



Faculty of Medicine and Health Sciences

**Antioxidant, Antimicrobial and Wound Healing Properties of
Clinacanthus nutans (Burm. f.) Lindau and *Strobilanthes crispus* (L.)
Blume Extracts**

Ban Weng Kit

**Master of Science
2023**

Antioxidant, Antimicrobial and Wound Healing Properties of *Clinacanthus nutans* (Burm.f.) Lindau and *Strobilanthes crispus* (L.) Blume Extracts

Ban Weng Kit

A thesis submitted

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

(Natural Products)

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: Ban Weng Kit

Matric No.: 18020132

Faculty of Medicine and Health Sciences

Universiti Malaysia Sarawak

Date : 10 JUNE 2023

ACKNOWLEDGEMENT

I would like to express my gratitude to my supervisors, Dr Isabel Fong Lim and Professor Dr. Khong Heng Yen for all the guidance and advice in accomplishing my research works. Besides, I also appreciate the assistance and ideas from Muhammad Mirza Ariffin and Joyce Phung Hui Yie throughout my study.

I would also like to extend my thanks to Mr. Raphael Chung Kok Ann, Madam Low Hee Ping, Mr. Lim Yu Seng, and Madam Jenny Ngo Yee Hoon for growing and providing the raw material of these two herbal plants for this study.

My sincere gratitude to the Faculty of Medicine and Health Sciences, for the advice and support given during my study period at Universiti Malaysia Sarawak.

Finally, I would like to thank the Centre for Graduate Studies of the Universiti Malaysia Sarawak for making it possible to complete my study here in Sarawak. Thank you all.

ABSTRACT

Clinacanthus nutans (*C. nutans*) and *Strobilanthes crispus* (*S. crispus*) both are well-known for their specific beneficial properties against different diseases. *C. nutans* is known to be anticancer and antiviral, especially against colon cancer, *varicella-zoster virus*, and herpes simplex virus. *S. crispus* has proven to be anti-diuretic and antidiabetic by multiple studies. In the previous studies, *S. crispus* extract showed promising results, capable of aiding in the wound healing process. Since *C. nutans* was commonly available as a balm in the traditional market, there is a high possibility that it contains antimicrobial and wound healing properties as well. Thus, this study aimed to confirm the medicinal benefits of *C. nutans* and *S. crispus*, specifically antimicrobial and wound healing properties. The *C. nutans* and *S. crispus* leaves were extracted with different polarity solvents; ethanol, acetone, and chloroform, through cold maceration and kept refrigerated at 2-8°C in the dark. The presence of phytochemicals, such as alkaloids, flavonoids, and saponin in the extracts were screened. These extracts were then assessed for their antioxidant potential by DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay using ascorbic acid as standard; determination of Total Phenolic Content (TPC) and Total Flavonoids Content (TFC) using Gallic Acid and Rutin as standard, respectively. Disk Diffusion Assay was performed to determine their antimicrobial properties. Modified scratch assay by co-incubating skin fibroblast with Methicillin-resistant *Staphylococcus aureus* (MRSA) and treated with the *C. nutans* and *S. crispus* extracts to determine their wound healing effects. These extracts were observed at 1, 3, 6, 10, 24, and 48 hours to investigate the cell migration activity depicting the closing up of wound. The phytochemical profiles for both *C. nutans* and *S. crispus* extracts were similar, except the *C. nutans* extracts have a higher saponin level compared with *S. crispus* extracts. The *C. nutans* acetone extract contained the highest level of antioxidant potential and TPC than the other

C. nutans extracts. However, the ethanol extract of *C. nutans* showed the highest TFC, which was corresponded to the flavonoid content obtained from the phytochemical screening. The highest TPC value was recorded by the *S. crispus* ethanol extract, while the TFC test revealed similar pattern results among these *S. crispus* extracts. The *C. nutans* extracts showed a zone of inhibition of 14 to 16 mm when treated on *Ps. aeruginosa*, while *S. crispus* extracts showed distinctive zone of inhibition range between 11 to 15 mm on *Ps. aeruginosa*. There was no positive result observed when the extracts were treated on other bacteria. Fibroblasts were sensitive to acetone extracts, and *S. crispus* extracts were proven to have higher toxicity during the MTT assay. During the scratch assay with extracts only, *C. nutans* extracts showed wound healing properties, whereas *S. crispus* extracts seemed to be delayed the wound healing activity. However, when MRSA was added for the co-incubation, *S. crispus* extracts showed inhibition against the growth of MRSA and aided the wound healing activity, but this situation did not demonstrate in *C. nutans* extracts. The *C. nutans* and *S. crispus* extracts exhibited strong antioxidant and antimicrobial properties. *C. nutans* extracts aids in the wound healing activity, but if MRSA was involved, *S. crispus* extracts were capable of inhibiting the growth and aiding in the wound healing activity.

Keywords: *Clinacanthus nutans*, *Strobilanthes crispus*, antioxidant, antimicrobial, wound healing.

Sifat-sifat Antioksidan, Antimikrobal dan Penyembuhan Luka *Clinacanthus nutans* (Burm.f.) Lindau dan *Strobilanthes crispus* (L.) Ekstrak

ABSTRAK

Clinacanthus nutans (*C. nutans*) dan *Strobilanthes crispus* (*S. crispus*) kedua-duanya terkenal dengan ciri-ciri khusus bermanfaat terhadap penyakit yang berlainan. *C. nutans* dikenali sebagai antikanser dan antivirus, terutama terhadap kanser usus, virus varicella-zoster dan virus herpes simplex. *S. crispus* telah terbukti anti-diuretik dan antidiabetik oleh pelbagai kajian. Dalam kajian yang lepas, ekstrak *S. crispus* menunjukkan hasil yang menjanjikan, dapat membantu proses penyembuhan luka. Memandangkan *C. nutans* biasanya diperolehi sebagai balsem di pasar tradisional, maka ada kemungkinan besar ia juga mengandung sifat antimikrob dan penyembuhan luka. Oleh itu, kajian ini bertujuan untuk membuktikan kebaikan perubatan *C. nutans* and *S. crispus*, khususnya sifat antimikrob dan penyembuhan luka. Daun *C. nutans* dan *S. crispus* diekstraksi dengan pelarut pelbagai polariti: etanol, aseton dan kloroform melalui maserasi sejuk dan disimpan dalam peti sejuk pada suhu 2-8 ° C dalam keadaan gelap. Kandungan fitokimia seperti alkaloid, flavonoid dan saponin dalam ekstrak disaring. Ekstrak ini kemudian diuji potensi antioksidan dengan uji pemungutan radikal bebas DPPH (2,2-diphenil-1-picrylhidrazil) dengan asid askorbik sebagai standard, penentuan Jumlah Kandungan Fenolik Total (TPC) dan Jumlah Kandungan Flavonoid (TFC) dengan Asid Gallik dan Rutin masing-masing sebagai standard. Kaedah resapan cakera digunakan untuk menentukan sifat antimikrob mereka. Uji gores yang diubahsuai dengan menginkubasi fibroblas kulit dan Methicillin Resistant *Staph. aureus* (MRSA) dengan ekstrak *C. nutans* dan *S. crispus* digunakan untuk menentukan kesan penyembuhan luka. Ekstrak ini diperhatikan pada 1 jam, 3 jam, 6 jam, 10 jam, 24 jam, dan 48 jam untuk menyiasat aktiviti migrasi sel. Profil fitokimia untuk kedua-dua ekstrak *C.*

nutans dan *S. crispus* adalah serupa, kecuali ekstrak *C. nutans* mempunyai kandungan saponin yang lebih tinggi berbanding dengan ekstrak *S. crispus*. Ekstrak *C. nutans* aseton mempunyai potensi antioksidan dan TPC yang lebih tinggi berbanding dengan ekstrak *C. nutans* yang lain. Walau bagaimanapun, ekstrak etanol *C. nutans* menunjukkan TFC tertinggi yang sepadan dengan jumlah flavonoid yang diperolehi dari penyaringan fitikimia flavonoid. Ekstrak *C. nutans* menunjukkan zon perencatan antara 14 hingga 16 mm ketika dirawat pada *Ps. aeruginosa*, sementara ekstrak *S. crispus* menunjukkan zon perencatan yang jelas antara 11 hingga 15 mm pada *Ps. aeruginosa*. Semasa ekstrak merawat pada bakteria lain, tiada keputusan positif diperhatikan. Fibroblas sensitif terhadap ekstrak aseton dan ekstrak *S. crispus* terbukti mempunyai ketoksikan yang lebih tinggi semasa ujian MTT. Semasa ujian calar dengan ekstrak sahaja, ekstrak *C. nutans* menunjukkan sifat penyembuhan luka, sedangkan ekstrak *S. crispus* melambatkan aktiviti penyembuhan luka. Namun, ketika MRSA ditambahkan untuk inkubasi bersama, ekstrak *S. crispus* menunjukkan perencatan terhadap pertumbuhan MRSA dan membantu aktiviti penyembuhan luka, tetapi keadaan ini tidak ditunjuk dalam ekstrak *C. nutans*. *C. nutans* dan *S. crispus* menunjukkan sifat antioksidan dan antimikroba yang kuat. Ekstrak *C. nutans* membantu dalam aktiviti penyembuhan luka tetapi jika MRSA terlibat, ekstrak *S. crispus* mampu merencat pertumbuhannya dan membantu dalam aktiviti penyembuhan luka.

Kata kunci: *Clinacanthus nutans*, *Strobilanthes crispus*, antioksidan, antimikrob, penyembuhan luka

TABLE OF CONTENTS

	Page
DECLARATION	i
ACKNOWLEDGEMENT	ii
ABSTRACT	iii
ABSTRAK	v
TABLE OF CONTENTS	vii
LIST OF TABLES	x
LIST OF FIGURES	xi
LIST OF ABBREVIATIONS	xiii
CHAPTER 1 INTRODUCTION	1
1.1 Study Background	1
1.1.1 Microenvironment on Wounds	3
1.1.2 Antimicrobial Resistance	4
1.1.3 Natural Products for Wound Healing Activities	4
1.2 Problem Statement	6
1.3 Objectives	7
1.4 Chapter Summary	8
CHAPTER 2 LITERATURE REVIEW	9
2.1 Acanthaceae Family	9

2.2	<i>Clinacanthus nutans</i> (Burm f.) Lindau	11
2.3	<i>Strobilanthes crispus</i> (L.) Blume	21
2.3.1	External (Topical) Application of <i>Clinacanthus nutans</i> and <i>Strobilanthes crispus</i>	28
CHAPTER 3	MATERIALS AND METHODS	34
3.1	Plant Collection	34
3.2	Extraction	34
3.3	Phytochemical Screening	35
3.3.1	Alkaloids Test	35
3.3.2	Flavonoids Test	35
3.3.3	Saponin Test	36
3.4	Total Phenolics Content (TPC)	36
3.5	Total Flavonoids Content (TFC)	37
3.6	Antioxidant (2,2-Diphenyl-1-picrylhydrazyl Assay)	37
3.7	Antimicrobial (Disk Diffusion Assay)	38
3.7.1	Nutrient broth (NB)	38
3.7.2	Mueller-Hinton Agar (MHA)	38
3.7.3	Mueller-Hinton Broth (MHB)	39
3.7.4	Antimicrobial Sensitivity Test (AST)	39
3.8	Wound Healing Activity (Scratch Assay)	40
3.8.1	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay	41

3.8.2	Bacteria Preparation	42
3.8.3	Co-incubation <i>in-vitro</i> Assay	43
CHAPTER 4	RESULTS	45
4.1	Determination of Total Flavonoid and Phenolics Contents.	45
4.2	Determination of Antimicrobial Properties of <i>C. nutans</i> and <i>S. crispus</i> Plant Extracts against Gram-positive and Gram-negative Bacteria.	48
4.3	Effects of <i>C. nutans</i> and <i>S. crispus</i> Extracts on Skin Fibroblasts.	50
4.4	Wound Healing Effects of <i>C. nutans</i> and <i>S. crispus</i> Extracts Treated on Pseudo-wound in the Presence of MRSA	58
CHAPTER 5	DISCUSSION	66
5.1	Phytochemical	66
5.2	Total Phenolic Content and Total Flavonoid Content of <i>Clinacanthus nutans</i> (<i>C. nutans</i>) and <i>Strobilanthes crispus</i> (<i>S. crispus</i>)	67
5.3	Antioxidant Properties	69
5.4	Antimicrobial Properties	72
5.5	Wound Healing Properties	74
CHAPTER 6	CONCLUSION AND RECOMMENDATIONS	79
6.1	Conclusion	79
6.2	Recommendations	80
REFERENCES		81
APPENDICES		96

