 minute. Finally, slides were dehydrated into 70 %, 80 %, 95 %, and 99 % ethanol for 1 minute each percentage. Before evaluation, slides were dipped into absolute xylene for 1 minute and mounted with cover slip using DPX mounting.

For evaluation purposes, the tissues were examined by light microscope and graded subjectively in terms of fibroblast proliferation, collagen formation, angiogenesis, and re-epithelisation process. For collagen formation, it would be graded into none, scant, moderate, or abundant. Effective wound healing treatment would result in abundance of collagen. Secondly, for epithelisation it would be graded into none, partial, thin complete, and mature complete epithelized cells. A good wound healing process would result in thick and mature epithelized cells as no more dead tissue would be accumulated on the healed wound. Lastly, fibroblasts proliferation also would be graded accordingly based on their proliferation rate; dominant fibroblasts showed a good wound healing process.

Statistical analysis

Statistical analysis was conducted with the Statistical

Package for the Social Sciences (SPSS) version 16. Data were expressed as the mean ± S.D. Significant differences between the treated groups and the control was determined by the One-way ANOVA test, followed by post-hoc Tukey's test, at a level of p-value < 0.05 is considered as statistically significant.

Results and Discussion

Phytochemicals screening showed that all ethanolic and aqueous extracts from rhizomes, leaves, and stems A. aureum contained tannins. Therefore, total tannin content was done to analyze its amount and from the results, it showed that ethanolic extracts have higher total tannin content than aqueous extracts (Table 1).

Table 1: Total tannin content of the extracts of *A. aureum.*

Type of extract	Average Absorbance at 725 nm	Total tannin content
EtOH rhizomes *A. aureum*	2.14 ± 0.28	118.56
EtOH stems *A. aureum*	3.04 ± 1.28	168.56
EtOH leaves *A. aureum*	1.85 ± 0.01	102.44
Aqueous rhizomes *A. aureum*	0.93 ± 0.08	51.33
Aqueous stems *A. aureum*	1.20 ± 0.46	66.33
Aqueous leaves *A. aureum*	1.25 ± 0.11	69.11

Wound contraction was measured as a percentage of the reduction in the wounded area. It was measured every three days until day 15. From the daily wound observation (Figure 1), the wound healing process occurred normally without any signs of inflammations or microbial infections that could render a normal wound healing process.

