



Faculty of Medicine and Health Sciences

Wound Healing Effects of *Litsea garciae* Extracts: *In vitro* Study on Fibroblast Cells

Ling Zinyin

**Master of Science
2023**

Wound Healing Effects of *Litsea garciae* Extracts: *In vitro* Study on
Fibroblast Cells

Ling Zinyin

A thesis submitted

In fulfillment of the requirements for the degree of Master of Science

(Biochemistry)

Faculty of Medicine and Health Sciences
UNIVERSITI MALAYSIA SARAWAK

2023

DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.



.....

Signature

Name: Ling Zinyin

Matric No.: 20020045

Faculty of Medicine and Health Sciences

Universiti Malaysia Sarawak

Date: 1 / 2 / 2024

ACKNOWLEDGEMENT

First and foremost, I would like to express my deepest gratitude to my main supervisor, Associate Professor Dr. Zunika binti Amit, for giving me the opportunity to be her student to complete this research project. All the difficulties that I encountered throughout this study had been solved under her patient guidance. I would also like to convey my sincere appreciation to my co-supervisor, Prof. Dr. Gabriele Ruth Anisah Frömming for her guidance and suggestions throughout this research journey. Besides, I would also like to thank Tun Zaidi Chair grant (F05/TZC/1916/2019) of Universiti Malaysia Sarawak, which supported the financial on purchasing all the consumables and reagents for this project. Moreover, I would like to thank my parents for their financial and spiritual support that motivated me to keep going without giving up on this long journey in achieving my dream.

Furthermore, I would like to extend my gratefulness to all the lab officers and lecturers who assisted me till completion of my lab work. Finally, I would like to appreciate the equipment and facilities provided by the Biochemistry Laboratory, Medical and Molecular Genetics Laboratory, Pharmacology Laboratory and Multipurpose Laboratory of Faculty of Medicine and Health Sciences and Gas Chromatography - Mass Spectrometry (GC-MS) Laboratory of Faculty of Resource Science and Technology, Universiti Malaysia Sarawak. I would not have done my cell culture work smoothly without the facilities and assistance from the people that I have met in Universiti Malaysia Sarawak. Hereby I wish to express my profound gratitude all of you.

ABSTRACT

Wound healing is a complex physiological process that involves regeneration of new cells and substitution of damaged cells after the onset of lesion. Wound healing involves the collaboration of many cell types and categorised into four overlapping main phases: haemostasis, inflammation, proliferation and remodelling. Although various wound healing treatments are currently available, the long-term usage of the medications has caused some adverse effects. To date, many traditional medicinal plants have been studied and widely used for various diseases due to their high efficacy, safety, and low cost. *Litsea garciae* (*L. garciae*) has many traditional medicinal uses and has been used by the indigenous people of Borneo Island to treat various diseases. However, its wound healing potential in skin wound therapy has not yet been investigated. Therefore, this study aimed to investigate whether the lipid and methanolic extracts of *Litsea garciae* could facilitate the process of *in vitro* wound healing and has a beneficial effect on the proliferation and migration of fibroblasts during *in vitro* wound healing. Total lipids were extracted using two different solvents: petroleum ether (PE) and Bligh and Dyer (BD). In addition, the crude methanolic extracts were prepared using 80% methanol. Firstly, the fatty acids composition of the lipid pulp and seed extracts of *L. garciae* were analysed using gas chromatography-mass spectrometry (GC-MS). The antimicrobial activities of the *L. garciae* lipid and methanolic pulp and seed extracts were evaluated through disk diffusion and broth microdilution assays. For the investigation of the potential wound healing properties of the *L. garciae* extracts, an *in vitro* study on the migration and chemotactic motility of the human dermal fibroblasts were conducted using wound scratch assay and transwell assay respectively. The effects of *L. garciae* extracts on the stimulation of the collagen type I, epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) expression levels were evaluated using

enzyme-linked immunosorbent assays (ELISA). This study showed that the prominent fatty acids in the *L. garciae* lipid seed extracts were lauric acid, palmitic acid, oleic acid, and linoleic acid. While for the *L. garciae* lipid pulp extracts, the predominant fatty acids were palmitic acid, stearic acid, oleic acid, and linoleic acid. All the *L. garciae* extracts exhibited mild antibacterial activities against the Gram-positive bacteria while Gram-negative bacteria exerted a weaker susceptibility towards the *L. garciae* extracts. The lipid pulp extracts were found to exhibit a stronger antimicrobial activity than the methanolic pulp extract while methanolic seed extract showed higher inhibition effect as compared to the lipid seed extracts. As for the wound healing property investigations, there were enhancement in the migration and chemotactic motility of fibroblasts treated with all the *L. garciae* pulp and seed extracts, but not with the methanolic seed extract. The expression levels of the collagen type I, EGF and VEGF by the wounded fibroblasts were ameliorated in the presence of the *L. garciae* extracts. These findings validated the ability of *L. garciae* extracts in promoting the wound healing process, which might be attributed to the presence of phytochemicals and fatty acids in the extracts. This study suggested that the *L. garciae* pulp and seed extracts might be a potent medication for skin wound healing.

Keywords: Wound healing, *Litsea garciae*, fibroblast, fatty acids, antimicrobial

Kesan-kesan Penyembuhan Luka bagi Ekstrak Litsea garciae: Kajian in vitro pada Sel Fibroblas

ABSTRAK

Penyembuhan luka merupakan proses fisiologi yang kompleks di mana ia melibatkan regenerasi sel-sel baru dan penggantian sel-sel yang rosak selepas kelukaan. Penyembuhan luka melibatkan kerjasama banyak jenis sel dan dikategorikan kepada empat tahap utama yang bertindih: hemostasis, inflamasi, proliferasi, dan pembentukan semula. Walaupun kini terdapat pelbagai cara bagi rawatan luka, tetapi penggunaan ubat-ubatan dalam jangka masa yang lama telah membawa kesan sampingan. Pada masa ini, banyak tumbuh-tumbuhan yang digunakan dalam perubatan tradisional telah dikaji dan digunakan secara meluas untuk merawat pelbagai penyakit disebabkan oleh keberkesanannya yang tinggi, keselamatan dan kos yang rendah. Litsea garciae (L. garciae) mempunyai banyak kegunaan dalam perubatan tradisional telah digunakan oleh bumiputera di Pulau Borneo untuk merawat pelbagai penyakit. Walau bagaimanapun, potensi tumbuhan ini dalam penyembuhan luka belum lagi dikaji. Oleh itu, kajian ini bertujuan untuk menyelidik sama ada ekstrak lipid dan metanol L. garciae dapat memudahkan proses penyembuhan luka dan mempunyai kesan yang bermanfaat terhadap proliferasi dan migrasi fibroblas dalam penyembuhan luka in vitro. Lipid diekstrak dengan menggunakan dua pelarut yang berbeza: eter petroleum (PE) dan “Bligh and Dyer” (BD). Selain itu, ekstrak metanol mentah disediakan dengan menggunakan 80% metanol. Pertama, komposisi asid lemak bagi ekstrak isi dan biji L. garciae yang diekstrak dengan PE dan kaedah BD telah dianalisa dengan menggunakan kromatografi gas-spektrometri jisim (GC-MS). Aktiviti antibakteria bagi ekstrak lemak dan metanolik L. garciae telah dinilai melalui ujian difusi cakera dan ujian mikrodilusi larutan. Bagi penyiasatan potensi ekstrak L. garciae atas sifat

penyembuhan luka, kajian *in vitro* pada migrasi dan motiliti kemotaktik bagi fibroblas kulit manusia telah dijalankan dengan menggunakan ujian goresan luka dan ujian “transwell” masing-masing. Kesan-kesan ekstrak *L. garciae* pada peningkatan tahap ekspresi kolagen jenis I, faktor pertumbuhan epidermis (EGF) dan faktor pertumbuhan endothelial vaskular (VEGF) telah dinilai melalui esei imunoserapan berkaitan enzim (ELISA). Hasil daripada penyelidikan ini mendapati bahawa asid lemak yang menonjol dalam ekstrak biji lipid *L. garciae* ialah asid laurik, asid palmitik, asid oleik dan asid linoleik. Manakala untuk ekstrak isi lipid *L. garciae*, asid lemak yang dominan ialah asid palmitik, asid stearik, asid oleik dan asid linoleik. Semua ekstrak *L. garciae* menunjukkan aktiviti antibakteria yang sederhana terhadap bakteria Gram-positif manakala bakteria Gram-negatif menunjukkan kerentanan yang lebih lemah terhadap ekstrak *L. garciae*. Ekstrak isi didapati mempunyai aktiviti antibakteria yang lebih kuat daripada ekstrak isi metanolik manakala ekstrak biji metanolik menunjukkan kesan perencatan yang lebih tinggi berbanding dengan ekstrak biji lipid. Bagi penyiasatan sifat penyembuhan luka, terdapat peningkatan dalam migrasi dan motiliti kemotaktik untuk fibroblas yang dirawat dengan semua ekstrak isi dan biji *L. garciae*, kecuali ekstrak biji metanolik. Tahap ekspresi kolagen jenis I, EGF dan VEGF yang dihasilkan oleh fibroblas telah meningkat apabila dirawat dengan ekstrak *L. garciae*. Dapatan kajian ini mengesahkan keupayaan ekstrak *L. garciae* dalam meningkatkan proses penyembuhan luka. Hal ini mungkin disebabkan oleh kewujudan fitokimia dan asid lemak dalam ekstrak tersebut. Kajian ini mencadangkan potensi ekstrak isi dan biji *L. garciae* sebagai ubat yang berkesan untuk penyembuhan luka kulit.

Kata kunci: Penyembuhan luka, *Litsea garciae*, fibroblas, asid lemak, antibakteria

TABLE OF CONTENTS

	Page
DECLARATION	i
ACKNOWLEDGEMENT	ii
ABSTRACT	iii
<i>ABSTRAK</i>	v
TABLE OF CONTENTS	vii
LIST OF TABLES	xii
LIST OF FIGURES	xiv
LIST OF ABBREVIATIONS	xviii
CHAPTER 1 INTRODUCTION	1
1.1 Background of Study	1
1.2 Problem Statement	2
1.3 Objectives of Research	4
CHAPTER 2 LITERATURE REVIEW	5
2.1 Structure of Skin	5
2.1.1 Wounds and Types of Wounds	6
2.1.2 Mechanism of Wound Healing	8
2.1.3 Wound Healing Treatment	14

2.1.4	Medicinal Plants with Wound Healing Properties	17
2.2	<i>Litsea garciae</i>	22
2.2.1	Geographical Distribution	23
2.2.2	Botany and Morphology	24
2.2.3	Applications	25
2.2.4	Chemical and Nutritional Compositions	27
2.2.5	Pharmacological Properties	35
2.3	Other Indigenous Plants in Sarawak	41
	CHAPTER 3 MATERIALS AND METHODS	43
3.1	Chemicals and Reagents	43
3.2	Equipment	45
3.3	<i>Litsea garciae</i>	46
3.3.1	<i>Litsea garciae</i> Fruits Collection	46
3.3.2	Methanolic Extraction of ground Pulp and Seed of <i>Litsea garciae</i>	48
3.3.3	Total Lipid Extraction of <i>Litsea garciae</i>	49
3.3.4	Collection of <i>Litsea garciae</i> Pulp and Seed Extracts by Rotary Evaporation	51
3.4	Fatty Acid Analysis	52
3.5	Antimicrobial Assay	54
3.5.1	Preparation of Mueller-Hinton, Nutrient and Blood Agar Medium and Nutrient Broth	54

3.5.2	Isolation of <i>S. aureus</i> , <i>S. epidermidis</i> , <i>E. coli</i> and <i>P. aeruginosa</i> Pure Culture	55
3.5.3	Preparation of Inoculum using Growth Method	56
3.5.4	Preparation of Diluted Sample	57
3.5.5	Disk Diffusion Assay	57
3.5.6	Broth Microdilution Assay	60
3.6	Cell Culture	62
3.6.1	Preparation for Cell Culture Work	62
3.6.2	Storage of Human Dermal Fibroblasts (HDF)	62
3.6.3	Human Dermal Fibroblasts (HDF) Culture	63
3.7	Cell Viability Analysis by MTT Assay	69
3.7.1	Solutions for MTT Assay	69
3.7.2	Cytotoxicity Tests for Extracts, Allantoin and DMSO	69
3.8	Investigation on the Wound Healing Potency of <i>Litsea garciae</i> Extracts	71
3.8.1	Wound Scratch Assay	71
3.8.2	Transwell Assay	72
3.8.3	Collagen 1 ELISA	75
3.8.4	Secretion of EGF	77
3.8.5	Secretion of VEGF	79
3.9	Statistical Analysis	81
	CHAPTER 4 RESULTS	82

4.1	Extraction of Samples	82
4.2	Fatty Acids Content	85
4.3	Antimicrobial Activities	95
4.3.1	Diameter of Zone of Inhibition	95
4.3.2	Minimum Inhibitory Concentration (MIC)	104
4.4	Cell Culture	110
4.5	Cell Viability	111
4.5.1	Effects of DMSO and allantoin on fibroblasts viability incubated in DMEM containing 10% FBS	111
4.5.2	Effects of <i>L. garciae</i> Extracts on Fibroblasts Viability	113
4.6	Wound Healing Properties of <i>L. garciae</i> Extracts	117
4.6.1	Effects of <i>L. garciae</i> Extracts on Fibroblast Cells Migration	117
4.6.2	Effects of <i>L. garciae</i> Extracts on Fibroblasts' Chemotactic Motility	130
4.6.3	Effects of <i>L. garciae</i> Extracts on Wound Healing Markers	136
CHAPTER 5 DISCUSSION		144
5.1	Extraction Yield of Pulp and Seed of <i>L. garciae</i>	144
5.2	Characterisation of Fatty Acids Extracted from Pulp and Seed of <i>L. garciae</i>	146
5.3	Antimicrobial Effects of Lipids Extracted from Pulp and Seed of <i>L. garciae</i>	151
5.4	Human Dermal Fibroblasts Cell Culture	157
5.5	Effects of Pulp and Seed Extracts of <i>L. garciae</i> on Human Dermal Fibroblasts Viability	159

5.6	Wound Healing Properties of Pulp and Seed Extracts of <i>L. garciae</i>	162
5.6.1	Effects of <i>L. garciae</i> Extracts on Fibroblast Cells Migration	162
5.6.2	Effects of <i>L. garciae</i> Extracts on Fibroblasts' Chemotactic Motility	164
5.6.3	Effect of <i>L. garciae</i> Extracts on Wound Healing Markers	167
5.7	Summary	170
	CHAPTER 6 CONCLUSION	175
6.1	Conclusion	175
6.2	Limitation of study and Future Work	176
	REFERENCES	179
	APPENDICES	217

LIST OF TABLES

	Page
Table 2.1 Growth factors involved in the wound healing process [Adapted from (Broughton et al., 2006; Enoch & Leaper, 2007)].	12
Table 2.2 Medicinal plants and their wound healing activities [Adapted from (Ning et al., 2022)].	20
Table 2.3 Nutrient contents of the <i>L. garciae</i> pulp/seed per 100 g pulp/seed portion.	28
Table 2.4 Phytochemical screening of <i>L. garciae</i> [Adapted from (Wulandari et al., 2018)].	32
Table 2.5 Total phenolic and flavonoid content of <i>L. garciae</i> [Adapted from (Wulandari et al., 2018)].	33
Table 2.6 The total phenolic, flavonoid and anthocyanin contents of different parts of <i>L. garciae</i> fruit extracts [Adapted from (Hassan et al., 2013)].	34
Table 2.7 The antioxidant properties of <i>L. garciae</i> fruit extracts from different parts using three different assays [Adapted from (Hassan et al., 2013)].	36
Table 2.8 Minimal Inhibitory Concentration (MIC) of <i>L. garciae</i> using antibacterial assay [Adapted from (Wulandari et al., 2018)].	38
Table 2.9 The percentage reduction of radial growth (mm) of <i>Colletotrichum gloeosporioides</i> (Cg) and <i>Colletotrichum capsici</i> (Cc) by varying concentrations of <i>L. garciae</i> leaf extracts in different solvents using antifungal assay [Adapted from (Johnny et al., 2010; Johnny et al., 2011)].	38
Table 2.10 Inhibitory concentration of barks and leaves methanolic extract on HeLa, MCF-7 and HT-29 [Adapted from Kutoi et al., (2012)].	39
Table 2.11 Lipxygenase (LO), Hyaluronidase (HO) and Xanthine Oxidase (XO) inhibitory activities [Adapted from Kutoi et al., (2012)].	40
Table 3.1 List of materials used for the experiments.	43
Table 3.2 List of equipment used for the experiments.	45
Table 4.1 Extraction yield of the pulp and seed extracts of <i>L. garciae</i> .	84
Table 4.2 Fatty acid composition of the pulp and seed extracts of <i>L. garciae</i> .	90

Table 4.3	Antimicrobial activities of the pulp and seed extracts of <i>L. garciae</i> against <i>Staphylococcus aureus</i> , <i>Staphylococcus epidermidis</i> , <i>Escherichia coli</i> and <i>Pseudomonas aeruginosa</i> by disk diffusion assay.	98
Table 4.4	Antimicrobial activities of the pulp and seed extracts of <i>L. garciae</i> against <i>Staphylococcus aureus</i> , <i>Staphylococcus epidermidis</i> , <i>Escherichia coli</i> and <i>Pseudomonas aeruginosa</i> by broth microdilution assay.	105

LIST OF FIGURES

	Page
Figure 2.1 The structure of skin [Adapted from (Voegeli, 2012)].	6
Figure 2.2 The wound healing stages involving cellular components [Adapted from (Wilkinson & Hardman, 2020)].	11
Figure 2.3 Transformation of the green, unripe <i>L. garciae</i> fruit into the red, ripe fruit.	22
Figure 2.4 Geographical distribution map of <i>L. garciae</i> [Adapted from (Google Maps, 2020)].	23
Figure 2.5 <i>L. garciae</i> tree bearing fruits (A) and flowers (B) [Adapted from (Bukbi, 2019)].	24
Figure 2.6 Different parts of the <i>L. garciae</i> fruit [Adapted from (Machabuca, 2008 and Herbs Encyclopedia, 2020)].	25
Figure 3.1 The sampling voucher of <i>Litsea garciae</i> (ZA-01).	47
Figure 3.2 Soxhlet (FAVORIT [®]) extraction apparatus used in this study. Picture was taken by the author in the Biochemistry laboratory of Faculty of Medicine and Health Sciences, UNIMAS.	49
Figure 3.3 Streaking pattern for pure culture isolation of bacteria using four-streak method.	56
Figure 3.4 Procedure of the disk diffusion assay (Tankeshwar, 2022).	59
Figure 3.5 Measurement of the zone of inhibition diameter (American Society for Microbiology, 2016).	59
Figure 3.6 Illustration for the broth microdilution assay [Adapted from (Starlab, 2021)].	61
Figure 3.7 Morphology of fibroblasts under an inverted microscope (Olympus IX73) at (a) 40x, (b) 100x, (c) 200x magnification.	64
Figure 3.8 Illustration for one of the corners of a hemacytometer.	67
Figure 3.9 Illustration for the transwell migration assay (ThermoFisher Scientific, 2021).	74
Figure 4.1 Optical appearance of PE, ME and BD extracts of the pulp and seed of <i>L. garciae</i> . Pictures were taken at room temperature (25°C) and at 40°C.	83

Figure 4.2	GC-MS chromatogram of (A) Petroleum ether and (B) Bligh and Dyer seed extracts of <i>L. garciae</i> .	88
Figure 4.3	GC-MS chromatogram of (C) Petroleum ether and (D) Bligh and Dyer pulp extracts of <i>L. garciae</i> .	89
Figure 4.4	Proportion (%) of saturated, monounsaturated and polyunsaturated fatty acids for (a) Petroleum ether and (b) Bligh and Dyer lipid pulp extracts of <i>L. garciae</i> .	91
Figure 4.5	Proportion (%) of saturated, monounsaturated and polyunsaturated fatty acids for (a) Petroleum ether and (b) Bligh and Dyer lipid seed extracts of <i>L. garciae</i> .	92
Figure 4.6	Proportion (%) of (a) saturated fatty acids, (b) monounsaturated fatty acids and (c) polyunsaturated fatty acids for petroleum ether and Bligh and Dyer lipid pulp and seed extracts of <i>L. garciae</i> .	94
Figure 4.7	Zone of inhibition of <i>L. garciae</i> pulp and seed extracts against <i>Staphylococcus aureus</i> .	100
Figure 4.8	Zone of inhibition of <i>L. garciae</i> pulp and seed extracts against <i>Staphylococcus epidermidis</i> .	101
Figure 4.9	Zone of inhibition of <i>L. garciae</i> pulp and seed extracts against <i>Escherichia coli</i> .	102
Figure 4.10	Zone of inhibition of <i>L. garciae</i> pulp and seed extracts against <i>Pseudomonas aeruginosa</i> .	103
Figure 4.11	Minimum Inhibitory Concentration (MIC) of <i>L. garciae</i> pulp and seed extracts against <i>Staphylococcus aureus</i> .	106
Figure 4.12	Minimum Inhibitory Concentration (MIC) of <i>L. garciae</i> pulp and seed extracts against <i>Staphylococcus epidermidis</i> .	107
Figure 4.13	Minimum Inhibitory Concentration (MIC) of <i>L. garciae</i> pulp and seed extracts against <i>Escherichia coli</i> .	108
Figure 4.14	Minimum Inhibitory Concentration (MIC) of <i>L. garciae</i> pulp and seed extracts against <i>Pseudomonas aeruginosa</i> .	109
Figure 4.15	Fibroblasts at different cell density were cultured in their growth media. For each cell density the mean absorptions (n=3) were calculated after 24 h of incubation.	110
Figure 4.16	Viability of fibroblasts treated with DMSO and allantoin. Fibroblasts at 2.2×10^4 cells/well in 200 μ L growth medium were incubated with (a) DMSO (0.05 - 10 %) and (b) Allantoin (50 - 500 μ g/mL) for 24 h. The cell viability was measured by MTT assay. The data were	

	presented as mean $\pm$ SD (n = 6). Significance was indicated with p<0.05 (*) by independent t-test.	112
Figure 4.17	Viability of fibroblasts treated with petroleum ether, methanolic and Bligh and Dyer pulp and seed extracts. Fibroblasts at 2.2×10^4 cells/well in 200 μ L growth medium were incubated with petroleum ether, methanolic and Bligh and Dyer (a) pulp and (b) seed extracts (31.25 - 500 μ g/mL) for 24 h. The cell viability was measured by MTT assay. The data were presented as mean $\pm$ SD (n = 6). Significance was indicated with p<0.05 (*) by independent t-test.	115
Figure 4.18	Viability of fibroblasts treated with petroleum ether and Bligh and Dyer pulp and seed extracts. Fibroblasts at 2.2×10^4 cells/well in 200 μ L growth medium were incubated with (a) petroleum ether pulp extracts (7.5 - 31.25 μ g/mL), (b) petroleum ether and Bligh and Dyer seed extracts (7.5 - 31.25 μ g/mL) for 24 h. The cell viability was measured by MTT assay. The data were presented as mean $\pm$ SD (n = 6). Significance was indicated with p<0.05 by independent t-test and there was no significant difference (p>0.05).	116
Figure 4.19	Migration of fibroblasts without treatment for 48h.	120
Figure 4.20	Migration of fibroblasts after treatment with 31.25 μ g/mL petroleum ether pulp extract for 48h.	121
Figure 4.21	Migration of fibroblasts after treatment with (a) petroleum ether (7.5 - 31.25 μ g/mL) and (b) Bligh and Dyer (31.25 - 125 μ g/mL) pulp extracts as compared with the negative control for 48h. The data were expressed as mean $\pm$ SD (n = 3) and were analysed using one-way ANOVA followed by Tukey's HSD test. There was no statistically significant difference (p>0.05) between all pulp extracts and negative control.	122
Figure 4.22	Migration of fibroblasts after treatment with methanolic pulp extracts (31.25 - 125 μ g/mL) as compared with the negative control for 48h. The data were expressed as mean $\pm$ SD (n = 3) and were analysed using one-way ANOVA followed by Tukey's HSD test. There was no statistically significant difference (p>0.05) between methanolic pulp extracts and negative control.	124
Figure 4.23	Migration of fibroblasts after treatment with (a) petroleum ether (7.5 - 31.25 μ g/mL), (b) Bligh and Dyer (7.5 - 31.25 μ g/mL) seed extracts as compared with the negative control for 48 h. The data were expressed as mean $\pm$ SD (n = 3) and were analysed using one-way ANOVA followed by Tukey's HSD test. There was a statistical significant difference (*p<0.05) between 31.25 μ g/mL Bligh and Dyer seed extract and negative control at 24 h while the other seed extracts were statistically insignificant different (p>0.05).	127

Figure 4.24	Migration of fibroblasts after treatment with methanolic seed extracts (31.25 – 125 µg/mL) as compared with the negative control for 48h. The data were expressed as mean ± SD (n = 3) and were analysed using one-way ANOVA followed by Tukey's HSD test. There was no statistically significant difference (p>0.05) between methanolic seed extracts and negative control.	128
Figure 4.25	Chemotactic motility of fibroblasts without treatment (growth media with and without FBS).	132
Figure 4.26	Transwell assay to detect the chemotactic motility of fibroblasts. Cells were treated with 31.25 µg/mL petroleum ether, Bligh and Dyer and methanolic pulp and seed extracts. The migrated cells on the lower chamber membrane were stained and counted. The number of cells were shown in the Figure 4.27.	134
Figure 4.27	Chemotactic motility of fibroblasts after treatment with petroleum ether, methanolic and Bligh and Dyer (a) pulp and (b) seed extracts (31.25 µg/mL) compared with the negative controls after 24 h incubation. The data were expressed as mean ± SD (n = 3) and were analysed using one-way ANOVA followed by Tukey's HSD test. The statistical significant difference (p<0.05) between extracts and negative controls (No FBS and 10% FBS) were represented by (*) and (#) respectively.	135
Figure 4.28	The effect of petroleum ether, Bligh and Dyer and methanolic (a) pulp and (b) seed extracts on the expression of collagen I by wounded fibroblasts. The data were expressed as mean ± SD (n = 3) and were analysed using one-way ANOVA followed by Tukey's HSD test. The statistical significant difference (p<0.05) between pulp and seed extracts and negative control were represented by (*).	138
Figure 4.29	The effect of petroleum ether, Bligh and Dyer and methanolic (a) pulp and (b) seed extracts on the secretion of EGF by wounded fibroblasts. The data were expressed as mean ± SD (n = 3) and were analysed using one-way ANOVA followed by Tukey's HSD test. There was no statistical significant difference (p>0.05) between all pulp and seed extracts and negative control.	141
Figure 4.30	The effect of petroleum ether, Bligh and Dyer and methanolic (a) pulp and (b) seed extracts on the secretion of VEGF by wounded fibroblasts. The data were expressed as mean ± SD (n = 2) and were analysed using independent t-test. The statistical significant difference (p<0.05) between pulp extracts and negative control were represented by (*) while seed extracts and negative control were statistically insignificant different (p>0.05).	143

LIST OF ABBREVIATIONS

AAP	<i>Auricularia auricula-judae</i>
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid
AEAC	Ascorbic acid equivalent antioxidant capacity
ALA	α -linolenic acid
ANOVA	Analysis of Variance
BD	Bligh and Dyer
BDPE	Bligh and Dyer pulp extract
BDSE	Bligh and Dyer seed extract
bFGF	Basic fibroblast growth factor
BHT	Butylated hydroxytoluene
BSC	Biological safety cabinet
CH ₃ Cl	Chloroform
CHD	Coronary heart disease
CLSI	Clinical and Laboratory Standards Institute
CO ₂	Carbon dioxide
CTGF	Connective tissue growth factor
DCF	<i>Digitaria ciliaris</i> flower
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
DPPH	2,2-Diphenyl-1-picryl-hydrazyl-hydrate
<i>E. coli</i>	<i>Escherichia coli</i>

ECM	Extracellular matrix
EFA	Essential fatty acids
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
Erk	Extracellular signal-regulated kinase
FabI	Enoyl-acyl carrier protein reductase
FAMEs	Fatty acid methyl esters
FBS	Foetal bovine serum
FD	Freeze-drying
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FRAP	Ferric reducing/antioxidant power
GC-MS	Gas chromatography-mass spectrometry
GML	Glycerol monolaurate
H ₂ O	Water
HCl	Hydrochloric acid
HDF	Human dermal fibroblasts
HO	Hyaluronidase
HRP	Horseradish peroxidase
HSD	Honestly Significant Different
IC ₅₀	Half maximal inhibitory concentration
IL-1	Interleukin-1
KGF	Keratinocyte growth factor
<i>L. garciae</i>	<i>Litsea garciae</i>
LA	Linoleic acid

LCFAs	Long-chain fatty acids
LC-PUFA	Long chain polyunsaturated fatty acids
LC-SFA	Long-chain saturated fatty acids
LDL	Low-density lipoprotein
LNO	<i>Lucuma</i> nut oil
LO	Lipoxygenase
MAPK	Mitogen-activated protein kinase
MCFAs	Medium-chain fatty acids
ME	Methanolic
MeOH	Methanol
MEPE	Methanolic pulp extract
MESE	Methanolic seed extract
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant <i>S. aureus</i>
MSC	Mesenchymal stem cells
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUFAs	Monounsaturated fatty acids
NaOH	Sodium hydroxide
NIST	National Institute of Standards and Technology
NO	Nitric oxide
NSAIDs	Nonsteroidal anti-inflammatory drugs
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
PDGF	Platelet-derived growth factor

PE	Petroleum ether
PEPE	Petroleum ether pulp extract
PESE	Petroleum ether seed extract
PFA	Paraformaldehyde
PUFAs	Polyunsaturated fatty acids
RDFP	Red dragon fruit peel
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<i>S. epidermidis</i>	<i>Staphylococcus epidermidis</i>
SCFAs	Short-chain fatty acids
SD	Standard deviation
SFAs	Saturated fatty acids
SFM	Serum-free medium
SHSD	Superheated-steam drying
SPP	Saudi pomegranate peel
SPSS	Statistical Package for the Social Science
TFA	Trifluoroacetic acid
TGF	Transforming growth factor
TMB	3,3' ,5,5' -Tetramethylbenzidine
TNF	Tumour necrosis factor
UNIMAS	Universiti Malaysia Sarawak
v/v	Volume/volume
VEGF	Vascular endothelial growth factor
VLCFAs	Very long chain fatty acids
XO	Xanthine oxidase

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Skin is a protective barrier against various pathogens and when this barrier becomes compromised, the body responds by initiating a sequential repair process known as wound healing. Wound healing is a complex and highly regulated biological process to replace and reestablishes impaired tissues. The normal wound healing event can be disrupted by some co-morbidities such as diabetes, obesity and malnutrition (Anderson & Hamm, 2014; Han & Ceilley, 2017). This may result in chronic wounds which brings a burden to the patients and health care system due to the high costing of chronic wound treatment.

Although there are a lot of wound healing treatments and medication options already available for wound healing, there is still a necessity to research new efficacious medicine, especially using a medicinal plant. The medications commonly used in wound care include antibiotics and nonsteroidal anti-inflammatory drugs (NSAIDs). However, there are adverse effects attributed to the long-term usage of antibiotics and NSAIDs. The popularity of researching medicinal plants could be due to the perception that plants will cause minimal unwanted side effects and are less costly and more efficacious.

Litsea garciae (*L. garciae*) is known locally as engkala. Previous studies showed that the bark, leaf and fruit of *L. garciae* possessed antioxidant, antimicrobial, antifungal, anti-inflammatory, and anticancer properties. In addition, *L. garciae* also contained various phytochemical components such as alkaloids, butenolide, terpenes, flavonoids, amides, lignans, steroids and fatty acids. The indigenous people in Sarawak claimed that the leaves,

shoots and barks of *L. garciae* had wound healing effects where these plant parts were used as wound healing dressings. However, no data was available on the wound healing potential of *L. garciae* fruit (pulp and seed).

Previous studies had revealed the potential role of lipids and methanolic extracts as treatment for the wound healing. Previous studies showed that plant lipids have anti-inflammatory, antimicrobial effects and are important modulators of the cell-cell interaction and intracellular signalling pathways which could contribute to wound healing process. The plant methanolic extracts also contained phytoconstituents that play a possible role in wound repair. Therefore, in current study, the potential wound healing activities of lipids and methanolic extracts from *L. garciae* pulp and seed extracts were evaluated via *in vitro* studies using the cell line human dermal fibroblasts to mimic the cells that were present in the dermis. The antimicrobial properties of the lipids and methanolic extracts were also investigated which would probably add on the wound healing activities of *L. garciae*. It was hypothesised that the lipids and methanolic extracts from pulp and seed extracts of *L. garciae* contribute to their wound healing and antibacterial properties.

1.2 Problem Statement

A rapid wound healing process is desirable as prolonged wound healing will increase the risk of wound infection. Open wounds prompt to encounter various foreign damaging compounds. There is a risk of the entry of microbes into the wounds, where the microbes may originate naturally from the skin (natural microflora) and from the external environment that may cause an infection. This issue is prevalent in clinical settings which has significant impacts on the patients and increases the cost of treatment that will bring a burden to the patients and the country's economy. The growing popularity of synthetic drugs in recent

years such as antibiotics and nonsteroidal anti-inflammatory drugs (NSAIDs) have been extensively used as wound healing medications. However, it has been reported that antibiotics and NSAIDs might cause some adverse effects in long-term treatment. Krischak et al. (2007) suggested that NSAIDs could cause a non-selective anti-proliferative effect on fibroblasts and reduce the biosynthesis of collagen. The antibiotics may lead to the development of bacterial resistance, which reduces the effectiveness of the antibiotics to an individual. Therefore, the use of natural sources such as medicinal plants has gained the interest of researchers to evaluate the potential therapeutic effects.

It was known that the antimicrobial activities of the medicinal plants were found to contribute to their wound healing properties. Since *L. garciae* has been used as a traditional wound healing treatment, hence it is a promising medicinal plant to be studied as an alternative treatment. The perception that the use of medicinal plants causes a very minimum adverse side effects as compared to the modern drugs (Krischak et al., 2007) had gained the interest of researchers to evaluate their potential therapeutic effects. *L. garciae* fruit was rich with nutrients and phytochemicals. However, its potential in wound healing has not been explored. Therefore, the potential of *L. garciae* fruit extracts as an alternative treatment for wound healing was explored in this study by using the fibroblasts cell line as the skin model. The findings revealed valuable information on the medicinal use of local fruit other than as a source of food. Hence, the commercial value of *L. garciae* fruit might be boosted and might contribute to Sarawak's economy.

1.3 Objectives of Research

The general objective of the current study was to evaluate the potential wound healing effects of *L. garciae* extracts by using fibroblasts cell line as the skin model.

The specific objectives of this research:

- i. To evaluate the fatty acids composition of petroleum ether and Bligh and Dyer pulp and seed extracts of *L. garciae* fruit.
- ii. To determine the antimicrobial properties of methanolic, petroleum ether and Bligh and Dyer pulp and seed extracts of *L. garciae* fruit.
- iii. To investigate the potential influence of methanolic, petroleum ether and Bligh and Dyer pulp and seed extracts of *L. garciae* on wound healing specifically the
 - a. effects on migration of fibroblasts.
 - b. effects on chemotactic motility of fibroblasts.
 - c. ability to stimulate the secretion of collagen type I, epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) by fibroblasts.

CHAPTER 2

LITERATURE REVIEW

2.1 Structure of Skin

Human skin composes three layers which are the epidermis (outermost layer), dermis (middle layer) and hypodermis (fatty layer). The epidermis comprises keratinised, stratified squamous epithelium which is called keratinocytes (Martin, 1997), that act as a protective barrier against harmful foreign invaders such as microorganisms from entering our body (Muhammad et al., 2013). There are five layers of epidermis, which are stratum basale (deepest layer), stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum (uppermost layer) as illustrated in Figure 2.1. Besides keratinocytes, epidermis also consists of melanocytes that synthesise melanin for skin pigmentation and protect against the UV radiation, and Langerhans cells, which act as the antigen-presenting cells and serve as the first line defence of the skin (Elder et al., 2009).

The dermis plays a crucial role that is responsible for the main functions of the skin, which provides the regulation of body temperature, defence mechanism, water retention and protective layer against mechanical injury. It contains extracellular matrix (ECM) with the abundance of proteins such as collagen and elastin. Collagen provides the tensile strength of the dermis while elastin gives elasticity to the skin. Also, the dermis contains fibroblasts, mast cells, macrophages and Langerhans cells that display immune response to combat infection (Voegeli, 2012). For the hypodermis, which is underneath the dermis, it is predominated with adipose tissue for the lipid's storage and functions as the thermal insulator and protective layer against an external force.

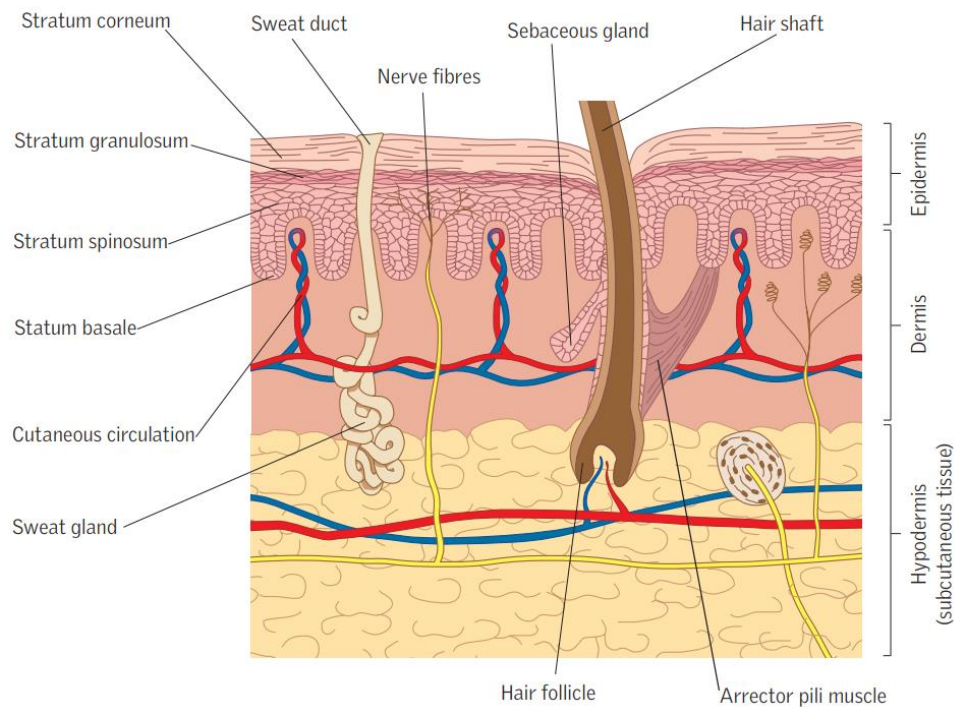


Figure 2.1: The structure of skin [Adapted from (Voegeli, 2012)].

2.1.1 Wounds and Types of Wounds

Definition of a wound is the disruption of biological tissue structural integrity and protective function caused by mechanical, chemical, and thermal injuries, which lead to the loss of functional continuity of epithelium (Firdous & Sautya, 2018; Nagori & Solanki, 2011). In addition, the wounds can be categorised into three main groups, which are mechanical injuries, burns and chemical injuries and chronic wounds. The mechanical injuries include abrasions, lacerations, skin tears, cuts, penetrating wounds, and bites. Abrasions, lacerations, and skin tears are caused by the friction force of a blunt object that strips off the skin epidermal layer. The cut, penetrating wounds and bites lead to deeper wounds, which are due to sharp objects and may cause the risk of infection. Besides, chemical injuries are caused by radiation while burns cause damage to the skin layers, which depend on the degree of burns (Black Country Healthcare, 2016).

The wound can be classified into acute or chronic depending on the duration of wound healing. The acute wounds will be healed within the predicted time without any complications while the repairing of chronic wounds may be halted at the inflammation phase that prolongs the wound healing time, which will cause some complications (Tipple, 2022). Additionally, wounds can be either open or closed wounds. A closed wound such as a bruise is an injury that impairs the underlying tissues without breaking the skin surface. In contrast, the open wound breaks the skin surface and may impair the underlying tissue. Abrasions, lacerations, punctures, burns and avulsions are some types of open wounds (Gonzalez, 2018).

Since the skin serves as a primary defence barrier, disruption of the protective barrier caused by wounds will initiate the first line of defence (innate immune response) that gives protection against the invasion of pathogens (Wilkinson & Hardman, 2020). Upon disruption of the epithelium, a rapid mechanism known as wound healing response with a cascade of events restores the damaged tissue to the normal anatomic structure and function (Lazarus et al., 1994). However, an individual with a weakened immune system is prompt to wound infection, in which the microbes deposit and multiply in the wound site. The causes of wound infection are due to the entry of microbes into the wounds through direct contact, airborne dispersal, or self-contamination. The direct contact of the hands of medical practitioner or surgical equipment and the airborne dispersal that refers to the contaminated air in the surrounding can result in the wound infection. While self-contamination is because one's endogenous flora which is originated from the skin or gastrointestinal tract, migrate to the wound site (Ngan, 2005).

2.1.2 Mechanism of Wound Healing

There is an involvement of a large amount of growth factors and different cell types, which include platelets, neutrophils, macrophages, lymphocytes, fibroblasts, keratinocytes, and endothelial cells at various phases of the wound healing process. Therefore, this process is influenced by multiple factors (local and systemic factors) which cause impact on distinct phases of the wound repair. The local factors are the factors affecting the wound site such as oxygenation, infection, and foreign body. Whereas systemic factors refer to the physical health condition of an individual that affects the wound healing ability (Broughton et al., 2006; Guo & DiPietro, 2010). For instance, one's age and gender, diseases, nutrition, sex hormones and stress.

Wound healing involves complex biological events that is categorised into four main phases which include haemostasis, inflammation, proliferation, and remodelling (Figure 2.2). The haemostasis phase takes place immediately following wounding and extravasation of blood to the wound site. Haemostasis involves vasoconstriction, primary haemostasis, and secondary haemostasis. Once the skin is wounded, the vasoconstriction of blood vessel walls resolves the bleeding. Next, primary haemostasis includes the accumulation of platelets and formation of platelet plug at the wound site while secondary haemostasis involves the initiation of coagulation cascade (Rodrigues et al., 2019). These events are vital to restrict blood loss (Enoch & Leaper, 2007; Wilkinson & Hardman, 2020). The platelets in the plug are activated by thrombin to degranulate and release alpha granules that act as a reservoir for the cytokines and growth factors secretion. Examples of growth factors include transforming growth factor (TGF)- β , platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF) and etc as demonstrated in Table 2.1 (Guo & DiPietro, 2010; Wilkinson & Hardman, 2020). These growth factors stimulate the

fibroblasts, macrophages, and endothelial cells and subsequently initiate the inflammatory response (Enoch & Leaper, 2007; Golebiewska & Poole, 2014). The activation of coagulation cascade transforms the soluble fibrinogen into insoluble fibrin and the combination of fibrin and platelet plug forms the thrombus. The thrombus stops the bleeding and serves as a provisional matrix for other cells in the inflammation phase (Rodrigues et al., 2019).

During the inflammation phase (day 1-3 after injury), chemotaxis occurs where there is infiltration of neutrophils and monocytes in which a few chemoattractants attracted them to the wound site. Monocytes are activated and differentiated into macrophages once they are recruited to the wound site (Enoch & Leaper, 2007; Guo & DiPietro, 2010). Neutrophils are a type of leukocytes which engulf, kill, and degrade the invading microbes by lysosomal enzymes via phagocytosis in order to prevent the infection caused by the bacterial contamination of the wound (Campos et al., 2008; Enoch & Leaper, 2007). Macrophages clear the apoptotic neutrophils and act as the main inflammatory regulator at the later stages of inflammation (Wilkinson & Hardman, 2020). Macrophages release enzymes such as collagenases for wound debridement, cytokines for instance, interleukins and tumour necrosis factor (TNF) for fibroblasts and angiogenesis stimulation, and TGF for keratinocytes stimulation, which are the growth factors to promote the proliferative phase (Campos et al., 2008; Enoch & Leaper, 2007).

The proliferative phase of wound healing usually commences after wounding (Campos et al., 2008), which is characterised by epithelialisation, angiogenesis, formation of granulation tissue, and collagen deposition. PDGF and TGF- β activate the migration of fibroblasts and endothelial cells to the lesion site, which play a prominent role in the

proliferative phase (Guo & DiPietro, 2010). The proliferation of fibroblasts is activated by PDGF while TGF- β stimulates the fibroblasts to synthesise collagen type I. The TGF- β also differentiates fibroblasts into myofibroblasts that are responsible for the wound contraction (Campos et al., 2008). The synthesis of glycosaminoglycans, proteoglycans, and collagen by fibroblasts within the wound aids in the construction of the new extracellular matrix (ECM) (Enoch & Leaper, 2007). During the formation of granulation tissue, angiogenesis occurs where new blood vessels are formed from pre-existing vessels. The pink, soft and granular appearance of granulation tissue is made up of fibroblasts, inflammatory cells, ECM, collagen fibres, blood vessels, proteoglycans, elastin, and hyaluronic acid, which acts as a scaffold for enhancement of wound healing via differentiation of endothelial and epithelial cells and angiogenesis (Enoch & Leaper, 2007; Wilkinson & Hardman, 2020).

The last phase of wound healing process, which is remodelling phase involves remodelling and scar maturation stage. This is featured by the collagen deposition in a well-organised manner starting from the first week to a prolonged period (Broughton et al., 2006). The ECM remodelling which approaches the steady state resembles the architecture and integrity of normal unwounded skin. It involves continuous generation and degradation of collagen (Guo & DiPietro, 2010). In granulation tissue, collagen fibres are thinner than the collagen fibres in unwounded skin, and they are oriented parallel to wounded skin (Campos et al., 2008). In normal unwounded skin, the collagen composition is made up of nearly 80 - 90% collagen type I and 10 - 20% collagen type III. While in granulation tissue of wounded skin, the approximate collagen composition changes to 30% collagen type III, and declines to 10% in the mature scar. A bundle of thin collagen fibres is eventually integrated into thicker collagen bundles by fibroblasts and collagen type I is organised along the stress line of the wound. Collagen type I replaces collagen type III progressively during the wound

healing process with the wound tensile strength escalation (Campos et al., 2008; Wilkinson & Hardman, 2020; Witte & Barbul, 1997). The remodelling of collagen fibres in the scar tissue takes up to one year. However, the organisation of collagen fibres and regained tensile strength will never approach normal as in intact skin (Diegelmann & Evans, 2004). Despite the collagen remodelling process, the degradation of collagen also takes place which is achieved by the specific collagenase enzymes, metalloproteinases. The metalloproteinases are synthesised by macrophages, fibroblasts, keratinocytes, and endothelial cells for the mediation of collagen cleavage into fragments (Enoch & Leaper, 2007).

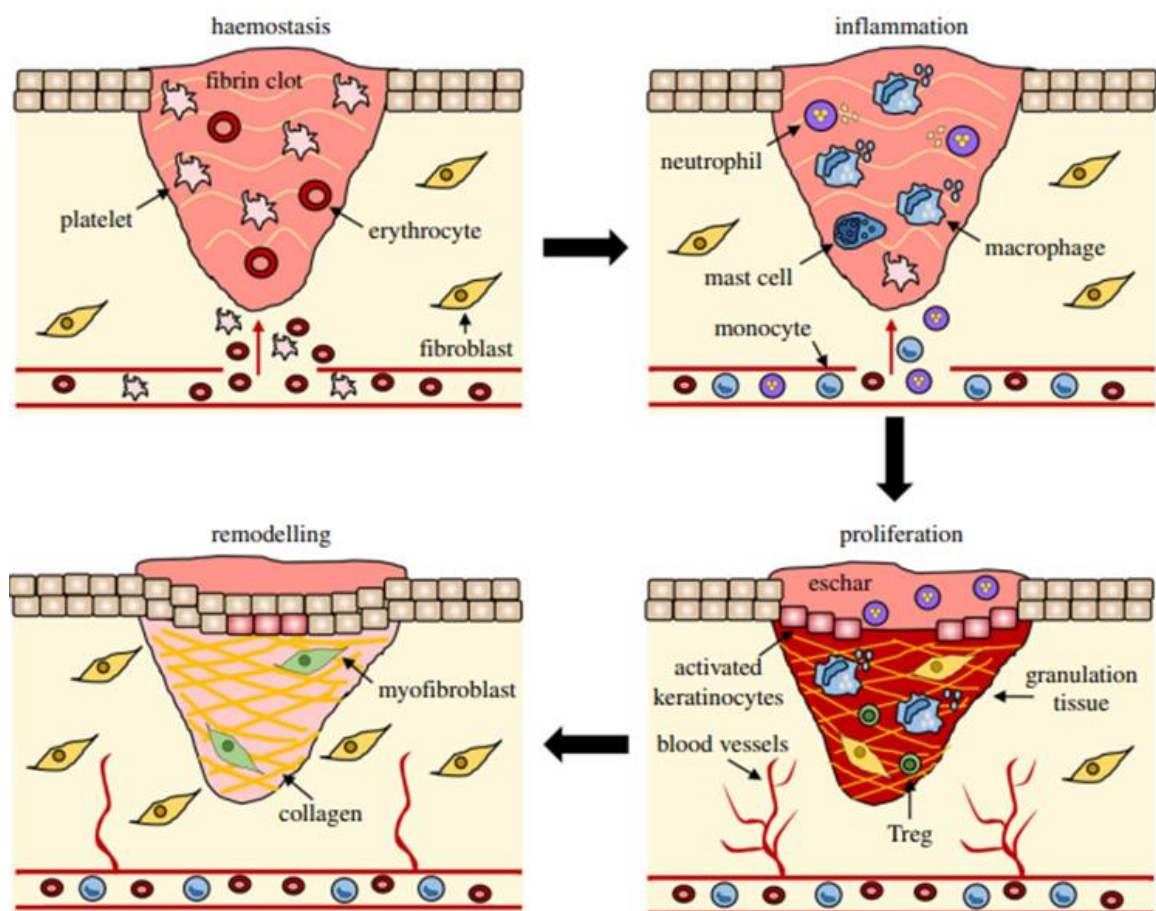


Figure 2.2: The wound healing stages involving cellular components [Adapted from (Wilkinson & Hardman, 2020)].

Table 2.1: Growth factors involved in the wound healing process [Adapted from (Broughton et al., 2006; Enoch & Leaper, 2007)].

Growth factor	Source	Function
EGF	Platelets, macrophages, keratinocytes	Stimulates differentiation, proliferation, migration and adhesion of keratinocytes
FGF	Fibroblasts, mast cells, endothelial cells, macrophages	Chemotactic and mitogenic for fibroblasts and keratinocytes; stimulates angiogenesis
IGF-1	Fibroblasts, plasma, liver	Fibroblast proliferation; stimulates the synthesis of collagen and sulphated proteoglycans
IL-1	Macrophages, lymphocytes, mast cells, keratinocytes	Fibroblast proliferation; neutrophil chemotaxis
KGF	Fibroblasts	Stimulates proliferation, migration and differentiation of keratinocytes
PDGF	Platelets, fibroblasts, endothelial cells, macrophages	Mitogenic for fibroblasts and endothelial cells; chemoattractant for neutrophils and fibroblasts; fibroblast proliferation and collagen metabolism
TGF- α	Activated macrophages, platelets, epithelial cells	Stimulates epithelial cells and fibroblasts proliferation; mitogenic for keratinocytes and

Table 2.1 continued

		fibroblasts; formation of granulation tissue
TGF- β	Platelets, macrophages, fibroblasts, keratinocytes, neutrophils	Mitogenic for fibroblasts and smooth muscle cells; chemotactic for macrophages; stimulates angiogenesis and collagen metabolism
TNF	Macrophages, mast cells, T-lymphocytes	Fibroblast proliferation; activates macrophages, stimulates angiogenesis
VEGF	Endothelial cells, fibroblasts, platelets, macrophages	Stimulates epithelialisation and production of collagen; stimulates angiogenesis

EGF, epidermal growth factor; FGF, fibroblast growth factor; IL-1, Interleukin-1; KGF, keratinocyte growth factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor.

2.1.3 Wound Healing Treatment

Most of the wounds can be healed naturally with time, but the proper treatment of wound will shorten the time of wound healing, which may avoid developing into chronic wound with various complications. The ways of wound management depend on the wound type, condition, and age, where condition refers to clean or infected wound while age indicates acute or chronic wound (Krahwinkel & Boothe, 2006).

For open wound, the inexpensive dry gauze wound dressing is still commonly used in medical field, however, frequent changing of the gauze may lead to an additional injury on the wound site (Han & Ceilley, 2017). There are many latest developments in wound dressings to function as a protective barrier to shield the wound from infection and speed up the wound healing process. For instance, incorporation of antimicrobial compounds into the traditional wound dressings (Percival et al., 2008). This new approach is more suitable for the chronic wounds with infection, while it is not applicable for a wide range of wound types.

At present, there are many different options of wound healing treatment with new technology inventions. For instance, silver dressing is an effective wound care management since ancient times, and it is modified with newer advances in technology where the concentration of silver can be maintained at abundance level to retain its antimicrobial efficacy with a minimum toxicity (Murphy & Evans, 2012). Although there is development of a little bacterial resistance to silver, silver is still functional to a broad range of antibiotic-resistant microorganisms (McHugh et al., 1975). However, a few studies have reported that lethal concentration of silver towards bacteria can contribute to the destruction of healthy skin cells. This is due to its toxic effect to fibroblasts (Liedberg & Lundeberg, 1989), causes

stagnant growth of keratinocytes (Lam et al., 2004) and hinders the reepithelialisation (Innes et al., 2001).

Despite the high technology approaches to hasten wound healing, systemic or topical antimicrobial therapy are more commonly used for the chronic wound treatment. Though the appropriate use of systemic medications is considered vital for the management of infected chronic wound (Krahwinkel & Boothe, 2006), however, some systemic medications have caused remarkable consequences on wound repair such as non-steroidal anti-inflammatory drugs (NSAIDs), chemotherapeutic drug and glucocorticoid steroids (Guo & DiPietro, 2010). NSAIDs which are used to reduce inflammation and relieve pain have been reported to hamper the wound healing process. The anti-proliferative effect of NSAIDs due to the reduction in fibroblasts number has resulted in the obstruction of wound healing (Krischak et al., 2007). Besides, glucocorticoid steroids act as an anti-inflammatory agent is revealed to suppress cellular wound responses by inhibiting proliferation of fibroblasts and reducing biosynthesis of collagen, and hence the wound repair is hampered (Anderson & Hamm, 2014). While for the chemotherapeutic drugs, they are utilised to inhibit a variety of pathways that are essential for a proper wound repair. The drugs bring negative impact on the cell migration to the lesion site and affect the wound contraction (Franz et al., 2007).

Apart from that, there are numerous types of topical antimicrobials which are extensively used in treating the infected chronic wounds. Basically, topical antimicrobials can be categorised into two groups, which are antiseptics and antibiotics (Lipsky & Hoey, 2009). Antiseptics are disinfectants that are used for inhibition of microorganisms or act as microbicides. They have a broad spectrum of antimicrobial effect where a wide diversity of microbes can be killed, however, they will cause toxicity to the skin components such as

fibroblasts and keratinocytes (Drosou, 2003). On the contrary, antibiotics are chemicals with a narrower antimicrobial spectrum that inhibit or kill specific microbes without causing toxicity to the skin, but there may have occurrence of bacterial resistance which lead to the loss of antimicrobial effectiveness (Sameh et al., 2018) and even increase the risk of allergic contact dermatitis (Draelos et al., 2011).

Furthermore, skin substitutes such as autograft, allograft and xenograft are widely used in the patients with large surface area of wound which are due to burns or chronic wound. Autograft is a better option as the tissue originates from an individual's own body. However, the patients may have a limited amount of tissue that can be used for grafting or their body conditions are not suitable for grafting. While for the allograft (from human) and xenograft (from animal), there are some issues such as rejection, transmission of diseases and cultural and ethical issues as the transplant tissue is from another person or different species (Murphy & Evans, 2012). Therefore, the advances in the bioengineering of skin substitutes have resolved the transplant rejection and transmission of infection. The bioengineered skin substitutes have been shown to exhibit comparable results with autograft, where the wound healing time is accelerated and patient's satisfaction for the treatment is higher (Pham et al., 2007). Although there are many benefits of bioengineered skin substitutes, the high costing of these products limits the widespread usage in the medical field.

Although there are high technology approaches and antimicrobial therapy that can treat the wounds, however there are many adverse effects on the wound healing with long-term usage. Therefore, it is necessary to seek for an alternative treatment for wound repair. Generally, the goals of wound healing are reducing time of healing, combating infection and

causing minimum pain to the patient (Bolton & Fattu, 1994). The killing effect of the antimicrobial agent is needed to target the foreign pathogens without damaging the healthy skin cells. Hence, many researchers have gained interest on the medicinal plants in order to evaluate their potential as an alternative wound healing medications with minimum side effects.

2.1.4 Medicinal Plants with Wound Healing Properties

Many studies have focused on the medicinal plants as an alternative treatment in wound healing potential. Table 2.2 shows numerous medicinal plants with notable wound healing properties that are used in the traditional Chinese medicine for wound healing therapy (Ning et al., 2022).

There are several medicinal plants that have been substantially investigated and have been proven to be efficacious clinically. Some of these medicinal plants have been commercialised as the wound healing treatment products, for instance, *Aloe vera*, Birch bark (genus *Betula*), *Calendula officinalis*, *Centella asiatica* and *Curcuma longa*. During ancient times, *Aloe vera* was beneficial in the treatment of exudative dermatitis and at present, it is a popular ingredient in skincare products. Study by Burusapat et al. (2018) revealed that *Aloe vera* could regulate the inflammatory response and improve the contraction and epithelialisation of wound (Sánchez et al., 2020). Besides, glucomannan (dietary fibre) was found to be the main component of *Aloe vera* that improved the fibroblasts proliferation and subsequently ameliorated the synthesis and secretion of collagen (Boudreau and Beland, 2006).

Birch bark originated from various birch species (genus *Betula*) and was used in traditional medicine to treat acute mastitis (breast tissue inflammation). Nowadays, it is

developed into a wound-healing gel, Oleogel-S10 (Episalvan[®]), which consists of triterpene extract from birch bark, and it is an approved drug in the European Union (Scheffler, 2019). A study by Ebeling et al. (2014) showed that triterpene extract from birch bark resulted in an enhancement of primary human keratinocytes migration and upregulation of mediators in the wound healing inflammatory phase and this caused a temporary inflammation. Temporary inflammation is a necessity in the process of wound repair while prolonged inflammation will result in chronic wounds (Landén et al, 2016).

Furthermore, according to Morin et al. (2012), *Calendula officinalis* (marigold) is one of the ingredients for Cicaderma[®] ointment which has been commercialised in Europe to treat wounds, burns and insect bites since 1930. The *in vitro* studies showed that fibroblast treated with *Calendula officinalis* had increased proliferation and migration rate, and increased growth factors secretion such as connective tissue growth factor (CTGF), TGFβ1, and basic fibroblast growth factor (bFGF) (Fronza et al., 2009; Dinda et al., 2015; Dinda et al., 2016). In addition, *in vivo* studies demonstrated that *Calendula officinalis* ameliorated re-epithelisation, angiogenesis, and deposition of collagen at the site of the wound (Parente et al., 2011; Preethi & Kuttan, 2009).

In addition, *Centella asiatica* was used to treat the ulcers and wounds in China during ancient times. Currently, the commercially available products such as Madecassol[®] and Emdecassol[®] are utilised to treat the skin problems. Study by Brinkhaus et al. (2000) showed that *Centella asiatica* improved the fibroblasts proliferation, enhanced the production of collagen, and exhibited anti-inflammatory response (Bylka et al., 2014). The *Centella asiatica* fresh leaves are made into an ointment in Iran, which is called centiderm. The later

is effective in healing the burn wounds with decrement of epithelialisation and healing time (Saeidinia et al, 2017).

Moreover, since ancient times, China and India have used *Curcuma longa* (turmeric) as a traditional medicine for treatment of wound healing. CumarGOLD Gel® with key composition of curcumin could enhance the new blood vessels formation and improve migration of fibroblasts and macrophages to the wound site (Akbik et al., 2014).

Table 2.2: Medicinal plants and their wound healing activities [Adapted from (Ning et al., 2022)].

Medicinal Plants	Wound Healing Activity and Potential Mechanism	References
<i>Angelica dahurica</i>	Promoted diabetic wound closure via inducing angiogenesis and granulation tissue formation.	Zhang et al. (2017)
<i>Angelica sinensis</i>	Stimulated wound healing with complete remodelling of the wounds and increased granulation tissue formation.	Zhao et al. (2006)
<i>Astragalus mongholicus</i>	Promoted cell proliferation and migration and accelerated wound healing via reducing inflammatory response and promoting the expression of TGF- β 1, bFGF and EGF.	Zhao et al. (2017)
<i>Calamus draco</i>	Reduced inflammation via inhibiting IL-1 α and TNF- α secretion. Increased VEGF and TGF expression, and collagen deposition.	Jiang et al. (2017)
<i>Panax notoginseng</i>	Promoted the neovascularisation accompanied with increased VEGF, PDGF, and FGF at either mRNA or protein levels. Reduced inflammation via downregulating TNF- α and IL-6 expressions.	Zhang et al. (2016)
<i>Arnebia euchroma</i>	Promoted fibroblast proliferation and migration via ERK 1/2 signaling pathway.	Imai et al. (2019)
<i>Bletilla striata</i>	Accelerated wound healing, suppressed macrophage infiltration, and promoted angiogenesis.	Zhao et al. (2021)
<i>Rehmannia glutinosa</i>	Better developed scars and epithelialisation, and improved formation of capillaries with enhanced VEGF expression.	Lau et al. (2009)
<i>Salvia miltiorrhiza</i>	Increased cell proliferation and promoted collagen synthesis.	Chen et al. (2014)
<i>Lilium lancifolium</i>	Upregulated ECM and cell adhesion related genes to regulate basic functions of cells in wound healing	Di et al. (2020)

Table 2.2 continued

<i>Glycyrrhiza uralensis</i>	Promoted the proliferation of capillaries and fibroblasts, and increased number of microvessels in wound site.	Hao et al. (2020)
<i>Zingiber officinale</i>	Upregulated the production of growth factor, and accelerated cellular proliferation, and migration.	Chen et al. (2013)
<i>Lonicera japonica</i>	Promoted wound healing, elevated the production of IL-10, and suppressed the production of TNF- α and IL-6	Chen et al. (2012)
<i>Portulaca oleracea</i>	Stimulated wound contraction and increased the strength of wound	Rashed et al. (2003)
<i>Hippophae rhamnoides</i>	Faster reduction in the wound area, increased collagen synthesis, promoted angiogenesis and increased levels of antioxidants in wounds.	Upadhyay et al. (2011)
<i>Urtica dioica</i>	Promoted the wound healing with shorten heal time.	Razika et al. (2017)
<i>Periploca forrestii</i>	Enhanced re-epithelialisation, promoting fibroblast proliferation, migration and stimulating the collagen synthesis.	Li et al. (2019)
<i>Streptocaulon juvenas</i>	Promoted wound healing via inducing the fibroblast proliferation and angiogenesis and inhibiting the inflammation.	Nguyen et al. (2017)
<i>Carthamus tinctorius</i>	Promoted wound closure and elevated the levels of VEGF and TGF- β 1 in treated wounds.	Gao et al. (2018)
<i>Reynoutria japonica</i>	Stimulated cell proliferation, migration, and collagen III synthesis.	Nawrot-Hadzick et al. (2021)
<i>Artemisia annua</i>	Encouraged the attachment, spreading and proliferation of the fibroblast cells.	Mirbehbahani et al. (2020)
<i>Acorus calamus</i>	Accelerated wound closure, less inflammatory cells, and higher collagen in treated wounds.	Jain et al. (2010)
<i>Triticum aestivum</i>	Promoted cell proliferation and migration and collagen synthesis in human dermal fibroblasts.	Sahin et al. (2019)

2.2 *Litsea garciae*

In this study, *Litsea garciae* (*L. garciae*) was selected as the potential medicinal plant for the wound healing treatment (Figure 2.3). It has a common name with different local languages in Sarawak, Malaysia, where it is known as engkala in the Malay language, enkala/pedar in Iban language, and ta'ang in the Bidayuh language (Poli & Assim, 2019). *L. garciae* is under the classification of the *Lauraceae* family and genus *Litsea*. The genus comprises of 136 species (Vidal, 2010). *L. garciae* has a flavour that is comparable to the *Lauraceae*, *Persea americana* with the common name of avocado and therefore it has the nickname of “Borneo avocado”. *L. garciae* is native and naturally grown in Borneo and it is now commercially cultivated in Kuching and Bau districts in Sarawak, Malaysia. The *L. garciae* tree plantation takes around five years for it to bear fruits and it is a seasonal plant that bears fruit once a year (Lim, 2012). *L. garciae* is an underutilised edible fruit in Borneo Island. However, it is not only just as food, but it also has some medicinal uses which are used by the natives. It is comprised of various chemical and nutritional compositions such as minerals and many chemical components which is beneficial for human health.



Figure 2.3: Transformation of the green, unripe *L. garciae* fruit into the red, ripe fruit.

2.2.1 Geographical Distribution

L. garciae is native to Borneo (Sabah and Sarawak in Malaysia, Indonesian Kalimantan and Brunei), Indonesia (Java and Bangka) and Philippines (Figure 2.4). *L. garciae* grow wildly throughout Borneo Island and commercially cultivated and available in the local markets in Borneo, Indonesia, Philippines, and Taiwan. It is cultivated in Peninsular Malaysia, but on a smaller scale (Lim, 2012). *L. garciae* is a sub-canopy, broadleaved evergreen tree that maintains its green leaves throughout the year. *L. garciae* is normally discovered in open, disturbed sites in mixed dipterocarp forests at an elevation of 200 metres. In addition, it is frequently found along rivers or on hillsides with sandy to clay soils (Lim, 2012).

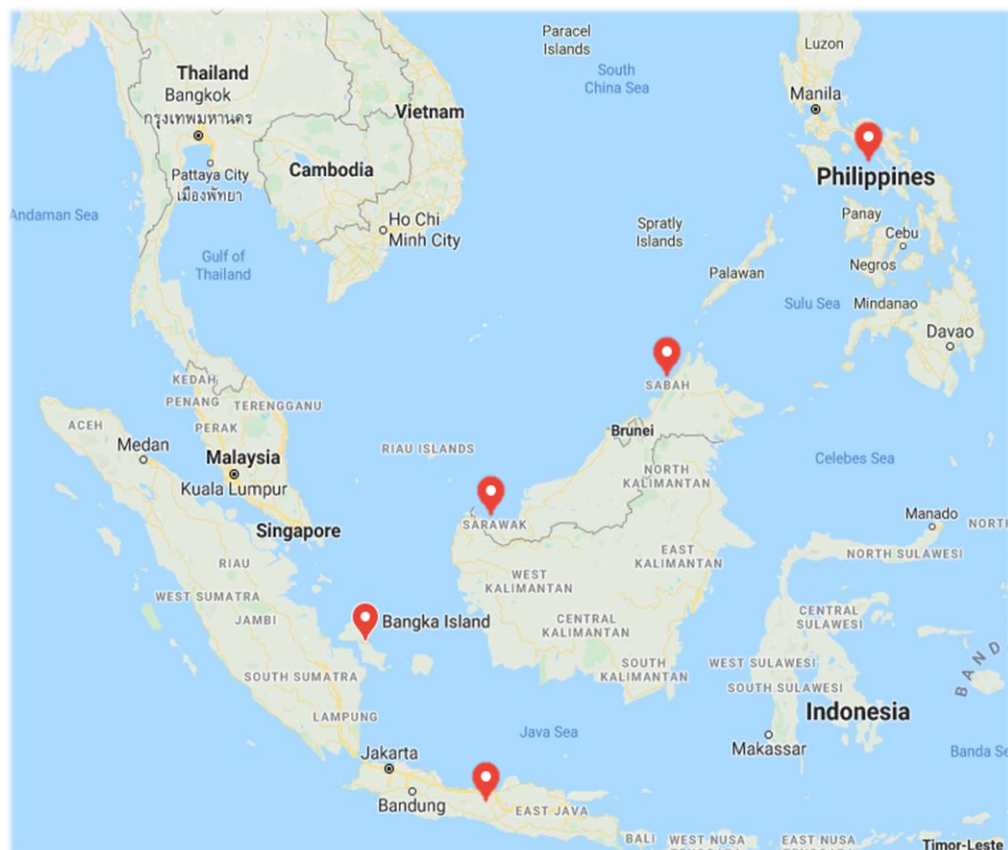


Figure 2.4: Geographical distribution map of *L. garciae* [Adapted from (Google Maps, 2020)].

2.2.2 Botany and Morphology

The tree of *L. garciae* can grow to 26 metres and the diameter of the trunk is about 40 – 50 cm. The arrangement of leaves is alternate, which is one leaf per plant node on alternate side. The leaf blade is simple, penni-veined, ovate-lanceolate, or obovate-lanceolate (25-40 × 6-15 cm) and glabrous (Huang et al., 2008). The flowers are small which is 15 mm in diameter and yellowish to white colour (Figure 2.5). The *L. garciae* fruit is oblate or globose in shape with 22-30 mm diameter across and 2.5-4.5 cm in diameter, which is placed on the green cup-shaped tube (cupule). The immature fruit peel colour is pale greenish white while the matured fruit peel is pink or red in colour (Figure 2.3). The fruit with a single seed has a diameter of 15-20 mm that is surrounded by creamy white pulp (Lim, 2012) as shown in Figure 2.6.



Figure 2.5: *L. garciae* tree bearing fruits (A) and flowers (B) [Adapted from (Bukbi, 2019)].