LIST OF TABLES

	Page
Table 2.1: The antioxidant potential of <i>C. nutans</i> extracts based on its DPPH free radical scavenging activities.	14
Table 2.2: IC ₅₀ of different solvent <i>C. nutans</i> extracts when treated on different cancer cell lines using various bioassays with their respectively study references.	18
Table 2.3: Antioxidant activity of <i>S. crispus</i> extracts based on DPPH free scavenging assay	23
Table 2.4: IC ₅₀ of MTT assays against different cancer cell lines on <i>C. nutans</i> solvent extracts.	27
Table 2.5: The Minimum Inhibition Concentration (MIC) of Gram-positive and Gram-negative bacteria when treated with different extracts of <i>C. nutans</i> .	32
Table 2.6: The Minimum Inhibition Concentration (MIC) of Gram-positive and Gram-negative bacteria when treated with different extracts of <i>S. crispus</i> .	33
Table 3.1: The preparation of culture media with different <i>C. nutans</i> and <i>S. crispus</i> extracts with MRSA	44
Table 4.1: The result of the phytochemical screening for different solvents extracts of <i>Clinacanthus nutans</i> and <i>Strobilanthes crispus</i> .	46
Table 4.2: The comparison of the antioxidant content (AAEAC), total phenolic content (TPC), and total flavonoids content (TFC) between <i>C. nutans</i> and <i>S. crispus</i> with different solvents extracts.	47
Table 4.3: Zone of inhibition when different <i>C. nutans</i> and <i>S. crispus</i> extracts treated on Gram-positive and Gram-negative bacteria on MHA.	49
Table 4.4: Cell Viability Assay of different concentrations of <i>C. nutans</i> extracts applied on the normal human skin fibroblast and incubated for 24 and 48 hours respectively.	51
Table 4.5: Cell Viability Assay of different concentrations of <i>S. crispus</i> extracts applied on the normal human skin fibroblast and incubated for 24 and 48 hours respectively.	55
Table 4.6: The Result of <i>in vitro</i> Scratch Assay with <i>C. nutans</i> extracts.	64
Table 4.7: The Result of <i>in vitro</i> Scratch Assay with <i>S. crispus</i> extracts.	65

LIST OF FIGURES

	Page
Figure 1.1: Stages of Wound Healing	2
Figure 2.1: <i>Clinacanthus nutans</i>	11
Figure 2.2: Bioactive Compunds in <i>Clinacanthus nutans</i> (<i>C. nutans</i>)	16
Figure 2.3: <i>Strobilanthes crispus</i>	21
Figure 4.1: Cell Viability Assay of different concentrations of <i>C. nutans</i> ethanol extract applied on the normal human skin fibroblast and incubated for 24 and 48 hours respectively.	52
Figure 4.2: Cell Viability Assay of different concentrations of <i>C. nutans</i> acetone extract applied on the normal human skin fibroblast and incubated for 24 and 48 hours respectively.	53
Figure 4.3: Cell Viability Assay of different concentrations of <i>C. nutans</i> chloroform extract applied on the normal human skin fibroblast and incubated for 24 and 48 hours respectively.	53
Figure 4.4: Cell Viability Assay of different concentrations of <i>S. crispus</i> ethanol extract applied on the normal human skin fibroblast and incubated for 24 and 48 hours respectively.	56
Figure 4.5: Cell Viability Assay of different concentrations of <i>S. crispus</i> acetone extract applied on the normal human skin fibroblast and incubated for 24 and 48 hours respectively.	57
Figure 4.6: Cell Viability Assay of different concentrations of <i>S. crispus</i> chloroform extract applied on the normal human skin fibroblast and incubated for 24 and 48 hours respectively.	57
Figure 4.7: Growth and coverage of MRSA over a duration of 24 hours when different concentration of MRSA were co-incubated with normal human skin fibroblasts.	58
Figure 4.8: Scratch Assay on normal human fibroblasts in the presence of <i>C. nutans</i> ethanol (E), chloroform (C) and acetone (A) extracts respectively. An <i>in vitro</i> wound healing process was observed in a time-dependent manner on normal human fibroblasts in the presence of ethanol (E), chloroform (C) and acetone (A), respectively.	60
Figure 4.9: Scratch Assay on normal human fibroblasts and MRSA. An <i>in vitro</i> wound healing process was observed in a time-dependent manner on	

human fibroblasts in the presence of ethanol (E), chloroform (C) and acetone (A) *C. nutans* extracts respectively. 61

Figure 4.10: Scratch Assay on human fibroblasts in the presence of *S. crispus* extracts. An *in vitro* wound healing process was observed in a time-dependent manner on human fibroblasts in the presence of ethanol (E), chloroform (C) and acetone (A) respectively 62

Figure 4.11: Scratch Assay on human fibroblasts in the presence of *S. crispus* extracts and MRSA. An *in vitro* wound healing process was observed in a time-dependent manner on human fibroblasts in the presence of ethanol (E), chloroform (C) and acetone (A) respectively. 63

LIST OF ABBREVIATIONS

µg	Microgram
µm	Micron
°C	Degree Celsius
<i>B. cereus</i>	<i>Bacillus cereus</i>
BHT	Butylated Hydroxytoluene
BT-474	Human Breast Tumour Cell Line
Caco-2	Human Colorectal Adenocarcinoma Cell Line
CaOx	Calcium Oxalate Crystals
CCD-18Co	Normal Human Colon Fibroblast Cell Line
CCK-8	Cell Counting Kit
Chang Liver Cell	Derived HeLa Cell Line
CO ₂	Carbon Dioxide
<i>C. nutans</i>	<i>Clinacanthus nutans</i>
D24	Human Melanoma Cancer Cell Line
DD	Disc Diffusion Method
DGDG	Digalactosyl Diglyceride
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
DMSO	Dimethyl Sulfoxide
<i>E. coli</i>	<i>Escherichia coli</i>
EC ₅₀	Half Maximal Effective Concentration
FDA	Food and Drug Administration
GAE	Gallic Acid Equivalent
HCT-116	Human Colon Cancer Cell Line

HeLa	Human Cervical Cancer Cell Line
HepG2	Human Liver Hepatocellular Carcinoma Cell Line
HIV	Human Immunodeficiency Virus
HSV-1	<i>Herpes simplex</i> virus 1
HSV-2	<i>Herpes simplex</i> virus 2
HT-29	Human Colon Adenocarcinoma Cell Line
HUVEC	Primary Human Umbilical Vein Endothelial Cells
IC ₅₀	Half-Maximal Inhibitory Concentration
IMR32	Human Neuroblastoma Cell Line
K562	Human Erythroleukemic Cell Line
<i>K. pneumoniae</i>	<i>Klebsiella pneumoniae</i>
Lev 30	Levofloxacin 30 µg
LS-174T	Human Colon Adenocarcinoma Cell Line
<i>M. luteus</i>	<i>Micrococcus luteus</i>
MHA	Mueller-Hinton Agar
MCF-7	Human Breast Cancer Cell Line
MDA-MB-231	Human Invasive Ductal Carcinoma Cell Line
MGDG	Monogalactosyl Diglyceride
MIC	Minimum Inhibitory Concentration
MM-418C1	Human Melanoma Cancer Cell Line
MRSA	Methicillin-Resistant <i>Staphylococcus aureus</i>
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl-2H-Tetrazolium Bromide
NCI-H23	Human Non-Small Cell Lung Carcinoma
NCI-H460	Human Non-Small Cell Lung Cancer Cell Line

NRU	Neutral Red Uptake
OD ₆₀₀	Optical Density at 600nm
OH	Hydroxy or Hydroxyl
ORAC	Oxygen Radical Absorbance Capacity
OUMS-36T-4F	Normal Human Skin Fibroblast
OX 1	Oxacillin 1 µg
Pen 10G	Penicillin 10 unit
<i>P. acnes</i>	<i>Propionibacterium acnes</i>
PBS	Phosphate-Buffered Saline
<i>Ps. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
PDT	Photodynamic Therapy
Raji	Human Continuous Cell Line
RE	Rutin Equivalent
ROS	Reactive Oxidative Species
<i>Staph. aureus</i>	<i>Staphylococcus aureus</i>
<i>Staph. epidermidis</i>	<i>Staphylococcus epidermidis</i>
<i>Strep. pneumoniae</i>	<i>Streptococcus pneumoniae</i>
<i>Strep. pyogenes</i>	<i>Streptococcus pyogenes</i>
<i>S. crispus</i>	<i>Strobilanthes crispus</i>
Saos-2	Human Osteosarcoma Cell Line
SNU-1	Poorly Differentiated Human Primary Carcinoma of Stomach
<i>spp.</i>	Species
SRB	Sulforhodamine B
T25	25m ² Tissue Culture Flask
T-47D	Human Breast Cancer Cell Line

TPC	Total Phenolic Content
TFC	Total Flavonoids Content
Vero	African Green Monkey Kidney Epithelial Cell Line
Van 30	Vancomycin 30 µg
VZV	<i>Varicella-zoster</i> virus

CHAPTER 1

INTRODUCTION

1.1 Study Background

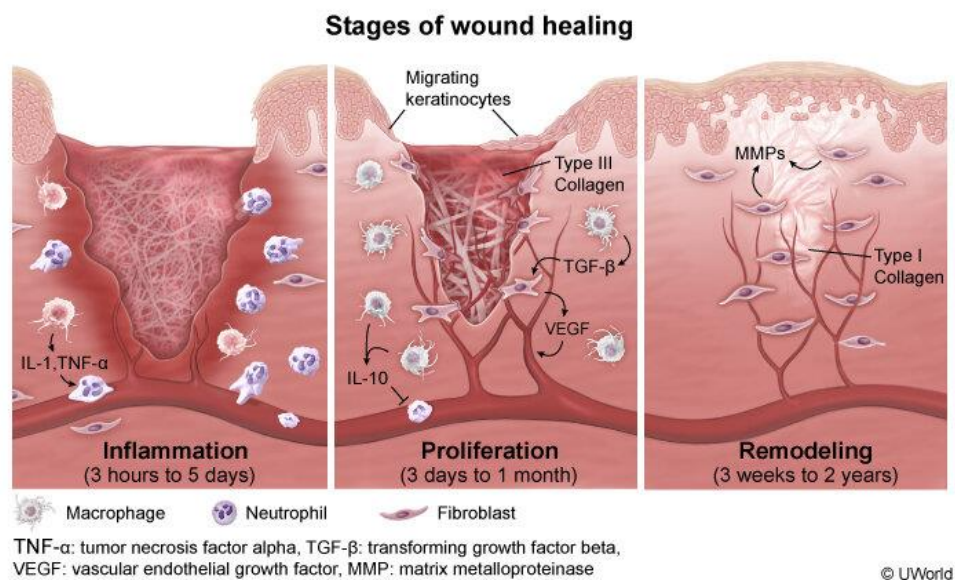
Wound healing, also known as a skin repairing process, is one of the most complicated processes in the human body. To close an open wound, multiple cells and stages are involved, including haemostasis, inflammation, angiogenesis, growth, re-epithelialization, and re-modelling (**Figure 1.1**) (Rodrigues et al., 2019). However, harmful substances or opportunistic microorganisms may act spontaneously and invade the body.

According to Grice and Segre (2011), bacteria such as *Propionibacterium acnes* (*P. acnes*), *Streptococcus pyogenes* (*Strep. pyogenes*), *Staphylococcus aureus* (*Staph. aureus*), *Enterococcus spp.* and *Pseudomonas aeruginosa* (*Ps. aeruginosa*) can be commonly isolated from the skin surface. They are usually the normal flora. Once the skin barrier is breached, persistent inflammation in the absence or delay of wound healing, persistent inflammation ensues, eventually leading to chronic wound.

A chronic wound is defined as an open wound that does not undergo an orderly and timely skin repair process. This process usually takes more than three months (Lanau-Roig et al., 2017). This situation commonly happens to people who have a weak immune system or patients with Human Immunodeficiency Virus (HIV). For example, diabetes is a chronic disease, and due to its high glucose level, the blood flow rate decreases. Thus, abrasions, open wounds, or other injuries tend to recover slower than to a healthy person (Chhibber et al., 2018). A chronic wound is a major public health challenge that presents multifactorial risk factors and complications that hinder the effective use of human resources, deplete the

healthcare system, and inadvertently emergence of resistance strain due to inappropriate use of antibiotics. The presence of methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most challenging issues when dealing with chronic wounds as colonization and proliferation of MRSA at the site of the wound have been found to delay the wound healing process (Manzuoerh et al., 2019).

Based on the market study done by the Fortune Business Insight (2021), chronic wound management incurs a weighty burden on the healthcare system and a hefty cost to the patient. The value of 2021 global chronic wound care market was approximately USD 11.61 billion and the projected to grow estimated USD 1.0 billion until 2029. Therefore, health care professionals are constantly looking for new approaches or medicine in chronic wound management.



(Adapted from Melissa, 2020)

Figure 1.1: Stages of Wound Healing

1.1.1 Microenvironment on Wounds

Skin is the largest organ in the human body. It is the outer defence system against environment factors and microbes. There is a plethora of microbes including opportunistic pathogens, commensal microbes, fungi, and viruses that human are exposed to every day. A cutaneous wound becomes a perfect entry site for these infectious agents (Kirchner et al., 2020). Besides these active infections, biofilm formation on the wound by certain pathogens will most likely delay the wound healing process and causes chronic wound.

Even though the initial triggered inflammation process causes the fibrin clot formation (Weisel & Litvonov, 2017) to achieve haemostasis and then the recruitment of immune cells to the site, the microbes have their own means to overcome the situation. According to the study by Thammavongsa (2015), *Staph. aureus* was commonly found on the skin and nasal area, it was capable of producing beta-barrel-forming toxins and promoting immune cells' lysis such as macrophages, neutrophils, and monocytes. Besides, the biofilm formation by the *Pseudomonas* is also reported to be one of the main issues of delaying the wound's closure and preventing the infiltration of immune cells towards the wound site (Ruffin & Brochiero, 2019).

Besides, fungi and viruses also affect the wound healing process through different mechanisms. *Candida albicans*, one of the common fungi found on the skin surface, once entered the body, can compromise the macrophage function, thus killing the immune cells (Uwamahoro et al., 2014). A few studies were performed on viruses in the skin ecosystem. Skin-tropic papillomaviruses could take over the wound healing process and infecting the wounded keratinocytes (Hufbauer & Akgul, 2017).

