ORIGINAL ARTICLES

The Effects of the Topical Application of Eurycoma Longifolia Jack (TA) Root Extract Hydrogel on Vascular Endothelial Growth Factor (VEGF) Expression during Wound Healing in vivo Excisional Wound Model

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ABSTRACT

INTRODUCTION: Medicinal plants are known for their positive impacts on wound healing by stimulating angiogenesis in the skin. Eurycoma longifolia Jack locally known as Tongkat Ali (TA), is a medicinal plant characterised by anti-inflammatory and antioxidant effects. **MATERIALS AND METHODS**: Excisional wound (15mm × 15mm) in diameter and (2mm) depth was created at the back of 20 male Sprague Dawley rats by incising the marked skin with sterilized surgical scalpel blade then excised the skin by surgical scissors and toothed forceps. The wounded rats were divided into 4 groups, each group contained 5 rats (n=5). Experimental groups were formed as follows: untreated (-ve) control, Hydrocyn® aqua gel (+ve) control, vehicle hydrogel, and TA hydrogel. All the treatments were applied twice daily for 5 days starting on the first day (wounding day). On Day 5 post-wounding, the granulation tissue was harvested from all groups and evaluated by immunohistochemistry assay for vascular endothelial growth factor (VEGF) expression. **RESULTS**: VEGF expression in granulation tissue of rat's skin treated with TA hydrogel and vehicle hydrogel increased significantly compared to that in the untreated (-ve) control group with p-values of 0.040 and 0.029, respectively. Although there was no significant difference in VEGF expression in granulation tissue of rat's skin treated with the TA group, Hydrocyn® aqua gel (+ve) control, and vehicle hydrogel groups, our study group showed higher expression of VEGF. **CONCLUSION**: Our study showed that the topical application of TA hydrogel increased the VEGF expression in granulation tissue of rat's skin, which is an essential growth factor for wound healing. Thus, there is great potential for TA hydrogel to be an effective wound-healing agent for managing cutaneous wounds.

Keywords
eurycoma longifolia jack, wound healing, vascular endothelial growth factor, immunohistochemistry.

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INTRODUCTION

The skin acts as a protective barrier between the body and the external environment against any physical injury, microbial attack, or loss of fluid and has an immune-neuroendocrine regulatory function that participates in the homeostasis maintenance of the body.[1] Wound healing is a vital process for all living creatures. It involves intricate reactions and integrated biological events between several types of cells, which are organised and regulated by several growth factors, chemokines, and cytokines. The hallmark of wound healing are cell proliferation and migration, and the essential contestants in these biological events are fibroblasts, endothelial cells, and keratinocytes.[2,3] The effective irrigation of the wound borders is an important factor for wound healing.[5]

Angiogenesis refers to the formation of new blood vessels from injured and pre-existing vasculature.[6] Angiogenesis is an essential biological process in the healing wound, it represents a vital subphase of the proliferation in the wound healing cascade. All wounds need angiogenesis for

healing, as new vasculature is demanded to restore microcirculation, re-establish tissue aspiration and supply nutrients and oxygen to stimulate cell growth and formation of granulation tissues, which is required for the regeneration of the damaged tissue.[1,4,7–9] The creation of new blood vessels depends on well-organised interaction between growth factors and different cell types and it is commanded by ischaemia and hypoxia.[10] Vascular endothelial growth factor (VEGF) plays a key role in the initiation and control of angiogenesis.[6]

VEGF is one of the most influential growth factors in the wound healing cascade. It is produced by keratinocytes, macrophages, and fibroblasts in response to pro-inflammatory cytokines at the early stage of wound healing and acts on angiogenesis and tissue granulation by stimulating the migration and propagation of endothelial cells.[11,12] Herbal medicines occupy a significant portion of the medicinal market in the world. Traditionally, medicinal plants have been used for curing ulcers, and infections and stimulating cutaneous wound healing.[13,14] Recently, many medicinal plants have been screened in tropical and subtropical regions of the world for their healing effects. Many studies proved that herbal drugs have been considered effective, affordable, and safe therapeutic agents.[2] It is estimated that 450 plant species have been recognised with expected healing effects.[15] Herbal medicines stimulate wound healing by several mechanisms, frequently by encouraging angiogenesis. Due to the availability of secondary metabolites and volatile compounds that have proangiogenic properties via upregulation of VEGF expression.[9]