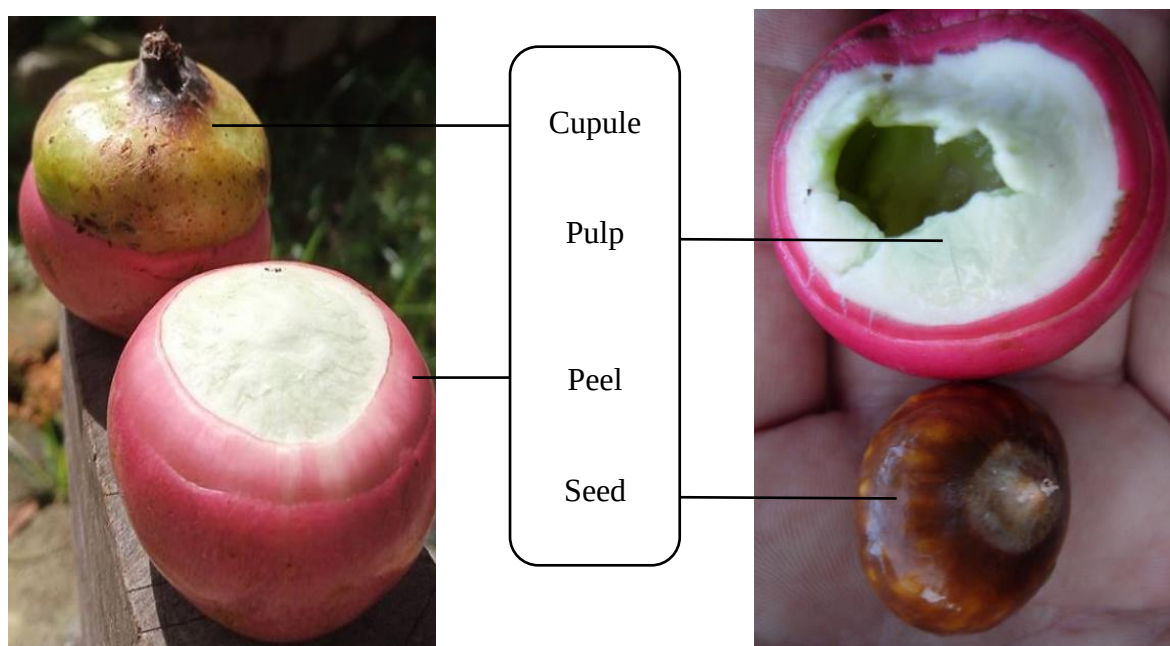


Figure 2.6: Different parts of the *L. garciae* fruit [Adapted from (Machabuca, 2008 and Herbs Encyclopedia, 2020)].

2.2.3 Applications

2.2.3.1 Edible Fruit

The fleshy part and the thin peel *L. garciae* fruit are edible. They can be eaten fresh, preserved, or used for food preparation. The most popular way to consume it was by soaking the fruit in warm water for 10 min. Then, water is discarded, and some salt is sprinkled over them. It is then ready to eat where the creamy white pulp has a soft texture and avocado-like flavour (Lim, 2012). Besides, the fruits can be consumed as a side dish with cooked rice and soy sauce. In addition to that, the unripe fruits can also be prepared by preservation as pickles. It is preserved by salt solution or vinegar and can be kept for a couple of days.

2.2.3.2 Traditional Medicinal Uses

In Borneo Island, there are different ethnic groups which are known as indigenous communities or natives. The indigenous peoples have used some parts of the plants such as leaves and bark for traditional medicinal uses. In Sarawak, Ibans use the ground bark of *L. garciae* as a dressing for the treatment of caterpillar stings and boils (bacterial skin infection) (Mirfat et al., 2018). They also utilise the bark to make a decoction to treat some diseases such as the medication for rectal bleeding (blood in stool). Besides, Selako people use a poultice of leaves and young shoots of *L. garciae* with a combination of shallot and fennel seed for the treatment of skin infections, diseases, and burns. Moreover, the Kayan people treat beri-beri by warming up a poultice of *L. garciae* leaves while the Kelabits make a cataplasm using the root bark to cure the sprains. Furthermore, Penans use pounded and warmed bark for the treatment of muscular aches and sprains. Moreover, the combination of *L. garciae* and durian bark can also be used as an antidote for snake bites (Lim, 2012).

2.2.3.3 Other Uses

The extracted oil from the *L. garciae* seeds can be utilised in the manufacturing of candles and soaps. Furthermore, *L. garciae* woods have been used as timbers in construction (Lim, 2012) and are also suitable for the plywood production and interior decoration such as flooring materials, furniture and panelling (Fern, 2019).

2.2.4 Chemical and Nutritional Compositions

2.2.4.1 Proximate and Minerals Compositions

Proximate compositions involve contents of moisture, ash, lipid, protein, and carbohydrates. While the minerals are sodium, phosphorus, calcium, magnesium, potassium, iron, zinc, and copper (Vaitkevičienė et al., 2022). Table 2.3 shows the proximate and minerals compositions of *L. garciae* pulp and seed obtained from Husen (2015). There was also another study about the proximate and minerals compositions of *L. garciae* pulp by Voon & Kueh (1999). The results from these studies varied slightly presumably attributed to the methodologies used and the plant collection sites.

Voon & Kueh (1999) reported that *L. garciae* pulp contained high moisture content (78.3%), which was in agreement with the results of Husen (2015) where the pulp consisted of 68.8% and seed comprised of 71.7% of moisture content. The importance of moisture content is to retain the refreshing character of the fruit. There was 6.8% of fat content while only 1.4% of protein content in the *L. garciae* pulp (Voon & Kueh, 1999). The creamy texture and buttermilk taste of *L. garciae* fruit is probably contributed by its fat content. Husen (2015) showed that *L. garciae* consisted of high carbohydrate content, where the pulp had 21.7% and seed had 18.6% of carbohydrate, and this may contribute to the high energy content of *L. garciae*.

The *L. garciae* pulp ash content was 2.5% while the seed was 1.4% as reported by Husen (2015). Ash content represents the total mineral content in foods. Generally, the *L. garciae* pulp mineral contents as reported by Husen (2015) were higher than by Voon and Kueh (1999). *L. garciae* pulp consisted of 652.9 mg of potassium and 91.5 mg of sodium per 100g of pulp portion while the *L. garciae* seed was lower in potassium and sodium (Husen, 2015). The United States Food and Drug Administration (FDA) claims that food

containing 350 mg of potassium and less than 140 mg of sodium may benefit the blood pressure control (FDA, 2000) and thus this shows that *L. garciae* is a healthy food to consume.

Vitamin C is necessary for the growth, development, and repair of body tissues, and also contributes to the antioxidant properties. Husen (2015) showed that *L. garciae* pulp comprised of a high content of vitamin C, where freeze-drying (FD) and superheated-steam drying (SHSD) methods contributed 11.8 mg and 34.7 mg of vitamin C content, respectively. The longer drying time in the FD method might affect the vitamin C content, where several days were needed to dry the samples as a comparison to the SHSD method which only required three hours.

Table 2.3: Nutrient contents of the *L. garciae* pulp/seed per 100 g pulp/seed portion.

Nutrient Contents	Pulp	Seed
Proximate		
Energy (kcal)	93.3	83.4
Moisture (%)	68.8	71.7
Protein (%)	2.6	3.3
Fat (%)	NA	NA
Carbohydrate (%)	21.7	18.6
Crude fibre (%)	4.3	5.0
Ash (%)	2.6	1.4
Minerals		
Phosphorus (mg)	NA	NA
Potassium (mg)	652.9	331.5
Calcium (mg)	7	2.4
Magnesium	3.7	1.8
Iron	4.9	1.1
Manganese (ppm)	NA	NA
Copper (ppm)	1.0	0.6
Zinc (ppm)	1.6	1.1
Sodium	91.5	6.3
Vitamin C	11.8 (FD)	4.8 (FD)
	34.7 (SHSD)	13 (SHSD)
Authors	Husen (2015)	Husen (2015)

NA= Not available FD = freeze dried SHSD = superheated-steam dried

2.2.4.2 Fatty Acid Composition

Fatty acids are classified depending on the length of fatty acids which are short-chain fatty acids (SCFAs), medium-chain fatty acids (MCFAs), long-chain fatty acids (LCFAs) and very long chain fatty acids (VLCFAs). SCFAs consist of aliphatic tails with less than six carbons while MCFAs contain aliphatic tails of 6 – 12 carbons. Besides, LCFA make up of 13 – 21 carbons in the aliphatic tails whereas VLCFAs have aliphatic tails that are longer than 22 carbons (Burdge & Calder, 2015). The functions of SCFAs are as the antimicrobial and antifungal agents. SCFAs and MCFAs also play an important role in the intracellular signalling and contribute to the regulation of cell metabolism (Schönfeld & Wojtczak, 2016). LCFAs act as an energy source for the cell which is involved in the fundamental cellular processes (Anderson & Stahl, 2013).

Moreover, fatty acids can also be categorised by saturation of fatty acids which are saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). SFAs are made up of single bonds while MUFAs consist of one carbon-carbon double bond and PUFAs contain two or more carbon-carbon double bonds (White, 2009). Consumption of SFAs with a higher carbon number (12, 14 or 16 carbons) has been reported to elevate the cholesterol level of low-density lipoprotein (LDL) (Mensink & Katan, 1992). With the exception, stearic acid (18 carbons) does not seem to increase LDL cholesterol level (Chait et al., 1993). While the intake of MUFAs helps to decrease the risk of getting coronary heart disease (CHD) by lowering the total cholesterol levels and preventing the LDL oxidation (Kris-Etherton, 1999).

PUFAs are the essential fatty acids that need to be acquired from daily diet as these fatty acids cannot be synthesised by human body (Kaur et al., 2014). PUFAs are the

component of phospholipids that are essential for the appearance and function of skin. Examples of PUFAs such as linoleic acid (LA) and α -linolenic acid (ALA) are the precursors for omega-6 and omega-3 fatty acids respectively. Both omega-3 and omega-6 fatty acids can modulate the inflammatory response in skin (Angelo, 2012). Omega-3 fatty acid helps to prevent wound infections and aids in early phase of wound healing (Alexander & Supp, 2014). A study proposed by Silva et al. (2018) demonstrated that omega-6 fatty acid could regulate the inflammatory phase of wound repair. According to the previous study by Huang et al. (2018), LA and ALA in fish oil were beneficial to be used in cosmetic products. The topical application of PUFAs were shown to exhibit photoprotective effect that could prevent and treat the skin aging, and improve cell migration with increment in wound closure that could promote the wound repair.

Furthermore, it was found that the *L. garciae* seed oil was very rich in saturated fatty acids (SFAs) ($76.94 \pm 1.50\%$), consisted of a moderate amount of monounsaturated fatty acids (MUFAs) ($16.23 \pm 1.20\%$), and a small proportion of polyunsaturated fatty acids (PUFAs) ($7.10 \pm 0.79\%$) (Poli & Assim, 2019). Lauric acid ($40.73 \pm 2.81\%$) was the predominant component of SFAs in *L. garciae* seed oil, and it was followed by myristic acid ($19.69 \pm 0.52\%$), palmitic acid ($7.20 \pm 0.63\%$), and capric acid ($6.07 \pm 2.01\%$). In addition, the remarkable quantities of MUFAs and PUFAs of *L. garciae* seed oil were contributed by oleic acid ($14.98 \pm 1.44\%$) and linoleic acid ($7.10 \pm 0.79\%$) respectively. The high contents of SFAs, MUFAs and PUFAs in pulp and seed of the *L. garciae* may contribute to their wound healing properties.

2.2.4.3 Phytochemicals

L. garciae contained a few alkaloids for instance, actinodaphnine, boldine, isodomeesticine, laurolitsine, and reticuline (Lee et al., 1995). According to Wulandari et al. (2018), the hexane, ethyl acetate and ethanol extracts of *L. garciae* branch, bark and leaf comprised of alkaloids and carotenoids (Table 2.4). Coumarin was present in all the extracts (Table 2.4) except for the ethyl acetate leaf extract. For triterpenoid and steroid, they were absent in all parts of n-hexane extracts and in ethanol extracts respectively. Besides, saponins were absent in all three solvents extraction. Saponins were glycosides of triterpenes and steroids (Mugford & Osbourn, 2012), and the absence of saponins in the *L. garciae* might be attributed to the undetected triterpenes and steroids in the extracts. The variation in the types of phytochemicals present could indicate that different solvents might extract different phytochemical compounds due to the polarity of the solvent used. The phytochemicals that exert anti-inflammatory and analgesic effect have been shown to improve wound healing process (Thangapazham et al, 2016).

Table 2.4: Phytochemical screening of *L. garciae* [Adapted from (Wulandari et al., 2018)].

Solvent	Part	Alk	Flav	Sap	Tan	Triter	Ste	Car	Cou	Caro
<i>n</i> -hexane	Branch	+	-	-	+	-	-	+	+	-
	Bark	+	+	-	-	-	+	+	+	-
	Leaf	+	+	-	+	-	-	+	+	-
Ethyl acetate	Branch	+	+	-	+	+	-	+	+	-
	Bark	+	-	-	-	+	-	+	+	+
	Leaf	+	-	-	+	-	+	+	-	+
Ethanol	Branch	+	+	-	+	+	-	+	+	-
	Bark	+	+	-	+	+	-	+	+	+
	Leaf	+	+	-	-	+	-	+	+	-

Remarks: (+) Present, (-) Absent, Alk; Alkaloids, Flav; Flavonoids, Sap; Saponins, Tan; Tannins, Triter; Triterpenoid, Ste; Steroid, Car; Carbohydrate, Cou; Coumarin, Caro; Carotenoids.

2.2.4.4 Phenolic and Flavonoid Content

The abundance of phenolic compounds in plants contributes to the high antioxidant contents. Phenolic compounds contain hydroxyl groups that can abstract hydrogen atom from the hydroxyl radicals, which lead to the conversion of phenol into a stable phenoxyl radical, and therefore radicals can be destroyed by the phenolic compounds (Aksoy et al., 2013). Besides, flavonoids are one of the most prevalent phenolics which comprised of catechins, proanthocyanins, anthocyanidins, flavons, flavonols, and their glycosides (Tungmunnithum et al., 2018). Flavonoids act as a reducing agent and hydrogen donor as they have redox potential which is a crucial criterion as an antioxidant. It is indicated that flavonoids consisted of anti-inflammatory, anti-allergic, and antithrombotic or vasoprotective effects (Panche et al., 2016).

A study by Wulandari et al. (2018) showed that all the *L. garciae* extracts contained a notable proportion of total phenolics and flavonoids (Table 2.5). The ethanol extracts of

all the plant parts contained the highest amount of total phenolic and flavonoid contents as opposed to the *n*-hexane and ethyl acetate extracts. As we know, flavonoid and phenolic compounds were shown to exhibit antioxidant and anti-inflammatory properties (Wang et al., 2016).

As stated in Table 2.6, a previous study by Hassan et al. (2013) reported the highest total phenolic contents of 80% methanol and aqueous extracts were in the cupule, followed by the seed and pulp. Likewise, flavonoid content was also shown to be the highest amount in the cupule and then followed by the seed and pulp from the 80% methanol and aqueous extracts. Moreover, the total anthocyanin content of the 80% methanol and aqueous extracts for *L. garciae* pulp was revealed to be the highest, followed by the seed and cupule. The flavonoid and phenolic compounds that exert antioxidant and anti-inflammatory properties can enhance the wound healing (Melguizo-Rodríguez et al., 2021).

Table 2.5: Total phenolic and flavonoid content of *L. garciae* [Adapted from (Wulandari et al., 2018)].

Solvent	Part	Phenolic ($\mu\text{g GAE/mg extract}$)	Flavonoid ($\mu\text{g GAE/mg extract}$)
<i>n</i> -hexane	Branch	30 ± 0.002	190 ± 0.004
	Bark	40 ± 0.002	170 ± 0.003
	Leaf	30 ± 0.001	110 ± 0.002
Ethyl acetate	Branch	30 ± 0.002	140 ± 0.005
	Bark	40 ± 0.004	160 ± 0.005
	Leaf	40 ± 0.004	210 ± 0.004
Ethanol	Branch	100 ± 0.001	1010 ± 0.002
	Bark	90 ± 0.009	800 ± 0.001
	Leaf	100 ± 0.001	240 ± 0.001

Table 2.6: The total phenolic, flavonoid and anthocyanin contents of different parts of *L. garciae* fruit extracts [Adapted from (Hassan et al., 2013)].

Chemical content	Sample	80% methanol extract			Aqueous extract		
		Cupule	Seed	Pulp	Cupule	Seed	Pulp
Total phenolic (mg GAE/g)		8.29 ± 0.70	8.09 ± 0.60	2.65 ± 0.11	3.71 ± 0.24	3.54 ± 0.17	2.01 ± 0.07
Total flavonoid (mg RE/g)		6.90 ± 0.61	5.73 ± 1.39	2.05 ± 0.21	2.88 ± 0.23	2.63 ± 0.29	1.46 ± 0.15
Total anthocyanin (mg CE/100g)		0.35 ± 0.00	2.11 ± 0.03	4.12 ± 0.10	0.22 ± 0.03	1.42 ± 0.10	2.83 ± 0.23

2.2.5 Pharmacological Properties

The pharmacological properties of *L. garciae* were similar to the other *Litsea* species, in which they exhibited antioxidant, antiplatelet, antitumour, anticonvulsant, antibacterial, antiviral, and antiplasmodial properties (Othman et al., 2019; Kamle et al., 2019).

2.2.5.1 Antioxidant Properties

Table 2.7 shows the antioxidant potency of *L. garciae* 80% methanol and aqueous extracts using three different assays (DPPH assay, FRAP assay and ABTS assay) (Hassan et al. 2013). The non-edible part (cupule and seed) of *L. garciae* had displayed a higher antioxidant property than the edible part (pulp) for both the 80% methanol and aqueous extracts in the FRAP and ABTS assays. The non-edible part of *L. garciae* exhibited the highest antioxidant activity, which was consistent with the DPPH assay results. For DPPH assay, the lower the IC₅₀ value, the stronger the antioxidant potency of extracts, in which their IC₅₀ values were in the order of cupule < seed < pulp.

In addition, the antioxidant activity of *L. garciae* was corresponding to the trend as exhibited by the total phenolic and flavonoid contents (Table 2.6), where it was in the order of cupule > seed > pulp. Not only that, the phytochemical screening for the *L. garciae* had detected the alkaloids, flavonoids, tannins, coumarin and carotenoids (Table 2.4). Therefore, *L. garciae* was suggested to serve as a superior source of natural antioxidants that possessed potential to act as therapeutic agents to prevent the oxidative-stress related diseases (Amit & Zinyin, 2021). Since oxidative stress causes impairment of wound healing, antioxidants aid in modulating the wound oxidative stress and hence ameliorate the wound repair process (Fitzmaurice et al., 2011).

Table 2.7: The antioxidant properties of *L. garciae* fruit extracts from different parts using three different assays [Adapted from (Hassan et al., 2013)].

Assays \ Sample	80% methanol extract			Aqueous extract		
	Cupule	Seed	Pulp	Cupule	Seed	Pulp
¹ DPPH assay	16.7 ± 0.6	17.3 ± 2.3	60.0 ± 3.5	20.0 ± 0.0	22.7 ± 2.3	62.7 ± 4.6
² FRAP assay	2,050.0 ± 28.5	1,910.0 ± 59.2	410.0 ± 54.1	1,650.0 ± 29.7	690.0 ± 17.0	210.0 ± 9.7
³ ABTS assay	25.05 ± 1.7	19.14 ± 1.7	4.05 ± 0.1	16.47 ± 2.0	6.86 ± 0.6	2.14 ± 1.0

Values are presented as mean ± SD (n = 3).

1. 2,2-Diphenyl-1-picryl-hydrazyl-hydrate assay (DPPH assay)
DPPH free radical scavenging activity was expressed as IC₅₀ (mg/mL).
2. Ferric reducing/antioxidant power (FRAP assay)
FRAP was expressed as µM ferric reduction to ferrous in 1 g of dry sample.
3. 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS assay)
ABTS assay free radical scavenging activity was expressed as mg ascorbic acid equivalent antioxidant capacity (AEAC) in 1 g of dry sample.

2.2.5.2 Antimicrobial and Antifungal Properties

Antibacterial activity of the *L. garciae* branch, leaf and bark extracts using three solvents (*n*-hexane, ethyl acetate and ethanol) against *Propionibacterium acnes* was investigated using broth microdilution test (Wulandari et al., 2018). From Table 2.8, it was revealed that the minimum inhibitory concentration (MIC) for *n*-hexane and ethanol extracts of the branch and ethyl acetate extract of the leaf was 312.5 ppm. In short, *L. garciae* had shown weak antibacterial activity with high MIC values of 312.5 to 1250 ppm, which might be due to the low amount of active compound.

Besides, the *L. garciae* crude leaf extract demonstrated some antifungal properties which were reported by Johnny et al. (2010) and Johnny et al. (2011) as illustrated in Table 2.9. From the study, methanol extracts exhibited a higher antifungal property against *C. capsici* as compared to *C. gloeosporioides*. For the chloroform extract, the radial growth inhibition had manifested a higher proportion on *C. gloeosporioides* than *C. capsici*. On the other hand, acetone extracts displayed a corresponding antifungal activity against both *C. gloeosporioides* and *C. capsici* at the concentrations of 1.00 µg/mL and 10.00 µg/mL. The antimicrobial and antifungal properties prevent wounds from infection in which the wound repair is accelerated (Paladini & Pollini, 2019).

Table 2.8: Minimal Inhibitory Concentration (MIC) of *L. garciae* using antibacterial assay [Adapted from (Wulandari et al., 2018)].

Solvent	Part	MIC (ppm)
<i>n</i> -hexane	Branch	625
	Bark	312.5
	Leaf	625
Ethyl acetate	Branch	625
	Bark	1250
	Leaf	312.5
Ethanol	Branch	625
	Bark	312.5
	Leaf	625

Table 2.9: The percentage reduction of radial growth (mm) of *Colletotrichum gloeosporioides* (*Cg*) and *Colletotrichum capsici* (*Cc*) by varying concentrations of *L. garciae* leaf extracts in different solvents using antifungal assay [Adapted from (Johnny et al., 2010; Johnny et al., 2011)].

Solvent	Mean $\pm$ S.E of % of Inhibition of Radial Growth (mm)				Fungus
	0.01 μ g/mL	0.10 μ g/mL	1.00 μ g/mL	10.00 μ g/mL	
Methanol	13.31 $\pm$ 0.49	15.17 $\pm$ 0.55	15.26 $\pm$ 0.58	17.22 $\pm$ 0.78	<i>Cg</i>
	13.59 $\pm$ 0.59	31.08 $\pm$ 0.77	33.77 $\pm$ 0.78	36.71 $\pm$ 1.41	<i>Cc</i>
Chloroform	14.24 $\pm$ 0.51	15.74 $\pm$ 0.67	17.73 $\pm$ 0.78	19.38 $\pm$ 0.48	<i>Cg</i>
	1.94 $\pm$ 1.70	5.84 $\pm$ 1.26	8.85 $\pm$ 1.99	9.68 $\pm$ 1.53	<i>Cc</i>
Acetone	18.88 $\pm$ 0.69	21.16 $\pm$ 0.50	21.78 $\pm$ 0.63	22.68 $\pm$ 0.56	<i>Cg</i>
	NI	NI	24.48 $\pm$ 1.38	25.48 $\pm$ 1.26	<i>Cc</i>

All the values represented the mean $\pm$ standard error. NI = no inhibition

2.2.5.3 Anticancer Activities

L. garciae exhibited moderate anticancer activity on three types of human cell lines using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay as stated in Table 2.10 (Kutoi et al. 2012). The *L. garciae* methanolic bark extract was shown to have moderate cytotoxicity against the human breast cancer (MCF-7) cell lines and human colorectal cancer (HT-29) cell lines, where the IC₅₀ values were 66 µg/mL and 77 µg/mL respectively. While there was a weak cytotoxic activity of bark extract against cervical cancer (HeLa) cell lines, in which the IC₅₀ value was 117 µg/mL. On the contrary, there was no cytotoxicity effect of methanolic leaf extract against HeLa cell lines. For the MCF-7 cell lines, there was a weak cytotoxic activity of leaf extract against the cells with an IC₅₀ value of 104 µg/mL, whereas leaf extract exhibited moderate cytotoxicity against the HT-29 cell lines with 73 µg/mL of IC₅₀ value. Most of the medicinal plants which accelerate the wound healing process have shown to possess anticancer activities (Shedoeva et al., 2019).

Table 2.10: Inhibitory concentration of barks and leaves methanolic extract on HeLa, MCF-7 and HT-29 [Adapted from (Kutoi et al., 2012)].

Part	Cytotoxicity activities against cell lines, IC ₅₀		
	HeLa	MCF-7	HT-29
Barks (µg/mL)	117	66	77
Leaves (µg/mL)	N/A	104	73

2.2.5.4 Anti-inflammatory Properties

L. garciae was also shown to exert anti-inflammatory properties (Kutoi et al. 2012). *L. garciae* methanolic crude extracts of barks, leaves, and fruits were shown to demonstrate some anti-inflammatory activities (Table 2.11). The inhibition of lipxygenase activities for the barks, leaves, and fruit extracts were 1.20%, 3.85% and 9.42% respectively. A slightly stronger hyaluronidase inhibitory activity was exhibited by barks, leaves and fruits with inhibition of 11.30%, 9.51% and 27.70% respectively. In addition, the xanthine oxidase inhibitory activities for the barks and leaves extracts were 2.19% and 2.26% respectively. The anti-inflammatory properties of *L. garciae* may enhance the wound healing process by regulating inflammatory cytokines during the inflammatory phase (Nguyen et al., 2017).

Table 2.11: Lipxygenase (LO), Hyaluronidase (HO) and Xanthine Oxidase (XO) inhibitory activities [Adapted from (Kutoi et al., 2012)].

Part	Anti-Inflammatory Assay (% of inhibition)		
	LO Assay	HO Assay	XO Assay
Barks	1.20	11.30	2.19
Leaves	3.85	9.51	2.26
Fruits	9.42	27.70	Not Active

2.3 Other Indigenous Plants in Sarawak

Besides *L. garciae*, there are several indigenous plants in Sarawak such as dabai, engkabang and red jerangau. These indigenous plants have been shown to contain nutritional values, antioxidant and antimicrobial properties and fatty acids. Dabai (*Canarium odontophyllum*) fruit has a bright yellow pulp with black colour skin and its flavour resembles the olive fruits. The previous study reported by Azlan et al. (2010) revealed that dabai fruit contained antioxidant capacities, with high contents of anthocyanins, flavonoids, and phenolic compound (Prasad et al., 2010). The methanol and acetone extracts of stem bark from dabai were found to exhibit the strongest antimicrobial activities against *Staphylococcus aureus* (Basri et al., 2017). According to the study proposed by Salleh et al. (2022), the dominant fatty acid in the dabai fruit was palmitic acid, followed by linoleic acid, oleic acid and stearic acid.

Moreover, there are a few species of engkabang that can be found on Borneo Island, which are *Shorea macrophylla* and *Shorea palembanica*. The genus *Shorea* is utilised as an ointment for skin diseases, cooking oil and butter in preparing the Sarawak cuisine (Shiva & Jantan, 1998). Rakman et al. (2022) revealed that there was a high content of minerals in the *S. macrophylla* and *S. palembanica* seeds such as potassium, calcium, magnesium, and sodium. It was discovered that the paramount fatty acids in *S. macrophylla* and *S. palembanica* seed oils were stearic acid, oleic acid, and palmitic acid. A study reported by Nur et al. (2012) demonstrated that the bark extract of *Shorea macroptera* inhibited moderately against the Gram-positive bacteria, *Staphylococcus aureus*, *Streptococcus anginosus*, *Streptococcus uberis*, and *Bacillus subtilis*.

Furthermore, red jerangau (*Boesenbergia stenophylla*) is originated from the family Zingiberaceae, which is commonly found in Sarawak highland areas. Red jerangau is utilised by the indigenous people to treat stomach aches and food poisoning (Toyat et al., 2015). According to the study of Vallirie (2018), the red jerangau stem extracts exhibited antimicrobial activities against the Gram-positive bacteria (*Staphylococcus aureus*) and Gram-negative bacteria (*Salmonella typhimurium*). Besides, red jerangau has been reported to contain some chemical constituents such as nerolidol and methyl cinnamate (Nor & Ibrahim, 2018). Nerolidol has been shown to exhibit anti-inflammatory, antinociceptive properties and gastroprotective activities (Klopell, 2007; Silva et al., 2014). It was discovered that methyl cinnamate displayed antioxidant activity and vasorelaxant effects (Vasconcelos-Silva et al., 2014; Vejayan et al., 2017).

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and Reagents

All the chemicals, reagents, and materials used for the experiments were listed in the Table 3.1 below.

Table 3.1: List of materials used for the experiments.

Materials	Manufacturer
0.22 μm syringe filter	Whatman [®] , UK
0.4% trypan blue solution	Sigma-Aldrich, UK
1.5 mL microcentrifuge tube	Axygen [®] , China
15 mL centrifuge tube	Corning, USA
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Amresco [®] , USA
50 mL centrifuge tube	Fisher Scientific, China
6-well plate	TrueLine, USA
75 cm ² culture flask	Nest Scientific, USA
96-well plate	Thermo Fisher Scientific, Denmark
Allantoin (C ₄ H ₆ N ₄ O ₃)	Sigma-Aldrich, USA
Amphotericin B (C ₄₇ H ₇₃ NO ₁₇)	Corning, USA
Blood agar base	Oxoid, UK
Butylated hydroxytoluene (BHT)	R&M Chemicals, UK
Chloramphenicol (C ₁₁ H ₁₂ Cl ₂ N ₂ O ₅)	Thermo Scientific [™] Oxoid [™] , UK
Chloroform (CH ₃ Cl)	Fisher Scientific, UK
Dimethyl sulfoxide (DMSO)	Amresco [®] , USA
Dulbecco's modified Eagle's medium (DMEM)/ Ham's F-12	Nacalai tesque, Japan
Dulbecco's phosphate buffered saline (DPBS)	Nacalai tesque, Japan
Filter paper	Whatman [®] , UK
Foetal bovine serum (FBS)	Tico Europe, Netherlands
Gentamicin	Thermo Scientific [™] Oxoid [™] , UK

Gram crystal violet	Thermo Scientific™ Remel™, USA
Hydrochloric acid (HCl)	Merck, Germany
Human EGF ELISA Kit (RAB0149)	Sigma-Aldrich, USA
Human Pro-Collagen I alpha 1 SimpleStep ELISA® Kit (ab210966)	Abcam, UK
Human VEGF-A ELISA Kit (RAB0507) ELISA Kit	Sigma-Aldrich, USA
Methanol (AR Grade)	HmbG® Chemicals, Malaysia
Mueller-Hinton agar (MHA)	Oxoid, UK
Mueller-Hinton broth (MHB)	Oxoid, UK
Nunc™ 25 cm ² cell culture flask	Thermo Fisher Scientific, Denmark
Nutrient agar	Oxoid, UK
Paraformaldehyde powder (PFA)	Sigma-Aldrich, USA
Penicillin-Streptomycin mixed solution	Pan Biotech, Germany
Petroleum ether	HmbG® Chemicals, Malaysia
Polycarbonate cell culture inserts with 8-micron pore size	Thermo Scientific™, USA
Serological pipette	TrueLine, USA
Sodium hydroxide (NaOH)	RCI Labscan Limited, Thailand
Trifluoroacetic acid (TFA)	Fisher Scientific, UK
Trypsin-EDTA solution	Nacalai tesque, Japan
V-vial	DWK Life Sciences, USA

3.2 Equipment

All the equipment used for the experiments was listed in the Table 3.2 below.

Table 3.2: List of equipment used for the experiments.

Equipment	Manufacturer
Analytical balance	Mettler Toledo, Switzerland
Autoclave machine	Tomy Seiko, Japan
Biosafety cabinet	Esco Micro Pte Ltd, Indonesia
Centrifuge machine	Kubota Corporation, Japan
CO ₂ incubator	Esco Scientific, Singapore
ELISA microplate reader	Molecular Devices, USA
Gas chromatograph coupled with mass spectrometer	Shimadzu, Japan
Heating mantle	Electrothermal, UK
Inverted microscope (Olympus IX70)	Olympus Optical Co., Ltd, Japan
Inverted microscope (Olympus IX73)	Olympus Corporation, Japan
Inverted microscope	Nikon Instrument Inc, USA
Liquid nitrogen storage tank	Thermo Scientific™, USA
Lyophiliser	Labconco, USA
Micropipette	Eppendorf, Germany
Mixer grinder	Panasonic, Malaysia
Multichannel pipette	Gilson, USA
Orbital shaker-incubator	Biosan, Latvia
Rotary evaporator	Tokyo Rikakikai, Japan
Soxhlet extractor chamber	FAVORIT®, Malaysia
Spectrophotometer	DeNovix Inc., USA
Stirring hotplate	FAVORIT®, Malaysia
Ultrapure water purification system	Elga LabWater, UK
Vortex mixer	Velp Scientifica, Italy
Water bath	JeioTech, Korea

3.3 *Litsea garciae*

3.3.1 *Litsea garciae* Fruits Collection

The *L. garciae* fruits were collected in July 2020 from the orchard at Kampung Buso, Bau, Sarawak, Malaysia. It was authenticated by a botanist, Dr Aida Shafreena binti Ahmad Puad from the Department of Plant Science and Environmental Ecology, Faculty of Resource Science and Technology, UNIMAS. The sampling voucher with number ZA-01, was kept at the Sarawak Herbarium, Forest Research Centre, Kuching, Sarawak (Figure 3.1). The collected fruits were pre-washed with running tap water. Then, cupule and peel of the fruits were removed from the pulp and were then discarded. Next, the pulp was separated from the seed and the pulp, and the seed were kept in two separate containers. The pulp and seed were kept at -20°C for at least 24 h before undergoing lyophilisation process. The frozen pulp and seed were freeze-dried using a lyophiliser (Labconco FreeZone 2.5 Litre Benchtop Freeze Dryer, USA) for 24 h and 18 h respectively. The freeze-dried pulp and seed were ground into fine powder by utilising a grinder (Panasonic MX-AC400 Mixer Grinder, Malaysia). The ground pulp and seed were then kept in screw-capped tubes at -20°C for extraction.



Figure 3.1: The sampling voucher of *Litsea garciae* (ZA-01).

Both ground pulp and seed were divided into three portions. Each portion (30 g) was subjected to three types of solvent extraction; methanolic extraction and total lipid extraction using petroleum ether and modified Bligh and Dyer (chloroform: methanol) extractions.

3.3.2 Methanolic Extraction of ground Pulp and Seed of *Litsea garciae*

The 80% methanolic extraction was conducted using the Soxhlet method according to Azwanida (2015) with minor modifications. Thirty grams of sample were weighed in a thimble, where the thimble was put in the Soxhlet extractor chamber (FAVORIT[®], Malaysia). The Soxhlet extractor chamber was connected to a reflux condenser (Figure 3.2) and then the Soxhlet extractor chamber was settled onto a round bottom flask containing 80% methanol (300 mL). The round bottom flask was then placed on the heating mantle and the temperature was set according to the boiling point of the solvent, where the boiling point of 80% methanol was 73.8°C (Johansen et al., 1996). The extracted solvent vaporised and condensed in the condenser and dripped back into the Soxhlet extractor chamber. The solvent in the Soxhlet extractor chamber emptied into the flask when the solvent level reached the siphon side arm and this cycle repeated. The Soxhlet apparatus was heated under reflux for 9 to 11 hours until the solvent in the Soxhlet extractor became colourless, indicating that all the samples were maximally extracted and collected in the round bottom flask. The collected sample extracts were then subjected to evaporation using a rotary evaporator (refer to Section 3.3.4).

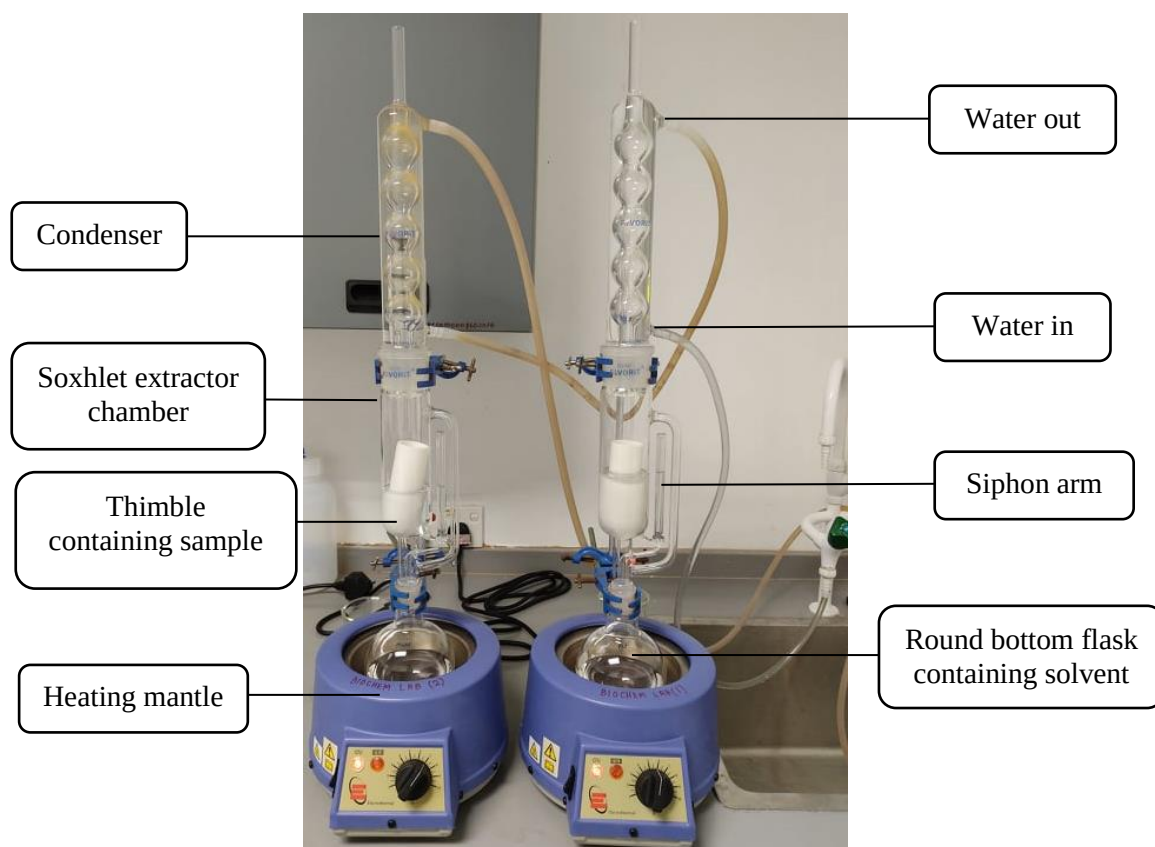


Figure 3.2: Soxhlet (FAVORIT[®]) extraction apparatus used in this study. Picture was taken by the author in the Biochemistry laboratory of Faculty of Medicine and Health Sciences, UNIMAS.

3.3.3 Total Lipid Extraction of *Litsea garciae*

3.3.3.1 Petroleum Ether Lipid Extraction

Total lipids of ground *L. garciae* pulp and seed were first extracted using petroleum ether as a solvent. This was carried out using the Soxhlet method adapted from Azwanida (2015) with slight modifications. Thirty grams of sample was filled in a thimble, and it was then put in the Soxhlet extractor chamber. A round bottom flask containing 300 mL of petroleum ether was placed on the heating mantle. Next, Soxhlet extractor chamber that was connected to a reflux condenser, was then placed onto the round bottom flask (Figure 3.2). The petroleum ether was boiled by setting the temperature of heating mantle at 60°C

(Scientific, 2020). The extraction process was continued for 9 to 11 h until the solvent in the Soxhlet extractor was observed to be colourless. The collected lipid extract in the round bottom flask was then subjected to evaporation using a rotary evaporator (refer to Section 3.3.4) and the total lipid was then dried under a stream of nitrogen gas.

3.3.3.2 Total Lipid Extraction Using Bligh and Dyer Method

The total lipids of the ground pulp and seed of *L. garciae* were also extracted using a monophasic neutral system by following the method of Bligh and Dyer (Bligh and Dyer, 1959). The chloroform (CHCl_3) and methanol (MeOH) were added to the sample in a final ratio of CHCl_3 : MeOH : sample suspension (1: 2: 0.8, vol/vol/vol). In this experiment, water (H_2O) was added to 40 g of the ground samples in order to extract the lipids by the monophasic system. There was addition of 25 mg/L of the antioxidant butylated hydroxytoluene (BHT) for all the solvents to prevent lipid degradation, except the solvent used for dissolving the sterols for GC analysis (Alma et al., 2003). After being stirred for at least 3 h at room temperature and under dark condition, CHCl_3 and H_2O were added to form a biphasic system (Folch et al., 1957) in order to purify the lipids from other compounds. At this stage, the ratio of chloroform: methanol: sample suspension was 2: 1: 0.75, vol/vol/vol.

The sample mixture was filtered using a Grade 1 filter paper with pore size of 11 μm (Whatman[®], UK) to remove the sample residues. The filtrate was transferred to a separatory funnel and was shaken vigorously for 5 min. After shaking, separatory funnel was then clamped to the retort stand and was left overnight for a good separation. The lower phase (chloroform layer) which contained lipids was dispensed into a beaker. A mixture of chloroform and methanol with volumetric ratios 2:1 (v/v) was added to the upper phase (methanol-water layer) for total extraction of total lipids. The separatory funnel was then

shaken and let to settle for 2 h. After that, lower phase was dispensed into the previous beaker and the solvent was evaporated using a rotary evaporator (refer to Section 3.3.4). The total lipid was then dried under a stream of nitrogen gas.

3.3.4 Collection of *Litsea garciae* Pulp and Seed Extracts by Rotary Evaporation

The collected sample extracts from different extraction methods were dried using the rotary evaporator (EYELA rotary evaporator N-1300E, Tokyo Rikakikai Co Ltd, Japan) by removing the solvents from the sample. According to the EYELA rotary evaporator guideline, the vacuum pressure for petroleum ether and chloroform was first set at 332 hPa and subsequently reduced to 266 hPa over time and the setting of heating bath temperature was at 35°C. Besides, vacuum pressure for 80% methanol was adjusted from 180 hPa to 73 hPa gradually and the temperature of heating bath was altered from 45°C to 40°C. Then, the sample extract was further evaporated using nitrogen gas to eliminate any remaining solvent. The evaporated sample extract was kept in a screw cap glass tube in -20°C for usage in various assays. The extraction yield of sample was calculated as the equation stated below:

$$\text{Extraction yield of sample (\%)} = \frac{\text{weight of extract after evaporating solvent}}{\text{dry weight of sample (g)}} \times 100$$

Equation 3.1

3.4 Fatty Acid Analysis

The fatty acid analysis of petroleum ether (PE) and Bligh and Dyer (BD) lipid pulp and seed extracts was conducted using gas chromatography-mass spectrometry (GC-MS). Derivatisation of fatty acids into fatty acid methyl esters (FAMES) was first carried out for the analysis of fatty acids content in *L. garciae* lipid extracts (David et al., 2002; Poli & Assim, 2019). A volume of 200 μ L of PE and BD total lipid extracts were dissolved in 1 mL of hexane in the screw cap test tubes respectively. Then, addition of 200 μ L of 2 M NaOH to the mixture of NaOH (0.8 g) in 10 mL of methanol was conducted and the mixture was vortexed for 10 sec. The mixture was then heated in the water bath at 50°C for 20 sec. After cooling down, it was vortexed again for another 10 sec. Then, the mixture was centrifuged (Kubota 5911, Kubota Corporation, Japan) at 4,000 rpm for 1 min separate the mixture into two distinct phases. After centrifugation, the upper phase of mixture containing FAMES (hexane phase) was transferred into a capped glass v-vial and stored at -20°C prior to GC-MS analysis.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

GC-MS analysis was performed using a gas chromatograph coupled with a mass spectrometer (Shimadzu GCMS-QP2010 Plus, Shimadzu, Japan). The GC separation was carried out using a BPX-5 capillary column (Trajan Scientific and Medical, Australia) (30.0 m $\times$ 0.25 mm [inner diameter]) coated with a fused silica material, 5% phenyl / 95% dimethyl polysiloxane (0.25 μ m). The column oven temperature was initially set at 50°C for 1 min and then increased to 260°C with an increment of temperature by 6°C/min and held for 10 min. The injection temperature was set at 280°C and the injection volume of 1 μ L derivatised sample was introduced to the GC autosampler with a split ratio of 1:20. The carrier gas was helium with a flow rate of 1.0 mL/min.

For the MS condition, the interface temperature was controlled at 280°C and the scan mass range of 45 – 550 m/z with 70 eV of electron impact ionisation energy was applied. Each sample was analysed in triplicate and the data processing unit recorded the peaks in their respective retention times and areas (Chowdhury, 2007). The relative percentage of FAMES was calculated using the average peak areas from three injections where the data was expressed as the mean of peak areas relative percentage $\pm$ standard deviation (SD). The FAMES of *L. garciae* lipid pulp and seed extracts were identified by matching the spectra against the National Institute of Standards and Technology (NIST) 17 mass spectral library which was incorporated to the data system (Samling et al., 2021).

3.5 Antimicrobial Assay

3.5.1 Preparation of Mueller-Hinton, Nutrient and Blood Agar Medium and Nutrient Broth

Mueller-Hinton agar (MHA) medium (Oxoid, UK) was prepared by solubilising 19 g of agar in 1000 mL of distilled water. Next, preparation of nutrient agar (Oxoid, UK) medium was performed by dissolving 28 g of agar powder in 1000 mL of distilled water. Each agar medium was boiled at 90°C and stirred well until the agar powder was totally dissolved and the agar medium was topped up to 1000 mL as the final volume. The agar medium was then sterilised by autoclaving (Tomy Seiko Autoclave SS-325, Japan) at 121°C for 15 min and let to cool to approximately 50°C. After that, the cooled agar medium was decanted into the petri dishes to a depth of 4 mm and was allowed to solidify at room temperature. The solidified agar plates were incubated overnight at 37°C for quality control purposes i.e., to ensure the media was prepared correctly and without any contamination. After checking for any possible contamination, the agar plates were sealed inside the airtight plastic bag in upside down position and placed in the 4°C fridge for future use. The agar plates were dried in a 37°C incubator for 15 min prior to use to eliminate the water droplets on the agar surface (Tendencia, 2004).

The preparation of blood agar medium was carried out by dissolving 40 g of blood agar base (Oxoid, UK) in 1000 mL of distilled water. Likewise, the procedure of blood agar preparation was similar to MHA and nutrient agar preparation. The only difference was the addition of 5% (vol/vol) sterile blood after the autoclaved agar medium was cooled down to 50°C to prevent haemolysis of blood and it was then mixed well before pouring into petri dishes.

Mueller-Hinton broth (MHB) (Oxoid, UK) was prepared by solubilising 21 g of MHB powder in 1000 mL of distilled water. The MHB was heated to boil and agitated frequently to dissolve the broth completely. The broth was adjusted to 1000 mL as the final volume. After that, it was autoclaved at 121°C for 15 min and was allowed to cool to room temperature. The broth was aliquoted by dispensing 3 mL of broth into bijou bottles using a repeater pipette. Then, they were kept at 4°C fridge until further use.

3.5.2 Isolation of *S. aureus*, *S. epidermidis*, *E. coli* and *P. aeruginosa* Pure Culture

The pure cultures of bacteria used in this study were obtained from the Multipurpose Laboratory of Faculty of Medicine and Health Sciences, Universiti Malaysia Sarawak. The Gram-positive bacteria *Staphylococcus aureus* (ATCC 700698) and *Staphylococcus epidermidis* (ATCC 12228), and the Gram-negative bacteria *Escherichia coli* (ATCC 11775) and *Pseudomonas aeruginosa* (ATCC 27853) were isolated using the four-streak method (Cox et al., 2017).

The isolated colonies were first cultured on a blood agar plate followed by subcultured on a nutrient agar plate. Firstly, a loopful of isolated colonies was inoculated gently onto the first quadrant of the blood agar plate's surface in a back and forth (zig-zag) motion. Next, the edge of first quadrant was streaked perpendicularly into the second quadrant and subsequently extended the streaks from the second quadrant to the third quadrant. The fourth quadrant was formed by streaking from the third quadrant into the centre of the blood agar plate. The streaking pattern was shown in Figure 3.3. The inoculated blood agar plates were covered with lids and incubated overnight at 37°C in an inverted position. Next, a loopful of isolated colonies from the blood agar plate was subcultured to the nutrient agar plate using the same procedures as above. The fresh culture obtained from

nutrient agar plate was used within 24 hours for the inoculum preparation (Ruangpan & Tendencia, 2004).

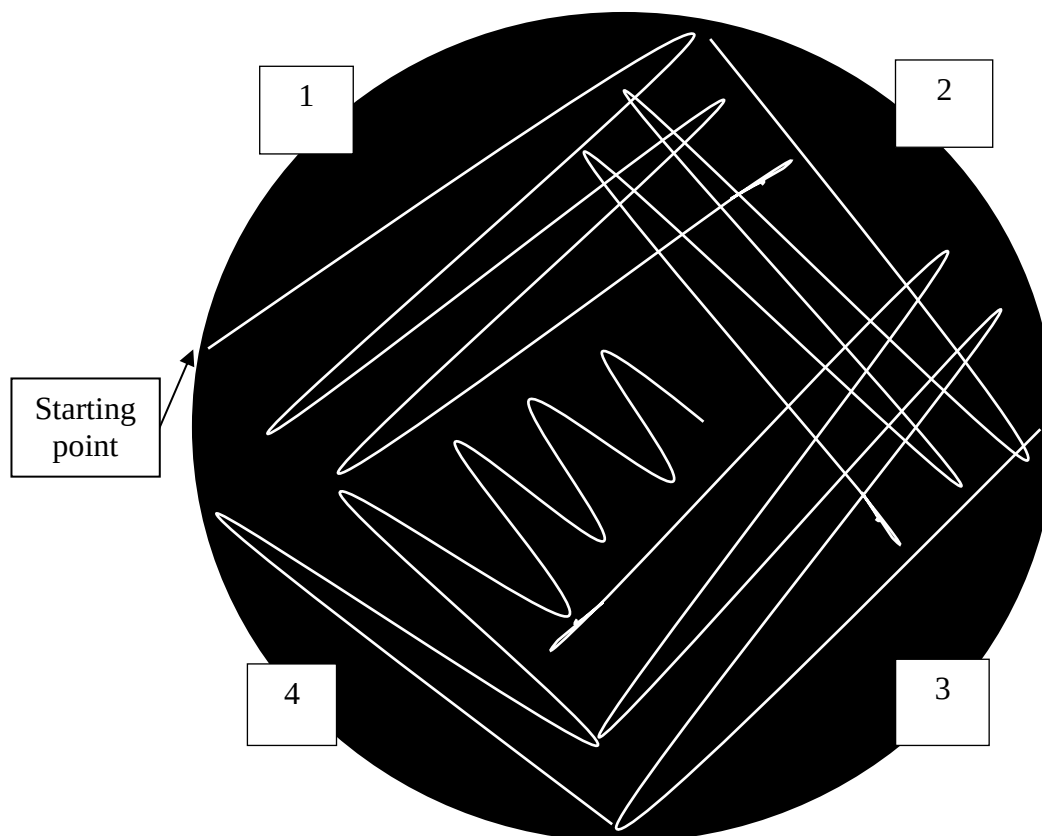


Figure 3.3: Streaking pattern for pure culture isolation of bacteria using four-streak method.

3.5.3 Preparation of Inoculum using Growth Method

Growth method was conducted based on the Clinical and Laboratory Standards Institute - Tenth Edition for inoculum preparation. Three to five well-isolated colonies with similar morphology were selected from the agar plates of two strains of Gram-positive bacteria *Staphylococcus aureus* and *Staphylococcus epidermidis* and two strains of Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*. The colonies from each bacterial agar plate were inoculated in a bijoux bottle containing 3 mL of MHB using a sterile inoculating loop. The broth culture was incubated at 37°C for 3 hours to achieve the turbidity

of 0.5 McFarland standard. The turbidity of 0.5 McFarland standard at OD₆₂₅ of between 0.08 to 0.13 was equivalent to bacterial suspension of approximately $1 \text{ to } 2 \times 10^8$ CFU/mL. (CLSI, 2015). The sterile broth was added to further dilute the bacterial suspension. The final inoculum of 1×10^8 CFU/mL must be used within 15 min for the inoculation of agar plates or broth. For broth microdilution assay, the final inoculum was further diluted by 1:100 dilution to form approximately 1×10^6 CFU/mL of inoculum (CLSI, 2015).

3.5.4 Preparation of Diluted Sample

For the disk diffusion assay, the stock solution of six sample extracts with the concentration of 1 g/mL were prepared in pure dimethyl sulfoxide (DMSO) (Amresco®, USA). After that, a stock solution of the sample was further diluted to 200, 400, 600 and 800 mg/mL using DMSO in respective bijou bottles and stored at -20°C. Moreover, the stock solution for each sample at the concentration of 100 mg/mL was used for broth microdilution assay. Two-fold serial dilutions were carried out in the 96-well microplate in which the first well was the stock solution. The stock solution was then dispensed to other wells across the column, which generated 50, 25, 12.5, 6.25, 3.125, 1.57 and 0.78 mg/mL solutions.

3.5.5 Disk Diffusion Assay

The antimicrobial susceptibility of methanolic, petroleum ether and Bligh and Dyer *L. garciae* pulp and seed extracts were determined using the disk diffusion assay (Figure 3.4), by referring to the procedure of Priscilia et al. (2020) and Clinical and Laboratory Standards Institute M02-A12 (CLSI, 2015). An aseptic technique was conducted throughout the process. The 5.5 mm Whatman filter paper disks were prepared and sterilised prior to use. Next, the paper disks were impregnated with 10 µL of sample extracts of different concentrations, a control (100% DMSO) and negative control (0.1% (v/v) Trifluoroacetic

acid, TFA) respectively. The final concentrations of extracts were 200, 400, 600, 800 and 1000 µg/mL. The standard antimicrobial susceptibility disk (antibiotic) chloramphenicol (30 µg; Thermo Scientific™ Oxoid™, UK) was the positive control for *E. coli* and *S. aureus* while for *P. aeruginosa* and *S. epidermidis*, the positive control used was gentamicin (10 µg; Thermo Scientific™ Oxoid™, UK).

Besides, the final inoculum ($1 \text{ to } 2 \times 10^8$ CFU/mL) that had achieved 0.5 McFarland standard turbidity was inoculated onto the surface of MHA plate using a sterile cotton swab. The inoculum was swabbed over the entire agar surface by streaking it twice, where the plate was rotated by 60° in order to distribute the inoculum evenly. Likewise, the rim of agar was swabbed to obtain a uniform growth of the bacterial lawn around the edge of agar. Then, agar plates were then let to dry for 5 min by leaving the lid open. Next, the impregnated paper disks and antibiotic disks were pressed gently onto the inoculated agar plate surface using a pair of sterile forceps for a thorough contact with the agar. Thus, each agar plate had six disks comprising of sample of five concentrations disks and an additional disk of either the control, positive or negative control. The plates were then inverted and incubated at 37°C for 18 hours. After 18 hours, the diameters of the zone of inhibition were measured with a ruler (Figure 3.5).

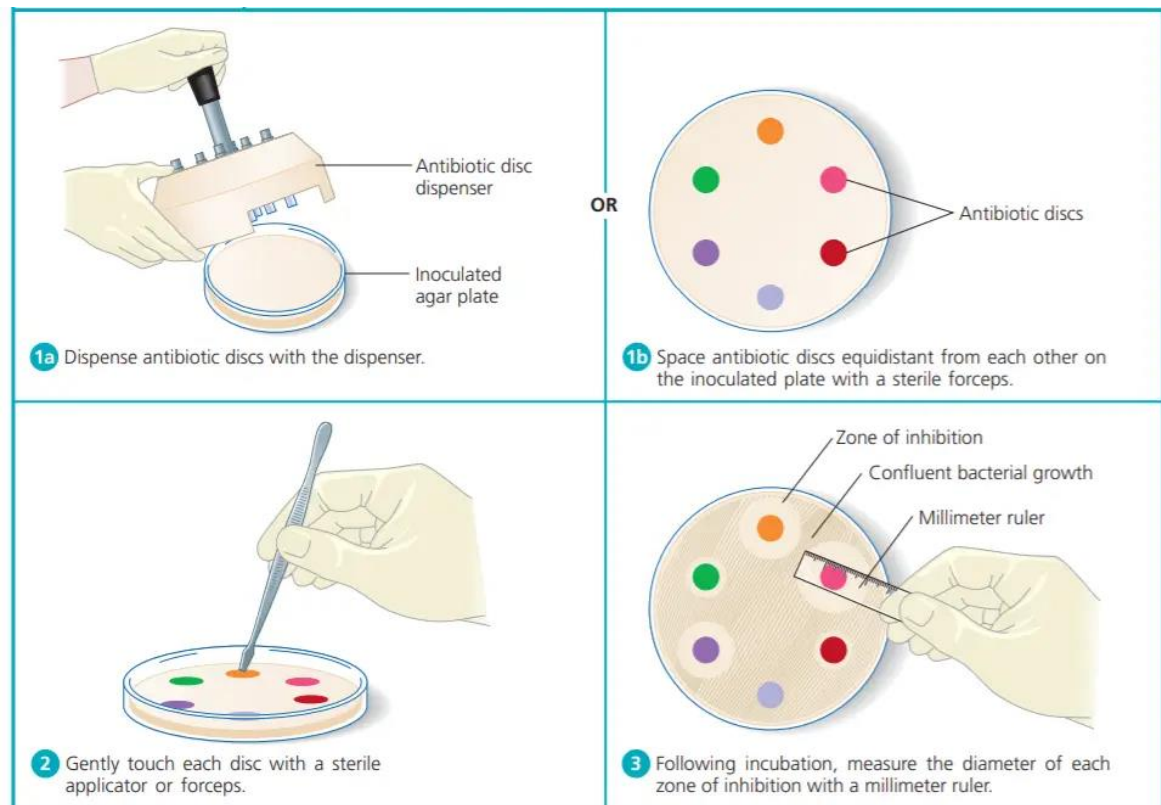


Figure 3.4: Procedure of the disk diffusion assay (Tankeshwar, 2022).

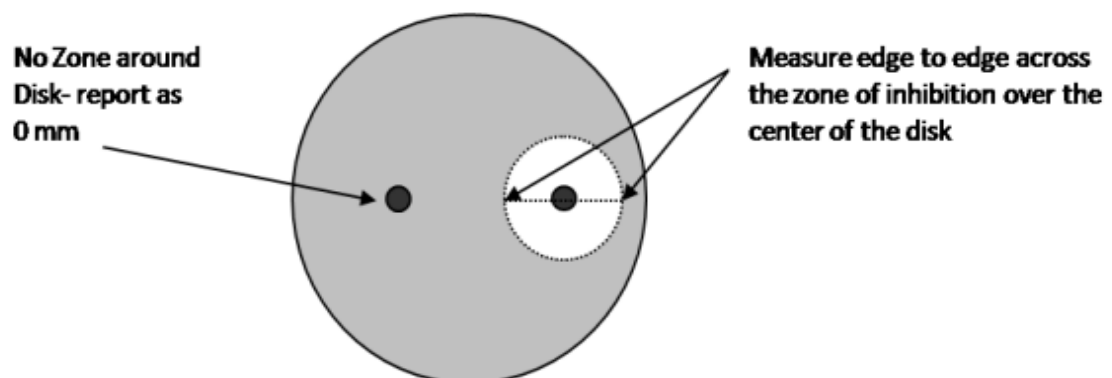


Figure 3.5: Measurement of the zone of inhibition diameter (American Society for Microbiology, 2016).

3.5.6 Broth Microdilution Assay

The evaluation of minimum inhibitory concentrations (MICs) of methanolic, petroleum ether and Bligh and Dyer *L. garciae* pulp and seed extracts against the bacteria (*S. aureus*, *S. epidermidis*, *E. coli* and *P. aeruginosa*) were performed using the broth microdilution assay that was adapted from the procedure of Clinical and Laboratory Standards Institute M07-A10 (CLSI, 2015) and Sundar & Koilpillai (2015). Throughout the process, the aseptic technique was utilised. In a 96-well microplate, 50 μL of Mueller-Hinton broth (MHB) was pipetted into B1 to H9 (rows B to H, columns 1 - 9) as illustrated in Figure 3.6. A volume of 100 μL of sample extract with concentration of 100 mg/mL was dispensed into the row A wells. Subsequently, 50 μL of sample extract and MHB mixture was pipetted into the next row well and mixed well. By establishing a serial dilution, the mixture was pipetted into the next row to form a concentration range of 50, 25, 12.5, 6.25, 3.13, 1.57, 0.78 and 0.39 mg/mL. Besides, the sterility control (uninoculated) wells were filled with 100 μL of MHB while 50 μL of MHB was dispensed into the respective wells which acted as a growth control.

In addition, 50 μL of 1×10^6 CFU/mL inoculum was inoculated into the sample extract and growth control wells. This resulted in 1:2 dilutions of both sample extracts and inoculum which brought the final concentration of extracts from 100 mg/mL to 50 mg/mL and the final inoculum to 5×10^5 CFU/mL. A lid was used to cover up the 96-well plate and it was sealed tightly with the parafilm. It was then incubated within 15 min of bacterial culture inoculation at 37°C for 18 hours. The MICs were determined by visual observation and reconfirmed with an ELISA microplate reader at 620 nm (OD_{620}). The turbidity indicated the growth of bacteria, and the turbidity of sample extracts was compared with the growth control for the determination of the lowest concentration for the sample with no

viability of bacteria. The sample extract well that was found with undetectable growth was recorded as the MIC for the respective sample extract.

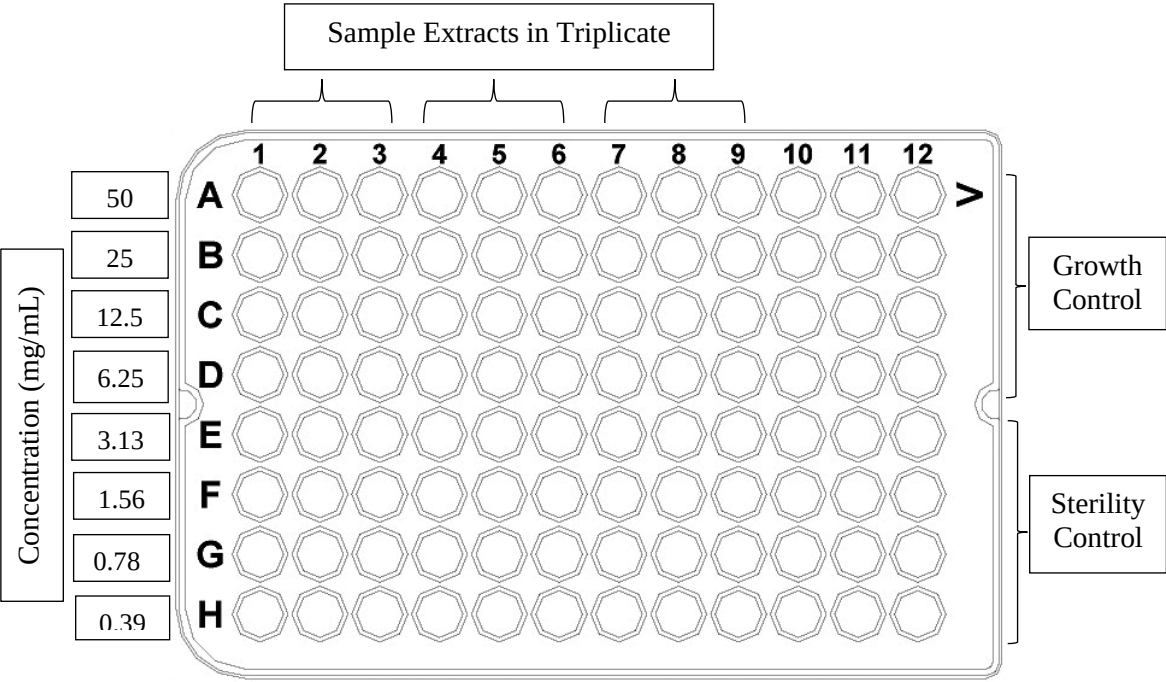


Figure 3.6: Illustration for the broth microdilution assay [Adapted from (Starlab, 2021)].

3.6 Cell Culture

3.6.1 Preparation for Cell Culture Work

Cell culture work was done in a class II biological safety cabinet (BSC) equipped with HEPA-filtered laminar airflow (Esco Micro Pte Ltd, Indonesia). The UV lamp of the BSC was switched on 30 min before commencing and after completing the cell culture work. The pipette tips, reagent reservoir and 1.5 mL microcentrifuge tubes were sterilised using an autoclave at 121°C for 15 min. The BSC and all the apparatus and materials were sterilised by spraying with 70% ethanol before putting them into the BSC. While for the cell culturing of fibroblasts, the cells were kept in the 5% CO₂ incubator at 37°C (Esco Scientific, Singapore).

The preparation of growth medium was performed using 500 mL of Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 supplemented with L-glutamine, sodium pyruvate and HEPES (Nacalai tesque, Japan), 50 mL of 10% foetal bovine serum (FBS) (Tico Europe, Netherlands), 5 mL of 1% penicillin-streptomycin mixed solution (Pan Biotech, Germany) and 1% of amphotericin B (Corning, USA). The growth medium was then aliquoted into 50 mL centrifuge tubes and stored at 4°C for further cell culture work.

3.6.2 Storage of Human Dermal Fibroblasts (HDF)

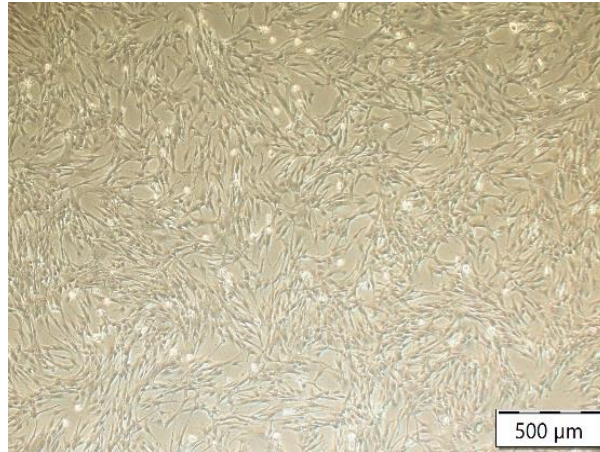
The fibroblast with cell's name OUMS-36T-4F and reference number JCRB1006.4F was obtained in a glass ampule from the Japanese Collection of Research Bioresources (JCRB) Cell Bank of National Institutes of Biomedical Innovation, Health, and Nutrition (NIBIOHN), Japan. Immediately, upon the arrival of the cell, the glass ampule was transferred to a liquid nitrogen storage tank where it was placed in the vapour phase of the liquid nitrogen tank.

3.6.3 Human Dermal Fibroblasts (HDF) Culture

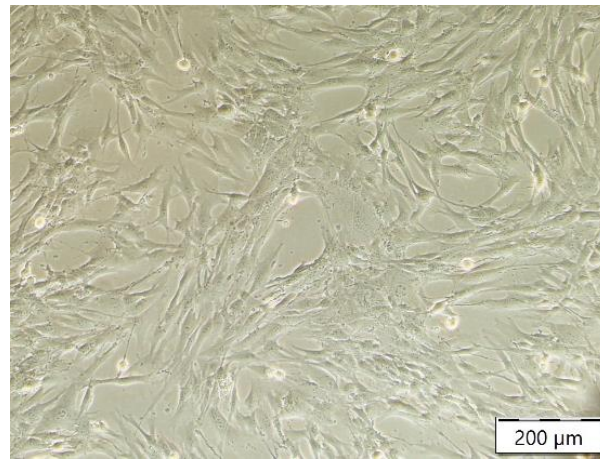
First Culturing of Fibroblasts

The glass ampule with fibroblasts was removed from the liquid nitrogen storage tank using safety gloves. It was then thawed for 2 min by shaking it in the 37°C water bath. Then, the entire surface of the glass ampule was sterilised with 70% ethanol. The neck of the glass ampule was wrapped with sterilised tissue and then snapped off under aseptic conditions. The fibroblast cell suspension was transferred to a 15 mL centrifuge tube and 10 mL of growth medium was added into the tube. The mixture was centrifuged at 1000 rpm for 5 min and the supernatant was discarded. The fibroblast cells were resuspended in 5 mL of growth medium by gently pipetting the cell suspension up and down a few times. The cells were cultured in a 25 cm² flask with a vented cap and the flask was shaken gently (north, south, east, west motion) for an even distribution of cells. The cells were incubated at 37°C in the incubator supplied with 5% CO₂ for 24 h (NIBIOHN JCRB Cell Bank, n.d.). The morphology of fibroblasts with cell density of 1×10^6 cells/mL was illustrated in Figure 3.7 after incubation for 24 h.

(a)



(b)



(c)

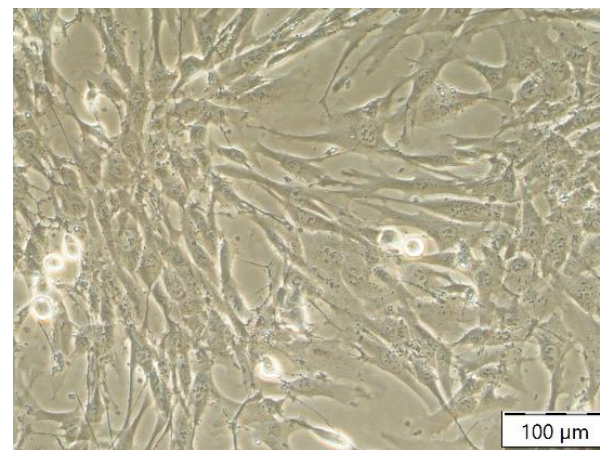


Figure 3.7: Morphology of fibroblasts under an inverted microscope (Olympus IX73) at (a) 40x, (b) 100x, (c) 200x magnification.

Subculturing of Fibroblasts

The cells were subcultured when the fibroblast cell culture had reached 80% confluency approximately after 24 h. The medium and trypsin-EDTA solution were pre-warmed in the water bath at 37°C. Under aseptic conditions, the used medium was removed from the 25 cm² culture flask using a serological pipette. Next, addition of 3 mL of Dulbecco's phosphate buffered saline (DPBS) (Nacalai tesque, Japan) to the culture flask was for the washing of the monolayer of cells. After the DPBS was discarded, addition of 1 mL of trypsin-EDTA solution (Nacalai Tesque, Japan) to the flask was done and it was incubated for 4 min at room temperature for the trypsinisation to take place. The flask was gently tapped to dissociate the cells from the flask bottom, and it was then observed under an inverted microscope (Olympus IX70, Olympus Optical Co., Ltd, Japan) to check if all the cells had been detached. The tapping was repeated at the side of the flask until all-rounded looking cells were detached from the flask bottom. Next, 5 mL of fresh growth medium was added into the flask and the cell suspension was transferred into a 15 mL centrifuge tube. The cell suspension was centrifuged at 1000 rpm for 5 min and the supernatant was discarded. Next, the cell pellet was re-suspended by adding 5 mL of medium with repeated gentle pipetting. The cell suspension was then cultured in a new 25 cm² culture flask and incubated at 37°C in a 5% CO₂ incubator (Merck, 2021).

Freezing of Fibroblasts

The freezing of fibroblast cells was performed when the cells reached 80% confluency. To prepare the freezing medium, growth medium was added with 5% of DMSO. Addition of 0.5 mL of DMSO to the growth medium was for the preparation of a 10 mL freezing medium. The freezing medium was sterilised using a 0.22 µm syringe filter

(Whatman[®], UK). The medium and trypsin-EDTA solution were pre-warmed in the water bath at 37°C while the freezing medium was cooled to room temperature.