1.1.2 Antimicrobial Resistance

The emergence of antimicrobial resistance can be attributed to a lack of awareness, and improper use of antibiotics, and genetic mutation over time (Akova, 2016). Antimicrobial resistance is often associated with high morbidity and mortality. It has always been a worldwide challenge after the discovery of antibiotics. It is because of the improper usage of antibiotics in the early days when the healthcare system still lacks proper procedures to identify of causative microbes (Akova, 2016). Until the year of 2000, only a systematic review first reported proper wound care management, which included the use of antibiotics (O'Meara et al., 2000).

Besides, the natural ability of *Staph. aureus* for survival, the environment can be one of the causes of antimicrobial resistance development. Furthermore, the low-level mupirocin resistance has increased from 0 % to 19 % when chlorhexidine baths and intranasal mupirocin were frequently applied to decolonize the MRSA in a 14-bed surgical ICU. (Cho et al., 2016)

1.1.3 Natural Products for Wound Healing Activities

There are many types of anecdotal medical plants available in different regions of the world, and numerous indigenous people have been using certain plants as medicine or herbal remedies for centuries. Yet, these are not scientifically documented as an official medicine registered under Food and Drug Administration (FDA). This triggers the initiatives of scientists to delve deeper into bioactive phytochemicals and study their pharmacological properties. In order to identify a plant that is efficacious in providing health benefits, there

are some properties required in the selection process, for example, antioxidant, antimicrobial, and wound healing properties.

Recently, there has been a strong drive towards naturally derived phytochemicals to address the above three different stages of wound healing. *Clinacanthus nutans* (Burm.f.) Lindau (*C. nutans*) and *Strobilanthes crispus* (L.) Blume (*S. crispus*) are among these plants that attracted much interest from researchers due to their medicinal properties (Ong et al., 2020).

Clinacanthus nutans (Burm.f.) Lindau (*C. nutans*), also known as Sabah snake grass, is well-known traditional medicine for natives in Southeast Asia, especially in Thailand and Malaysia. The locals in Malaysia usually sun-dried the *C. nutans* leaves and brewed them in hot water to serve as tea. It is believed that daily consuming of *C. nutans* tea can cure diabetes, dysuria and diarrhoea (P'ng et al., 2012). According to Wanikiat (2008), *C. nutans* was used to cure oxidative stress-related diseases and skin lesions *in vivo* caused by herpes simplex virus and *varicella-zoster* virus. Other than that, some colon cancer survivors testified that consuming *C. nutans* over the long period of time could ease the chemotherapy effects and aid in the cancer illness recovery process. Subsequently, *C. nutans* was officially recognized as the principal medicinal plant for primary healthcare by the Ministry of Public Health in Thailand (Shim et al., 2012).

Strobilanthes crispus (L.) Blume (*S. crispus*) originated in Madagascar and slowly distributed to the Malay Archipelago region (Burkill, 1935). *S. crispus* is a shrub with darker green colour on its upper surface than underneath the leaf. Because of this characteristic, the Chinese called it “Black-Faced General”. In addition, the underside of the leaves is rough, contrary to the upper surface which is smooth hence the Malays named it “Jin Batu” or

“Pecah Beling”. *S. crispus* is traditionally used as an antidiabetic, antilytic, laxative, anticancer and diuretic agent. Due to its high calcium carbonate content, when *S. crispus* is brewed in water, it causes the water to be slightly alkaline and capable of easing urination (Nurraihana et al., 2013). In Malaysia, the natives chew and swallow the fresh *S. crispus* leaves to strengthen their immune system (Samuel et al., 2010).

Many studies have been conducted on *C. nutans* and its properties such as antioxidant, antiviral, and anti-inflammatory properties. *C. nutans* is commonly applied externally and available as a balm in the traditional market (Shin et al., 2013). Nevertheless, the studies on antimicrobial properties of *C. nutans* in relation to wound healing properties are minimal. A histological study on *S. crispus* reported by Al-Henhena and team (2011) demonstrated the ethanolic leaves extract of *S. crispus* capable of accelerating the wound healing process in rats (Al-Henhena et al., 2011). As such, this study aimed to assess the efficacy of *C. nutans* and *S. crispus* antimicrobial properties in relation to wound healing.

1.2 Problem Statement

Many studies have shown that different extraction methods and solvent polarity affect the outcome of the different bioassays (Khoo et al., 2018). There was little information on the use of a solvent with different polarity correlated in the same bioassays for *C. nutans* and *S. crispus*, respectively. Previous studies on *C. nutans* and *S. crispus*, both had demonstrated their high antioxidant contents and antimicrobial properties against some microbes such as *Staph. aureus* and *Strep. pneumoniae* (Lim et al., 2015; Ong et al., 2020). *S. crispus* was also reported to aid wound healing activity (Al-Henhena et al., 2011). However, only a few intensive studies on wound healing properties (Khoo et al., 2018;

Nurraihana et al., 2013). Besides, the wound healing assay done in the previous study were in-vitro scratch assay under fixed condition, which does not represent an actual wound healing process.

Hence, this study investigates the wound healing properties of different polarity solvent extracts of *C. nutans* and *S. crispus*, respectively, using a modified scratch assay on keratinocytes co-incubated with MRSA at different time points microscopically.

1.3 Objectives

This study aimed to investigate the wound healing efficacy of *C. nutans* and *S. crispus* extracts to provide the scientific data for the claim of ethnobotany use. The specific objectives are outlined as below:

- i. To determine the antioxidant content, Total Phenolics Content (TPC) and Total Flavonoids Content (TFC) of *Clinacanthus nutans* (*C. nutans*) and *Strobilanthes crispus* (*S. crispus*) extracts, respectively.
- ii. To evaluate the antimicrobial properties of *C. nutans* and *S. crispus* extracts respectively against Gram-positive and Gram-negative bacteria based on Disk Diffusion assay.
- iii. To investigate the wound healing properties of different polarity solvent extracts of *C. nutans* and *S. crispus*, using a modified scratch assay on keratinocytes co-incubated with Methicillin-resistant *Staphylococcus aureus* (MRSA).

1.4 Chapter Summary

This chapter introduces the main idea of the study and provides an idea of how this study will be beneficial to our society. Natural products have been used over generations in wound care, which including *Clinacanthus nutans* and *Strobilanthes crispus*. However, there are only a few intensive studies on wound healing properties (Khoo et al., 2018; Nurraihana et al., 2013). Hence, this study investigates the wound healing efficacy of *C. nutans* and *S. crispus* extracts, respectively, using a modified scratch assay on keratinocytes co-incubated with MRSA. In addition, this study also evaluates the phytochemical contents, antioxidant and antimicrobial activities to understand their correlations further to wound healing properties.

CHAPTER 2

LITERATURE REVIEW

2.1 Acanthaceae Family

Anecdotal medicinal plants for traditional herbal remedies from the Acanthaceae family often attract more attention due to their purported medicinal properties. Many studies have been carried out and reported active biological and pharmacological activities. Hussain and Ghani (2008) had studied a few herbal remedies used by the Kaghan Valley natives to cure gastrointestinal disorders. Among these herbals was *Justicia adhatoda* L. from Acanthaceae family. In the study, *J. adhatoda* L. was reported to heal respiratory tract diseases and the cataplasm was used on the fresh wound to alleviate inflammatory swellings. When the *J. adhatoda* L. leaves were prepared according to the traditional herbal remedy, the drinks could relieve stomach pain and dysenteric bleeding (Hussain & Ghani, 2008).

In addition, a study by Prasad (2014) on seven species from Acanthaceae family, namely *Nilgirianthus* sp., *Justicia gendurussa*, *Barleriaprionities*, *Adhatoda zylanica*, and *Hemigraphis colorata* showed promising results in the phytochemical studies. These plants exhibited significant antimicrobial activities against *Pseudomonas* spp., *Staph. aureus* and *E. coli* (Prasad, 2014). Apart from the antimicrobial properties reported, the anti-diabetic properties of Acanthaceae plants were also delineated. Moreover, 16 plants from this Acanthaceae family showed favourable results, such as exhibiting hypocholesterolemic and hypotriglyceridemic properties when the plant extracts were treated on diabetic rats (Kavitha et al., 2015).

Anecdotal medicinal plants usually originated from multiple time-tested properties with varying efficacies due to minimal accessibility and use of technology. For example, *Barleria cristata* L. is known and traditionally used to treat diabetes and respiratory diseases. However, only recently the antioxidant property of the plant was determined and subsequently manufactured as cosmetic and hair products which promote scalp and skin health (Khan et al., 2017). Besides, *B. cristata* L. also showed an anti-inflammation property and it is applied externally (Gambhire et al., 2009).

Acanthus ilicifolius L is another example from Acanthaceae family. According to Indian folklore, it was believed to be aphrodisiac, blood purifier and a treatment for many ailments including skin diseases, snakebites, stomach pain, leukaemia, and asthma (Velmani et al., 2016). Further extensive investigations have extended the use of this plant to treat dyspepsia and paralysis (Singh & Aeri, 2013). According to Velmani et al. (2016), *A. ilicifolius* L exhibited multiple efficacious properties such as anti-inflammatory, anti-cancer, anti-protozoal, anti-microbial, and anti-osteoporotic activities. Due to the variety of efficacies, *A. ilicifolius* L was also known as the “holy leaved acanthus” and commonly used by the Indians and Chinese as traditional medicine (Velmani et al., 2016). Additionally, studies that had been performed were not limited to those benefits to the human body but also the environment. Shackira et al. (2017) investigated the phytostabilization of zinc in the atmosphere by this plant. They discovered it could accumulate a large amount of zinc in the roots of *A. ilicifolius* L with a relatively smaller amount of zinc absorbed in the stem and leaves. Therefore, *A. ilicifolius* L was highly recommended as a promising candidate for zinc phytostabilization in polluted wetlands (Shackira et al., 2017).

While plenty of species from the Acanthaceae family that exhibited numerous beneficial properties, some of them were not found in the folklore. Therefore, in discovering of a new potential natural product, it is pertinent to determine the known efficacious properties alluded by the traditional knowledge of the indigenous people and their folklores, but also prudent to investigate further the existence of other yet to be discovered and documented characteristics and properties.

2.2 *Clinacanthus nutans* (Burm f.) Lindau

Clinacanthus nutans (Burm.f.) Lindau (*C. nutans*) is one of the plants from the Acanthaceae family. It is known by different names in Southeast Asia. In Malaysia, it is called ‘Sabah snake grass’, ‘*Belalai Gajah*’ or ‘憂遁草’. *C. nutans* is a perennial plant that can grow up to 1-meter tall with narrow and lanceolate leaves. *C. nutans* is commonly used by the locals as a traditional medicine remedy for internal and external uses. It is purported to cure oxidative stress-related diseases, snake and insect bites, and skin lesions *in vivo* caused by *herpes simplex* virus (HSV) and *varicella-zoster* virus (VZV) (Shim et al., 2012).



Above photographs showed the close up of *C. nutans* (left) and the bush itself (right).
Photographs courtesy of Mr. Lim Yu Seng.

Figure 2.1: *Clinacanthus nutans*

There have been multiple studies conducted to investigate the antioxidant potential in *C. nutans*. According to Alam et al. (2016), the IC₅₀ of the ethanolic crude extract was 110.4 µg/mL when tested with 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. Besides, chloroform *C. nutans* extract was reported to contain the highest level of antioxidant potential (7852.63 ± 449.90 µg Trolox equivalent/g extract) when tested for their DPPH scavenging activity (Yong et al., 2013). Furthermore, aqueous *C. nutans* extract was effective (32.33 ± 0.97 % scavenging activity at 100 µg/mL) in nitric oxide scavenging activity while methanolic *C. nutans* extract was effective (~34 % scavenging activity at 100 µg/mL) in hydrogen peroxide scavenging activity (Yong et al., 2013). Based on Pongmuangmul et al. (2016) study, monogalactosyl diglyceride (MGDG) (**Figure 2.2: 1**) and digalactosyl diglyceride (DGDG) (**Figure 2.2: 2**) extracted from *C. nutans* have similar effects on Herpes Simplex Virus (HSV-1 and HSV-2) comparable to the commercially available synthetic compounds, such as acyclovir. There were also studies conducted to investigate the cytotoxic effects of *C. nutans* extracts against multiple human cancer cell lines, including liver cancer cell line HepG2, neuroblastoma cell line IMR32, stomach carcinoma cell line SNU-1, cervical adenocarcinoma cell line HeLa, colorectal cancer cell line LS-174T, chronic myeloid leukaemia cell line K562, Burkitt's lymphoma cell line Raji, and its effects in relation to normal primary umbilical vein endothelial cell HUVECs. Based on these results, chloroform extract was the most superior in inhibiting most of the human cancer cell lines, except IMR32. Therefore, chloroform extract of *C. nutans* has been recommended as an alternate adjunctive regimen for cancer prevention or treatment (Yong et al., 2013).

Oxidative stress-related diseases usually involve an imbalance of reactive oxygen species (ROS) built up in our bodies and damage our cells. The most efficacious means to

overcome this issue is by increasing the antioxidants present in our body to remove these ROS. The most effective and practical way is through diet intake. Most plants and fruits, especially those rich in flavonoids contain significant antioxidants (Muflihah et al., 2021, Itam et al., 2021).

