THE EFFECTS OF *EURYCOMA LONGIFOLIA* JACK TONGKAT ALI ROOT EXTRACT HYDROGEL ON HISTOLOGICAL AND IMMUNOHISTOCHEMICAL PARAMETERS OF WOUND HEALING

BY

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ABSTRACT

Wound management is one of the significant health problems throughout the world. Medicinal plants have been used widely in wound management. *Eurycoma longifolia* Jack which is called Tongkat Ali (TA), is one of the Malaysian popular plants. Many studies have proved that TA has effective medicinal impacts. Our study aimed to investigate the possible effects of TA roots extract in the acceleration wound healing via enhancing wound contraction, epithelialization, histopathological parameters of wound healing and VEGF expression. TA roots were authenticated by microscopic techniques and extracted by ethanol and Soxhlet technique. *In vitro* cytotoxicity assay was conducted to determine the maximum safe dose for its topical application. TA extract was chemically analyzed by GC-MS to indicate its phytochemicals. TA roots extract was prepared in a hydrogel for *in vivo* healing studies. *In vivo* studies were conducted on 40 Sprague Dawley rats. The rats were divided into two batches; 5-days and 21-day application. The rats in each batch were arranged into 4 groups (n=5): untreated (-ve) control, Hydrocyn® aqua gel (+ve), vehicle (xanthan) and TA hydrogels. A full-thickness circular excisional wound (15×15) mm was created on the rat's back. The wounded area was measured and photographed on days 3,6,9,12,15and 18 post wounding to determine the wound contraction percentage and epithelialization period for the rats of 21-day batch. For the histopathological studies, 5-days application batch was appointed to investigate the effect of TA hydrogel on wound healing parameters and VEGF expression by immunohistochemistry assay. 21-day application was appointed to investigate the effect of TA hydrogel on collagen formed in the healed skin by measuring the epidermal/dermal thicknesses and evaluating the fibres orientation. Crude sample presents the authentic microscopical features to that of *E.l.*J taproot. Extract weight was 45.3 gm and Ic_{50} was 118.5 µg/mL. TA extract contains several antioxidant/anti-inflammatory phytochemicals, which are necessary for wound healing according to GC-MS. The hydrogel was prepared using 2% XG and TA ethanol extract for wound application. From the *in vivo* studies, no irritation was reported from TA hydrogel and it showed significant raise in wound contraction percentage compared with the other groups on day 3. Although in the later healing stages and epithelialization, TA hydrogel did not show a statistical difference, yet it was comparable to a medically certified wound healing agent. Histologically, TA hydrogel group on day 5 showed a significant increase in fibroblast number and collagen density and it showed significant VEGF expression compared with the (-ve) group by IHC. On day 21, TA hydrogel improved collagen production through increasing the epidermis/dermis thicknesses compared with other experimental groups and the collagen fibres were oriented in a mixed pattern. These positive results might be attributed to the availability of phytochemicals in TA roots with antioxidant/anti-inflammatory effects which are the main mechanisms for an effective healing process. This is the first study that indicates the possibility of preparation of TA roots in a hydrogel and proved its positive impact on wound healing. TA hydrogel can be considered as a promising and effective wound healing agent.