Firstly, the removal of used medium from the culture flask was conducted using a serological pipette. Next, the monolayer cells were washed with 3 mL of DPBS. Then, 1 mL of trypsin-EDTA solution was added to the flask and incubated for 4 min at room temperature. The flask was gently tapped to detach the cells from the flask and rounded cells should be seen under the microscope (Olympus IX70, Olympus Optical Co., Ltd, Japan). Then, 5 mL of fresh medium was added to the flask and the cell suspension was then transferred into a 15 mL centrifuge tube. The tube was then centrifuged at 1000 rpm for 5 min. The supernatant was discarded, and the cell pellet was re-suspended in 1 mL of freezing medium per vial (Nacalai Tesques, n.d.). The cryovial was labelled properly with the cell name, passage number, date, and name. The cryovial with $1 \times 10^6 - 5 \times 10^6$ cells/mL of cell density was cryopreserved in the liquid nitrogen storage. The slow freezing method was performed which was from -20°C at 1 h, followed by -80°C for overnight and finally to -196°C of liquid nitrogen storage.

Fibroblast Cell Counting

One hundred μ L fibroblast cell suspension was transferred to a 1.5 mL microcentrifuge tube and 200 μ L of DPBS and 200 μ L of 0.4% trypan blue solution (Sigma-Aldrich, UK) were added to the tube. This made up a 5x dilution for cell counting. The cell suspension mixture was mixed evenly by gently suspending the solution up and down. It was then let to stand for 3 min at room temperature for 3 min before doing the cell counting.

The hemacytometer and cover glass were sterilised using 70% ethanol and a Kimwipe[®] was used to wipe away excess fluid. The cover glass was placed on the

hemacytometer and slid back and forth to remove air pockets. After that, the cover glass was pressed against the hemacytometer until a Newton's ring was seen. The stained cell suspension mixture was injected into both chambers of the hemacytometer using a 100 μL micropipette. Approximately 50 μL of cell suspension mixture was transferred in between the hemacytometer and cover glass. This step was done gently to avoid air bubbles and over-injection of cells that would cause miscounting of cells. The number of cells was counted on the four corners and the centre of a hemacytometer using a cell counter (Figure 3.8). The cells in each of the 1 mm^2 partitions were counted with a microscope which is set at 100x magnification. For the cells that were located on the lines, the cells that were included for counting were the cells on the upper and right sidelines (Nacalai Tesques, n.d.). The number of viable cells in original cell suspension was calculated using the following formula:

$$\begin{aligned} \text{Number of viable cells} &= \frac{\text{total counted number of viable cells}}{\text{counted number of partitions}} \times 10^4 \times \text{dilution ratio} \\ &= \frac{\text{total counted number of viable cells}}{5} \times 10^4 \times 5 \end{aligned} \quad \text{Equation 3.2}$$

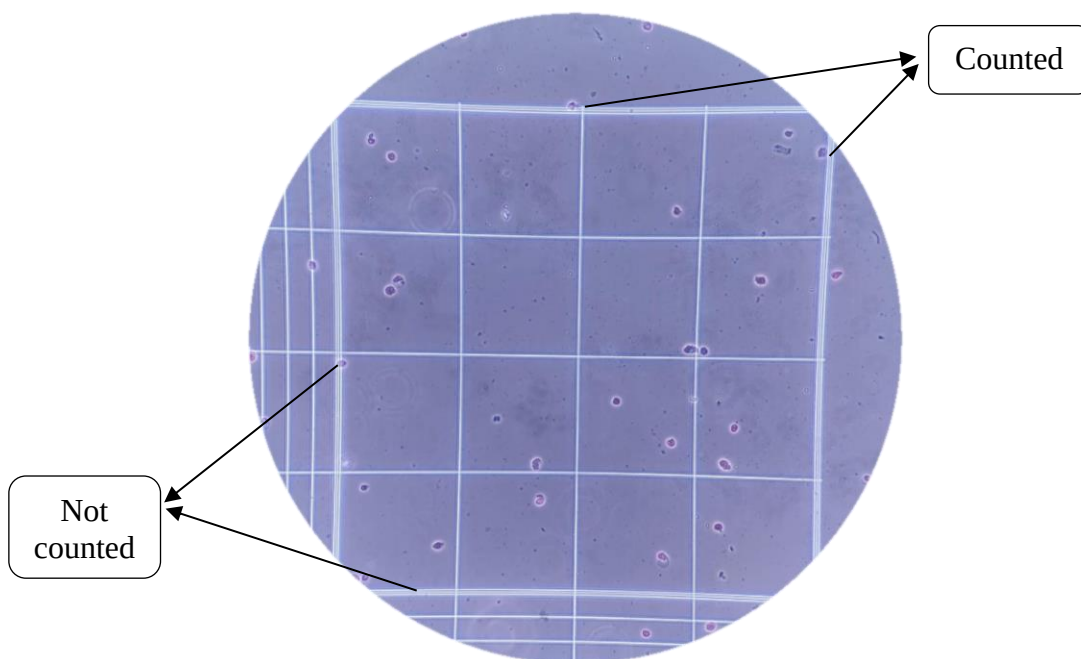


Figure 3.8: Illustration for one of the corners of a hemacytometer.

Fibroblast Cell Seeding Density

The desired cell seeding density was pre-determined before conducting the cell culture work. The total cells/mL were counted using a hemacytometer in which the trypan blue solution was used to mix with the cell suspension. The volume of cell suspension required and the volume of medium required to make up the desired cell density were calculated using the following formula:

$$(1) \text{ Volume of cell suspension needed (mL)} = \text{Total number of wells} \times \frac{\text{Desired cell density}}{\text{Total cells/mL}}$$

$$(2) \text{ Total volume of medium needed (mL)} = \text{Total number of wells} \times \text{Volume per well}$$

$$(3) \text{ Volume of medium needed to top-up (mL)} = \text{Total volume of medium needed} - \text{Volume of cell suspension needed}$$

Equation 3.3

Fibroblast Cell Seeding Density Optimisation

The optimisation of fibroblast cell seeding density was evaluated using the MTT assay. The seeding of fibroblast cells in a 96-well plate was performed with different cell densities, which were 2.5×10^3 , 2.5×10^4 and 2.5×10^5 cells/well. The cells were cultured for 24 h at 37°C in a 5% CO₂ incubator until the confluency of 80% was reached. The used growth medium was replaced with the 180 µL fresh growth medium and 20 µL of 5 mg/mL MTT solution, followed by 3 h of incubation in a CO₂ incubator. The supernatant was aspirated after incubation and 100 µL DMSO was used to solubilise the formazan crystals formed by incubating in an orbital shaker at 37 °C for 15 min. The absorbance was measured at 570 nm using a microplate reader. A well with DMSO was acted as a blank. The experiment was done in triplicate.

3.7 Cell Viability Analysis by MTT Assay

3.7.1 Solutions for MTT Assay

The preparation of 5 mg/mL MTT solution was done through diluting 150 mg of MTT powder in 30 mL of DPBS. The MTT solution was vortexed and sterilised by sterile filtration. It was then stored at - 20°C in a 15 mL tube and wrapped with aluminium foil.

3.7.2 Cytotoxicity Tests for Extracts, Allantoin and DMSO

The cytotoxicity of DMSO, allantoin (positive control) and *L. garciae* extracts on fibroblast cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Amresco®, USA) (Bolla et al., 2019; Li et al., 2019). The seeding of cells at a density of 2.2×10^4 cells/well in 200 µL growth medium was conducted in a 96-well plate. All the outer wells of the 96-well plate were filled with sterilised water to prevent an edge effect which was an evaporation of the medium that could probably affect the results. Therefore, only 60 wells were used for experiments. The cells were then cultured for 24 h at 37°C. The used growth medium was discarded from the wells using a vacuum aspirator. The washing of cells was performed using DPBS prior to the addition of a fresh growth medium and DMSO, allantoin or extracts.

Cytotoxicity of DMSO on fibroblasts was determined using a range of DMSO concentrations which were 0.05, 1.25, 2.5, 5 and 10%. While cytotoxicity of allantoin on fibroblasts was tested using a range of concentrations of 50, 100, 125, 200, 250 and 500 µg/mL. For the screening of the non-toxic concentration of methanolic, petroleum ether and Bligh and Dyer *L. garciae* pulp and seed extracts, the concentrations such as 31.25, 62.5, 125, 250 and 500 µg/mL were used. The extracts were prepared fresh before the experiment. The additional concentrations (7.5 and 15 µg/mL) for the petroleum ether pulp and seed

extracts and Bligh and Dyer seed extract were determined after the cytotoxicity screening of these extracts.

A volume of 180 μ L growth medium and 20 μ L of DMSO, allantoin or extracts were pipetted into the 96-well plate. For the negative control, there was an addition of 200 μ L of growth medium to the wells. Then, the cells were incubated with DMSO, allantoin or extracts for 24 h at 37°C. The used growth medium was aspirated after 24 h and the cells were washed by DPBS. A volume of 180 μ L fresh growth medium and 20 μ L of 5 mg/mL MTT solution were added to the wells and incubated for 3 h at 37°C. After 3 h, the supernatant was discarded and then 100 μ L of DMSO was added to solubilise the formazan crystals. The plate was placed in an orbital shaker at 37 °C for 15 min to ensure that the formazan crystals were totally solubilised. Then, the absorbance was measured at 570 nm using a microplate reader where DMSO was used as a blank. The experiment was repeated 6 times and the data were presented as mean $\pm$ SD (n = 6). The negative control was considered as 100% viable. The percentage of cell viability was calculated using the formula:

$$\text{The percentage of cell viability} = \frac{\text{Mean absorbance of test sample}}{\text{Mean absorbance of negative control}} \times 100\%$$

Equation 3.4

3.8 Investigation on the Wound Healing Potency of *Litsea garciae* Extracts

3.8.1 Wound Scratch Assay

The effect of *L. garciae* pulp and seed extracts on fibroblast cell migration was evaluated using the scratch assay (Buranasukhon et al., 2017; Ado et al., 2013). The seeding of fibroblast cells at a density of 1×10^5 cells/well in a 12-well plate was then cultured in 1 mL of growth medium for 24 h at 37°C, 5% CO₂ incubator. The fibroblast cell monolayer which had achieved 80% confluence after 24 h incubation was scratched gently with a 200 µL sterile pipette tip. Then, aspiration of the used medium was done using a vacuum aspirator and the washing of cells were conducted once with DPBS for the removal of cell debris.

Then, the cells were then treated with methanolic, petroleum ether and Bligh and Dyer *L. garciae* pulp and seed extracts at different concentrations depending on the non-toxic concentration of each extract evaluated from the cytotoxicity assay. For methanolic pulp and seed extracts, and Bligh and Dyer pulp extract, the concentrations of 31.25, 62.5 and 125 µg/mL were used. For petroleum ether pulp and seed extracts, and Bligh and Dyer seed extract, lower concentrations (7.5, 15 and 31.25 µg/mL) were utilised. Allantoin at 125 µg/mL (Sigma-Aldrich, USA) was used as a positive control while the growth medium was used as a negative control. All the extracts and allantoin were pre-diluted with the serum-reduced medium (5% of serum) to suppress the fibroblasts proliferation.

After the addition of 1mL media containing the extract, allantoin or negative control, the 12-well plate was then incubated for 24 h at 37°C in a 5% CO₂ incubator. The scratched area was monitored over 48 h for closure of wound area. The images of wound closure were captured at intervals of 0, 6, 12, 24 and 48 h using a digital camera (Nikon Eclipse TS100,

Nikon Instrument Inc, USA) connected to the inverted microscope. The area of wound closure was measured using the built-in software of Nikon camera. The experiments were performed in triplicate and the percentage of wound closure was calculated and compared with baseline value using the equation stated below:

$$\% \text{ Wound closure} = \frac{W_{0h} - W_{xh}}{W_{0h}} \times 100\% \quad \text{Equation 3.5}$$

W_{0h} = Wound at 0 hour

W_{xh} = Wound at 'x' hour; 'x' = 0, 6, 12, 24 and 48 hours

3.8.2 Transwell Assay

3.8.2.1 Preparation of 4% Paraformaldehyde (PFA)

The preparation of 4% PFA was done in a fume hood. A volume of 80 mL of DPBS was heated until 60°C in a beaker and 4 g of PFA (Sigma-Aldrich, USA) was added and stirred until dissolved. The mixture turned cloudy after addition of PFA and thus, 1N NaOH was added dropwise to form a clear solution. The solution was removed from the heat and the pH of solution was adjusted to 7.4 using 1N HCl. The solution was made up to 100 mL using DPBS and was aliquoted and stored at – 20°C until use.

3.8.2.2 Preparation of 0.2% Crystal Violet Solution

The 1% crystal violet stain (Gram crystal violet, Thermo Scientific™ Remel™, USA) was diluted to 0.2% crystal violet solution using DPBS.

3.8.2.3 Transwell Assay

The evaluation of transwell assay was for the detection of chemotactic mobility of the *L. garciae* pulp and seed extracts on fibroblast cells (Mapoung et al., 2021; Li et al.,

2019; ThermoFisher Scientific, 2021). This assay was conducted in the 6-well transwell plate (Thermo Scientific™, USA) which consisted of the upper and bottom chambers. The upper chamber of the polycarbonate cell culture insert contained an 8-micron pore size (Figure 3.9). Methanolic, petroleum ether and Bligh and Dyer *L. garciae* pulp and seed extracts with a concentration of 31.25 µg/mL were diluted with the complete medium (with 10% of foetal bovine serum). Two types of negative controls were employed which were complete medium and serum-free medium (SFM).

Before the transwell assay, fibroblasts were cultured with a SFM for 24 h. Serum starvation was required to increase the sensitivity of cells towards chemoattractant and hence improve cell migration. Then, the addition of 1 mL of serum starved fibroblast cell suspension with a density of 3×10^5 cells/well to the upper chamber of the transwell plate was carried out. Then, a volume of 2 mL of extracts (which had been diluted with a complete medium) or negative controls were added into the bottom chamber and the cells were incubated for 24h at 37°C. Next, the media was aspirated from the upper and lower chambers, followed by washing the chambers with DPBS.

The cells that had not migrated and were still attached to the upper surface of the membrane were gently removed with cotton swabs. The migrating cells on the lower surface of the microporous membrane were fixed with 4% PFA solution at room temperature for 15 min. Addition of 1 mL of 4% PFA to the upper chamber was performed while the bottom chamber was filled with 2 mL of 4% PFA. Both sides of the membrane were washed with DPBS twice after fixing. The membranes were then stained with 0.2% crystal violet solution for 10 min where the upper chamber was filled with 1 mL of 0.2% crystal violet solution while the bottom chamber was filled with 2 mL of the staining solution. The staining solution

was discarded and washed thoroughly with distilled water. The images of stained cells on the lower surface of the membrane were captured under phase-contrast microscopy (Olympus IX73, Olympus Corporation, Japan). The number of cells that migrated through the microporous membrane was calculated and the experiments were performed in triplicate.

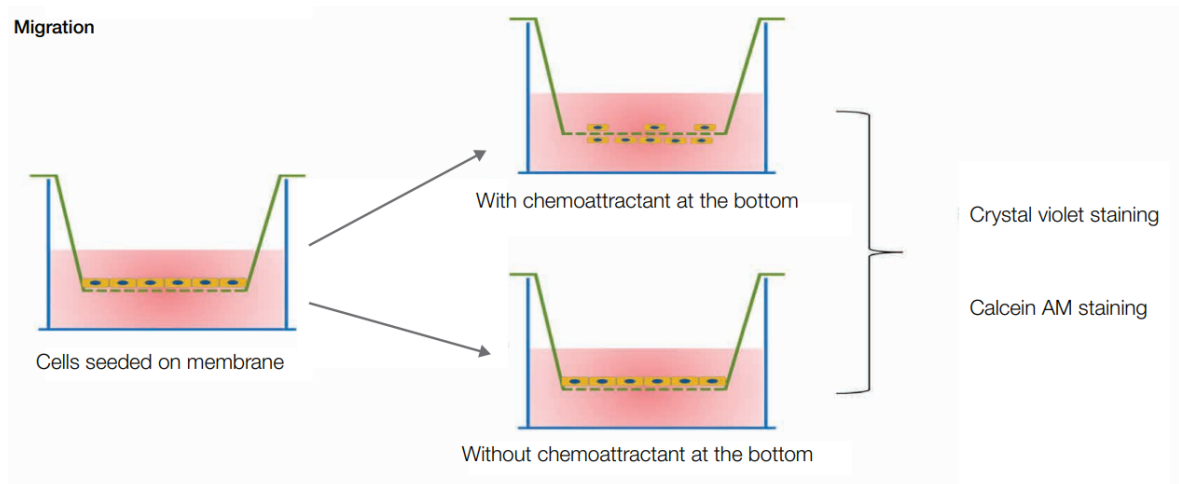


Figure 3.9: Illustration for the transwell migration assay (ThermoFisher Scientific, 2021).

3.8.3 Collagen 1 ELISA

Collagen 1 in fibroblasts was quantified with human Pro-Collagen I alpha 1 SimpleStep ELISA® Kit (ab210966) (Abcam, UK) according to the manufacturer's instructions (Abcam, 2021). This ELISA kit consisted of sample diluent NS, human Pro-Collagen I alpha 1 lyophilised recombinant protein (standard), 10X human Pro-Collagen I alpha 1 capture antibody, 10X human Pro-Collagen I alpha 1 detector antibody, antibody diluent CPI, 10X wash buffer PT, 3,3',5,5'-Tetramethylbenzidine (TMB) development solution, stop solution, pre-coated 96 well microplate, and plate seal.

The fibroblast cells were cultured in a 12-well plate with a seeding density of 1×10^5 cells/well incubated at 37°C for 24 h. The effect of pulp and seed of *L. garciae* extracts, allantoin and negative control on the expression of collagen type I was assessed by treating the fibroblast cells with different concentrations (according to the wound scratch assay) of methanolic, petroleum ether and Bligh and Dyer *L. garciae* pulp and seed extracts for 24 h. Then, the growth medium was collected into a 1.5 mL microcentrifuge tube and then centrifuged at 1000 rpm for 5 min at 4°C. The supernatants of cells were then aliquoted and stored at -80°C for further analysis.

The standards were prepared according to the manufacturer's instruction. Firstly, the human Pro-Collagen I alpha 1 standard was reconstituted with 1 mL of sample diluent NS. The mixture was mixed gently and left at room temperature for 10 min. This made the 31250 pg/mL stock standard solution. Next, eight tubes of 1.5 mL microcentrifuge tubes were prepared and labelled as standards 1-8. In the tube labelled as standard 1, 486 µL of sample diluent NS was added and 150 µL of sample diluent NS was added into the tubes labelled as standards 2-8. Then, a volume of 32 µL of 31250 pg/mL stock standard solution was pipetted

into the standard 1 and 150 μL of solution was pipetted from standard 1 into standard 2. A serial dilution was performed from standards 1 - 7 which produced the working standards with concentrations of 0, 31.25, 62.5, 125, 250, 500, 1000 and 2000 pg/mL . The standard 8 was the blank control with 0 pg/mL of protein.

Next, 3 mL antibody cocktail was prepared by combining 300 μL 10X capture antibody, 300 μL 10X detector antibody and 2.4 mL antibody diluent CPI with a thorough mixing. The preparation of 50 mL 1X wash buffer PT was by diluting the 5 mL 10X wash buffer PT with 45 mL distilled water and mixed thoroughly.

The cell supernatants were diluted with the sample diluent in a ratio of 1:200. The 50 μL cell supernatants or working standards were added into the 96 well-plate. The 50 μL antibody cocktail was then added to the wells and the plate was sealed and incubated at room temperature for 1 h on an orbital shaker set at 130 rpm. After incubation, the media were aspirated, and the wells were washed with 350 μL 1X wash buffer PT for three times. The plate was inverted and tapped gently against the clean paper towel during each wash to remove excess liquid. Next, 100 μL TMB development solution was added to the wells and the plate was incubated at room temperature for 10 min in the dark on an orbital shaker set at 130 rpm. Then, 100 μL of stop solution was added into the wells to enhance the signal intensity and the plate was shaken on an orbital shaker for 1 min to enable proper mixing of the mixtures. The absorbance was measured at 450 nm using an ELISA microplate reader (Molecular Devices SpectraMax iD3, USA) with an endpoint reading setting. All the standards were assayed in duplicate, while controls and samples were assayed in triplicate.

3.8.4 Secretion of EGF

The secretion of the epidermal growth factor (EGF) level was detected by enzyme-linked immunosorbent assay (ELISA) (Li et al., 2019). The EGF secretion level in cell supernatants was measured using the Human EGF ELISA Kit (RAB0149) (Sigma-Aldrich, USA). This ELISA kit comprised of sample diluent buffer A (1X), sample diluent buffer B (5X), lyophilised human EGF protein standard, colorimetric TMB reagent (Horseradish peroxidase (HRP) substrate), 20X wash buffer, stop solution, HRP-Streptavidin, biotinylated human detection antibody, pre-coated 96 well microplate and plate seal.

The fibroblast cells were grown at a density of 1×10^5 cells/well in 1 mL of growth medium in a 12-well plate and incubated in 5% CO₂ incubator at 37°C for 24 h. The different concentrations (according to the wound scratch assay) of methanolic, petroleum ether and Bligh and Dyer *L. garciae* pulp and seed extracts were added into 12-well plates and incubated at 37°C with 5% CO₂ for 24 h. The growth medium with *L. garciae* extracts was transferred into a 1.5 mL microcentrifuge tube and centrifuged at 1000 rpm for 5 min at 4°C to remove the debris. The collected cell supernatants were aliquoted and stored at -80°C for future use. The aliquoted cell supernatants were diluted into the sample diluent buffer in a ratio of 1:2. Moreover, 60 mL 1X wash buffer was prepared by mixing the 3 mL 20X wash buffer with 57 mL distilled water.

The standard was prepared by first reconstituting lyophilised EGF with 1000 µL of 1X sample diluent buffer B. The mixture was left to stand at the room temperature for 10 min and was then mixed thoroughly and gently to form the 130 ng/mL stock standard solution. Eight 1.5 mL microcentrifuge tubes were labelled as standards 1-8. Then, 1298 µL sample diluent buffer B was added to tube labelled as standard 1 and 300 µL of sample

diluent buffer B was added into tubes labelled as standards 2-8. From the 130 ng/mL stock standard solution, 2 μ L solution was pipetted into the standard 1 and a series of dilution was performed by transferring 200 μ L of solution from standards 1 - 7. The concentrations of working standard were 0, 0.82, 2.05, 5.12, 12.8, 32, 80 and 200 pg/mL. The standard 8 was acted as the blank control without protein.

All the reagents and cell supernatants were thawed to room temperature before use. The 100 μ L cell supernatants from *L. garciae* extracts, allantoin and negative control or working standards were added into the 96 well-plate. The wells were covered and incubated at room temperature for 2.5 h with gentle shaking on an orbital shaker. The solution was aspirated, and the wells were washed with 300 μ L 1X wash buffer for 4 times. For every wash, complete removal of the remaining wash buffer was done, and the plate was inverted and blotted against the clean paper towel. Next, 100 μ L of 1X prepared detection antibody was added to the wells and the wells were covered up and incubated at room temperature for 1 h with gentle shaking. The solution was discarded, and the wells were washed with 300 μ L 1X wash buffer for 4 times. Then, 100 μ L of streptavidin solution was added to the wells, followed by incubation, and shaking of the plate for 45 min at room temperature. The discarding and washing steps were repeated before the addition of 100 μ L of TMB one-step substrate reagent to the wells. The 96-well plate was covered and incubated at room temperature for 30 min in the dark condition on an orbital shaker. A volume of 50 μ L stop solution was added into the wells and the absorbance was measured at 450 nm immediately using an ELISA microplate reader (Molecular Devices SpectraMax iD3, USA). All the standards and samples were assayed in triplicate (Merk, 2021).

3.8.5 Secretion of VEGF

The secretion of the vascular endothelial growth factor (VEGF) level was determined by enzyme-linked immunosorbent assay (ELISA) (Li et al., 2019). The VEGF secretion level in cell supernatants was quantified using the Human VEGF-A ELISA Kit (RAB0507) ELISA Kit (Sigma-Aldrich, USA). This ELISA kit consisted of sample diluent buffer A (1X), sample diluent buffer B (5X), lyophilised human VEGF protein standard, colorimetric TMB reagent (HRP substrate), 20X wash buffer, stop solution, HRP-Streptavidin, biotinylated human detection antibody, pre-coated 96 well microplate and plate seal.

The fibroblast cells with density of 1×10^5 cells/well were cultured in 1 mL of growth medium in a 12-well plate and incubated at 37°C for 24 h. The methanolic, petroleum ether and Bligh and Dyer *L. garciae* pulp and seed extracts with different concentrations were added into 12-well plates and incubated at 37°C with 5% CO₂ for 24 h. The growth medium containing *L. garciae* extracts were collected with 1.5 mL microcentrifuge tubes and centrifuged at 1000 rpm for 5 min at 4°C to remove the debris. The collected cell supernatants were aliquoted and stored at -80°C until use. The VEGF assay was conducted by diluting the aliquoted cell supernatants with sample diluent buffer in a ratio of 1:2. Next, a 60 mL 1X wash buffer was prepared by mixing the 3 mL 20X wash buffer with 57 mL distilled water.

The standard for VEGF was prepared by reconstituting lyophilised VEGF with 640 µL of 1X sample diluent buffer B. The mixture was mixed gently and thoroughly to generate 50 ng/mL of stock standard solution. Standards 1 - 8 tubes were prepared, and a microcentrifuge tube labelled as standard 1 was filled with 440 µL sample diluent buffer B while 400 µL of sample diluent buffer B was pipetted into tubes labelled as standards 2 – 7.

A volume of 60 μL stock standard solution was transferred to standard 1 tube and a series of working standards were formed by transferring 200 μL of solution from standard 1 – 7 tubes. The standard 8 was the blank control without protein. The working standard concentrations were 0, 8.23, 24.69, 74.07, 222.2, 666.7, 2000 and 6000 pg/mL .

All the reagents and cell supernatants were thawed to room temperature before use. Then, 100 μL of working standards or cell supernatants from *L. garciae* extracts, allantoin and negative control were added into the 96 well-plate. The wells were sealed and incubated at room temperature for 2.5 h with gentle shaking on an orbital shaker. After incubation, the solution was aspirated, and the wells were washed with 300 μL 1X wash buffer for 4 times. A complete removal of the wash buffer was performed by inverting the plate and the plate was blotted against the clean paper towel. Next, addition of 100 μL of 1X prepared detection antibody to the wells was done and the wells were sealed and incubated at room temperature for 1 h with gentle shaking. The solution was then discarded, and the wells were washed with 300 μL 1X wash buffer for 4 times. Next, 100 μL of streptavidin solution was transferred to the wells, which was followed by incubation and shaking of the plate for 45 min at room temperature. The wells were then washed for 4 times before the addition of 100 μL of TMB one-step substrate reagent to the wells. The plate was sealed and incubated in dark at room temperature for 30 min with gentle shaking. The reaction was stopped by adding 50 μL of stop solution into the wells and the absorbance was measured at 450 nm immediately using an ELISA microplate reader (Molecular Devices SpectraMax iD3, USA). All the standards and samples were assayed in duplicate (Merk, 2021).

3.9 Statistical Analysis

Each assay was performed at least in triplicate except for the VEGF ELISA assay which were done in duplicate. The data was presented as mean $\pm$ standard deviation of the mean (SD). The data was analysed statistically using the Statistical Package for the Social Science (SPSS). The data was analysed by using one-way Analysis of Variance (ANOVA) followed by Tukey's Honestly Significant Different (HSD) test, whereby $p < 0.05$ was set as a significance level. For the cell viability and VEGF ELISA assays, independent t-test was used to analyse the data and $p < 0.05$ indicated there was statistical significant difference.

CHAPTER 4

RESULTS

4.1 Extraction of Samples

The petroleum ether (PE), methanolic (ME) and Bligh and Dyer (BD) pulp and seed extracts of *L. garciae* were illustrated in Figure 4.1. It was shown that PE and BD pulp extracts exhibited similar colour which was green at room temperature (25°C) and greenish-brown at 40°C. On the contrary, the methanolic pulp extract was reddish-brown in colour at both room temperature and 40°C. All the pulp extracts were in a semi-liquid state and sticky with high viscosity. Furthermore, PE seed extract appeared to be white solid at room temperature while it turned into light yellowish semi-liquid at 40°C. For methanolic seed extract, dark brown semi-liquid of the extract was observed at both room temperature and 40°C. The BD seed extract was light yellowish colour solid at room temperature and changed to milky, dark yellowish semi-liquid after heating at 40°C. Table 4.1 showed the extraction yield in percentage of the *L. garciae* pulp and seed extracts. The extraction yield of the methanolic pulp extract was the highest (39.23%) as compared to another two extractions of pulp, which was followed by BD (32.68%) and PE (25.63%) pulp extracts. Besides, the highest extraction yield of seed was BD extraction (44.83%) while the PE extract showed quite similar yield to BD extract which was 41.70%. In contrast, methanolic seed extract exhibited the lowest extraction yield (5.90%) which was extremely lower than other extracts.

Room Temperature (25°C)		40°C	
Pulp	Seed	Pulp	Seed
			
Petroleum ether extracts			
			
Bligh and Dyer extracts			
			
Methanolic extracts			

Figure 4.1: Optical appearance of PE, ME and BD extracts of the pulp and seed of *L. garciae*. Pictures were taken at room temperature (25°C) and at 40°C.

Table 4.1: Extraction yield of the pulp and seed extracts of *L. garciae*.

Sample Extracts	Extraction yield (%)	Colour		Physical state	
		25°C	40°C	25°C	40°C
Petroleum ether pulp extract (PEPE)	25.63	Green	Greenish-brown	Semi-liquid, sticky with high viscosity	
Bligh and Dyer pulp extract (BDPE)	32.68	Green	Greenish-brown	Semi-liquid, sticky with high viscosity	
Methanolic pulp extract (MEPE)	39.23	Reddish-brown	Reddish-brown	Semi-liquid, sticky with high viscosity	
Petroleum ether seed extract (PESE)	41.70	White	Light yellowish	Solid	Semi-liquid, low viscosity
Bligh and Dyer seed extract (BDSE)	44.83	Light yellowish	Milky, dark yellowish	Solid	Semi-liquid, low viscosity
Methanolic seed extract (MESE)	5.90	Dark brown	Dark brown	Semi-liquid, low viscosity	

30 g of dried powder of *L. garciae* pulp and seed underwent extraction using petroleum ether, and Bligh and Dyer methods to obtain the total lipid and for its phytochemical components using methanol as solvent.

4.2 Fatty Acids Content

The lipids extracted using PE and BD solvents were analysed for fatty acid components using GC-MS. Results from GC-MS chromatograms (Figure 4.2 and Figure 4.3) showed that there were 22 fatty acids detected in PE and BD lipid pulp and seed extracts of *L. garciae*. Table 4.2 revealed the components of fatty acids found in *L. garciae* lipid extracts, however, not every extract consisted of same fatty acids content.

Generally, *L. garciae* lipid pulp extract contained a comparable proportion of saturated and unsaturated fatty acids (Table 4.2, Figure 4.4). The PE and BD pulp consisted of $49.90 \pm 0.85\%$ and $52.83 \pm 0.92\%$ of saturated fatty acids respectively. The unsaturated fatty acids were divided into monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs), where PE and BD pulp consisted of $41.46 \pm 4.09\%$ and $38.30 \pm 2.03\%$ of MUFAs while PUFAs were $7.21 \pm 0.00\%$ and $8.86 \pm 1.62\%$ respectively (Figure 4.4). The unsaturated fatty acids/saturated fatty acids ratio were 0.98 and 0.89 for PE and BD pulp extracts respectively.

On the other hand, the lipid seed extract comprised much higher saturated fatty acids than unsaturated fatty acids (Table 4.2 and Figure 4.5). The saturated fatty acids in PE seed were $89.33 \pm 3.28\%$ while BD seed contained $81.46 \pm 8.98\%$ of saturated fatty acids. For unsaturated fatty acids, PE and BD seed contained $7.96 \pm 3.63\%$ and $11.67 \pm 2.76\%$ of MUFAs whereas PUFAs were $2.71 \pm 0.40\%$ and $6.87 \pm 1.56\%$ respectively. The unsaturated fatty acids/saturated fatty acids ratio for PE and BD seed extracts were 0.12 and 0.23 respectively.

Both PE and BD lipid pulp extracts comprised of lauric acid, myristic acid, palmitic acid, margaric acid, stearic acid, arachidic acid, heneicosylic acid and behenic acid for

saturated fatty acids (Table 4.2). The predominant saturated fatty acids were palmitic acid, followed by stearic acid for both PE and BD pulp extracts. PE pulp extracts composed of $33.05 \pm 0.85\%$ palmitic acid and $8.97 \pm 0.65\%$ of stearic acid. Similarly, BD pulp extract consisted of $33.42 \pm 4.12\%$ of palmitic and $8.20 \pm 3.81\%$ of stearic acid. Moreover, there were some fatty acids that were undetected in PE extract but found in the BD pulp extracts and vice versa. For instance, capric acid and nonadecylic acid were not detected in PE pulp extract while there was a trace amount of these fatty acids in BD pulp extract. The undetected fatty acids in BD pulp extract were pentadecylic acid, tricosylic acid, and lignoceric acid whereas PE pulp extract contained a trace amount of them. Most of the fatty acids identified in PE and BD pulp extracts were of similar percentages except lauric acid which has a significantly lower percentage in PE extract ($0.11 \pm 0.01\%$) compared to BD pulp extract ($6.86 \pm 4.44\%$).

For the saturated fatty acids of seed extracts, both PE and BD lipid seed extracts constituted of capric acid, undecylic acid, lauric acid, tridecylic acid, myristic acid, pentadecylic acid, palmitic acid, stearic acid, nonadecylic acid and heneicosylic acid. The PE and BD lipid seed extracts had a higher content of lauric acid which were $75.31 \pm 5.25\%$ and $63.98 \pm 5.25\%$ respectively, followed by palmitic acid which were $7.39 \pm 2.51\%$ and $10.36 \pm 2.95\%$ respectively. Furthermore, the non-detected fatty acids for PE seed extract were tricosylic acid and lignoceric acid while there were small proportion found in BD seed extract. Behedic acid was detected in PE seed extract with a marginal amount whereas it was undetected in BD seed extract.

Besides, the highest proportion of MUFAs was represented by oleic acid for all the extracts. The PE and BD lipid pulp extracts contained $40.73 \pm 4.08\%$ and $37.71 \pm 1.95\%$ of

oleic acid respectively, while the PE and BD lipid seed extracts consisted of $7.73 \pm 3.56\%$ and $11.10 \pm 2.69\%$ respectively. Paullinic acid and brassidic acid were not detected in PE pulp extract while the BD pulp extract consisted of a trace level of them. A little percentage of margaroleic acid was detected in PE pulp extract, however, it was not found in BD pulp extract. In addition, there was a little percentage of brassidic acid detected in PE seed extract, whereas it was undetected in BD seed extract. Apart from that, the most abundant polyunsaturated fatty acid in all extracts was linoleic acid, in which both PE and BD lipid pulp extracts consisted of $7.21 \pm 0.00\%$ and $8.84 \pm 1.63\%$ whereas both seed extracts made up of $2.71 \pm 0.40\%$ and $6.87 \pm 1.56\%$. There was a negligible amount of arachidonic acid in BD pulp extract while it was not discovered in PE pulp and seed extracts and BD seed extract.

In summary, *L. garciae* lipid pulp and seed extracts were discovered to have a comparable fatty acids profile between the two types of lipid extraction (PE and BD methods). The percentage composition of fatty acids was not significantly different using these two different solvent extractions for both the pulp and seed extracts. Furthermore, there was no significant difference between the percentage of saturated fatty acid components and unsaturated fatty acids components ($p > 0.05$) for pulp extract (Figure 4.4). However, for the seed extract there was a significantly different percentage in the composition between the saturated and unsaturated fatty acids contents ($p < 0.05$) for both PE and BD seed extracts (Figure 4.5). This was probably contributed by the high percentage composition of saturated fatty acids, lauric acid. Furthermore, the seed extracts contained more saturated fatty acids than the pulp extract (Figure 4.6 (a)). Finally, the pulp extracts contained more monounsaturated and polyunsaturated fatty acids as compared to the seed extracts (Figure 4.6 (b) and (c)).

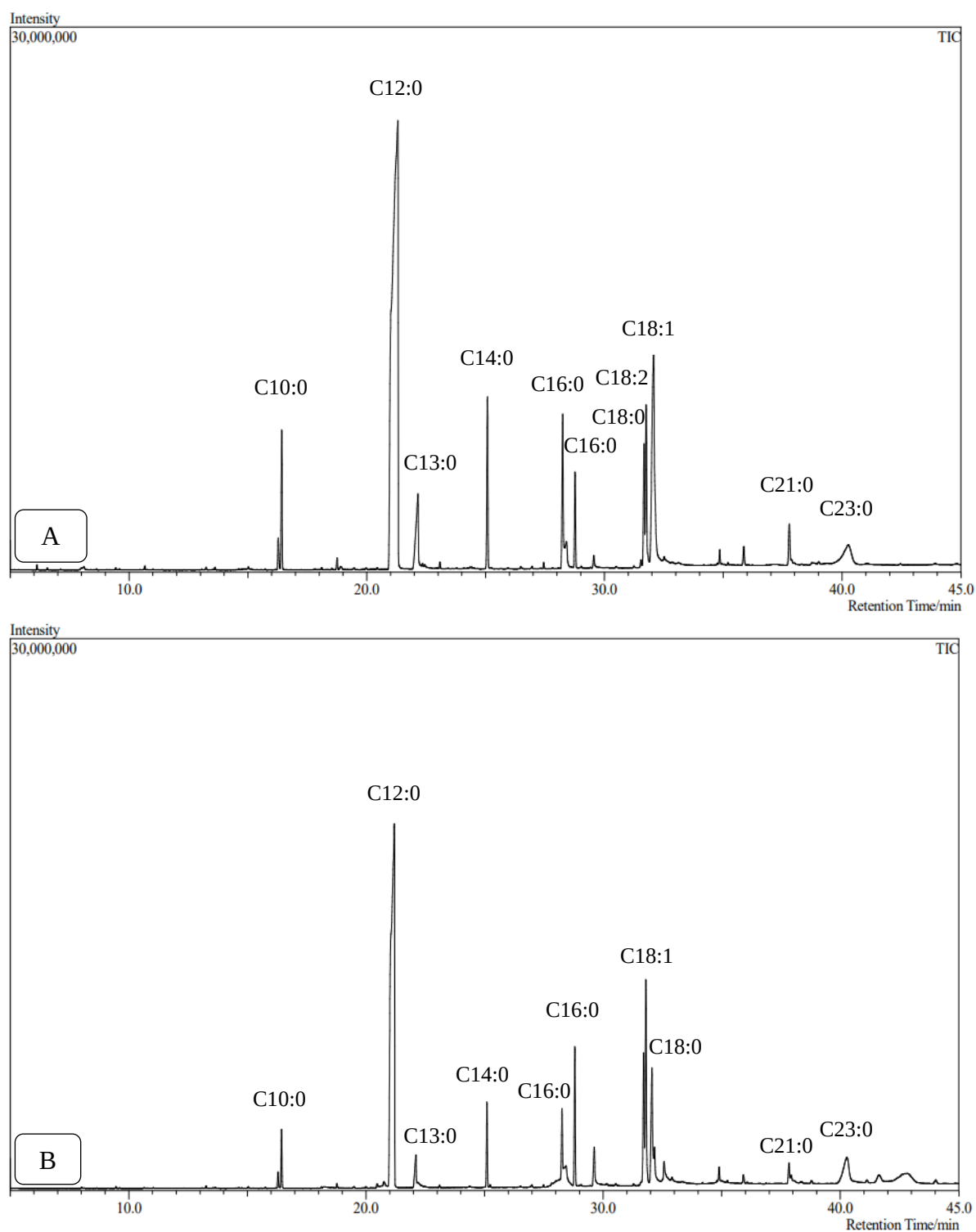


Figure 4.2: GC-MS chromatogram of (A) Petroleum ether and (B) Bligh and Dyer seed extracts of *L. garciae*.

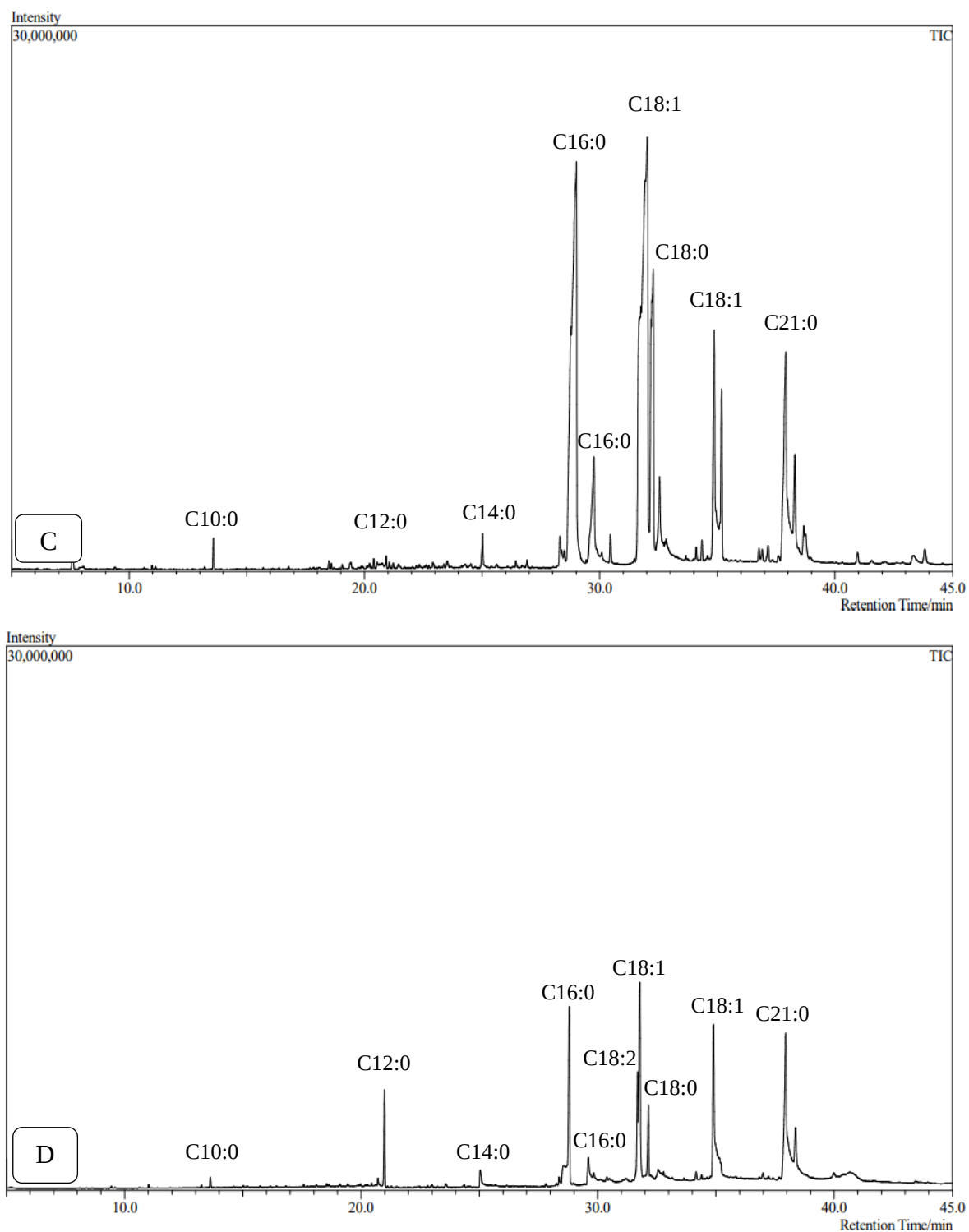


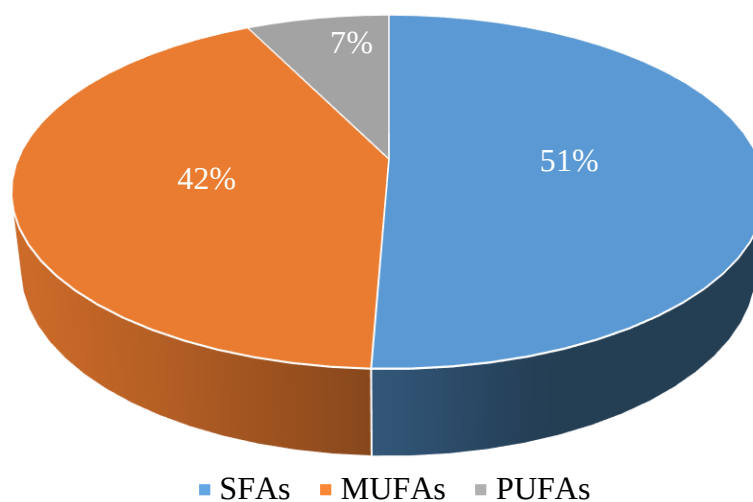
Figure 4.3: GC-MS chromatogram of (C) Petroleum ether and (D) Bligh and Dyer pulp extracts of *L. garciae*.

Table 4.2: Fatty acid composition of the pulp and seed extracts of *L. garciae*.

No.	Fatty acid	No. of carbon atom	Chemical formula	Area (%) *			
				PEPE ^e	BDPE ^f	PESE ^g	BDSE ^h
1	Capric acid	C10:0	C ₁₀ H ₂₀ O ₂	ND	0.06 ± 0.00	2.07 ± 0.93	1.99 ± 0.23
2	Undecylic acid	C11:0	C ₁₁ H ₂₂ O ₂	ND	ND	0.20 ± 0.06	0.14 ± 0.02
3	Lauric acid	C12:0	C ₁₂ H ₂₄ O ₂	0.11 ± 0.01	6.86 ± 4.44	75.31 ± 5.25	63.98 ± 5.25
4	Tridecylic acid	C13:0	C ₁₃ H ₂₆ O ₂	ND	ND	0.12 ± 0.01	0.08 ± 0.00
5	Myristic acid	C14:0	C ₁₄ H ₂₈ O ₂	2.84 ± 0.37	2.19 ± 3.27	2.66 ± 1.71	3.25 ± 0.24
6	Pentadecylic acid	C15:0	C ₁₅ H ₃₀ O ₂	0.06 ± 0.00	ND	0.07 ± 0.00	0.11 ± 0.02
7	Palmitic acid	C16:0	C ₁₆ H ₃₂ O ₂	33.05 ± 0.85	33.42 ± 4.12	7.39 ± 2.51	10.36 ± 2.95
8	Margaric acid	C17:0	C ₁₇ H ₃₄ O ₂	0.47 ± 0.10	0.28 ± 0.00	ND	ND
9	Stearic acid	C18:0	C ₁₈ H ₃₆ O ₂	8.97 ± 0.65	8.20 ± 3.81	1.00 ± 1.37	2.41 ± 0.00
10	Nonadecylic acid	C19:0	C ₁₉ H ₃₈ O ₂	ND	2.27 ± 0.00	0.64 ± 0.77	0.08 ± 0.00
11	Arachidic acid	C20:0	C ₂₀ H ₄₀ O ₂	2.63 ± 0.06	2.28 ± 0.00	ND	ND
12	Heneicosylic acid	C21:0	C ₂₁ H ₄₂ O ₂	1.18 ± 0.21	0.72 ± 0.20	0.17 ± 0.00	0.30 ± 0.08
13	Behenic acid	C22:0	C ₂₂ H ₄₄ O ₂	1.30 ± 0.00	0.12 ± 0.00	0.41 ± 0.00	ND
14	Tricosylic acid	C23:0	C ₂₃ H ₄₆ O ₂	0.20 ± 0.02	ND	ND	0.33 ± 0.16
15	Lignoceric acid	C24:0	C ₂₄ H ₄₈ O ₂	0.36 ± 0.02	ND	ND	0.35 ± 0.07
		Total SFA ^a		49.90 ± 0.85	52.83 ± 0.92	89.33 ± 3.28	81.46 ± 8.98
16	Palmitoleic acid	C16:1	C ₁₆ H ₃₀ O ₂	0.66 ± 0.06	0.49 ± 0.00	ND	ND
17	Margaroleic acid	C17:1	C ₁₇ H ₃₂ O ₂	0.17 ± 0.00	ND	ND	ND
18	Oleic acid	C18:1	C ₁₈ H ₃₄ O ₂	40.73 ± 4.08	37.71 ± 1.95	7.73 ± 3.56	11.10 ± 2.69
19	Paullinic acid	C20:1	C ₂₀ H ₃₈ O ₂	ND	0.69 ± 0.00	0.34 ± 0.00	0.57 ± 0.07
20	Brassicidic acid	C22:1	C ₂₂ H ₄₂ O ₂	ND	0.61 ± 0.00	0.33 ± 0.00	ND
		Total MUFA ^b		41.46 ± 4.09	38.30 ± 2.03	7.96 ± 3.63	11.67 ± 2.76
21	Linoleic acid	C18:2	C ₁₈ H ₃₂ O ₂	7.21 ± 0.00	8.84 ± 1.63	2.71 ± 0.40	6.87 ± 1.56
22	Arachidonic acid	C20:4	C ₂₀ H ₃₂ O ₂	ND	0.06 ± 0.00	ND	ND
		Total PUFA ^c		7.21 ± 0.00	8.86 ± 1.62	2.71 ± 0.40	6.87 ± 1.56
		U/S Ratio ^d		0.98	0.89	0.12	0.23

* Mean of relative percentage peak areas (peak area of each fatty acid/total peak areas of all fatty acids). All the values were analysed using Student's t-test and were expressed as mean ± SD (n = 3). ND = Not detected; ^a Saturated fatty acids; ^b Monounsaturated fatty acids; ^c Polyunsaturated fatty acids; ^d Unsaturated fatty acids/Saturated fatty acids ratio; ^e Petroleum ether pulp extract; ^f Bligh and Dyer pulp extract; ^g Petroleum ether seed extract; ^h Bligh and Dyer seed extract.

(a)



(b)

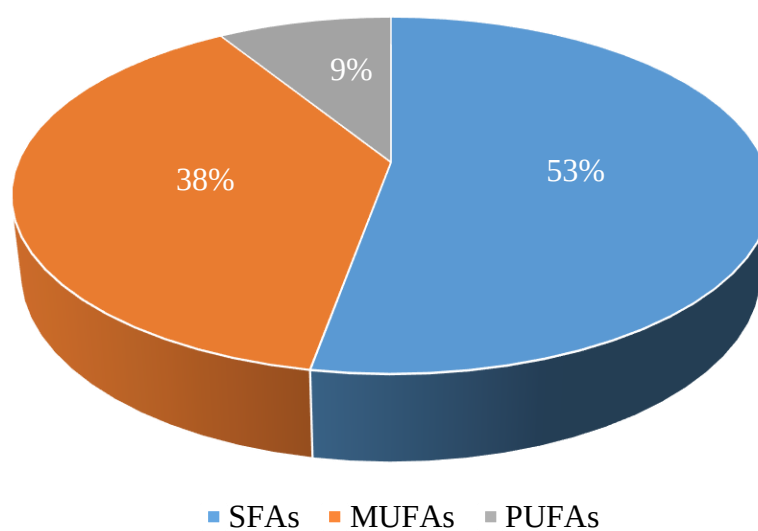
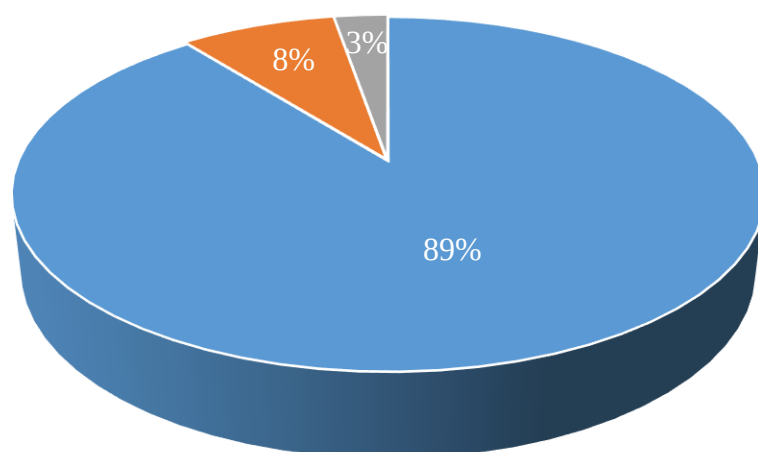


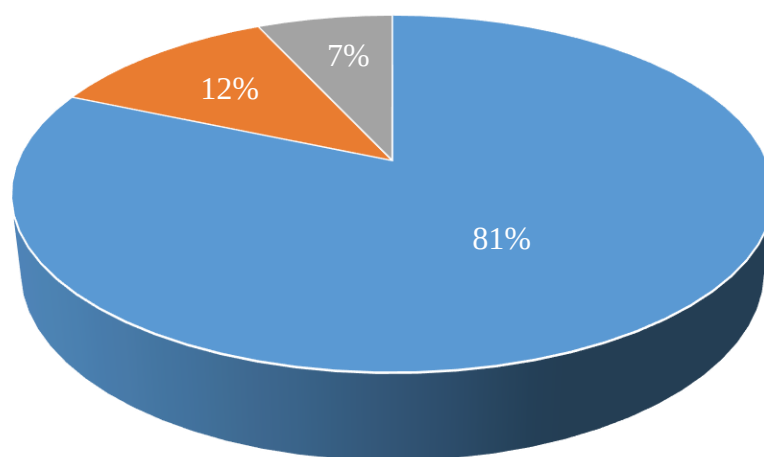
Figure 4.4: Proportion (%) of saturated, monounsaturated and polyunsaturated fatty acids for (a) Petroleum ether and (b) Bligh and Dyer lipid pulp extracts of *L. garciae*.

(a)



■ SFAs ■ MUFAs ■ PUFAs

(b)

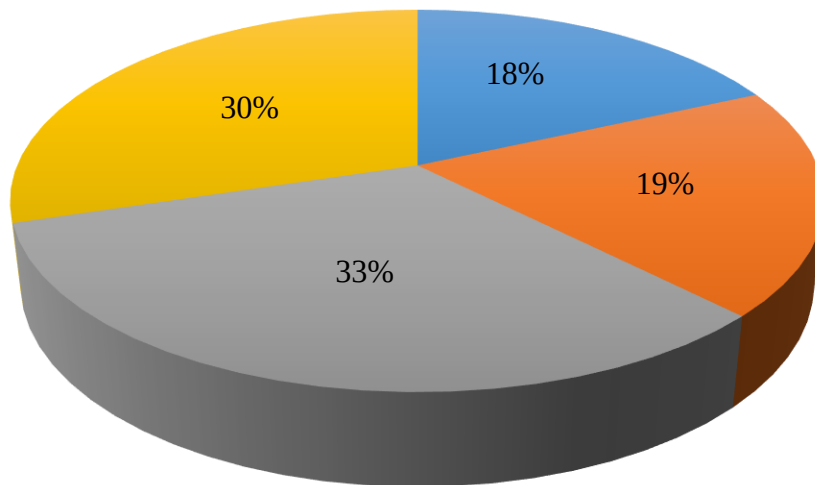


■ SFAs ■ MUFAs ■ PUFAs

Figure 4.5: Proportion (%) of saturated, monounsaturated and polyunsaturated fatty acids for (a) Petroleum ether and (b) Bligh and Dyer lipid seed extracts of *L. garciae*.

(a)

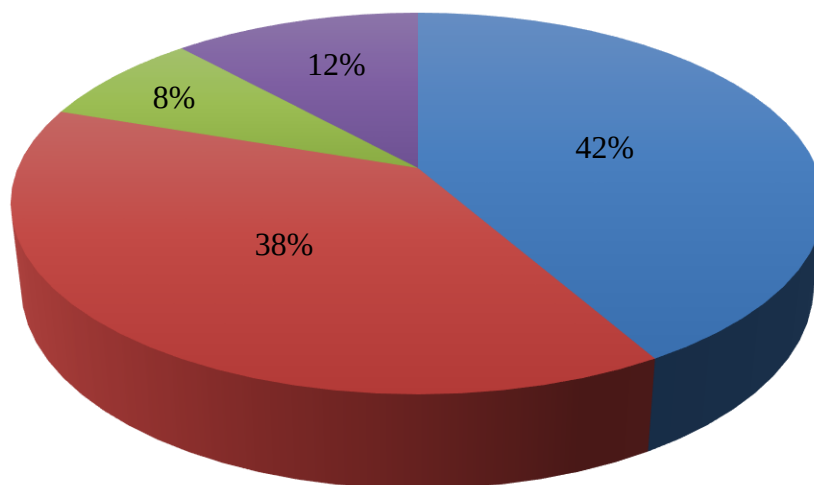
SFAs



■ PE pulp ■ BD pulp ■ PE seed ■ BD seed

(b)

MUFAs



■ PE pulp ■ BD pulp ■ PE seed ■ BD seed

(c)

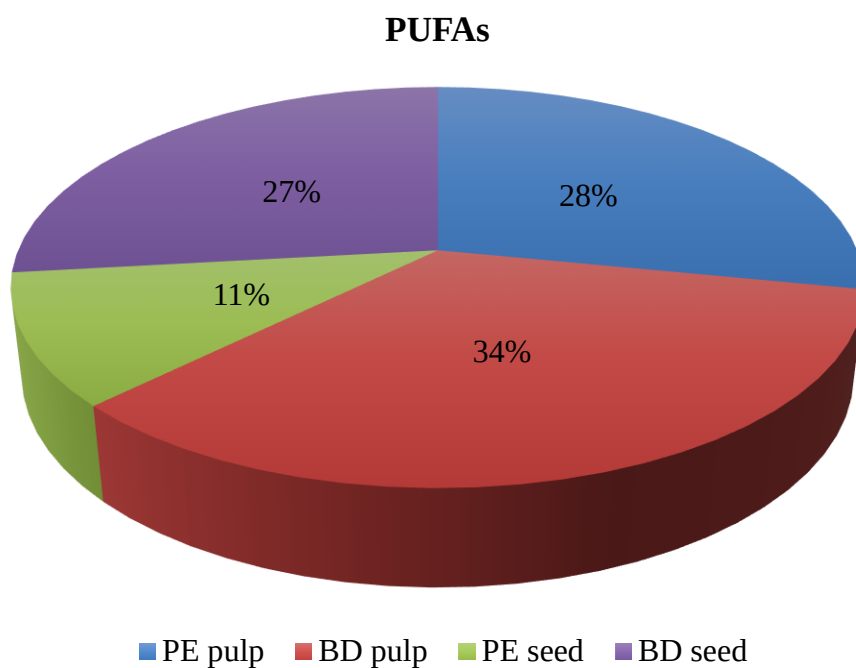


Figure 4.6: Proportion (%) of (a) saturated fatty acids, (b) monounsaturated fatty acids and (c) polyunsaturated fatty acids for petroleum ether and Bligh and Dyer lipid pulp and seed extracts of *L. garciae*.

4.3 Antimicrobial Activities

4.3.1 Diameter of Zone of Inhibition

The skin supports the growth of many commensal bacteria and serves as a physical barrier for the prevention of the foreign pathogens' invasion. However, a breakage of the skin or wound disrupts the normal environment and makes the skin favourable for microbes to colonise and establish infection. Previous studies had demonstrated that fatty acids and methanolic plant or fruit extracts (Casillas-Vargas et al., 2021; Kang et al., 2011) contained antimicrobial activities. Having antimicrobial activities was found to contribute to the wound-healing properties of the studied extracts (Ghildiyal et al., 2015).

Gram-positive bacteria (*Staphylococcus aureus* and *Staphylococcus epidermidis*) and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) that were used for experiments in this study were bacteria that could normally be found on human skin. Table 4.3 showed the results of susceptibility of Gram-positive bacteria (*Staphylococcus aureus* and *Staphylococcus epidermidis*) and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) towards various *L. garciae* pulp and seed extracts using the disk diffusion assay. The determination of the susceptibility of bacteria was conducted through the zone of inhibition diameter. The concentrations of 200 – 1000 mg/disk were utilised for petroleum ether (PE), methanolic and Bligh and Dyer (BD) pulp and seed extracts.

It was discovered that both Gram-positive bacteria (*S. aureus* and *S. epidermidis*) were susceptible to the PE lipid pulp extract. There was an increasing trend of susceptibility of *S. aureus* and *S. epidermidis* to PE lipid pulp extract from 200 to 800 mg/disk. The highest inhibitory activity was manifested by 800 mg/disk of PE lipid pulp extract against *S. aureus* (8.67 ± 0.58 mm) and *S. epidermidis* (9.83 ± 0.29 mm). Furthermore, methanolic pulp extract

exhibited increasing inhibition against *S. aureus* over the concentrations of 200 – 1000 mg/disk, where 1000 mg/disk of methanolic pulp extract displayed the highest inhibitory effect (8.00 ± 0.00 mm). On the contrary, *S. epidermidis* was resistant to the methanolic pulp extract (200 – 400 mg/disk) while it was susceptible to 600 – 1000 mg/disk of the methanolic pulp extract with a lower inhibition (7.00 ± 0.76 mm at 1000 mg/disk).

Moreover, BD lipid pulp extract had shown a weak inhibitory ability as compared to other pulp extracts. It started to inhibit *S. aureus* at the concentrations of 600 mg/disk and 1000 mg/disk of the BD lipid pulp extract revealed 7.00 ± 0.00 mm of inhibition, whereas there was totally no inhibition towards *S. epidermidis*. As the *S. epidermidis* bacteria are commensal bacteria that grow naturally on human skin (Byrd et al., 2018), therefore no inhibition of BD lipid pulp extract is beneficial for *S. epidermidis* to fight against the pathogenic microbes on the skin. Contradictorily, Gram-negative bacteria were generally less susceptible to all *L. garciae* pulp extracts. Among the pulp extracts, only PE lipid pulp extract was able to inhibit *P. aeruginosa* from 200 – 1000 mg/disk and *P. aeruginosa* was highly susceptible to the PE lipid pulp extract (10.00 ± 1.00 mm) at the concentration of 600 and 800 mg/disk. In addition, *P. aeruginosa* manifested a little susceptibility against the 1000 mg/disk methanolic pulp extract (6.00 ± 0.00 mm) while BD lipid pulp extract was unable to inhibit *P. aeruginosa*. Apart from that, all the pulp extracts did not exert inhibition towards the *E. coli*.

Additionally, the susceptibility of bacteria against *L. garciae* seed extracts was different in comparison to the pulp extracts. For Gram-positive bacteria, *S. epidermidis* and *S. aureus* had shown resistance towards the PE lipid seed extract. Besides, methanolic seed extract displayed a good inhibitory ability from 200 – 1000 mg/disk against both *S. aureus*

and *S. epidermidis*. *S. aureus* had revealed the highest susceptibility towards 1000 mg/disk methanolic seed extract (11.00 ± 0.00 mm) as compared to other extracts while methanolic seed extract manifested highest inhibition against *S. epidermidis* (9.33 ± 0.58 mm) at 1000 mg/disk. As a comparison, the BD lipid seed extract showed a slight inhibitory activity against *S. epidermidis* (6.00 ± 0.00 mm) at 800 mg/disk while *S. epidermidis* was not susceptible to BD lipid seed extract from 200 – 600 mg/disk. Likewise, BD lipid seed extract also did not exhibit inhibition against *S. aureus*. In contrast, all the *L. garciae* seed extracts showed lower inhibitory ability towards Gram-negative bacteria. The PE lipid seed extract only possessed a little inhibitory ability against *P. aeruginosa* from 600 – 1000 mg/disk, which the 800 mg/disk exhibited 6.33 ± 0.29 mm of inhibition. Furthermore, *P. aeruginosa* was susceptible to 400 – 1000 mg/disk of methanolic seed extract and the highest inhibitory effect was 1000 mg/disk (7.83 ± 0.29 mm). All the seed extracts could not show inhibitory effect towards *E. coli* while *P. aeruginosa* was also resistant to the BD lipid seed extract.

On top of this, all the *L. garciae* pulp and seed extracts displayed lower antimicrobial activities as compared to the standard antibiotics. By comparing to the positive controls (chloramphenicol and gentamicin), they exhibited higher inhibition against *S. epidermidis* and *S. aureus* respectively, where the zone of inhibition diameters were 24.33 ± 1.15 mm and 24.67 ± 0.58 mm. For *P. aeruginosa* and *E. coli*, chloramphenicol and gentamicin displayed 19.00 ± 1.00 mm and 23.33 ± 2.89 mm for the zone of inhibition diameters respectively. The DMSO was acted as a control and Trifluoroacetic acid (TFA) was the negative control with no inhibition against all the bacteria.

Table 4.3: Antimicrobial activities of the pulp and seed extracts of *L. garciae* against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli* and *Pseudomonas aeruginosa* by disk diffusion assay.

Sample	Mean diameter of zone of inhibition (mm)*			
	Gram-positive bacteria		Gram-negative bacteria	
	<i>Staphylococcus aureus</i>	<i>Staphylococcus epidermidis</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>
PEPE^a (mg/disk)				
200	8.00 ± 0.00	7.50 ± 0.50	NI	8.33 ± 0.58
400	8.33 ± 0.58	8.67 ± 0.58	NI	9.17 ± 1.04
600	8.67 ± 0.58	9.50 ± 0.50	NI	10.00 ± 1.00
800	8.67 ± 0.58	9.83 ± 0.29	NI	10.00 ± 0.50
1000	8.33 ± 0.58	7.33 ± 0.58	NI	6.33 ± 0.58
MEPE^b (mg/disk)				
200	6.00 ± 0.00	NI	NI	NI
400	6.17 ± 0.29	NI	NI	NI
600	6.33 ± 0.29	6.00 ± 0.00	NI	NI
800	7.17 ± 0.29	6.67 ± 0.58	NI	NI
1000	8.00 ± 0.00	7.00 ± 0.76	NI	6.00 ± 0.00
BDPE^c (mg/disk)				
200	NI	NI	NI	NI
400	NI	NI	NI	NI
600	6.00 ± 0.00	NI	NI	NI
800	6.67 ± 0.29	NI	NI	NI
1000	7.00 ± 0.00	NI	NI	NI
PESE^d (mg/disk)				
200	NI	NI	NI	NI
400	NI	NI	NI	NI
600	NI	NI	NI	6.00 ± 0.00

Table 4.3 continued

800	NI	NI	NI	6.33 ± 0.29
1000	NI	NI	NI	6.33 ± 0.58
MESE^e (mg/disk)				
200	7.00 ± 0.00	6.83 ± 0.29	NI	NI
400	8.50 ± 0.50	7.33 ± 0.29	NI	6.00 ± 0.00
600	9.67 ± 0.58	8.00 ± 0.00	NI	6.50 ± 0.00
800	10.30 ± 0.58	8.67 ± 0.29	NI	7.00 ± 0.00
1000	11.00 ± 0.00	9.33 ± 0.58	NI	7.83 ± 0.29
BDSE^f (mg/disk)				
200	NI	NI	NI	NI
400	NI	NI	NI	NI
600	NI	NI	NI	NI
800	NI	6.00 ± 0.00	NI	NI
1000	NI	6.00 ± 0.00	NI	NI
Chloramphenicol (30 µg/disk)	24.33 ± 1.15	NT	19.00 ± 1.00	NT
Gentamicin (10 µg/disk)	NT	24.67 ± 0.58	NT	23.33 ± 2.89
Control (100% DMSO)	NI	NI	NI	NI
Negative control (0.1% TFA)	NI	NI	NI	NI

*The diameter of the zone of inhibition was expressed as mean ± SD (n=3) including the disk diameter of 5.5 mm. NI = No inhibition; NT = Not tested; ^a Petroleum ether pulp extract; ^b Methanolic pulp extract; ^c Bligh and Dyer pulp extract; ^d Petroleum ether seed extract; ^e Methanolic seed extract; ^f Bligh and Dyer seed extract.

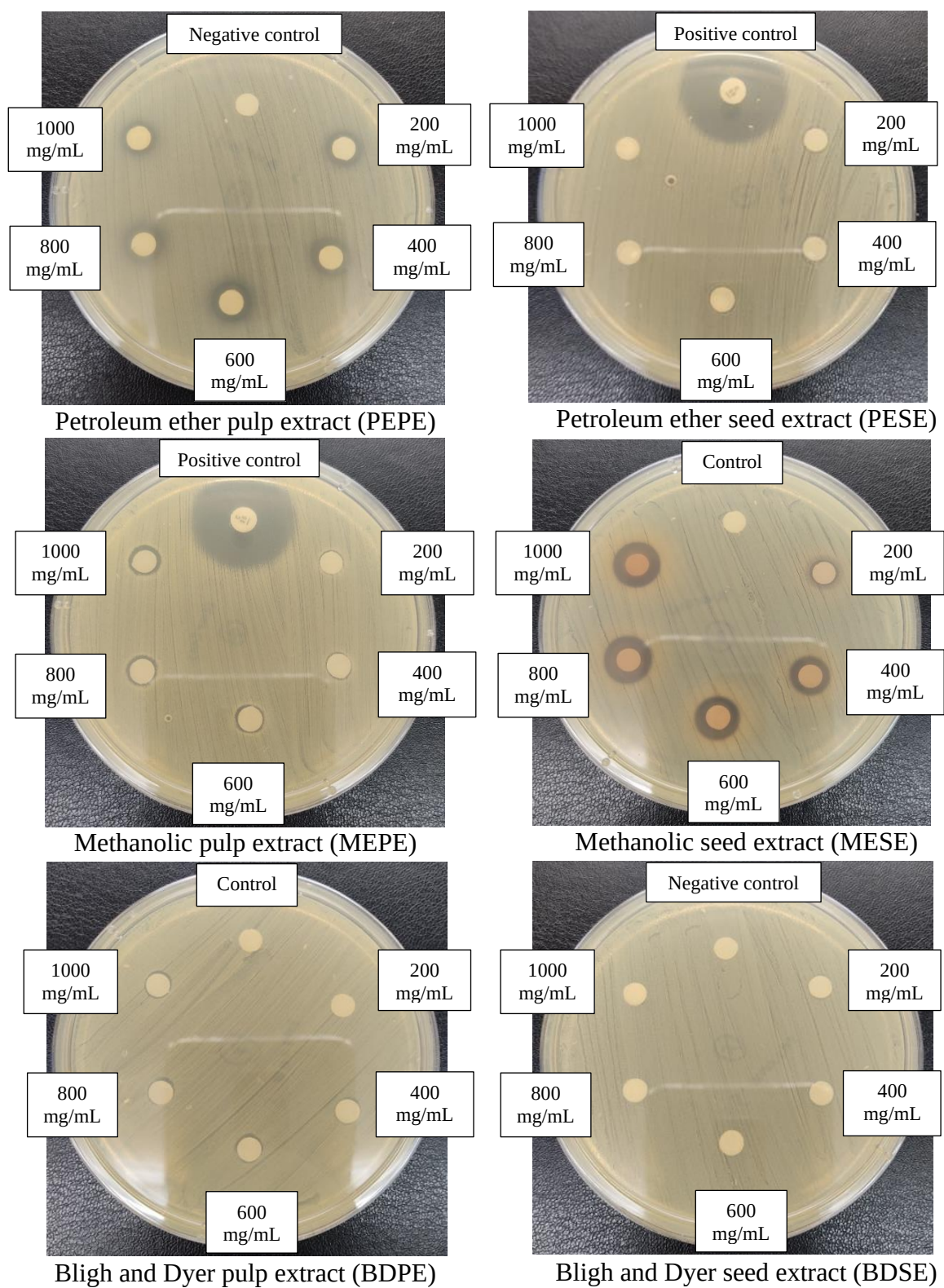


Figure 4.7: Zone of inhibition of *L. garciae* pulp and seed extracts against *Staphylococcus aureus*.