Since *C. nutans* has been attributed to its ability to cure oxidative stress-related diseases (Alam et al., 2016, Yong et al., 2013), it is germane associated the relatively high levels of antioxidants in *C. nutans*. There are many studies conducted to investigate the antioxidants present in *C. nutans*. The highest IC₅₀ value recorded was 240.1±15.3 mg/mL from a study with the *C. nutans* samples from Thailand (Wanikiat et al., 2008) (Table 2.1). It was followed by a study done in Indonesia, with the IC₅₀ of 178.4 mg/mL (Agustina, 2011) and 114.5 mg/mL from Tinh (2014). The highest IC₅₀ value recorded with the *C. nutans* samples from Malaysia was 16 mg/mL (Wong et al., 2014), whereas the others were equal to or lower than 4 mg/mL. This demonstrated that geographical variation might contribute to the varying efficacy of *C. nutans* as a potent antioxidant.

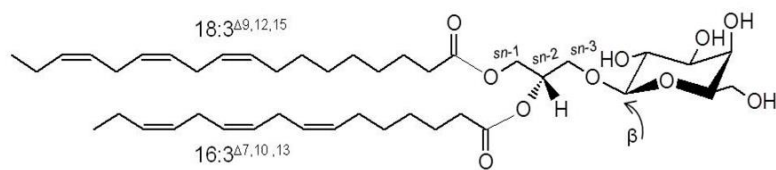
Table 2.1: The antioxidant potential of *C. nutans* extracts based on its DPPH free radical scavenging activities.

Plant source		Control	Extraction Solvent	IC ₅₀ Values	Reference
Country	State				
Malaysia	-		Hot water	16 mg/mL	Wong et al. (2017)
	Seremban	Quercetin	Methanol	1.33±0.0001 mg/mL	Peng et al. (2014)
	Seremban	Quercetin	Methanol	1126.63 µg/mL (Leaves) 1548.89 µg/mL (Stem)	Lee et al. (2014)
	Serdang	BHT, Caffeic acid and α -tocopherol	Methanol	1-year old bud: 64.6 µg/mL 1-year old leaf: 112.1 µg/mL	Ghasemzadeh et al. (2014)
	Taiping		Methanol, petroleum ether, ethyl acetate	4 mg/mL	Arullappan et al. (2014)
	Perak	Green tea	70 % ethanol	Not determined	Shiuan et al. (2012)
	-	-	80 % aqueous methanol	200-300 µg/mL	Tan et al. (2019)
Thailand	-	Trolox	Methanol	240.1±15.3 mg/mL	Wanikiat et al. (2008)
	-	Ascorbic acid	50 % ethanol	110.4±6.59 µg/mL	Pannangpetch et al. (2007)
Vietnam	-	-	Methanol	114.50 mg/mL	Tinh (2014)
Indonesia	-	BHT	Ethyl acetate	178.40 mg/L	Agustina (2011)

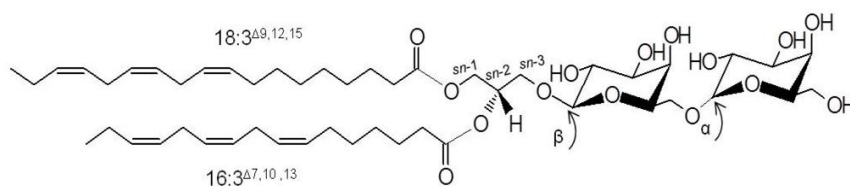
The IC₅₀ values of DPPH free radical scavenging activities of *C. nutans* extracts were summarized in Table 2.1. The *C. nutans* extracts exhibited the antioxidant potential based on the geographical area where it was planted, positive controls used, type of solvent extraction conducted.

Besides, *C. nutans*'s high antioxidant free radical scavenging activities might be related to the presence of different types of bioactive compounds in the extracts, including C-glycosyl flavones (**Figure 2.2: 3**), orientin (**Figure 2.2: 4**) and isoorientin (**Figure 2.2: 5**), in accordance with the report by Alam et al. (2016).

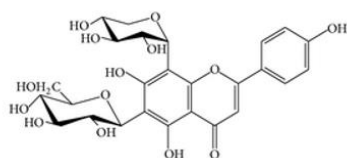
There are many studies have been reported on the investigation of the anticancer properties of *C. nutans* (Ong et al., 2020). The latest study has involved Vero kidney cell lines with multiple *C. nutans* extracts. Using the Neutral Red Uptake (NRU) cytotoxicity assay, the ethyl acetate *C. nutans* extract showed the lowest IC₅₀ of 141.8 µg/mL, followed by hexane and chloroform extracts with IC₅₀ of 560.7 µg/mL and 593.4 µg/mL respectively. Furthermore, ethanol, methanol, and water extracts were investigated. The IC₅₀ recorded for ethanol extract was higher than 640 µg/mL while both methanol and water extracts recorded no result (Ong et al., 2020). Based on the results by Fong (2015), a cell counting kit (CCK-8) assay was used to determine the cold and hot water extracts against human melanoma cell lines (D24 and MM-418C1), human breast adenocarcinoma (MCF-7), and human breast carcinoma (BT-474). The IC₅₀ recorded were 1.63 mg/mL for D24 cell line and 2.0 mg/mL for both MM-418C1 and MCF-7 cell lines respectively. However, there was no IC₅₀ recorded for BT-474.



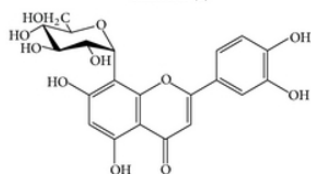
(1) Monogalactosyl diglyceride (MGDG)



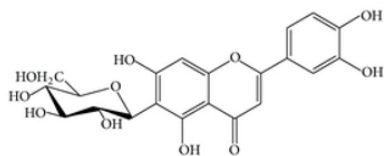
(2) Digalactosyl diglyceride (DGDG)



(3) C-glycosyl flavones



(4) Orientin



(5) Isoorientin

Figure 2.2: Bioactive Compunds in *Clinacanthus nutans* (*C. nutans*)

Two apoptosis assays, namely MTT and Sulforhodamine B (SRB) assays, were conducted on the MCF-7 cell line. The MTT assay for the methanol and ethyl acetate extracts recorded IC₅₀ values of 35.0 µg/mL and 30.0 µg/mL, respectively (Teoh et al., 2017). In addition, the SRB assay carried out by Dan (2014) also showed the methanolic *C. nutans* extract exhibited moderate cytotoxicity with an IC₅₀ value of 66.57 µg/mL. Dan (2014) study also revealed that *C. nutans* demonstrated anti-cancer activities against other cell lines namely, human liver hepatocellular carcinoma cell line (HepG2) (IC₅₀ = 36.80 µg/mL), human cervical cancer cell line (HeLa) (IC₅₀ = 91.08 µg/mL), and human lung carcinoma cell line (NCI-H460) (IC₅₀ = 57.36 µg/mL). Previous studies done on HeLa cell line reported significant variation in IC₅₀ values (**Table 2.2**). Different solvent extracts, namely methanol and petroleum ether results exhibited different cytotoxicity. For example, the methanolic *C. nutans* extract recorded the weakest cytotoxicity with the highest IC₅₀ value (IC₅₀ = 91.08 µg/mL) when it was conducted using SRB assay (Dan, 2014) and MTT assay (IC₅₀ = 56.8 µg/mL) (Ghasemzadeh et al., 2014). The petroleum ether extract revealed higher cytotoxicity with a lower IC₅₀ value of 18.0 µg/mL based on the MTT assay (Arullappan et al., 2014). However, according to Teoh (2017), when 50 µg/mL of methanol and petroleum ether *C. nutans* extracts were treated on HeLa cells, only an estimated 25 % inhibition was recorded for both extracts with MTT assay, respectively. These varying results showed that more studies needed to be done by comparing different solvent extracts with different extraction duration despite treating the same cell lines to identify the effectiveness of *C. nutans* extracts regarding cytotoxicity properties.

Table 2.2: IC₅₀ of different solvent *C. nutans* extracts when treated on different cancer cell lines using various bioassays with their respectively study references.

Solvent	Bioassay	IC ₅₀ Values when Treated on Cell Lines	References
Cold Water	CCK-8	D24: 1.63 mg/mL ; MM-418C1 : 2 mg/mL ; MCF-7 : 2 mg/mL ; BT-474 : -ve	Fong (2015)
How Water		MM-418C1 : 2 mg/mL ; MCF-7 : 2 mg/mL ; BT-474 : -ve	
N/A	MTT	K-562 : -ve ; HCT-116 : -ve	Esmaili et al. (2013)
Chloroform	MTT	K-562 : 47.7 µg/mL ; Raji : 47.31 µg/mL ; IMR-32 : -ve	Yong et al. (2013)
Methanol	MTT	HeLa : 56.8 µg/mL	Ghasemzadeh et al. (2014)
N/A	CCK-8	D24 : 0.95 mg/mL (24 hrs) ; 0.77 mg/mL (72 hrs)	Fong et al. (2016)
Methanol	SRB	MCF-7 : 66.57 µg/mL ; Hep-G2 : 36.80 µg/mL ; HeLa : 91.08 µg/mL ; NCI-H460 : 57.36 µg/mL	Dan (2014)
Methanol	MTT	Hep-G2 : 76.66 µg/mL (after 10 mins of PDT)	Tan et al. (2019)
N/A	MTT	-ve	Liew et al. (2012)

Table 2.2 continued

Methanol	MTT	Hep-G2 : 43.93 µg/mL	Abd Hamid & Yahaya (2016)
Chloroform		Hep-G2 : 55.61 µg/mL	
Ethyl acetate		Hep-G2 : 62.06 µg/mL	
Hexane		Hep-G2 : 68.38 µg/mL	
Methanol	MTT	MCF-7 : 35.0 µg/mL ; HeLa : 50.0 µg/mL (~25 % inhibition)	Teoh et al. (2017)
Ethyl Acetate		MCF-7 : 30.0 µg/mL	
Petroleum ether		HeLa : 50 µg/mL (~25 % inhibition)	
Petroleum ether	MTT	K-562 : 20.0 µg/mL ; HeLa : 18.0 µg/mL	Arullappan et al. (2014)
Hexane	NRU	Vero : 560.7 µg/mL	Ong et al. (2020)
Chloroform		Vero : 593.4 µg/mL	
Ethyl acetate		Vero : 141.8 µg/mL	
Ethanol		Vero : >640 µg/mL	

Note:

CCK-8: Cell Counting Kit; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl-2H-Tetrazolium Bromide; SRB: Sulforhodamine B;

NRU: Neutral Red Uptake; D24: Human Melanoma Cancer Cell Line; MM-418C1: Human Melanoma Cancer Cell Line;

MCF-7: Human Breast Cancer Cell Line; BT-474: Human Breast Tumour Cell Line; K562: Human Erythroleukemic Cell Line;

HCT-116: Human Colon Cancer Cell Line; Raji: Human Continuous Cell Line; IMR-32: Neuroblastoma Cell Line;

HeLa: Human Cervical Cancer Cell Line; HepG2: Human Liver Hepatocellular Carcinoma Cell Line;

NCI-H460: Human Non-Small Cell Lung Cancer Cell Line; Vero: African Green Monkey Kidney Epithelial Cell Line.

2.3 *Strobilanthes crispus* (L.) Blume

Strobilanthes crispus (*S. crispus*) (**Figure 2.3**) is also from the *Acanthaceae* family. It is a shrub that can be easily differentiated from the others due to the leaves colour and texture. *S. crispus* leaves are usually darker in colour and smooth on the upper surface, but the other surface is lighter in colour and rough. Therefore, *S. crispus* is also known as “Black-Faced General, 黑面将军” in Chinese, “*Jin Batu*” or “*Pecah Beling*” in the Malay language by the local folks. *S. crispus* is believed to be originated from Madagascar and is distributed to Southeast Asia gradually over time (Burkill, 1935). The locals often dried the leaves and used them in traditional medicine or infused fresh leaves. They believed that consuming *S. crispus* daily, can strengthen their immunity and overall health (Chua et al., 2019).



The photographs above showed the close up (left) of *Strobilanthes crispus* (*S. crispus*) and its shrubs (right). Photographs courtesy of Mr. Lim Yu Seng.

Figure 2.3: *Strobilanthes crispus*

Previous studies on *S. crispus* revealed it exhibited various beneficial properties including antioxidant, antimicrobial, anti-diabetic, anti-diuretic and anticancer (**Table 2.3**). According to Abu Bakar et al. (2006), old *S. crispus* leaves contained higher antioxidants compared than fermented leaves. Besides, different extraction solvent extracts would result in different antioxidants scavenging activities. For example, the antioxidant scavenging potential obtained from the aqueous extract was 28.5 %, while that of the ethanol extract was 14.5 % (Qader et al., 2011).

Interestingly, the antioxidant content of *S. crispus* distributed and grown all over Malaysia differed widely, indicative of the high likelihood that geography and its surrounding environmental characteristics, including soil properties, affected *S. crispus* chemical profiles and subsequently its bio-profiles (Ghasemzadeh et al., 2015). According to Ghasemzadeh et al. (2015), *S. crispus* demonstrated antioxidant and anticancer properties. For instance, when *S. crispus* extract was tested against the HeLa cell line, it triggered a strong apoptotic reaction. It also showed a promising result with IC_{50} of 182.5 $\mu\text{g/mL}$ from the aqueous crude extract. Tan et al. (2019) investigated its anticancer properties and found that *S. crispus* was able to kill HepG2 liver cancer cells when they were exposed to light. *C. nutans* was also assessed in this study and was found to show the same properties. Nonetheless, *S. crispus* was superior to *C. nutans* and is a promising candidate as a photosensitizing agent.