خلاصة البحث

معالجة الجروح هي واحدة من المشاكل الطبية ذات الأهمية حول العالم. تم استخدام النباتات الطبية على نطاق واسع في معالجة الجروح. يُعد *Eurycoma longifolia* Jack الذي يُطلق عليه اسم تونكات علي ت.أ, أحد أشهر النباتات الماليزية. أثبتت العديد من الدراسات أن جذور تونكاتعلي لها تأثيرات طبية فعالة. الهدف من هذه الدراسة هو بحث التأثيرات المحتملة لمستخلص جذور تونكات علي في تسريع التئام الجروح من خلال تعزيز تقلص الجرح و عودَة التَّظَهْرُن والمعلمات النسيجية المرضية لالتئام الجروح وتعبير عامل النمو البطاني الوعائي. تمت المصادقة على جذور تونكات علي ت.أ. بتقنيات مجهرية واستخلاصها باستخدام تقنية الإيثانول وسوكسلت. تم إجراء فحص السمية الخلوية في المختبر لتحديد الجرعة الآمنة القصوى لتطبيقه الموضعي. تم تحليل مستخلص تونكات علي كيميائيًا بواسطة كروماتوغرافيا الغاز – مطياف الكتلة للإشارة إلى المواد الكيميائية النباتية الخاصة به. تم تحضير مستخلص جذور تونكات علي في هيدروجيل للاستخدام في دراسة معالجة الجروح في الجسم الحي .أجريت دراسات في الجسم الحي على ٤٠ جرذ ذكر من سلالة سبراكو داولي. تم تقسيم الجرذان إلى دفعتين: تطبيق الهيدروجل لمده ٥ أيام و ٢١ يوم. تم ترتيب الجرذان في كل دفعة الى 4 مجموعات (ن = 5): مجموعة المراقبة غير المعالجة , مجموعه جل , (Hydrocyn® aqua) مجموعه المادة الحاملة للمستخلص (زانثان) هيدروجل و مجموعة ت.أ هيدروجل. جراحيا تم إنشاء جرح استئصالي دائري كامل السماكة (15 × 15) ملم على ظهر الجرذان. تم قياس منطقه الجرح وتصويرها في الأيام 3, 6, 9, 12, 15 و 18 بعد الجرح لتحديد نسبة تقلص الجرح وفترة عودَة التَّظَهْرُن بتشكل النسيج الظهاري لدفعة الجرذان التي تبلغ مده علاجها 21 يومًا. أما بالنسبة لدراسة الأنسجة المرضية فقد تم تعيين دفعة الجرذان التي تبلغ مده علاجها 5 أيام للتحقيق في تأثير هيدروجيل تونكات علي على معايير التئام الجروح وتعبير عامل النمو البطاني الوعائي عن طريق مقايسة الكيمياء الهيستولوجية المناعية. تم تعيين دفعة الجرذان التي تبلغ مده علاجها 21 يومًا للتحقق من تأثير هيدروجيل تونكات علي على الكولاجين المتكون في الجلد الملتئم عن طريق قياس سماكة البشرة / الجلد وتقييم اتجاه الألياف. قدمت العينة الأولية السمات الميكروسكوبية الأصلية لتلك الخاصة بـ .*E.l*J taproot كان وزن المستخلص 45.3 غرام وتركيز المادة الموافق للتثبيط النصفي 118.5 ميكروغرام / مل. يحتوي مستخلص تونكات علي على العديد من المواد الكيميائية النباتية المضادة للأكسدة / المضادة للالتهابات ، والتي تعد ضرورية لالتئام الجروح وفقًا لكروماتوغرافيا الغاز – مطياف الكتلة. تم تحضير الهيدروجيل باستخدام صمغ الزانثان (2%) و المستخلص الإيثانولي لتونكات علي لتطبيقه على الجرح. من الدراسات التي أجريت في الجسم الحي , لم يتم الإبلاغ عن أي تهيج من هيدروجيل تونكات علي وأظهر زيادة معنوية في نسبة تقلص الجرح مقارنة بالمجموعات الأخرى في اليوم الثالث. على الرغم من أنه في مراحل الشفاء اللاحقة و عَودَةُ التَّظَهْرُن , لم يُظهر هيدروجيل تونكات علي فارقًا إحصائيًا , ومع ذلك كان يمكن مقارنته بعامل التئام الجروح المعتمد طبيًا. من الناحية النسيجية ، أظهرت مجموعة هيدروجيل تونكات علي في اليوم الخامس زيادة معنوية في عدد الخلايا الليفية وكثافة الكولاجين وأظهرت تعبيرًا كبيرًا في عامل النمو البطاني الوعائي مقارنة بمجموعة المراقبة بواسطة مقايسة الكيمياء الهيستولوجية المناعية. في اليوم الحادي والعشرين ، حسّن هيدروجيل تونكات علي إنتاج الكولاجين من خلال زيادة سماكة البشرة / الأدمة مقارنةً بالمجموعات التجريبية الأخرى وتم توجيه ألياف الكولاجين بنمط مختلط. يمكن أن تُعزى هذه النتائج الإيجابية إلى توافر المواد الكيميائية النباتية في جذور تونكات علي ذات التأثيرات المضادة للأكسدة / المضادة للالتهابات والتي تعد الآليات الرئيسية لعمليه التئام الجروح بصورة صحيحة. هذه هي الدراسة الأولى التي تشير إلى إمكانية تحضير هيدروجيل من جذور تونكات علي وأثبتت تأثيرها الإيجابي على التئام الجروح. يمكن اعتبار هيدروجيل تونكات علي كعامل واعد وفعال في التئام الجروح.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Master of Medical Sciences.

