Collagen is the key component of the extracellular matrix that plays a critical role in the strength and quality of the healed wound. *Eurycoma longifolia* Jack root extract (TA) has been documented as an anti-inflammatory, antioxidant, and antimicrobial agent that may improve the quality of healed wounds. The objective of this study is to investigate the effect of topical application of TA hydrogel on the quality of the healed skin in rats. MATERIALS AND METHODS: Twenty male Sprague Dawley rats were grouped into 4 groups: Negative control, Hydrocyn® aqua gel positive control, vehicle (Xanthan) hydrogel, and *Eurycoma longifolia* Jack (TA) hydrogel. Treatments were applied twice daily starting on wounding day until day 21. An excisional wound was created on the back of 20 rats. Tissue samples of the healed skin were collected for histological examination by measuring the epidermal and dermal thicknesses and evaluating the collagen fibre quality by VVG stain. RESULTS: The TA hydrogel group had the thickest newly formed epidermis compared with the other experimental groups. For the dermal thickness, compared with the vehicle (xanthan) hydrogel group, TA hydrogel, and Hydrocyn aqua® gel positive control groups showed significantly increased thickness with p values 0.020 and 0.045, respectively. Histologically TA hydrogel group showed a significant increase in mixed-oriented collagen fibres, and fascicular collagen bundles and showed profound collagen density. CONCLUSION: TA hydrogel improved the quality of healed skin by increasing the epidermal/dermal thicknesses and enhancing the quality of newly produced collagen fibres. It can be considered a promising and effective wound-healing agent.
biocompatibility, and safety. About 70% to 95% of the population in most developing nations and 70% to 90% of people in well-developed countries utilise orthodox medicine in their initial healthcare to manage their medical problems. The key to the treatment of skin injury is to provide the outermost barrier, the epidermis, prevent infection, stop bleeding, moisturise and relieve pain. Eurycoma longifolia Jack is native to Southeast Asian countries such as Thailand, India, Malaysia, and Vietnam. The roots of this plant are used by local folks in Malaysia as an aphrodisiac, to improve libido and energy, for hypertension and fever treatment. Many studies have confirmed that the root and root bark of Eurycoma longifolia Jack (TA) have many pharmacological effects such as anticancer, antimalarial, anti-inflammatory, and antioxidant properties.

As well as the ethanol extract of TA roots, has been confirmed in previous studies to show antibacterial and antifungal effects. All these pharmacological effects are assigned to the presence of important phytochemicals such as quassinoids, alkaloids, terpenoids, tannins, polysaccharides, glycosides, phenolic compounds, and other important bioactive compounds which are heavily concentrated in the roots. In our previous study, we prepared the ethanol extract of Eurycoma longifolia Jack (TA) roots in a hydrogel for in vivo wound healing studies. The current study aims to explore the effect of the topical application of TA hydrogel on the quality of the healed skin by measuring the epidermal and dermal thicknesses and evaluating the orientation of collagen fibres in the excisional wound model in Sprague Dawley rats.

**Materials and Methods**

**Animal and Housing**

20 adult male Sprague Dawley rats of 160-180 g weight were used in this study and were handled carefully. The animal study was approved by The Institutional Animal Care and Use Committee of International Islamic University Malaysia (IACUC–IIUM) [approval number: IIUM/504/14/2/IACUC]. All details regarding animal care and housing are illustrated in our previous publication.

**Study Design and Surgical Procedure**

The excisional wound model as designated by Morton and Malone was conducted in this study (Morton and Malone 1972; Bektas et al. 2020). After administering the anaesthesia to rats, a full-thickness circular wound of 15 mm×15 mm in diameter and 2mm depth was created surgically at the dorsal interscapular region of each rat. All details regarding the surgical procedure and calculation of the number of rats in each group are illustrated in our previous publication (Al-Bayati et al. 2022). All the 20 wounded rats were arranged into 4 studying groups (n=5): Group 1 untreated (negative control), Group 2 Hydrocyn® aqua gel (positive control) control, Group 3 vehicle hydrogel (2% w/w xanthan) and Group 4 Eurycoma longifolia Jack (TA) hydrogel (xanthan-based hydrogel containing 0.12%w/w TA) as shown in. After getting haemostasis on a wounding day, all the types of treatments (Hydrocyn aqua gel, Vehicle hydrogel, and TA hydrogel) were applied twice daily for 21 days.