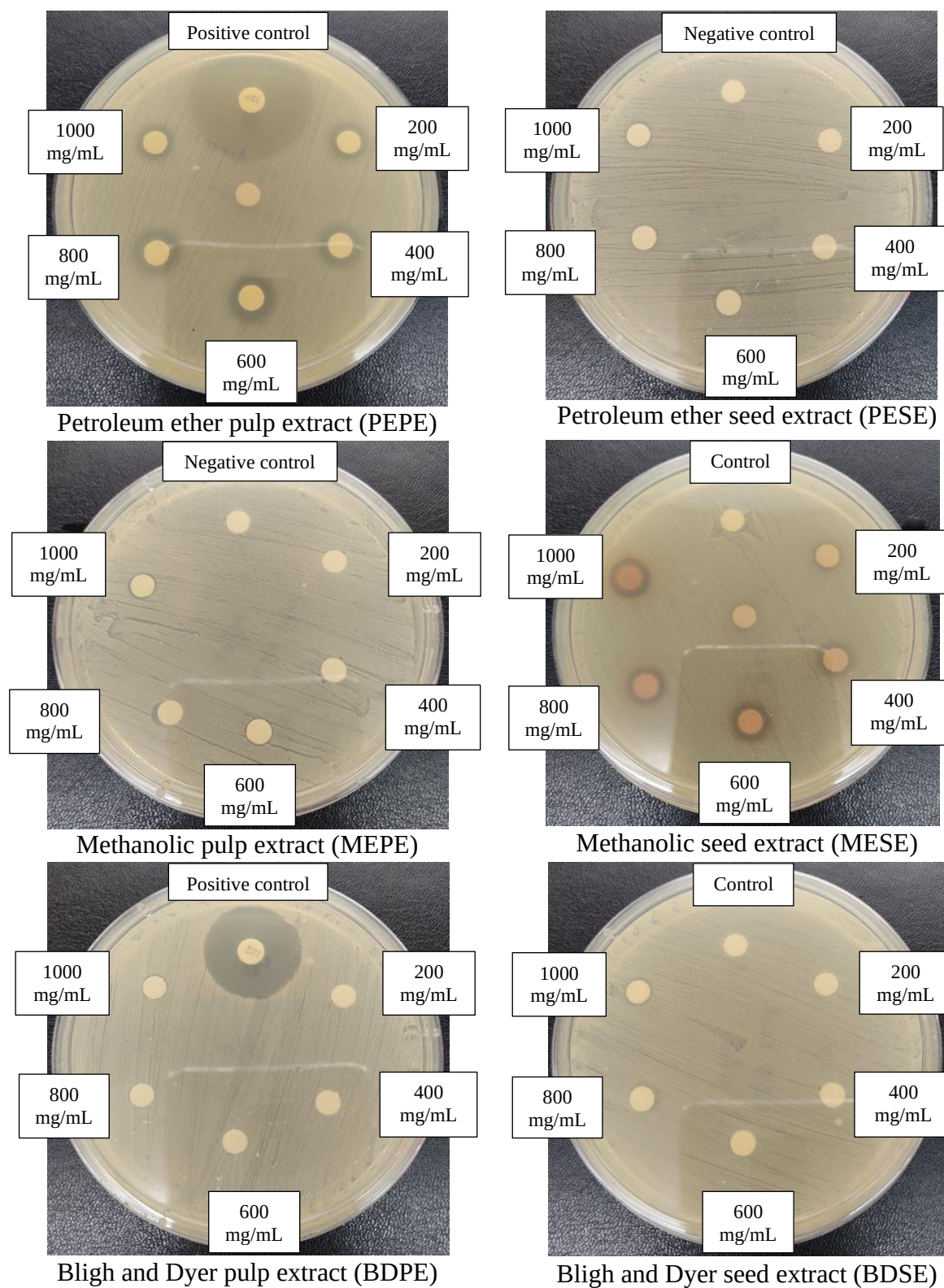


Figure 4.8: Zone of inhibition of *L. garciae* pulp and seed extracts against *Staphylococcus epidermidis*.

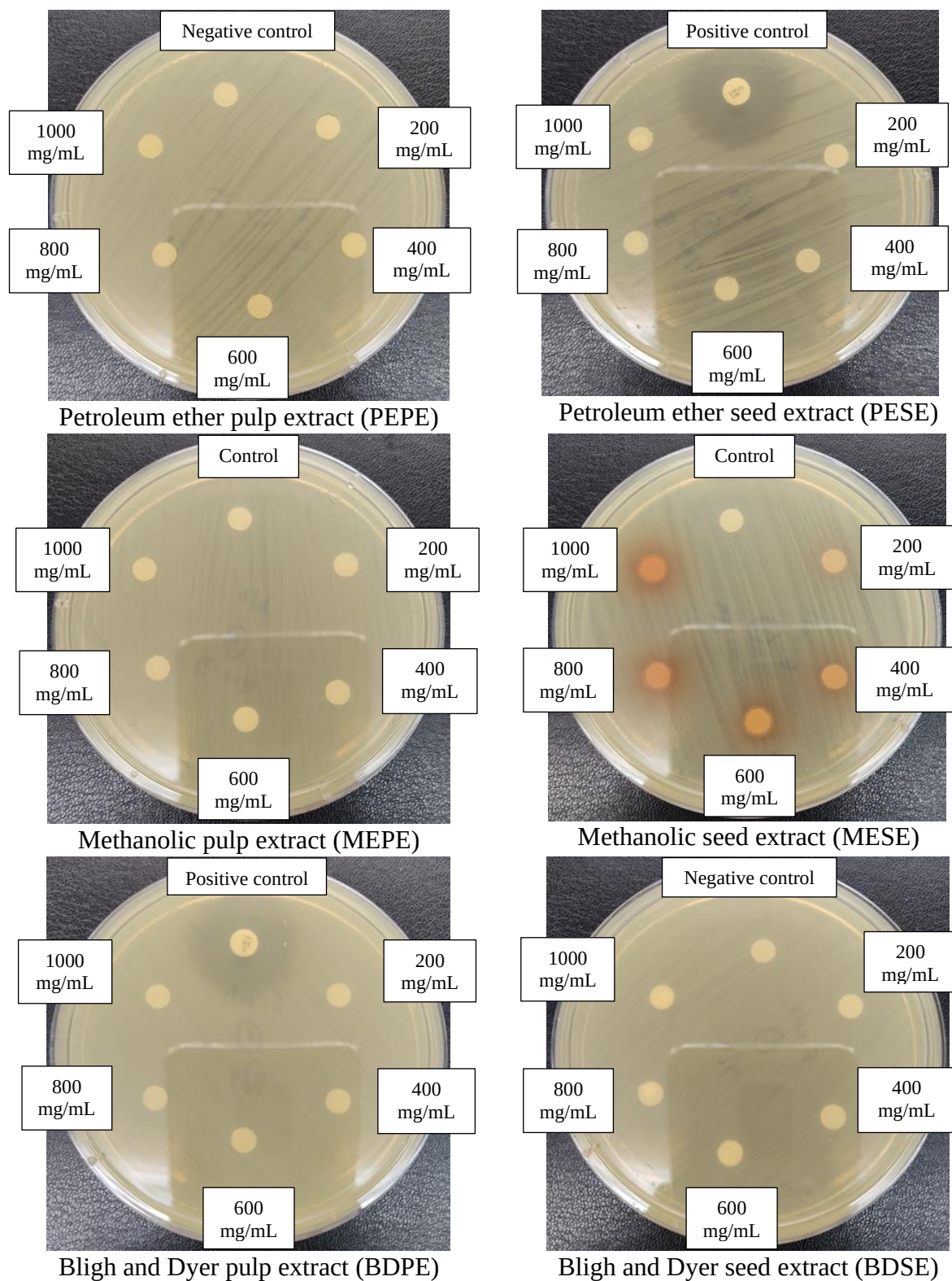


Figure 4.9: Zone of inhibition of *L. garciae* pulp and seed extracts against *Escherichia coli*.

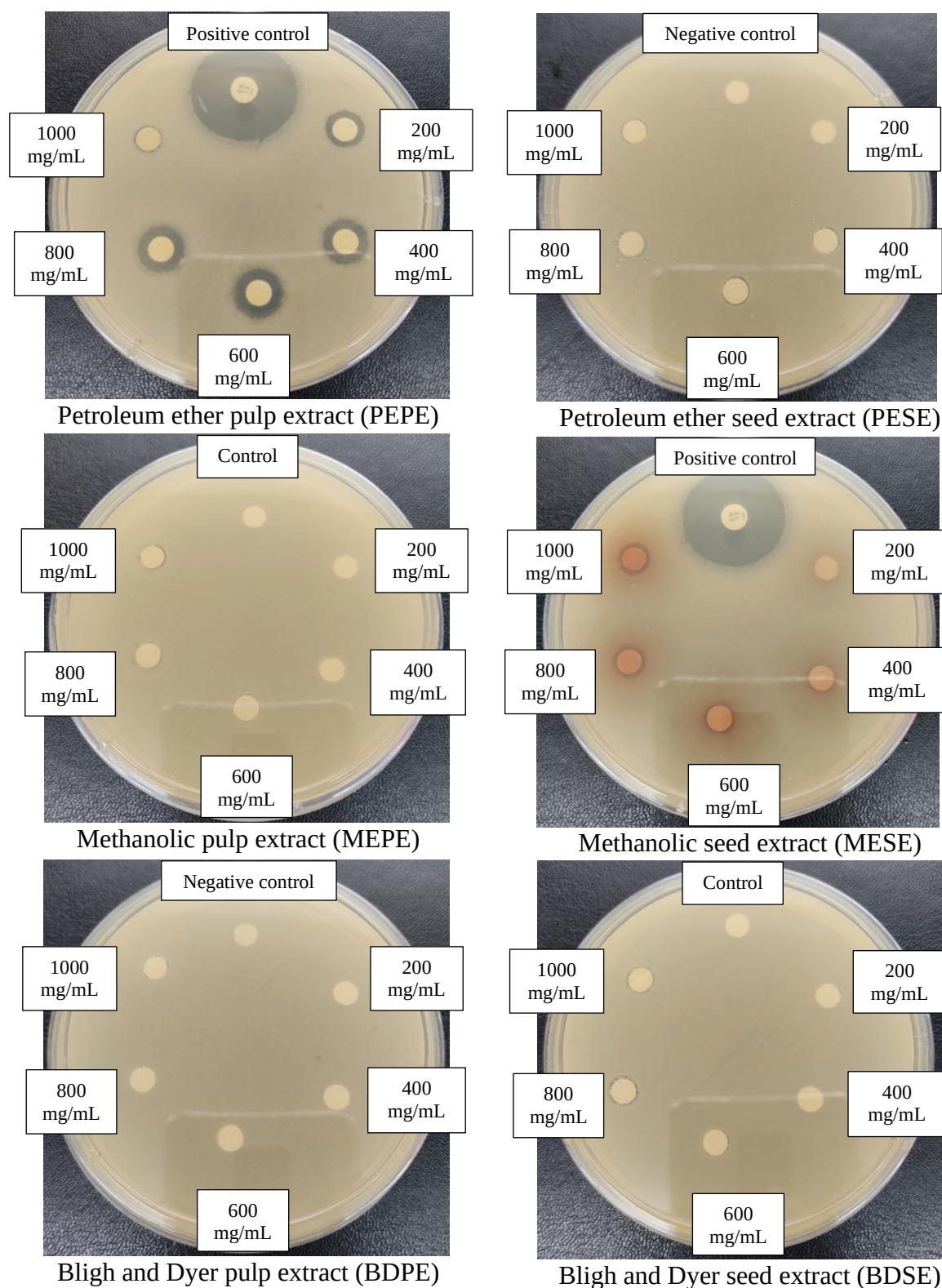


Figure 4.10: Zone of inhibition of *L. garciae* pulp and seed extracts against *Pseudomonas aeruginosa*.

4.3.2 Minimum Inhibitory Concentration (MIC)

Table 4.4 showed the antimicrobial activities of various pulp and seed extracts of *L. garciae* through the determination of minimum inhibitory concentrations (MICs) by broth microdilution assay. The final concentrations of *L. garciae* extracts applied in this assay were 0.39, 0.78, 1.57, 3.13, 6.25, 12.5, 25 and 50 mg/mL as stated in Figure 4.11 - Figure 4.14.

For Gram-positive bacteria, PE lipid pulp extract was effective against *S. aureus* and *S. epidermidis* with MIC values of 12.5 mg/mL and 3.13 mg/mL respectively. In addition, the MIC value of BD lipid pulp extract was 12.5 mg/mL for both Gram-positive bacteria. Both *S. aureus* and *S. epidermidis* were susceptible to the methanolic pulp extract with a higher MIC value of 25 mg/mL. Besides, the PE lipid pulp extract exerted stronger antibacterial potential against Gram-negative bacteria (*E. coli* and *P. aeruginosa*) with the MIC values of 12.5 mg/mL and 0.78 mg/mL respectively. Moreover, both the methanolic and BD pulp extracts exhibited antimicrobial effects against *E. coli* and *P. aeruginosa* with MIC value of 12.5 mg/mL.

Apart from that, the PE, methanolic and BD seed extracts had demonstrated the minimum inhibition against both *S. aureus* and *S. epidermidis* at a higher MIC value of 25 mg/mL. Furthermore, both PE and BD lipid seed extracts exhibited a weaker inhibition against *E. coli* with higher MIC value of 25 mg/mL, whereas they exerted stronger inhibitory activity against *P. aeruginosa* at the lower MIC value of 12.5 mg/mL. In addition, both *E. coli* and *P. aeruginosa* displayed susceptibility towards methanolic seed extract at 12.5 mg/mL of MIC value.

Table 4.4: Antimicrobial activities of the pulp and seed extracts of *L. garciae* against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli* and *Pseudomonas aeruginosa* by broth microdilution assay.

Microorganisms	MIC (mg/mL)					
	PEPE	BDPE	MEPE	PESE	BDSE	MESE
<i>Staphylococcus aureus</i>	12.5	25	25	25	25	25
<i>Staphylococcus epidermidis</i>	3.13	25	25	25	25	25
<i>Escherichia coli</i>	12.5	12.5	12.5	25	25	12.5
<i>Pseudomonas aeruginosa</i>	0.78	12.5	12.5	12.5	12.5	12.5

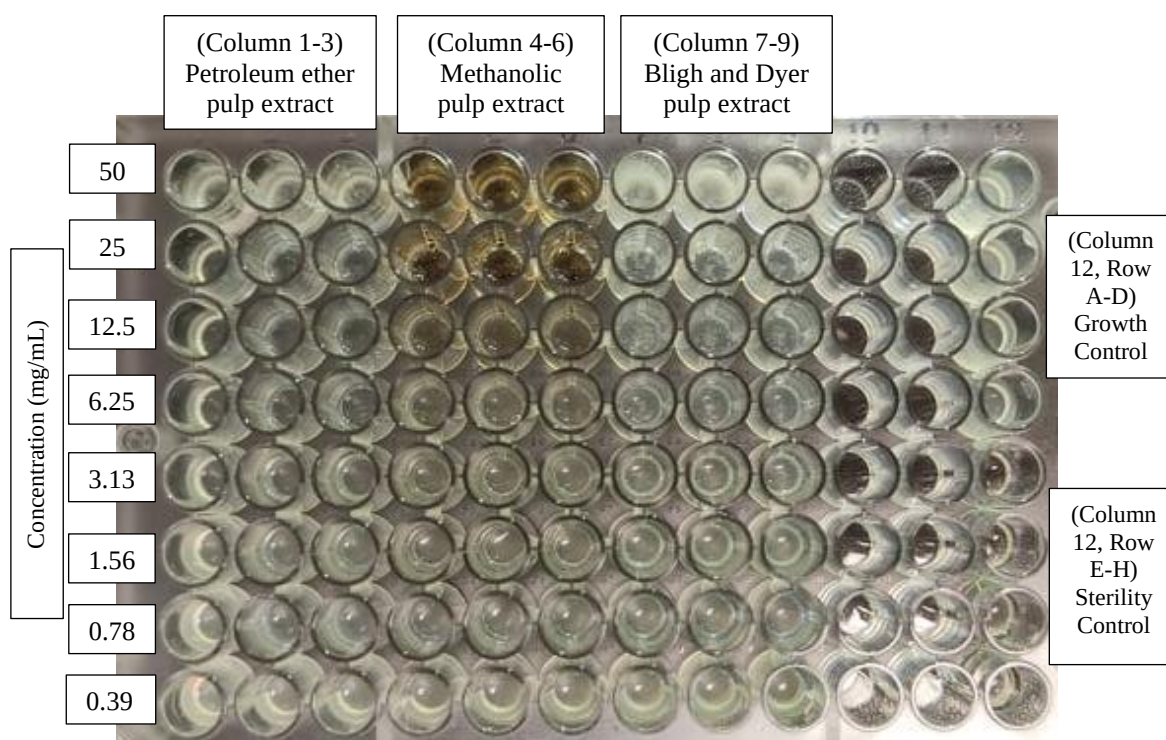
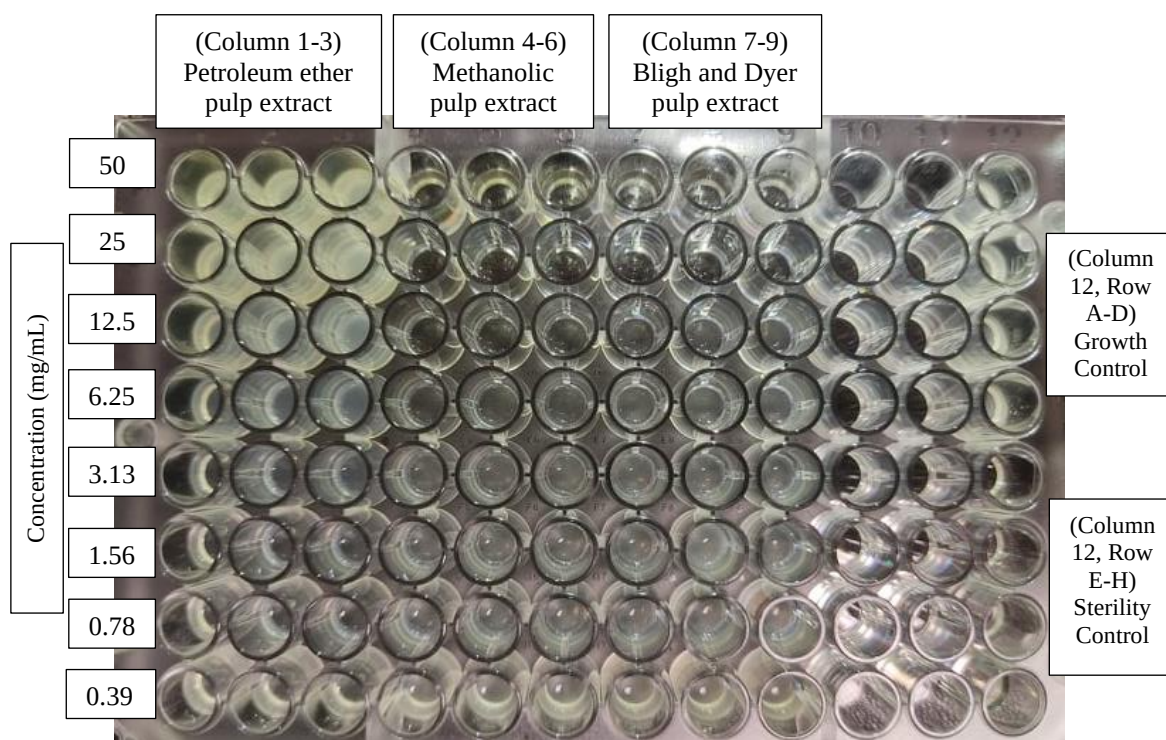


Figure 4.11: Minimum Inhibitory Concentration (MIC) of *L. garciae* pulp and seed extracts against *Staphylococcus aureus*.

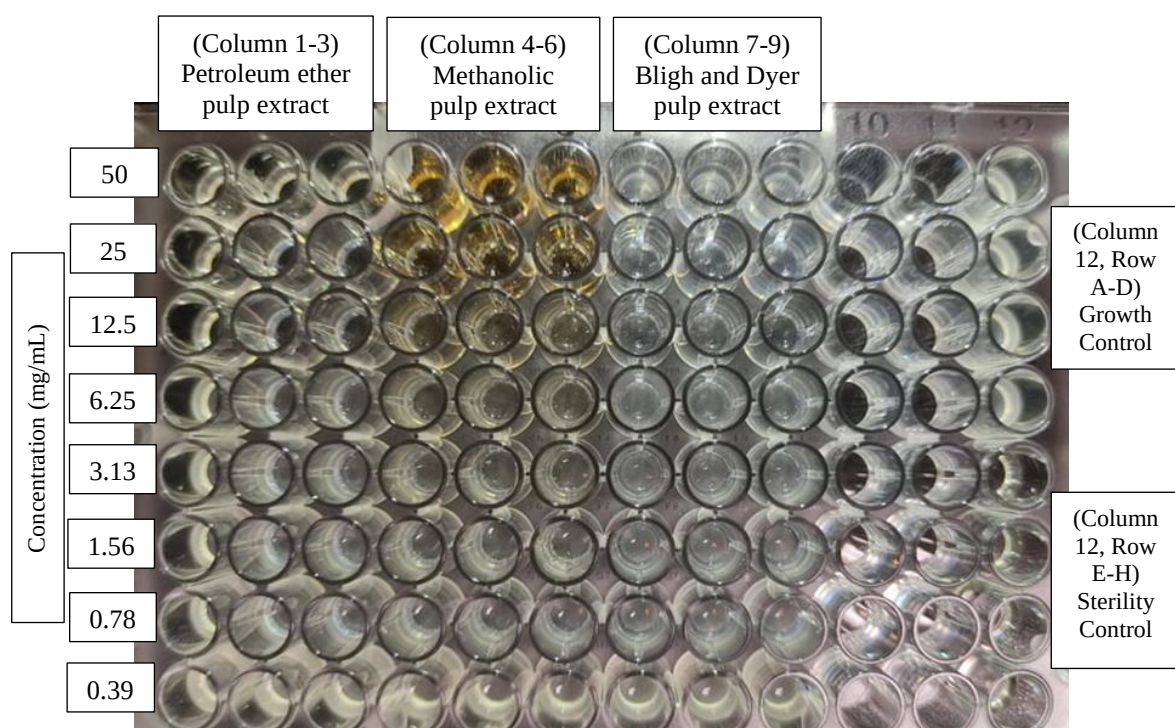
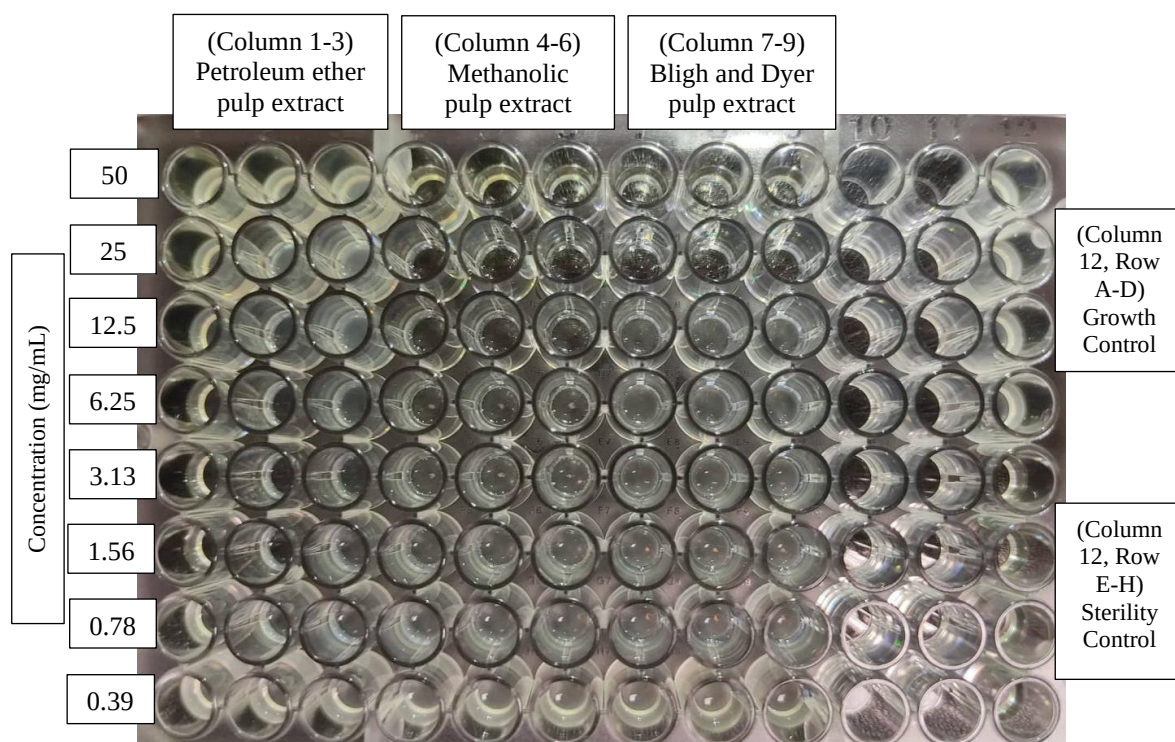


Figure 4.12: Minimum Inhibitory Concentration (MIC) of *L. garciae* pulp and seed extracts against *Staphylococcus epidermidis*.

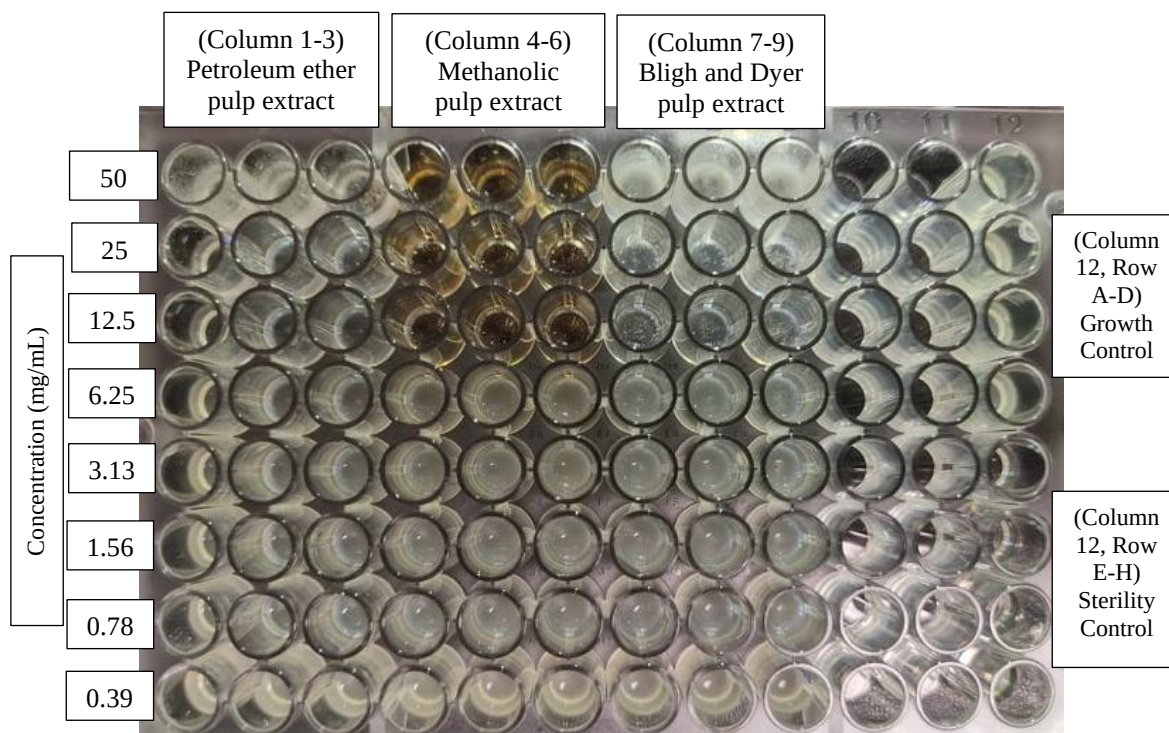
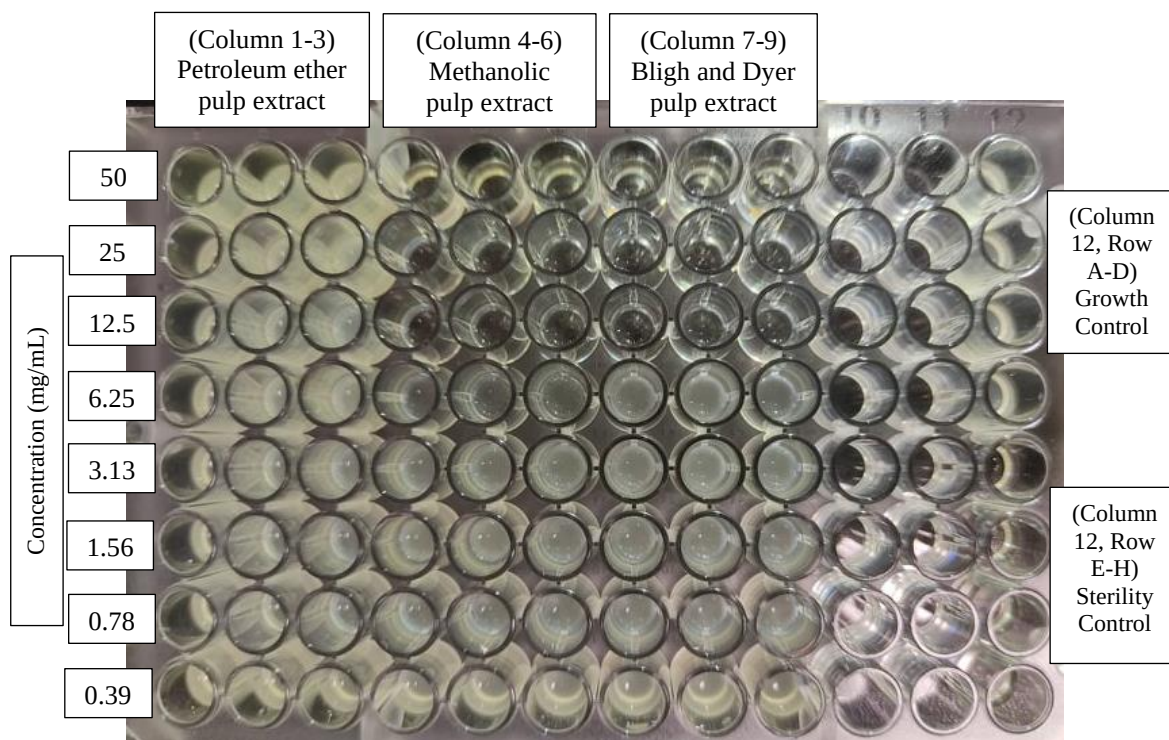


Figure 4.13: Minimum Inhibitory Concentration (MIC) of *L. garciae* pulp and seed extracts against *Escherichia coli*.

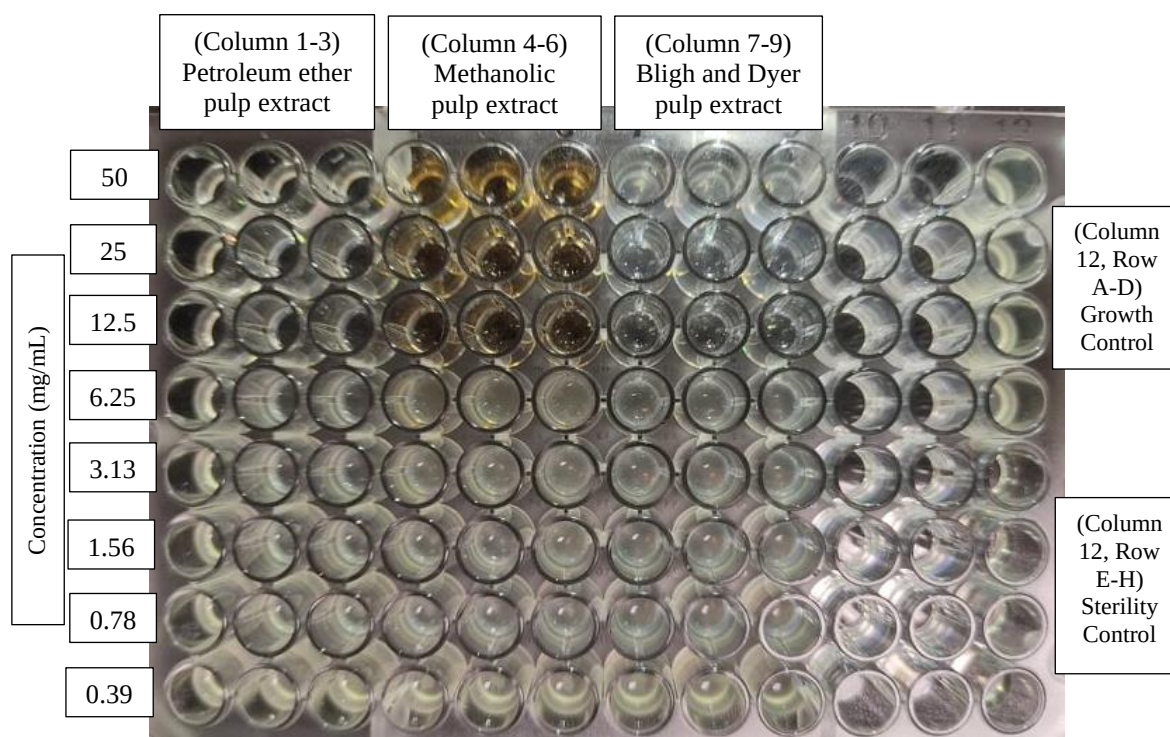
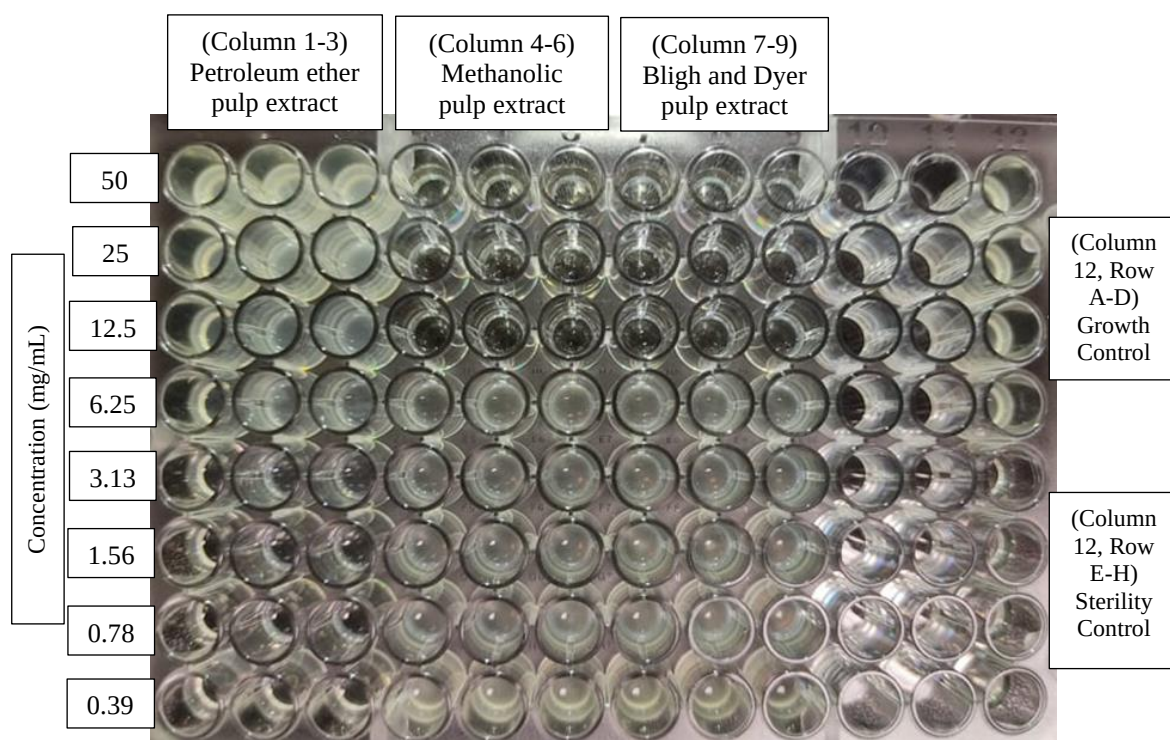


Figure 4.14: Minimum Inhibitory Concentration (MIC) of *L. garciae* pulp and seed extracts against *Pseudomonas aeruginosa*.

4.4 Cell Culture

Cell Seeding Density Optimisation

Three different fibroblast cell densities i.e. 2.5×10^3 cells/well, 2.5×10^4 cells/well and 2.5×10^5 cells/well were used to determine the fibroblast cell seeding density that achieved optimal growth after 24 h of culturing. Figure 4.15 illustrated the cell growth curve for fibroblasts where the growth curve was divided into lag phase, exponential (log) phase and stationary phase. The cell density of 2.5×10^3 cells/well was discovered to be the lag phase of fibroblasts while stationary phase was represented by 2.5×10^5 cells/well. The exponential (log) phase was the desired growth phase where cells were at their optimal growth. In this study, the exponential (log) phase was the 2.5×10^4 cells/well of cell seeding density. This was almost similar to the recommended fibroblast cell density by JCRB Cell Bank which was 2.2×10^4 cells/well. Hence, 2.2×10^4 cells/well of cell seeding density was utilised for the MTT assay.

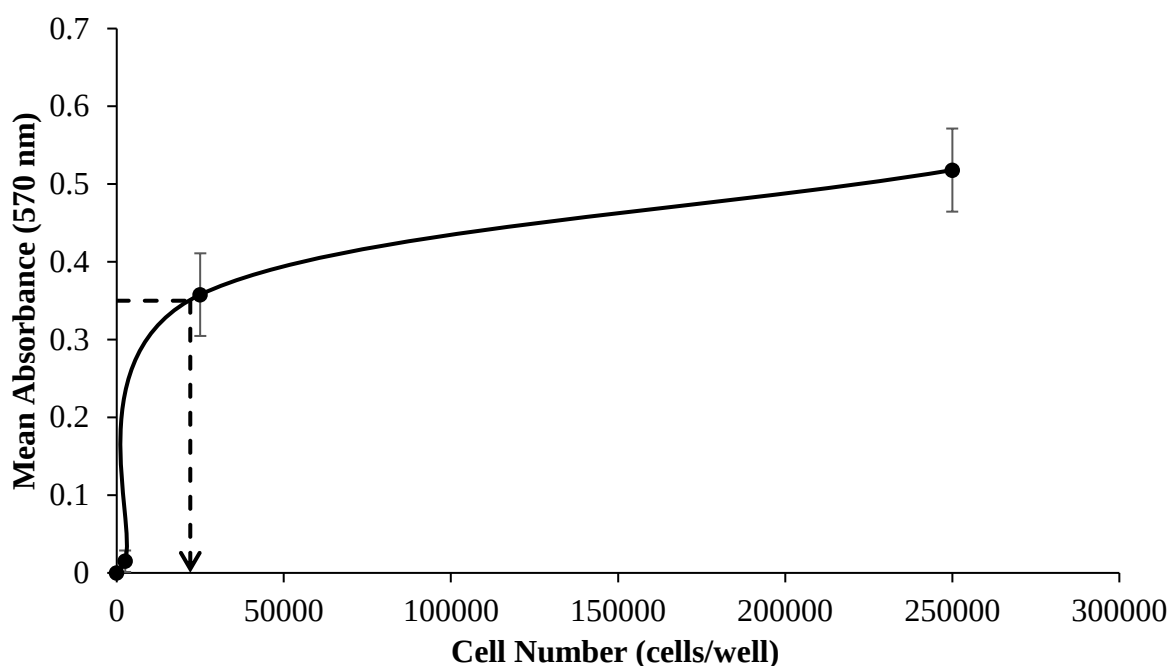


Figure 4.15: Fibroblasts at different cell density were cultured in their growth media. For each cell density the mean absorptions (n=3) were calculated after 24 h of incubation.

4.5 Cell Viability

4.5.1 Effects of DMSO and allantoin on fibroblasts viability incubated in DMEM containing 10% FBS

The cytotoxicity effect of DMSO on fibroblasts was first evaluated as it was the screening test to determine the percentage of DMSO used to dissolve the *L. garciae* pulp and seed extracts (Figure 4.16) and therefore, it was critical to determine the non-toxic concentration of DMSO on fibroblasts. Figure 4.16 (a) showed that 10% DMSO was very toxic to the fibroblasts where only $57.78 \pm 0.01\%$ of fibroblasts were viable. Fibroblasts treated with DMSO concentrations of 2.5% and 5%, had cell viability of $84.07 \pm 0.02\%$ and $79.83 \pm 0.02\%$ respectively. While fibroblasts treated with 0.05% and 1.25% DMSO had cell viability of $96.69 \pm 0.02\%$ and $94.25 \pm 0.04\%$ respectively. Since the cell viability of fibroblasts treated with 0.05% DMSO was $96.69 \pm 0.02\%$ which was nearly 100%, therefore this concentration was then selected as the non-toxic concentration to dilute the *L. garciae* extracts.

The wound healing activities were compared with the allantoin, which was a positive control drug that was known to possess good wound healing properties. Therefore, the cytotoxicity effect of allantoin on fibroblasts was determined to find the safe dosage of allantoin on fibroblasts (Figure 4.16 (b)). The 500 $\mu\text{g/mL}$ of allantoin was extremely toxic to the fibroblasts where it caused $97.55 \pm 0.00\%$ loss in cell viability. Treatment of fibroblast with allantoin concentrations of 200 $\mu\text{g/mL}$ and 250 $\mu\text{g/mL}$ led to approximately 20% loss in cell viability and this still caused cytotoxic effect to the fibroblasts. Treatment of fibroblasts with 50 $\mu\text{g/mL}$, 100 $\mu\text{g/mL}$ and 125 $\mu\text{g/mL}$ concentrations of allantoin gave cell viability in the range of 97.60 – 99.08%. Therefore, 125 $\mu\text{g/mL}$ of allantoin with cell viability

of $98.50 \pm 0.02\%$ was selected to be a positive control for the wound scratch assay as it was the minimum non-toxic concentration.

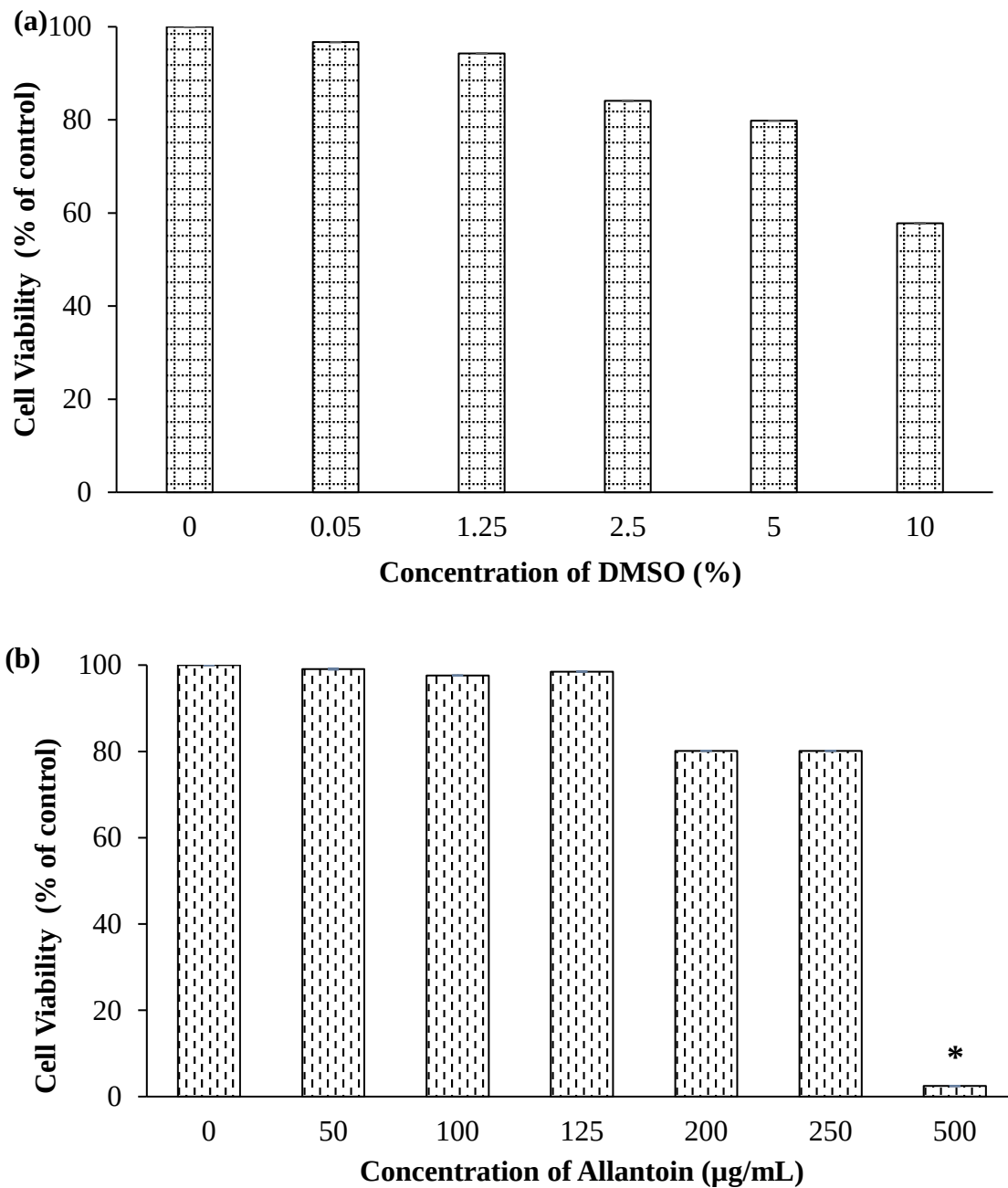


Figure 4.16: Viability of fibroblasts treated with DMSO and allantoin. Fibroblasts at 2.2×10^4 cells/well in 200 µL growth medium were incubated with (a) DMSO (0.05 - 10 %) and (b) Allantoin (50 - 500 µg/mL) for 24 h. The cell viability was measured by MTT assay. The data were presented as mean $\pm$ SD (n = 6). Significance was indicated with $p < 0.05$ (*) by independent t-test.

4.5.2 Effects of *L. garciae* Extracts on Fibroblasts Viability

The experiments were carried out for the determination of non-toxic concentrations of PE, methanolic and BD pulp and seed extracts of *L. garciae* on fibroblasts. These concentrations would be used for further downstream experiments. Firstly, the 500 µg/mL PE lipid pulp extract was mildly cytotoxic to the fibroblasts where the cell viability was $80.15 \pm 0.04\%$. Fibroblasts treated with 62.5 - 250 µg/mL concentrations had cell viability of 85.48 – 93.24% (Figure 4.17(a)). Since the viability was not nearly 100%, two additional concentrations of PE lipid pulp extracts, 7.5 µg/mL and 15 µg/mL were then analysed, in which the cell viabilities were 101.84% and 100.58% respectively (Figure 4.18 (a)).

All the concentrations of BD lipid pulp extract used in these experiments were non-toxic to the fibroblasts. Fibroblasts treated with BD pulp extract with concentrations of 31.25 – 125 µg/mL had cell viability of 98.62 – 113.92%, whereas treatment with 250 µg/mL and 500 µg/mL showed cell viability of $96.76 \pm 0.03\%$ and $94.93 \pm 0.02\%$ respectively.

Similarly, all the concentrations of methanolic pulp extract were not toxic to the fibroblasts. The cell viability of fibroblasts treated with 250 µg/mL and 500 µg/mL of methanolic pulp extract were $96.33 \pm 0.03\%$ and $97.21 \pm 0.03\%$ respectively, while fibroblasts treated with the concentrations of 31.25 – 125 µg/mL of methanolic pulp extracts had cell viability of 102.66 – 110.18%.

Fibroblasts treated with PE lipid seed extract of concentrations 62.5 – 500 µg/mL had cell viability in the range of 85.65 – 91.32% which was far from 100% cell viability (Figure 4.17 (b)). Therefore, additional concentrations of 7.5 µg/mL and 15 µg/mL were evaluated, where there were $104.75 \pm 0.04\%$ and $106.10 \pm 0.04\%$ of viable cells respectively

while the cell viability of fibroblasts treated with 31.25 $\mu\text{g/mL}$ was $103.69 \pm 0.04\%$ (Figure 4.18 (b)).

Next, 500 $\mu\text{g/mL}$ of BD lipid seed extract was very toxic to the fibroblasts where fibroblasts treated with 500 $\mu\text{g/mL}$ had cell viability of $69.27 \pm 0.03\%$ (Figure 4.17 (b)). Both BD lipid seed extracts of 125 $\mu\text{g/mL}$ and 250 $\mu\text{g/mL}$ were mildly toxic to fibroblasts where the cell viability were $80.47 \pm 0.03\%$ and $79.93 \pm 0.01\%$ respectively. Fibroblasts with the treatment of 31.25 $\mu\text{g/mL}$ and 62.5 $\mu\text{g/mL}$, had shown viability of $100.02 \pm 0.04\%$ and $90.51 \pm 0.05\%$ respectively. Although 62.5 $\mu\text{g/mL}$ of BD lipid seed extracts was not toxic to the fibroblasts, the cell viability was not close to the 100% viable cells. Hence, fibroblasts were treated with additional concentrations of BD lipid seed extracts, 7.5 $\mu\text{g/mL}$ and 15 $\mu\text{g/mL}$, where there were $100.11 \pm 0.04\%$ and $99.42 \pm 0.06\%$ of viable cells respectively.

Apart from that, fibroblasts treated with methanolic seed extracts with concentrations of 31.25 – 250 $\mu\text{g/mL}$ showed cell viability of 100.17 – 109.83%. For 500 $\mu\text{g/mL}$ of methanolic seed extract, the cell viability was $88.19 \pm 0.03\%$ which was also non-toxic to the fibroblasts but since it was far from the 100% viable cells, hence it was not considered to be included for the downstream cell culture assays.

In conclusion, 7.5 $\mu\text{g/mL}$, 15 $\mu\text{g/mL}$ and 31.25 $\mu\text{g/mL}$ of PE lipid pulp and seed extracts and BD lipid seed extracts were adopted for the wound healing assays. On the contrary, methanolic pulp and seed extracts and BD lipid pulp extracts with concentrations of 31.25 $\mu\text{g/mL}$, 62.5 $\mu\text{g/mL}$ and 125 $\mu\text{g/mL}$ were used for the evaluation of the wound healing assays.

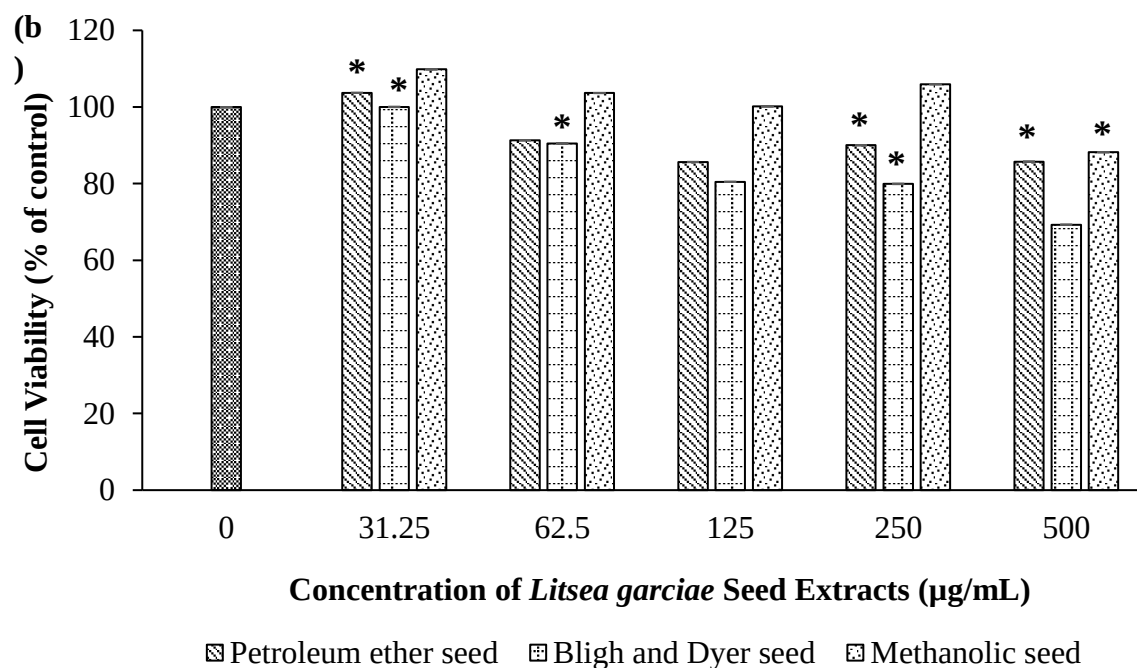
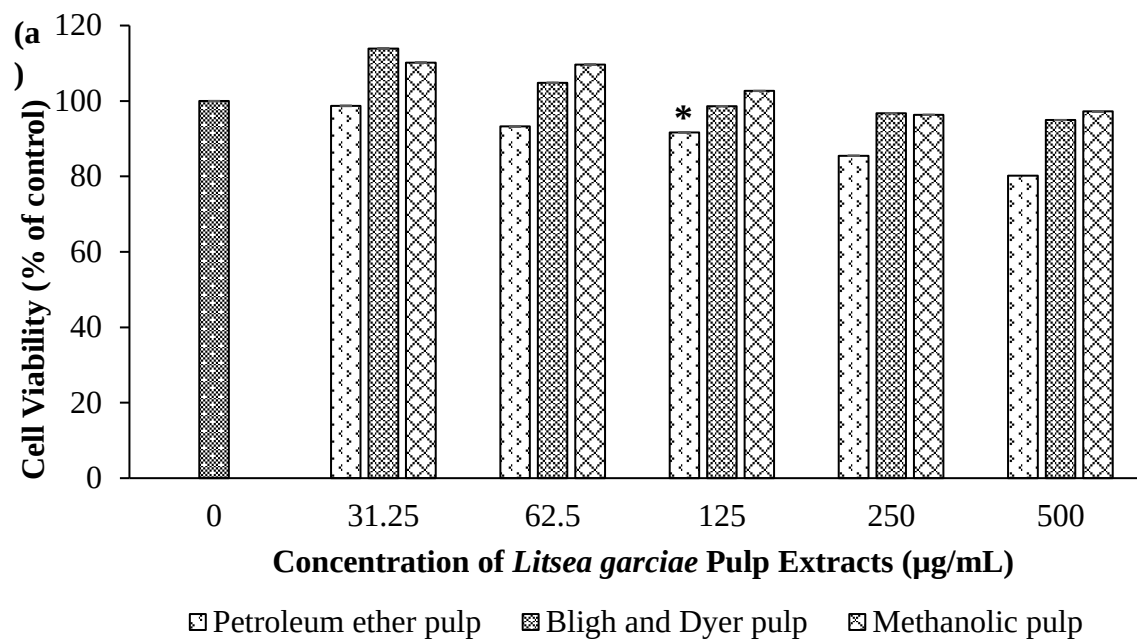


Figure 4.17: Viability of fibroblasts treated with petroleum ether, methanolic and Bligh and Dyer pulp and seed extracts. Fibroblasts at 2.2×10^4 cells/well in 200 µL growth medium were incubated with petroleum ether, methanolic and Bligh and Dyer (a) pulp and (b) seed extracts (31.25 - 500 µg/mL) for 24 h. The cell viability was measured by MTT assay. The data were presented as mean $\pm$ SD (n = 6). Significance was indicated with $p < 0.05$ (*) by independent t-test.

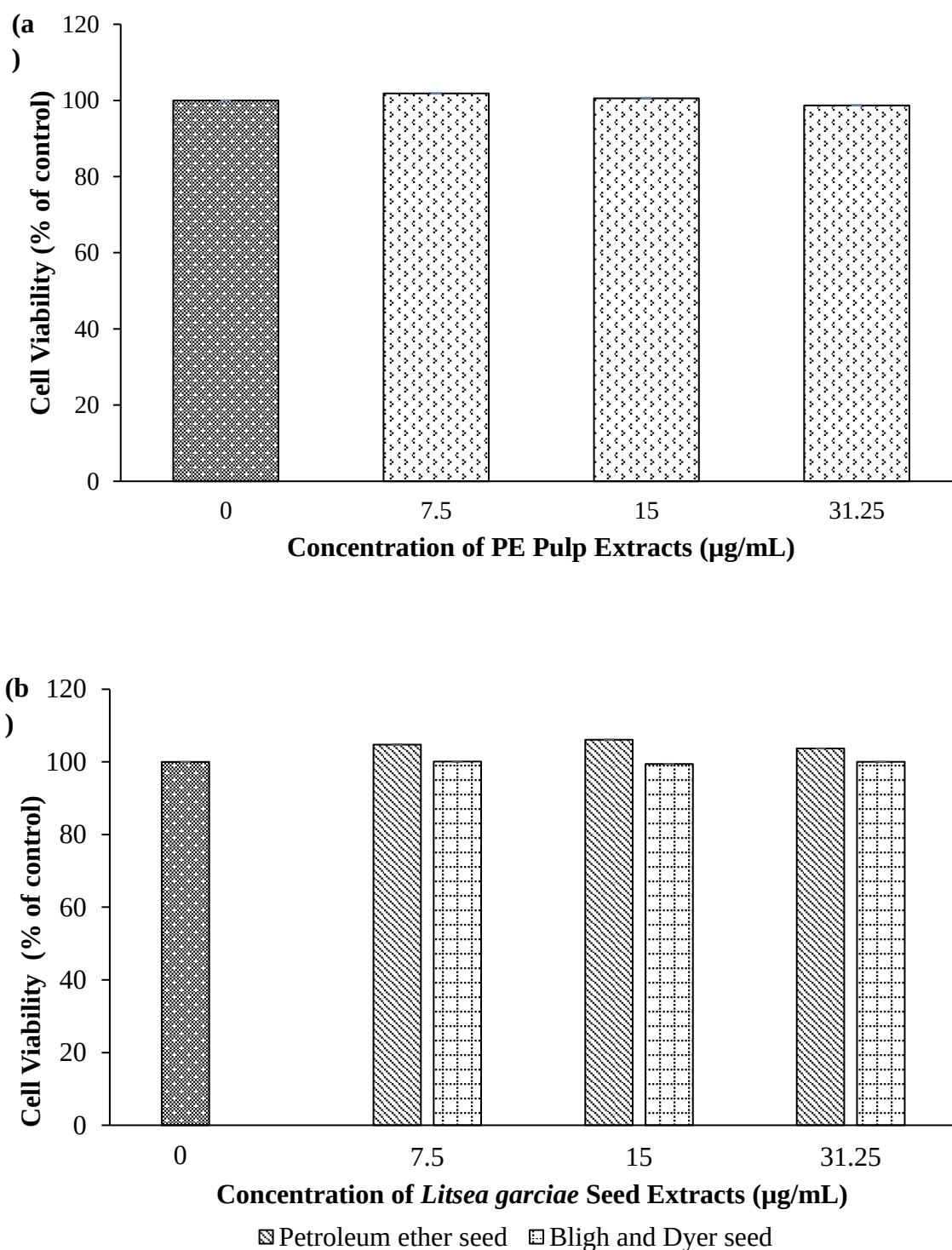


Figure 4.18: Viability of fibroblasts treated with petroleum ether and Bligh and Dyer pulp and seed extracts. Fibroblasts at 2.2×10^4 cells/well in 200 µL growth medium were incubated with (a) petroleum ether pulp extracts (7.5 - 31.25 µg/mL), (b) petroleum ether and Bligh and Dyer seed extracts (7.5 - 31.25 µg/mL) for 24 h. The cell viability was measured by MTT assay. The data were presented as mean $\pm$ SD (n = 6). Significance was indicated with $p < 0.05$ by independent t-test and there was no significant difference ($p > 0.05$).

4.6 Wound Healing Properties of *L. garciae* Extracts

4.6.1 Effects of *L. garciae* Extracts on Fibroblast Cells Migration

During wound healing, skin cells will migrate from the wound edges to the wound site to re-establish normal skin structure. The effect of *L. garciae* extracts on the migration activities of fibroblasts was evaluated using a scratch assay. The later measures the migration rate i.e., the progression of the wound closure on scratch wounded cells.

Figure 4.21 showed the effect of lipid pulp extracts on fibroblast cells migration in comparison with the untreated cells, designated as the negative control (Figure 4.19). After 6 h of incubation, (Figure 4.21 (a)), there was a slight increase in migration rate observed in the negative control i.e., $10.06 \pm 4.15\%$ when compared to 0 h. There was an enhancement in the cell migration rate when fibroblasts were incubated with $7.5 \mu\text{g/mL}$, $15 \mu\text{g/mL}$, and $31.25 \mu\text{g/mL}$ of PE lipid pulp extract, where the migration rates were $11.54 \pm 2.71\%$, $17.45 \pm 4.30\%$ and $19.38 \pm 4.73\%$ respectively. The cells that were treated with $31.25 \mu\text{g/mL}$ of PE lipid pulp extract manifested the highest increment in migration rate which was 9.32% when compared to the negative control. There was a similar cell migration rate in cells treated with $31.25 \mu\text{g/mL}$ in comparison with the positive control ($125 \mu\text{g/mL}$ allantoin).

For 12 h incubation, only cells with the treatment of $31.25 \mu\text{g/mL}$ of the PE lipid pulp extract (Figure 4.20) exerted a notable enhancement in cell migration, where the wound closure rate was $33.08 \pm 7.14\%$ when compared to $25.02 \pm 9.37\%$ of the negative control. Thus, there was an increment of 8.06% in cell migration versus the negative control. The effect of treatment with PE lipid extract at $31.25 \mu\text{g/mL}$ on the cell migration was better than the positive control.

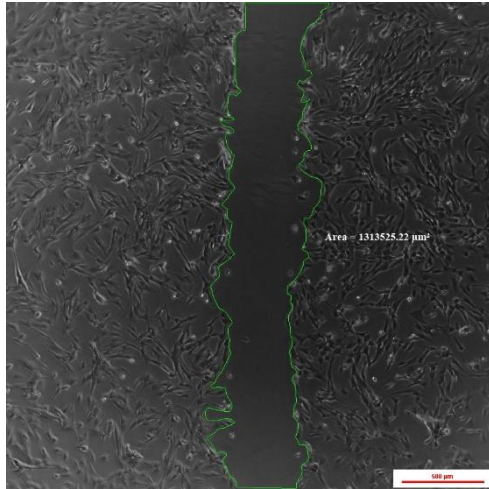
After 24 h incubation, cells with the treatment of 7.5 $\mu\text{g/mL}$ and 31.25 $\mu\text{g/mL}$ of the PE lipid pulp extracts, showed cell migration rates of $82.27 \pm 3.78\%$ and $91.27 \pm 8.35\%$ respectively in contrast to the $77.02 \pm 6.32\%$ of the negative control. Therefore, there was an enhancement of 5.25% and 14.25% respectively in the wound closure as opposed to the negative control. When comparing to the positive control, incubation of cells with 31.25 $\mu\text{g/mL}$ of the PE lipid pulp extract displayed a better improvement in cell migration.

After 48 h incubation, the cells appeared to exhibit a full wound closure for all the concentrations of the PE lipid pulp extracts where their closure rates were similar to the positive and negative controls. Incubation of the cells with BD lipid pulp extracts (31.25 $\mu\text{g/mL}$, 62.5 $\mu\text{g/mL}$ and 125 $\mu\text{g/mL}$) for 6 h showed an enhancement in cell migration rate with the wound closure rates of $16.83 \pm 2.41\%$, $15.31 \pm 0.91\%$ and $13.79 \pm 1.74\%$ respectively (Figure 4.21 (b)). As a comparison to the negative control, the incubation of the cells with concentration of 31.25 $\mu\text{g/mL}$ of extract exhibited the greatest enhancement of cell migration which was 6.77%. The cell migration rate of the fibroblasts after treatment with 31.25 $\mu\text{g/mL}$ of the extract was similar to the positive control treatment.

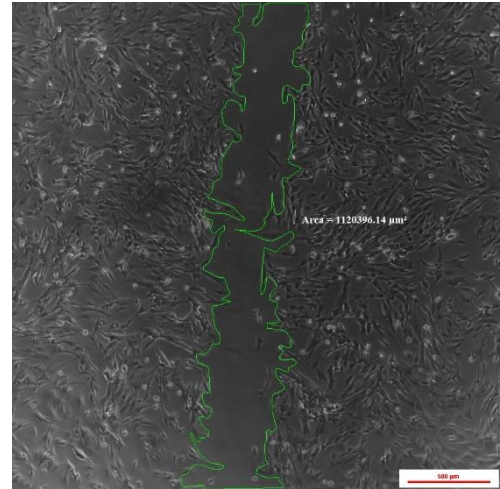
Next, 12 h incubation of the cells with 31.25 $\mu\text{g/mL}$, 62.5 $\mu\text{g/mL}$ and 125 $\mu\text{g/mL}$ of BD lipid pulp extracts appeared to ameliorate the cell migration, where the wound closure rates were $33.46 \pm 10.59\%$, $29.62 \pm 3.96\%$ and $26.40 \pm 3.75\%$ respectively. Similar to the 6 h incubation period, it was found that treatment with a concentration of 31.25 $\mu\text{g/mL}$ BD lipid pulp extract improved cell migration the most, which was 8.44% in contradiction to the negative control. In addition, the increase in the cell migration rate was slightly higher for the treatment with 31.25 $\mu\text{g/mL}$ of the extract as opposed to the positive control.

After 24 h of incubation, all the BD lipid pulp extracts at 31.25 µg/mL, 62.5 µg/mL and 125 µg/mL caused increment in the cell migration rate at $84.72 \pm 6.75\%$, $85.81 \pm 7.11\%$ and $84.25 \pm 4.18\%$ respectively as compared to the negative control. There was a slightly higher wound closure rate in the cells with the treatment of 62.5 µg/mL extract than other extracts and the elevation of cell migration was 8.79% in contradiction with the negative control. All the concentrations of BD lipid pulp extracts exhibited better enhancement in cell migration than the positive control. After treatment for 48 h, the wounds treated by all the BD lipid pulp extracts were fully sealed, which resembled the wound closure rates of the negative control and positive control.

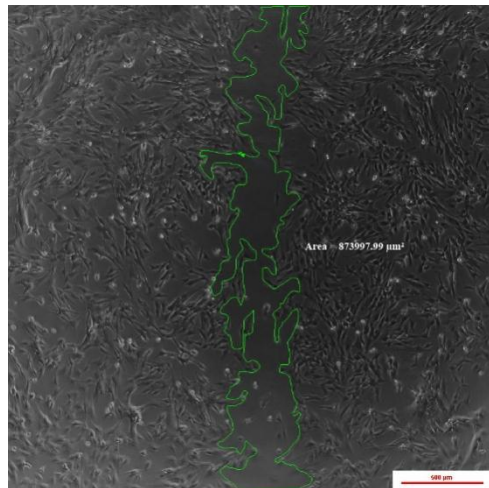
0h



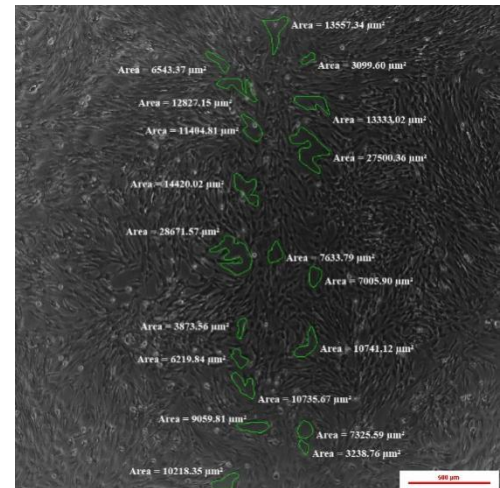
6h



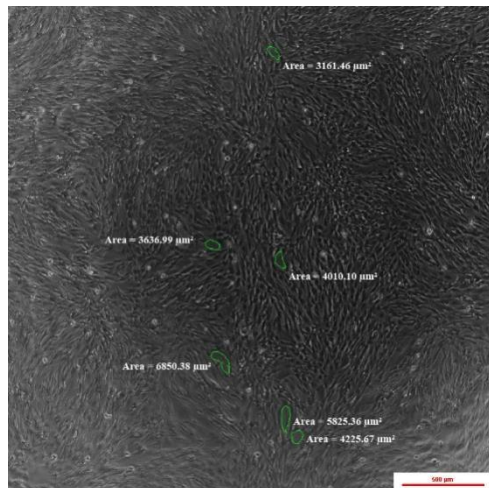
12h



24h



36h



48h

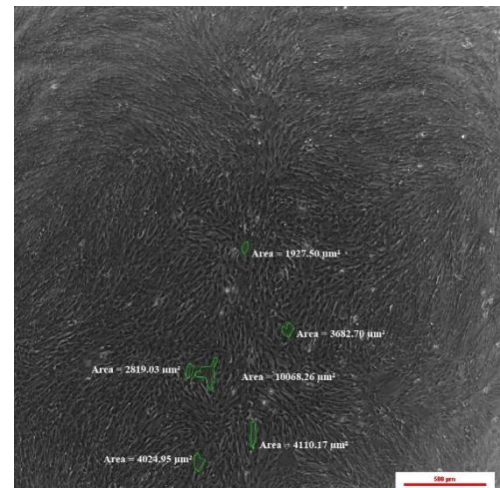
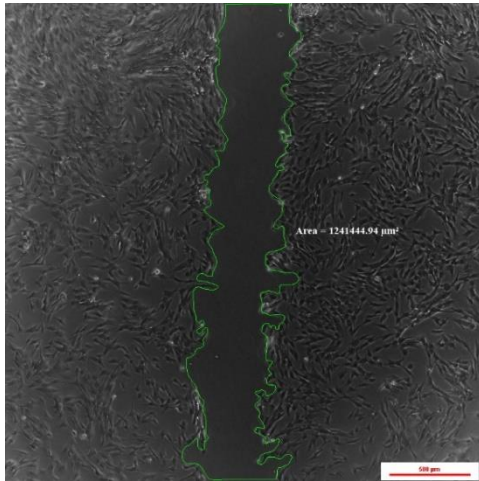
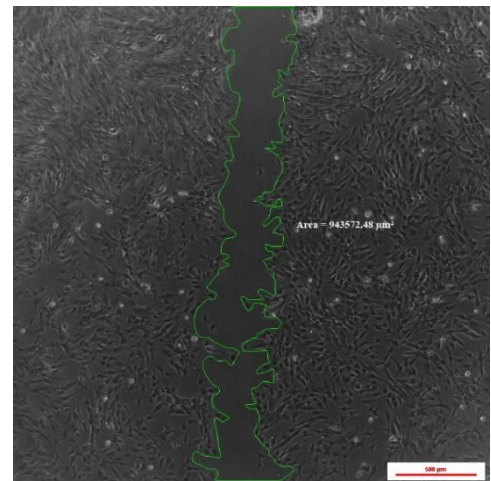


Figure 4.19: Migration of fibroblasts without treatment for 48h.