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DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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"In the name of Allah, Most Gracious, Most Merciful "

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LIST OF ABBREVIATION

A. fumigatus	*Aspergillus fumigatus*
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
ATCC	American type culture collection
B. cereus	*Bacillus cereus*
C.albicans	*Candida albicans*
CO2	Carbon dioxide
COX-2	Cyclooxygenase -2
DAB	3,3′-Diaminobenzidine
DM	Diabetes mellitus
DMSO	Dimethyl sulfoxide
DPX	Dibutylphthalate Polystyrene Xylene
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
E.l. J	*Eurycoma longifolia* Jack
FGF	Fibroblast growth factor
GC-MS	Gas Chromatography–Mass Spectrometry
GFs	Growth Factors
HCL	Hydrochloric acid
H&E	Hematoxylin and Eosin
HGF	Human Gingival Fibroblast
HIF-1	Hypoxia Inducible Factor -1
5-HMF	5-Hydroxymethylfurfural
HRP	Horseradish Peroxidase
I-ACUC	IIUM ANIMAL CARE AND USE COMMITTEE
IHC	Immunohistochemistry
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
IQR	Interquartile Range
KPI	Key Performance Indicator
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinases
MMP	Matrix Metalloproteinase
MOH	Ministry of Health (Malaysia)
mTOR	Mammalian Target of Rapamycin
NF-$_K$B	Nuclear Factor Kappa B
NMPC	Natural Medicinal Product Centre
OA	Oleic Acid
OD	Optical Density
OECD	Organization for Economic Cooperation and Development
PDGF	Platelet Derived Growth Factor
PII	Primary Irritation Index
ROS	Reactive Oxygen Species
S.aureus	*Staphylococcus aureus*

S.mutans	*Streptococcus mutans,*
STD	Standard Deviation
S.typhi	*Salmonella typhi*
TA	Tongkat Ali
TGF-B1	Transforming Growth Factor – B1
TNF	Tumour Necrosis Factor
VEGF	Vascular Endothelial Growth Factor
VVG	Verhoeff-Van Gieson
WHO	World Health Organization
WHS	Wound Healing Society
Wi	Initial Quantity of Ground Sample Used
Wp	Quantity of Extract Produced
XG	Xanthan Gum

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Skin is the largest and pivotal organ in the body with three main functions: thermoregulation, sensation and protection. It works as a physical barrier, which protect us from infection, thus when it is traumatized, microbes and elements can have a direct entrance to the underlying tissues. As well as skin performs other functions such as hemostasis, metabolic and immunologic actions (Heidari et al., 2018). Preserving these important functions needs effective and powerful actions to maintain the skin from any injury and to restore and repair skin functions when lost or destroyed (Shedoeva et al., 2019). The wound refers to the damage in the coherence of the skin's layers as a result of physical, chemical or thermal injuries. Wounded tissues are characterized by lacking the functional and anatomical solidarity of the living organ. Wounds treatment put a lot of financial, social and economic burdens on care givers, health organizations, patients and their families. Wounds represent a worldwide health challenge (Boakye et al., 2018).