Natural products' most common efficacious property is their antioxidant potential, a plethora of studies had been conducted to assess the antioxidant content. However, as shown in **Table 2.3**, the extraction solvents and standard control for DPPH were often not standardised. The results ranged from EC_{50} of 12.6 $\mu\text{g/mL}$ to 100 $\mu\text{g/mL}$ or 12.59 % inhibition to 63.21 % inhibition.

Table 2.3: Antioxidant activity of *S. crispus* extracts based on DPPH free scavenging assay

Solvent	Standard Reference Used	DPPH Free Scavenging Activity	Reference
Methanol	Gallic acid; ascorbic acid; quercetin; Beta Hydroxy Acid (BHA)	EC ₅₀ : 12.6 µg/mL; 25.5 µg/mL; 15.3 µg/mL; 21.9 µg/mL	Muslim et al. (2010)
Aqueous	Gallic acid	28.5 % Inhibition	Qader et al. (2011)
Ethanol	Gallic acid	14.5 % Inhibition	
Hot water	Green tea; Black tea	Young Leaves: (Unfermented) 61.22 % Inhibition (Fermented) 12.59 % Inhibition Old Leaves: (Unfermented) 63.21 % Inhibition (Fermented) 27.58 % Inhibition	Abu Bakar et al. (2006)
Methanol	N/A	EC ₅₀ : ~100 µg/mL	Tan et al. (2019)
Aqueous	Butylated Hydroxytoluene (BHT)	IC ₅₀ : 78.3 µg/mL (Penang, Malaysia) IC ₅₀ : 58.2 µg/mL (Selangor, Malaysia) IC ₅₀ : 44.1 µg/mL (Kelantan, Malaysia)	Ghasemzadeh et al. (2015)
Ethanol		IC ₅₀ : 146.5 µg/mL (Penang, Malaysia) IC ₅₀ : 117.6 µg/mL (Selangor, Malaysia) IC ₅₀ : 80.5 µg/mL (Kelantan, Malaysia)	

Note: EC₅₀: Half-Maximal Effective Concentration; IC₅₀: Half-Maximal Inhibitory Concentration.

There were two common extraction solvents involved: aqueous and ethanol. The aqueous extract showed a lower IC₅₀ value compared with the ethanol extracts. The lowest IC₅₀ for aqueous extract recorded was 44.1 µg/mL (Kelantan), followed by 58.2 µg/mL (Selangor) and 78.3 µg/mL (Penang). For ethanol extracts, Kelantan extracts showed IC₅₀ of 80.5 µg/mL, followed by Selangor and Penang with IC₅₀ of 117.6 µg/mL and 146.5 µg/mL, respectively. Even though involved different solvents, both showed the same trend as the extracts from Kelantan were superior to those of Selangor and Penang. Similarly, both extracts from Kelantan exhibited higher antioxidants than the extracts from Selangor and Penang (**Table 2.3**). Previous studies also demonstrated the ecology affected the antioxidant contents as reported by Ghasemzadeh (2015) with *S. crispus* (**Table 2.1**).

Even though the local did not know *S. crispus* for cancer treatment, a plethora of anti-cancer studies had been conducted on various parts of the *S. crispus* plant. These studies demonstrated that different solvent extracts of *S. crispus* exhibited cytotoxicity activities against a few cancer cell lines, including MCF-7, Hep-G2, and MCF-7 (**Table 2.4**). The ethanolic *S. crispus* extract with the lowest IC₅₀ value of 30.0 µg/mL was superior to other solvent extracts against MCF-7 (Chong et al., 2012). It was followed by hot water extract of fermented old *S. crispus* leaves at IC₅₀ of 72.5 µg/mL, and hot water extract of unfermented old *S. crispus* leaves at IC₅₀ of 80.5 µg/mL. The latter were considered to have weaker effects on the MCF-7 cell line. For young *S. crispus* leaves, unfermented and fermented leaves extracts were recorded with IC₅₀ higher than 100 µg/mL (Abu Bakar et al., 2006). The resultant *S. crispus* extracts also demonstrated different cytotoxic properties when different polarity solvents were used for extraction. According to Muslim et al. (2010), the aqueous extract recorded IC₅₀ of 120.7 µg/mL, and the methanolic *S. crispus* extract recorded IC₅₀ of

160.16 $\mu\text{g/mL}$. On the contrary, Endrini et al. (2007) and Rahmat et al. (2006) found no effect against these cell lines with chloroform and methanol *S. crispus* extracts.

However, *S. crispus* extracts was shown to be potential anti-cancer chemopreventive candidates against Hep-G2 and Caco-2 cell lines. From **Table 2.4**, the chloroform extract of *S. crispus* showed a moderate effect when exposed to Hep-G2 with the IC_{50} of 28.0 $\mu\text{g/mL}$ (Endrini et. al., 2007) while Rahmat et al. (2006) showed that the methanolic *S. crispus* extract exhibited a similar performance with IC_{50} of 29.3 $\mu\text{g/mL}$ and 28.0 $\mu\text{g/mL}$ for chloroform extract respectively. However, the IC_{50} was higher than 200 $\mu\text{g/mL}$ with methanol and acetone extracts by Muslim et al. (2010). Thus, the weakest performance was recorded.

The lowest IC_{50} recorded with the strongest effect on Hep-G2 was from Tan (2019), which was 8.51 $\mu\text{g/mL}$, but there was an extra procedure prior to the MTT assay. After the methanol extract was applied to the Hep-G2 for 2 hours, it underwent a 10-minute photodynamic therapy (PDT) before another 24 hours of incubation for the MTT assay. This study by Tan (2019) showed a high possibility that PDT was able to improve the performance of the extracts when the combination was applied to the cancer cell lines. Therefore, more studies should be done on different cancer cell lines.

According to Endrini et al. (2007), when tested on Caco-2 cell line, the chloroform extract showed IC_{50} of 25.1 $\mu\text{g/mL}$, similarly Rahmat et al. (2006) recorded an IC_{50} of 25.1 $\mu\text{g/mL}$ with the chloroform extract. However, the methanol *S. crispus* extract obtained a lower IC_{50} value of 22.3 $\mu\text{g/mL}$ when tested on Caco-2 cell line. In addition, Rahmat et al. (2006) tested the methanolic *S. crispus* extract on MDA-MB-231 cell line and the IC_{50} recorded was 27.2 $\mu\text{g/mL}$.

According to Chong et al. (2012), ethanolic *S. crispus* extracts were applied on various cancer cell lines, which including MCF-7, MDA-MB-231, HeLa and HT-29. The ethanolic *S. crispus* extract performed the best on MCF-7, followed by HT-29 ($IC_{50} = 52.0 \mu\text{g/mL}$), HeLa ($78 \mu\text{g/mL}$) and lastly MDA-MB-231 ($>100 \mu\text{g/mL}$) showed the least effect. Ismail et al. (2012) also investigated extracts from different parts of the *S. crispus* plant on human colorectal cell line HT-29. The results were promising as most of the extracts showed competitive results. The ethyl acetate extract of the leaves and flowers showed IC_{50} of $70.2 \mu\text{g/mL}$ and $42.0 \mu\text{g/mL}$. Furthermore, the methanol extract of the leaves obtained IC_{50} of $59.0 \mu\text{g/mL}$, while the dichloromethane extract of the flower showed IC_{50} of $90.3 \mu\text{g/mL}$.

These findings supported the geographical factor that may affect the results of the extracts. The study collected different *S. crispus* samples from different regions of Malaysia, resulting in different cytotoxicity against the HeLa cell lines. The findings are in agreement with Ghasemzadeh et al. (2015); *S. crispus* extract from Kelantan recorded the lowest IC_{50} value of $182.5 \mu\text{g/mL}$, followed by that of Selangor ($IC_{50} = 266.4 \mu\text{g/mL}$) and Penang ($IC_{50} = 331.5 \mu\text{g/mL}$).

In addition, *S. crispus* was proven to be an anti-diuretic. The accumulation of calcium oxalate crystals (CaOx) in our body was believed to be one of the main inorganic salts that caused urolithiasis. Gul et al. (2020) reported that the *S. crispus* methanol extract could inhibit the formation of CaOx by 50.54 % while the ethyl acetate extract could breakdown the CaOx crystals effectively by 52.5 %. Therefore, the methanol and ethyl acetate extracts of *S. crispus* exhibited anti-diuretic properties. More studies are needed to investigate the mechanism behind these anti-diuretic processes.

Table 2.4: IC₅₀ of MTT assays against different cancer cell lines on *C. nutans* solvent extracts.

Solvent	IC ₅₀ values when treated on cell lines	References
Hot Water	MCF-7: [Young leaves] Unfermented & fermented: >100 µg/mL [Old Leaves] Unfermented: 80.5 µg/mL Fermented: 72.5 µg/mL MDA-MB-231 : [Young Leaves & Old Leaves] Unfermented & fermented:>100 µg/mL	Abu Bakar et al. (2006)
Methanol	MCF-7: 160.16 µg/mL HCT-116: >200.0 µg/mL Hep-G2: >200.0 µg/mL T-47D: 121.53 µg/mL NCI-H23: >200.0 µg/mL CCD-18Co: >200.0 µg/mL	Muslim et al. (2010)
Aqueous	MCF-7: 120.7 µg/mL HCT-116: >200.0 µg/mL Hep-G2: >200.0 µg/mL T-47D: >200.0 µg/mL NCI-H23: >200.0 µg/mL CCD-18Co: >200.0 µg/mL	
Chloroform	MCF-7: -ve MDA-MB-231: -ve Hep-G2: 28.0 µg/mL Caco-2: 25.1 µg/mL Chang Liver Cell: -ve	Endrini et al. (2007)
Methanol	MCF-7: -ve MDA-MB-231: 27.2 µg/mL Hep-G2: 29.3 µg/mL Caco-2: 22.3 µg/mL Chang Liver Cell: -ve	Rahmat et al. (2006)
Chloroform	Hep-G2: 28.0 µg/mL Caco-2: 25.1 µg/mL	
Ethanol	MCF-7: 30.0 µg/mL MDA-MB-231: >100 µg/mL HeLa: 78.0 µg/mL HT-29: 52.0 µg/mL	Chong et al. (2012)
Ethyl acetate	HT-29: 70.2 µg/mL (Leaves) 42.0 µg/mL (Flowers)	Ismail et al. (2012)
Methanol	HT-29: 59.0 µg/mL (Leaves)	
Dichloromethane	HT-29: 90.3 µg/mL (Flowers)	
Methanol	Hep-G2: 8.51 µg/mL (After 10 mins PDT)	Tan et al. (2019)
Ethanol	HeLa: 331.5 µg/mL (Penang) 266.4 µg/mL (Selangor) 182.5 µg/mL (Kelantan)	Ghasemzadeh et al. (2015)

Note:

MCF-7: Human Breast Cancer Cell Line; MDA-MB-231: Human Invasive Ductal Carcinoma Cell Line

HCT-116: Human Colon Cancer Cell Line; HepG2: Human Liver Hepatocellular Carcinoma Cell Line;

T-47D: Human Breast Cancer Cell Line ; NCI-H23: Human Non-Small Cell Lung Carcinoma ;

CCD-18Co: Normal Human Colon Fibroblast Cell Line ;

CaCo-2: Human Colorectal Adenocarcinoma Cell Line ;

Chang Liver Cell: Derived HeLa Cell Line ; HeLa: Human Cervical Cancer Cell Line;

HT-29: Human Colon Adenocarcinoma Cell Line

2.3.1 External (Topical) Application of *Clinacanthus nutans* and *Strobilanthes crispus*

All *C. nutans* balms are available in the traditional markets. It is used topically to ease skin irritation, insect bites, scorch, itches, and eczema rash (Shin et al., 2013). On the other hand, *S. crispus* is usually consumed as tea and hardly used topically. However, this does not indicate that *S. crispus* does not exhibit antimicrobial or wound healing properties.

From **Table 2.5**, Yang and his co-researchers (2013) assayed the antimicrobial activities of methanolic *C. nutans* extracts against *Staph. aureus*, *B. cereus*, *P. acnes*, *Staph. epidermidis*, and *E. coli*. The findings revealed *C. nutans* showed an inhibitory effect against four bacteria strains and was highly potent against *Staph. aureus* and *E. coli* with MIC values of 12.50 mg/mL respectively (Yang et al., 2013). Nevertheless, another study against *Staph. aureus* recorded a higher MIC value of 62.5 mg/mL (Tinh, 2014). Besides, Tinh also reported that *C. nutans* methanol extract exhibited strong antimicrobial activity against *Salmonella typhi*, a Gram-negative bacterium at a MIC of 125 mg/mL (Tinh, 2014). These findings were also supported by Sekar and Rashid (2016). They reported that *C. nutans* methanol extract with a concentration of 25 mg/mL showed active inhibitory effects against two Gram-positive and two Gram-negative bacteria. The most significant zone of inhibition

(ZOI) was observed in *Staph. aureus* with a diameter of 23 mm, followed by *E. coli* (D = 10 mm), *Ps. aeruginosa* (D = 9.33 mm), and *B. cereus* (D = 8 mm), respectively.