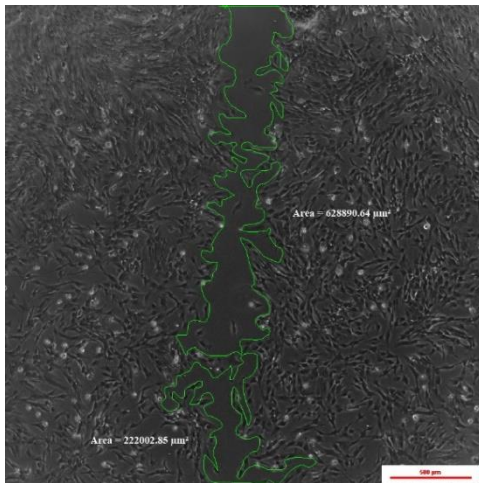
0h



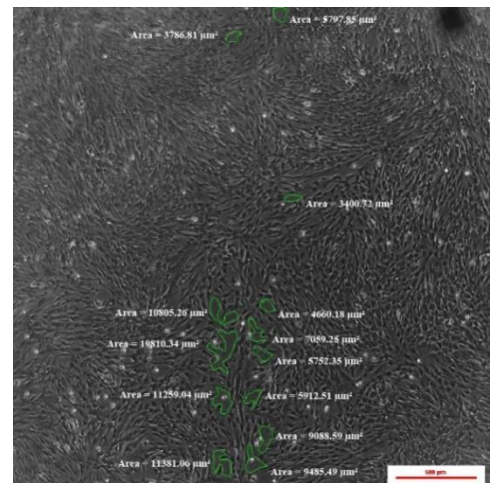
6h



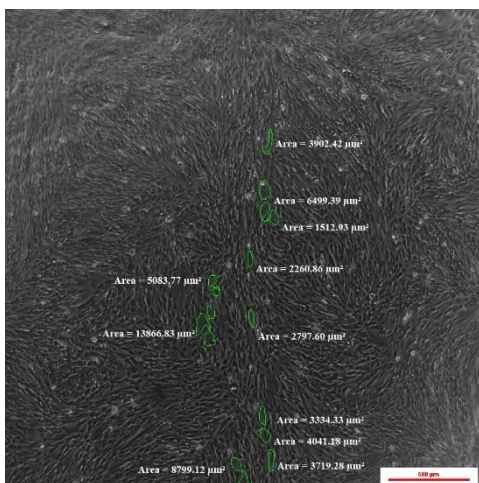
12h



24h



36h



48h

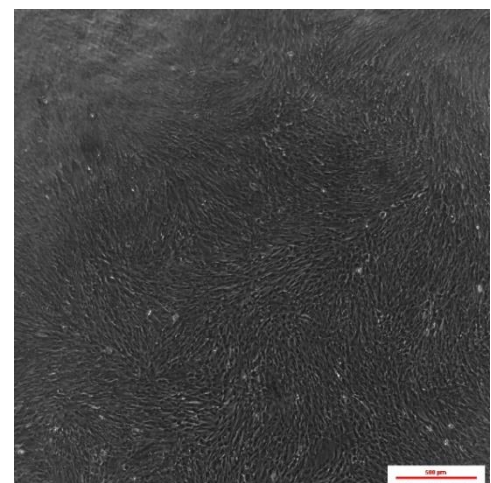


Figure 4.20: Migration of fibroblasts after treatment with 31.25 $\mu\text{g/mL}$ petroleum ether pulp extract for 48h.

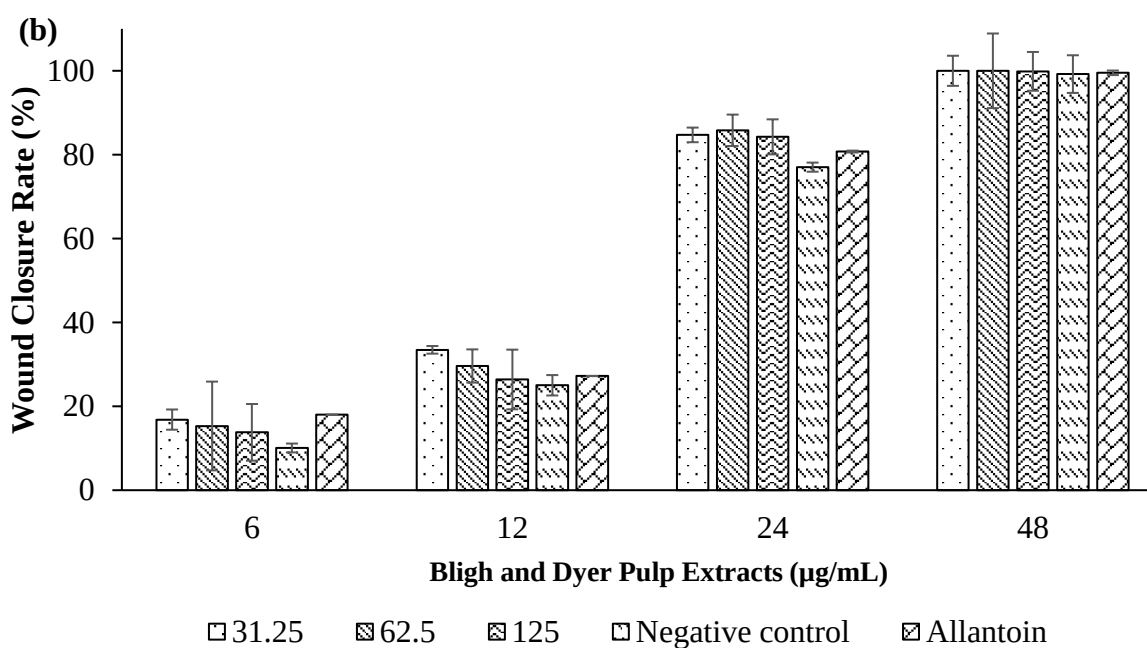
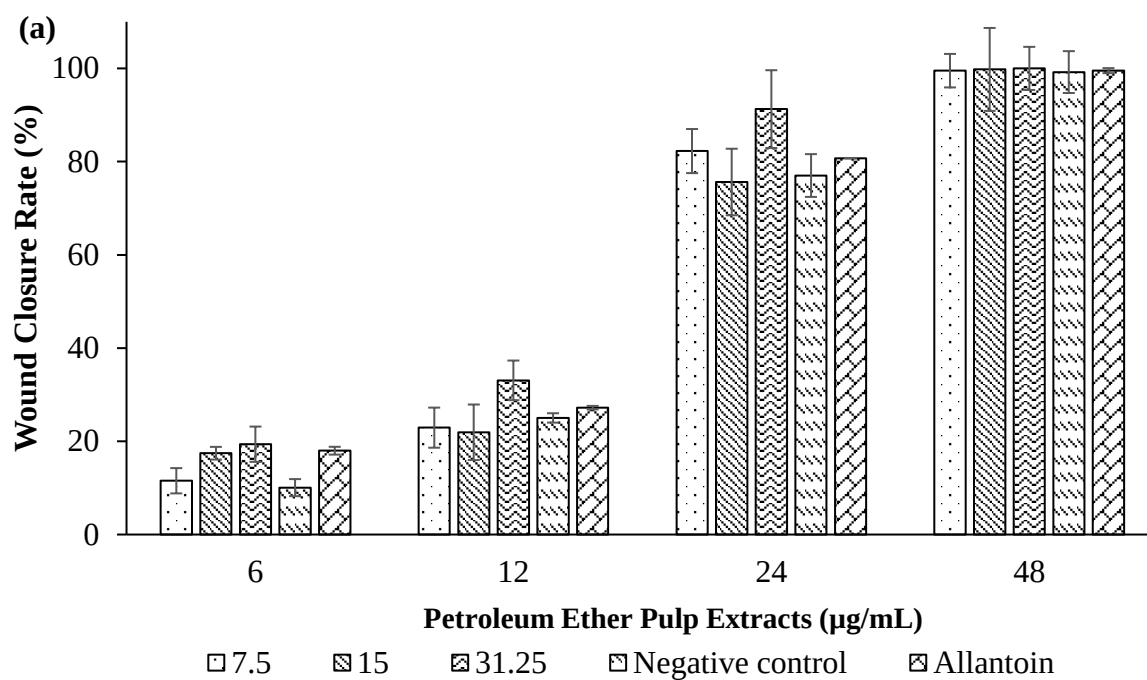


Figure 4.21: Migration of fibroblasts after treatment with (a) petroleum ether (7.5 - 31.25 $\mu\text{g/mL}$) and (b) Bligh and Dyer (31.25 - 125 $\mu\text{g/mL}$) pulp extracts as compared with the negative control for 48h. The data were expressed as mean $\pm$ SD ($n = 3$) and were analysed using one-way ANOVA followed by Tukey's HSD test. There was no statistically significant difference ($p > 0.05$) between all pulp extracts and negative control.

Figure 4.22 showed that after 6 h incubation with methanolic pulp extracts (31.25 µg/mL, 62.5 µg/mL, and 125 µg/mL), there were increment in wound closure rates which were $14.00 \pm 10.26\%$, $16.88 \pm 6.93\%$ and $12.54 \pm 2.20\%$ respectively. The 62.5 µg/mL of the extract was revealed to be the highest cell migration rate with an increment of 6.81% in contrast with the negative control. While for the positive control, it was found that the cell migration rate was closely resembled to the 62.5 µg/mL of extract.

After 12 h of incubation, only methanolic pulp extract at 62.5 µg/mL caused improvement in the cell migration, which was $27.71 \pm 7.81\%$ of wound closure rate. There was an improvement of 2.69% as opposed to the negative control. The cell migration rate after treatment with 62.5 µg/mL of the extract was identical to the positive control.

After treatment for 24 h, the wound closure rates of cells treated with methanolic pulp extracts at concentrations of 31.25 µg/mL and 62.5 µg/mL were $81.48 \pm 11.96\%$ and $85.47 \pm 11.06\%$ respectively. The wound closure rate for cells with the treatment of methanolic pulp extracts with a concentration of 62.5 µg/mL was increased by 8.45% as opposed to the negative control. Cells treated with the extract at this concentration (62.5 µg/mL) of extract had enhanced the cell migration rate slightly greater than the positive control.

After 48 h of incubation, the wounds treated with methanolic pulp extracts at different concentrations were fully recovered. The cell migration rates of the extracts were the same as the negative control and positive control.

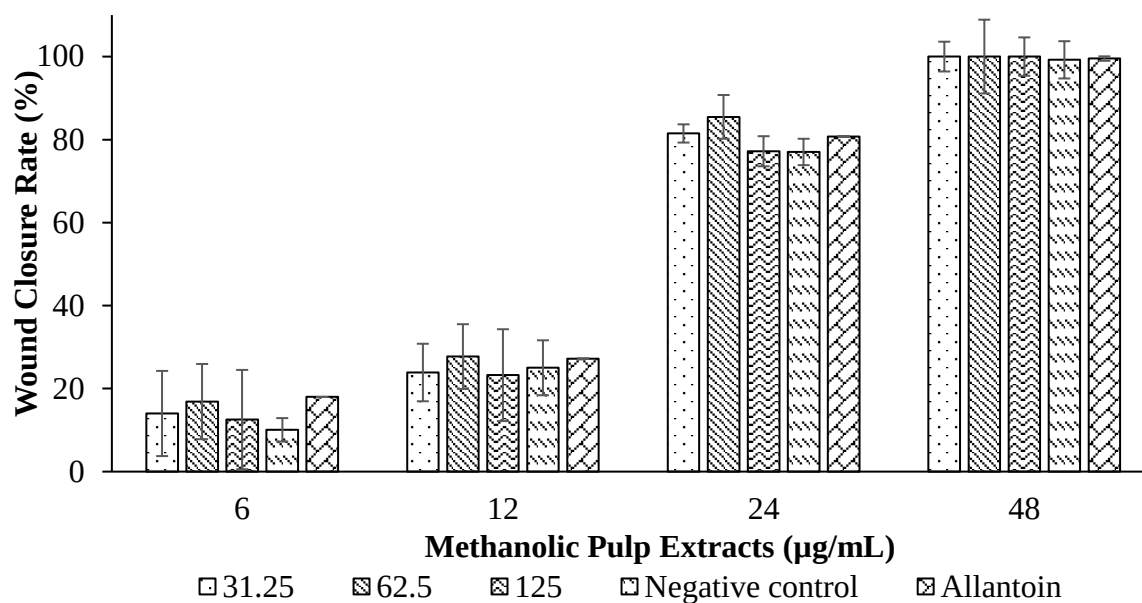


Figure 4.22: Migration of fibroblasts after treatment with methanolic pulp extracts (31.25 - 125 µg/mL) as compared with the negative control for 48h. The data were expressed as mean $\pm$ SD (n = 3) and were analysed using one-way ANOVA followed by Tukey's HSD test. There was no statistically significant difference ($p > 0.05$) between methanolic pulp extracts and negative control.

The effect of *L. garciae* lipid seed extracts on fibroblast cells migration was illustrated in Figure 4.23. After incubation for 6 h, all the PE lipid seed extracts (7.5 µg/mL, 15 µg/mL, and 31.25 µg/mL) had demonstrated improvement in cell migration, where there were $22.28 \pm 1.24\%$, $17.22 \pm 1.55\%$ and $21.81 \pm 11.37\%$ of wound closure rates respectively. Incubation of cells with 7.5 µg/mL of PE lipid seed extracts caused the most increment in cell migration rate, which was 12.22% in contrast to the negative control. For the positive control, the cell migration rate was similar to the cells treated with 7.5 µg/mL of PE lipid seed extract.

After 12 h of treatment, there was an elevation in wound closure rates in all the cells treated with all the PE lipid seed extracts, which were $34.11 \pm 3.51\%$, $34.24 \pm 5.42\%$ and $40.87 \pm 13.93\%$ respectively. This gave an enhancement of 15.85% in the cell migration rate for the cells after treatment with 31.25 µg/mL of extract against the negative control. All the PE lipid seed extracts caused a higher enhancement in the cell migration rate than the positive control.

After treatment for 24 h, the wound closure rates for the cells treated with all the PE lipid seed extracts were $90.62 \pm 3.12\%$, $89.38 \pm 5.43\%$ and $86.61 \pm 9.68\%$ respectively. The cells with the treatment of 7.5 µg/mL PE lipid extract exhibited an elevation of 13.60% in cell migration in contradiction with the negative control. There was a better improvement in cell migration rate for all the PE lipid seed extracts against the positive control. After 48 h of incubation, the full closure of wounds treated by all the PE lipid seed extracts were observed which resembled the negative control and positive control.

Apart from that, after treatment for 6 h, there were amelioration in the wound closure rates for the cells treated with all the BD lipid seed extracts (7.5 µg/mL, 15 µg/mL, and 31.25 µg/mL). Cells incubated with 15 µg/mL of BD lipid seed extract had manifested the highest increment in cell migration, in which the wound closure rate was $18.67 \pm 4.30\%$ with 8.61% of increment versus the negative control. Cells treated with 15 µg/mL of BD lipid seed extract exhibited the same cell migration rate as the positive control.

After incubation for 12 h, the wound closure rates for the cells treated with all the concentrations of BD lipid seed extracts were ameliorated, which were $29.28 \pm 10.38\%$, $29.26 \pm 4.25\%$ and $28.63 \pm 2.27\%$ respectively. The greatest enhancement in the cell migration was revealed by the cells with the treatment of 15 µg/mL of BD lipid seed extract, where there was 4.24% of improvement in the wound closure rate as opposed to the negative control. The cells treated with all the BD lipid seed extracts had displayed similar cell migration rate with the positive control.

After treatment for 24 h, the cells with the treatment of all the BD lipid seed extracts had demonstrated an elevation in the cell migration with the wound closure rates of $83.22 \pm 4.01\%$, $85.81 \pm 2.77\%$ and $92.52 \pm 6.67\%$ respectively. Incubation with 31.25 µg/mL of BD lipid seed extract had improved the cell migration by 15.50% in contrast with the negative control. It was shown that incubation with 31.25 µg/mL of the extract ameliorated the cell migration rate greater than the positive control. After incubation for 48 h, the cells treated with all the BD lipid seed extracts exhibited a complete wound closure which was similar to the negative control and positive control.

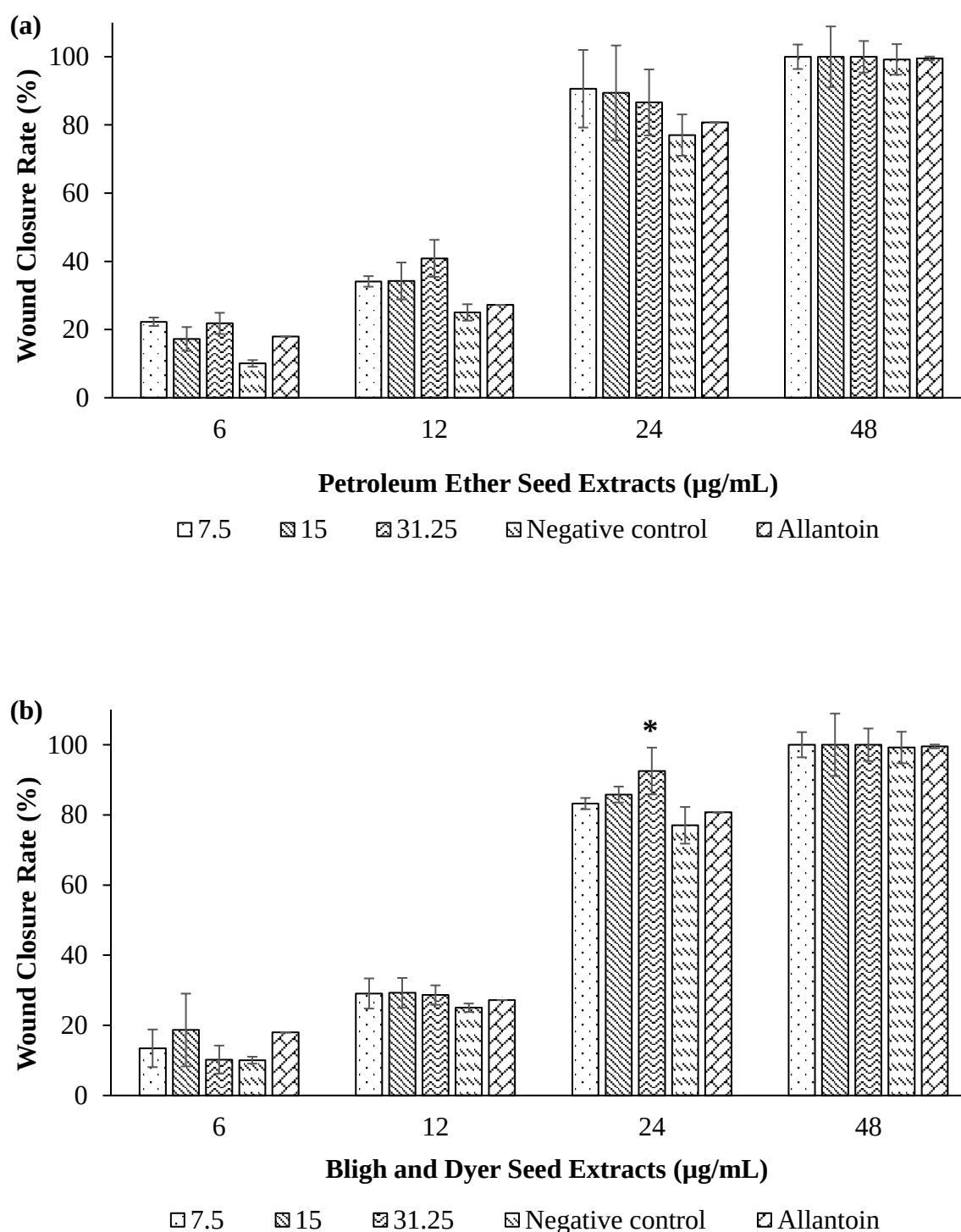


Figure 4.23: Migration of fibroblasts after treatment with (a) petroleum ether (7.5 - 31.25 µg/mL), (b) Bligh and Dyer (7.5 - 31.25 µg/mL) seed extracts as compared with the negative control for 48 h. The data were expressed as mean $\pm$ SD (n = 3) and were analysed using one-way ANOVA followed by Tukey's HSD test. There was a statistical significant difference (*p<0.05) between 31.25 µg/mL Bligh and Dyer seed extract and negative control at 24 h while the other seed extracts were statistically insignificant different (p>0.05).

In addition to that, after incubation for 6 h to 24 h, all the methanolic seed extracts (31.25 $\mu\text{g/mL}$, 62.5 $\mu\text{g/mL}$, and 125 $\mu\text{g/mL}$) did not show any improvement in the cell migration rate, as demonstrated in Figure 4.24. The positive control manifested enhancement in cell migration as compared to all the methanolic seed extracts. After 48 h of incubation, the wounds that incubated with all the methanolic seed extracts were found to exhibit the same cell migration rate as the negative control and positive control.

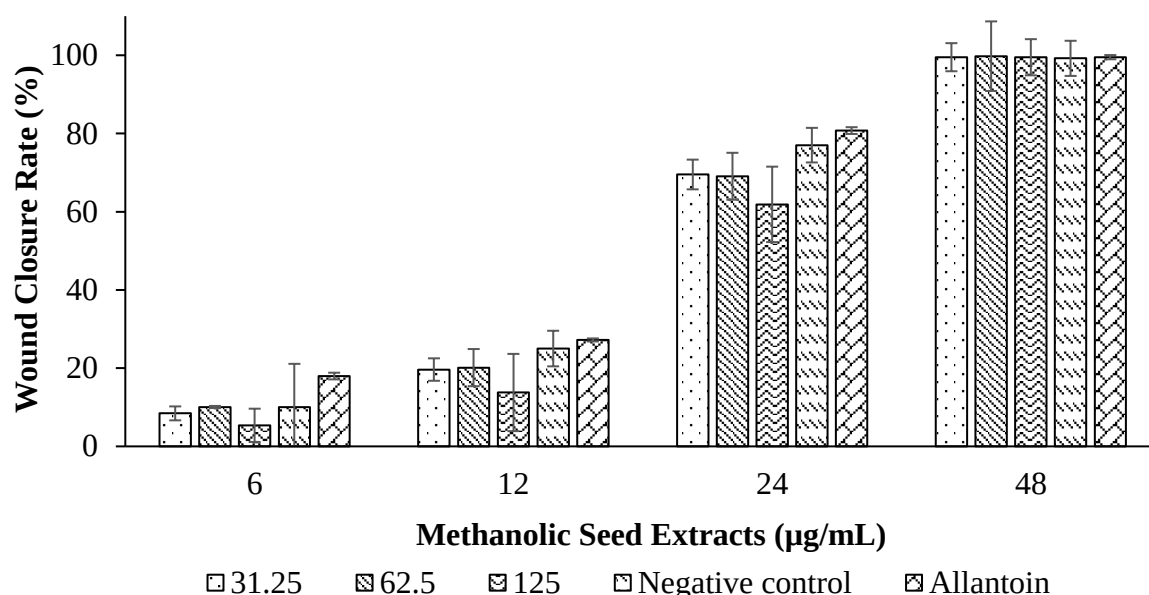


Figure 4.24: Migration of fibroblasts after treatment with methanolic seed extracts (31.25 – 125 $\mu\text{g/mL}$) as compared with the negative control for 48h. The data were expressed as mean $\pm$ SD ($n = 3$) and were analysed using one-way ANOVA followed by Tukey’s HSD test. There was no statistically significant difference ($p > 0.05$) between methanolic seed extracts and negative control.

Besides, after 6 h of incubation, the allantoin which acted as a positive control had improved the cell migration by 7.93%, where the wound closure rate was $17.99 \pm 3.59\%$ on contrary to the negative control. After treatment for 12 h, the wound closure rate of the cells after the treatment of allantoin was $27.22 \pm 8.89\%$ with an enhancement of 2.20% when comparing to the negative control. After 24 h of incubation, the cell migration of the cells treated allantoin had increased to $80.75 \pm 4.63\%$ against the negative control, with the improvement of 3.73% in the wound closure rate. After 48 h of treatment, the wound was fully recovered which was identical to the negative control.

Generally, with the exception methanolic seed extract, the *L. garciae* pulp and seed extracts had caused improvement in the cell migration rate. There was a statistical significant difference ($p < 0.05$) in the cell migration for the cells treated with 31.25 $\mu\text{g/mL}$ BD lipid seed extract after treatment for 24 h.

In short, incubation of cells with 7.5 $\mu\text{g/mL}$ of the PE lipid seed extract had demonstrated the highest increment in cell migration (12.22%) after 6 h of treatment. After incubation for 12 h, PE lipid seed extract at 31.25 $\mu\text{g/mL}$ caused a notable enhancement in cell migration (15.85%). In addition, cells treated with 31.25 $\mu\text{g/mL}$ of BD lipid seed extract, caused significant amelioration in cell migration after 24 h of treatment which was 15.50%. On top of that, it was found that some of the *L. garciae* pulp and seed extracts had caused a greater improvement in cell migration than the allantoin at every time points.

4.6.2 Effects of *L. garciae* Extracts on Fibroblasts' Chemotactic Motility

The transwell migration assay was done for an assessment of the effect of pulp and seed extracts of *L. garciae* on the chemotactic motility of the fibroblasts. Apart from methanolic seed extract, the scratch assays (section 4.6.1) showed that *L. garciae* pulp and seed extracts at a concentration of 31.25 µg/mL increased the cell migration. Therefore, 31.25 µg/mL was selected as the concentration for the transwell assay. In this experiment, two types of negative controls were employed where the cells were cultured in complete medium (10% FBS) and in serum-free medium (SFM). Fibroblasts were cultured in a SFM for 24 h before the transwell assay to increase the sensitivity of cells towards the extracts and therefore improved the cell migration.

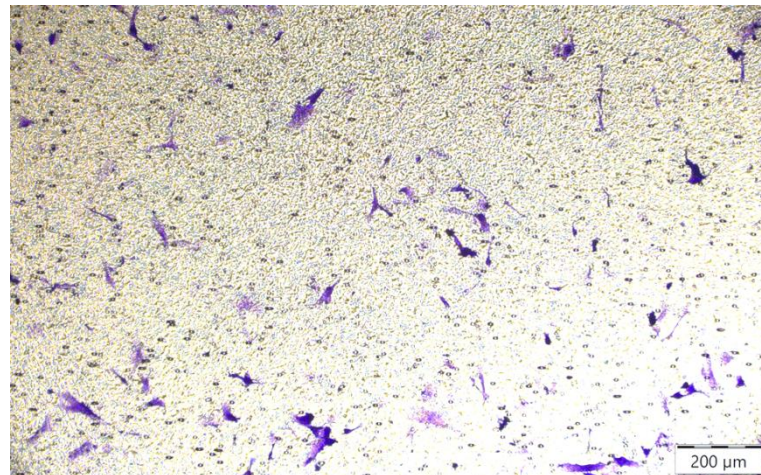
Figure 4.26 and Figure 4.27 (a) showed that all the *L. garciae* pulp extracts (PE, BD and methanolic pulp) enhanced the chemotactic motility of the fibroblasts as opposed to the negative controls. The cell number of negative control (SFM) was 524 ± 90.07 while there were 897 ± 123.06 cells for the negative control (10% FBS) (Figure 4.25). The incubation with PE lipid pulp extract had significantly increased the number of fibroblasts that migrated through a transwell insert to 1326 ± 244.31 which was an increment of 153% against the negative control (SFM) and an increment of 47.84% against the negative control (10% FBS). Similarly, there was a significant improvement in the chemotactic motility of the fibroblasts that were treated with BD lipid pulp extract where the cell number increased to 1279 ± 88.39 . The increment in the chemotactic motility was 144.08% compared to the negative control (SFM) and there was an increment of 42.64% in contradiction with the negative control (10% FBS). Besides, methanolic pulp extract was also shown to significantly enhanced the cell chemotactic motility where the cell number increased by 134.35% and 36.95% in contrast

to the negative control (SFM) and negative control (10% FBS) respectively, which gave the cell number of 1228 ± 209.89 .

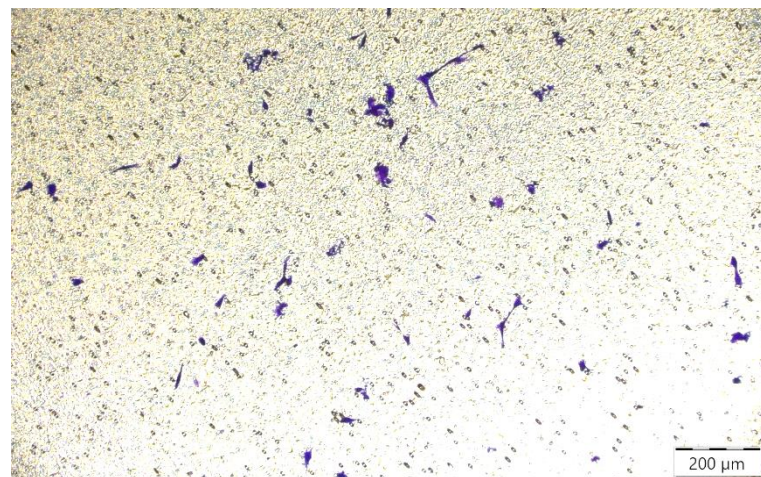
Similarly, all the *L. garciae* seed extracts (PE, BD and methanolic seed) demonstrated an improvement in chemotactic motility of the fibroblasts (Figure 4.26 and Figure 4.27 (b)) when compared to the negative control (SFM). On the contrary, only PE and BD lipid seed extracts had shown amelioration in the chemotaxis of fibroblasts against the negative control (10% FBS). For the PE lipid seed extract, there was 2016 ± 232.50 of fibroblasts migrated, in which the cell number had elevated by 284.73% and 124.83% in contrast to the negative control (SFM) and negative control (10% FBS) respectively. Apart from that, the cell number of BD lipid seed extract was 1708 ± 284.23 , where it had manifested an increment in the chemotaxis of fibroblasts on contrary to the negative control (SFM) and negative control (10% FBS) by 226.02% and 90.52% respectively. For the methanolic seed extract, the cell number was 739 ± 121.93 where there was an increment of 41.09% in chemotactic motility of fibroblasts versus the negative control (SFM). In contrast, methanolic seed extract did not display enhancement in the chemotaxis of fibroblasts when comparing to the negative control (10% FBS).

In summary, the seed extracts, PESE and BDSE could induce a higher chemotactic response as compared to all the pulp extracts. In addition, the presence of 10% FBS influenced the fibroblasts' chemotactic motility where it caused enhancement in the fibroblast's chemotactic motility but still with lower enhancement effects than the extracts. In conclusion, with the exception of methanol seed extract, all the *L. garciae* pulp and seed extracts could induce a chemotactic response and increase the fibroblast cells motility. This

result presumably suggests one of the action mechanisms of the *L. garciae* extracts in promoting wound healing.

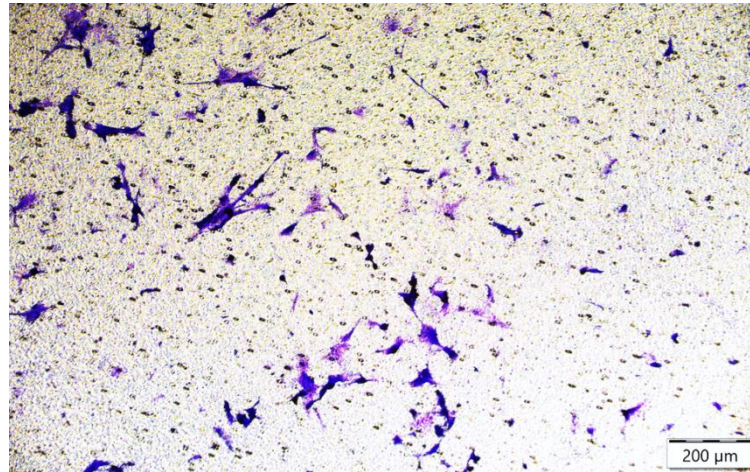


Negative control (10% FBS)

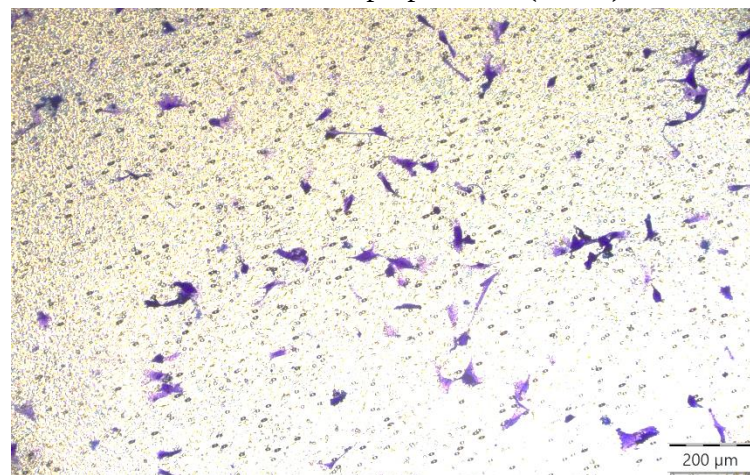


Negative control (SFM)

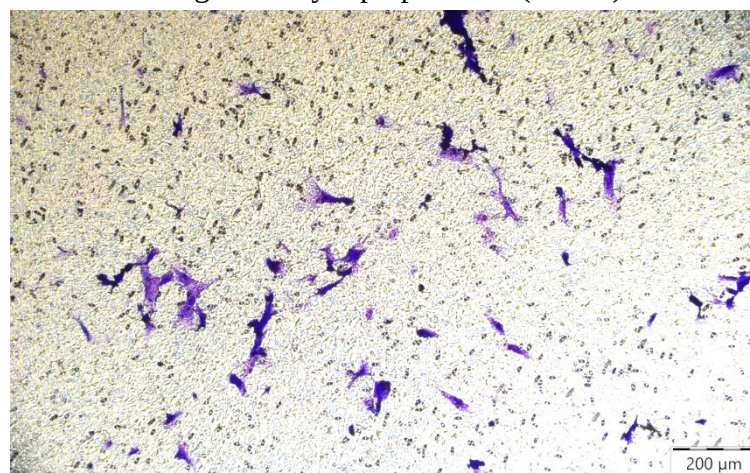
Figure 4.25: Chemotactic motility of fibroblasts without treatment (growth media with and without FBS).



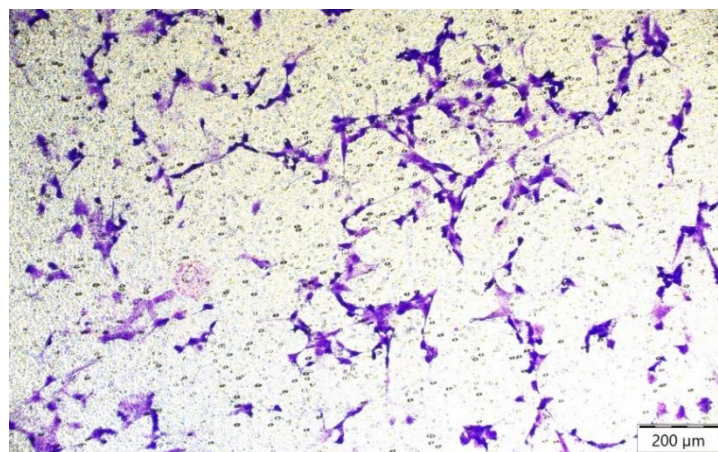
Petroleum ether pulp extract (PEPE)



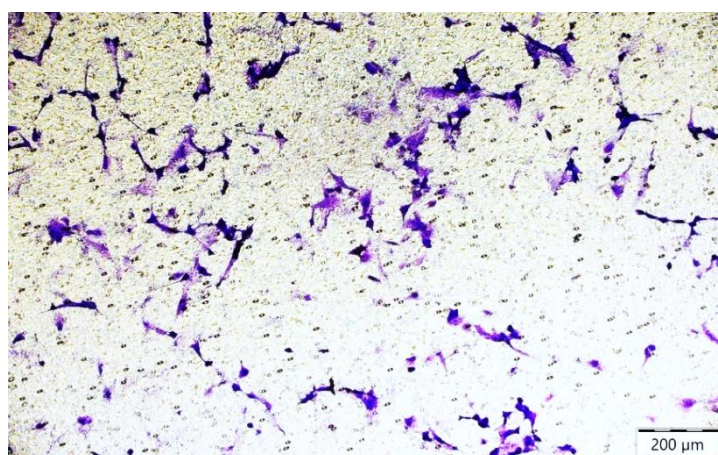
Bligh and Dyer pulp extract (BDPE)



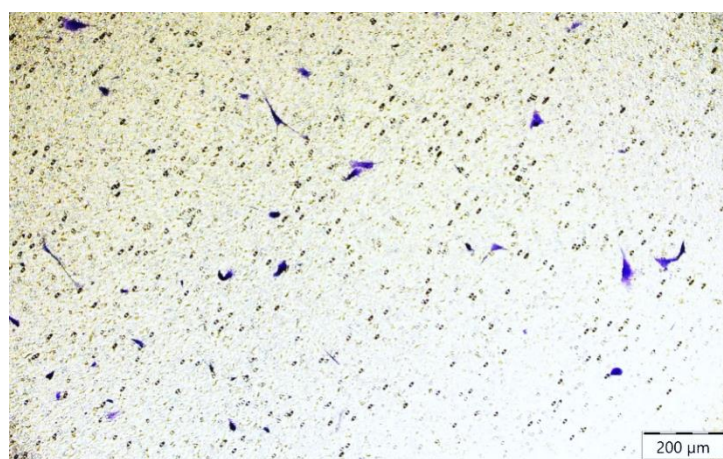
Methanolic pulp extract (MEPE)



Petroleum ether seed extract (PESE)



Bligh and Dyer seed extract (BDSE)



Methanolic seed extract (MESE)

Figure 4.26: Transwell assay to detect the chemotactic motility of fibroblasts. Cells were treated with 31.25 $\mu\text{g/mL}$ petroleum ether, Bligh and Dyer and methanolic pulp and seed extracts. The migrated cells on the lower chamber membrane were stained and counted. The number of cells were shown in the Figure 4.27.

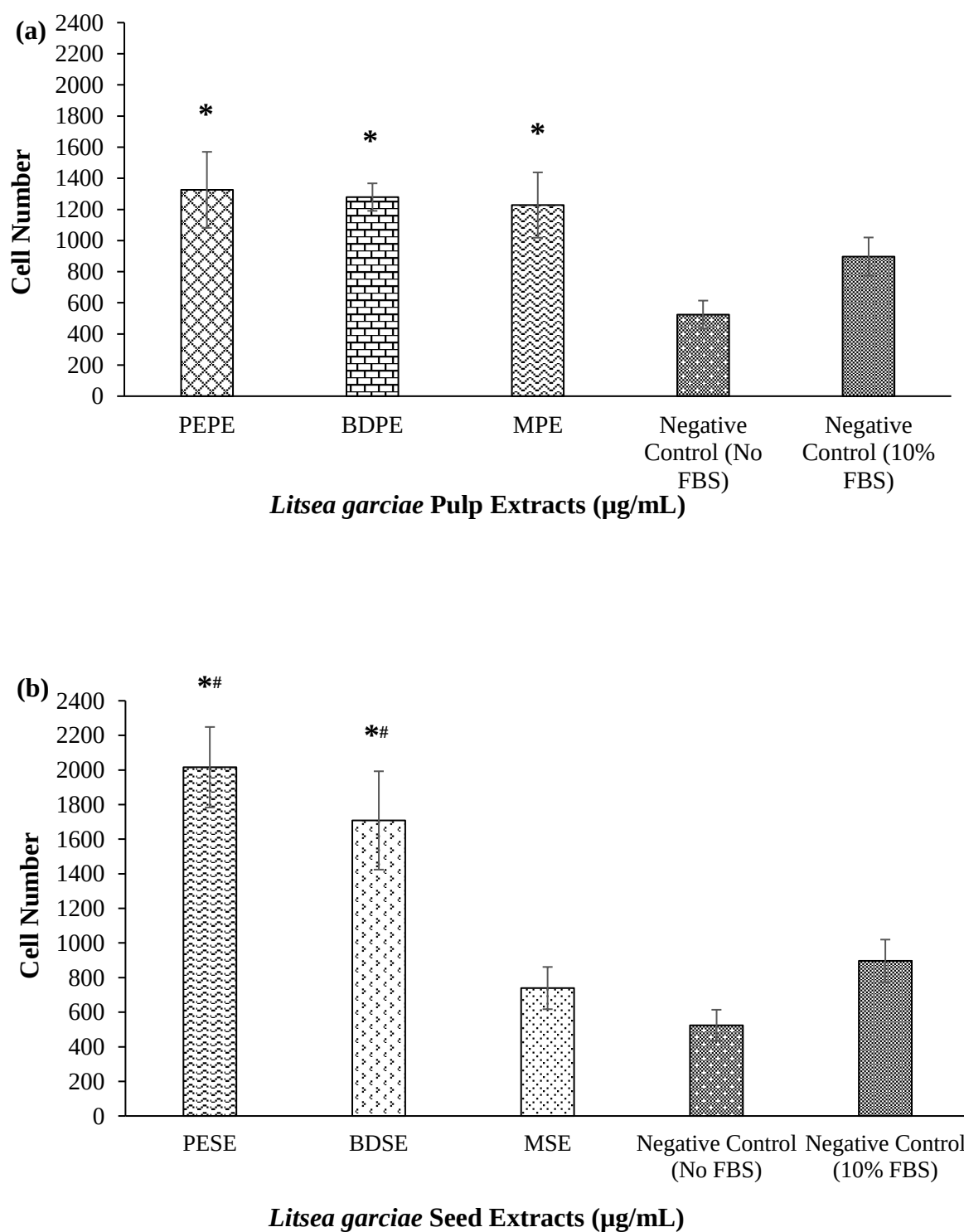


Figure 4.27: Chemotactic motility of fibroblasts after treatment with petroleum ether, methanolic and Bligh and Dyer (a) pulp and (b) seed extracts ($31.25 \mu\text{g/mL}$) compared with the negative controls after 24 h incubation. The data were expressed as mean $\pm$ SD ($n = 3$) and were analysed using one-way ANOVA followed by Tukey's HSD test. The statistical significant difference ($p < 0.05$) between extracts and negative controls (No FBS and 10% FBS) were represented by (*) and (#) respectively.

4.6.3 Effects of *L. garciae* Extracts on Wound Healing Markers

4.6.3.1 Effects of *L. garciae* Extracts on the Synthesis of Collagen Type I

In the process of wound healing, the function of collagen is for the attraction of fibroblasts and stimulation of new collagen deposition to the lesion site. Collagen type I is the most abundant collagen type. It occupies more than 90% of the tissue and it is vital in the wound healing process. Optimum deposition of collagen is essential during connective tissue formation and prevention of scarring.

The effects of the *L. garciae* pulp and seed extracts on the collagen type I synthesis by wounded fibroblasts from the wound scratch assay were quantified using the human Pro-Collagen I alpha 1 ELISA. The treatment of fibroblast with 31.25 µg/mL of PE lipid pulp (Figure 4.28) significantly increased the collagen type I synthesis by 74.26% in contrast with the negative control. The concentration of collagen type I synthesised by the negative control was $1.17 \times 10^5 \pm 0.02$ pg/mL and $2.03 \times 10^5 \pm 0.37$ pg/mL by fibroblasts treated with 31.25 µg/mL of PE lipid pulp.

Moreover, incubation of fibroblasts with BD lipid pulp extracts of concentrations 31.25 µg/mL, 62.5 µg/mL and 125 µg/mL also enhanced the synthesis of collagen type I, with an increase in collagen type I concentrations by 127.87%, 107.74% and 20.23% respectively against the negative control. The concentrations of collagen type I were $2.66 \times 10^5 \pm 0.31$ pg/mL, $2.42 \times 10^5 \pm 0.23$ pg/mL and $1.40 \times 10^5 \pm 0.14$ pg/mL when treated with 31.25 µg/mL, 62.5 µg/mL and 125 µg/mL respectively compared to $1.17 \times 10^5 \pm 0.02$ pg/mL of control. Furthermore, the treatment of fibroblasts with methanolic pulp extracts at concentrations of 31.25 µg/mL and 62.5 µg/mL also elevated the synthesis of collagen type I contents by 53.84% and 48.96% respectively in contradiction with the negative control.

The concentration of collagen type I synthesised by the fibroblasts treated with methanolic pulp extracts at 31.25 µg/mL was $1.79 \times 10^5 \pm 0.03$ pg/mL and by fibroblasts treated with 62.5 µg/mL of methanolic pulp extracts was $1.74 \times 10^5 \pm 0.26$ pg/mL.

For the seed extracts, only the PE lipid seed extract at 7.5 µg/mL, BD lipid seed extract at 15 µg/mL and methanolic seed extract at 31.25 µg/mL caused increased synthesis of collagen type I synthesis. Incubation of fibroblasts with 7.5 µg/mL PE lipid seed extract caused an increment in the collagen type I synthesis by 32.65%, which was $1.55 \times 10^5 \pm 0.15$ pg/mL as compared to the negative control. Besides, 15 µg/mL of BD lipid seed extract with was found to enhance the collagen type I content by 11.47%, in which the concentration of collagen type I was $1.30 \times 10^5 \pm 0.00$ pg/mL as compared to the negative control. Incubation of fibroblasts with 31.25 µg/mL of the methanolic seed extract increased the synthesis of collagen type I to $1.32 \times 10^5 \pm 0.24$ pg/mL, with an amelioration of 13.54% in contrast with the negative control. Interestingly, incubation of fibroblasts with the positive control, allantoin did not show increment in the collagen type I synthesis in contradiction with the negative control.

In conclusion, the lipid and methanolic pulp and seed extracts caused a significant increase in the collagen type I synthesis for the wounded fibroblast cells. However, the increment in collagen type I synthesis was more prominent for the fibroblast cells incubated with the pulp lipid and methanolic extracts than in the fibroblast cells treated with the seed extracts. The results could also suggest that *L. garciae* could promote wound healing by increasing the synthesis of collagen type I.

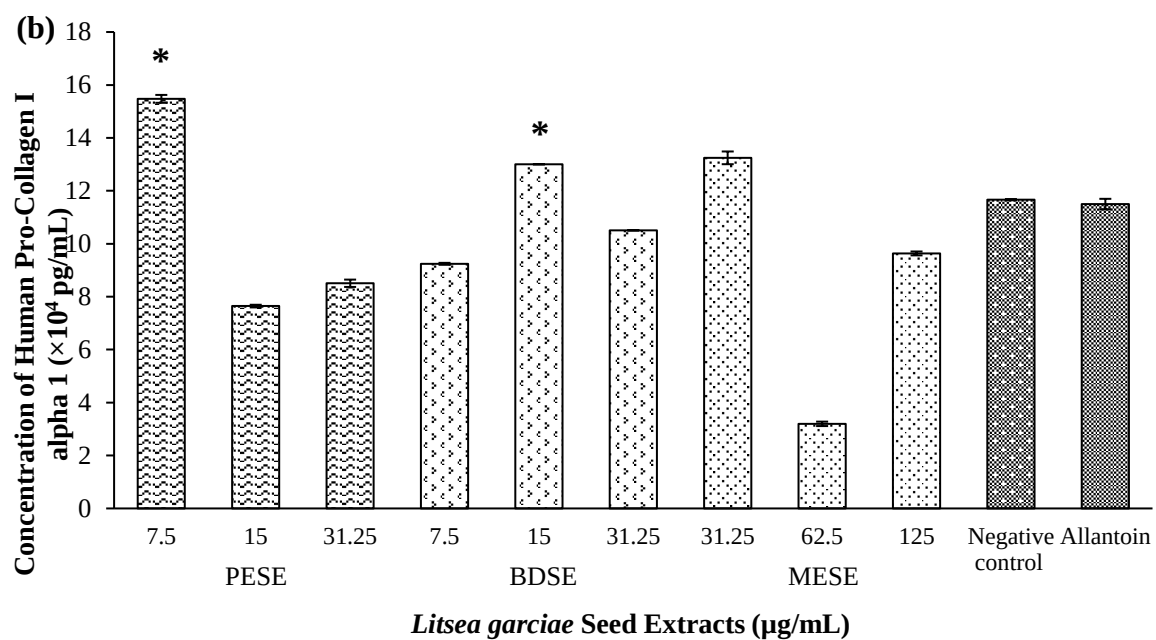
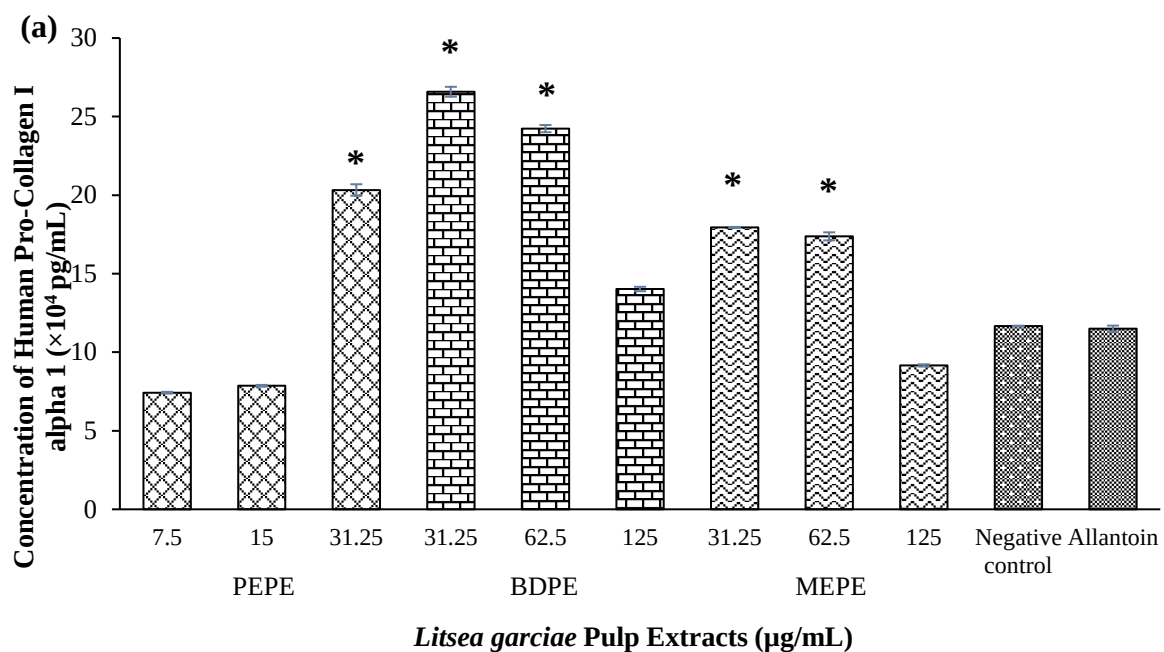


Figure 4.28: The effect of petroleum ether, Bligh and Dyer and methanolic (a) pulp and (b) seed extracts on the expression of collagen I by wounded fibroblasts. The data were expressed as mean $\pm$ SD ($n = 3$) and were analysed using one-way ANOVA followed by Tukey's HSD test. The statistical significant difference ($p < 0.05$) between pulp and seed extracts and negative control were represented by (*).

4.6.3.2 Effects of *L. garciae* Extracts on the Secretion of Epidermal Growth Factor (EGF)

EGF is essential to initiate the proliferation and migration of fibroblasts during wound healing, where the collagen synthesis and regeneration of skin are stimulated through the keratinocyte-fibroblast interactions (Kim et al., 2015). The effects of *L. garciae* extracts on the secretion of EGF by the wounded fibroblasts were evaluated using Human EGF ELISA.

The secretion of EGF by wounded fibroblasts was improved after treatment with different concentrations of PE lipid pulp extracts. In comparison to the negative control, incubation of fibroblasts with 7.5 µg/mL, 15 µg/mL and 31.25 µg/mL caused an increment in the secretion of EGF by 8.77%, 24.00% and 15.38% respectively (Figure 4.29). Similarly, incubation of fibroblasts with BD lipid pulp extract with concentrations of 31.25 µg/mL, 62.5 µg/mL and 125 µg/mL showed an increased trend in secretion of EGF by 8.77%, 8.77% and 10.00% respectively in comparison to control. Furthermore, incubation of fibroblasts with the methanolic pulp extracts at concentrations of 31.25 µg/mL 62.5 µg/mL and 125 µg/mL also demonstrated an increment in the secretion of EGF level by 13.69%, 7.54% and 11.69% which were 0.28 pg/mL, 0.26 pg/mL and 0.27 pg/mL respectively. Moreover, there was no effect on EGF secretion for the PE lipid seed extract.

For the *L. garciae* seed extract, only the treatment with 15 µg/mL and 31.25 µg/mL of BD lipid seed extracts increased the secretion of EGF level to 0.31 pg/mL and 0.29 pg/mL respectively compared to 0.24 pg/ml by the negative control. Thus, there were 27.85% and 18.15% increases in the EGF secretion level respectively in contrast to the negative control. Interestingly, incubation of fibroblasts with the positive control, allantoin did not show

improvement in the secretion of EGF levels (Figure 4.29). Apart from that, methanolic seed extracts were revealed to show no effect on the EGF secretion level.

In conclusion, there was an increased trend in the secretion of EGF by fibroblasts incubated with *L. garciae* lipid and methanolic pulp extracts. However, for *L. garciae* seed extract only treatment with BD lipid seed extract improved the secretion of EGF. Nevertheless, these results indicated that *L. garciae* extracts could promote wound healing by increasing the secretion of EGF.

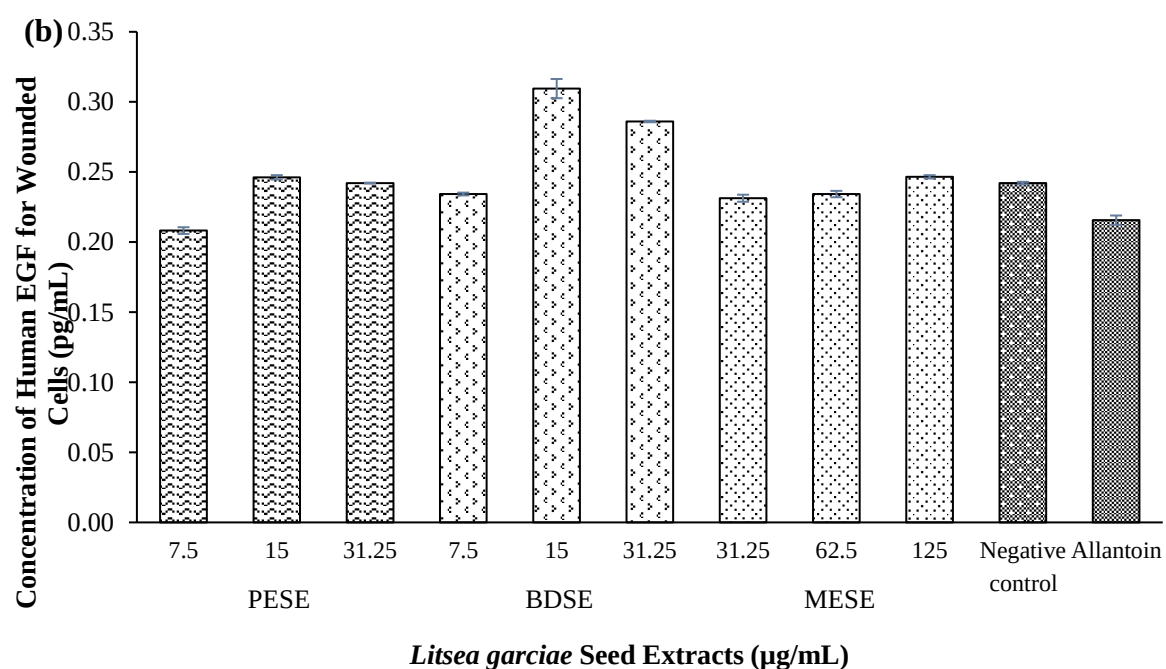
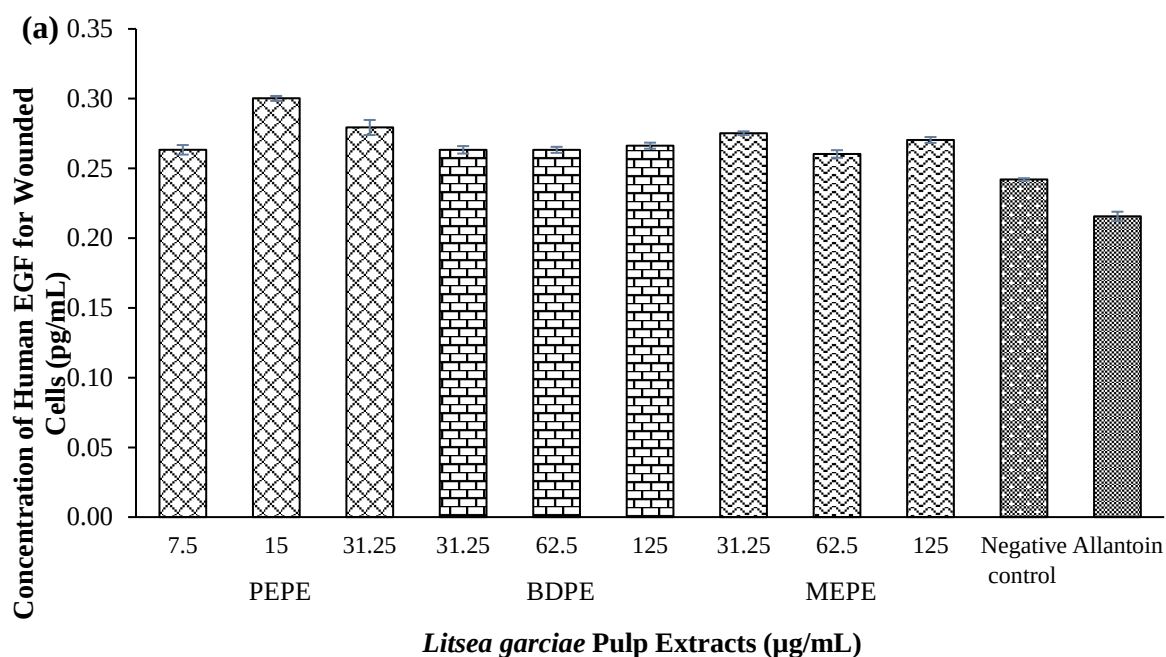


Figure 4.29: The effect of petroleum ether, Bligh and Dyer and methanolic (a) pulp and (b) seed extracts on the secretion of EGF by wounded fibroblasts. The data were expressed as mean $\pm$ SD ($n = 3$) and were analysed using one-way ANOVA followed by Tukey's HSD test. There was no statistical significant difference ($p > 0.05$) between all pulp and seed extracts and negative control.

4.6.3.3 Effects of *L. garciae* Extracts on the Secretion of Vascular Endothelial Growth Factor (VEGF)

VEGF stimulates wound healing by increasing vascular permeability, altering the extracellular matrix components, and promoting angiogenesis. It promotes collagen deposition and epithelialisation as well. The effects of the *L. garciae* pulp and seed extracts on the secretion of VEGF level of wounded fibroblasts by the *L. garciae* pulp and seed extracts were determined using Human VEGF-A ELISA (Figure 4.30).

Firstly, incubation of fibroblasts with PE lipid pulp extracts did not show enhancement in the VEGF secretion. In contrast, incubation of fibroblasts with BD lipid pulp extracts (31.25 µg/mL 62.5 µg/mL and 125 µg/mL) had manifested improvement in the VEGF secretion level which were 525.27 pg/mL, 522.80 pg/mL and 617.73 pg/mL. There were 2.67%, 2.19% and 20.75% of elevation respectively for all BD lipid pulp extracts as compared to the negative control (511.60 pg/mL). Furthermore, all the methanolic pulp extracts (31.25 µg/mL 62.5 µg/mL and 125 µg/mL) were demonstrated to elevate and enhance the secretion of VEGF by 5.56%, 22.52% and 8.07% respectively, where there were 540.07 pg/mL, 626.80 pg/mL and 552.87 pg/mL of VEGF secretion as opposed to the negative control.

On the contrary, incubation of fibroblasts with all the seed extracts did not increase the VEGF secretion level. Besides, the positive control allantoin did not cause any improvement in the secretion of VEGF of the wounded fibroblasts. In short, only the BD lipid pulp extract and the methanolic pulp extracts enhance the secretion of VEGF.

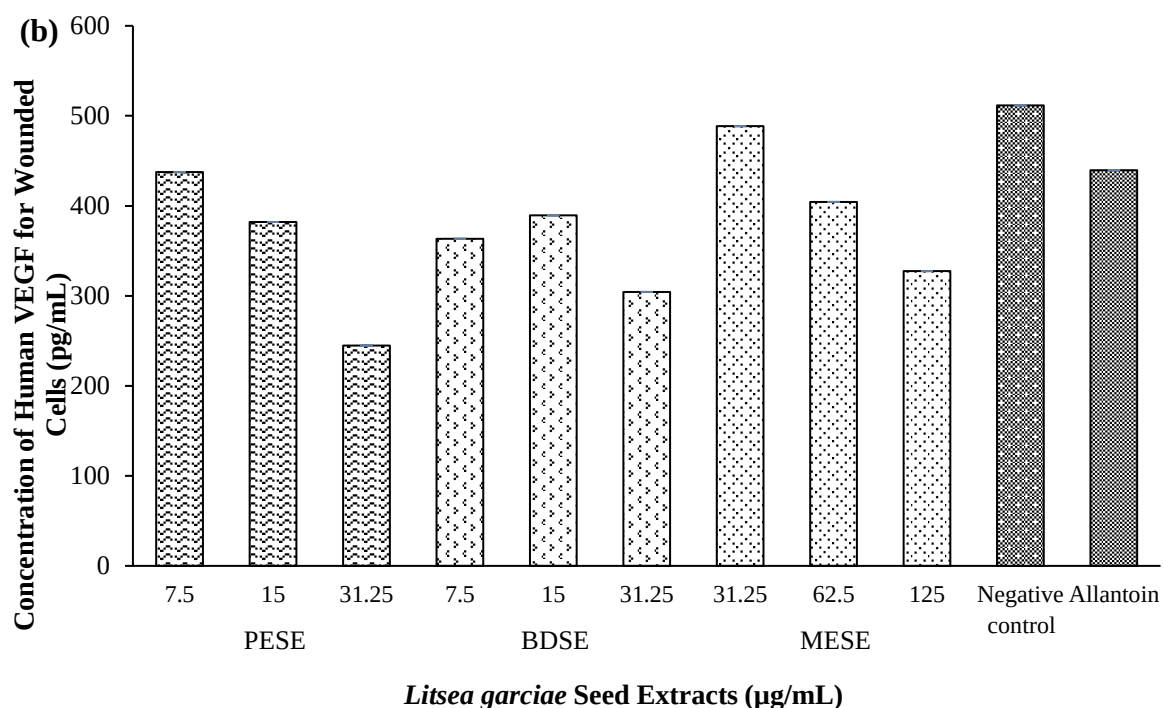
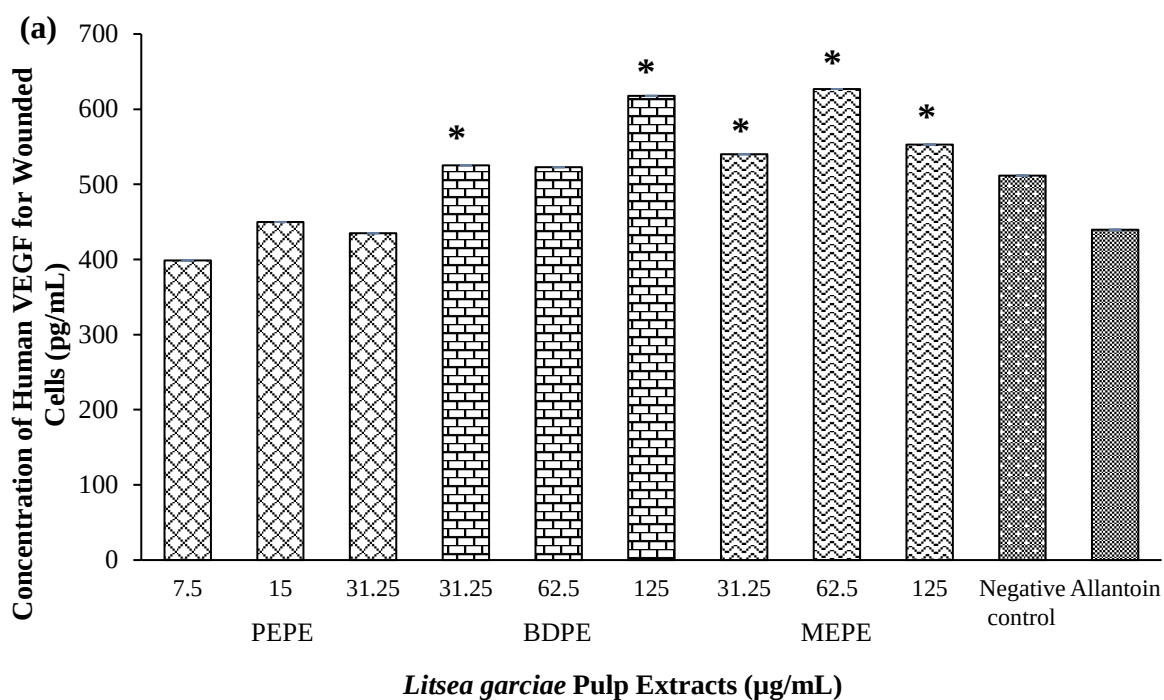


Figure 4.30: The effect of petroleum ether, Bligh and Dyer and methanolic (a) pulp and (b) seed extracts on the secretion of VEGF by wounded fibroblasts. The data were expressed as mean $\pm$ SD ($n = 2$) and were analysed using independent t-test. The statistical significant difference ($p < 0.05$) between pulp extracts and negative control were represented by (*) while seed extracts and negative control were statistically insignificant different ($p > 0.05$).

CHAPTER 5

DISCUSSION

5.1 Extraction Yield of Pulp and Seed of *L. garciae*

In this study, three types of solvent extraction were utilised, which were PE and BD extractions for total lipid extraction and 80% methanol for total phenolic content extraction. At room temperature, the appearance of PE and BD lipid pulp and seed extracts was consistent with the finding of Pui (2015), which were green semi-liquid for pulp and light yellowish solid for seed extracts. At 40°C, the PE and BD lipid seed extracts turned from solid into semi-liquid which could be attributed to the high percentage of saturated fatty acids in the *L. garciae* seed that contributed to its high melting point (Davis & Dimick, 1989).

In this study, the yield of PE and BD lipid seed extracts were 41.70% and 44.83% respectively. These values were higher than the yield of lipid extract of dried seed powder of *L. garciae* using hexane as a solvent which was 34.70% (Pui, 2015), which could suggest that PE and BD were superior solvents than hexane in extracting the lipids. Pui (2015) had also shown that the Soxhlet extracted fresh pulp without air-dried yielded only 2.65% of lipids. This was in contradiction with our findings where the yields of PE and BD lipid pulp extracts were higher when freeze-dried samples were used. It might be due to the high moisture content and smaller total surface area per unit volume of fresh sample that affects the extraction yield. The high moisture level was shown to obstruct the solubility of lipids in the non-polar solvents while the smaller total surface area interfered with the solvent penetration (Efthymiopoulos et al., 2019).

In addition, the appearance of methanolic *L. garciae* pulp and seed extracts was in line with the study reported by Adfa et al. (2021), where the appearance of methanolic avocado seed extract was reddish-brown semi-liquid. The high colour intensity of methanolic extracts was attributed to the polar solvent which would generate a darker extract with an abundance of phenolic compounds (Tan et al., 2014). Furthermore, the slightly higher yield of methanolic pulp extract might be due to the great quantity of phytochemicals being extracted using the polar solvent (Adam et al., 2019; Naczk & Shahidi, 2006). In contrast, the low yield of methanolic seed extract might be due to the low amount of polar constituents in the seed.

Apart from that, for the comparison between PE and BD lipid extraction methods, it was found that the yield of BD lipid pulp and seed extracts was slightly higher than the PE lipid extracts. This was probably due to PE lipid extraction using the Soxhlet method required operating under high temperature, that might cause thermal degradation of long chain polyunsaturated fatty acids (LC-PUFA) (Schlechtriem et al., 2003). In addition, BD lipid extraction used the chloroform: methanol mixture in a ratio of 1:2 which might contribute to high efficiency of lipid extraction. The efficiency of lipid extraction is contributed by the polarity of solvents and van der Waals forces of attraction between lipid molecules and solvent molecules, hence, the ratio of chloroform: methanol mixture plays a vital role in the lipid extraction (Kaur et al., 2018).

5.2 Characterisation of Fatty Acids Extracted from Pulp and Seed of *L. garciae*

Consumption of fats and oils may impart some good or bad effects on human health, which depend on the fatty acids profile of certain foods. Therefore, categorisation of fatty acids is essential to determine the nutritional potential of the fruit (Dubois et al., 2007). This thesis was the first study that determined the fatty acids composition of *L. garciae* lipid pulp extract using PE and BD solvents. The fatty acids composition of *L. garciae* lipid pulp and seed extracts were identified and classified according to the length and saturation of fatty acids, for instance, medium-chain fatty acids, long-chain chain fatty acids, saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids.

In this study, short-chain fatty acids (SCFAs) were undetected in the pulp and seed extracts of *L. garciae*. The medium-chain fatty acids (MCFAs) found in the *L. garciae* pulp and seed extracts were capric acid (C10:0), undecylic acid (C11:0) and lauric acid (C12:0). While the long-chain fatty acids (LCFAs) detected in the *L. garciae* extracts were tridecylic acid (C13:0), myristic acid (C14:0), pentadecylic acid (C15:0), palmitic acid (C16:0), palmitoleic acid (C16:1), margaric acid (C17:0), margaroleic acid (C17:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), nonadecylic acid (C19:0), arachidic acid (C20:0), paullinic acid (C20:1), arachidonic acid (C20:4) and heneicosylic acid (C21:0). Behenic acid (C22:0), brassidic acid (C22:1), tricosylic acid (C23:0) and lignoceric acid (C24:0) were the very long chain fatty acids (VLCFAs) discovered in the *L. garciae* extracts.

