

IN VITRO STUDY OF *POLYGONUM MINUS* EXTRACT EFFECT ON SKIN
CELLS HEALING CAPACITY

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Specially dedicated to *my parents* and *my dear husband*

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ABSTRACT

Non-healing chronic wounds represent a common irritating health problem especially to diabetic patients and those with vascular insufficiency. Untreated chronic wounds may lead to serious complications like severe persistent pain, septicaemia or amputations. With the increasing ratios of aging population, obesity and the high prevalence of diabetic ulcers, chronic wounds are reflected as a considerable burden to the community and healthcare professionals. *Polygonum minus* (*P. minus*) leaves or “Kesum” as commonly known in Malaysia was found to have anti-oxidant properties, rich in some essential vitamins and minerals and have anti-ulcer and antimicrobial effects. *In vitro* study was carried out to determine the efficacy of different extracts of *P. minus* leaves on wound healing. Aqueous, ethanol and diethyl ether extracts of *P. minus* were obtained under reduced pressure in a rotary vacuum evaporator. Their total phenolic content and radical scavenging activity were evaluated by using Folin-Ciocalteu and DPPH (2, 2-diphenyl-1-picrylhydrazyl) assays, respectively. MTT assay was used to determine the cytotoxicity of the different extract solvents on human skin fibroblast cells (HSF1184). The enhancing capacity of *P. minus* extracts on cellular migration at their maximum and minimum non-toxic doses was evaluated using Scratch assay. Both aqueous (31µg/ml) and ethanol extract (63µg/ml) of *P. minus* leaves enhanced cellular migration and wound closure by 71% and 65% in day 1 respectively, comparing to 42% closure with the control. The induced scratch was completely healed in day 2 with aqueous and ethanol extracts while the control scratch healed by 79%. Moreover, aqueous extract exhibited the highest total phenolic content (217.1 mg GAE/l) and the highest antioxidant ability (IC₅₀ = 56 µg/ml). Therefore, aqueous extract of *P. minus* leaves may play a role in improving the healing capacity of skin cells and in the same time provide a safer, more natural and costly effective remediation. Further *in vivo* studies may be needed to ascertain its effectiveness.

ABSTRAK

Luka kronik yang sukar sembuh merupakan masalah kesihatan yang menjengkelkan, terutamanya kepada penghidap-penghidap diabetes dan yang lemah sistem saluran dalam tubuh. Luka kronik ini, jika tidak dirawat, boleh menyebabkan komplikasi serius seperti sakit parah, septisemia atau amputasi/kekudungan. Berikutan peningkatan kadar populasi warga berumur, obesiti dan kekerapan ulser diabetes, luka kronik disenaraikan sebagai masalah yang perlu diberi perhatian oleh komuniti dan perunding professional kesihatan. Daun *P. minus*, atau dikenali sebagai daun kesum di Malaysia, didapati mengandungi ciri-ciri antioksidan, kaya dengan vitamin dan mineral penting, serta kesan anti-ulser dan anti-mikrob. Kajian *in vitro* telah dijalankan untuk mengenalpasti keberkesanan beberapa ekstrak daun *P. minus* ke atas penyembuhan luka. Ekstrak akueus, etanol dan dietil ether telah diletakkan di bawah tekanan rendah di dalam mesin pengewap berputar vakum. Jumlah kandungan fenolik dan aktiviti carian radikal telah diuji menggunakan assay Folin-Ciocalteu dan DPPH. Assay MTT telah digunakan untuk mengenalpasti kesan 'cytotoxicity' setiap larutan ekstrak ke atas sel *fibroblast* kulit manusia (HSF1184). Kapasiti peningkatan daripada ekstrak *P. minus* ke atas migrasi selular pada dos maksimum dan minimum telah diuji menggunakan scratch assay. Kedua-dua akueus (31µg/ml) dan ekstrak etanol (63µg/ml) daripada daun *P. minus* telah meningkatkan migrasi selular dan penutupan parut luka dengan 71% dan 65% dalam hari pertama, berbanding penutupan 41% oleh larutan kawalan. Luka calar didapati sembuh sepenuhnya dalam 2 hari kesan ekstrak akueus dan etanol, manakala larutan kawalan hanya sembuh 79%. Tambahan pula, ekstrak akueus menunjukkan jumlah tertinggi kandungan fenolik (217.1mg GAE/l) dan ciri-ciri antioksidan ($IC_{50} = 56\mu\text{g/ml}$). Kesimpulannya, ekstrak akueus daun *P. minus* berpotensi untuk mempercepatkan penyembuhan sel kulit, di samping sebagai rawatan yang lebih selamat, semulajadi dan efektif dari segi kos. Kajian *in vivo* selanjutnya mungkin diperlukan untuk kepastian keberkesanan ekstrak daun ini.