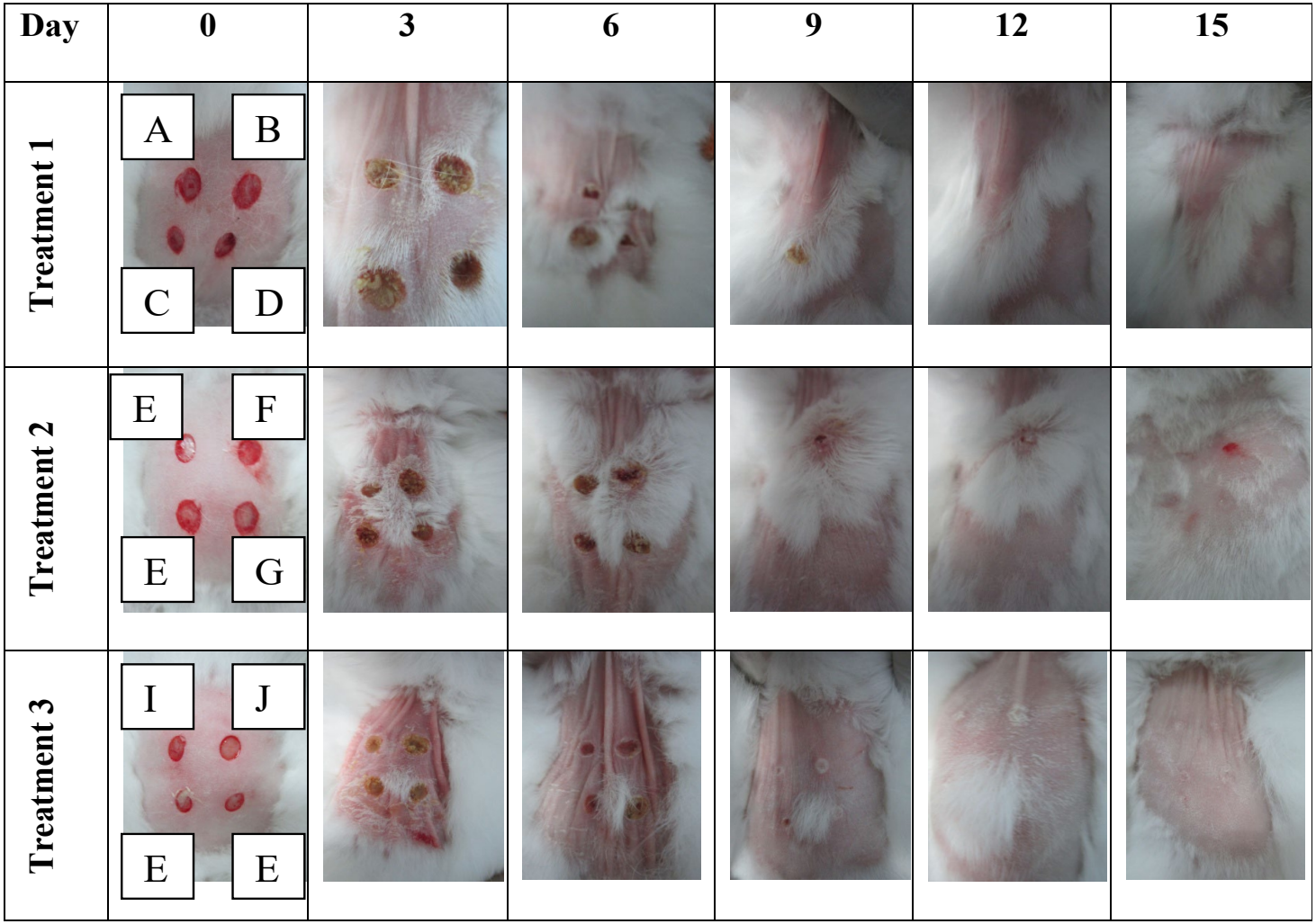
On day 9, the wounds treated with 5 % and 10 % aqueous extracts of leaves of *A. aureum* have been fully healed compared with negative control (68.75 %). This is statistically significant ($p < 0.05$) and showed that leaves also could enhance the wound healing process. Percentage wound contraction (%) of different extracts on exsicional wound in rabbits for three interval days. On day 9, treatment with 5 % and 10 % aqueous extracts of leaves of *A. aureum* shows statistically significant percentage wound contraction compared to negative control (Figure 2).

The epithelisation period was measured as the number of days required for the falling of the eschar (dead-tissue remnants) without any residual raw wound. The results (Figure 3) showed that three extracts have significant effect on epithelisation period which were 10 % aqueous extract of rhizomes *A. aureum*, 5 % and 10 % aqueous extract of leaves *A. aureum* compared to negative control. These findings were essential as a faster epithelisation period means that the treatment could enhance the wound healing process compared to normal healing without any treatment.

Malaysia is a promising natural resource for medicinal plants. Some plants have been studied for wound healing properties such as *Plantago major* (Mahmood & Phipps, 2006) and *Rafflesia hasseltii* (Mahmood et al., 2009). Therefore, this study aims to add more depth in Malaysian medicinal plants used for wound healing treatment. Due to lack of published reports on their wound healing properties, this present study will justify the medicinal use of *A. aureum* rhizome extracts in wound healing.

The phytochemicals screening was done for all aqueous and ethanolic extracts of *A. aureum*. Based on our result, all extracts of *A. aureum* possessed tannins. The finding of tannins presence in all extracts was in conformance with previous study done by Bandaranayake (2002) and Hemayet et al. (2012). Since the target compound in wound healing study was tannins, total tannin content was done to further analyse the quantitative amount of tannins in each part and extract from *A. aureum*.

From the result, the highest total tannin content could be found in ethanolic extract of stems *A. aureum* (168.56 mg TAE/ g dry extract) while the lowest was found in aqueous extract of rhizomes *A. aureum* which was only 51.33 mg TAE/ g dry extract. Tannins were known to have astringent properties that would draw and contract tissues together by precipitating protein (Getie et al., 2002). Ong (2004) has reported that tannins could be used to reduce inflammation, stop bleeding and heal wounds as a topical application. Tannins have been targeted for years by researchers around the world for wound healing study. A study by Agyare et al. (2011) has successfully isolated bioactive compounds from tannins such as furosin and geraniin that increased the proliferation rate of fibroblasts and stimulated collagen synthesis. Besides, tannins also were reported to have an angiogenic effect or promote the formation of new blood vessels in wound areas (Li et al., 2011). Theoretically, angiogenesis starts when cell contact relaxes and outer pericytes layer is disrupted. Subsequently, there will be a migration and proliferation of endothelial cells that lead to the formation of new blood vessels (Moon et al., 1999).