**Histopathologic Examination**

On day 21 after wound creation, rats were anesthetized and biopsies of healed skin were collected and processed by staining with H&E and Verhoeff’s Van Gieson stain (VVG) to investigate histologically the effect of (TA) hydrogel on the collagen formed in the healed skin by histopathologic evaluation of collagen fibres characteristics and measurement of epidermal and dermal thicknesses. The thickness of the newly formed epidermis and dermis was measured using Leica Aperio ImageScope - Pathology Slide Viewing Software 12.4.3 on histologic sections stained by H&E stain.

The characteristics of collagen fibers in healed skin were evaluated semi-quantitatively by staining the tissue with VVG stain and observing 5 HPF in each slide. The collagen fibers were observed for the following characteristics; orientation, pattern, and density, Figure 7. Collagen density was assessed into ‘minimal’ where collagen fibers were sparse and widely spaced, ‘moderate’ where collagen fibers were loosely packed and ‘profound’ where collagen fibers were tightly packed. The data was analysed using a Chi-Square statistical analysis test to compare the percentage of fields having each score in the
different studied groups.

RESULTS

Histopathologic evaluation of the healed skin on day 21 post-wounding included investigating the effect of the topical application of TA hydrogel on the quality of the healed skin in terms of the epidermal and dermal thicknesses and collagen fibers characteristics in the healed skin.

A. Measurement of Epidermal and Dermal Thicknesses of Healed Skin

Figure 1 shows the measurement of the epidermal/dermal thickness in the studied groups. The average thicknesses of the newly formed epidermis in untreated (negative control), Hydrocyn aqua® gel (positive control), vehicle (xanthan), and Eurycoma longifolia Jack (TA) hydrogels were 67.92 µm, 90.78 µm, 113.41 µm, and 118.05 µm respectively as shown in table 1 that Eurycoma longifolia Jack (TA) hydrogel group showed the thickest newly formed epidermis compared with the other experimental groups, however, the difference was not sizable enough to show significance, $p=0.055$ Kruskal-Wallis test.

Whereas the average thicknesses of the newly generated dermis in untreated (negative control), Hydrocyn aqua® gel (positive control), vehicle (xanthan) and Eurycoma longifolia Jack (TA) hydrogels were 876.84 µm, 1182.7 µm, 543.3 µm and 1106 µm respectively as shown in table 1 that Eurycoma longifolia Jack (TA) hydrogels and Hydrocyn aqua® gel (positive control) control groups showed significantly increase in dermal thicknesses compared with the vehicle (xanthan) hydrogel group with $p=0.020$ and 0.045 respectively. Kruskal-Wallis test (n=5) *p<0.05.

Table 1: Thickness of the newly formed epidermis and dermis (µm) on Day 21 post-wounding in the 4 experimental groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Epidermal thickness</th>
<th>Dermal thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated negative control</td>
<td>67.92 ± 34.50 µm</td>
<td>876.84 ± 151.7 µm</td>
</tr>
<tr>
<td>Hydrocyn aqua® gel positive control</td>
<td>90.78 ± 41.44 µm</td>
<td>1182.7 ± 435.4 µm</td>
</tr>
<tr>
<td>Vehicle (xanthan) hydrogel</td>
<td>113.41 ± 2.13 µm</td>
<td>543.3 ± 27 µm</td>
</tr>
<tr>
<td>Eurycoma longifolia Jack (TA) hydrogel group</td>
<td>118.05 ± 9.86 µm</td>
<td>1106 ± 23.8 µm</td>
</tr>
</tbody>
</table>

B. Histopathological Evaluation of Collagen Fibers Characteristics by VVG Stain

Figure 2 shows the histological picture of the different studied groups stained with VVG stain to show the characteristics of collagen fibers.
Table 2 shows the results for collagen fiber orientation. TA hydrogel group showed the highest percentage of mixed and horizontal orientation 56% and 36%, respectively. The percentage of horizontal fibers in the TA group is significantly higher than in the negative control group with a p-value of 0.0106.

<table>
<thead>
<tr>
<th>Groups</th>
<th>1=vertical</th>
<th>2=mixed</th>
<th>3=horizontal</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control, no treatment</td>
<td>5 (20%)</td>
<td>20 (80%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Positive control Hydrocyn® aqua gel</td>
<td>8 (32%)</td>
<td>13 (52%)</td>
<td>4 (16%)</td>
<td>0.0106</td>
</tr>
<tr>
<td>Vehicle hydrogel</td>
<td>7 (28%)</td>
<td>18 (72%)</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Tongkat Ali (TA) hydrogel</td>
<td>2 (8%)</td>
<td>14 (56%)</td>
<td>9 (36%)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3 shows the results of the pattern of collagen fibers. TA hydrogel group showed the highest results of the ‘fascicle’ pattern compared to the other groups. The percentage of fascicle pattern in the TA group is significantly higher than the negative control group with a p-value of 0.0015.

