WOUND HEALING PROPERTIES OF METHANOLIC FRACTION OF *CENTELLA ASIATICA* EXTRACT

BY

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ABSTRACT

Asiaticoside from *Centella asiatica* is claimed as a bioactive compound capable of wound healing. In order to ensure that the pharmacological activity of the extract is traceable and measurable, this present study attempted to evaluate the bioactivity of fractionated extract of rich asiaticoside. The ability of the extract in accelerating wound healing by facilitating the healing process had been evaluated via antioxidant activity test, antibacterial activity test, *in vitro* scratch assay study of cell migration, and *in vivo* wound excision study. The result of extraction showed that only methanol fraction of extract contains about 2.4% of asiaticoside. The methanol fraction exhibited antioxidant activity with IC_{50} value of 370.51 µg/mL as DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenger while IC_{50} value of 399.07 µg/mL in ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging capacities. The methanol fraction was found to be less active against the test organisms which were *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumonia* (ATCC 700603), *Bacillus subtilis* (IMR B 145/11C), *Streptococcus pyogenes* (ATCC 19615), and *Salmonella typhimurium* (IMR S 974/05B). In the *in vitro* scratch assay, methanol fraction of extract with concentration of 0.2 and 100 µg/mL showed significant effect of cell migration on human dermal fibroblast and human dermal keratinocyte as compared to positive control ($p < 0.05$). From *in vivo* study, it was shown that the methanol fraction (40%, 10% and 2.5%) induced collagen synthesis. Histopathology data also concluded that there was dose dependant effect of the tested extract as wound healer. Taken together, recent findings suggest that methanolic fraction of *C. asiatica* extract demonstrated remarkable polyvalent activity, thus has potential as an effective wound healer. In conclusion, the claim on the presence of wound healing properties in *C. asiatica* had been well supported based on the results obtained in this study.