Eurycoma longifolia Jack which is locally known in Malaysia as Tongkat Ali (TA) is a widely used medicinal plant in south-east Asian countries, which belongs to the Simaroubaceae family. Each part of the plant is used as a therapeutic agent due to its various medicinal values and the most important part of the plant is the root.[16] Traditionally, the use of TA root extracts as an anti-inflammatory and analgesic therapeutic agent is well confirmed. Previous studies had proved that TA root extracts have antioxidant, anti-inflammatory, antipyretic, antimalarial, cytotoxic, aphrodisiac, and antimicrobial properties.[16–23] In our previous study, ethanol extract of TA root was incorporated into the hydrogel to be used as a delivery system for TA for in vivo wound healing.[24] Therefore, the current study aims to investigate the impact of the topical application of TA hydrogel on VEGF expression in granulation tissue of the healing excisional wound in rats model by immunohistochemistry assay.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS AND ETHICAL APPROVAL

The animal study was approved by the Institutional Animal Care and Use Committee of the International Islamic University Malaysia (IACUC-IIUM), approval number: IIUM/504/14/2/IACUC. The animal study was conducted on 20 Male Sprague-Dawley rats (160-180 g). All details regarding animal care and housing are illustrated in our previous publication.[25]

WOUND CREATION AND STUDY DESIGN

In this study, the excisional wound model as designated by Morton and Malone was used.[13,26] After administering the anaesthesia to rats, a full-thickness circular wound of 15 mm× 15mm in diameter and 2mm depth was made surgically at the dorsal interscapular region of each rat as shown in Figure 1. The wound was created by incising the marked skin with a sterilised surgical scalpel blade then excising the skin with surgical scissors and toothed forceps. The rats were divided into four groups: Group 1 untreated (-ve) control, Group 2 Hydrocyn® aqua gel (+ve) 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 Figure 2. All details regarding the surgical excision, study design, and preparation of TA hydrogel are explained and clarified in our previous publications.[24,25]

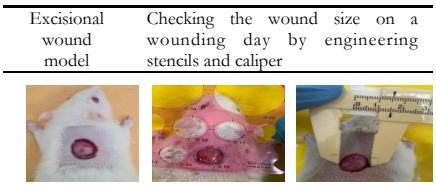

Figure 1: Excisional wound model, a full-thickness circular wound of 15 mm× 15mm in diameter and 2mm depth at the back of a male Sprague Dawley rat

TREATMENT APPLICATION

TA hydrogel was authenticated, extracted, and prepared in a hydrogel as a delivery system for the medicinal plant.[24] Wounds were left undressed and all treatments for all groups (Hydrocyn® aqua gel, vehicle hydrogel, and TA hydrogel) except the untreated (-ve) control group were applied for 5 days twice daily morning and evening with the amount of 1 g of each treatment for each rat.

TISSUE COLLECTION

On day 5 post wounding the granulation tissue was harvested from all the rats to study the influence of TA hydrogel application on VEGF expression by immunohistochemistry (IHC) assay. The rats were weighed using the METTLER TOLEDO balance and anaesthetised by intraperitoneal injection with 10 mL of anaesthesia cocktail. The anaesthesia cocktail consisted of 8.75 mL of ketamine (100 g/mL) mixed with 1.25 mL of xylazine (100 g/mL). The dose was identified based on the body weight, 0.1 mL of the cocktail for every 100 g of body weight was injected at the peritoneal site of each rat. The entire granulation tissue of the wound with an area of 5 mm of unwounded skin was demarcated and excised surgically by scissors, scalpel, and tissue forceps.

Figure 2 shows the images of excisional wounds of four groups on a wounding day, three days after treatment, and 5 days post-treatment before collecting the biopsy of the granulation tissue. Each specimen was kept in an individual cassette and fixed in a 10% buffered formalin at neutral pH (7.4) for six hours before putting them in LEICA ASP 6025 (GmbH, Germany) auto tissue processor overnight. Before impregnation in the wax, each specimen was divided into two halves in order to be positioned perpendicular to the metal mould.