Fatty acids composition of PE and BD lipid seed extract of *L. garciae* in this study was compatible with the study by Poli and Assim (2019), where the percentage of saturated fatty acids (SFAs) of PE seed oil was in the range of 75.50 – 79.05%, while the percentage of monounsaturated and polyunsaturated fatty acids (MUFAs and PUFAs) ranged from

14.44 – 17.31% and 6.49 – 8.16% respectively. In addition, Pui (2015) showed a higher composition of SFAs and lower proportion of MUFAs and PUFAs, where the hexane extracted *L. garciae* dried seed powder comprised of 99.28% of SFAs, 0.45% of MUFAs and 0.27% of PUFAs.

Besides, this study had revealed that MCFAs, lauric acid and palmitic acid were the major SFAs for both PE and BD lipid seed extracts. Our findings were in agreement with the study by Pui (2015) that showed hexane extracted dried seed powder of *L. garciae* was predominated by lauric acid (95.19%) and followed by palmitic acid (2.82%). Nonetheless, our results were in contradiction to the findings of Poli and Assim (2019), where they reported that the major SFAs of *L. garciae* seed oil were lauric acid (38.64 – 44.79%) and myristic acid (18.99 – 20.11%). In this study, the most prevalent MUFA and PUFA detected was consistent with the findings of Poli and Assim (2019) and Pui (2015), which were oleic acid (13.17 – 16.68% and 0.45% respectively) and linoleic acid (6.49 – 8.16% and 0.27% respectively).

Furthermore, Chowdhury et al. (2007) stated that coconut oil manifested a high content of SFAs, where the predominant fatty acid was lauric acid (51.02%), followed by myristic acid (18.94%) and palmitic acid (8.62%). The composition of fatty acids of coconut oil was similar to the *L. garciae* lipid seed extracts in this study, where the coconut oil consisted of lower amount of MUFA, oleic acid (5.84%) and PUFA, linoleic acid (1.28%).

The main fatty acids found in both pulp extracts were the MUFA, oleic acid, followed by SFA, palmitic acid and PUFA, linoleic acid. In this study, the findings were not in accordance with the study by Pui (2015), where it had displayed the hexane extracted *L.*

garciae fresh pulp contained high percentages of MUFA, palmitoleic acid (40.36%), followed by SFA, palmitic acid (23.11%) and PUFA, oleic acid (18.67%).

In contrast to our findings, Bora et al. (2001) reported that avocado seed oil possessed a lower percentage of SFAs (32.50%), where palmitic acid (20.85%) was the most prominent SFA. The unsaturated fatty acids contributed most to the total amount of the total fatty acids with 67.44% such that there were 20.71% of MUFAs and 46.73% of PUFAs (Bora et al., 2001). The predominant MUFA was represented by oleic acid (17.41%) while PUFA was dominated by the linoleic acid (38.89%), where the findings were compatible with our results. It was known that avocado contained high amount of good fat which was characterised by the high content of unsaturated fatty acids. A diet with high unsaturated fatty acids content could reduce the risk of cardiovascular disease by 30% which was stated in the study by Estruch et al. (2013).

Nevertheless, the proportional distribution of fatty acids in the *L. garciae* lipid pulp extract was comparable to that of avocados as reported in many past studies. A previous study reported by Carvalho et al. (2015) had revealed that oleic acid was the most dominant fatty acid in avocados regardless of the plantation altitude and avocado maturity stage. This was in accordance with the study proposed by Yanty et al. (2011), where oleic acid was the most abundant fatty acid (43.65 – 51.22%), followed by palmitic acid (26.41 – 30.37%) and linoleic acid (12.75 – 17.45%) in three cultivars of avocados. Moreover, according to the findings of Bora et al. (2001), the highest abundance of fatty acid in avocado pulp oil was oleic acid (64.44%), followed by palmitic acid (21.31%) and linoleic acid (9.15%), which was in agreement with our findings.

The *L. garciae* lipid pulp extract also resembled the fatty acids composition of palm oil (Sambanthamurthi et al., 2000), where there was a similar proportion of saturated and unsaturated fatty acids in palm oil. The SFAs of Malaysian palm oil was 49.40%, where palmitic acid (43.50%) was the most abundant, followed by stearic acid (4.30%), myristic acid (1.10%), lauric acid (0.30%) and arachidic acid (0.20%). The MUFAs in palm oil was accounted for 40.00% of the total fatty acids, in which oleic acid was the major MUFA with 39.80% and there was only 0.20% of palmitoleic acid. There was 10.50% of PUFAs in the palm oil with 10.20% of linolenic acid and followed by 0.30% of linoleic acid.

A study by Naghshineh et al. (2010) reported that blending the saturated fatty acids in the oil which was rich with unsaturated fatty acids increased the oxidative stability of the oil. Therefore, relatively equal and low unsaturated fatty acids/saturated fatty acids ratio for *L. garciae* pulp and seed extracts respectively might contribute to their chemically stable properties that could reduce the risk of oxidation.

Furthermore, the most dominant fatty acids in *Canarium odontophyllum* (dabai) pulp and kernel oil were similar to the *L. garciae* lipid pulp extracts, which were oleic, linoleic and palmitic acids (Azlan et al., 2010). The *C. odontophyllum* pulp oil was shown to contain an equal amount of saturated and monounsaturated fatty acids while *C. odontophyllum* kernel oil consisted of a higher percentage of saturated fatty acids than unsaturated fatty acids. According to the study of Rakman et al. (2022), the fatty acids in *Shorea macrophylla* and *Shorea palembanica* oils were predominated by stearic acid, followed by oleic acid and palmitic acid.

Additionally, there was no significant difference in fatty acids composition between *L. garciae* PE and BD lipid pulp and seed extracts. Although the fatty acid components were

similar for the two solvent systems, but the fatty acids were in different proportions. These findings were in accordance with the study reported by Shreelalitha et al. (2011), where the components of fatty acids in split beans of *Canavalia maritima* and *Canavalia cathartica* were similar but in different percentages. This might indicate that the fatty acid profile of *L. garciae* lipid extracts were not affected by the type of extraction method.

5.3 Antimicrobial Effects of Lipids Extracted from Pulp and Seed of *L. garciae*

Human skin serves as the first line of defence of the body against harmful pathogens. In maintaining healthy skin, the diverse communities of commensal skin bacteria that colonise the skin will naturally trigger the immune response of the skin to prevent the invasion of harmful microbes (Byrd et al., 2018). The colonisation of commensal bacteria on intact skin is beneficial to humans. However, the disruption of the skin barrier may alter the benign bacteria into pathogenic ones, which will impair the wound healing process (Brown & Horswill, 2020). The impaired skin may lead to an infected wound, where wound infections are mostly caused by the *Staphylococcus aureus*, *Streptococcus pyogenes*, *Enterococci* and *Pseudomonas aeruginosa* (Ngan, 2005).

Besides the benefits of some dietary fatty acids that bring to human health, lipids containing various fatty acids also exhibit antimicrobial properties and possess wound healing potential (Ferreira et al., 2012). SCFAs, for example acetate, propionate and butyrate can inhibit microbial growth on the skin and control the balance of skin microbiota (Tedelind et al., 2007). MCFAs which include caproic acid, caprylic acid, capric acid and lauric acid have been found to hasten wound healing in human skin (Magalhães et al., 2008; Yang et al., 2020). LCFAs, for example palmitic acid, stearic acid and oleic acid play a key role in the maintenance of the skin barrier's normal function, which blocks the entrance of pathogens into the skin (Frommer et al., 2019; Nakamura et al., 2014). Essential fatty acids (EFAs) that are mainly comprised of linoleic acid, linolenic acid, and arachidonic acid help to retain the structure of the epidermal cell membrane, where the abnormal epidermal tissue may result in skin diseases (Yang et al., 2020).

In this study, the antimicrobial activities of *L. garciae* pulp and seed extracts were investigated against the Gram-positive bacteria (*Staphylococcus aureus* and *Staphylococcus epidermidis*) and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) as they are the common microflora found on the human skin. According to the study proposed by Cau et al. (2021), a high abundance of pathogenic *S. epidermidis* and *S. aureus* was shown to cause impairment of epidermal barrier and increase the severity of inflammation in the mice with atopic dermatitis skin. *E. coli* and *P. aeruginosa* are pathogens that can cause the formation of biofilm on infected wound sites, which allows the colonisation of bacteria and results in chronic wounds (Oliveira et al., 2018).

This thesis was the first study that determined the antimicrobial activities of *L. garciae* pulp and seed extracts, as previous studies on *L. garciae* were on the other parts of the plant such as branch, bark, and leaf. According to Wulandari et al. (2018), the branch, bark, and leaf extracts of *L. garciae* extracted with three types of solvents, *n*-hexane, ethyl acetate and ethanol solvents exhibited mild antibacterial properties against the Gram-positive bacteria, *Propionibacterium acnes*. The findings reported by Wulandari et al. (2018) were in accordance with this study, where all the *L. garciae* pulp and seed extracts demonstrated mild antibacterial activities against the Gram-positive bacteria. The pulp and seed extracts of *L. garciae* did not possess strong antibacterial properties, probably because the bioactive compounds might present in an inadequate amount that leads to a weaker antibacterial activity (Parekh & Chanda, 2007).

In this study, the *E. coli* was totally resistant to all *L. garciae* pulp and seed extracts while the *P. aeruginosa* was susceptible to the PE pulp and seed extracts and methanolic seed extract as shown by the disk diffusion assay. The *L. garciae* pulp and seed extracts

might inhibit other bacterial species that were not tested in this study (Shale et al., 1999). In addition, the extracts in this study were not purified components where purified active components in the extracts might increase the antibacterial effect (Fabry et al., 1998). Generally, the Gram-negative bacteria showed lower susceptibility to the pulp and seed extracts of *L. garciae* in contrast with the Gram-positive bacteria. This was in accordance with the finding of Hao et al. (2021), where *Litsea cubeba* essential oil had shown a stronger antibacterial effect against the Gram-positive bacteria in contradiction with the Gram-negative bacteria. This might be due to the presence of hydrophilic lipopolysaccharide component in the outer membrane of Gram-negative bacteria, which shields the hydrophobic compounds from penetrating the bacterial cell membrane (Shakeri et al., 2014). Therefore, the resistance of Gram-negative bacteria against the *L. garciae* pulp and seed extracts might be attributed to the rich contents of hydrophobic long-chain fatty acids.

Furthermore, the *L. garciae* pulp and seed extracts were found to exert stronger antimicrobial potential as compared to the *Canarium odontophyllum* (dabai) fruit extracts. The acetone, hexane, aqueous and methanol extracts from *C. odontophyllum* fruit were not able to show inhibition against *S. aureus*, methicillin-resistant *S. aureus* (MRSA), *E. coli* and *P. aeruginosa*. However, *C. odontophyllum* acetone and hexane pulp extracts inhibited the yeast, *Candida glabrata* (Basri et al., 2014). Moreover, the acetone extract from *Shorea macroptera* (engkabang) stem bark exhibited antimicrobial activity against the Gram-positive bacteria (*S. aureus*, *S. anginosus*, *S. uberis*, and *B. subtilis*). While for the Gram-negative bacteria, *S. typhimurium*, *E. coli* and *P. aeruginosa* were not susceptible to the *S. macroptera* extract. These findings from Nur et al. (2012) were comparable to our findings for *L. garciae* extracts, where the Gram-positive bacteria exerted higher susceptibility against the *L. garciae* extracts in comparison with the Gram-negative bacteria. In addition,

the stem hexane and ethyl acetate extracts of *Boesenbergia stenophylla* (red jerangau) were shown to display inhibition towards the *S. aureus* and *S. typhimurium* (Vallirie, 2018).

In addition to the antibacterial activity, Johnny et al. (2011) also described the antifungal property of *L. garciae* leaf extracted with methanol, chloroform, and acetone solvents. The study showed that the *L. garciae* methanolic leaf extract exhibited a stronger antifungal property against *Colletotrichum capsici* than chloroform and acetone leaf extracts. On the other hand, the study by Johnny et al. (2010) revealed that *L. garciae* acetone leaf extract had shown a greater antifungal activity against *Colletotrichum gloeosporioides* in contrast to methanolic and chloroform leaf extracts. The high antifungal property was probably attributed to the presence of active compounds in the plant, for instance, alkaloids, flavones, flavanones, propenylphenols, terpenes and steroids (Johann et al., 2007).

The genus *Litsea* comprises more than 400 species and has been used as traditional medicine since ancient times. The biological and pharmacological activities of several species of genus *Litsea* have been studied (Azhar & Salleh, 2020). The active chemical components found in the fruit oil of *L. glaucescens*, *L. glutinosa*, *L. laevigata* and *L. monopetala*, include lauric acid, monoterpenes, sesquiterpenes and aldehydes (Arif et al., 2008; Choudhury et al., 1996; Choudhury et al., 1997; Chowdhury et al., 2008). According to Arif et al. (2008), *L. laevigata* fruit essential oil had demonstrated growth inhibition against the Gram-positive bacteria, *Streptococcus albus*. Moreover, Li et al. (2014) described that the chemical constituents of *L. cubeba* essential oil comprised of high abundance of aldehydes and a little quantity of alcohols and alkenes, which contributed to its moderate antibacterial effect. These findings were consistent with the present study, hence the

antimicrobial properties of the pulp and seed extracts of *L. garciae* might be attributed to its chemical compositions.

The *L. garciae* methanolic pulp and seed extracts from the previous study had demonstrated that there was antioxidant activity displayed by the extracts (Hassan et al., 2013). The antioxidant properties of extracts might be contributed by the phytochemicals such as flavonoids and phenolics. The synergistic effect among the flavonoids and phenolics would as well lead to the antimicrobial properties (Hassan et al., 2013; Naqvi et al., 2019). In addition, Wulandari et al. (2018) revealed that the phytochemicals such as alkaloids, coumarin, carotenoids, flavonoids and tannins were discovered in the branch, bark, and leaf parts of *L. garciae*. Therefore, the antimicrobial properties of methanolic pulp and seed extracts of *L. garciae* in this study might be contributed by the presence of phytochemicals.

Lauric acid had been proven to exhibit strong antimicrobial activity against Gram-positive bacteria in comparison with other MCFAs (Batovska et al., 2009). According to the findings of Fischer et al. (2014), lauric acid effectively inhibits the Gram-positive bacteria (*S. aureus*) growth. It was discovered that lauric acid disrupted the Gram-positive bacterial cell membrane, prevented the biofilm formation, and induced reactive oxygen species synthesis (Yang et al., 2018). On contrary, lauric acid was ineffective towards the Gram-negative bacteria (*E. coli* and *P. aeruginosa*). Nonetheless, there were some exceptions whereby lauric acid could inhibit other variety of Gram-negative bacteria such as *Fusobacterium nucleatum* (Signat et al., 2011) and thus lauric acid might have ability to maintain the protective functions of the skin against foreign pathogens. In addition, lauric acid monoglyceride derivative, i.e., glycerol monolaurate (GML) was found to be more potent than lauric acid in the inhibition of Gram-positive bacteria growth (Kabara et al.,

1972). The PE and BD lipid seed extracts of *L. garciae* were predominated by MCFAs, for instance, capric acid and lauric acid. Hence, this could suggest that the antimicrobial activities against the Gram-positive and Gram-negative bacteria in this study is contributed by the presence of MCFAs in the extracts.

Furthermore, the *L. garciae* PE and BD lipid pulp extracts contained a greater proportion of LCFAs (palmitic acid, stearic acid, oleic acid, and linoleic acid) in contrast with the seed extracts. The long-chain polyunsaturated fatty acids (LC-PUFAs) exhibited antimicrobial potency against the Gram-positive bacteria, *P. acnes* and *S. aureus* as reported by Desbois & Lawlor (2013). The antimicrobial activities of *L. garciae* pulp extracts might be contributed by the LC-PUFAs such as oleic acid and linoleic acid (Chen et al., 2011; Zheng et al., 2005). Oleic acid showed inhibitory effect towards *S. aureus* by destroying the bacterial cell wall. Chen et al., 2011 also stated the ability of oleic acid to cure the infection of methicillin-resistant *S. aureus* (MRSA) in mouse skin model. Besides, linoleic acid was shown to exert growth inhibition against the *S. aureus* by improving the permeability of bacterial membrane. This would affect the metabolism of bacteria where the oxygen uptake and generation of macromolecules were obstructed (Greenway & Dyke, 1979). In addition, Zheng et al., 2005 revealed that the antimicrobial actions of LC-PUFAs (oleic acid and linoleic acid) were more effective than the long-chain saturated fatty acids (LC-SFAs) (palmitic acid and stearic acid). It was shown that bacterial enoyl-acyl carrier protein reductase (FabI) of bacteria was suppressed by LC-PUFA through selective inhibition while LC-SFA was inactive towards FabI.

5.4 Human Dermal Fibroblasts Cell Culture

Human dermal fibroblasts (HDF) are the main cell types present at the dermis of the skin layer. The crucial roles of fibroblasts in wound healing are producing several regulatory molecules and crosstalk with other immune cells involved in the healing process. Fibroblasts are the source of new extracellular matrix (ECM) and collagen structures for supporting other cells associate with effective wound healing and thus restore the function of the skin (Kim et al., 2007; Singer & Clark, 1999). The *in vitro* cell culture of HDF is usually used to mimic the living skin for various studies such as cytotoxicity tests, cell migration and chemotactic motility of fibroblasts (Coulomb, 1989).

In this study, good practices of cell culture techniques were strictly followed to obtain optimal growth of cells, To minimise the possibility of cell culture contaminations, biological safety cabinet (BSC) equipped with HEPA-filtered air and aseptic cell culture techniques were used when handling the cells. The standard basal medium contained a high concentration of glucose, amino acids, vitamins, and inorganic salts, which provide the energy source and regulate the osmotic balance of cells for proper cell growth and proliferation. In addition, the supplementation of medium with serum was the source of growth factors and hormones that were necessary for the growing cells. Moreover, antibiotics were added to the media to further minimise the risk of entry of contaminants into the cultured cells (Segeritz & Vallier, 2017). Phenol red in the media acted as a pH indicator to monitor the pH changes due to the release of metabolites by the cells. Also, the pH level was stabilised by growing the cells in the 5% CO₂ incubator at 37°C for retaining the optimal pH range of 7.2 – 7.4 (Schwartz et al., 1989).

For the optimisation of fibroblast cell seeding density, the phases of cell growth were important to take into consideration. There were four phases in the cell growth curve which were lag, log, stationary and decline phases. For the lag phase, there was no cell proliferation as the cells were undergoing metabolic activity while the commencement of cell proliferation increased the cell number exponentially during the log phase. During the stationary phase, the cells were still metabolically active, but the proliferation rate remained constant as there was accumulation of waste products from cell metabolism. Lastly, the cells were lack of nutrients and subsequently the cell death occurred (Nagabhushanam, 2020). Therefore, the fibroblast cell seeding density in this study was optimised at 2.5×10^4 cells/well, which was at the log phase of cell growth. Since the recommended fibroblast cell density by JCRB Cell Bank is 2.2×10^4 cells/well, which was similar to our finding on the optimal fibroblast cell density hence 2.2×10^4 cells/well was utilised for the determination of cytotoxicity effect of *L. garciae* pulp and seed extracts.

5.5 Effects of Pulp and Seed Extracts of *L. garciae* on Human Dermal Fibroblasts Viability

Cell viability measurement is used to determine the number of viable cells in a population while cell proliferation involves the cell division that can measure the cellular metabolic changes of viable cells (Adan et al., 2016). Cell viability and proliferation assays are used to detect the cytotoxicity effects of certain substances during drug screening. Cytotoxicity is defined as the toxicity of healthy cells that is attributed to toxic agents and the percentage of cell viability below 80% is considered cytotoxic (ISO, 2009; Mukherjee, 2019). There are various assays to assess the cytotoxicity of the potential drug, one of them is the MTT assay which was used in this study to examine the cytotoxic effects of *L. garciae* pulp and seed extracts on fibroblasts. The MTT assay is the metabolic cell proliferation assay whereby the mitochondrial activity of cells is determined through the concentration of formazan that is formed. Since only viable cells consist of mitochondrial enzymes that can convert the MTT into formazan crystals, thus a higher number of viable cells will form a higher concentration of formazan (van Meerloo et al., 2011).

Dimethyl sulfoxide (DMSO) acts as a vehicle control that is capable to solubilise both polar and non-polar compounds in plant extracts and therefore it is widely used in the drug screening of mammalian cells (Balakin et al., 2006; Timm et al., 2013). According to Singh et al. (2017), the concentrations of DMSO that were greater than 3% were toxic to the fibroblasts and led to cell death, while concentrations of DMSO in the range of 0.001 – 1% were non-cytotoxic. This was in agreement with our findings, where fibroblasts treated with 0.05% of DMSO had cell viability of $96.69 \pm 0.02\%$ and therefore this was a safe concentration to dissolve the *L. garciae* extracts for the treatment of fibroblasts in our study.

Drug allantoin was used as a positive control for this study. It has been widely used in the cosmetic and pharmaceutical industries as it has a wound healing effect and stimulates epithelial cell growth (Araújo et al., 2010). The concentration of allantoin in the range of 0.25 – 250 µg/mL was shown to be non-toxic to the fibroblasts as proposed by Jorge et al. (2008). This was in contradiction to our finding where allantoin with a concentration of 200 µg/mL was still mildly cytotoxic to the fibroblasts. At the concentration of 125 µg/mL, the cell viability ($98.50 \pm 0.02\%$) was the highest with no cytotoxic effect and thus it was selected for the future assay.

In this study, all the PE and BD lipid pulp extracts with concentrations of 31.25 – 250 µg/mL were not cytotoxic to the fibroblasts. Besides, all the concentrations of PE lipid seed extracts were not toxic to the fibroblasts while the BD lipid seed extracts with concentrations ranging from 125 – 500 µg/mL were cytotoxic. The findings for the PE lipid pulp extracts and BD lipid pulp and seed extracts were similar to the study reported by Kyland et al. (2020), where the viability of fibroblasts treated with the red dragon fruit peel (RDFP) ethanolic extract was the lowest for 500 µg/mL of the extract. The concentrations of 250 and 500 µg/mL of the RDFP extracts were cytotoxic to the fibroblasts whereas 7.81 – 125 µg/mL of the extracts were not cytotoxic to the fibroblasts. Although the 61.25 and 125 µg/mL of PE lipid pulp and seed extracts and BD lipid seed extracts were not cytotoxic, however, the loss of viable cells was around 20%. Hence, lower concentrations of extracts which were 7.5 and 15 µg/mL were chosen for subsequent experiments to maximise the cell viability and ensure the safest concentration to treat the fibroblasts. Furthermore, the cell viability of fibroblasts with the treatment of methanolic pulp extracts at the concentrations from 31.25 – 500 µg/mL was not affected. For the 31.25 – 500 µg/mL of methanolic seed extracts, there was no cytotoxic effect on the fibroblasts.

In addition, Addis et al. (2020) demonstrated that the *Calendula arvensis* and *Helichrysum italicum* waste extracts significantly increased the cell viability of the human foreskin fibroblasts in contrast with the untreated cells. It was found that the waste extracts at a lower concentration exhibited higher cell viability than the extracts at a higher concentration. The findings were in line with the present study, which had shown that most of the *L. garciae* extracts at the concentration of 31.25 µg/mL were detected to enhance the cell viability of the fibroblasts as compared to the untreated cells. According to the previous study proposed by Siri wattanasatorn et al. (2020), it had shown that the cell proliferation rate could be determined using the cell viability of fibroblasts. Therefore, the amelioration in the cell viability might infer that *L. garciae* extracts could promote the proliferation of fibroblasts.

Furthermore, Huttner et al. (1978) revealed that the low concentration of oleic acid at 18 µM promoted the proliferation of human fibroblasts. It was found that oleic acid was the most potent fatty acid in inducing the cell proliferation by hampering the synthesis of prostaglandin E₂ (Pace-Asciak & Wolfe, 1968). This is due to the prostaglandin E₂ mediates the normal functions of fibroblasts and inhibits the proliferation of fibroblasts (Huang et al., 2009). Therefore, inhibiting the production of prostaglandin E₂ helps to enhance the cell proliferation. In current study, high abundance of oleic acid in the *L. garciae* extracts might result in an increment in the cell viability and proliferation of fibroblasts.

5.6 Wound Healing Properties of Pulp and Seed Extracts of *L. garciae*

5.6.1 Effects of *L. garciae* Extracts on Fibroblast Cells Migration

The migration of fibroblasts plays a main role in wound healing, which takes part in the initiation of the proliferative phase in the wound healing process (Addis et al, 2020; Tschumperlin, 2013). Fibroblast migration is a rate-determining step in wound healing as it is vital for the emergence of granulation tissue (Cen et al., 2022). The production of new granulation tissue involves the modulation of subsequent phases of the wound healing process such as angiogenesis, inflammation, and re-epithelialisation. During tissue remodelling, the granulation tissue will develop into mature connective tissue with a full recovery of function and structure (Häkkinen et al., 2011). Therefore, defective in granulation tissue generation will result in prolonged wound healing that might eventually lead to chronic wounds.

In this study, except for the methanolic seed extract, all the *L. garciae* pulp and seed extracts were found to enhance the fibroblast migration rate. The enhancement of fibroblast migration rate might be attributed to the fatty acids content in the *L. garciae* extracts, as de Oliveira et al. (2013) reported that avocado oil improved the re-epithelialisation process in the rat model due to the presence of phytochemicals and fatty acids such as oleic, linoleic, and linolenic acids. The presence of phytochemicals and fatty acids could stimulate the migratory activity of fibroblasts which induces the production of granulation tissue and accelerates the wound healing process (Guidoni et al., 2019). Moreover, Li et al. (2019) revealed that *Periplaneta americana* crude ethanol extract improved the fibroblast migration rate. It was demonstrated that fibroblasts treated with 0.5 mg/mL of *P. americana* extract exhibited a greater rate of cell migration after 6 h and 12 h of incubation whereas the

concentration of 0.05 mg/mL of the extract was found to enhance the cell migration at the highest rate after incubation for 24 h as compared to the untreated cells.

Besides, according to previous study described by Rojo et al. (2010), *Pouteria lucuma* nut oil (LNO) was revealed to exhibit wound healing effect by improving the migratory activity of fibroblasts. The promotion in the migration of fibroblasts might be attributed to the fatty acid mixture in the LNO, for instance, linoleic acid, oleic acid, palmitic acid, stearic acid, and linolenic acid, where linoleic acid and oleic acid were the major fatty acids in the LNO. It was found that the fibroblasts treated with only oleic acid or linoleic acid did not increase the cell migration as effective as the LNO. Therefore, it is hypothesised that the combination of linoleic acid and oleic acid in the *L. garciae* extracts might enhance the migration of fibroblasts in the present study.

In contrast, the methanolic seed extract did not cause any increment in the rate of fibroblast migration. The previous study by Ramos-Jerz et al. (2013) demonstrated a similar finding to our study, where treatment of human dermal fibroblasts with avocado seeds extracted with methanol and water mixture led to a reduction in the proliferation rate of the human dermal fibroblasts. It was found that avocado seeds contained chlorogenic acid which exerted an anti-proliferative effect on the fibroblasts and minimised the cell migration rate (De Leonardis et al., 2008). The study of Seal et al. (2020) demonstrated that *Litsea cubeba* methanolic fruit extracts consisted of chlorogenic acid. As *L. garciae* is under the same genus as *L. cubeba*, hence there is a possibility that chlorogenic acid was present in the *L. garciae* fruit which led to no enhancement in the fibroblast migration rate.

In addition, it was shown that some of the *L. garciae* pulp and seed extracts exhibited similar or slightly higher increments in the fibroblast migration as compared to allantoin.

This finding was in agreement with the previous study reported by Namchaiw et al. (2021), where the wound closure rates of the cells treated with *Coccinia grandis* methanolic leaf extracts were similar to or slightly higher than the allantoin-treated cells. Therefore, the consistent result with allantoin indicates the ability of *L. garciae* extracts to improve the migratory activity of fibroblasts, which in turn hastens the wound healing process.

5.6.2 Effects of *L. garciae* Extracts on Fibroblasts' Chemotactic Motility

The chemotactic motility or chemotaxis of fibroblasts refers to the migration of fibroblasts towards the chemoattractant such as plant extracts (Stock & Baker, 2009). It has a vital role in the wound healing process, where the chemotactic gradients control the migration of fibroblasts and the synthesis of extracellular matrix (Postlethwaite, 1987). The study on chemotaxis was conducted using a transwell migration assay. A transwell plate consisted of two chambers that were divided by a microporous membrane. The fibroblasts were plated on the membrane inserts of the upper chamber while the chemoattractant was added to the bottom chamber (Zhang et al., 2016). The number of migrated cells towards the chemoattractant was determined to examine the effects of *L. garciae* extracts on the fibroblasts' chemotactic motility.

The cell migration assay results (section 4.6.1) showed that there was a considerable improvement in the cell migration rate when the fibroblasts were treated with *L. garciae* extracts (31.25 µg/mL) for 24 h. Therefore, this concentration was used for the chemotactic motility assay. In addition, two types of negative controls were utilised where the culture of fibroblasts were performed in the complete medium (10% FBS) and serum-free medium (SFM) respectively. This was because the FBS also acted as a chemoattractant which improved the cell migration as reported by Zhang et al. (2016). Thus, the utilisation of two

types of negative controls validated that the increment in the fibroblasts' chemotactic motility was attributed to the *L. garciae* extracts only.

In this study, all the pulp and seed extracts of *L. garciae* with a concentration of 31.25 µg/mL demonstrated an enhancement in the fibroblasts' chemotactic motility as compared with the negative control (SFM). In addition, the *L. garciae* pulp and seed extracts were shown to increase the chemotactic motility of fibroblasts as opposed to the negative control (10% FBS). However, there was no improvement in the chemotactic motility of the fibroblasts treated with methanolic seed extract. This finding on the effect of methanolic seed extract in fibroblasts' chemotactic motility was consistent with the previous part of this study, where the migration of fibroblasts after the treatment of methanolic seed extract had not been ameliorated in the wound scratch assay. These findings were in agreement with the previous study as reported by Mapoung et al. (2021), where the medicinal mushroom *Auricularia auricula-judae* (AAP) with the concentration of 25 µg/mL had induced the greatest chemotactic motility in the transwell assay. The result of the transwell assay was similar to that of the scratch assay, in which the 25 µg/mL of AAP exerted the highest cell migration. It was shown that the induction of cell migration by the AAP could presumably promote wound healing activity. According to the study by Li et al. (2019), the crude ethanol extract of *Periplaneta americana* exhibited a chemotactic effect and increased the migration rate of human skin fibroblasts. Hence, a significant increase in the fibroblasts' chemotactic motility and migration by the *L. garciae* extracts could suggest their potential in enhancing the proliferation of fibroblasts during the wound healing process.

Moreover, a previous study from Seo et al. (2016) revealed that the ethanol extract of *Polygonum aviculare* comprised phenolic compounds that include quercetin, gallic acid,

and rutin. These compounds were responsible for the stimulation of the Wnt/ β -catenin signalling pathway and accelerated the motility of fibroblasts. The activation of the Wnt/ β -catenin signalling pathway regenerated the wound healing activities such as the proliferation of cells and tissue remodelling (Choi et al., 2022). A study by Raduan et al. (2022) showed that the *L. garciae* bark extracts contained phenolic and flavonoid compounds. The total phenolic and flavonoid contents are quantified as gallic acid and quercetin or rutin equivalents respectively (Raduan et al., 2022; Wulandari et al, 2018). In addition, quercetin was found to exert antimicrobial, antioxidant and anti-inflammatory properties and initiated the migration of cells (Comino-Sanz et al., 2021; Nguyen & Bhattacharya, 2022). Furthermore, Chittasupho et al. (2022) demonstrated having antimicrobial, antioxidant and cell migration-inducing properties might result in the wound healing improvement and the skin restoration. Hence, the promotion of fibroblasts' chemotactic motility might be attributed to the presence of quercetin, gallic acid and rutin which plays an essential role in activating the Wnt/ β -catenin signalling pathway and ameliorates the wound healing process.

In addition, Smith et al. (2012) demonstrated that unsaturated fatty acids such as linoleic and oleic acids improved the chemotactic motility of mesenchymal stem cells (MSC). Since the MSC can be differentiated into different types of cell lineages such as fibroblasts (Caplan, 2005), hence the enhancement in the fibroblasts' chemotaxis might be due to the high proportion of linoleic and oleic acids in the *L. garciae* extracts. In short, the migration and chemotactic motility of fibroblasts might be ameliorated by the *L. garciae* extracts and these findings affirmed the capability of *L. garciae* extracts to improve the wound healing process.

5.6.3 Effect of *L. garciae* Extracts on Wound Healing Markers

Wound healing can be regulated by collagen type I and growth factors such as epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF). Wound healing is a complex biological process that involves three overlapping stages: inflammation, proliferation and remodelling. Fibroblasts play a critical role in all three stages by secreting growth factors, cytokines, collagens, and other extracellular matrix (ECM) components (Schäfer & Werner, 2007). Collagen type I is the dominant protein for the skin ECM formation (Tracy et al., 2016) and modulates the wound healing process by controlling cellular mitogenesis, differentiation, proliferation, and migration. The proliferation and migration of fibroblasts were also mediated by the Erk1/2 and p38 MAPK signalling pathways (Ranzato et al., 2009). The deposition of collagens contributes to structural support to the connective tissue, which enhances the tensile strength of the wound (Rangaraj et al., 2011). Moreover, growth factors are responsible for triggering the inflammation phase and stimulating cellular proliferation, angiogenesis, and migration (Vaidyanathan, 2021). The EGF induces the proliferation and migration of fibroblasts that leads to wound contraction, where the area of the wound site is reduced (Bodnar, 2013). In addition, VEGF is a key initiator of angiogenesis in which the formation of new blood vessels is affected by the VEGF production level (Cross & Claesson-Welsh, 2001). The VEGF also triggers the proliferation and migration of the endothelial cells which hastens the wound healing process (Galiano et al., 2004).

In the present study, all the *L. garciae* lipid and methanolic pulp extracts at the concentration of 31.25 µg/mL showed a significant increment in the collagen type I synthesis. While for the seed extracts, the PE lipid seed extract at 7.5 µg/mL, BD lipid seed extract at 15 µg/mL and methanolic seed extract at 31.25 µg/mL demonstrated a notable

improvement in the collagen type I level. The previous study of Park et al. (2020), showed that the fibroblasts treated with the *Digitaria ciliaris* flower (DCF) absolute extracted with hexane caused an increase in collagen type I levels. It was found that the promotion in the generation of collagen type I by the DCF absolute was enhanced by an extracellular signal-regulated kinase (Erk) 1/2 and p38 mitogen-activated protein kinase (MAPK) (Park et al., 2020). Furthermore, Bolla et al. (2019) showed that mouse fibroblasts treated with *Aristolochia saccata* methanolic leaf extract had improved collagen type I expression. The upregulation in the expression of collagen type I was assumed to initiate the migration of fibroblasts, which indicated that the *A. saccata* extract was a potent wound healing agent. This was attributed to the presence of phytochemicals in the *A. saccata* extract such as flavonoids and triterpenoids, which possessed antioxidant properties and ameliorated the wound healing process (James & Friday, 2010). Furthermore, Ribeiro Barros Cardoso et al. (2004) stated that linoleic acid could promote a greater production of nitric oxide (NO) at the wound site of the mice. It was shown that an increment in NO production improved the synthesis of collagen by the fibroblasts (Frank et al., 2002). Therefore, the increment in the expression of collagen type I in this study might be attributed to the phytochemical constituents and linoleic acid that was present in the *L. garciae* extracts.

Moreover, in this study, all the concentrations of *L. garciae* lipid and methanolic pulp extracts and BD lipid seed extracts at the concentrations of 15 µg/mL and 31.25 µg/mL manifested an enhancement in the secretion of EGF. Similarly, the VEGF secretion levels of the fibroblasts treated with the BD lipid and methanolic pulp extracts were also increased. These findings were in agreement with the results reported by Li et al. (2019), in which the crude ethanol extract of *Periplaneta americana* was found to ameliorate the EGF and VEGF secretion levels in the keratinocytes. Besides, the enhancement in the expression of EGF and

VEGF, *P. americana* extract also caused increased proliferation and migration of fibroblasts and keratinocytes, induced neovascularisation, and accelerated the rate of reepithelialisation of the wound, which would eventually hasten the process of wound healing (Li et al., 2019).

Besides, Karim et al. (2021) described that the methanolic extract of Saudi pomegranate peel (SPP) had elevated the expression of EGF and VEGF in the rat wound lysates. It was known that NO plays a key role in the regular wound healing process, where it facilitates the proliferation and migration of fibroblasts, angiogenesis and modulates the synthesis of collagen (Witte & Barbul, 2002). Although the release of NO at the optimum level aids in wound repair, however, the overproduction of NO over a period of time may cause the wound to develop into chronic wounds (Soneja et al, 2005). According to the study of Karim et al. (2021), the rats treated with SPP extract exhibited lower NO levels in the wound than the control group over 7 days of treatment and hence the wound repair was ameliorated. In addition, Rodrigues et al. (2016) stated that the wound of rats treated with linoleic acid had increased VEGF concentration, which induced the new blood vessels formation. In addition, Ili & Sari (2023) demonstrated that egg yolk oil improved the wound healing by increasing the EGF and VEGF secretion at the lesion site. This finding was attributed to the fatty acids composition in egg yolk oil which were predominated by the oleic, palmitic, and linoleic acids. Therefore, the improvement in expression of the EGF and VEGF levels in current study might be attributed to the presence of oleic, palmitic, and linoleic acids in the *L. garciae* extracts.

5.7 Summary

The lipids and phytochemical constituents of the pulp and seed extracts of *L. garciae* were believed to exert antimicrobial and wound healing effects on human dermal fibroblasts. The present study showed that extraction of lipids using PE and BD as the extraction solvent produced higher lipid yield than using hexane as done by Pui (2015). Besides, the lipid yield using PE as extraction solvent produced slightly lower lipid yield than using BD as extraction solvent. This might be attributed to the thermal degradation of long chain polyunsaturated fatty acids in the PE extraction since using the Soxhlet apparatus subjected the extracts to heat. In addition, a higher lipid yield using BD as extraction solvent might be contributed by the forces of attraction between the lipid molecules and the solvent mixture containing polar and non-polar molecules. While for the methanolic extraction, the yield of *L. garciae* pulp extract was higher than the seed extract, which was probably due to a higher concentration of phytochemicals in the pulp extract.

This study also identified the fatty acids composition of *L. garciae* lipid pulp and seed extracts. The most prominent MCFA and LCFA were lauric acid and palmitic acid respectively for both PE and BD lipid seed extracts. Whereas in PE and BD pulp extracts, the prominent LCFA were palmitic acid and stearic acid. Oleic acid and linoleic acid were found to be the most abundant MUFA and PUFA respectively. It was shown that the fatty acids profile of *L. garciae* lipid seed extracts was comparable with the coconut oil (Chowdhury et al., 2007) and avocado seed oil (Bora et al., 2001).

The fatty acids of *L. garciae* seed extracts had been studied by others (Poli and Assim, 2019) but this thesis is the first to elucidate the fatty acid contents of *L. garciae* lipid pulp where PE and BD were used as solvents. The major fatty acids of *L. garciae* lipid pulp

extracts were the MUFA, oleic acid, followed by SFA, palmitic acid and PUFA, linoleic acid. It was found that the predominant fatty acids of *L. garciae* lipid pulp extracts were similar to that of the avocado pulp oil (Bora et al., 2001; Yanty et al., 2011). Furthermore, there was a similar proportion of saturated and unsaturated fatty acids for the *L. garciae* lipid pulp extract, which resembled the proportion distribution of fatty acids in the palm oil (Sambanthamurthi et al., 2000). Lastly, the fatty acids content of *L. garciae* lipid pulp and seed extracts was similar for both PE and BD extraction methods, but with different proportions of fatty acids.

Control of microbial infection of wound is vital for better wound healing. In the present study, the antimicrobial properties of the PE and BD lipid and methanolic pulp and seed extracts of *L. garciae* were evaluated against the common human skin microflora, Gram-positive bacteria (*Staphylococcus aureus* and *Staphylococcus epidermidis*) and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). It was demonstrated that all the *L. garciae* pulp and seed extracts exhibited mild antibacterial activities against the Gram-positive bacteria. This was presumably due to an inadequate quantity of bioactive compounds in the *L. garciae* extracts. Apart from that, Gram-negative bacteria were revealed to exert a weaker susceptibility towards the *L. garciae* extracts as compared to the Gram-positive bacteria. This was probably due to the presence of high content of hydrophobic long-chain fatty acids in the *L. garciae* extracts that might lead to the resistance of some Gram-negative bacteria as their hydrophilic outer membranes prevent the penetration of hydrophobic compounds.

Generally, the lipid pulp extracts could inhibit the Gram-positive and Gram-negative bacteria better than the methanolic pulp extract. The PE and BD lipid pulp extracts consisted

of LCFA such as oleic acid and linoleic acid, where the oleic acid could destroy the cell wall of *S. aureus* while linoleic acid might improve the permeability of the membrane of *S. aureus* that would affect the metabolism of bacteria. On top of that, high abundance of MCFA in the PE and BD lipid seed extracts such as capric acid and lauric acid, might play a role in contributing to the antimicrobial properties against the Gram-positive and Gram-negative bacteria. While the methanolic seed extract exerted a stronger inhibition towards the Gram-positive and Gram-negative bacteria in comparison with the lipid seed extracts. Moreover, the methanolic seed extract at a higher concentration had shown a slightly stronger inhibition effect towards the bacteria as compared to the methanolic pulp extract. The antimicrobial activities for all the *L. garciae* pulp and seed extracts might be contributed by the phytochemicals such as alkaloids, coumarin, carotenoids, flavonoids, propenylphenols, tannins, terpenes, and steroids (Johann et al., 2007; Wulandari et al., 2018).

The wound healing properties of *L. garciae* pulp and seed extracts were determined by examining the effects of *L. garciae* extracts on the migration of fibroblasts, chemotactic motility of fibroblasts and wound healing markers. First, all the *L. garciae* pulp and seed extracts were shown to improve the migration rate of fibroblasts, except the methanolic seed extract. It was found that the presence of phytochemicals and fatty acids such as oleic, linoleic, and linolenic acids (de Oliveira et al., 2013) in the *L. garciae* would improve the migratory activity of fibroblasts by inducing the synthesis of granulation tissue. Also, the synergistic effect among the linoleic acid and oleic acid in the *L. garciae* extracts might contribute to an enhancement in cell migration. Contrarily, there was no improvement in the migration rate for the fibroblasts treated with the methanolic seed extract, which might be due to the presence of chlorogenic acid in the *L. garciae* fruit that exhibited an anti-proliferative effect and decreased the fibroblasts migration rate (De Leonardis et al., 2008;

Seal et al., 2020). Furthermore, some concentrations of the PE and BD lipid pulp and seed extracts and methanolic pulp extracts demonstrated a comparable enhancement in the migration rate of fibroblasts with the allantoin. This confirms the capability of *L. garciae* extracts in promoting the migration of fibroblasts which is required for wound healing.

In addition, *L. garciae* pulp and seed extracts at 31.25 µg/mL enhanced the chemotactic motility of fibroblasts as opposed to the negative control (SFM). With the exception to methanolic seed extract, all *L. garciae* pulp and seed extracts at 31.25 µg/mL also improved the chemotactic motility of fibroblasts when comparing to the negative control (10% FBS). The increment in the fibroblasts' chemotactic motility might be contributed by the presence of quercetin, gallic acid and rutin (Raduan et al., 2022; Wulandari et al, 2018) that activated the Wnt/ β -catenin signalling pathway. Also, the high percentage of linoleic and oleic acids in the *L. garciae* extracts might result in an enhancement in the chemotactic motility of fibroblasts.

Lastly, the wound healing markers such as collagen type I, epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) had been increased in the presence of the *L. garciae* extracts. The 31.25 µg/mL of *L. garciae* lipid and methanolic pulp extracts demonstrated a noteworthy enhancement in the collagen type I synthesis. Not only that, 7.5 µg/mL of PE lipid, 15 µg/mL of BD lipid and 31.25 µg/mL of methanolic seed extracts increased the expression of collagen type I significantly. *L. garciae* extracts might trigger the Erk1/2 and p38 MAPK signalling pathways (Ranzato et al., 2009), which promotes the proliferation and migration of fibroblasts and collagen type I expression level. Hence, the improvement in the synthesis of collagen type I might be due to the presence of phytochemical constituents and linoleic acid in the *L. garciae* extracts.

In addition to that, the 15 µg/mL and 31.25 µg/mL of the *L. garciae* lipid and methanolic pulp extracts and BD lipid seed extracts promoted the secretion level of EGF. All the concentrations of BD lipid and methanolic pulp extracts used in this study enhanced the VEGF secretion level. The elevation in the EGF and VEGF expression levels had been shown to ameliorate the proliferation and migration of fibroblasts, induce formation of blood vessels and hasten the reepithelialisation of wound (Li et al., 2019). Moreover, the presence of oleic, palmitic and linoleic acids in the *L. garciae* extracts might increase the secretion of EGF and VEGF. In summary, the enhancement in the wound healing process of fibroblasts might be contributed by the phytochemicals and fatty acids in the *L. garciae* extracts in which they exerted antimicrobial properties, improved the migration and chemotactic motility of fibroblasts, and enhanced the expression levels of collagen type I, EGF and VEGF.

Generally, the wound healing capabilities of *L. garciae* lipid seed extracts were better than the methanolic seed extract. The migration rate and chemotactic motility of fibroblasts had been enhanced significantly after treatment with the lipid seed extracts. While for the methanolic seed extract, there was no improvement being observed in the fibroblast's migration and chemotactic motility. The expression of collagen type I and EGF also promoted at a higher level for the fibroblasts treated with the lipid seed extracts as opposed to the methanolic seed extract. In addition, for the *L. garciae* pulp extracts, all the lipid and methanolic pulp extracts exhibited a similar extent of wound healing abilities. All the pulp extracts had shown an increment in the fibroblast migration rate and a significant improvement in the fibroblast chemotactic motility. For the secretion of collagen type I, EGF and VEGF, all the pulp extracts demonstrated amelioration in the secretion levels.

CHAPTER 6

CONCLUSION

6.1 Conclusion

Generally, the objectives of this study had been achieved in which the pulp and seed extracts of *L. garciae* had shown to exhibit the wound healing properties. Throughout this study, the fatty acids composition of lipid pulp and seed extracts of *L. garciae* using the PE and BD extraction methods were evaluated. The predominant fatty acids found in the *L. garciae* lipid seed extracts were lauric acid, palmitic acid, oleic acid, and linoleic acid. While for the *L. garciae* lipid pulp extracts, it was dominated by palmitic acid, stearic acid, oleic acid, and linoleic acid. All the *L. garciae* pulp and seed extracts exerted mild antibacterial activities against the Gram-positive bacteria. In contradiction, the Gram-negative bacteria were discovered to be less susceptible towards the *L. garciae* extracts as opposed to the Gram-positive bacteria.

Furthermore, most of the *L. garciae* extracts ameliorated the migration and chemotactic motility of the fibroblasts. The secretion of collagen type I, EGF and VEGF by the fibroblasts also improved after treatment with the *L. garciae* extracts. These findings could be attributed to the presence of phytochemical contents and fatty acids in the *L. garciae* extracts that contribute to their wound healing properties. To conclude, *L. garciae* pulp and seed extracts might be a potent wound healing drug to be used as a topical medication to apply on the wounded skin. Besides, the large-scale cultivation of *L. garciae* fruit will boost Sarawak's economy as the *L. garciae* fruit is not only as food, its high nutritional values and medicinal benefits can bring contribution to the society and scientific community. Hence,

the commercial value of this product may provide more job opportunities to the low-income communities in Sarawak.

6.2 Limitation of study and Future Work

The further investigations on this current study can be conducted to solve the limitation of study and produce more thorough research findings. First, the cold-pressed extraction of *L. garciae* lipid extract can be performed to maintain the temperature of extracted oil below 50°C (Dimić, 2005). This extraction method without using high temperature can preserve the bioactive compounds and fatty acids which bring benefits to the wound repair (Rabrenović et al., 2014). According to the study of Nevin & Rajamohan (2010), the cold-pressed coconut oil applied on the excision wounds in young rats had accelerated the epithelialisation and improved the fibroblast proliferation and neovascularisation, which resulted in an enhancement in the process of wound healing in contrast with the untreated control group.

Moreover, other species of Gram-negative bacteria can be utilised to investigate the antimicrobial properties of the *L. garciae* extracts, for instance, *Bartonella henselae*, *Pasteurella multocida* and *Klebsiella pneumoniae*. These bacteria do not belong to the normal skin microflora, but they will lead to the skin infection. Several studies had shown that the medicinal plants exhibited antimicrobial activities towards the Gram-negative bacteria. Ma et al. (2019) described that the essential oils from the plants such as genus *Citrus*, *Origanum*, *Cinnamomum*, *Pelargonium* and *Melaleuca* could kill the *B. henselae* cells. According to the findings of Ashraf et al. (2015), the fresh *Euphorbia royleana* extracted with methanol, hexane and water inhibited the growth of the *P. multocida* better than the *E. coli*. Not only that, *K. pneumoniae* was found to be susceptible to the

dichloromethane, methanol and aqueous extracts of *Andrographis paniculate* (Sule et al., 2010).

In addition, other experimental approaches can be performed to evaluate the antimicrobial potency of *L. garciae* extracts. As MIC assay is an antimicrobial susceptibility screening test for the antimicrobial lipids against a target bacterial species, it is unable to visualise the destabilisation of bacterial cell membrane by the antimicrobial lipids. Therefore, electron microscopy can be used to observe and visualise the morphological changes and antimicrobial effects of antimicrobial lipids against the target bacteria. Not only that, another useful approach such as supported lipid bilayer is a model membrane system that is used to monitor the interactions of antimicrobial lipids with the phospholipid membranes at the molecular level (Yoon et al., 2018).

Furthermore, an *in vitro* study of the effect of *L. garciae* extracts on the human keratinocytes can be conducted as keratinocytes also act as the principal cells that are located at the epidermis layer of skin. The keratinocytes serve as a barrier to minimise the loss of water and avoid penetration of dermis by foreign substances (Pastar et al., 2008). The study of Namchaiw et al. (2021) stated that the *Coccinia grandis* methanol leaf extract promoted the migration rate of keratinocytes, which brought beneficial effect on the wound repair. In addition to that, an *in vivo* wound healing activity of the *L. garciae* extracts can be further evaluated to confirm the safety and effectiveness of the extracts that might develop into a potent drug.

Additionally, the 3D cell culture model can be utilised for the *in vitro* study of human skin fibroblasts and *in vivo* wound healing evaluation in rats. A study by Intini et al. (2018) had demonstrated the use of 3D-printed chitosan-based scaffolds for the determination of

the biocompatibility and toxicity towards human fibroblasts and keratinocytes. The 3D-printed scaffolds were also used in the wound healing evaluation of streptozotocin-induced diabetic rats, where the 3D-printed scaffolds had shown to promote the tissue regeneration and functionality that suggested the capability of the scaffolds in the wound healing treatment.

Besides, there are numerous growth factors involve in the wound healing such as platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF- β), where the effects of *L. garciae* extracts can be tested on the secretion of these growth factors. PDGF and TGF- β are essential for the initiation of phenotypic change in fibroblasts, where fibroblasts will be converted into myofibroblasts that arrange along with the ECM to generate the contraction force which enhances the wound closure (Barrientos et al., 2008).

REFERENCES

- Abcam. (2021). *ab210966 – Human Pro-Collagen I alpha 1 SimpleStep ELISA® Kit*.
[online] Available at: <https://www.abcam.com/ps/products/210/ab210966/>
- Adam, O. A. O., Abadi, R. S. M., & Ayoub, S. M. H. (2019). The effect of extraction method and solvents on yield and antioxidant activity of certain sudanese medicinal plant extracts. *The Journal of Phytopharmacology*, 8(5), 248-252.
- Adan, A., Kiraz, Y., & Baran, Y. (2016). Cell proliferation and cytotoxicity assays. *Current Pharmaceutical Biotechnology*, 17(14), 1213-1221.
- Addis, R., Cruciani, S., Santaniello, S., Bellu, E., Sarais, G., Ventura, C., & Pintore, G. (2020). Fibroblast proliferation and migration in wound healing by phytochemicals: Evidence for a novel synergic outcome. *International Journal of Medical Sciences*, 17(8), 1030-1042.
- Adfa, M., Gusatyana, N. M. C., Mudyanto, A., Avidlyandi, A., Oktiarni, D., & Yudha, S. S. (2021). Combined effect of methanol extract of *Persea americana* seeds and *Cassia alata* leaves against *Staphylococcus epidermidis*. *Advances in Biological Sciences Research*, 13, 36-40.
- Ado, M. A., Abas, F., Mohammed, A. S., & Ghazali, H. M. (2013). Anti-and pro-lipase activity of selected medicinal, herbal and aquatic plants, and structure elucidation of an anti-lipase compound. *Molecules*, 18(12), 14651-14669.
- Akbik, D., Ghadiri, M., Chrzanowski, W., & Rohanizadeh, R. (2014). Curcumin as a wound healing agent. *Life Sciences*, 116(1), 1-7.
- Aksoy, L., Kolay, E., Ağılönü, Y., Aslan, Z., & Kargıoğlu, M. (2013). Free radical

- scavenging activity, total phenolic content, total antioxidant status, and total oxidant status of endemic *Thermopsis turcica*. *Saudi Journal of Biological Sciences*, 20(3), 235-239.
- Alexander, J. W., & Supp, D. M. (2014). Role of arginine and omega-3 fatty acids in wound healing and infection. *Advances in Wound Care*, 3(11), 682-690.
- Alma, M. H., Mavi, A., Yildirim, A., Digrak, M., & Hirata, T. (2003). Screening chemical composition and *in vitro* antioxidant and antimicrobial activities of the essential oils from *Origanum syriacum* L. growing in Turkey. *Biological and Pharmaceutical Bulletin*, 26(12), 1725-1729.
- American Society for Microbiology. (2016). *Kirby-Bauer Disk Diffusion Susceptibility Test Protocol*. [online] Available at: <https://asm.org/getattachment/2594ce26-bd44-47f6-8287-0657aa9185ad/Kirby-Bauer-Disk-Diffusion-Susceptibility-Test-Protocol-pdf.pdf>. [Accessed on 30 January 2023].
- Amit, Z., & Zinyin, L. (2021). A mini review on the nutritional compositions and pharmacological properties of *Litsea garciae*. *Malaysian Applied Biology*, 50(1), 29-39.
- Anderson, C. M., & Stahl, A. (2013). SLC27 fatty acid transport proteins. *Molecular Aspects of Medicine*, 34(2-3), 516-528.
- Anderson, K., & Hamm, R. L. (2012). Factors that impair wound healing. *Journal of the American College of Clinical Wound Specialists*, 4(4), 84-91.
- Angelo. (2012). *Essential Fatty Acids*. [online] Available at: <https://lpi.oregonstate.edu/mic/other-nutrients/essential-fatty-acids>. [Accessed on 8 March 2023].

- Araújo, L. U., Grabe-Guimarães, A., Mosqueira, V. C. F., Carneiro, C. M., & Silva-Barcellos, N. M. (2010). Profile of wound healing process induced by allantoin. *Acta Cirurgica Brasileira*, 25, 460-461.
- Arif, M. M., Raj, S. M., Jirovetz, L., & Shafi, M. P. (2008). Composition and antimicrobial analysis of the essential oil of *Litsea laevigata* Nees.(Lauraceae). *Natural Product Communications*, 3(7), 1069-1072.
- Ashraf, A., Sarfraz, R. A., Rashid, M. A., & Shahid, M. (2015). Antioxidant, antimicrobial, antitumor, and cytotoxic activities of an important medicinal plant (*Euphorbia royleana*) from Pakistan. *Journal of Food and Drug Analysis*, 23(1), 109-115.
- Azhar, M. A. M., & Salleh, W. M. N. H. W. (2020). Chemical composition and biological activities of essential oils of the genus *litsea* (Lauraceae)—A review. *Agriculturae Conspectus Scientificus*, 85(2), 97-103.
- Azlan, A., Prasad, K. N., Khoo, H. E., Abdul-Aziz, N., Mohamad, A., Ismail, A., & Amom, Z. (2010). Comparison of fatty acids, vitamin E and physicochemical properties of *Canarium odontophyllum* Miq.(dabai), olive and palm oils. *Journal of Food Composition and Analysis*, 23(8), 772-776.
- Azwanida, N. N. (2015). A review on the extraction methods use in medicinal plants, principle, strength and limitation. *Med Aromat Plants*, 4(196), 1-6.
- Balakin, K. V., Savchuk, N. P., & Tetko, I. V. (2006). In silico approaches to prediction of aqueous and DMSO solubility of drug-like compounds: trends, problems and solutions. *Current Medicinal Chemistry*, 13(2), 223-241.

- Barrientos, S., Stojadinovic, O., Golinko, M. S., Brem, H., & Tomic-Canic, M. (2008). Growth factors and cytokines in wound healing. *Wound Repair and Regeneration*, 16(5), 585-601.
- Basri, D. F., Mustari, N. F., Alamin, Z. A. Z. & Zin, N. M. (2017). Stem bark of *Canarium odontophyllum* Miq.(Dabai) as potential source of antimicrobial agent. *Jurnal Sains Kesihatan Malaysia*, 15(1), 1-6.
- Batovska, D. I., Todorova, T., Tsvetkova, V., & Najdenski, H. M. (2009). Antibacterial study of the medium chain fatty acids and their 1-monoglycerides: individual effects and synergistic relationships. *Polish Journal of Microbiology*, 58(1), 43-47.
- Black Country Healthcare. (2016). *Wound Management Guidelines*. [online] Available at: <https://www.bcpft.nhs.uk/documents/policies/w/1444-wound-management-guidelines/file> [Accessed on 6 June 2023].
- Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, 37(8), 911-917.
- Bodnar, R. J. (2013). Epidermal growth factor and epidermal growth factor receptor: the Yin and Yang in the treatment of cutaneous wounds and cancer. *Advances in Wound Care*, 2(1), 24-29.
- Bolla, S. R., Al-Subaie, A. M., Al-Jindan, R. Y., Balakrishna, J. P., Ravi, P. K., Veeraraghavan, V. P., & Surapaneni, K. M. (2019). *In vitro* wound healing potency of methanolic leaf extract of *Aristolochia saccata* is possibly mediated by its stimulatory effect on collagen-1 expression. *Heliyon*, 5(5), 1-7.
- Bolton, L., & Fattu, A. J. (1994). Topical agents and wound healing. *Clinics in Dermatology*, 12(1), 95-120.

- Bora, P. S., Narain, N., Rocha, R. V., & Paulo, M. Q. (2001). Characterization of the oils from the pulp and seeds of avocado (cultivar: Fuerte) fruits. *Grasas y Aceites*, 52(3-4), 171-174.
- Boudreau, M. D., & Beland, F. A. (2006). An evaluation of the biological and toxicological properties of *Aloe barbadensis* (miller), Aloe vera. *Journal of Environmental Science and Health Part C Environmental Carcinogenesis & Ecotoxicology Reviews*, 24, 103-154.
- Brinkhaus, B., Lindner, M., Schuppan, D., & Hahn, E. G. (2000). Chemical, pharmacological and clinical profile of the East Asian medical plant *Centella asiatica*. *Phytomedicine*, 7(5), 427-448.
- Brown, M. M., & Horswill, A. R. (2020). *Staphylococcus epidermidis*—Skin friend or foe? *PLoS Pathogens*, 16(11), 1-6.
- Bukbi, S. (2019). *Pokok engkalak kebun kamek baruk bebua*h. [online] Available at: <https://www.facebook.com/salbiah.bukbi/photos> [Accessed on 17 October 2021].
- Buranasukhon, W., Athikomkulchai, S., Tadtong, S., & Chittasupho, C. (2017). Wound healing activity of *Pluchea indica* leaf extract in oral mucosal cell line and oral spray formulation containing nanoparticles of the extract. *Pharmaceutical Biology*, 55(1), 1767-1774.
- Burdge, G. C., & Calder, P. C. (2015). Introduction to fatty acids and lipids. *Intravenous Lipid Emulsions*, 112, 1-16.
- Burusapat, C., Supawan, M., Pruksapong, C., Pitiseree, A., & Suwantemee, C. (2018). Topical *Aloe vera* gel for accelerated wound healing of split-thickness skin graft donor sites: A double-blind, randomized, controlled trial and systematic

- review. *Plastic and Reconstructive Surgery*, 142(1), 217-226.
- Bylka, W., Znajdek-Awiżeń, P., Studzińska-Sroka, E., Dańczak-Pazdrowska, A., & Brzezińska, M. (2014). *Centella asiatica* in dermatology: an overview. *Phytotherapy Research*, 28(8), 1117-1124.
- Byrd Allyson, L., Yasmine, B., & Segre Julia, A. (2018). The human skin microbiome [J]. *Nature Reviews Microbiology*, 16, 143-155.
- Campos, A. C., Groth, A. K., & Branco, A. B. (2008). Assessment and nutritional aspects of wound healing. *Current Opinion in Clinical Nutrition & Metabolic Care*, 11(3), 281-288.
- Caplan, A. I. (2005). Mesenchymal stem cells: cell-based reconstructive therapy in orthopedics. *Tissue Engineering*, 11(7-8), 1198-1211.
- Carvalho, C. P., Bernal E, J., Velásquez, M. A., & Cartagena V, J. R. (2015). Fatty acid content of avocados (*Persea americana* Mill. cv. Hass) in relation to orchard altitude and fruit maturity stage. *Agronomía Colombiana*, 33(2), 220-227.
- Casillas-Vargas, G., Ocasio-Malavé, C., Medina, S., Morales-Guzmán, C., Del Valle, R. G., Carballeira, N. M., & Sanabria-Ríos, D. J. (2021). Antibacterial fatty acids: An update of possible mechanisms of action and implications in the development of the next-generation of antibacterial agents. *Progress in Lipid Research*, 82,1-28.
- Cau, L., Williams, M. R., Butcher, A. M., Nakatsuji, T., Kavanaugh, J. S., Cheng, J. Y., & Gallo, R. L. (2021). *Staphylococcus epidermidis* protease EcpA can be a deleterious component of the skin microbiome in atopic dermatitis. *Journal of Allergy and Clinical Immunology*, 147(3), 955-966.

- Cen, R., Wang, L., He, Y., Yue, C., Tan, Y., Li, L., & Lei, X. (2022). Dermal fibroblast migration and proliferation upon wounding or lipopolysaccharide exposure is mediated by stathmin. *Frontiers in Pharmacology*, 12, 1-14.
- Chait, A., Brunzell, J. D., Denke, M. A., Eisenberg, D., Ernst, N. D., Franklin Jr, F. A., & Mullis, R. M. (1993). Rationale of the diet-heart statement of the American Heart Association. Report of the Nutrition Committee. *Circulation*, 88(6), 3008-3029.
- Chen, C. H., Wang, Y., Nakatsuji, T., Liu, Y. T., Zouboulis, C. C., Gallo, R. L., & Huang, C. M. (2011). An innate bactericidal oleic acid effective against skin infection of methicillin-resistant *Staphylococcus aureus*: a therapy concordant with evolutionary medicine. *Journal of Microbiology and Biotechnology*, 21(4), 391-399.
- Chen, W. C., Liou, S. S., Tzeng, T. F., Lee, S. L., & Liu, I. M. (2012). Wound repair and anti-inflammatory potential of *Lonicera japonica* in excision wound-induced rats. *BMC Complementary and Alternative Medicine*, 12(1), 1-9.
- Chen, W. C., Liou, S. S., Tzeng, T. F., Lee, S. L., & Liu, I. M. (2013). Effect of topical application of chlorogenic acid on excision wound healing in rats. *Planta Medica*, 79(08), 616-621.
- Chen, Y. S., Lee, S. M., Lin, Y. J., Chiang, S. H., & Lin, C. C. (2014). Effects of danshensu and salvianolic Acid B from *Salvia miltiorrhiza* Bunge (*Lamiaceae*) on cell proliferation and collagen and melanin production. *Molecules*, 19(2), 2029-2041.
- Chittasupho, C., Manthaisong, A., Okonogi, S., Tadtong, S., & Samee, W. (2022). Effects of quercetin and curcumin combination on antibacterial, antioxidant, *in vitro* wound healing and migration of human dermal fibroblast cells. *International Journal of Molecular Sciences*, 23(1), 1-16.

- Choi, S., Yoon, M., & Choi, K. Y. (2022). Approaches for regenerative healing of cutaneous wound with an emphasis on strategies activating the Wnt/ β -catenin pathway. *Advances in Wound Care*, 11(2), 70-86.
- Choudhury, S. N., Ghosh, A. C., Choudhury, M., & Leclercq, P. A. (1997). Essential oils of *Litsea monopetala* (Roxb.) Pers. A new report from India. *Journal of Essential Oil Research*, 9(6), 635-639.
- Choudhury, S. N., Singh, R. S., Ghosh, A. C., & Leclercq, P. A. (1996). *Litsea glutinosa* (Lour.) CB Rob., a new source of essential oil from northeast India. *Journal of Essential Oil Research*, 8(5), 553-556.
- Chowdhury, J. U., Bhuiyan, M. N. I., & Nandi, N. C. (2008). Aromatic plants of Bangladesh: Essential oils of leaves and fruits of *Litsea glutinosa* (Lour.) CB Robinson. *Bangladesh Journal of Botany*, 37(1), 81-83.
- Chowdhury, K., Banu, L. A., Khan, S., & Latif, A. (2007). Studies on the fatty acid composition of edible oil. *Bangladesh Journal of Scientific and Industrial Research*, 42(3), 311-316.
- Clinical and Laboratory Standards Institute. (2015). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard*, 10th ed., Wayne, PA: Clinical and Laboratory Standards Institute.
- Clinical and Laboratory Standards Institute. (2015). *Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard*, 12th ed., Wayne, PA: Clinical and Laboratory Standards Institute.

- Comino-Sanz, I. M., López-Franco, M. D., Castro, B., & Pancorbo-Hidalgo, P. L. (2021). The role of antioxidants on wound healing: A review of the current evidence. *Journal of Clinical Medicine*, 10(16), 1-22.
- Coulomb, B., Lebreton, C., & Dubertret, L. (1989). Influence of human dermal fibroblasts on epidermalization. *Journal of Investigative Dermatology*, 92(1), 122-125.
- Cox, N. A., Richardson, L. J., Cosby, D. E., Berrang, M. E., Wilson, J. L., & Harrison, M. A. (2017). A four-quadrant sequential streak technique to evaluate *Campylobacter* selective broths for suppressing background flora in broiler carcass rinses. *Journal of Food Safety*, 37(2), 1-3.
- Cross, M. J., & Claesson-Welsh, L. (2001). FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition. *Trends in Pharmacological Sciences*, 22(4), 201-207.
- David, F., Sandra, P., & Wylie, P. L. (2002). Improving the analysis of fatty acid methyl esters using retention time locked methods and retention time databases. *Agilent Technologies—Application*, 1-11.
- Davis, T. R., & Dimick, P. S. (1989). Lipid composition of high-melting seed crystals formed during cocoa butter solidification. *Journal of the American Oil Chemists Society*, 66(10), 1494-1498.
- De Leonardis, A., Pizzella, L., & Macciola, V. (2008). Evaluation of chlorogenic acid and its metabolites as potential antioxidants for fish oils. *European Journal of Lipid Science and Technology*, 110(10), 941-948.
- de Oliveira, A. P., Franco, E. D. S., Rodrigues Barreto, R., Cordeiro, D. P., de Melo, R. G., de Aquino, C. M. F., & Maia, M. B. D. S. (2013). Effect of semisolid formulation of

- Persea americana* Mill (avocado) oil on wound healing in rats. *Evidence-Based Complementary and Alternative Medicine*, 2013, 1-8.
- Desbois, A. P., & Lawlor, K. C. (2013). Antibacterial activity of long-chain polyunsaturated fatty acids against *Propionibacterium acnes* and *Staphylococcus aureus*. *Marine Drugs*, 11(11), 4544-4557.
- Di, R., Murray, A. F., Xiong, J., Esposito, D., Komarnytsky, S., Gianfagna, T. J., & Munafo Jr, J. P. (2020). Lily steroidal glycoalkaloid promotes early inflammatory resolution in wounded human fibroblasts. *Journal of Ethnopharmacology*, 258, 1-7.
- Diegelmann, R. F., & Evans, M. C. (2004). Wound healing: an overview of acute, fibrotic and delayed healing. *Frontiers in Bioscience*, 9(1), 283-289.
- Dimić, E. (2005). Cold pressed oils. *Novi Sad: University of Novi Sad, Faculty of Technology. str*, 170-172.
- Dinda, M., Dasgupta, U., Singh, N., Bhattacharyya, D., & Karmakar, P. (2015). PI3K-mediated proliferation of fibroblasts by *Calendula officinalis* tincture: implication in wound healing. *Phytotherapy Research*, 29(4), 607-616.
- Dinda, M., Mazumdar, S., Das, S., Ganguly, D., Dasgupta, U. B., Dutta, A., Jana, K., & Karmakar, P. (2016). The water fraction of *Calendula officinalis* hydroethanol extract stimulates in vitro and in vivo proliferation of dermal fibroblasts in wound healing. *Phytotherapy Research*, 30(10), 1696-1707.
- documents/Human-Pro-Collagen-I-alpha-1-ELISA-Kit-protocol-book-v2
ab210966%20(website).pdf. [Accessed on 19 March 2022].
- Draelos, Z. D., Rizer, R. L., & Trookman, N. S. (2011). A comparison of postprocedural wound care treatments: do antibiotic-based ointments improve outcomes?. *Journal*

of the American Academy of Dermatology, 64(3), 23-29.

Drosou, A. (2003). Antiseptics on wounds: an area of controversy. *Wounds*, 15, 149-166.

Dubois, V., Breton, S., Linder, M., Fanni, J., & Parmentier, M. (2007). Fatty acid profiles of 80 vegetable oils with regard to their nutritional potential. *European Journal of Lipid Science and Technology*, 109(7), 710-732.

Ebeling, S., Naumann, K., Pollok, S., Wardecki, T., Vidal-y-Sy, S., Nascimento, J. M., Boerries, M., Schmidt, G., Brandner, J. M., & Merfort, I. (2014). From a traditional medicinal plant to a rational drug: understanding the clinically proven wound healing efficacy of birch bark extract. *PloS One*, 9(1), 1-18.

Efthymiopoulos, I., Hellier, P., Ladommatos, N., Kay, A., & Mills-Lampsey, B. (2019). Effect of solvent extraction parameters on the recovery of oil from spent coffee grounds for biofuel production. *Waste and Biomass Valorization*, 10, 253-264.

Elder, D. E., Elenitsas, R., Johnson, B. L., Murphy, G. F. & Xu, X. (2009). *Lever's Histopathology of the Skin*, 10th ed., Philadelphia PA: Lippincott Williams & Wilkins.

Enoch, S., & Leaper, D. J. (2008). Basic science of wound healing. *Surgery (Oxford)*, 26(2), 31-37.