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LIST OF SYMBOLS / ABBREVIATIONS

Abs	-	Absorbance
B.subtilis	-	<i>Bacillus subtilis</i>
°C	-	Degree Celsius
CO ₂	-	Carbon dioxide
DFU	-	Diabetic Foot Ulceration
DMEM	-	Dulbecco's Modified Eagle's Medium
DMSO	-	Dimethyl sulfoxide
DPPH	-	2,2-Diphenyl-1-picrylhydrazyl
E.coli	-	<i>Escherichia coli</i>
<i>et al.</i>	-	Et alia
FBS	-	Foetal Bovine Serum
F-C	-	Folin Ciocalteu
GAE	-	Gallic acid equivalent
g	-	Gram
h	-	Hour
HCl	-	Hydrochloric acid
HSF 1184	-	Human skin fibroblast 1184
IC ₅₀	-	Inhibition concentration at 50%
mg	-	Milligram
mg/L	-	Milligram per Litre
mg/ml	-	Milligram per millilitre
min	-	Minute
mL/g	-	Mili Litre per gram

MNTD	-	Maximum non-toxic dose
MTT	-	Methyl tetazolium
NHMS	-	National Health Morbidity Survey
nm	-	Nanometre
PBS	-	Phosphate Buffered Saline
PGE2	-	Prostaglandin E2
pH	-	Hydrogen concentration
ROS	-	Reactive Oxygen Radicals
S.aureus	-	Staphylococcus aureus
SD	-	Standard deviation
SOD	-	Super oxide dismutase
TPC	-	Total Phenolic Content
µg	-	Microgram
µl	-	Microliter
%	-	Percentage

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CHAPTER 1

INTRODUCTION

1.1 Research background

Non-healing chronic wounds represent a common irritating health problem especially to diabetic patients and those with vascular insufficiency (Balaji *et al.*, 2012; Trøstrup *et al.*, 2013). Untreated chronic wounds may lead to serious complications like severe persistent pain, septicaemia or amputations (Trøstrup, *et al.*, 2013). With the increasing ratios of aging population, obesity and the high prevalence of diabetes, chronic wounds are reflected as a considerable economic burden (Balaji *et al.*, 2014).

For optimum healing of skin wounds, a balanced integration between biological and molecular processes is needed which affects cellular proliferation, migration, extracellular matrix deposition and remodelling, in addition to neovascularisation (Mildner *et al.*, 2013). Dysfunctional angiogenesis and hypoxic microenvironment of the wound bed are considered the impeding factors for normal tissue remodelling and healing, in addition to the infiltration of T-cells and macrophages that leads to a cascade of tissue toxicity and increased oxidative stress (Tregrove *et al.*, 1999).

P. minus is one of the well-known Malaysian plants, and locally is known as “Kesum”. It is part of the Polygonaceae family and found mainly in countries of Southeast Asia (Gattuso, 2001). For ages, its leaves were used widely as a flavouring ingredient in many Malaysian dishes. It was used also as a traditional medication for treatment of digestive disorders, dandruff and eyesight problems (George *et al.*, 2014b). *P. minus* has been recognized by the Malaysian Government in the herbal product blueprint as an essential oil-producing plant (Yaacob, 1987).