AA: *A. aureum*, A: Solcoseryl jelly; B: 5 % Rhizomes AA; C: Aqua cream; D: 10 % Rhizomes AA; E: Control; F: 5 % Stems AA; G: 10 % Stems AA; I: 5 % Leaves AA, J: 10 % Leaves AA.
Figure 1: Photographic observation of wound contraction every three interval days from day 0 to day 15. There were no signs of inflammations and bacterial infection throughout the experiment.

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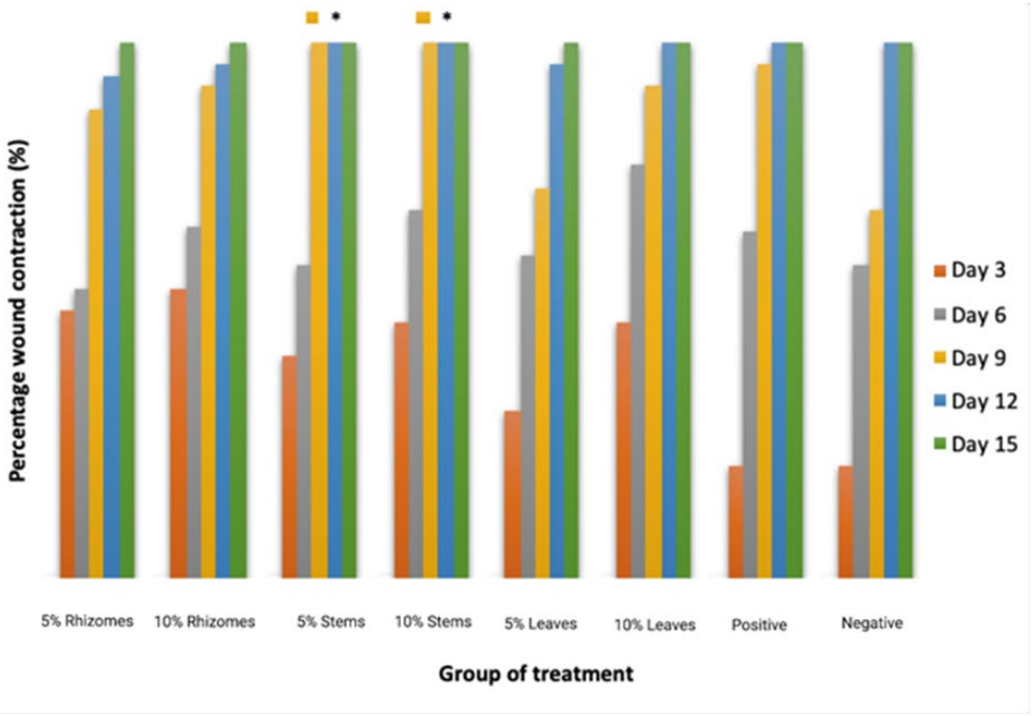

Figure 2. Percentage of wound contraction (%) of different extracts on excisional wound in rabbits for three-day interval. On day 9, treatment with 5% aqueous extracts of leaves of *A. aureum* and 10% aqueous extracts of leaves of *A. aureum* showed statistically significant percentage wound contraction compared to the negative control (Positive control: solcoseryl jelly; Negative control: aqua cream), *: $p < 0.05$