IMMUNOHISTOCHEMICAL STAINING

An immunohistochemistry assay was used to identify the expression of VEGF in the granulation tissue of the healing excisional wound model in rats.[7,11,27] Anti-

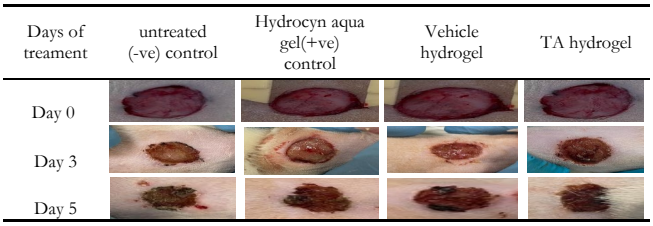

Figure 2: Gross images of excisional wounds of four groups over 5 days post-treatment: Group 1: untreated (-ve) control, Group 2: Hydrocyn® aqua gel (+ve) control, Group 3: vehicle (xanthan) hydrogel and Group 4:Eurycoma longifolia Jack (TA) hydrogel. Day 0 is the wounding day before treatment application, day 3 after three days of treatment, and day 5 of treatment and before collecting the biopsy of granulation tissue.

VEGF receptor 1 antibody [Y103] was used in this study to evaluate VEGF expression after TA hydrogel applicat ion. According to the datasheets of ABCAM, the anti-VEGF antibody is rabbit monoclonal (ab182457), and it is localised in the cell membrane and secreted. Human gastric carcinoma was used as a positive control.

Based on the Abcam guidelines, all the sections of granulation tissue were put in Leica auto Stainer (XL) ST5010 (GmbH, Germany) for dewaxing (program 2:22 minutes and 10 seconds). The sections were agitated in xylene and then dipped in absolute alcohol and 90% alcohol after that all the sections were washed with running tap water and distilled water.

After dewaxing, antigen retrieval was conducted by a pressure cooker (BioCare Medical, USA). The sections were placed inside a container filled with antigen retrieval solution pH 6, then the container was placed inside the pressure cooker, which was filled with distilled water. The pressure cooker was set at a temperature of 121°C for 30 seconds and 95°C for 10 seconds, for antigen retrieval purposes. The sections were left to cool down for 20 minutes and were washed with DAKO wash buffer three times. After that, the sections were incubated with enough drops of hydrogen peroxide block for 10 minutes, then they were washed with DAKO wash buffer. Then, the primary antibody was prepared according to the following formula:

The concentration of primary antibody was 1/100, and 50 µL was added to each section, So, 50/100=0.5 µL of primary antibody +49.5 µL of antibody diluent.

Therefore, 50 µL of diluted antibody was added to each section and incubated overnight in the fridge at a temperature of 2-4°C. After that, horseradish peroxidase (HRP) was applied to the sections for indirect detection for 15 minutes then wash 4 times with buffer solution. HRP is an enzyme used in IHC as it conjugated secondary antibodies against the primary antibody used. Eventually, 30 µL of DAB chromogen was mixed with 1.5 mL of DAB substrate and applied to the sections for 10 minutes to produce brown discolouration, which indicates the localisation or expression of VEGF. After that, the sections were counterstained in LEICA Xl AUTO STAINER (program 3: 30 minutes and 28 seconds). Lastly, the slides were mounted with DPX and glass coverslip and left them overnight for drying. All the steps of the immunohistochemistry assay were conducted in the Department of Pathology and Laboratory Medicine (PALM) at Sultan Ahmad Shah Medical Centre, International Islamic University Malaysia (SASMEC@IIUM).

QUANTIFICATION OF THE EXPRESSION OF VEGF BY IHC ASSAY

The objective of IHC was to investigate the impact of the topical application of TA hydrogel on the expression of VEGF in the granulation tissue of healing wounds in Sprague Dawley rats. Semi-quantitative evaluation of the positive staining was conducted by using the free software ImageJ. Based on the previous studies, positive immunostaining of VEGF in wound healing is usually expressed in endothelial cells, keratinocytes, and fibroblasts of the granulation tissue[8,11] Immunostaining for VEGF was assessed in five fields to each section at 40x magnification. All images were viewed using an optical microscope OLYMPUS BX51 with INFINITY lite Camera connected to the microscope and captured using INFINITY CAPTURE LITE B -203084 software.

Image analysis of IHC was used to quantify the H-DAB and it was implemented by ImageJ software version number 1.53c using the plugin colour deconvolution. The IHC images were decomposed using the software into three basic colours: brown (immunohistochemical stained), purple (Mayer's hematoxylin), and green (glass slide background). The morphometric analysis, corresponding to the brown colour, was done using the Threshold Colour plugin, and antibodies/markers were measured as the percentage of total pixels in each image.[28,29] Data were reported as the percentage area of positive brown discolouration.[7,11,27,28]

STATISTICAL ANALYSIS

Data were analysed by T-test using Minitab version 19 and plotting was done with GraphPad Prism 8 considering p-value<0.05 as an indication of significant difference. Data are expressed as the mean ± standard error (SE).