Estruch, R., Ros, E., Salas-Salvadó, J., Covas, M. I., Corella, D., Arós, F., & Martínez-González, M. A. (2013). Primary prevention of cardiovascular disease with a Mediterranean diet. *New England Journal of Medicine*, 368(14), 1279-1290.

Fabry, W., Okemo, P. O., & Ansorg, R. (1998). Antibacterial activity of East African medicinal plants. *Journal of Ethnopharmacology*, 60(1), 79-84.

FDA. (2000). *Health Claim Notification for Potassium Containing Foods*. [online]

- Available at: <https://www.fda.gov/food/food-labeling-nutrition/health-claim-notification-potassium-containing-foods> [Accessed on 16 January 2023].
- Fern, K., 2019. *Tropical Plants Database*. [online] Available at: <http://tropical.theferns.info/viewtropical.php?id=Litsea+garciae> [Accessed on 9 June 2020].
- Ferreira, A. M., Souza, B. M. V. D., Rigotti, M. A., & Loureiro, M. R. D. (2012). The use of fatty acids in wound care: an integrative review of the Brazilian literature. *Revista da Escola de Enfermagem da USP*, 46, 752-760.
- Firdous, S. M., & Sautya, D. (2018). Medicinal plants with wound healing potential. *Bangladesh Journal of Pharmacology*, 13(1), 41-52.
- Fischer, C. L., Blanchette, D. R., Brogden, K. A., Dawson, D. V., Drake, D. R., Hill, J. R., & Wertz, P. W. (2014). The roles of cutaneous lipids in host defense. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, 1841(3), 319-322.
- Fitzmaurice, S. D., Sivamani, R. K., & Isseroff, R. R. (2011). Antioxidant therapies for wound healing: a clinical guide to currently commercially available products. *Skin Pharmacology and Physiology*, 24(3), 113-126.
- Folch, J., Lees, M., & Stanley, G. S. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *Journal of Biological Chemistry*, 226(1), 497-509.
- Frank, S., Kämpfer, H., Wetzler, C., & Pfeilschifter, J. (2002). Nitric oxide drives skin repair: novel functions of an established mediator. *Kidney International*, 61(3), 882-888.
- Franz, M. G., Steed, D. L., & Robson, M. C. (2007). Optimizing healing of the acute wound by minimizing complications. *Current Problems in Surgery*, 44(11), 691-763.

- Frommer, K. W., Hasseli, R., Schäffler, A., Lange, U., Rehart, S., Steinmeyer, J., & Neumann, E. (2019). Free fatty acids in bone pathophysiology of rheumatic diseases. *Frontiers in Immunology*, 10, 1-10.
- Fronza, M., Heinzmann, B., Hamburger, M., Laufer, S., & Merfort, I. (2009). Determination of the wound healing effect of *Calendula* extracts using the scratch assay with 3T3 fibroblasts. *Journal of Ethnopharmacology*, 126(3), 463-467.
- Galiano, R. D., Tepper, O. M., Pelo, C. R., Bhatt, K. A., Callaghan, M., Bastidas, N., & Gurtner, G. C. (2004). Topical vascular endothelial growth factor accelerates diabetic wound healing through increased angiogenesis and by mobilizing and recruiting bone marrow-derived cells. *The American Journal of Pathology*, 164(6), 1935-1947.
- Gao, S. Q., Chang, C., Niu, X. Q., Li, L. J., Zhang, Y., & Gao, J. Q. (2018). Topical application of hydroxysafflor yellow A accelerates the wound healing in streptozotocin induced T1DM rats. *European Journal of Pharmacology*, 823, 72-78.
- George Broughton, I. I., Janis, J. E., & Attinger, C. E. (2006). Wound healing: an overview. *Plastic and Reconstructive Surgery*, 117(7S), 1-32.
- Ghildiyal, S., Gautam, M. K., Joshi, V. K., & Goel, R. K. (2015). Wound healing and antimicrobial activity of two classical formulations of *Laghupanchamula* in rats. *Journal of Ayurveda and Integrative Medicine*, 6(4), 241-247.
- Golebiewska, E. M., & Poole, A. W. (2015). Platelet secretion: From haemostasis to wound healing and beyond. *Blood Reviews*, 29(3), 153-162.
- Gonzalez, A. (2018). *Open wound*. [online] Available at: <https://www.healthline.com/health/open-wound> [Accessed on 23 January 2023].
- Google Maps. (2020). *Malaysia, Indonesia, Philipines, 1: 200,000*. Google Maps. [online]

Available at: <https://www.google.com.my/maps> [Accessed on 22 May 2020].

- Greenway, D. L. A., & Dyke, K. G. H. (1979). Mechanism of the inhibitory action of linoleic acid on the growth of *Staphylococcus aureus*. *Microbiology*, 115(1), 233-245.
- Guidoni, M., de Christo Scherer, M. M., Figueira, M. M., Schmitt, E. F. P., De Almeida, L. C., Scherer, R., & Fronza, M. (2019). Fatty acid composition of vegetable oil blend and *in vitro* effects of pharmacotherapeutical skin care applications. *Brazilian Journal of Medical and Biological Research*, 52, 1-8.
- Guo, S. A., & DiPietro, L. A. (2010). Factors affecting wound healing. *Journal of Dental Research*, 89(3), 219-229.
- Häkkinen, L., Larjava, H., & Koivisto, L. (2011). Granulation tissue formation and remodeling. *Endodontic Topics*, 24(1), 94-129.
- Han, G., & Ceilley, R. (2017). Chronic wound healing: a review of current management and treatments. *Advances in Therapy*, 34, 599-610.
- Hao, B., Wang, X., Ma, X., Jin, Y., Fan, W., Laba, C., & Liang, J. (2020). Preparation of complex microcapsules of soluble polysaccharide from *Glycyrrhiza uralensis* and its application in wound repair and scar inhibition. *International Journal of Biological Macromolecules*, 156, 906-917.
- Hao, K., Xu, B., Zhang, G., Lv, F., Wang, Y., Ma, M., & Si, H. (2021). Antibacterial activity and mechanism of *Litsea cubeba* L. essential oil against *Acinetobacter baumannii*. *Natural Product Communications*, 16(3), 1-7.
- Hassan, S. H. A., Fry, J. R., & Bakar, M. F. A. (2013). Antioxidant and phytochemical study on pengolaban (*Litsea garciae*), an edible underutilized fruit endemic to Borneo. *Food Science and Biotechnology*, 22, 1-7.

- Herbs Encyclopedia. (2020). *Engkala trees (Litsea garciae)*. [online] Available at: <https://www.picuki.com/media/2207426604878230941> [Accessed on 23 May 2020].
- Huang, P. H., Li, J., Li, X. W., & van der Werff, H. H. (2008). *Litsea*. *Flora of China*, 7, 102-254.
- Huang, S. K., White, E. S., Wettlaufer, S. H., Grifka, H., Hogaboam, C. M., Thannickal, V. J., & Peters-Golden, M. (2009). Prostaglandin E2 induces fibroblast apoptosis by modulating multiple survival pathways. *The FASEB Journal*, 23(12), 4317–4326.
- Huang, T. H., Wang, P. W., Yang, S. C., Chou, W. L., & Fang, J. Y. (2018). Cosmetic and therapeutic applications of fish oil's fatty acids on the skin. *Marine Drugs*, 16(8), 1-20.
- Husen, R. (2015). Potential use of superheated-steam treatment in underutilised fruit of engkala (*Litsea garciae*) and evaluation of its antioxidant capacity. In *PhD's Thesis, Kyushu Institute of Technology, Japan*, (pp. 9-80).
- Huttner, J. J., Milo, G. E., Panganamala, R. V., & Cornwell, D. G. (1978). Fatty acids and the selective alteration of *in vitro* proliferation in human fibroblast and guinea-pig smooth-muscle cells. *In vitro*, 14, 854-859.
- Ili, P., & Sari, F. (2023). Egg yolk oil accelerates wound healing in streptozotocin induced diabetic rats. *Biotechnic & Histochemistry*, 98(2), 94-111.
- Imai, K., Kato, H., Taguchi, Y., & Umeda, M. (2019). Biological effects of shikonin in human gingival fibroblasts via ERK 1/2 signaling pathway. *Molecules*, 24(19), 1-13.
- Innes, M. E., Umraw, N., Fish, J. S., Gomez, M., & Cartotto, R. C. (2001). The use of silver coated dressings on donor site wounds: a prospective, controlled matched pair

- study. *Burns*, 27(6), 621-627.
- Intini, C., Elviri, L., Cabral, J., Mros, S., Bergonzi, C., Bianchera, A., & McConnell, M. (2018). 3D-printed chitosan-based scaffolds: An *in vitro* study of human skin cell growth and an *in-vivo* wound healing evaluation in experimental diabetes in rats. *Carbohydrate Polymers*, 199, 593-602.
- ISO. (2009). *Biological evaluation of medical devices—Part 5: Tests for in vitro cytotoxicity*. International Organization for Standardization: Geneva, Switzerland.
- Jain, N., Jain, R., Jain, A., Jain, D. K., & Chandel, H. S. (2010). Evaluation of wound-healing activity of *Acorus calamus* Linn. *Natural Product Research*, 24(6), 534-541.
- James, O., & Friday, E. T. (2010). Phytochemical composition, bioactivity and wound healing potential of *Euphorbia heterophylla* (Euphorbiaceae) leaf extract. *International Journal on Pharmaceutical and Biomedical Research*, 1(1), 54-63.
- Jiang, X. W., Qiao, L., Liu, L., Zhang, B. Q., Wang, X. W., Han, Y. W., & Yu, W. H. (2017). *Dracorhodin perchlorate* accelerates cutaneous wound healing in wistar rats. *Evidence-Based Complementary and Alternative Medicine*, 2017, 1-9.
- Johann, S., Pizzolatti, M. G., Donnici, C. L., & Resende, M. A. D. (2007). Antifungal properties of plants used in Brazilian traditional medicine against clinically relevant fungal pathogens. *Brazilian Journal of Microbiology*, 38, 632-637.
- Johansen, H. N., Glitsø, V., & Bach Knudsen, K. E. (1996). Influence of extraction solvent and temperature on the quantitative determination of oligosaccharides from plant materials by high-performance liquid chromatography. *Journal of Agricultural and Food Chemistry*, 44(6), 1470-1474.

- Johnny, L., Yusuf, U. K., & Nulit, R. (2010). The effect of herbal plant extracts on the growth and sporulation of *Colletotrichum gloeosporioides*. *Journal of Applied Biosciences*, 34, 2218-2224.
- Johnny, L., Yusuf, U. K., & Nulit, R. (2011). Antifungal activity of selected plant leaves crude extracts against a pepper anthracnose fungus, *Colletotrichum capsici* (Sydow) butler and bisby (Ascomycota: Phyllachorales). *African Journal of Biotechnology*, 10(20), 4157-4165.
- Jorge, M. P., Madjarof, C., Ruiz, A. L. T. G., Fernandes, A. T., Rodrigues, R. A. F., de Oliveira Sousa, I. M., & de Carvalho, J. E. (2008). Evaluation of wound healing properties of *Arrabidaea chica* Verlot extract. *Journal of Ethnopharmacology*, 118(3), 361-366.
- Kabara, J. J., Swieczkowski, D. M., Conley, A. J., & Truant, J. P. (1972). Fatty acids and derivatives as antimicrobial agents. *Antimicrobial Agents and Chemotherapy*, 2(1), 23-28.
- Kamle, M., Mahato, D. K., Lee, K. E., Bajpai, V. K., Gajurel, P. R., Gu, K. S., & Kumar, P. (2019). Ethnopharmacological properties and medicinal uses of *Litsea cubeba*. *Plants*, 8(6), 150.
- Kang, C. G., Hah, D. S., Kim, C. H., Kim, Y. H., Kim, E., & Kim, J. S. (2011). Evaluation of antimicrobial activity of the methanol extracts from 8 traditional medicinal plants. *Toxicological Research*, 27, 31-36.
- Karim, S., Alkreathy, H. M., Ahmad, A., & Khan, M. I. (2021). Effects of methanolic extract based-gel from Saudi pomegranate peels with enhanced healing potential on excision wounds in diabetic rats. *Frontiers in Pharmacology*, 12, 1-12.

- Kaur, N., Chugh, V., & Gupta, A. K. (2014). Essential fatty acids as functional components of foods-a review. *Journal of Food Science and Technology*, 51, 2289-2303.
- Kaur, R., Mahajan, A., & Bhatia, A. (2018). Comparison of different methods of lipid extraction from microalgae *Chlorella pyrenoidosa*. *Journal of Emerging Technologies and Innovative Research*, 5(5), 115-120.
- Kim, D., Kim, S. Y., Mun, S. K., Rhee, S., & Kim, B. J. (2015). Epidermal growth factor improves the migration and contractility of aged fibroblasts cultured on 3D collagen matrices. *International Journal of Molecular Medicine*, 35(4), 1017-1025.
- Kim, W. S., Park, B. S., Sung, J. H., Yang, J. M., Park, S. B., Kwak, S. J., & Park, J. S. (2007). Wound healing effect of adipose-derived stem cells: a critical role of secretory factors on human dermal fibroblasts. *Journal of Dermatological Science*, 48(1), 15-24.
- Klopell, F. C., Lemos, M., Sousa, J. P. B., Comunello, E., Maistro, E. L., Bastos, J. K., & Andrade, S. F. D. (2007). Nerolidol, an antiulcer constituent from the essential oil of *Baccharis dracunculifolia* DC (Asteraceae). *Zeitschrift für Naturforschung C*, 62(7-8), 537-542.
- Krahwinkel, D. J., & Boothe, H. W. (2006). Topical and systemic medications for wounds. *Veterinary Clinics: Small Animal Practice*, 36(4), 739-757.
- Krischak, G. D., Augat, P., Claes, L., Kinzl, L., & Beck, A. (2007). The effects of non-steroidal anti-inflammatory drug application on incisional wound healing in rats. *Journal of Wound Care*, 16(2), 76-78.
- Kris-Etherton, P. M. (1999). Monounsaturated fatty acids and risk of cardiovascular disease. *Circulation*, 100(11), 1253-1258.

- Kutoi, C. J., Yen, K. H., & Seruji, N. M. U. (2012). Pharmacology evaluation of *Litsea garciae* (Lauraceae). In *2012 IEEE Business, Engineering & Industrial Applications Colloquium (BEIAC)*, (pp. 31-33).
- Kylanel, A. N., Sugiaman, V. K., & Pranata, N. (2020). Fibroblast viability test toward red dragon fruit (*Hylocereus polyrhizus*) peel ethanolic extract. *Systematic Reviews in Pharmacy*, *11*(12), 356-360.
- Lam, P. K., Chan, E. S. Y., Ho, W. S., & Liew, C. T. (2004). In vitro cytotoxicity testing of a nanocrystalline silver dressing (Acticoat) on cultured keratinocytes. *British Journal of Biomedical Science*, *61*(3), 125-127.
- Landén, N. X., Li, D., & Ståhle, M. (2016). Transition from inflammation to proliferation: a critical step during wound healing. *Cellular and Molecular Life Sciences*, *73*, 3861-3885.
- Lau, T. W., Lam, F. F. Y., Lau, K. M., Chan, Y. W., Lee, K. M., Sahota, D. S., & Lau, C. B. S. (2009). Pharmacological investigation on the wound healing effects of *Radix Rehmanniae* in an animal model of diabetic foot ulcer. *Journal of Ethnopharmacology*, *123*(1), 155-162.
- Lazarus, G. S., Cooper, D. M., Knighton, D. R., Margolis, D. J., Percoraro, R. E., Rodeheaver, G., & Robson, M. C. (1994). Definitions and guidelines for assessment of wounds and evaluation of healing. *Wound Repair and Regeneration*, *2*(3), 165-170.
- Lee, T. H., Lee, G. W., Park, K. H., Mohamed, M. A. A., Bang, M. H., Baek, Y. S., & Kim, J. (2014). The stimulatory effects of *Stewartia koreana* extract on the proliferation and migration of fibroblasts and the wound healing activity of the extract in

- mice. *International Journal of Molecular Medicine*, 34(1), 145-152.
- Li, J., Chen, L., Xu, J., Xie, Z., Xu, Y., Jiang, P., & Liu, W. (2019). Effects of *Periploca forrestii* Schltr on wound healing by Src mediated Mek/Erk and PI3K/Akt signals. *Journal of Ethnopharmacology*, 237, 116-127.
- Li, L. J., Wang, M. Z., Yuan, T. J., Xu, X. H., Dad, H. A., Yu, C. L., & Peng, L. H. (2019). The crude ethanol extract of *Periplaneta americana* L. stimulates wound healing in vitro & in vivo. *Chinese Medicine*, 14(1), 1-9.
- Li, W. R., Shi, Q. S., Liang, Q., Xie, X. B., Huang, X. M., & Chen, Y. B. (2014). Antibacterial activity and kinetics of *Litsea cubeba* oil on *Escherichia coli*. *PLoS One*, 9(11), 1-6.
- Liedberg, H., & Lundeberg, T. (1989). Assessment of silver-coated urinary catheter toxicity by cell culture. *Urological Research*, 17(6), 359-360.
- Lim, T. K. (2012). *Edible medicinal and non-medicinal plants* (Vol. 1, pp. 656-687), Dordrecht, The Netherlands: Springer.
- Lipsky, B. A., & Hoey, C. (2009). Topical antimicrobial therapy for treating chronic wounds. *Clinical Infectious Diseases*, 49(10), 1541-1549.
- Ma, X., Shi, W., & Zhang, Y. (2019). Essential oils with high activity against stationary phase *Bartonella henselae*. *Antibiotics*, 8(4), 246.
- Machabuca. (2008). *Litsea garciae* – *Engkala*. [online] Available at: <https://www.flickr.com/photos/69028902@N00/13517463885/in/photostream/> [Accessed on 23 May 2020].

- Magalhães, M. S. F., Fechine, F. V., Macedo, R. N. D., Monteiro, D. L. S., Oliveira, C. C., Brito, G. A. D. C., & Moraes, M. O. D. (2008). Effect of a combination of medium chain triglycerides, linoleic acid, soy lecithin and vitamins A and E on wound healing in rats. *Acta Cirurgica Brasileira*, 23, 262-269.
- Mapoung, S., Umsumarng, S., Semmarath, W., Arjsri, P., Thippraphan, P., Yodkeeree, S., & Limtrakul, P. (2021). Skin wound-healing potential of polysaccharides from medicinal mushroom *Auricularia auricula-judae* (Bull.). *Journal of Fungi*, 7(4), 1-16.
- Martin, P. (1997). Wound healing--aiming for perfect skin regeneration. *Science*, 276(5309), 75-81.
- Mchugh, G. L., Moellering, R., Hopkins, C., & Swartz, M. (1975). *Salmonella typhimurium* resistant to silver nitrate, chloramphenicol, and ampicillin: A new threat in burn units?. *The Lancet*, 305(7901), 235-240.
- Melguizo-Rodríguez, L., Illescas-Montes, R., Costela-Ruiz, V. J., Ramos-Torrecillas, J., de Luna-Bertos, E., García-Martínez, O., & Ruiz, C. (2021). Antimicrobial properties of olive oil phenolic compounds and their regenerative capacity towards fibroblast cells. *Journal of Tissue Viability*, 30(3), 372-378.
- Mensink, R. P., & Katan, M. B. (1992). Effect of dietary fatty acids on serum lipids and lipoproteins. A meta-analysis of 27 trials. *Arteriosclerosis and Thrombosis: A Journal of Vascular Biology*, 12(8), 911-919.
- Merck. (2021). *Human Dermal Fibroblasts (HDF) Culture Protocol*. [online] Available at: <https://www.sigmaaldrich.com/MY/en/technical-documents/protocol/cell-culture->

and-cell-culture-analysis/primary-cell-culture/human-dermal-fibroblasts. [Accessed on 17 June 2021].

Merck. (2021). *ELISA Protocols*. [online] Available at: <https://www.sigmaaldrich.com/MY/en/technical-documents/protocol/protein-biology/elisa/elisa-protocols>. [Accessed on 19 June 2021].

Mirbehbahani, F. S., Hejazi, F., Najmoddin, N., & Asefnejad, A. (2020). *Artemisia annua* L. as a promising medicinal plant for powerful wound healing applications. *Progress in Biomaterials*, 9, 139-151.

Mirfat, A. H. S., Amin, I., Kartinee, K. N., Muhajir, H., & Shukri, M. A. M. (2018). Underutilised fruits: A review of phytochemistry and biological properties. *Journal of Food Bioactives*, 1, 2-30.

Morin, C., Roumegous, A., Carpentier, G., Barbier-Chassefiere, V., Garrigue-Antar, L., Caredda, S., & Courty, J. (2012). Modulation of inflammation by Cicaderma ointment accelerates skin wound healing. *Journal of Pharmacology and Experimental Therapeutics*, 343(1), 115-124.

Mugford, S. T., & Osbourn, A. (2013). Saponin synthesis and function. *Isoprenoid Synthesis in Plants and Microorganisms*, 405-424.

Muhammad, A. A., Pauzi, N. A. S., Arulselvan, P., Abas, F., & Fakurazi, S. (2013). In vitro wound healing potential and identification of bioactive compounds from *Moringa oleifera* Lam. *BioMed Research International*, 2013, 1-10.

Mukherjee, P. K. (2019). *Quality control and evaluation of herbal drugs: Evaluating natural products and traditional medicine*. Elsevier.

Murphy, P. S., & Evans, G. R. (2012). Advances in wound healing: a review of current

- wound healing products. *Plastic Surgery International*, 2012, 1-8.
- Nacalai Tesques. (n.d.). *Cell Cultivation version 2.2*. [online] Available at: www.nacalai.com. [Accessed on 14 June 2021].
- Naczek, M., & Shahidi, F. (2006). Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis. *Journal of Pharmaceutical and Biomedical Analysis*, 41(5), 1523-1542.
- Nagabhushanam, R. (2020). *Fouling Organisms of the Indian Ocean*. CRC Press.
- Naghshineh, M., Ariffin, A. A., Ghazali, H. M., Mirhosseini, H., & Mohammad, A. S. (2010). Effect of saturated/unsaturated fatty acid ratio on physicochemical properties of palm olein–olive oil blend. *Journal of the American Oil Chemists' Society*, 87, 255-262.
- Nagori, B. P., & Solanki, R. (2011). Role of medicinal plants in wound healing. *Research Journal of Medicinal Plant*, 5(4), 392-405.
- Nakamura, M. T., Yudell, B. E., & Loor, J. J. (2014). Regulation of energy metabolism by long-chain fatty acids. *Progress in Lipid Research*, 53, 124-144.
- Namchaiw, P., Jaisin, Y., Niwaspragrit, C., Malaniyom, K., Auvuchanon, A., & Ratanachamnong, P. (2021). The leaf extract of *Coccinia grandis* (L.) Voigt accelerated *in vitro* wound healing by reducing oxidative stress injury. *Oxidative Medicine and Cellular Longevity*, 2021, 1-10.
- Naqvi, S. A. R., Nadeem, S., Komal, S., Naqvi, S. A. A., Mubarik, M. S., Qureshi, S. Y., & Aslam, N. (2019). Antioxidants: natural antibiotics. In *Antioxidants*. IntechOpen.
- Nawrot-Hadzik, I., Matkowski, A., Pitułaj, A., Sterczała, B., Olchow, C., Szewczyk, A., & Choromańska, A. (2021). *In vitro* gingival wound healing activity of extracts from

- Reynoutria japonica* houtt rhizomes. *Pharmaceutics*, 13(11), 1-18.
- Nevin, K. G., & Rajamohan, T. (2010). Effect of topical application of virgin coconut oil on skin components and antioxidant status during dermal wound healing in young rats. *Skin Pharmacology and Physiology*, 23(6), 290-297.
- Ngan, V. (2005). *Wound Infection*. [online] Available at: <https://dermnetnz.org/topics/wound-infections>. [Accessed on 10 July 2023].
- Nguyen, M. C., Le, D. T., Kamei, K., & Dang, T. P. T. (2017). Wound healing activity of *Streptocaulon juventas* root ethanolic extract. *Wound Repair and Regeneration*, 25(6), 956-963.
- Nguyen, T. L. A., & Bhattacharya, D. (2022). Antimicrobial activity of quercetin: an approach to its mechanistic principle. *Molecules*, 27(8), 1-13.
- Nguyen, V. L., Truong, C. T., Nguyen, B. C. Q., Van Vo, T. N., Dao, T. T., Nguyen, V. D., & Bui, C. B. (2017). Anti-inflammatory and wound healing activities of calophyllolide isolated from *Calophyllum inophyllum* Linn. *PloS One*, 12(10), 1-16.
- NIBIOHN JCRB Cell Bank. (n.d.). *Successful cell culture*. [online] Available at: <https://cellbank.nibiohn.go.jp/english/successful-cell-culture.html>. [Accessed on 26 November 2020].
- Ning, S., Zang, J., Zhang, B., Feng, X., & Qiu, F. (2022). Botanical Drugs in Traditional Chinese Medicine With Wound Healing Properties. *Frontiers in Pharmacology*, 13, 1-14.
- Nor, N. A. M., & Ibrahim, H. (2018). Chemical constituents of essential oils of *Boesenbergia armeniaca* and *B. stenophylla* (Zingiberaceae) endemic to Borneo. *Pakistan Journal of Botany*, 50(5), 1917-1922.

- Nur, A., Norizan, A., Mashita, A., Norrizah, J. S., & Syed, A. (2012). Antioxidant, antimicrobial and cytotoxic activities of resveratrol oligomers of *Shorea macroptera* dyer. *Australian Journal of Basic and Applied Sciences*, 6(8), 431-436.
- Oliveira, A., Sousa, J. C., Silva, A. C., Melo, L. D., & Sillankorva, S. (2018). Chestnut honey and bacteriophage application to control *Pseudomonas aeruginosa* and *Escherichia coli* biofilms: evaluation in an ex vivo wound model. *Frontiers in Microbiology*, 9, 1-13.
- Othman, L., Sleiman, A., & Abdel-Massih, R. M. (2019). Antimicrobial activity of polyphenols and alkaloids in middle eastern plants. *Frontiers in Microbiology*, 10, 911.
- Pace-Asciak, C., & Wolfe, L. S. (1968). Inhibition of prostaglandin synthesis by oleic, linoieic and linoienic acids. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, 152(4), 784-787.
- Paladini, F., & Pollini, M. (2019). Antimicrobial silver nanoparticles for wound healing application: progress and future trends. *Materials*, 12(16), 2540.
- Panche, A. N., Diwan, A. D., & Chandra, S. R. (2016). Flavonoids: an overview. *Journal of Nutritional Science*, 5, 1-15.
- Parekh, J., & Chanda, S. (2007). Antibacterial and phytochemical studies on twelve species of Indian medicinal plants. *African Journal of Biomedical Research*, 10(2), 175-181.
- Parente, L. M., Andrade, M. A., Brito, L. A., Moura, V. M., Miguel, M. P., LinoJúnior, R. S., Tresvenzol, L. F., Paula, J. R., & Paulo, N. M. (2011). Angiogenic activity of *Calendula officinalis* flowers L. in rats,". *Acta Cirurgica Brasileira*, 26(1), 19-24.

- Park, S. M., Won, K. J., Hwang, D. I., Kim, H. B., Li, Y., & Lee, H. M. (2020). Potential beneficial effects of *Digitaria ciliaris* flower absolute on the wound healing-linked activities of fibroblasts and keratinocytes. *Planta Medica*, 86(05), 348-355.
- Pastar, I., Stojadinovic, O., & Tomic-Canic, M. (2008). Role of keratinocytes in healing of chronic wounds. *Surgical Technology International*, 17, 105-112.
- Percival, S. L., Bowler, P., & Woods, E. J. (2008). Assessing the effect of an antimicrobial wound dressing on biofilms. *Wound Repair and Regeneration*, 16(1), 52-57.
- Pham, C., Greenwood, J., Cleland, H., Woodruff, P., & Maddern, G. (2007). Bioengineered skin substitutes for the management of burns: a systematic review. *Burns*, 33(8), 946-957.
- Poli, F. M., & Assim, Z. (2019). Fatty acid profiles in the kernel oils of *Artocarpus odoratissimus* and *Litsea garciae*. *Journal of Engineering and Applied Sciences*, 14(3), 6135-6138.
- Postlethwaite, A. E., Keski-Oja, J., Moses, H. L., & Kang, A. H. (1987). Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor beta. *The Journal of Experimental Medicine*, 165(1), 251-256.
- Prasad, K. N., Chew, L. Y., Khoo, H. E., Kong, K. W., Azlan, A., & Ismail, A. (2010). Antioxidant capacities of peel, pulp, and seed fractions of *Canarium odontophyllum* Miq. fruit. *Journal of Biomedicine and Biotechnology*, 1-8.
- Preethi, K. C., & Kuttan, R. (2009). Wound healing activity of flower extract of *Calendula officinalis*. *Journal of Basic and Clinical Physiology and Pharmacology*, 20(1), 73-80.

- Priscilia, B., Novita, H., & Elya, B. (2020). Antioxidant and antibacterial assay against fish pathogen bacteria of *Kjellbergiodendron celebicum* (Koord.) Merr. leaf extract. *Pharmacognosy Journal*, 12(1), 173-179.
- Pui, J. S. W. (2015). Characterisation of lipids from *Litsea graciae* and *Litsea cubeba* fruits and their biological activities. In *Final Year Project's Thesis, Universiti Malaysia Sarawak*, (pp. 31-37).
- Rabrenović, B. B., Dimić, E. B., Novaković, M. M., Tešević, V. V., & Basić, Z. N. (2014). The most important bioactive components of cold pressed oil from different pumpkin (*Cucurbita pepo* L.) seeds. *LWT-Food Science and Technology*, 55(2), 521-527.
- Raduan, S. Z., Ahmed, Q. U., Kasmuri, A. R., Rusmili, M. R. A., Mia, M. A. R., Sulaiman, W. M. A. W., & Shaikh, M. F. (2022). Antioxidant capabilities of *Litsea garciae* bark extracts and their relation to the phytochemical compositions. *Malaysian Applied Biology*, 51(1), 99-118.
- Rakman, S. A., Assim, Z., & Ahmad, F. B. (2022). Oilseeds and Seed Oils of *Shorea macrophylla* and *Shorea palembanica*: Evaluation of Proximate, Antinutritive Factors and Chemical Composition. *Malaysian Journal of Chemistry*, 25(1), 11-29.
- Ramos-Jerz, M. D. R., Villanueva, S., Jerz, G., Winterhalter, P., & Deters, A. M. (2013). *Persea americana* Mill. seed: fractionation, characterization, and effects on human keratinocytes and fibroblasts. *Evidence-Based Complementary and Alternative Medicine*, 2013, 1-12.
- Rangaraj, A., Harding, K., & Leaper, D. (2011). Role of collagen in wound management. *Wounds UK*, 7(2), 54-63.

- Ranzato, E., Mazzucco, L., Patrone, M., & Burlando, B. (2009). Platelet lysate promotes in vitro wound scratch closure of human dermal fibroblasts: different roles of cell calcium, P38, ERK and PI3K/AKT. *Journal of Cellular and Molecular Medicine*, 13(8b), 2030-2038.
- Rashed, A. N., Afifi, F. U., & Disi, A. M. (2003). Simple evaluation of the wound healing activity of a crude extract of *Portulaca oleracea* L.(growing in Jordan) in *Mus musculus* JVI-1. *Journal of Ethnopharmacology*, 88(2-3), 131-136.
- Razika, L., Thanina, A. C., Nadjiba, C. M., Narimen, B., Mahdi, D. M., & Karim, A. (2017). Antioxidant and wound healing potential of saponins extracted from the leaves of Algerian *Urtica dioica* L. *Pakistan Journal Of Pharmaceutical Sciences*, 30(3), 1023-1029.
- Ribeiro Barros Cardoso, C., Aparecida Souza, M., Amália Vieira Ferro, E., Favoreto Jr, S., & Deolina Oliveira Pena, J. (2004). Influence of topical administration of n-3 and n-6 essential and n-9 nonessential fatty acids on the healing of cutaneous wounds. *Wound Repair and Regeneration*, 12(2), 235-243.
- Rodrigues, H. G., Vinolo, M. A., Sato, F. T., Magdalon, J., Kuhl, C. M., Yamagata, A. S., & Curi, R. (2016). Oral administration of linoleic acid induces new vessel formation and improves skin wound healing in diabetic rats. *PloS One*, 11(10), 1-19.
- Rodrigues, M., Kosaric, N., Bonham, C. A., & Gurtner, G. C. (2019). Wound healing: a cellular perspective. *Physiological Reviews*, 99(1), 665-706.
- Rojo, L. E., Villano, C. M., Joseph, G., Schmidt, B., Shulaev, V., Shuman, J. L., & Raskin, I. (2010). Original Contribution: Wound-healing properties of nut oil from *Pouteria lucuma*. *Journal of Cosmetic Dermatology*, 9(3), 185-195.

- Ruangpan, L., & Tendencia, E. A. (2004). Bacterial isolation, identification and storage. In *Laboratory manual of standardized methods for antimicrobial sensitivity tests for bacteria isolated from aquatic animals and environment*, pp. 3-11. Iloilo, Philippines: Aquaculture Department, Southeast Asian Fisheries Development Center.
- Saeidinia, A., Keihanian, F., Lashkari, A. P., Lahiji, H. G., Mobayyen, M., Heidarzade, A., & Golchai, J. (2017). Partial-thickness burn wounds healing by topical treatment: a randomized controlled comparison between silver sulfadiazine and centiderm. *Medicine*, 96(9), 1-9.
- Sahin, S., Ozmen, I., Bastemur, G. Y., & Ozkorucuklu, S. P. (2019). Development of voltammetric Glucose-6-phosphate biosensors based on the immobilization of Glucose-6-phosphate dehydrogenase on Polypyrrole-and chitosan-coated Fe₃O₄ nanoparticles/polypyrrole nanocomposite films. *Applied Biochemistry and Biotechnology*, 188, 1145-1157.
- Salleh, S. F., Ajibola, O. O., Nolasco-Hipolito, C., Husaini, A., Zarrabal-Octavio, C., Lihan, S., & Saat, R. (2022). Fatty acid profile and antioxidant capacity of Dabai (*Canarium odontophyllum* L.): Effect of origin and fruit component. *Molecules*, 27(12), 1-16.
- Sambanthamurthi, R., Sundram, K., & Tan, Y. A. (2000). Chemistry and biochemistry of palm oil. *Progress in Lipid Research*, 39(6), 507-558.
- Sameh, N., Aly, U. F., Abou-Taleb, H. A., & Abdellatif, A. A. H. (2018). Prospective role of simvastatin on wound healing: review of the literature. *Journal of Bioequivalence & Bioavailability*, 10(2), 36-42.
- Samling, B. A., Assim, Z., Tong, W. Y., Leong, C. R., Ab Rashid, S., Kamal, N. N. S. N.

- M., & Tan, W. N. (2021). *Cynometra cauliflora* L.: An indigenous tropical fruit tree in Malaysia bearing essential oils and their biological activities. *Arabian Journal of Chemistry*, 14(9), 1-12.
- Sánchez, M., González-Burgos, E., Iglesias, I., & Gómez-Serranillos, M. P. (2020). Pharmacological update properties of *Aloe vera* and its major active constituents. *Molecules*, 25(6), 1-37.
- Schäfer, M., & Werner, S. (2007). Transcriptional control of wound repair. *Annual Review of Cell and Developmental Biology*, 23, 69-92.
- Scheffler, A. (2019). The wound healing properties of betulin from birch bark from bench to bedside. *Planta medica*, 85(07), 524-527.
- Schlechtriem, C. H., Focken, U., & Becker, K. (2003). Effect of different lipid extraction methods on $\delta^{13}\text{C}$ of lipid and lipid-free fractions of fish and different fish feeds. *Isotopes in Environmental and Health Studies*, 39(2), 135-140.
- Schönfeld, P., & Wojtczak, L. (2016). Short-and medium-chain fatty acids in energy metabolism: the cellular perspective. *Journal of Lipid Research*, 57(6), 943-954.
- Schwartz, M. A., Both, G., & Lechene, C. (1989). Effect of cell spreading on cytoplasmic pH in normal and transformed fibroblasts. *Proceedings of the National Academy of Sciences*, 86(12), 4525-4529.
- Scientific, T. (2020). *Thomas Scientific*. [online] Available at: Petroleum Ether, ACS Reagent: https://www.thomassci.com/Chemicals/Solvents-P/_/Petroleum-Ether-ACS-Reagent4?q=Petroleum%20Ether. [Accessed on 30 November 2020].

- Seal, T., Chaudhuri, K., Pillai, B., Chakrabarti, S., Mondal, T., & Auddy, B. (2020). Evaluation of antioxidant activities, toxicity studies and the DNA damage protective effect of various solvent extracts of *Litsea cubeba* fruits. *Heliyon*, 6(3), 1-9.
- Segeritz, C. P., & Vallier, L. (2017). Cell culture: Growing cells as model systems *in vitro*. In *Basic science methods for clinical researchers*, pp. 151-172. Academic Press.
- Seo, S. H., Lee, S. H., Cha, P. H., Kim, M. Y., Min, D. S., & Choi, K. Y. (2016). *Polygonum aviculare* L. and its active compounds, quercitrin hydrate, caffeic acid, and rutin, activate the Wnt/ β -catenin pathway and induce cutaneous wound healing. *Phytotherapy Research*, 30(5), 848-854.
- Shakeri, A., Khakdan, F., Soheili, V., Sahebkar, A., Rassam, G., & Asili, J. (2014). Chemical composition, antibacterial activity, and cytotoxicity of essential oil from *Nepeta ucrainica* L. spp. *kopetdagensis*. *Industrial Crops and Products*, 58, 315-321.
- Shale, T. L., Stirk, W. A., & van Staden, J. (1999). Screening of medicinal plants used in Lesotho for anti-bacterial and anti-inflammatory activity. *Journal of Ethnopharmacology*, 67(3), 347-354.
- Shedoeva, A., Leavesley, D., Upton, Z., & Fan, C. (2019). Wound healing and the use of medicinal plants. *Evidence-Based Complementary and Alternative Medicine*, 2019, 1–30.
- Shreelalitha, S. J., Supriya, P., Sridhar, K. R., & Nareshkumar, S. (2011). Fatty acid profile of ripened *Canavalia* split beans of the coastal sand dunes. *Sand dunes: Ecology, Geology and Conservation*, 43-67.

- Shiva, M.P., & Jantan, I. (1998). Non-Timber Forest Product from Dipterocarps. In: Appanah, S., Turnbull, M. J. (Eds.). A Review of Dipterocarps: Taxonomy, Ecology and silviculture. Bogor: Center for International Forestry Research.
- Signat, B., Roques, C., Poulet, P., & Duffaut, D. (2011). Role of *Fusobacterium nucleatum* in periodontal health and disease. *Current Issues in Molecular Biology*, 13(2), 25-36.
- Silva, J. R., Burger, B., Kühl, C., Candreva, T., Dos Anjos, M. B., & Rodrigues, H. G. (2018). Wound healing and omega-6 fatty acids: From inflammation to repair. *Mediators of Inflammation*, 2018, 1-17.
- Silva, M. P., Oliveira, G. L., De Carvalho, R. B., De Sousa, D. P., Freitas, R. M., Pinto, P. L., & de Moraes, J. (2014). Antischistosomal activity of the terpene nerolidol. *Molecules*, 19(3), 3793-3803.
- Singer, A. J., & Clark, R. A. (1999). Cutaneous wound healing. *New England Journal of Medicine*, 341(10), 738-746.
- Singh, M., McKenzie, K., & Ma, X. (2017). Effect of dimethyl sulfoxide on *in vitro* proliferation of skin fibroblast cells. *Journal of Biotech Research*, 8, 78-82.
- Siriwattanasatorn, M., Itharat, A., Thongdeeying, P., & Ooraikul, B. (2020). *In vitro* wound healing activities of three most commonly used thai medicinal plants and their three markers. *Evidence-Based Complementary and Alternative Medicine*, 2020, 1-11.
- Smith, A. N., Muffley, L. A., Bell, A. N., Numhom, S., & Hocking, A. M. (2012). Unsaturated fatty acids induce mesenchymal stem cells to increase secretion of angiogenic mediators. *Journal of Cellular Physiology*, 227(9), 3225-3233.
- Soneja, A., Drews, M., & Malinski, T. (2005). Role of nitric oxide, nitroxidative and oxidative stress in wound healing. *Pharmacological Reports*, 57, 108-119.

- Starlab. (2021). *Polystyrene Microplates*. [online] Available at: <https://www.starlabgroup.com/GB-en/product/polystyrene-microplates-pf-sl-155275.html>. [Accessed on 30 January 2023].
- Stock, J. B., & Baker, M. D. (2009). Chemotaxis. In *Encyclopedia of Microbiology, Third Edition*, pp. 71-78. Elsevier.
- Sule, A., Ahmed, Q. U., Samah, O. A., & Omar, M. N. (2010). Screening for antibacterial activity of *Andrographis paniculata* used in Malaysian folkloric medicine: a possible alternative for the treatment of skin infections. *Ethnobotanical Leaflets*, 2010(4), 445-456.
- Sundar, S., & Koilpillai, Y. J. (2015). Antimicrobial potential of petroleum ether extract and active column fractions of the *Solanum incanum* leaves. *Journal of Pure and Applied Microbiology*, 9(4), 2949-2954.
- Tan, N. A. H., Siddique, B. M., Muhamad, I. I., & Kok, F. S. (2014). The effect of solvents on the soxhlet extraction of omega 3 from perah oil. *Jurnal Teknologi*, 67(4), 51-54.
- Tankeshwar, A. (2022). *Modified Kirby-Bauer Disc Diffusion Method*. [online] Available at: <https://microbeonline.com/antimicrobial-susceptibility-testing-procedure-modified-kirby-bauer-method/>. [Accessed on 30 January 2023].
- Tedelind, S., Westberg, F., Kjerrulf, M., & Vidal, A. (2007). Anti-inflammatory properties of the short-chain fatty acids acetate and propionate: a study with relevance to inflammatory bowel disease. *World Journal of Gastroenterology*, 13(20), 2826.
- Tendencia, E. A. (2004). Disk diffusion method. In *Laboratory manual of standardized methods for antimicrobial sensitivity tests for bacteria isolated from aquatic animals and environment*, pp. 13-29. Tigbauan, Iloilo, Philippines: Aquaculture Department,

Southeast Asian Fisheries Development Center.

Thangapazham, R. L., Sharad, S., & Maheshwari, R. K. (2016). Phytochemicals in wound healing. *Advances in Wound Care*, 5(5), 230-241.

ThermoFisher Scientific. (2021). *In vitro* cell migration and invasion assays using Nunc Polycarbonate Cell Culture Inserts. [online] Available at: <https://assets.thermofisher.com/TFS-Assets/BID/Application-Notes/cell-migration-invasion-assays-cell-culture-inserts-app-note.pdf>. [Accessed on 12 September 2022].

Timm, M., Saaby, L., Moesby, L., & Hansen, E. W. (2013). Considerations regarding use of solvents in in vitro cell based assays. *Cytotechnology*, 65, 887-894.

Tipple, A. (2022). *What are the different types of wounds?* [online] Available at: <https://lfafirstresponse.com.au/blog/what-are-the-different-types-of-wounds/> [Accessed on 6 June 2023].

Toyat, A. J., Abdullah, N. A. P., Go, R., Abdullah, T. L., Saleh, G., Jiwan, M., & Magiman, M. M. (2015). Conservation strategies for jerangau merah (*Boesenbergia stenophylla*) using DNA profiling and micropropagation. *International Journal of Agriculture, Forestry and Plantation*, 1, 89-101.

Tracy, L. E., Minasian, R. A., & Caterson, E. J. (2016). Extracellular matrix and dermal fibroblast function in the healing wound. *Advances in Wound Care*, 5(3), 119-136.

Tschumperlin, D. J. (2013). Fibroblasts and the ground they walk on. *Physiology*, 28(6), 380-390.

Tungmunthum, D., Thongboonyou, A., Pholboon, A., & Yangsabai, A. (2018). Flavonoids and other phenolic compounds from medicinal plants for pharmaceutical and medical

- aspects: An overview. *Medicines*, 5(3), 93.
- Upadhyay, N. K., Kumar, R., Siddiqui, M. S., & Gupta, A. (2009). Mechanism of wound-healing activity of *Hippophae rhamnoides* L. leaf extract in experimental burns. *Evidence-Based Complementary and Alternative Medicine*, 2011, 1-9.
- Vaidyanathan, L. (2021). Growth Factors in Wound Healing—A Review. *Biomedical and Pharmacology Journal*, 14, 1469-1481.
- Vaitkevičienė, N., Sapronaitė, A., & Kulaitienė, J. (2022). Evaluation of proximate composition, mineral elements and bioactive compounds in skin and flesh of beetroot grown in Lithuania. *Agriculture*, 12(11), 1-12.
- Vallirie, E. J. (2018). Antimicrobial activity of red jerangau (*Boesenbergia stenophylla*) extract against *Staphylococcus aureus*, *Salmonella typhimurium*, and *Listeria monocytogenes*. In *Final Year Project's Thesis, Universiti Malaysia Sarawak*, (pp. 19-24).
- Vasconcelos-Silva, A. A., de Lima, F. J. B., de Brito, T. S., Lahlou, S., & Magalhães, P. J. C. (2014). Vasorelaxation induced by methyl cinnamate, the major constituent of the essential oil of *Ocimum micranthum*, in rat isolated aorta. *Clinical and Experimental Pharmacology and Physiology*, 41(10), 755-762.
- van Meerloo, J., Kaspers, G. J., & Cloos, J. (2011). Cell sensitivity assays: the MTT assay. *Cancer Cell Culture: Methods and Protocols*, 237-245.
- Vejayan, J., Selladuri, N. E., Ibrahim, H., Shuib, A. S., & Yusoff, M. M. (2017). Biological activities of essential oils hydrodistilled from two closely related ginger species: *Alpinia malaccensis* var. *nobilis* and *Alpinia latilabris* leaves. *Journal of Essential Oil Bearing Plants*, 20(4), 959-971.

- Vidal, S. (2010). *The Plant List*. [online] Available at: <http://www.theplantlist.org/browse/A/Lauraceae/Litsea/> [Accessed on 14 May 2020].
- Voegeli, D. (2012). Understanding the main principles of skin care in older adults. *Nursing Standard*, 27(11), 59-68.
- Voon, B. H. & Kueh, H. S. (1999). The nutritional value of indigenous fruits and vegetables in Sarawak. *Asia Pacific Journal of Clinical Nutrition*, 8(1), 24-31.
- Wang, Y. S., Wen, Z. Q., Li, B. T., Zhang, H. B., & Yang, J. H. (2016). Ethnobotany, phytochemistry, and pharmacology of the genus *Litsea*: An update. *Journal of Ethnopharmacology*, 181, 66-107.
- White, B. (2009). Dietary fatty acids. *American Family Physician*, 80(4), 345-350.
- White, L. A., Mitchell, T. I., & Brinckerhoff, C. E. (2000). Transforming growth factor β inhibitory element in the rabbit matrix metalloproteinase-1 (collagenase-1) gene functions as a repressor of constitutive transcription. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression*, 1490(3), 259-268.
- Wilkinson, H. N., & Hardman, M. J. (2020). Wound healing: Cellular mechanisms and pathological outcomes. *Open Biology*, 10(9), 1-14.
- Witte, M. B., & Barbul, A. (1997). General principles of wound healing. *Surgical Clinics of North America*, 77(3), 509-528.
- Wulandari, I., Kusuma, I. W., & Kuspradini, H. (2018). Antioxidant and antibacterial activity of *Litsea garciae*. In *IOP Conference Series: Earth and Environmental Science*, (Vol. 144, No. 1, pp. 1-7).

- Yang, H. T., Chen, J. W., Rathod, J., Jiang, Y. Z., Tsai, P. J., Hung, Y. P., & Huang, I. H. (2018). Lauric acid is an inhibitor of *Clostridium difficile* growth in vitro and reduces inflammation in a mouse infection model. *Frontiers in Microbiology*, 8, 1-16.
- Yang, M., Zhou, M., & Song, L. (2020). A review of fatty acids influencing skin condition. *Journal of Cosmetic Dermatology*, 19(12), 3199-3204.
- Yanty, N. A. M., Marikkar, J. M. N., & Long, K. (2011). Effect of varietal differences on composition and thermal characteristics of avocado oil. *Journal of the American Oil Chemists' Society*, 88, 1997-2003.
- Yoon, B. K., Jackman, J. A., Valle-González, E. R., & Cho, N. J. (2018). Antibacterial free fatty acids and monoglycerides: biological activities, experimental testing, and therapeutic applications. *International Journal of Molecular Sciences*, 19(4), 1-40.
- Zhang, C., Barrios, M. P., Alani, R. M., Cabodi, M., & Wong, J. Y. (2016). A microfluidic transwell to study chemotaxis. *Experimental Cell Research*, 342(2), 159-165.
- Zhang, X. N., Ma, Z. J., Wang, Y., Li, Y. Z., Sun, B., Guo, X., Pan, C., & Chen, L. M. (2016). The four-herb Chinese medicine formula Tuo-Li-Xiao-Du-San accelerates cutaneous wound healing in streptozotocin-induced diabetic rats through reducing inflammation and increasing angiogenesis. *Journal of Diabetes Research*, 2016, 1-11.
- Zhang, X. N., Ma, Z. J., Wang, Y., Sun, B., Guo, X., Pan, C. Q., & Chen, L. M. (2017). *Angelica dahurica* ethanolic extract improves impaired wound healing by activating angiogenesis in diabetes. *PLoS One*, 12(5), 1-18.
- Zhao, B., Zhang, X., Han, W., Cheng, J., & Qin, Y. (2017). Wound healing effect of an *Astragalus membranaceus* polysaccharide and its mechanism. *Molecular Medicine*

Reports, 15(6), 4077-4083.

Zhao, H., Mortezaei, R., Wang, Y., Sheng, X., Aria, F., & Bojanowski, K. (2006). SBD. 4 stimulates regenerative processes in vitro, and wound healing in genetically diabetic mice and in human skin/severe-combined immunodeficiency mouse chimera. *Wound Repair and Regeneration*, 14(5), 593-601.

Zhao, Y., Wang, Q., Yan, S., Zhou, J., Huang, L., Zhu, H., Chen, L., & Zheng, T. (2021). *Bletilla striata* polysaccharide promotes diabetic wound healing through inhibition of the NLRP3 inflammasome. *Frontiers in Pharmacology*, 12, 1-14.

Zheng, C. J., Yoo, J. S., Lee, T. G., Cho, H. Y., Kim, Y. H., & Kim, W. G. (2005). Fatty acid synthesis is a target for antibacterial activity of unsaturated fatty acids. *FEBS Letters*, 579(23), 5157-5162.

APPENDICES

Appendix A: Journal Publications

1. **Amit, Z., & Zinyin, L.** (2021). A mini review on the nutritional compositions and pharmacological properties of *Litsea garciae*. *Malaysian Applied Biology*, 50(1), 29-39.

Malays. Appl. Biol. (2021) 50(1): 29–39

A MINI REVIEW ON THE NUTRITIONAL COMPOSITIONS AND PHARMACOLOGICAL PROPERTIES OF *Litsea garciae*

ZUNIKA AMIT* and LING ZINYIN

*Department of Basic Medical Sciences, Faculty of Medicine and Health Sciences,
Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia*

*E-mail: zunika@unimas.my

Accepted 8 May 2021, Published online 30 June 2021

ABSTRACT

Litsea garciae is an underutilized plant found in certain parts of South East Asia. The plant part has been traditionally used to treat, among others, skin infections, boil, rectal bleeding, muscular pain, and sprains. Besides its medicinal properties, its seasonal fruit is consumed for its avocado-like flavor. This article aims to provide information on what is known so far about the nutritional composition and pharmacological properties of *Litsea garciae*.

Key words: Antioxidant, *Litsea garciae*, pharmacological, phytochemical, underutilized fruit

2. **Ling, Z.,** Gabriele, R. A. F., & Zunika, A. (2022). Fatty acids composition and antimicrobial activities of *Litsea garciae* pulp and seed extracts. *Medicinal Plants-International Journal of Phytomedicines and Related Industries*, 14(2), 301-311.

Medicinal Plants
Vol. 14 (2), June 2022, 301-311

doi : 10.5958/0975-6892.2022.00035.1

IndianJournals.com
A product of Diva Enterprises Pvt. Ltd.



Research Article

Fatty acids composition and antimicrobial activities of *Litsea garciae* pulp and seed extracts

Zinyin Ling, Gabriele Ruth Anisah Frömming and Zunika Amit*

Department of Basic Medical Sciences, Faculty of Medicine and Health Sciences, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia

Received: April 13, 2022; Accepted: May 07, 2022

ABSTRACT

Litsea garciae is a native plant to Borneo Island. The current study aimed to identify and compare the fatty acids composition and antimicrobial activities of the pulp and seed extracts of *L. garciae*. The total lipids of *L. garciae* pulp and seed were extracted with petroleum ether (PE) and Bligh-Dyer (BD) methods and the fatty acids were analysed using gas chromatography. The fatty acids of seed extracts consisted of more than 80% saturated fatty acids whereas the pulp extracts contained a similar percentage of saturated and unsaturated fatty acids (approximately 50%). The predominant saturated fatty acids were palmitic acid followed by stearic acid for both PE and BD lipid pulp extracts. In contrast, the PE and BD lipid seed extracts had a high content of lauric acid followed by palmitic acid. The predominant monounsaturated fatty acid was oleic acid while polyunsaturated fatty acid was linoleic acid for all extracts. As for the antimicrobial activities, PE lipid pulp extract had higher antimicrobial activity against *S. aureus*, *P. aeruginosa*, and *S. epidermidis* than other extracts in both antimicrobial assays. This study showed that the PE and BD lipid pulp and seed extracts had similar major components of fatty acids but with different proportions. In addition, the components of fatty acids might contribute to the antibacterial activities of *L. garciae*.

Keywords: *Litsea*, lipids, GC-MS, petroleum ether, Bligh and Dyer, disk diffusion assay, broth microdilution assay

Appendix B: Conference

1. The Regional Conference and Exhibition of Natural Products, Traditional Medicines and Herbs on 9-11 December 2021 in Borneo Convention Centre Kuching (BCKK).

Oral Presentation

FATTY ACID COMPOSITION AND ANTIMICROBIAL ACTIVITY OF *Litsea garciae* PULP AND SEED EXTRACTS

Ling Zinyin, Gabriele Ruth Anisah Frömming And Zunika Amit*

Department of Basic Medical Sciences, Faculty of Medicine and Health Sciences, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia.

*Corresponding author's: Fax: +6082665152; Tel: +608258100 ext 7455; E-mail: zunika@unimas.my

ABSTRACT

Litsea garciae is a native plant to Borneo Island. The current study identified and compared the fatty acids composition and antimicrobial activities of the pulp and seed extracts of *L. garciae*. The total lipids of *L. garciae* pulp and seed were extracted with petroleum ether (PE) and Bligh-Dyer (BD) methods and the fatty acids were analysed using gas chromatography. The antimicrobial activities of the total lipids were determined using disk diffusion and broth microdilution assays. The predominant saturated fatty acids were palmitic acid followed by stearic acid for both PE and BD lipid pulp extracts. In contrast, the PE and BD lipid seed extracts had a high content of lauric acid followed by palmitic acid. The predominant monounsaturated fatty acid was oleic acid while polyunsaturated fatty acid was linoleic acid for all extracts. As for the antimicrobial activities, PE lipid pulp extract had higher antimicrobial activity against *S. aureus*, *P. aeruginosa* and *S. epidermidis* than other extracts in both antimicrobial assays. This study shows that the PE and BD lipid pulp and seed extracts had a similar major components of fatty acids but with different proportions. In addition, the components of fatty acids might contribute to the antibacterial activity of *L. garciae*.

Keywords: *Litsea garciae*, fatty acid, antimicrobial activity, gas chromatography.

Appendix C: Images of wound closure in the wound scratch assay

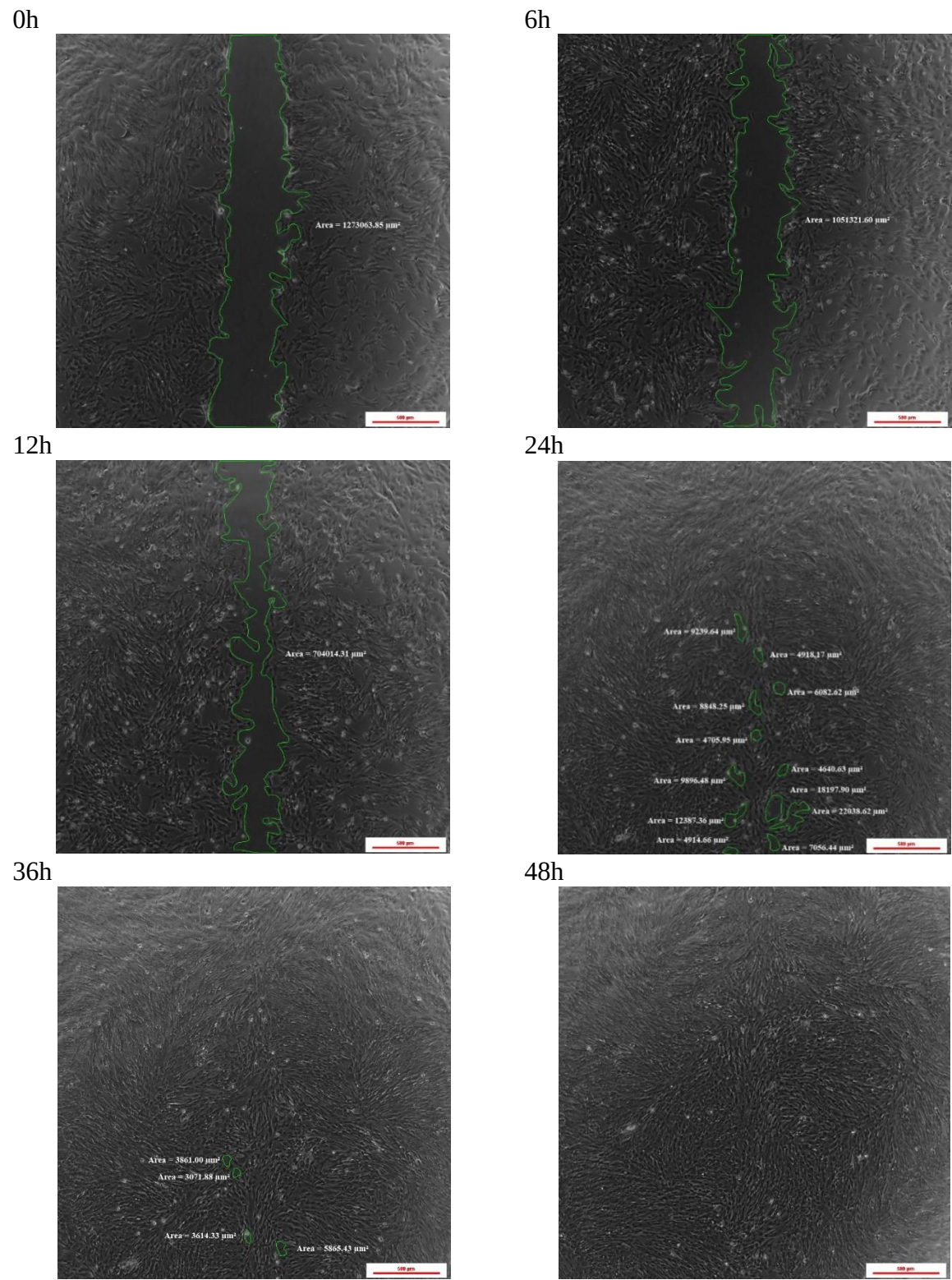
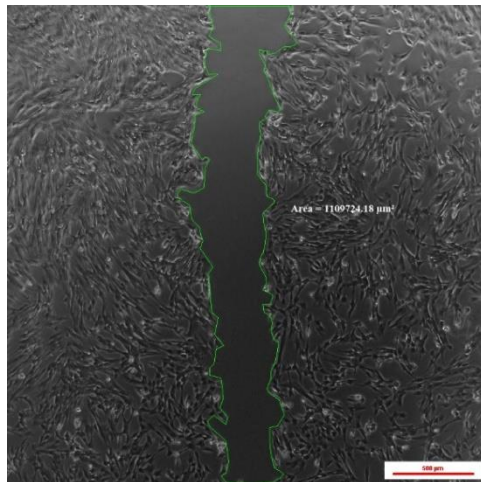
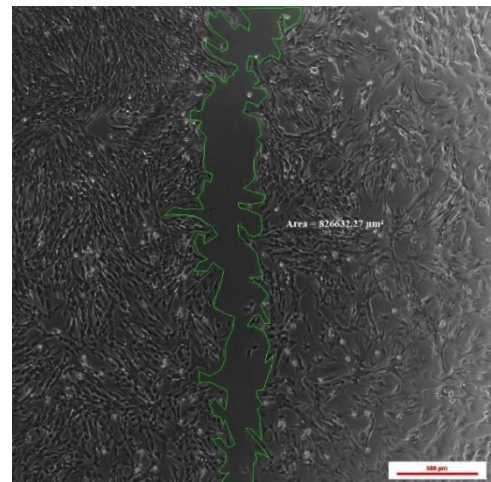


Figure 1: Migration of fibroblasts after treatment with 31.25 $\mu\text{g/mL}$ Bligh and Dyer pulp extract for 48h.

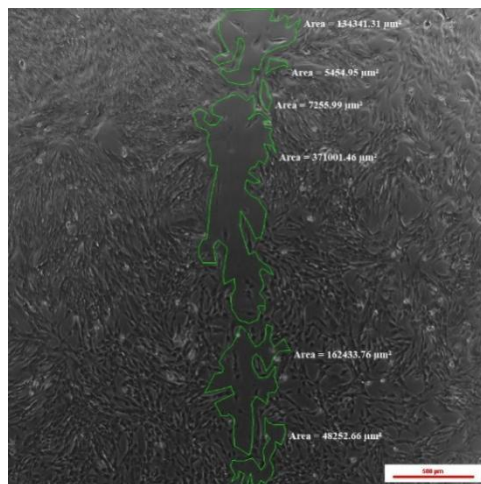
0h



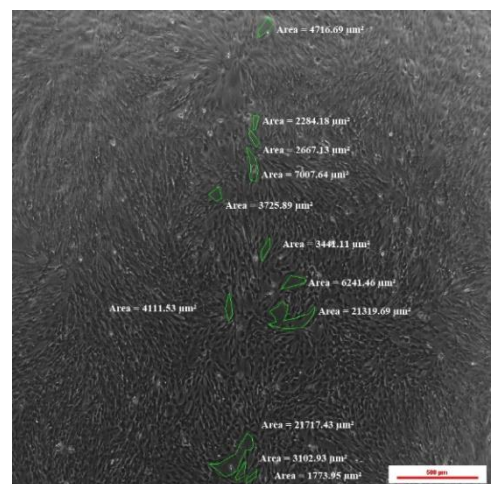
6h



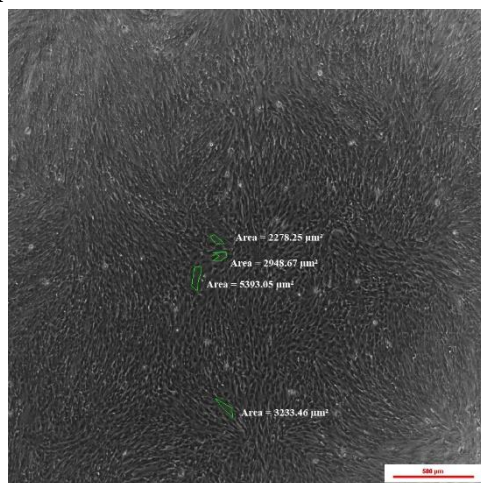
12h



24h



36h



48h

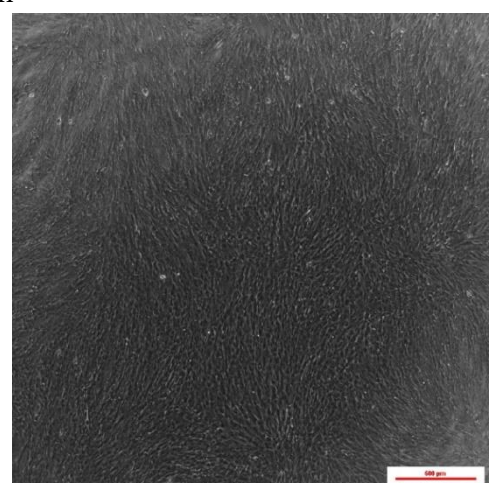
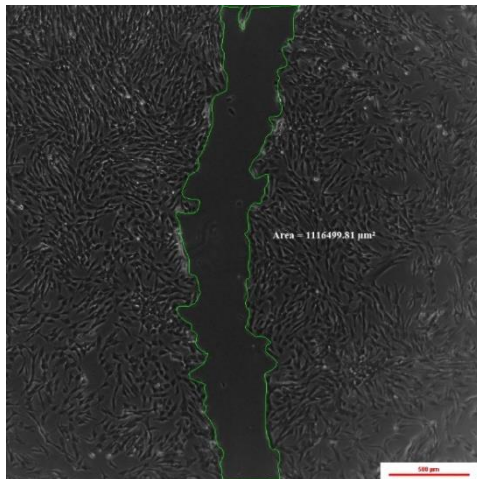
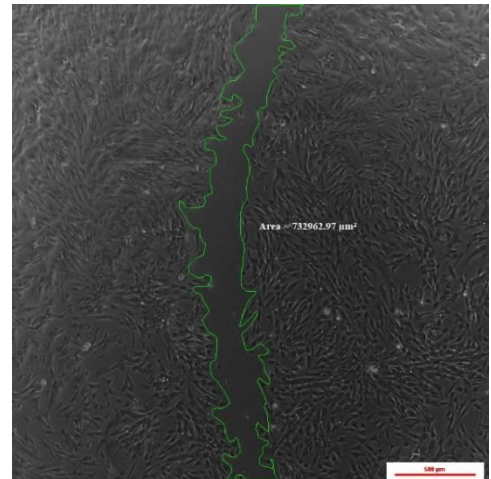


Figure 2: Migration of fibroblasts after treatment with 31.25 $\mu\text{g/mL}$ methanolic pulp extract for 48h.

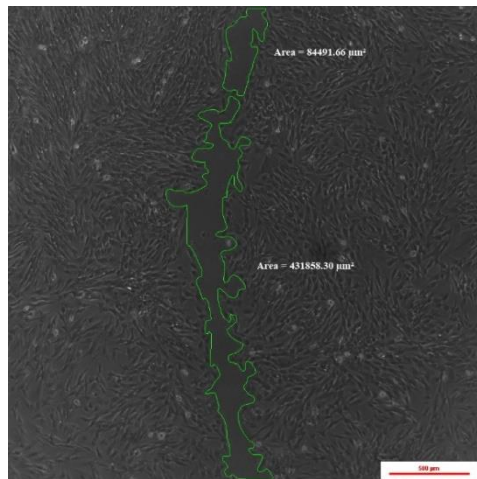
0h



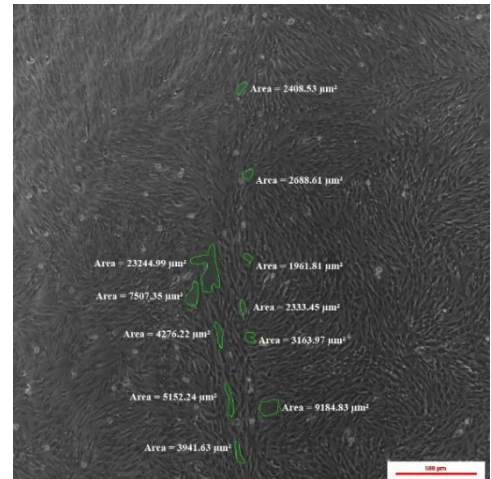
6h



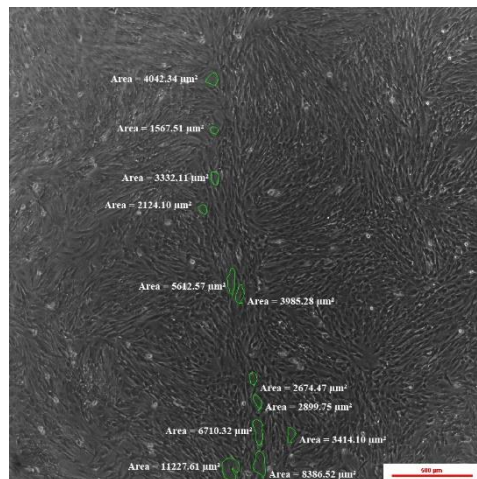
12h



24h



36h



48h

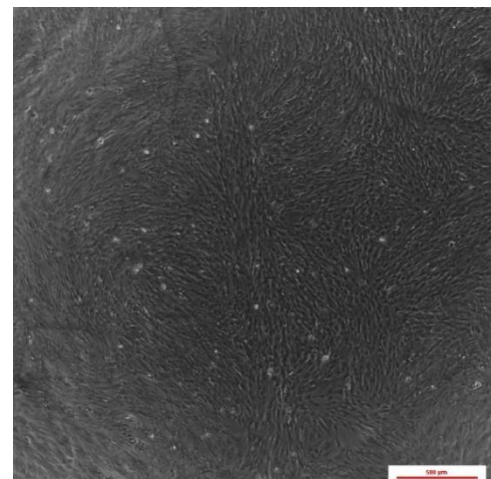
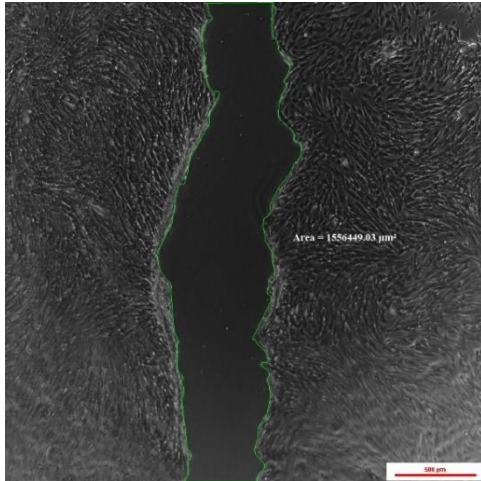
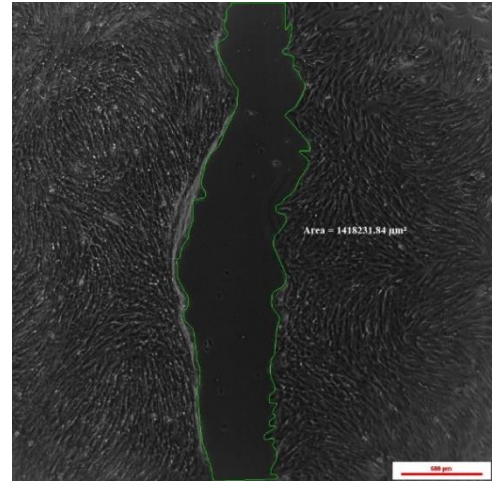


Figure 3: Migration of fibroblasts after treatment with 31.25 $\mu\text{g/mL}$ petroleum ether seed extract for 48h.