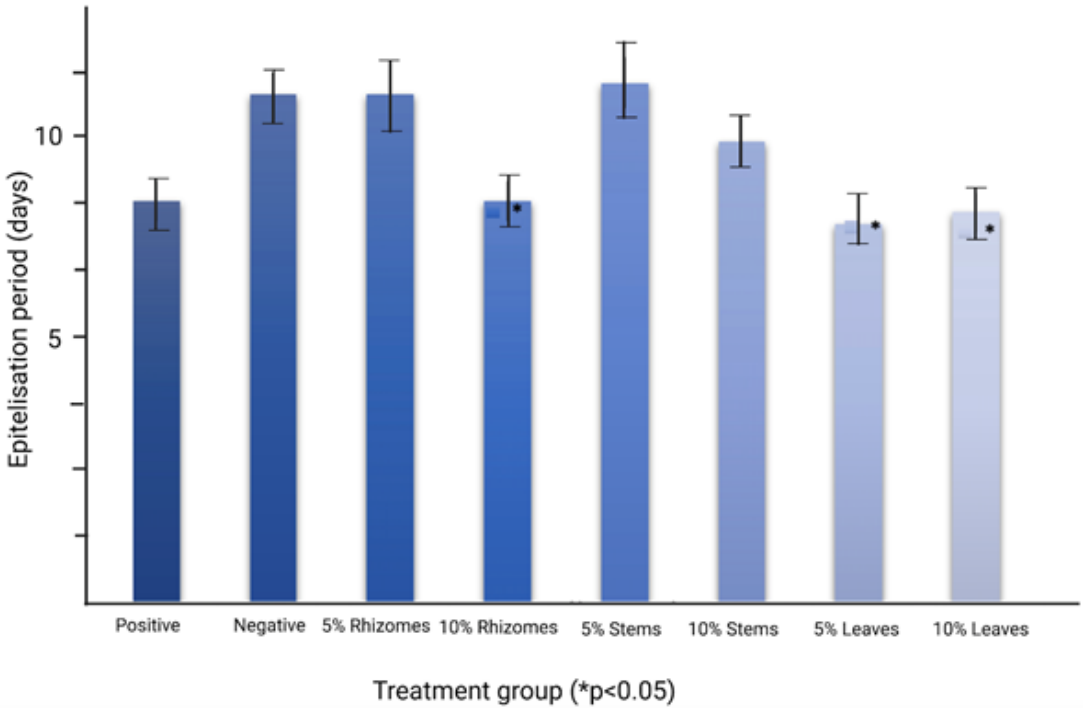

Figure 3: Epithelisation period vs treatment group in which 10 % rhizomes *A. aureum*, 5 % and 10 % leaves *A. aureum* showed significant faster epithelisation period compared to negative control. AA: *A. aureum;* *: $p < 0.05$. (Positive control: salcoseryl jelly; Negative control: aqua cream).