ملخص البحث

نبات *Centella asiatica* هو نبات تقليي تبين أن له تأثيرات دوائية في علاج الجروح الجلدية. لتأكيد أن الفعالية العلاجية لخلاصة هذا النبات ممكنة التحقق والقياس هدفت هذه الدراسة لتقييم الفعالية الحيوية للخلاصة الغنية بالآسياتيكوسيد. تم تقييم قدرة الخلاصة على تسريع علاج الجروح بتسهيلها لعملية الشفاء من خلال فحص الفعالية المضادة للأكسدة، الفعالية المضادة للبكتيريا، فحص الخدش في الزجاج لهجرة الخلايا، و دراسة الاستئصال الجراحي في الحي. تم تقسيم الخلاصة الإيتانولية إلى سبعة أجزاء بطريقة الكروماتوغرافيا السائلة الفراغية. بينت النتائج أن الخلاصة المتانولية فقط احتوت على حوالي 2.4% آسياتيكوسيد. هذا الجزء أظهر أيضاً فعالية مضادة للأكسدة بقمية IC_{50} تساوي 370.51 µg/mL كمنظف لـ-2-(1,1-diphenyl DPPH (picrylhydrazyl بينما قيمة IC_{50} تساوي 399.07 µg/mL في القدرة المنظفة للجذر -(2,2' ABTS ((azino-bis(3-ethylbenzothiazoline-6-sulfonic acid. تم فحص التأثير المضاد للبكتيريا للخلاصة الغنية بالآسياتيكوسيد باستخدام طريقة نفوذية القرص. تم أيضاً تحديد التركيز الأصغري المثبط MIC باستخدام طريقة التمديد. تم استخدام سبعة أنواع من البكتيريا وهي *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumonia* (ATCC 700603), *Bacillus subtilis* (IMR B 145/11C), *Streptococcus pyogenes* (ATCC 19615), and *Salmonella typhimurium* (IMR S 974/05B). من بين تراكيز الخلاصة المجزأة لحد 600 µg/mL، فقط قيم MICs لنوعين من البكتيريا ت تم تحديدها. قيمة الـMICs للخلاصة المقاسة ضد *Bacillus subtilis* كان 300 µg/mL بينما ضد *Streptococcus pyogenes* كان 500 µg/mL. تم اختبار الخلاصة الغنية بالآسياتيكوسيد على خلايا جلد بشرية human dermal fibroblast (HDF) و human dermal keratinocyte (HaCaT). بناء على فحص السمية على الخلايا، كلا النوعين من الخلايا أظهر تحريضاً مهماً لحيوية الخلايا عند تطبيق الجزء المتانولي من الخلاصة بتركيز 100 µg/mL و0.19 µg/mL. إضافة إلى ذلك، لم تظهر الخلاصة تقريباً أي سمية بالتراكيز المختبرة حيث أن قيمة الـIC_{50} لم تكن قابلة للقياس بتركيز يتراوح بين 0.19 µg/mL و100 µg/mL. حيث أن كل التراكيز المستخدمة أظهرت أكثر من 80% في فحص حيوية الخلايا، تم اختيار التركيز في فحص الخدش عشوائياً كأعلى قيمة بمعنى 100 µg/mL، قيمة وسطية 6 µg/mL وقيمة دنيا 0.2 µg/mL. في فحص الخدش في الزجاج أظهر الجزء المتانولي بتركيز 0.2 و100 µg/mL تأثيراً مهماً على هجرة خلايا HDF وHaCaT مقارنة للناظم الموجب ($p<0.05$). من خلال الفحص في الحي تبين أن الجزء المتانولي (40%, 10% and 2.5%) من الخلاصة المعايرة حرضت تشكل الكولاجين. أظهرت البيانات النسيجية أيضاً أن هناك تأثيراً مرتبطاً بالجرعة للخلاصة المختبرة من أجل شفاء الجروح. تقترح كل هذه النتائج مجتمعة أن الخلاصة المعيارية لنبات *C. asiatica* تملك تأثيرات متعددة وبالتالي لها قابلية قوية في شفاء الجروح. كخلاصة، الادعاء الحالي بامتلاك هذا النبات خواص شافية للجروح هو ادعاء مدعوم علمياً من خلال نتائج هذه الدراسة.

ABSTRAK

Asiatikosia adalah sebatian bioaktif dalam *Centella asiatica* yang bertanggungjawab ke atas penyembuhan luka. Bagi memastikan aktiviti farmakologi ekstrak *C. asiatica* dapat ditentukan, kajian ini dijalankan dengan menilai bioaktiviti fraksi ekstrak kaya asiatikosia. Keupayaan ekstrak ini dalam proses penyembuhan luka telah dinilai berdasarkan ujian antioksidan, antibakteria, sel migrasi melalui kaedah luka *in vitro*, dan luka secara *in vivo*. Keputusan proses pengekstrakan menunjukkan hanya fraksi metanol mengandungi asiatikosia iaitu sebanyak 2.4%. Fraksi ini menunjukkan kesan antioksidan dengan nilai IC_{50} sebanyak 370.51 μg/mL dalam kaedah DPPH (1,1-diphenyl-2-picrylhydrazyl) dan 399.07 μg/mL dalam kaedah ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid). Fraksi metanol didapati kurang aktif terhadap bakteria yang telah digunakan iaitu *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumonia* (ATCC 700603), *Bacillus subtilis* (IMR B 145/11C), *Streptococcus pyogenes* (ATCC 19615), dan *Salmonella typhimurium* (IMR S 974/05B). Dalam kaedah luka secara *in vitro*, fraksi metanol ekstrak dengan kepekatan 0.2 μg/mL dan 100 μg/mL menunjukkan kesan positif yang signifikan ke atas migrasi sel dermal fibroblast manusia dan sel keratinocyte dermal manusia jika dibandingkan dengan kontrol positif (p <0.05). Berdasarkan keputusan eksperimen *in vivo*, fraksi metanol (40%, 10% and 2.5%) telah berjaya meningkakan penghasilan kolagen dalam kulit arnab. Data histopatologi menunjukkan kenaikan secara bergantung dos sebagai penyembuh luka. Keputusan-keputusan daripada ekperimen-ekperimen yang telah dijalankan mencadangkan bahawa ekstrak *C. asiatica* menunjukkan kesan aktiviti polivalen yang mengagumkan dan dengan ini mencadangkan bahawa *C. asiatica* mempunyai potensi sebagai penyembuh luka yang berkesan. Secara konklusinya, keupayaan *C. asiatica* untuk membantu menyembuhkan luka telah terbukti dan disokong sepenuhnya berdasarkan keputusan-keputusan positif yang dicapai hasil daripada penyelidikan ini.