RESULTS

VEGF expression was calculated semi-quantitively by image J software as shown in Figures 3 and 4 and Table 1. TA hydrogel and vehicle hydrogel group showed significantly higher expression compared with the untreated (-ve) control group with p-values 0.040 and 0.029, respectively. Although the TA hydrogel group showed higher expression than Hydrocyn® aqua gel (+ve) and vehicle (xanthan) hydrogel groups, the difference was not significant with p-values of 0.161 and 0.256, respectively.

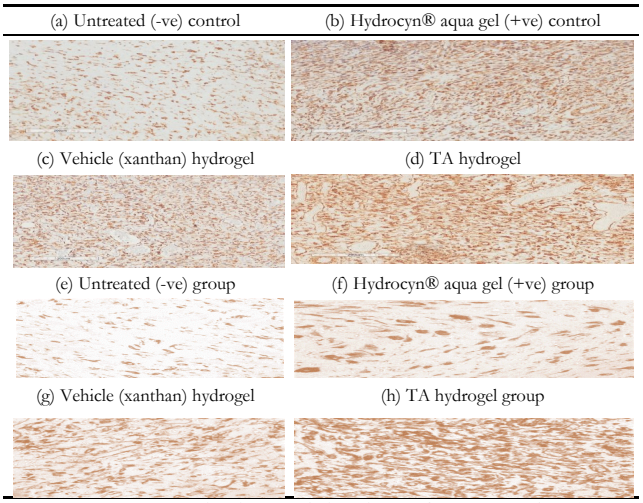

Figure 3 VEGF expression in granulation tissue of (a) untreated (-ve) control, (b) Hydrocyn® aqua gel (+ve) control, (c) Vehicle (xanthan) hydrogel, and (d) TA hydrogel rat's skin on day 5 post wounding at 20x and 200 µm scale. Slides were scanned by an Aperio CS2 image capture device. Deconvolution of immunohistochemistry image of (e) untreated (-ve) control, (f) Hydrocyn® aqua gel (+ve) control, (g) Vehicle (xanthan) hydrogel, and (h) TA hydrogel. Extraction of brown discolouration (DAB staining) to quantify the VEGF expression in granulation tissue of healing wound in Sprague Dawley rat on day 5 post wounding by image J software at magnification 40x.

Table 1 The percentage of the positive area of VEGF immunostaining within the granulation tissue of healing wound of four experimental groups determined by Image J analysis software. Data are expressed as the mean ± standard error (n=5).

Treatment group	Untreated (-ve) control	Hydro-cyn® aqua gel (+ve) control	Vehicle (xanthan) hydrogel	TA hydrogel
VEGF positive staining area percentage (%)	7.47 ± 2.36	11.59 ± 4.05	16.71± 5.67	20.73 ± 8.45

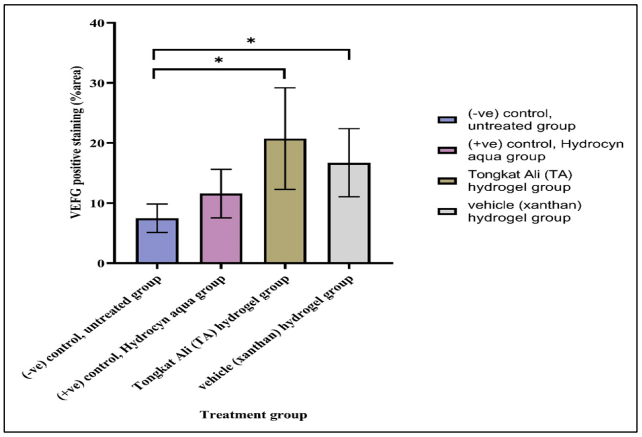

Figure 4 Immunohistochemical analysis of the presence of VEGF in excisional wounds of the rats in the four experimental groups on Day 5 post-wounding. Values are expressed as means ± standard error of the mean (n = 5). A p-value of <0.05 was obtained between groups compared to negative control by T-test using Minitab version 19.