These findings were consistent and verified by Ong et al. (2020), who reported *C. nutans* extracted with methanol, chloroform, ethyl acetate, hexane, ethanol, and water respectively exhibited strong antimicrobial activity against *Ps. aeruginosa* with an IC₅₀ value of 0.63 mg/mL. In addition, the hexane and ethyl acetate extracts demonstrated active inhibitory effects against all the bacteria involved, namely *Staph. aureus*, *B. cereus*, *Ps. aeruginosa*, *K. pneumoniae*, and *E. coli*. The MIC recorded for *K. pneumoniae* was 0.63 mg/mL with ethyl acetate *C. nutans* extract, 1.25 mg/mL for hexane extract and 2.5 mg/mL for ethanol extract. Furthermore, both hexane and ethyl acetate *C. nutans* extracts showed MIC of 0.63 mg/mL when treated on *E. coli*. The Gram-negative bacteria were more susceptible to the *C. nutans* extracts than Gram-positive bacteria (Ong et al., 2020).

There were many more studies performed with other extraction solvents, and some of them showed promising results. The ethyl acetate *C. nutans* extract exhibited a MIC of 6.31 mg/mL when treated on *B. cereus* while the ethyl acetate *C. nutans* extract showed a MIC of higher than 100 mg/mL when treated on *Salmonella typhi* and *E. coli* (Arullappan et al., 2014). Wong et al. (2013) investigated the hot water *C. nutans* extract on *Staph. aureus*, *M. luteus*, *Ps. aeruginosa* and *E. coli*, and all of them showed MIC higher than 50 mg/mL. According to Chomnawang et al. (2005), when ethanol *C. nutans* extract was tested on bacteria were commonly found on human skin surfaces, namely *P. acnes* and *Staph. epidermidis*, the MIC for both was higher than 5 mg/mL. Chomnawang et al. (2005) repeated the test of the ethanol *C. nutans* extract on MRSA in 2008 and found the MIC was higher than 5 mg/mL.

There were several reports on the antimicrobial properties of *S. crispus* (**Table 2.6**) but the data obtained were inconsistent. The earliest study conducted on the *S. crispus* antimicrobial properties was by Abou Muamar (1999). The study involved *Staph. aureus*, *Ps. aeruginosa*, *Salmonella typhi* and *Vibrio cholera*. The results showed that when the concentration of methanol extract at 38.481 mg/kg and 35.539 mg/kg were effective in inhibiting the growth of *Staph. aureus* and *Salmonella typhi*, respectively but no inhibition was observed in the other bacteria. Muskhazli (2009) conducted agar well diffusion assay on medicinal plants demonstrated that the methanol extract of *S. crispus* exhibited strong antimicrobial activity against *Bacillus cereus* with a MIC value of 2 mg/mL. Another study by Lim (2015) on two Gram-positive and two Gram-negative bacteria revealed that the ethanol extracts of *S. crispus* were only effective against Gram-positive bacteria such as *Staph. aureus* and *Strep. pneumoniae* with MIC values of <50 mg/mL and 100 mg/mL, respectively.

A monumental study by Al-Henhena et al. (2011) changed the perspective toward *S. crispus*. In the histological examination, ethanolic *S. crispus* extract was tested on rats to accelerate the wound healing. The intrasite gel was used as a control. When 200 mg/mL of ethanolic *S. crispus* extract was applied to the wound, the healing time recorded was similar to that of the control. However, no histology study had yet to be done on *C. nutans* extracts.

Overall, both *C. nutans* and *S. crispus* exhibited efficacious properties such as antioxidant, anticancer, and antimicrobial properties. Nonetheless, there is still a knowledge gap between the comparison of extracts from different extraction solvents and their effects on the specific treatments, such as cytotoxicity on cancer cell lines. Albeit there were many reports on antimicrobial properties of *C. nutans*, there is still no *in vivo* experimental model has been performed on *C. nutans* to determine the wound healing property. While a

histological study was done on *S. crispus* extract and showed that the ethanol *S. crispus* extract was capable of accelerating the wound healing process (Al-Henhena et al., 2011), there is still no study done on co-incubation of bacteria on skin fibroblasts before and after exposure to these extracts. Therefore, this study aims to delineate the efficacy of these two plants' extracts as ROS scavengers, their ability to inhibit bacteria growth, and their ability to promote *in vitro* wound healing.

Table 2.5: The Minimum Inhibition Concentration (MIC) of Gram-positive and Gram-negative bacteria when treated with different extracts of *C. nutans*.

Reference	Gram-positive bacteria							Gram-negative bacteria			
	<i>Staph. aureus</i>	<i>MRSA</i>	<i>M. luteus</i>	<i>B. cereus</i>	<i>Strep. pneumoniae</i>	<i>P. acnes</i>	<i>Strep. epidermidis</i>	<i>Ps. aeruginosa</i>	<i>Salmonella typhi</i>	<i>K. pneumoniae</i>	<i>E. coli</i>
<i>Clinacanthus nutans</i> (<i>C. nutans</i>)											
Arullappan et al. (2014)	-	-	-	EA: 6.31 mg/mL (MIC)	-	-	-	-	EA: >100 mg/mL (MIC)	-	EA: >100 mg/mL (MIC)
Sekar & Rashid (2016)	M: 25 mg/mL (23.0 mm inhibition zone)	-	-	M: 25 mg/mL (8.0 mm inhibition zone)	-	-	-	M: 25 mg/mL (9.33 mm inhibition zone)	-	-	M: 25 mg/mL (10.0 mm inhibition zone)
Yang et al. (2013)	M: 12.50 mg/mL (MIC)	-	-	M: >12.50 mg/mL (MIC)	-	M: >12.50 mg/mL (MIC)	M: >12.50 mg/mL (MIC)	-	-	-	M: 12.50 mg/mL (MIC)
Chomnawang et al. (2005)	-	-	-	-	-	E: >5 mg/mL (MIC)	E: >5 mg/mL (MIC)	-	-	-	-
Chomnawang et al. (2009)	-	E: >5 mg/mL (MIC)	-	-	-	-	-	-	-	-	-
Tinh (2014 cited in Khoo, 2018)	M: 62.5 mg/mL (MIC)	-	-	-	-	-	-	-	M: 125 mg/mL (MIC)	-	-
Wong et al. (2013)	HW: >50 mg/mL (MIC)	-	HW: >50 mg/mL (MIC)	-	-	-	-	HW: >50 mg/mL (MIC)	-	-	HW: >50 mg/mL (MIC)
Ong C.W. (2020)	H: 2.5 mg/mL EA: 2.5 mg/mL (MIC)	-	-	H: 1.25 mg/mL C: 2.5 mg/mL EA: 0.63 mg/mL E: 2.5 mg/mL (MIC)	-	-	-	M: 0.63 mg/mL H: 1.25 mg/mL C: 0.63 mg/mL EA: 0.63 mg/mL E: 0.63 mg/mL W: 2.5 mg/mL (MIC)	-	H: 1.25 mg/mL EA: 0.63 mg/mL E: 2.5 mg/mL (MIC)	H: 0.63 mg/mL EA: 0.63 mg/mL (MIC)

Table 2.6: The Minimum Inhibition Concentration (MIC) of Gram-positive and Gram-negative bacteria when treated with different extracts of *S. crispus*.

Reference	Gram-positive bacteria							Gram-negative bacteria			
	<i>Staph. aureus</i>	<i>MRSA</i>	<i>M. luteus</i>	<i>B. cereus</i>	<i>Strep. pneumoniae</i>	<i>P. acnes</i>	<i>Strep. epidermidis</i>	<i>Ps. aeruginosa</i>	<i>Salmonella typhi</i>	<i>K. pneumoniae</i>	<i>E. coli</i>
<i>Strobilanthes crispus</i> (<i>S. crispus</i>)											
Abou Muamar (1999)	M: 38.481 mg/kg (MIC)	-	-	-	-	-	-	-ve	M: 35.539 mg/kg (MIC)	-	-
Muskhazli (2009)	-	-	-	M: 2 mg/mL (MIC)	-	-	-	-	-	-	-
Lim V.H. et al. (2015)	E: <50 mg/mL (MIC)	-	-	-	E: 100 mg/mL (MIC)	-	-	-ve	-	-ve	-

CHAPTER 3

MATERIALS AND METHODS

3.1 Plant Collection

The raw plant materials, namely *Clinacanthus nutans* (*C. nutans*) and *Strobilanthes crispus* (*S. crispus*) were collected from different housing areas in Kuching, Sarawak. The leaves were cleaned and left to dry at room temperature for three weeks. A domestic grinder (Sharp, Osaka, Japan) was used to ground the hand-picked leaves to powder form and packed in a zip-lock bag. It was kept in the dark for future use.

3.2 Extraction

The extraction used three solvents with different polarities: ethanol, acetone and chloroform, using the cold maceration method. A sample of 50 g dried *C. nutans* powder was weighed and mixed with 500 mL of ethanol. The mixture was stirred constantly with a magnetic stirrer. After 24 hours, the mixture was filtered with Whatman© No. 93 filter paper (Whatman, Buckinghamshire, United Kingdom) and transferred to a rotary evaporator (BUCHI, Flawil, Switzerland). The rotary evaporator was used to remove the solvent in the filtrate. The extract was removed from the rotary evaporator and transferred into an amber centrifuge tube (Biologix, Jinan, China), and wrapped in aluminium foil (Diamond, Malaysia). It was kept in a refrigerator at 4-8°C prior to use.

The procedures were repeated with acetone and chloroform on the powdered samples of *C. nutans*. The powdered samples of *S. crispus* were extracted with ethanol, acetone and chloroform with the same procedure as well.

3.3 Phytochemical Screening

3.3.1 Alkaloids Test

Dragendorff's reagent (potassium iodide-bismuth nitrate) is used to detect the presence of alkaloids (Shah and Hossain, 2011). A stock solution was prepared by mixing 0.5 g of bismuth (III) nitrate (Sigma-Aldrich, Missouri, USA) dissolved in 8 mL of distilled water and 2 mL concentrated hydrochloric acid, 4 M (Fisher Chemical, Massachusetts, USA). To this stock solution, 5 g of potassium iodide (Sigma-Aldrich, Missouri, USA) were dissolved with distilled water and topped up to the calibration mark in a 10 mL volumetric flask to achieve a 50 % w/v potassium iodide solution. This stock solution was kept in an amber glass bottle. The potassiumtetraiodobismuthate ($K[BiI_4]$) combined with the ammonium salt in the alkaloid would form insoluble complex salt (Raaijmakers et al., 2020). When the sample containing alkaloid was mixed with this Dragendorff's reagent (2:1) and left to stand for 5 minutes, these ion pairs would form an insoluble orange-red coloured complex visible to the naked eye. The observations were recorded based on the colour intensity, the strongest colour intensity will be recorded as 4+.

3.3.2 Flavonoids Test

The presence of flavonoids was tested according to Gul et al. (2017) with slight modifications. The extracts were mixed with 2 mL of sodium hydroxide and resulted in a yellow mixture. The mixture was then added with hydrochloric acid, 4 M (Fisher Chemical, Massachusetts, USA) slowly until colourless. The stronger the yellow colour intensity of the yellow mixture, more hydrochloric acid will be required to be added in order for the mixture to turn colourless, thus the value recorded will be bigger.

3.3.3 Saponin Test

The ethanol *C. nutans* extract was transferred into a test tube, and 5 mL of distilled water was added into the tube. The tube was sealed with Parafilm® (Sigma-Aldrich, Missouri, USA) and shaken vigorously before it was left to stand for 5 minutes. The foam was observed and recorded its height. This was repeated for the other extracts from *C. nutans* and *S. crispus* respectively.

3.4 Total Phenolics Content (TPC)

Folin-Ciocalteu Reagent (Sigma-Aldrich, Missouri, USA) was used to determine the total phenolics content (TPC) according to Chan et al. (2012). A 10 mg ethanolic *C. nutans* extract sample was dissolved with 1 mL of ethanol in a test tube. From the mixture, 200 µL was transferred into a new tube and made up to 3 mL. It was mixed with 0.5 mL of 50 % (v/v) Folin-Ciocalteu Reagent (Sigma-Aldrich, Missouri, USA) and 2.0 mL of 20 % (w/v) sodium bicarbonate (Sigma-Aldrich, Missouri, USA) solution before incubating in the dark for 60 minutes at room temperature. The wavelength of 650 nm was used to measure the absorbance of the mixtures with a spectrophotometer (DeNovix DS-11 FX, Wilmington, USA) and the standard reference used was Gallic acid (Sigma-Aldrich, Missouri, USA). All the extracts for *C. nutans* and *S. crispus* respectively, and standard solutions were prepared using a serial dilution. All the experiments were conducted in triplicates.

3.5 Total Flavonoids Content (TFC)

The aluminium colourimetric method was used to access the total flavonoid content (TFC) of the extracts, based on Baba and Malik (2015) with slight modifications. A sample of 10.0 mg plant extract was dissolved in 1.0 mL of methanol (Fisher Chemical, Massachusetts, USA). Once dissolved, 500 μ L of the mixture were transferred into a new tube and made up to 5.0 mL. After the mixing, 1.0 mL of the mixture was transferred into a new tube with 1.0 mL of 2 % (w/v) aluminium chloride (AlCl_3) (Sigma-Aldrich, Missouri, USA). It was then incubated in the dark for 10 minutes before the absorbance was measured at a wavelength of 420 nm with a spectrophotometer (DeNovix DS-11 FX, Wilmington, USA). The standard reference used was Rutin (Sigma-Aldrich, Missouri, USA), and the samples were carried out in triplicates.