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 thesis for the Master Degree of Pharmaceutical Sciences (Pharmaceutical Technology).

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DECLARATION

I hereby declare that this thesis is the result of my own investigation, 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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Signature……. Date ….....

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Signature Date

This thesis is dedicated to my parents for laying the foundation of what I turned out to be in life.

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LIST OF SYMBOLS

µg	Microgram
µL	Microliter
µm	Micrometer
g	Gram
mg	Milligram
mL	Milliliter
mm	Millimeter
mM	MilliMolar
ng	Nanogram
nm	Nanometer
rpm	Revolutions per Minute
w/w	Weight per weight

LIST OF ABBREVIATIONS

ABTS	2,2-azinobis-(3ethylbenzothiazoline-6-sulphonic acid)
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
EC	Efficient Concentration
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
GACP	Good Agricultural and Collection Practices
GMP	Good Manufacturing Practices
H&E	Hematoxylin and Eosin
HaCaT	Human Epidermal Keratinocyte
HCl	Hydrochloric Acid
HDF	Human Dermal Fibroblast
IC	Inhibitry Concentration
IL-1/IL-6	Interleukin-1/ Interleukin-6
MF	Methanol Fraction
MF0.2	0.2 µg/mL of Methanol Fraction
MF100	100 µg/mL of Methanol Fraction
MF6	6 µg/mL of Methanol Fraction
MIC	Minimal Inhibitory Concentration
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide
n.d.	No Date
ND	Not Detected
NaOH	Sodium Hydroxide
PAF	Platelet-Activating Factor
PBS	Phosphate Buffer Saline
PDGF	Platelet-Derived Growth Factor
ROSs	Reactive Oxygen Species
SD	Standard Deviation
SEM	Standard Error of Mean
TGF-β	Transforming Growth Factor-Beta
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 RESEARCH BACKGROUND

Wounds are perhaps, an unavoidable and inescapable part of our life. According to Director General of Health, Malaysia Health Ministry, Datuk Dr Noor Hisham Abdullah, wounds are a major problem in Malaysia since seven hospitals nationwide spent almost RM1 million in 2013 to manage patients with wounds (Veno, 2014). Wounds may be generated by physical, chemical, thermal, microbial or immunological insult to the tissue (Thakur, Jain, Pathak, and Sandhu, 2011). Different types of wounds may require different types of treatments.

The human body is created with a complex self-healing mechanism. The healing process is stepwise, which consists of four different phases that overlap with each other. These phases are the haemostasis, inflammation, proliferation, and remodelling or maturation phase. Normally, the phases of wound healing progress in a predictable and timely manner. Abnormal progression of these phases leads to poor healing of wounds, resulting in either a chronic wound or pathological scarring (Thakur et al., 2011).

Factors that commonly lead to abnormal progression of wound healing are microbial infection, diabetic condition and poor blood circulation (Thakur et al., 2011). Under these conditions, the management of wounds can become complicated and sometimes costly. In order to overcome these problems, various products have appeared in the market to heal wounds in the shortest time possible and to increase patient compliance by minimizing pain, discomfort and scarring. However, it is important to recognize that wound care should always support the natural healing