Wound healing is a complicated biological process characterized by multiple cellular and biological events to restore the missing layers of the injured tissue (Shedoeva et al., 2019). It consists of multiple cellular and biochemical courses in order to replace the injured tissue and re-establish the structural and functional integrity of the skin (Tottoli et al., 2020). Wound healing is arranged into four superimposed phases: hemostasis, inflammation, proliferation, and remodeling. Immediately after cutaneous injury, cellular elements such as fibroblasts, keratinocytes, macrophages, and other immune cells rapidly proliferate and emigrate to the wound bed and commence the healing process. Closing the wound and re-establishing tissue homeostasis initiates rapidly by keratinocytes, which reform a functional epidermis [re epithelialization]. Fibroblast is one of the most important cells in the skin with its an essential role in granulation tissue formation during healing process. Extracellular matrix proteins and new blood vessels are the main components of granulation tissue (Shedoeva et al.,

2019). As well as, angiogenesis is an important cellular event in the restoration of the injured tissue, since it re-establishes the normal blood circulation, provides nutrients for the cells, restores oxygen to the injured tissue and expel the cellular waste. Any disturbance in the regulation of neovascularization can lead to dehiscence and chronic wound (Guerra et al., 2018). Impairment in the regulation in any stage of the wound healing process retards healing and may lead to several skin pathologies, such as non-healing or chronic wound (Shedoeva et al., 2019). Time reduction and avoiding the inappropriate consequences are the main mechanisms for improving the wound healing process. Antibiotics, antiseptics and de sloughing agents have been utilized to hasten the healing process and decrease the inappropriate consequences. These agents are confronted with limitations due to their undesirable side effects and high costs. Currently, research is trending to harness the natural resources such as medicinal plants in managing and treating many kinds of the wounds because of their availability, nontoxicity, biocompatibility and safety (Canyon Hydro et al., 2013; V. L. Nguyen et al., 2017).

About 70% to 95% of population in most developing nations and 70% to 90% of people in well-developed countries utilize an orthodox medicine in their initial healthcare to manage their medical problems. Many medicinal plants have scientifically proven to be used as wound healing agents for the treatment of the wounds (Demilew et al., 2018). The medicinal plants follow several mechanisms to display their healing efficiencies which are; antioxidant, anti-inflammatory, anti-microbial effects, having the ability to increase collagen production and stimulate fibroblast and keratinocytes proliferation. Therefore, any medicinal plant that possesses two or more of these important biological features, there will be a great possibility to be an effective wound healing factor (Canyon Hydro et al., 2013; Shah & Amini-Nik, 2017)

In Malaysia, manufacturing medicinal plants is a promising and upcoming industrial sector and the Malaysian medicinal plants market undergoes an astonishing development with manufacturing effective, high-quality and safe medical products for consumption by human. The tropical plants are known as an excellent recourse of phytochemicals as they produce these biological and chemical compounds as a means

of defense against ailments, pests and predators (Jantan, 2004; Khanam et al., 2015; Sekar et al., 2014).

Eurycoma longifolia Jack which is generally called as Tongkat Ali (TA) is the one of the most popular and important medicinal plant in the Malaysian market (Aida et al., 2016). *Eurycoma longifolia* Jack is native to Southeast Asian countries such as Thailand, India, Malaysia, and Vietnam. *Eurycoma longifolia* Jack, bird's nest and tin are the three national treasures of Malaysia. The root of this plant is used by local folks in Malaysia for aphrodisiac, improve libido and energy, hypertension and fever treatment. Many studies have been 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 (Ruan et al., 2019; Varghese et al., 2013). As well as the ethanol extract of TA roots has been confirmed in previous studies to show antibacterial and antifungal effects (Alloha et al., 2019; Faisal et al., 2015, 2016). All these pharmacological effects are assigned to the presence of important phytochemicals such as: quassinoids, alkaloids, terpenoids, tannins, polysaccharides, glycosides and phenolic compounds and other important bioactive compounds which are heavily concentrated in the roots (Abubakar et al., 2017; Khanam et al., 2015; Rehman et al., 2016; Ruan et al., 2019).