3.6 Antioxidant (2,2-Diphenyl-1-picrylhydrazyl Assay)

The method used was described by Yong et al. (2013) and Chan et al. (2012) with slight modifications to determine the 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, Missouri, USA) free radical scavenging activity. The extracts were prepared with concentrations of 10, 20, 40, 60, 80, and 100 μ g/mL with a serial dilution. The total volume should be 1 mL for each concentration. One mL of DPPH ethanolic solution (0.2 mM) was added to each tube and sealed. The mixtures were swirled gently for 1 minute and incubated in the dark for 30 minutes before the absorbance was measured using a spectrophotometer (DeNovix DS-11 FX, Wilmington, USA). The wavelength used for the absorbance measurement was 517 nm. A mixture of ethanol (Fisher Chemical, Massachusetts, USA) and DPPH solution (Sigma-Aldrich, Missouri, USA) was used as a negative control for this

experiment. Ascorbic acid (Sigma-Aldrich, Missouri, USA) was used as the standard reference. All the samples and standards were performed in triplicates. After determining the EC₅₀ of the standard reference and the extracts, the results recorded will be in Ascorbic Acid Equivalent to Antioxidant Content (AAEAC).

3.7 Antimicrobial (Disk Diffusion Assay)

3.7.1 Nutrient broth (NB)

The Nutrient Broth (NB) was prepared by mixing 13.0 g NB powder (Oxoid, Basingstoke, England) in 1 L distilled water in a 1 L Schott bottle (Favorit, Puchong, Malaysia). The mixture was brought to boil and aliquot into 3 mL Bijou bottles (Biologix, Jinan, China) before these aliquots were autoclaved at 121°C for 15 minutes. The NB was stored at 4°C, ready for use.

3.7.2 Mueller-Hinton Agar (MHA)

The Mueller-Hinton agar (MHA) was prepared by mixing 38.0 g of MHA powder (Oxoid, Basingstoke, England) in 1 L distilled water in a 1 L Schott bottle. The mixture was brought to boil to dissolve it completely with a constant stirring magnetic stirrer on a heated magnetic hot plate (IKA, Staufen, Germany). The MHA was then autoclaved at 121°C for 15 minutes. The MHA was left to cool in a 60°C water bath and poured into PT90 Petri dish Favorit, Puchong, Malaysia). Once the MHA had solidified, it was incubated overnight to monitor for contamination before storing at 4°C, ready for use.

3.7.3 Mueller-Hinton Broth (MHB)

The Mueller-Hinton Broth (MHB) was prepared by mixing 21.0 g of MHB powder in 1 L distilled water in a 1 L Schott bottle. The mixture was brought to boil and aliquot into 25 mL Universal bottles (Biologix, Jinan, China) before these aliquots were autoclaved at 121°C for 15 minutes. The MHB was stored at 4°C, ready for use.

3.7.4 Antimicrobial Sensitivity Test (AST)

A total of 8 strains of Gram-positive and Gram-negative bacteria that were of clinical importance were used in this study. Some of these were normal flora of the body albeit opportunistic pathogens. The Gram-positive bacteria were *Strep. pyogenes* (ATCC No. 25663, Manassas, USA), *Staph. aureus* (ATCC No. 25923, Manassas, USA), and *Methicillin-resistant Staph. aureus* (ATCC No. 33591, Manassas, USA). The Gram-negative bacteria involved were *Ps. aeruginosa* (ATCC No. 109246, Manassas, USA), *E. coli* (ATCC No. 1100101, Manassas, USA), *Shigella species* (ATCC No. 23354, Manassas, USA), *Salmonella typhi* (ATCC No. 14028, Manassas, USA), and *K. pneumoniae* (ATCC No. BAA-1705, Manassas, USA). The NB was used to promote bacterial growth. The bacteria were freshly cultured 24 hours prior plating as bacterial lawn on MHA to perform the disk diffusion test.

The Disk Diffusion method was conducted according to Vora et al. (2018) with slight modifications. Each bacterium was spread uniformly on a sterile MHA plate with a sterile cotton swab on an inoculating turntable (Fisher Scientific, Massachusetts, USA). The concentration of the extracts used was 1 mg/mL. The sterile blank discs (Oxoid, Basingstoke, England) were placed on the planned area on the bacterial lawn in the MHA plate. The

extract pipetted onto the sterile blank discs was 20 μ L. The method was repeated for the other 7 strains of bacteria in triplicates. Levofloxacin 5 μ g disc (Oxoid, Basingstoke, England) was used as the positive control because it was effectiveness against most Gram-positive and Gram-negative bacteria. It was placed along on each strain of bacterial lawn and performed in triplicates. The MHA plates were incubated at 37°C and observed at 24, 48, and 72 hours. The inhibition diameter for each extract was observed and recorded in mm unit.

3.8 Wound Healing Activity (Scratch Assay)

Skin fibroblast (OUMS-36T-4F, Westmead, Australia) was decided for the *in-vitro* scratch assay. Dulbecco's modified Eagle's medium (Gibco, Waltham, USA) with 10 % Fetal Bovine Serum (Gibco, Waltham, USA) was used for the cell culture. Scratch assays without extracts and bacteria were performed to discover the optimum observation time point and duration for the wound healing activity before the others.

The method conducted was based on Grada et al. (2017) with modification. T25 culture flask (Biologix, Jinan, China) was used to culture the skin fibroblasts for 24 hours. When the adherent cells reached the confluency of 80-85 %, the culture media was removed, and a scratch was created on the marked area to create a pseudo-wound with a sterile 1-mL pipette tip. A volume of 2 mL fresh culture media was added to the T25 flask and incubated at 37°C and 5 % CO₂. The cells were observed under the microscope at intervals of 1-, 3-, 6-, 10-, 24-, and 48-hours.

Based on the preliminary results, the duration sufficient for the cells to migrate and cover up the pseudo-wound was 24 hours incubation. However, observation at a time point of 48 hours was necessary to observe activities of co-incubation of bacteria and cells. The scratch assay was repeated with the respective extracts added to the culture media before adding into the T25 flasks containing the adherent cells.

3.8.1 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

The minimum inhibition concentration (MIC) for the extracts was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Merck, Massachusetts, USA) assay. The working stock was prepared as a 10 % MTT reagent in phosphate buffered saline (PBS) (Sigma-Aldrich, Missouri, USA).

Cell optimisation was conducted, and it was to observe the condition of the cells after incubation of 24- and 48-hour. The density of the cells was started with 10×10^4 cells and serial dilution to a lower density of 6.25×10^3 cells per well in 96-well flat-bottomed culture plate (Biologix, Jinan, China). These cells were incubated at 37°C and 5 % CO₂ for 24 hours., The media was replaced with fresh media after 24 hours of incubation and 20 µL of 10 % MTT reagent was added to these adherent cells. The plate was incubated for another 3 hours in the dark before 100 µL Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Missouri, USA) was added and mixed thoroughly. It was to make sure the formazan crystals were solubilized before the result was read by using a microplate reader (SpectraMax iD3 Multi-Mode Microplate Reader, Molecular Devices, San Jose, USA) at wavelength 570 nm. The cell viability was calculated according to the formula below (Equation 3.1):

$$\text{Cell viability (\%)} = \frac{\text{Absorbance 570 nm of sample}}{\text{Absorbance 570 nm of control}} \times 100 \% \quad \text{Equation 3.1}$$

According to the preliminary result obtained, the most optimum density of 2.2×10^4 cells was selected to determine the MIC of the extracts with the MTT assay. Subsequently, a density of 2.2×10^4 cells per well was seeded in 96 well plates (Biologix, Jinan, China) for 24 hours. The extracts were dissolved with the fresh culture media, and a sterile $0.45 \mu\text{m}$ hydrophilic filter (Sartorius, Göttingen, Germany) was used to filter the mixture.

There were total of 8 test extracts with different concentration involved. To test each extract, fresh culture media containing the extracts replaced the existing culture media from the starting concentration of $500 \mu\text{g/mL}$, with 1:2 serial dilution, the lowest concentration involved was $3.906 \mu\text{g/mL}$. These treated cells were incubated at 37°C with 5 % CO_2 for 24 and 48 hours.

After each incubation time point, the culture media was removed and $200 \mu\text{L}$ fresh culture media and $20 \mu\text{L}$ of 5 mg/mL 10 % MTT reagent were added and incubated in the dark. After incubation for 3 hours, a $100 \mu\text{L}$ DMSO were added and mixed thoroughly to solubilize the formazan crystals before the absorbance was measured. The data was recorded using a microplate reader (SpectraMax iD3 Multi-Mode Microplate Reader, Molecular Devices, San Jose, USA) at 570 nm.

3.8.2 Bacteria Preparation

The bacteria decided for the co-incubation *in-vitro* assay was MRSA as it was well-known as one of the most common pathogens that is difficult to treat with open wounds. From the observation during the preliminary scratch assay, the pseudo-wound closed after

24 hours of incubation. Therefore, the density of MRSA initially added was adjusted and lowered to prevent it from fully taking over the cells before the incubation period of 48 hours was reached. The Optical Density (OD) of the MRSA was recorded at a wavelength of 600 nm with a spectrophotometer (DeNovix DS-11 FX, Wilmington, USA). An inoculation loop with the size of 10 μ L was used. MRSA was transferred into 3 mL fresh NB and incubated overnight.

Scratch assay was repeated with different concentration of MRSA. The concentration of MRSA added into the culture media was recorded based on the volume of MRSA in the total volume of the 2 mL mixture used. The different volumes of MRSA added were 100 μ L (5 %), 20 μ L (1 %), 10 μ L (0.5 %), 5 μ L (0.25 %) and 2 μ L (0.1 %), respectively. These were incubated at 37°C with 5 % CO₂. The observations were recorded after incubation time points of 1 hour, 3 hours, 6 hours, 10 hours, 24 hours, and 48 hours, respectively.

3.8.3 Co-incubation *in-vitro* Assay

From the preliminary MTT assay, the extracts with different polarity solvents exhibited different MIC. The concentration lower than the MIC for the extracts was selected to avoid extracts having any effect on the skin fibroblasts. The concentrations used for *C. nutans* and *S. crispus* were 7 μ g/mL and 70 μ g/mL, respectively.

For the scratch assay with MRSA, the MRSA selected OD₆₀₀ ranges between 0.4 to 0.5 to obtain the most relevant results for 48 hours assay. A 2 μ L of MRSA added corresponding to a negligible 0.1 % of NB was added to the fibroblast culture media.

The scratch assay was conducted according to Grada et al. (2017) with modifications during the co-incubation of MRSA. Skin fibroblasts were cultured in a T25 flask. The flask was then incubated for 24 hours to ensure the fibroblasts reached a confluency of 80-85 %. Scratch assay was performed with a sterile 1 mL pipette tip on the designed area. The media preparation with extracts and MRSA to make up a final volume of 2 mL is summarised in **Table 3.1**. The cells were incubated at 37°C and 5 % CO₂. They were observed immediately after incubation for 1 hour, 3 hours, 6 hours, 10 hours, 24 hours, and 48 hours. Microphotography was conducted to record the wound healing progression at different time points.

Table 3.1: The preparation of culture media with different *C. nutans* and *S. crispus* extracts with MRSA

Plant	<i>Clinacanthus nutans</i>			<i>Strobilanthes crispus</i>		
	Ethanol	Acetone	Chloroform	Ethanol	Acetone	Chloroform
Extracts (1 mg/mL) / μ L	14	14	14	140	140	140
MRSA / μ L	2	2	2	2	2	2
DMEM + 10 % FBS / μ L	1984	1984	1984	1858	1858	1858
Total volume / μ L	2000	2000	2000	2000	2000	2000

CHAPTER 4

RESULTS

The wound healing progression process involved intricate stages including the inflammation stage that produces reactive oxygen species (ROS) (Rodrigues et al., 2019). Both Gram-positive and Gram-negative bacteria are often isolated from these wounds in chronic cases. The ability of a plant extract to elicit antioxidant activity has attributed to its protective effects against ROS addition to its antimicrobial properties. In order to determine the antioxidant potential and antimicrobial properties of *C. nutans* and *S. crispus*, the total phenolic and total flavonoid contents were assessed, and disc diffusion assay was conducted. The wound healing properties of *C. nutans* and *S. crispus* extracts of different solvent polarities solvents using a modified scratch assay on human keratinocytes co-incubated with MRSA at different time points microscopically was performed.

4.1 Determination of Total Flavonoid and Phenolics Contents.

Once the dried powder leaves from *C. nutans* and *S. crispus* were extracted with different polarity solvents, alkaloid, flavonoid and saponin tests were performed. The triplicate results were recorded (**Table 4.1**). In the alkaloid test, the ethanol and acetone extracts for *C. nutans* and *S. crispus* showed intense precipitation of 4+. For the flavonoids test, *C. nutans* ethanol extract exhibited a more vigorous colour intensity of 3, followed by acetone extract and chloroform extract, respectively. On the other hand, the *S. crispus* acetone extracts with higher flavonoid content showed the stronger colour intensity of 3, followed by chloroform extract and ethanolic extract, respectively. For the saponin test, only

C. nutans and *S. crispus* ethanolic extracts were dissolved fully in water. The *C. nutans* ethanolic extract formed a 10 mm foam layer indicative of more saponin than the *S. crispus* ethanolic extract which only yielded a 5 mm foam layer. The acetone and chloroform extracts were only partially dissolved in water, so no observations were done with these four samples.