DISCUSSION

The skin is the body's first line of defence against harmful substances, such as radiation, heat, and microorganisms.[30,31] It is therefore important that the various treatments that are performed on cutaneous wounds are designed to restore skin integrity. This is done through the development of effective therapeutic agents that can minimise the risk of developing complications. Despite the availability of numerous non-invasive and effective treatments, it is still important to develop new products that can help accelerate wound healing.[8,32] Herbal medicines have been used to treat wounds throughout the ages. They can help regenerate the skin's natural healing environment through secondary metabolites and phytochemicals by providing an appropriate healing atmosphere.[14,33]

TA is one of the most popular herbal medicines in the countries of Southeast Asia. Traditionally, TA roots are used for many disorders and diseases.[21,22,34] Furthermore, recently in the West, Eurycoma longifolia has shown significant effect as a complementary and alternative medical therapy.[21] In our previous studies, the ethanol extract of TA roots was prepared in the form of hydrogel[24] and investigated its effect on wound contraction and reepithelialisation in an excisional wound model in Sprague Dawley rats.[25] In this study, TA hydrogel enhanced VEGF expression in granulation tissue on Day 5 post-wounding in a rat model, which is an essential growth factor for wound healing. IHC is an effective method for the evaluation of protein indication within the tissues.[29]

IHC assay of the granulation tissue of excisional wound in the rat model showed a significant increase in VEGF expression in TA and vehicle (xanthan) hydrogels treated groups compared to (-ve) untreated group with a p-value of 0.040 and 0.029, respectively. Although there was no significance in VEGF expression between TA hydrogel, vehicle (xanthan) hydrogel, and Hydrocyn® aqua gel (+ve) control-treated groups, the former showed higher expression than the two latter groups (Table 1). The positive impact of TA hydrogel on VEGF expression could be attributed to the availability of phytochemicals with anti-inflammatory and antioxidant properties such as terpenoids, alkaloids, flavonoids, phenolic compounds, cardiac glycosides, and proteins [21,34,35].

According to Kasote et al., (2015), any medicinal plant that contains tannins, terpenoids, flavonoids, and polyphenols will positively affect the wound-healing process by promoting angiogenesis through the upregulation of VEGF expression.[8] Furthermore, our ethanol extract of TA roots contains 26% 5-hydroxymethylfurfural (5-HMF) which have been proven in previous studies to possess proangiogenic effects in the healing process.[36] Kong et al., (2019) proved that 5-HMF significantly increased the expression of VEGF in wounded rat skin.[36] Additionally, the high expression of VEGF in the vehicle (xanthan) hydrogel group could be attributed to the fact that this polymer is a natural polysaccharide.[37] Natural polysaccharides are commonly known for their ability to enhance wound healing via inflammatory reduction and angiogenesis stimulation due to the availability of Glycosaminoglycans (GAGs).[37,38]

Recently, xanthan gum has been widely used in many pharmaceutical industries.[39] Many studies have shown the effectiveness of xanthan gum's incorporation in medicinal topical applications, tissue engineering, and cosmetic products due to its biological properties of sustainability, permeation, biocompatibility, and good spreadability.[38–40]

Thus, it is speculated that the reason for the highest expression of VEGF in the TA hydrogel group could be attributed to the synergistic effects of anti-inflammatory/ antioxidant phytochemicals available in its chemical structure[21,22,34,35] and the good physical and rheological properties of the vehicle (xanthan gum) of which TA extract was incorporated into it. There is also a great possibility that the availability of glycosaminoglycans in the chemical structure of xanthan gum, as it is a natural polysaccharide, might be one of the reasons for the positive effects of both TA and vehicle hydrogels.[38,41-43] Therefore, these results are in agreement with published reports of many in vitro and in vivo studies that have claimed the positive effect of medicinal plants in significant upregulation of VEGF and enhancing the wound healing process via promoting angiogenesis.[8,11,36]

CONCLUSION

This study proved that the topical application of Eurycoma longifolia Jack (TA) hydrogel showed proangiogenic effects in the wound healing process via significantly upregulated VEGF expression in the rat wound model compared with the untreated (-ve) control group. This positive impact might be attributed to the availability of phytochemicals with anti-inflammatory/ antioxidant properties, which are the main mechanisms for enhancing the wound healing process. Thus, TA hydrogel might be regarded as a promising wound healing agent for cutaneous wounds as it showed a proangiogenic effect by elevating VEGF expression, which is a key factor for angiogenesis initiation.

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CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

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