<table>
<thead>
<tr>
<th>Groups</th>
<th>1=reticular</th>
<th>2=moderate</th>
<th>3=fascicle</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control, no treatment</td>
<td>7 (28%)</td>
<td>17 (68%)</td>
<td>1 (4%)</td>
<td></td>
</tr>
<tr>
<td>Positive control Hydrocyn® aqua gel</td>
<td>15 (60%)</td>
<td>8 (32%)</td>
<td>2 (8%)</td>
<td>0.001572</td>
</tr>
<tr>
<td>Vehicle hydrogel</td>
<td>8 (32%)</td>
<td>17 (68%)</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Tongkat Ali (TA) hydrogel</td>
<td>3 (12%)</td>
<td>14 (56%)</td>
<td>8 (32%)</td>
<td></td>
</tr>
</tbody>
</table>

Table 4 shows the results for collagen fiber density. TA hydrogel group showed the highest percentage 24% of ‘profound’ density. The percentage of ‘profound’ density in the TA group is significantly higher than the negative control group with p=0.018.

<table>
<thead>
<tr>
<th>Groups</th>
<th>1=vertical</th>
<th>2=moderate</th>
<th>3=profound</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control, no treatment</td>
<td>2 (8%)</td>
<td>25 (96%)</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Positive control Hydrocyn® aqua gel</td>
<td>6 (24%)</td>
<td>19 (76%)</td>
<td>0%</td>
<td>0.01803</td>
</tr>
<tr>
<td>Vehicle hydrogel</td>
<td>1 (4%)</td>
<td>24 (96%)</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Tongkat Ali (TA) hydrogel</td>
<td>0%</td>
<td>19 (76%)</td>
<td>6 (24%)</td>
<td></td>
</tr>
</tbody>
</table>

DISCUSSION

Collagen is the fundamental element of the extracellular matrix that plays an essential role in the regeneration of injured skin.17 Immediately once the skin is injured, the fibroblasts migrate quickly to the wounded site and stimulate the production of new tissue via proliferation.18 Meanwhile, skin fibroblasts can secrete a wide variety of growth factors and produce collagen.19 The thickness of the newly generated epidermis and dermis is very important for keeping the healed area. Without the support of a fully developed dermal matrix, the newly formed epidermis is breakable and fragile.20 Our testing substance showed the thickest epidermal layer with (118.05 µm ± 9.86) compared with the other experimental groups; untreated negative control (67.928 µm ± 34.50), Hydrocyn aqua® gel positive control (90.78 µm ± 41.44) and vehicle (xanthan) hydrogel (113.41 µm ± 2.13), however, the difference was not sizable enough to show statistical significance.

Surprisingly, TA hydrogel and Hydrocyn aqua® gel positive control groups showed a significant increase in the thickness of the newly formed dermis compared with vehicle (xanthan) hydrogel groups with p=0.020 and 0.045 respectively. These results are consistent with its positive effects on wound contraction and re-epithelialisation16 as well as with its significant effect on VEGF expression in the healing wound.21 Thus, TA hydrogel is safe and effective to apply on wounded skin as it stimulates collagen production in a comparable way to the reference wound healing agent, Hydrocyn aqua® gel. A relatively thicker epidermis and a significant increase in dermal thickness produced by TA hydrogel indicate the positive effect of topical application of TA hydrogel in the healing process of cutaneous wounds and might point out a superior skin barrier and our testing substance probably better than the others. As collagen is the key element of the dermal layer22, the significant effect of Eurycoma longifolia Jack (TA) hydrogel on collagen production in the healing wound might be attributed to the availability of phytochemicals with wound healing activity such as alkaloids, flavonoids, glycosides, terpenes, 5-HMF, oleic acid, and palmitic acids.6,23

Many previous studies have proved that those phytochemicals stimulate collagen production and cell proliferation through antioxidant and anti-inflammatory properties.7,8,24 This study also investigated the effect of the topical application of Eurycoma longifolia Jack (TA)
Topical application of *Eurycoma longifolia* Jack (TA) hydrogel improved the quality of the healed skin by significantly stimulating collagen production by increasing the epidermal and dermal thicknesses of the healed skin and improving the organisation of deposited collagen fibres in a pattern similar to normal skin. *Eurycoma longifolia* Jack (TA) hydrogel could be an effective, affordable, and safe wound healing agent for improving the quality of the healed skin.

### ACKNOWLEDGEMENT

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