Studies on its wound healing potential are lacking. Our study aims to explore how these important phytochemicals of the TA root with their anti-inflammatory, antioxidant and antimicrobial effects can improve the healing process through different mechanisms such as efficiency of re-epithelialization, level of angiogenesis, expression of important growth factors such as (VEGF), and the production of collagen in the healed skin.

1.2 PROBLEM STATEMENT AND JUSTIFICATION OF THE STUDY

Wound healing is a vital and convoluted process give rise to the re-establishing of the structural and functional integrity of injured tissue. It consists of four overlapping processes: homeostasis, inflammation, proliferation and remodeling, a complicated signaling mechanism involves various cytokines, chemokines, and growth factors

control all these processes (Bektas et al., 2020; Kong, Fan, et al., 2019; Shedoeva et al., 2019). To provide an appropriate condition and moist environment for healing, many synthetic topical products and wound dressings are used. However, some of these products are expensive and unaffordable by the patients and have harmful side effects including allergic reaction and antimicrobial resistance associated with infection (Lordani et al., 2018; Umar et al., 2021). It is estimated that around 70% of pathogens existed in US hospitals obtained resistance for at least one antibiotic leading to the death of more than 14,000 patients yearly from infections (Khanam et al., 2015). In order to solve the issue of the side effects of synthetic topical agents, developing wound healing agents from medicinal plants is one of the effective approaches to avoid using the synthetic drugs with some of their drawbacks (Sharma et al., 2021; Umar et al., 2021).

Recently there is great interest in medicinal plants in wound healing due to the vast diversity of phytochemicals with anti-inflammatory, antioxidant and immunomodulatory effects (Heidari et al., 2019). Antioxidant, anti-inflammatory, anti-bacterial, increase collagen formation and stimulation fibroblasts and keratinocytes proliferation and differentiation are biological properties of the wound healing agent. Any medicinal plant that possesses two or more of these biological properties can be considered as a wound healing agent (Boakye et al., 2018).

Eurycoma longifolia Jack, Tongkat Ali (TA) is one of the most popular traditional medicines in Southeast Asia. In many Asian countries the roots of this plant have been used traditionally to treat many disorders such as fever, hypertension aphrodisiac etc. In the west, *Eurycoma longifolia* Jack (TA) has performed a good role in the herbal therapy as an alternative and complementary medicine (Rehman et al., 2016).

Recent studies have proved that the TA roots have antioxidant, anti-inflammatory effects (Rehman et al., 2016; Ruan et al., 2019; Varghese et al., 2013) and antimicrobial effects (Alloha et al., 2019; Faisal et al., 2015, 2016). These biological properties due to availability of important phytochemicals such as quassinoids , alkaloids , flavonoids , tannins , terpenoids , steroids , glycosides and polysaccharides (Izzany et al., 2018; Rehman et al., 2016).

According to the literature review, an alcoholic extract of *Eurycoma longifolia* Jack (TA) roots might be an effective wound healing agent through anti-inflammatory, antimicrobial and antioxidant properties of its phytochemicals.

1.3 GENERAL OBJECTIVE

To investigate the effect of *Eurycoma longifolia* Jack (TA) root extract on wound healing process.

1.4 SPECIFIC OBJECTIVES

1. To investigate the effect of *Eurycoma longifolia* Jack (TA) root extract hydrogel on wound contraction.
2. To evaluate the effect of *Eurycoma longifolia* Jack (TA) root extract hydrogel on re-epithelialization period.
3. To assess histologically the effect of *Eurycoma longifolia* Jack (TA) root extract hydrogel on different histopathological parameters of wound healing.
4. To determine immunohistochemically the expression of angiogenic growth factor VEGF in wounds treated by *Eurycoma longifolia* Jack (TA) root extract hydrogel.
5. To investigate the effect of *Eurycoma longifolia* Jack (TA) root extract hydrogel on collagen production in the healed skin through histochemical staining.

1.5 RESEARCH QUESTIONS

1. What is the effect of topical application of *Eurycoma longifolia* Jack (TA) root extract hydrogel on wound contraction?
2. What is the effect of topical application of *Eurycoma longifolia* Jack (TA) root extract hydrogel on re-epithelialization period?