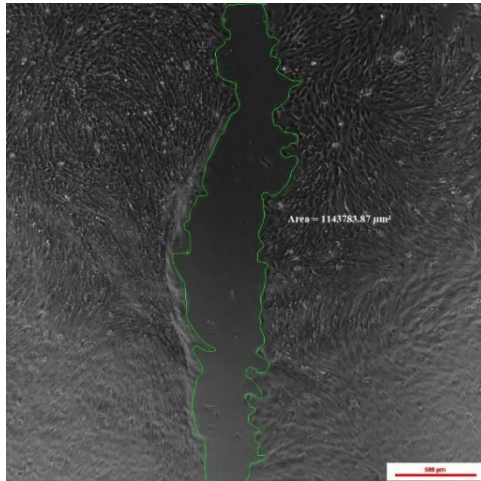
0h



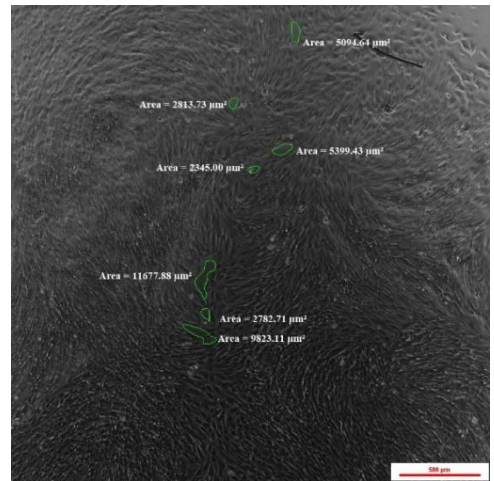
6h



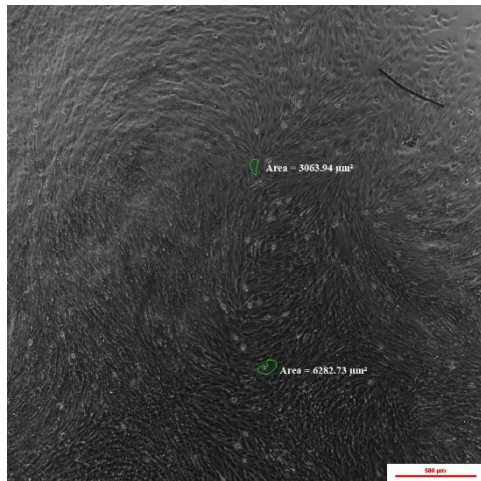
12h



24h



36h



48h

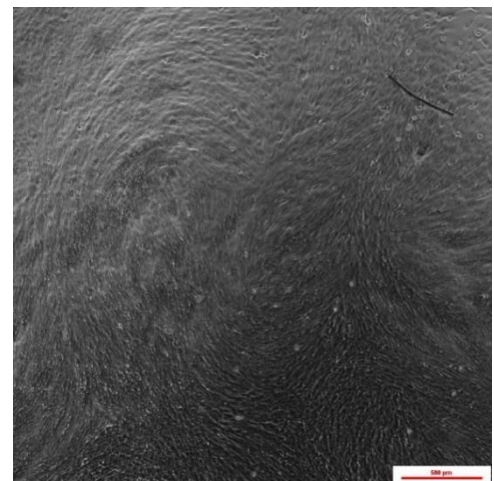
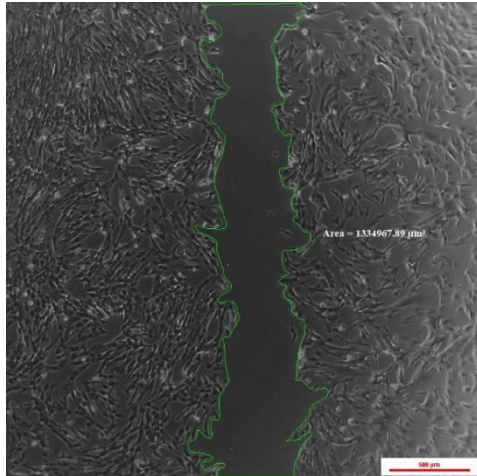
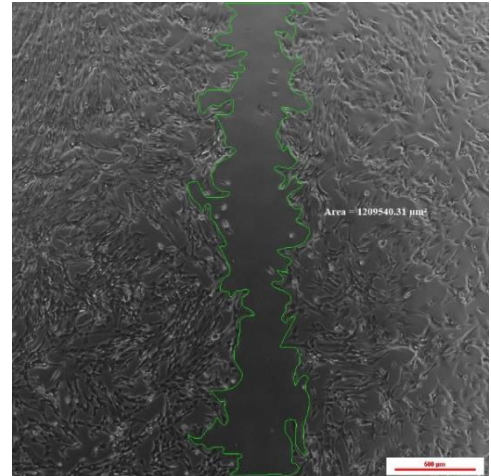


Figure 4: Migration of fibroblasts after treatment with 31.25 $\mu\text{g/mL}$ Bligh and Dyer seed extract for 48h.

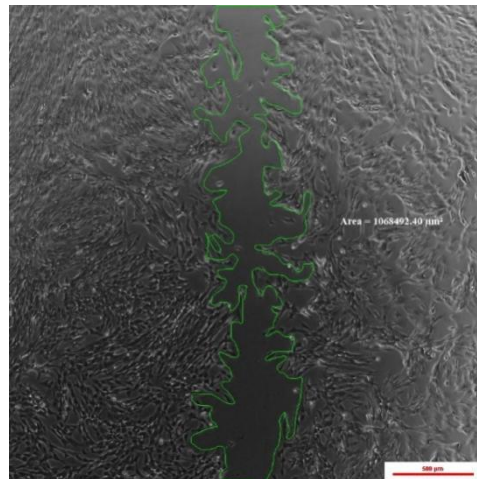
0h



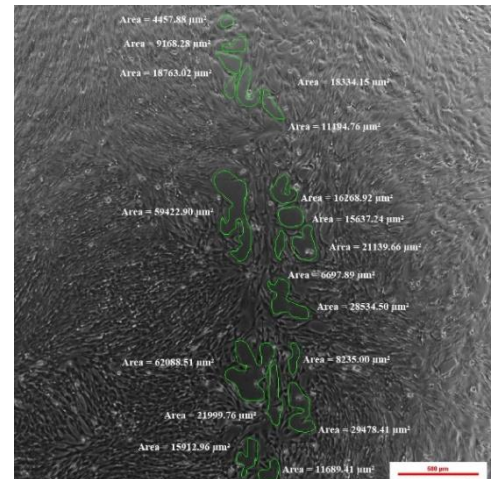
6h



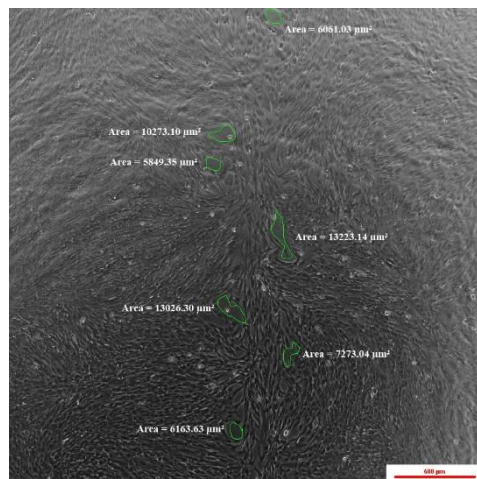
12h



24h



36h



48h

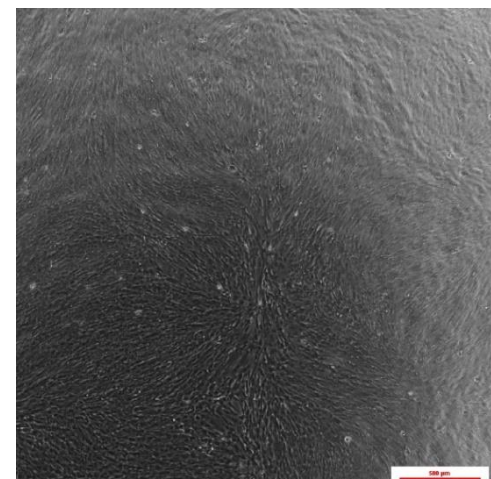
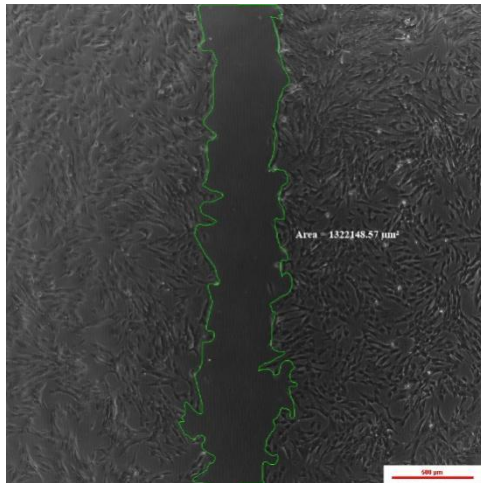
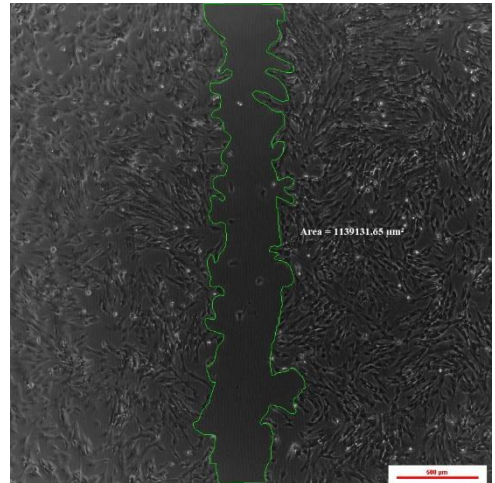


Figure 5: Migration of fibroblasts after treatment with 31.25 $\mu\text{g/mL}$ methanolic seed extract for 48h.

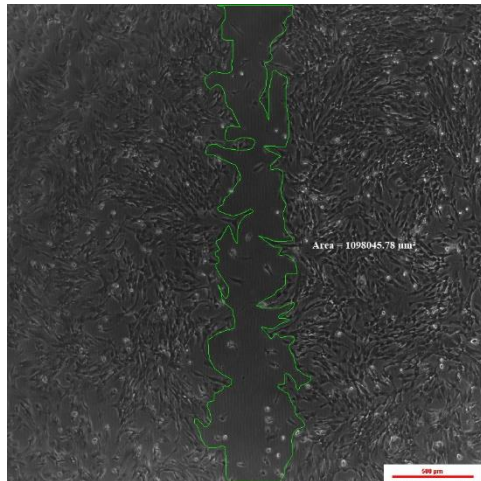
0h



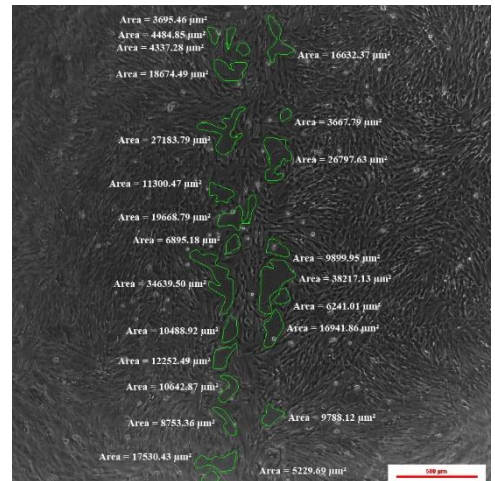
6h



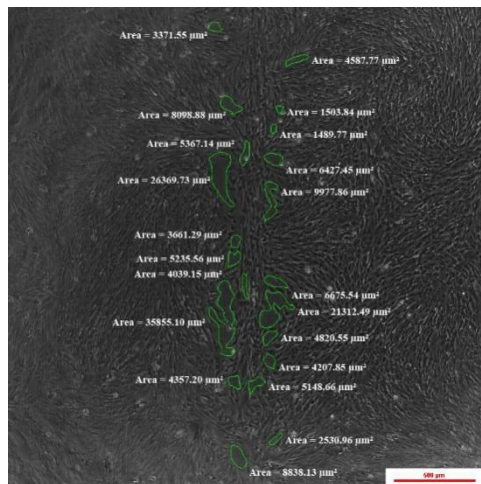
12h



24h



36h



48h

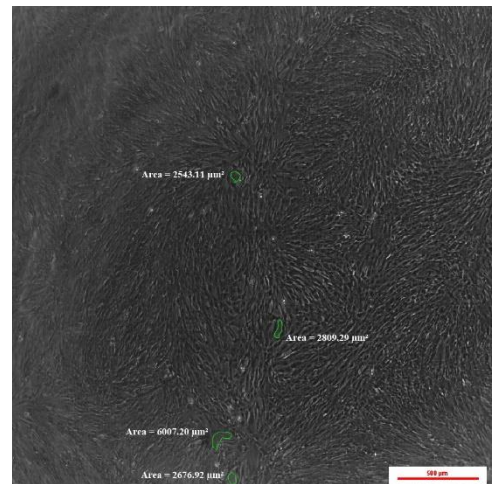
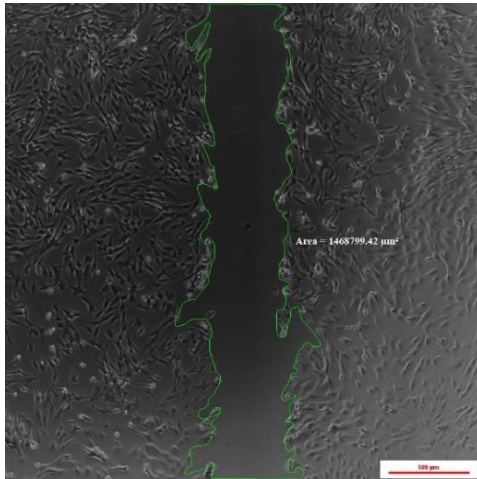
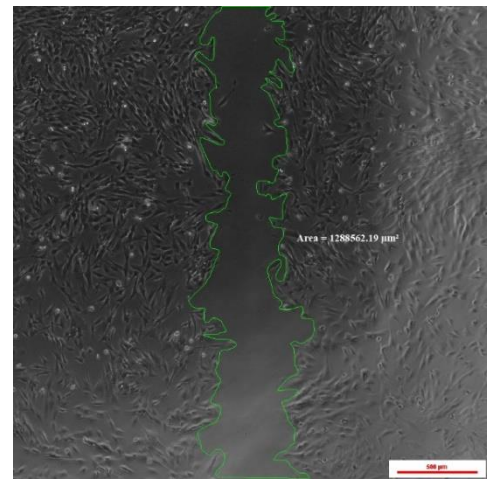


Figure 6: Migration of fibroblasts after treatment with 125 $\mu\text{g/mL}$ allantoin for 48h.

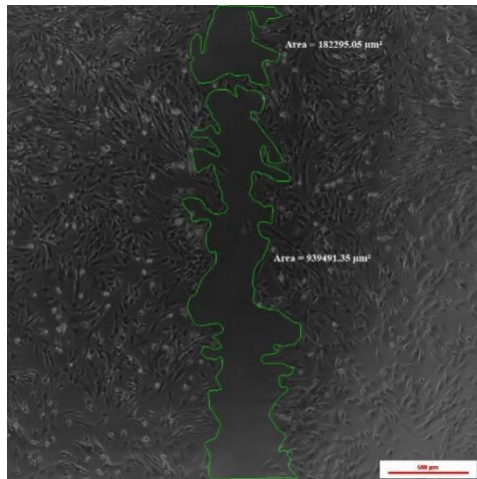
0h



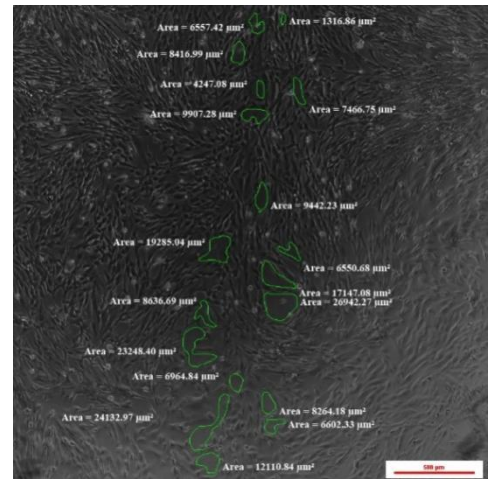
6h



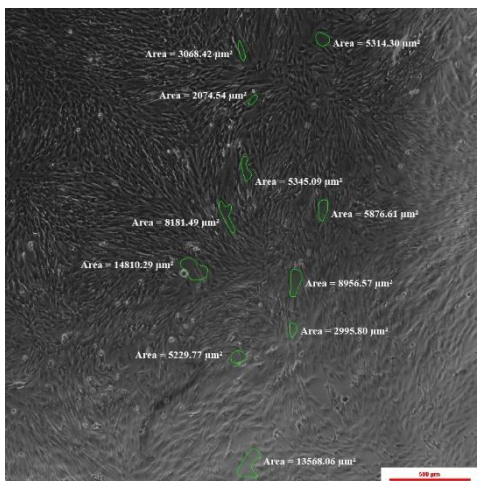
12h



24h



36h



48h

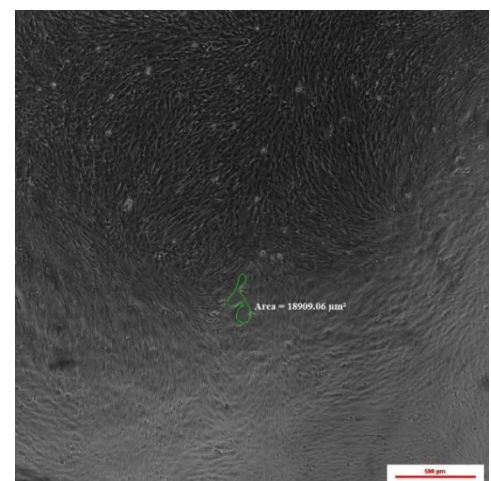
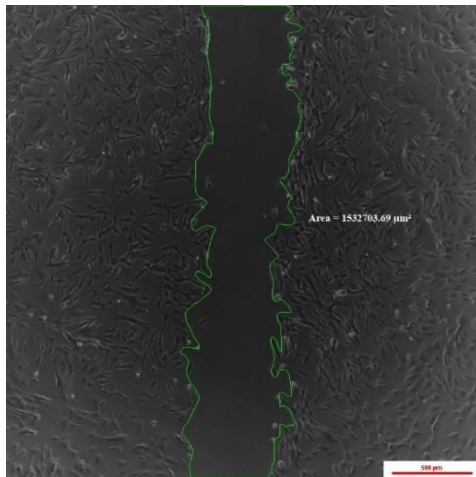
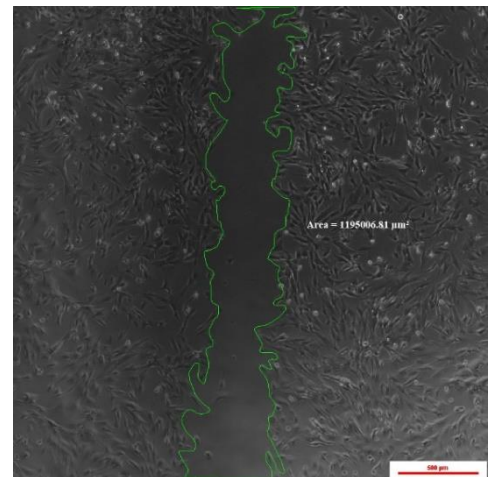


Figure 7: Migration of fibroblasts after treatment with 7.5 $\mu\text{g/mL}$ petroleum ether pulp extract for 48h.

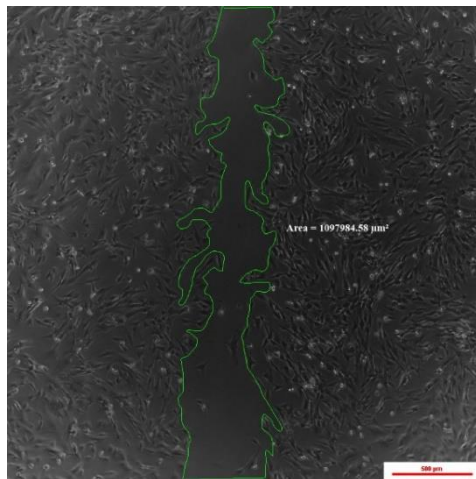
0h



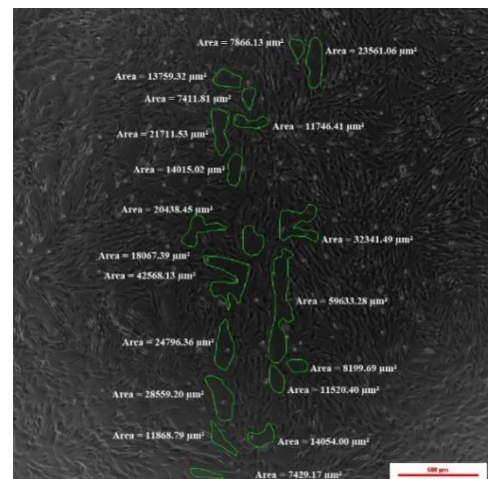
6h



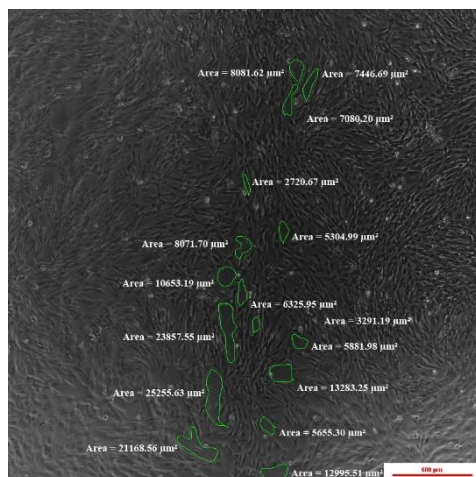
12h



24h



36h



48h

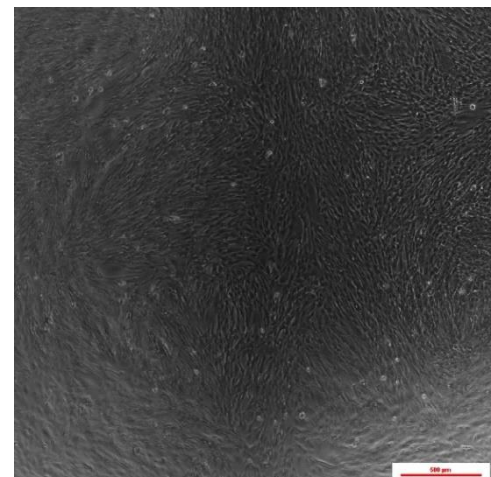
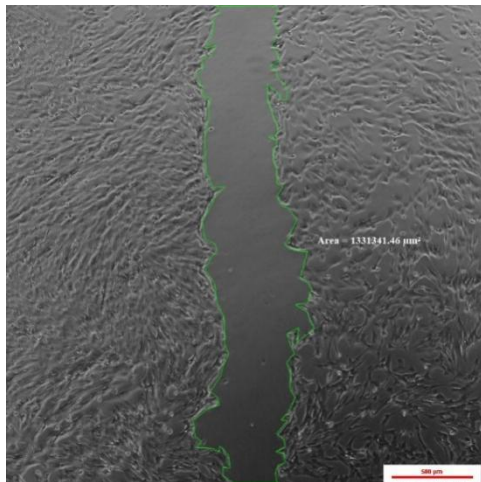
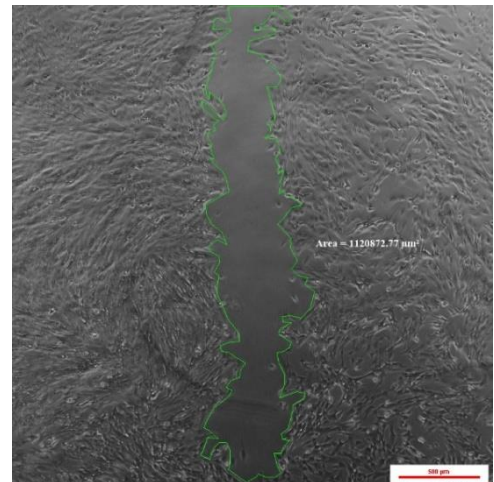


Figure 8: Migration of fibroblasts after treatment with 15 $\mu\text{g/mL}$ petroleum ether pulp extract for 48h.

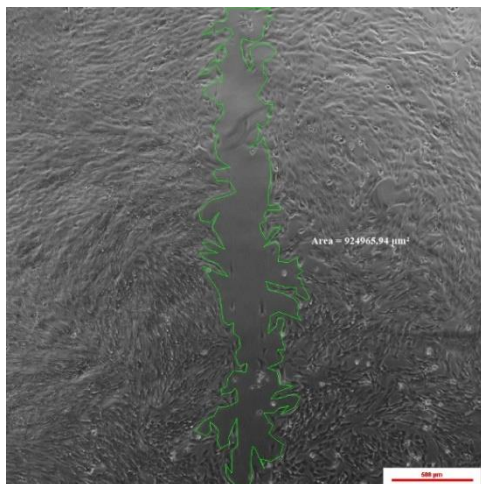
0h



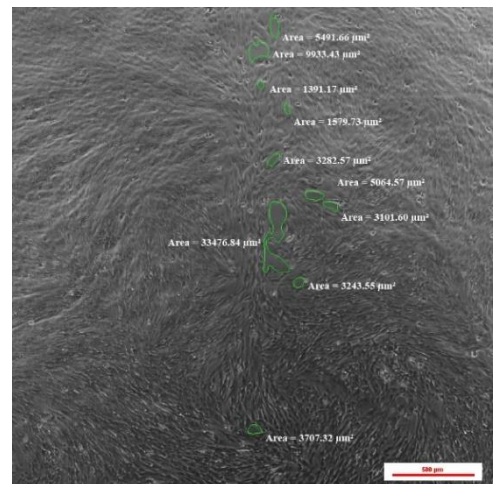
6h



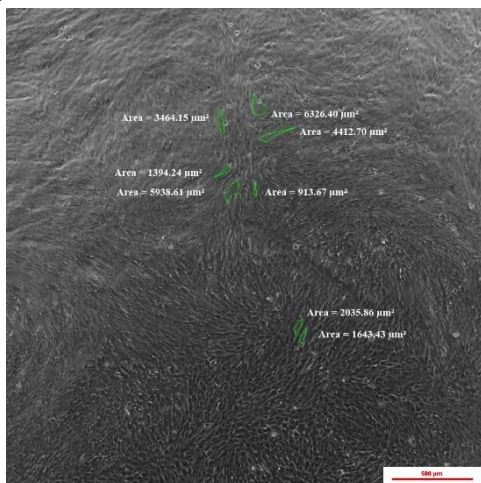
12h



24h



36h



48h

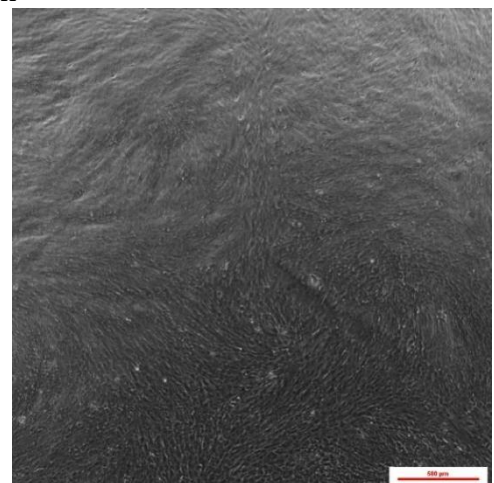
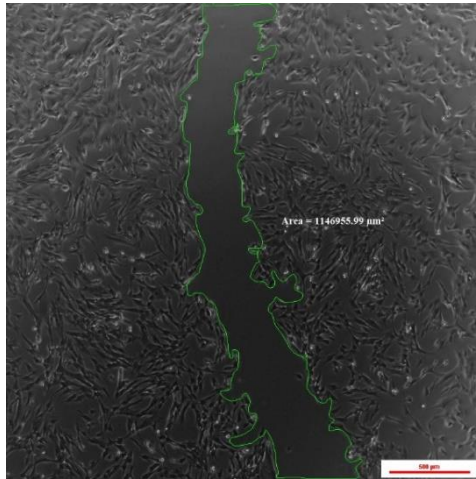
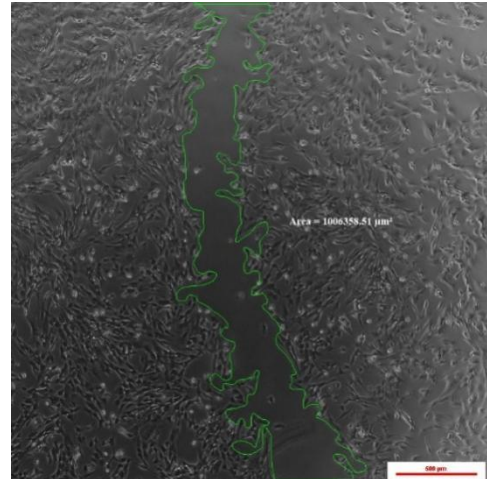


Figure 9: Migration of fibroblasts after treatment with 62.5 $\mu\text{g/mL}$ methanolic pulp extract for 48h.

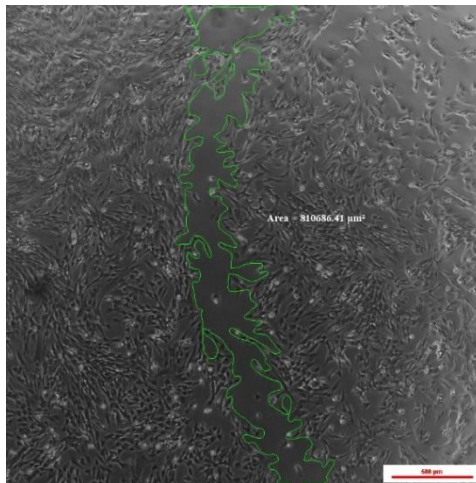
0h



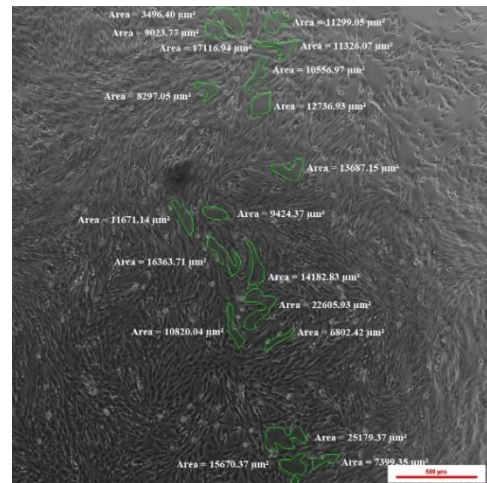
6h



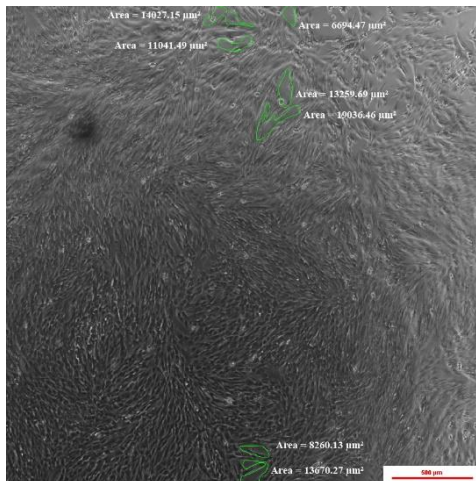
12h



24h



36h



48h

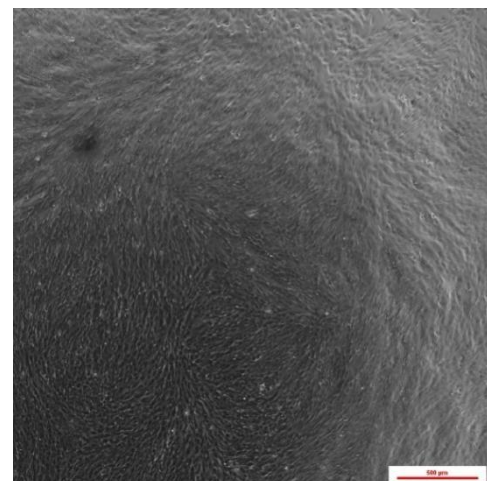
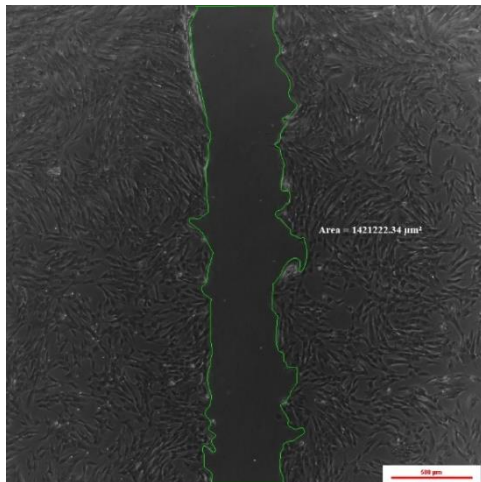
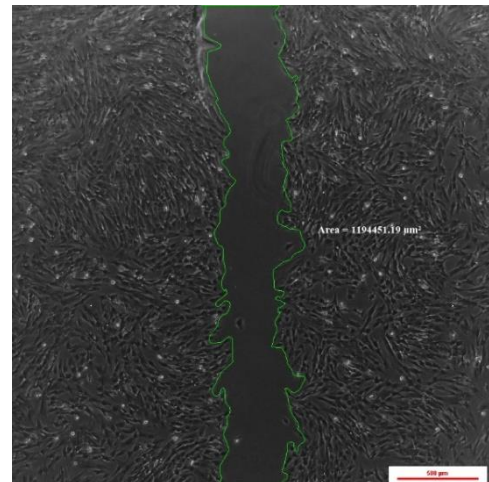


Figure 10: Migration of fibroblasts after treatment with 125 $\mu\text{g/mL}$ methanolic pulp extract for 48h.

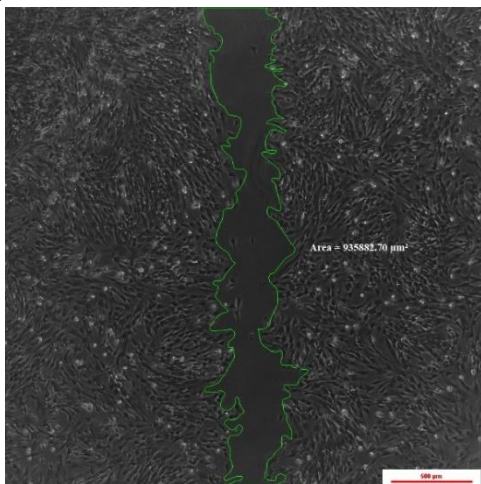
0h



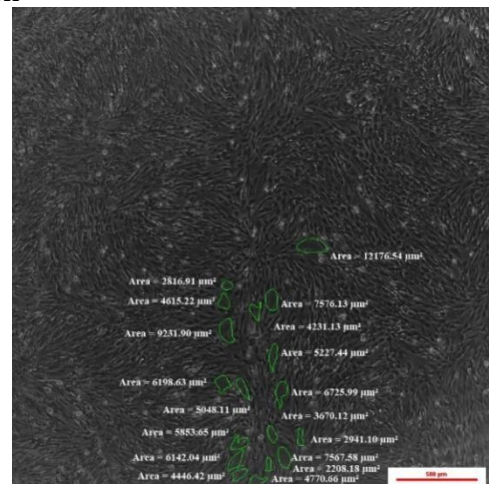
6h



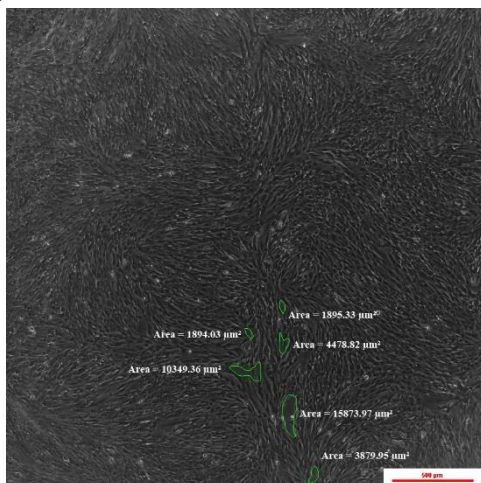
12h



24h



36h

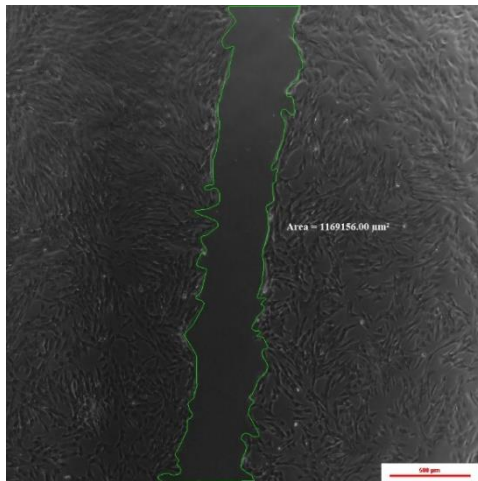


48h

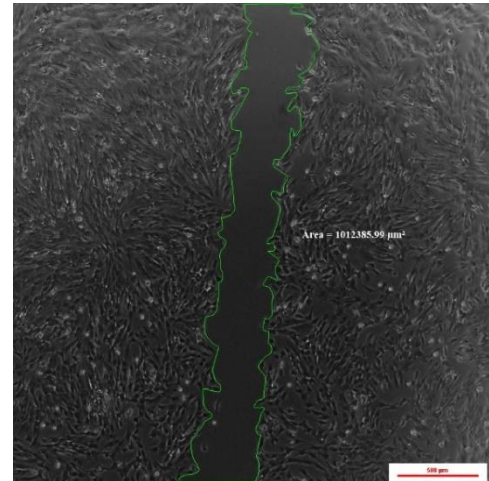


Figure 11: Migration of fibroblasts after treatment with 62.5 $\mu\text{g/mL}$ Bligh and Dyer pulp extract for 48h.

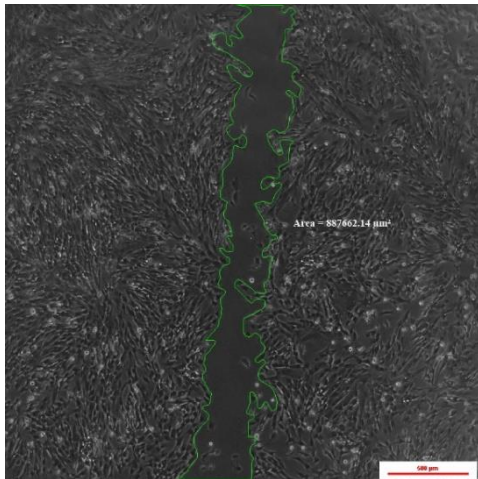
0h



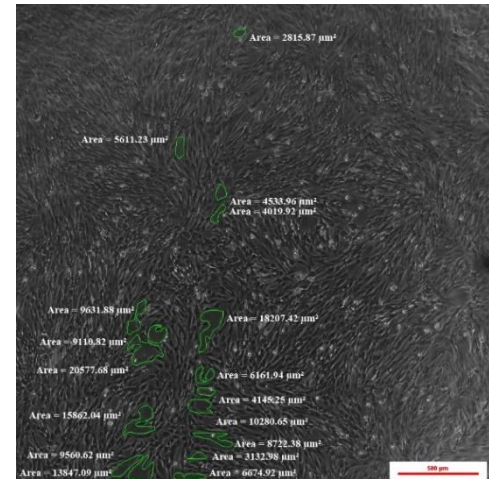
6h



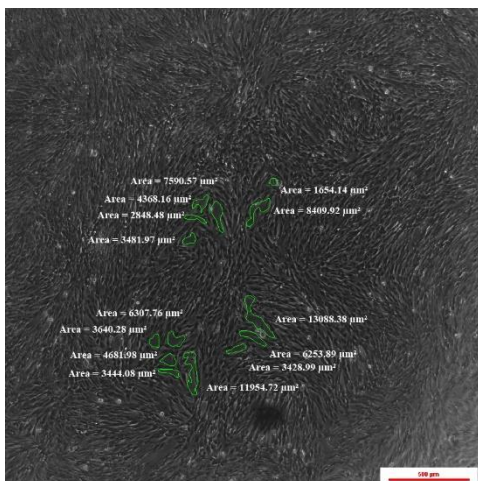
12h



24h



36h



48h

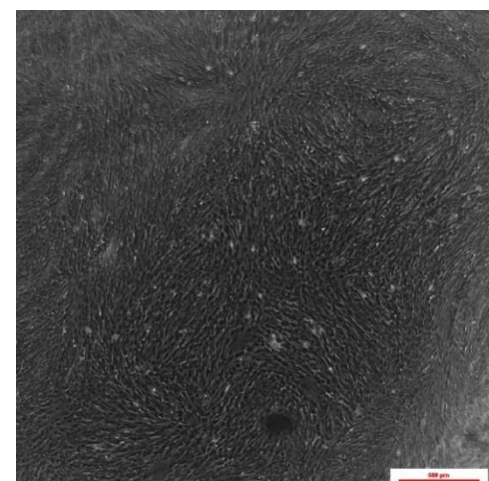
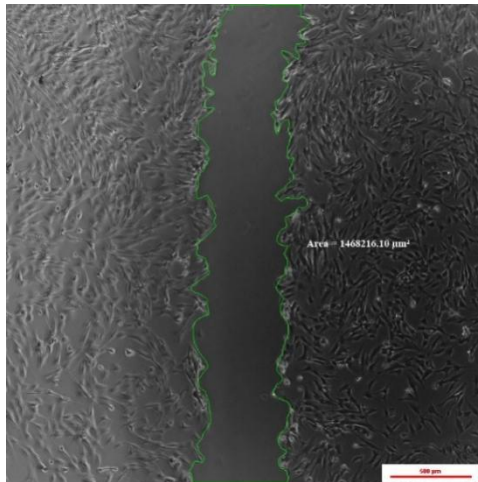
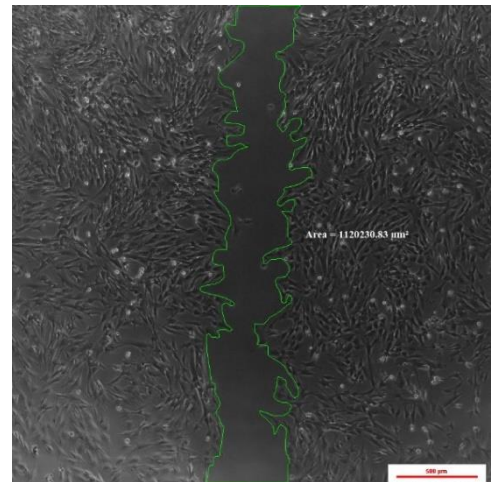


Figure 12: Migration of fibroblasts after treatment with 125 $\mu\text{g}/\text{mL}$ Bligh and Dyer pulp extract for 48h.

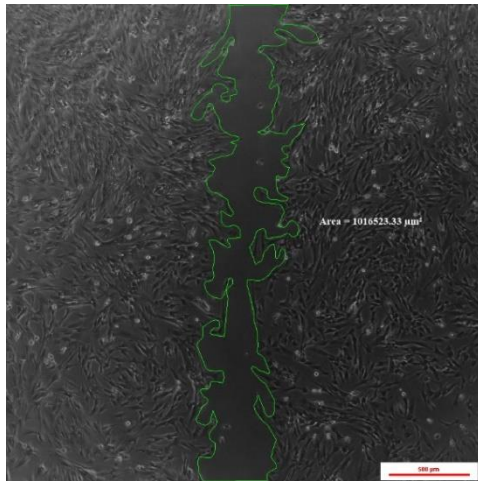
0h



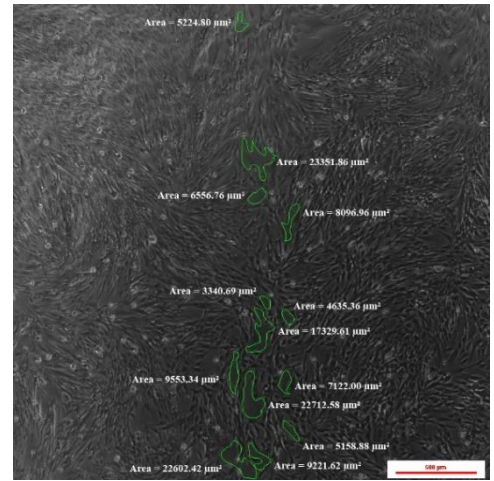
6h



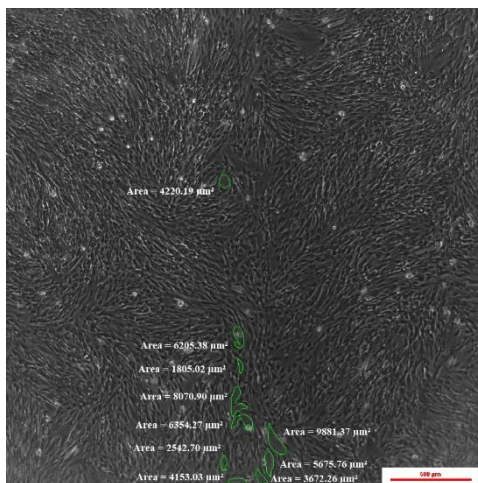
12h



24h



36h



48h

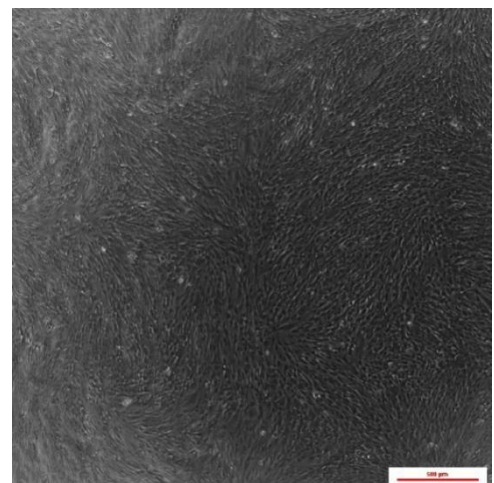
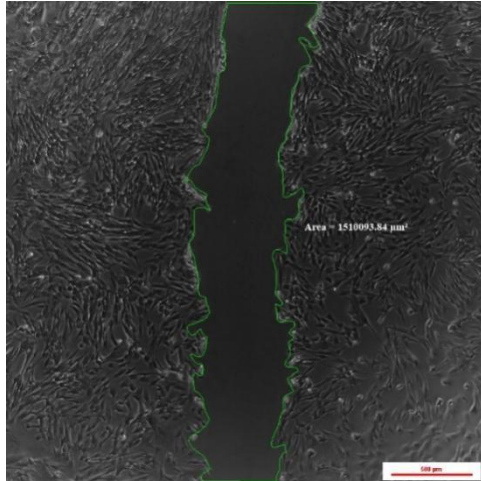
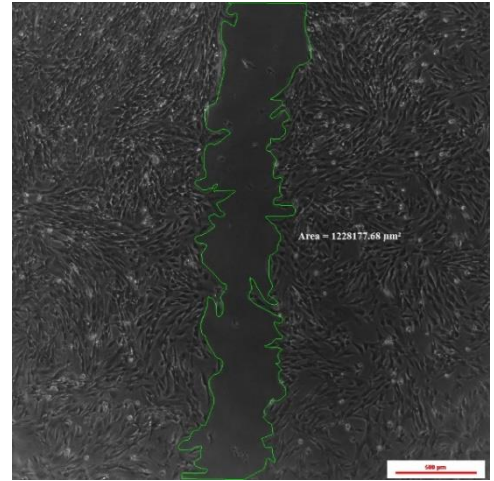


Figure 13: Migration of fibroblasts after treatment with 7.5 $\mu\text{g/mL}$ petroleum ether seed extract for 48h.

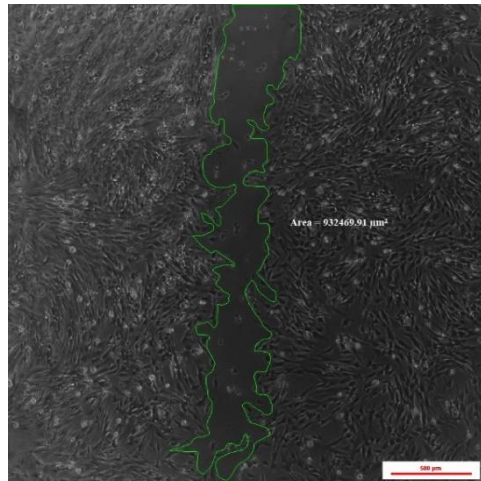
0h



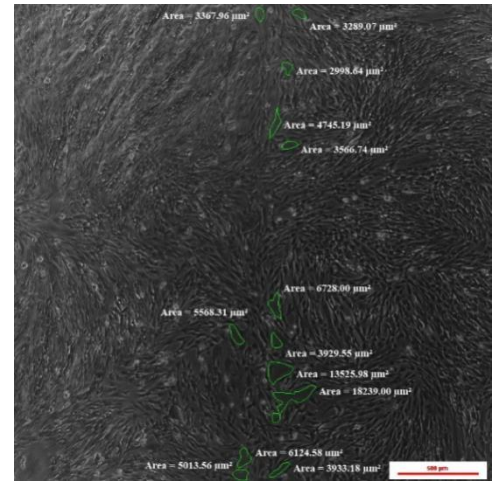
6h



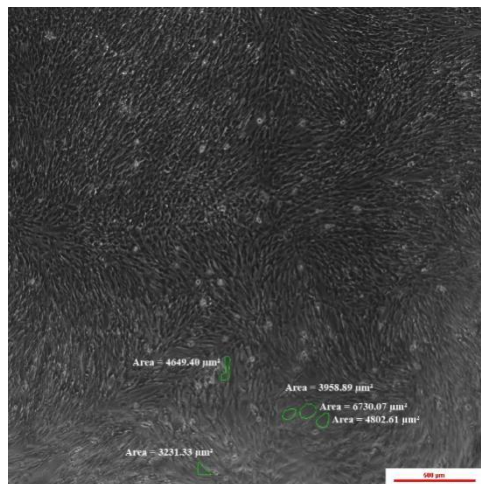
12h



24h



36h



48h

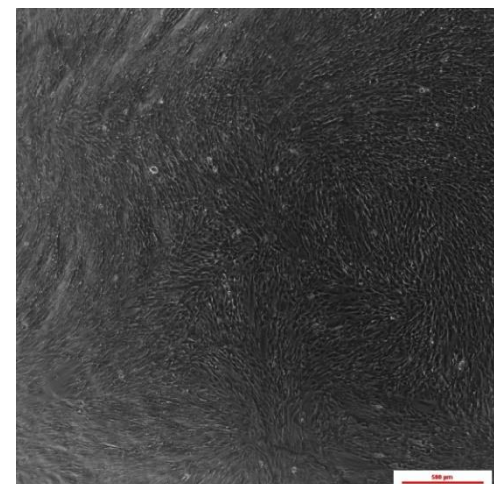
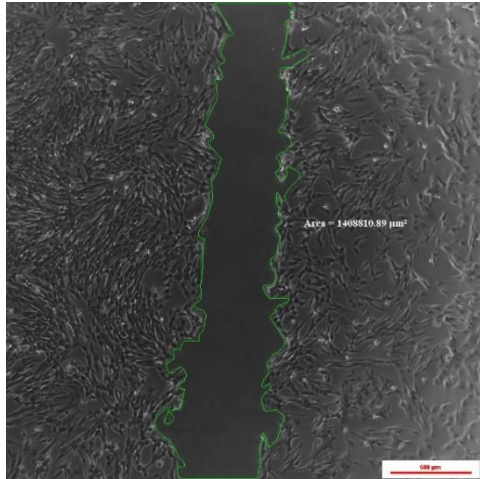
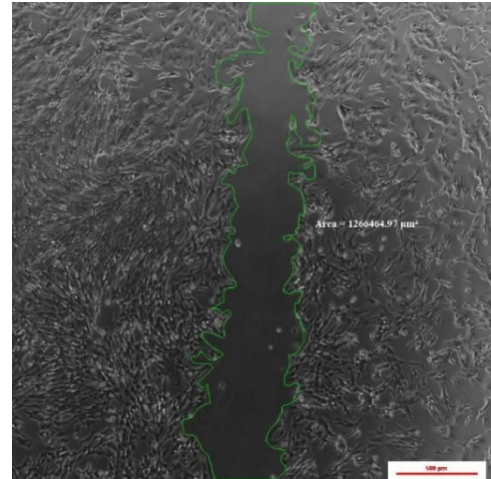


Figure 14: Migration of fibroblasts after treatment with 15 $\mu\text{g/mL}$ petroleum ether seed extract for 48h.

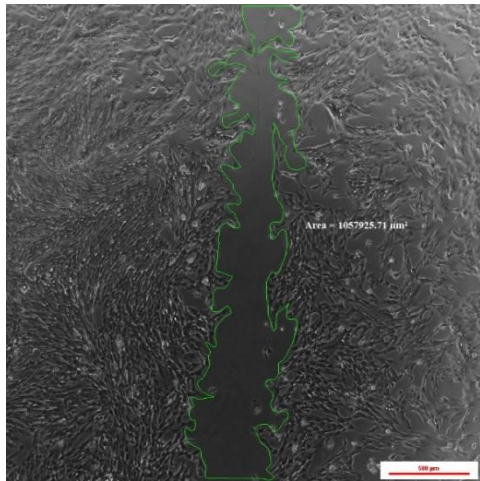
0h



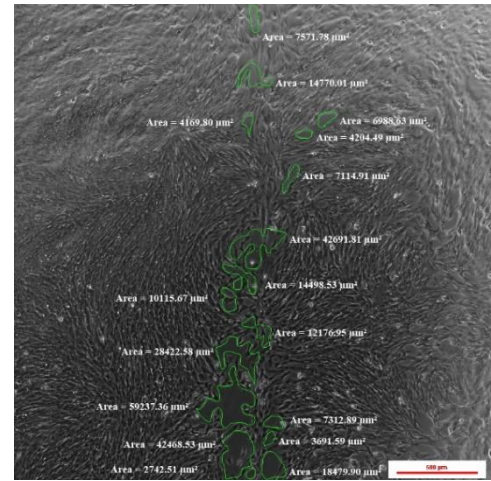
6h



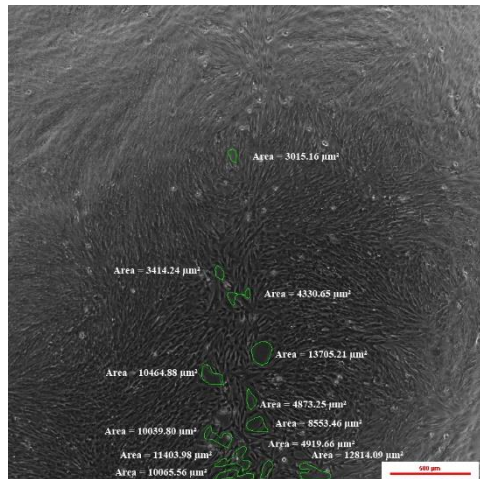
12h



24h



36h



48h

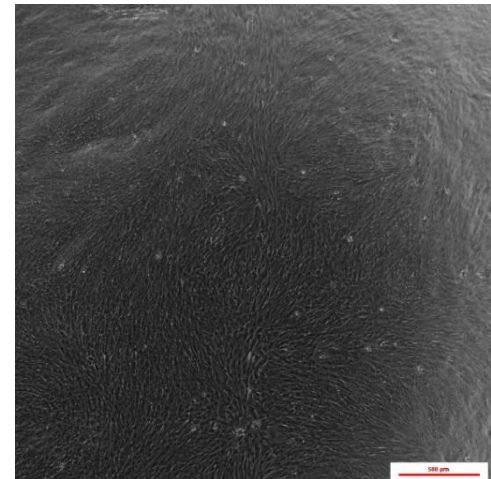
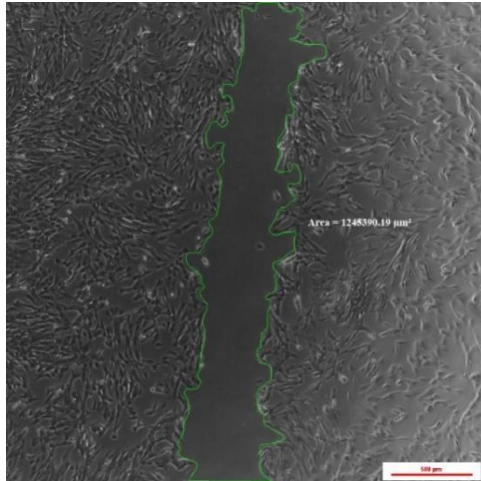
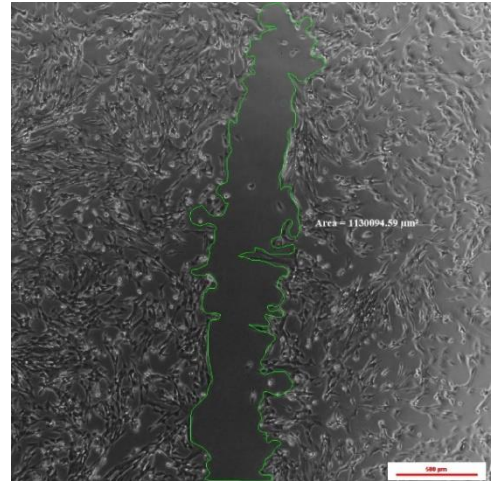


Figure 15: Migration of fibroblasts after treatment with 62.5 $\mu\text{g/mL}$ methanolic seed extract for 48h.

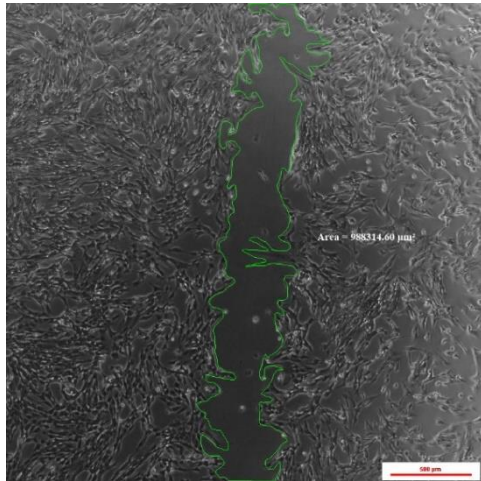
0h



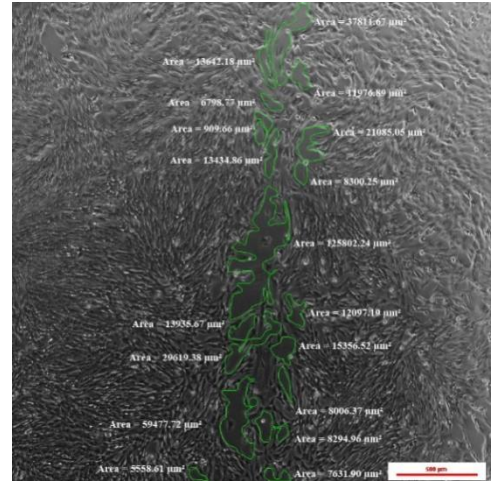
6h



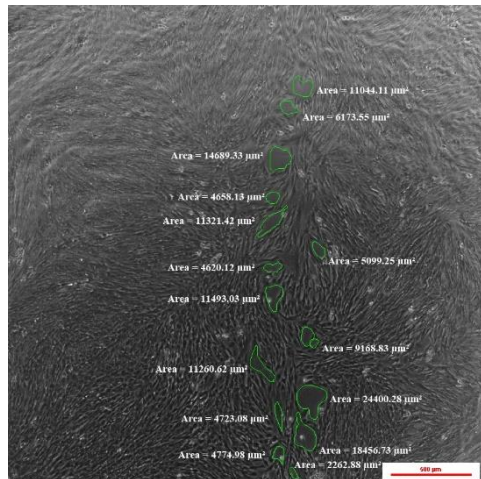
12h



24h



36h



48h

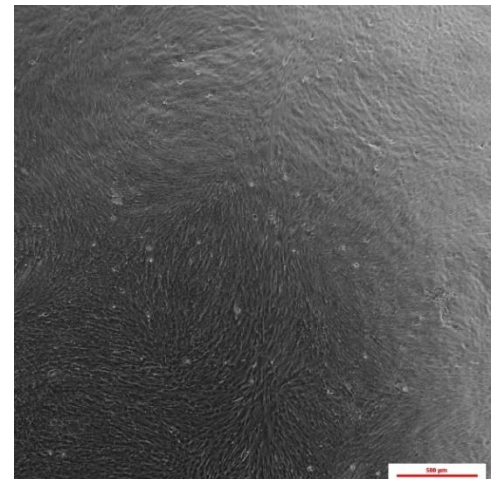
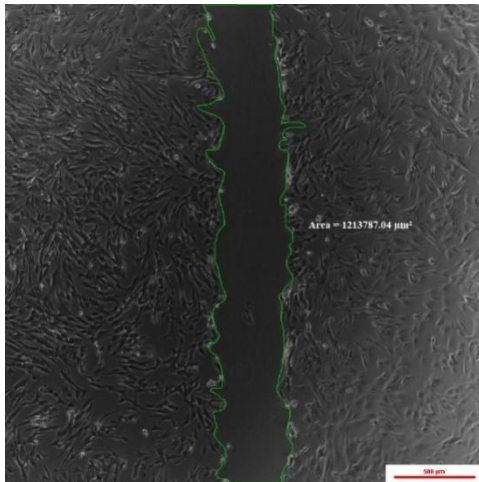
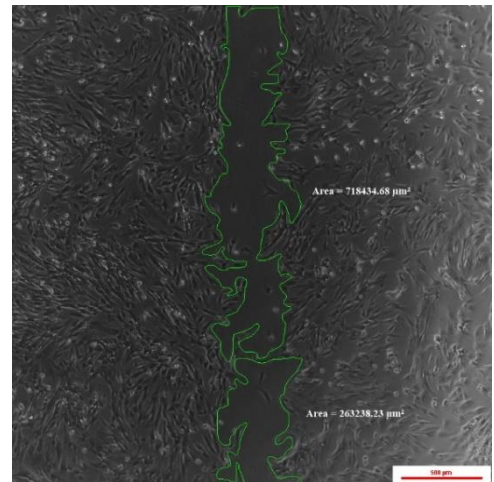


Figure 16: Migration of fibroblasts after treatment with 125 $\mu\text{g/mL}$ methanolic seed extract for 48h.

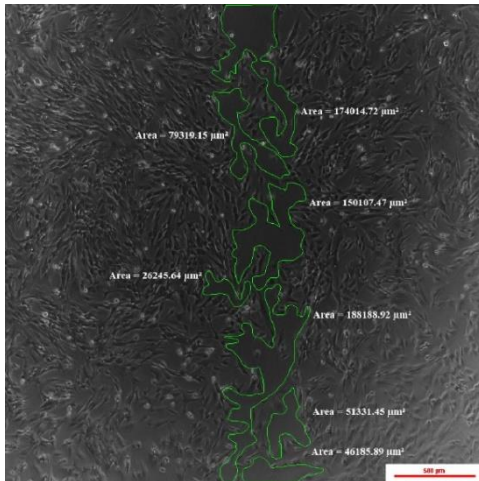
0h



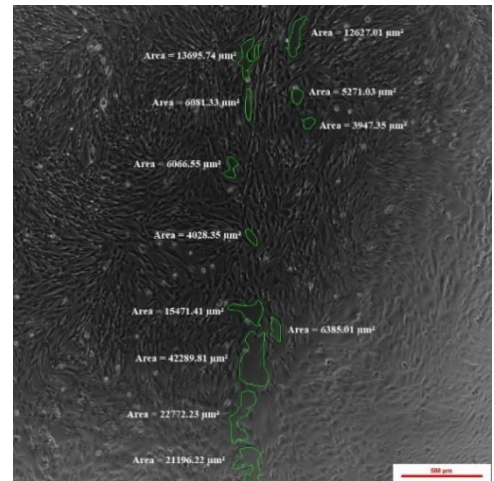
6h



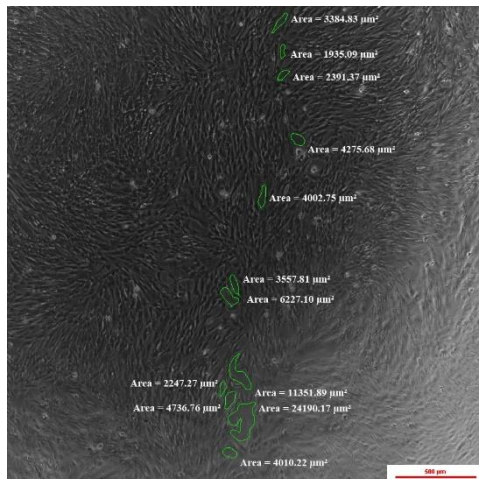
12h



24h



36h



48h

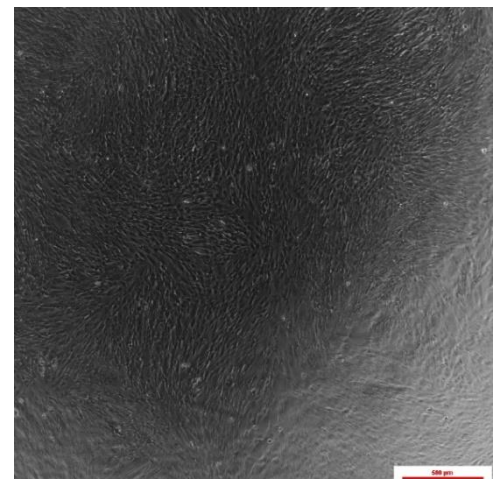
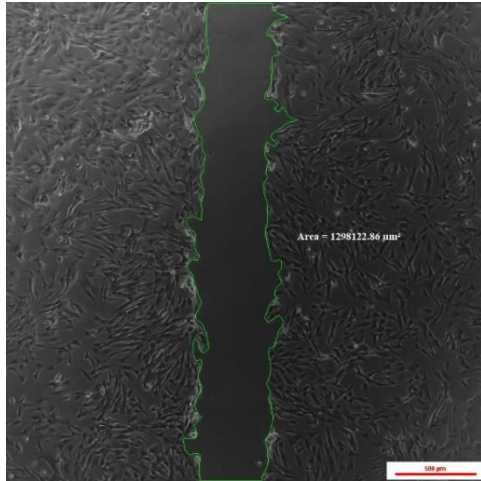
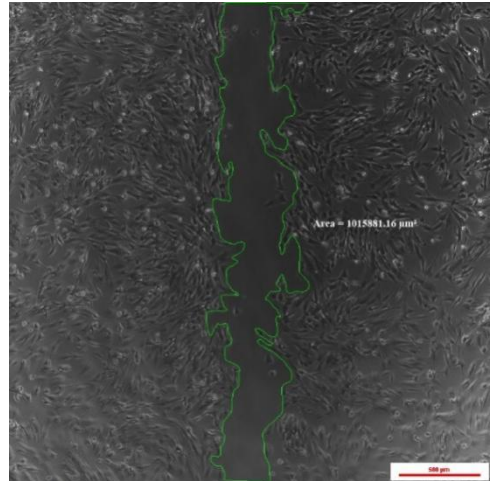


Figure 17: Migration of fibroblasts after treatment with 7.5 $\mu\text{g/mL}$ Bligh and Dyer seed extract for 48h.

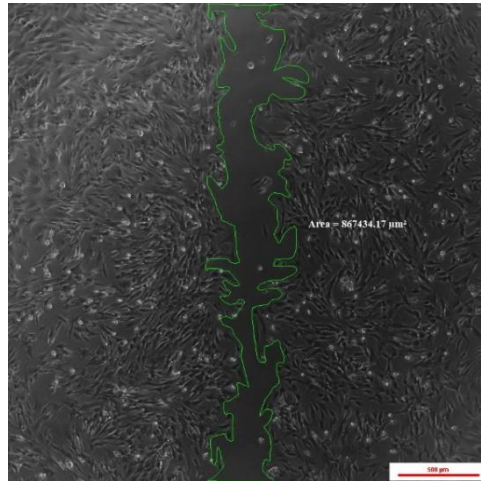
0h



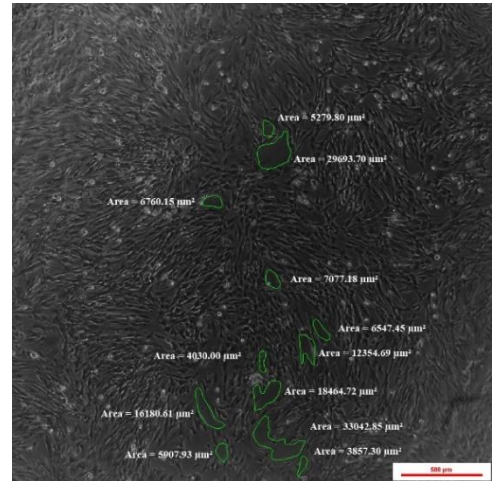
6h



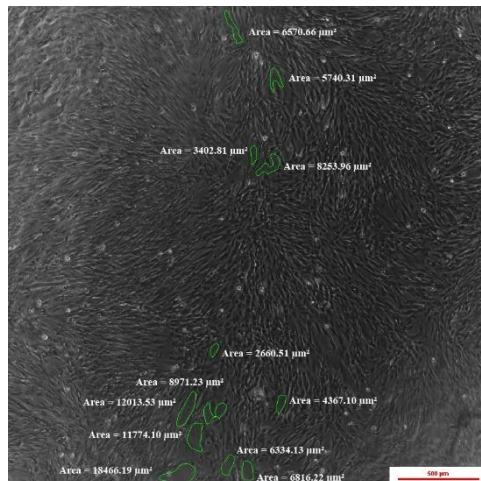
12h



24h



36h



48h

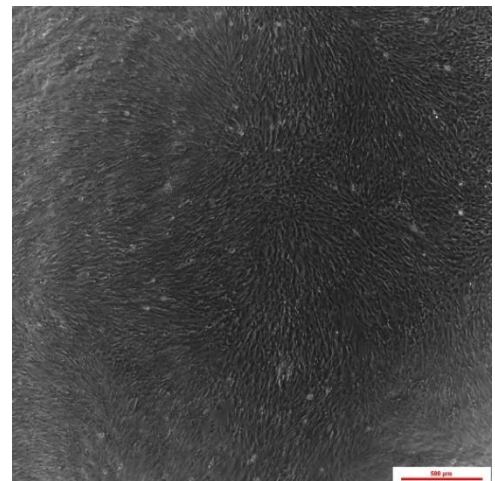


Figure 18: Migration of fibroblasts after treatment with 15 $\mu\text{g/mL}$ Bligh and Dyer seed extract for 48h.