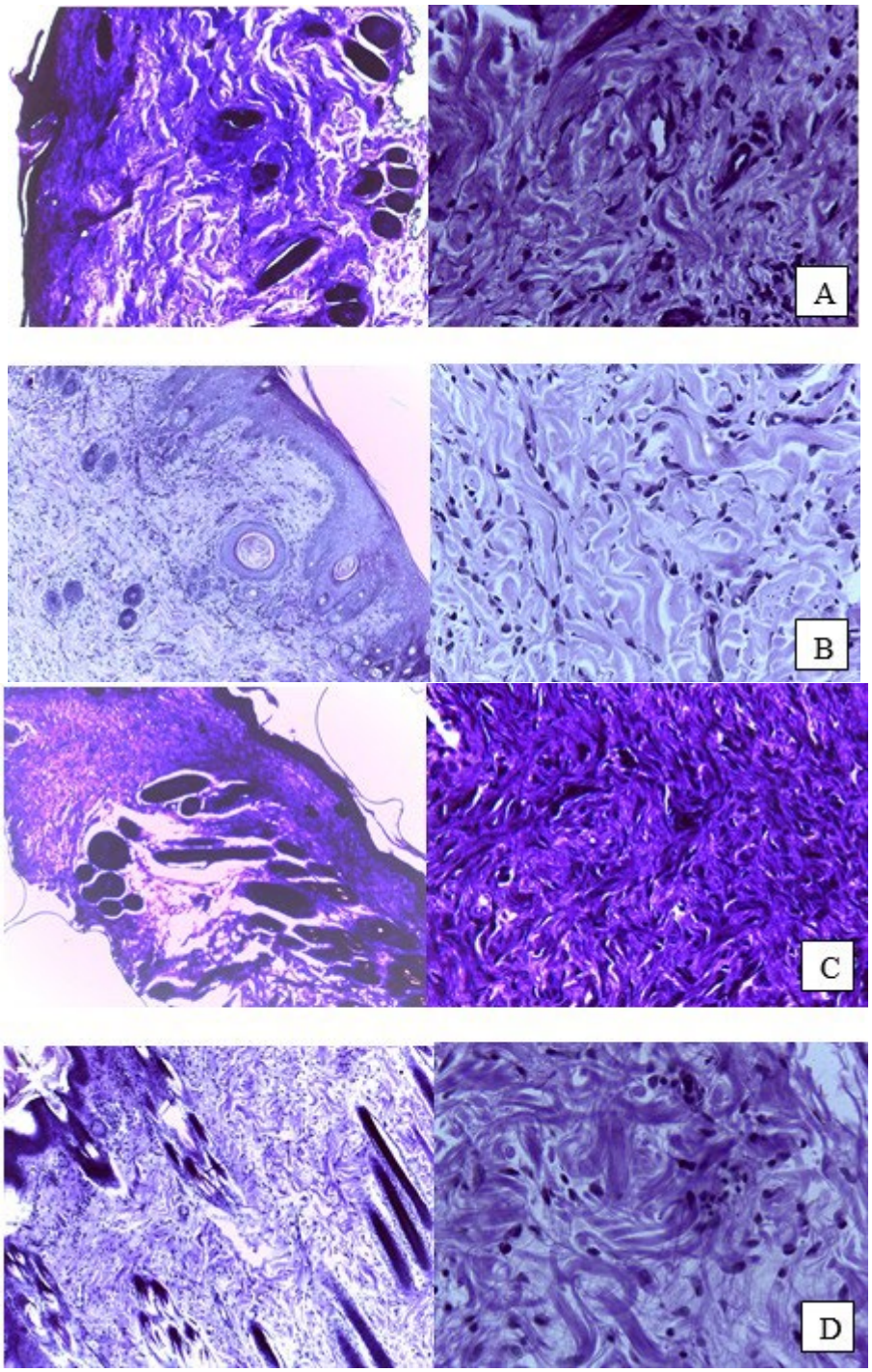

Figure 4: Histopathological findings showed that treatment with 10 % aqueous extract of rhizomes *A. aureum* (Plate A) produced complete epithelisation, moderate amount of collagens and fibroblasts while treatment with 5 % aqueous extract of leaves *A. aureum* (Plate B) produced complete epithelisation, moderate amount of collagens and predominat fibroblasts. Plate C was positive treatment with Solcoseryl® jelly while Plate D was negative treatment with Aqua cream.

Based on the result, on day 9, treatment with 5 % and 10 % aqueous extracts of leaves of *A. aureum* shows statistically significant percentage wound contraction compared to negative control. Meanwhile, epithelisation period was reduced in three groups which were 10 % aqueous extract of rhizomes *A. aureum*, 5 % and 10 % aqueous extract of leaves *A. aureum* compared to negative control (10.75 ± 0.96 days). The fastest epithelisation period among them was treatment with 5 % aqueous extract of leaves *A. aureum* (8.00 ± 0.00 days) followed by 10 % aqueous extract of leaves *A. aureum* (9 days) and 10 % aqueous extract of rhizomes *A. aureum* (10 days). Different concentration has given different epithelisation period thus it was important to study both low and high dose to obtain the best concentration (Figure 4).

Fibroblasts proliferation was one of the parameters measured in the histopathological study. From the photomicrographs, the appearance of fibroblasts could be easily recognised by large nucleoli indicating active protein synthesis, extensive and purple-stained cytoplasm, and appearance of the granular (Young et al., 2006). Of all photomicrographs, the most abundant fibroblasts could be seen in skin specimens treated with 5 % and 10 % of aqueous extract of leaves *A. aureum*. This is solid evidence of their effectiveness as a wound healing agent compared to negative and positive control.

Based on the scoring method of histopathological slide, it has shown that Aqua cream as a negative control asserted minimal effect to the skin regeneration process with poor proliferation of collagen, fibroblast, and macrophage. It was different when the wound was treated with 10 % aqueous extract of rhizomes and leaves *A. aureum* that showed better and more production of collagens, fibroblasts, and macrophages. The epithelial cells also were completely formed and it demonstrated that the wound has undergone complete recovery and regeneration of wounded cells.

Collagens as the largest component of the extracellular matrix are responsible for more tensile strength and support. Type 1 collagen is the most abundant collagen normally enhancing wound healing process by promoting keratinocyte attachment and migration (Ling et al., 2011). This is similar with a study done by Ionita et al. (2009) that an intense collagen fibre was found throughout the dermal thickness due to the fibroblasts stimulation enhanced by the extract treatment. Besides, another parameter to be observed is a sign of inflammation in the dermis layer because a good treatment would prevent inflammation (Moghbel et al., 2005). Therefore, the aqueous extract of leaves and rhizomes *A. aureum* have been concluded to be a powerful wound healing agent through high percentage wound contraction, rapid epithelisation period, and increasing of fibroblasts and collagen proliferation and building faster epithelised cells.

Conclusion

This study has proven the wound healing properties of aqueous extract of rhizomes and leaves *A. aureum* by accelerating wound contraction and epithelisation period significantly compared to negative and positive control. This finding provided significant support for the use of medicinal plants among the Malays people. Further isolation of pure compounds responsible for wound healing properties need to be done before this plant could be commercialized as a new wound healing agent in Malaysia.

Conflict of Interest

The authors declare no conflict of interests.

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