About 80% of the world population was found to be depending on traditional herbs for treatment of several health problems (Grover and Yadav, 2004). Recently, *P. minus* was profusely studied for its various medicinal properties. Nearly fifty-three compounds or secondary metabolites were isolated from *P. minus* leaves such as flavonoids, terpenoids, geranial, gerniol, aldehydes and phenolic compounds (Narasimhulu and Mohamed, 2014). These secondary metabolites are the responsible factors for the biological useful properties of that plant such as its antioxidant, antiviral, antiulcer, antifungal and antimicrobial activities (Qader *et al.*, 2012a; Vimala *et al.*, 2012).

Previous work done by Blanche *et al.* (2014) has revealed the effectiveness of *P. minus* extract on regeneration of skin cells and its anti-ageing effects. Their results were significant as the number and length of crows’ feet wrinkles in 32 patients decreased by 20% and 25%, respectively.

P. minus was chosen for this research work because of its enormous medicinal properties. Many studies have reported its anti-oxidant and anti-ulcer activities (Qader *et al.*, 2011; Wasman *et al.*, 2010). The plant is a rich source of miscellaneous minerals such as calcium, sodium and zinc, as well as some vitamins like vitamin C and E (Ching and Mohamed, 2001). It was proposed as a constituent of some nutraceutical and cosmeceutical products (Blanche *et al.*, 2014; Vimala *et al.*, 2011). In addition, (Shanmugam and Tan, 2004) suggested that it may increase

the blood circulation. Due to all of these previously mentioned properties, *P. minus* may have a good potential to improve the healing process of skin wounds efficiently as recommended by Qader *et al.* (2012b).

1.2 Problem statement

Non-healing chronic wounds represent a common irritating health problem especially to diabetic patients and those with vascular insufficiency. Despite of diversity of the available medications and treatment protocols, successful treatment of chronic wounds is unsatisfactory, particularly in case of chronic wounds with insufficient blood flow like diabetic or vascular ulcers. *P. minus* was found to have anti-oxidant properties, rich in some essential vitamins and minerals and moreover has anti-ulcer effects (Qader *et al.*, 2012a; Vimala *et al.*, 2012). Therefore, it may play a role in improving the healing capacity of skin cells and in the same time provide a safer, more natural and costly effective remediation.

1.3 Research objectives

The objectives of the present study are:

1. To evaluate the antioxidant capacity and total phenolic contents of different extracts of *P. minus* leaves.
2. To evaluate the viability of cultured human skin fibroblasts with *P. minus* extracts using MTT cytotoxicity assay.

3. To determine the ability of the plant extracts to enhance migration of cultured skin cells for closure of an induced wound using Scratch assay.

1.4 Scope of the study

This study was conducted to elucidate the healing capacity of *P. minus* leaves. The antioxidant capacity of different extracts of the plant leaves was evaluated using DPPH assay. Their total phenolic contents was measured as well. *P. minus* extracts were added to a monolayer of cultured fibroblasts to monitor their induced effects on the proliferation and cytotoxicity of the cells and to determine their maximum non-toxic doses. Finally, *P. minus* extracts were added to a confluent monolayer of fibroblasts after inducing a horizontal Scratch to the cells to observe their effect on cell migration for wound closure.

1.5 Significance of the study

Chronic non-healing wounds are characterized by high levels of free radicals especially diabetic ulcers when hyperglycaemia add to the oxidative stress by increasing the levels of reactive oxygen species (Woo *et al.*, 2007). *P. minus* is a widely available and well-known traditional herb among Southeast Asian countries. Its toxicity levels were studied and proven to be safe for human consumption (Ming *et al.*, 2013; Ibrahim *et al.*, 2010). *P. minus* has anti-oxidant and anti-ulcer effects in addition to its ability to provide several essential vitamins (Wasman *et al.*, 2010; Qader *et al.*, 2011). Thus, it may help in improving the healing capacity of skin cells in an efficient and safe way.

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