Table 4.1: The result of the phytochemical screening for different solvents extracts of *Clinacanthus nutans* and *Strobilanthes crispus*.

	<i>Clinacanthus nutans</i>			<i>Strobilanthes crispus</i>		
	Ethanol	Acetone	Chloroform	Ethanol	Acetone	Chloroform
Alkaloids Test (precipitate)	4+	4+	0	4+	4+	0
Flavonoids Test (colour intensity)	3	2	1	1	3	2
Saponin Test (foam layer / cm)	1.0	N/A	N/A	0.5	N/A	NA

Note: N/A stated for Not Available

When assessed for the antioxidant potential in scavenging ROS, acetone extract of *C. nutans* exhibited the highest level of antioxidant potential (25 %) than ethanolic *C. nutans* extract (17.74 %) and chloroform extract of *C. nutans* (11.96 %) relative to ascorbic acid. Interestingly, all the extracts from *S. crispus* exhibited a significantly higher antioxidant capacity than *C. nutans* extracts by reducing more than 55 % of the ROS relative to ascorbic acid. When these extracts were assessed for their TPC, acetone *C. nutans* extract showed the highest TPC (4.335 mg GAE/g defatted material) at approximately 1.48-fold more in comparison with chloroform *C. nutans* extract (2.934 mg GAE/g defatted material) and more than 16.8 fold higher than *C. nutans* ethanolic extract (0.258 mg GAE/g defatted material) respectively. For *S. crispus* extracts, the ethanolic extract showed the highest TPC (1.878

mg GAE/g defatted material) followed by chloroform (0.207 mg GAE/g defatted material) and the ethanolic extract was approximately 11.4 fold more than acetone (0.165 mg GAE/g defatted material) extracts respectively. The TFC for these extracts was assessed and the results showed *C. nutans* ethanolic extract contained the highest amount of flavonoid at 0.472 mg RE/g defatted material, followed by acetone *C. nutans* extract (0.190 mg RE/g defatted material) and chloroform *C. nutans* extract (0.080 mg RE/g defatted material) respectively. In the *S. crispus* extracts, the TFC was relatively similar for chloroform, acetone, and ethanolic extracts at 0.313, 0.278, and 0.252 mg RE/g defatted material, respectively.

Table 4.2: The comparison of the antioxidant content (AAEAC), total phenolic content (TPC), and total flavonoids content (TFC) between *C. nutans* and *S. crispus* with different solvents extracts.

	<i>Clinacanthus nutans</i>			<i>Strobilanthes crispus</i>		
	Ethanol	Acetone	Chloroform	Ethanol	Acetone	Chloroform
AAEAC (%)	17.74	25.00	11.96	>55	>55	>55
TPC (mg GAE/g defatted material)	0.258 ±0.063	4.335 ±0.342	2.934 ±0.506	1.878 ±0.525	0.165 ±0.114	0.207 ±0.821
TFC (mg RE/g defatted material)	0.472 ±0.021	0.190 ±0.006	0.080 ±0.004	0.2524 ±0.007	0.2777 ±0.014	0.3127 ±0.008

Note:

- (1) Ascorbic Acid Equivalent Antioxidant Content = AAEAC
- (2) TPC recorded as mg Gallic Acid Equivalent/g defatted material
- (3) TFC recorded as mg Rutin Equivalent/g defatted material

4.2 Determination of Antimicrobial Properties of *C. nutans* and *S. crispus* Plant Extracts against Gram-positive and Gram-negative Bacteria.

When the *C. nutans* and *S. crispus* extracts were treated on the Gram-positive and Gram-negative bacteria on MHA, only *Ps. aeruginosa* showed a distinct zone of inhibition. When *C. nutans* ethanol extract was treated on *Ps. aeruginosa*, an average zone of inhibition of 16 mm was recorded, which showed better antibacterial potential than that of the acetone (14 mm) and chloroform extracts (14 mm), respectively. Despite all the zones of inhibition recorded with *S. crispus* extracts were smaller, ethanol extract with 14 mm, acetone extract with 12 mm, and chloroform extract with 12 mm, these zones of inhibition were more distinguishable than those of *C. nutans* extracts. However, compared to the zone of inhibition of positive control, Levofloxacin 5 mg, the zone of inhibition was 25 mm, which was larger than *C. nutans* and *S. crispus* extracts. This was expected as these *C. nutans* and *S. crispus* extracts were crude and from raw material without fractionation, purification, or modification. Nonetheless, by virtue of its crude nature and yet the ability to deliver these antibacterial properties, making these extracts are promising candidates for future drug development.

Table 4.3: Zone of inhibition when different *C. nutans* and *S. crispus* extracts treated on Gram-positive and Gram-negative bacteria on MHA.

Bacteria	<i>Clinacanthus nutans</i>									<i>Strobilanthes crispus</i>									Control		
	Ethanol			Acetone			Chloroform			Ethanol			Acetone			Chloroform			Levofloxacin 5mg		
	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72
<i>Strep. pyogenes</i>	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	43	42	43
<i>Staph. aureus</i>	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	10	11	11
<i>Methicillin-Resistant Staph. aureus</i>	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	7	X	X
<i>E. coli</i>	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	42	41	39
<i>Ps. aeruginosa</i>	15±1	16±1	16±1	14±1	14±1	15±1	14±1	14±1	14±1	15±1	14±1	13±1	12±1	12±1	12±1	11±1	11±1	11±1	27	25	24
<i>Salmonella typhi</i>	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	30	31	31
<i>Shigella sp.</i>	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	30	33	34
<i>K. pneumoniae</i>	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	24	28	27

Note:

(1) the unit used is in mm

4.3 Effects of *C. nutans* and *S. crispus* Extracts on Skin Fibroblasts.

Cell viability assay show the number of healthy living cells survive under certain condition. It is significant to determine the cell viability when different concentration of *C. nutans* and *S. crispus* extracts applied on the skin fibroblasts, because if the concentration of extracts used in the scratch assay is too high and cause the initial cell viability lower than 100%, it might affect the final observation. Therefore, the Minimum Inhibition Concentration (MIC) of *C. nutans* and *S. crispus* extracts need to be determined and the concentration to proceed must not affect the cell viability to be lower than 100 %.

When different concentrations of ethanolic *C. nutans* extract were treated on normal skin fibroblasts, there was little or no effect on the cell viability up to 62.5 µg/mL at 24 and 48hours incubation (**Table 4.4, Figure 4.1**). At higher concentrations of 125 µg/mL onwards, a decrease in cell viability was observed in the 24 hours samples. On the other hand, a similar trend of decreased cell viability could only be observed at the highest concentration of 500 µg/mL for both 24 and 48 hours samples. The *C. nutans* acetone extract was shown to be toxic to fibroblasts as when the concentration of *C. nutans* acetone extract was higher than 7.81 µg/mL. The cell viability started to drop in a dose-dependent manner (**Table 4.4, Figure 4.2**). At 48 hours incubation, the drop in cell viability of normal skin fibroblasts was more pronounced at lower concentrations of 7.81 µg/mL. Overall, *C. nutans* acetone extracts exhibited earlier and lowered dose-dependent trends. The MTT results for *C. nutans* chloroform extract were similar to *C. nutans* ethanol extract. In chloroform extracts, the fibroblast cell viability remained approximately at 100 % at 62.5 µg/mL or below when incubated for 24 hours and 125 µg/mL if incubated for 48 hours (**Table 4.4, Figure 4.3**).

Table 4.4: Cell Viability Assay of different concentrations of *C. nutans* extracts applied on the normal human skin fibroblast and incubated for 24 and 48 hours respectively.

Concentration of <i>C. nutans</i> extracts ($\mu\text{g/mL}$)	24 Hours			48 Hours		
	Ethanol	Acetone	Chloroform	Ethanol	Acetone	Chloroform
0	100.0 $\pm$ 0	100.0 $\pm$ 0	100.0 $\pm$ 0	100.0 $\pm$ 0	100.0 $\pm$ 0	100.0 $\pm$ 0
3.91	109.7 $\pm$ 47.7	108.9 $\pm$ 5.4	107.3 $\pm$ 23.8	111.7 $\pm$ 13.7	107.6 $\pm$ 2.6	120.8 $\pm$ 8.8
7.81	97.9 $\pm$ 6.8	103.9 $\pm$ 6.9	99.1 $\pm$ 15.0	108.2 $\pm$ 5.5	80.8 $\pm$ 8.0	117.6 $\pm$ 11.8
15.63	118.2 $\pm$ 16.6	93.5 $\pm$ 16.3	98.8 $\pm$ 7.8	102.1 $\pm$ 2.9	94.1 $\pm$ 12.5	115.8 $\pm$ 11.7
31.25	121.9 $\pm$ 21.4	79.8 $\pm$ 9.1	98.6 $\pm$ 10.0	99.0 $\pm$ 13.5	100.9 $\pm$ 20.0	114.1 $\pm$ 9.1
62.5	99.7 $\pm$ 21.8	85.3 $\pm$ 13.4	100.3 $\pm$ 17.4	104.2 $\pm$ 15.4	84.3 $\pm$ 17.2	113.9 $\pm$ 15.7
125	76.7 $\pm$ 13.8	73.0 $\pm$ 1.9	91.6 $\pm$ 27.9	100.5 $\pm$ 20.6	61.6 $\pm$ 15.2	111.9 $\pm$ 11.9
250	84.6 $\pm$ 18.4	44.5 $\pm$ 0.5	59.5 $\pm$ 14.2	95.1 $\pm$ 7.0	48.5 $\pm$ 5.3	77.5 $\pm$ 28.9
500	76.3 $\pm$ 30.4	55.6 $\pm$ 30.2	69.9 $\pm$ 15.4	78.0 $\pm$ 11.8	24.9 $\pm$ 7.4	58.7 $\pm$ 12.2

Therefore, a standardised *C. nutans* extract concentration was selected at 7 µg/mL to proceed with co-incubation of normal human fibroblasts with MRSA to study the effects of dose and time from different *C. nutans* extracts, respectively.

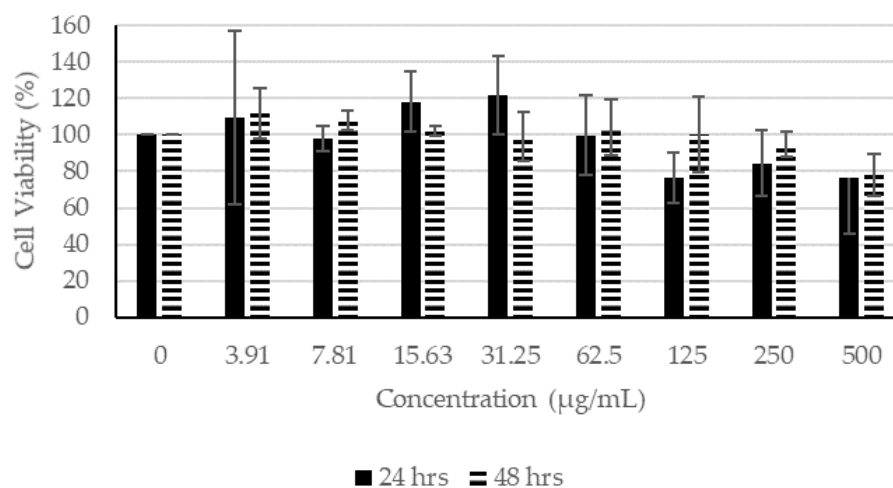


Figure 4.1: Cell Viability Assay of different concentrations of *C. nutans* ethanol extract applied on the normal human skin fibroblast and incubated for 24 and 48 hours respectively.

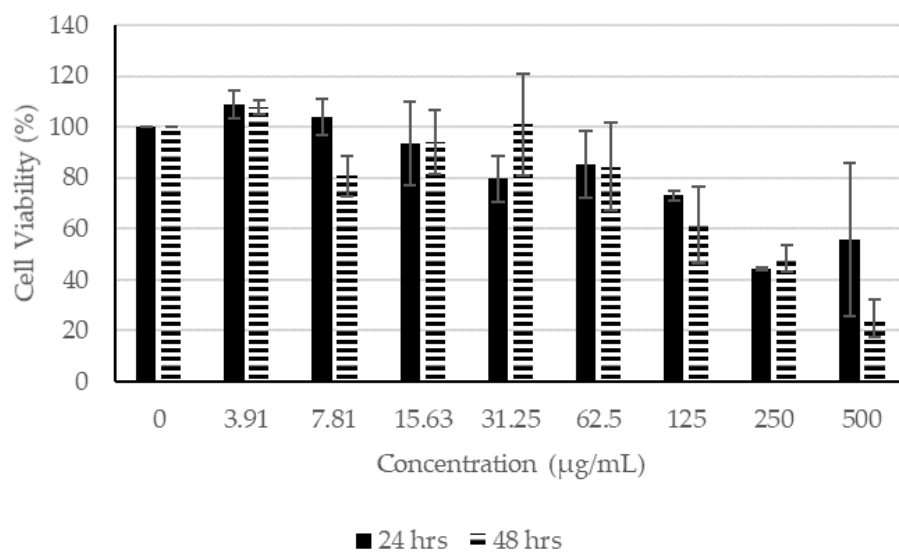


Figure 4.2: Cell Viability Assay of different concentrations of *C. nutans* acetone extract applied on the normal human skin fibroblast and incubated for 24 and 48 hours respectively.

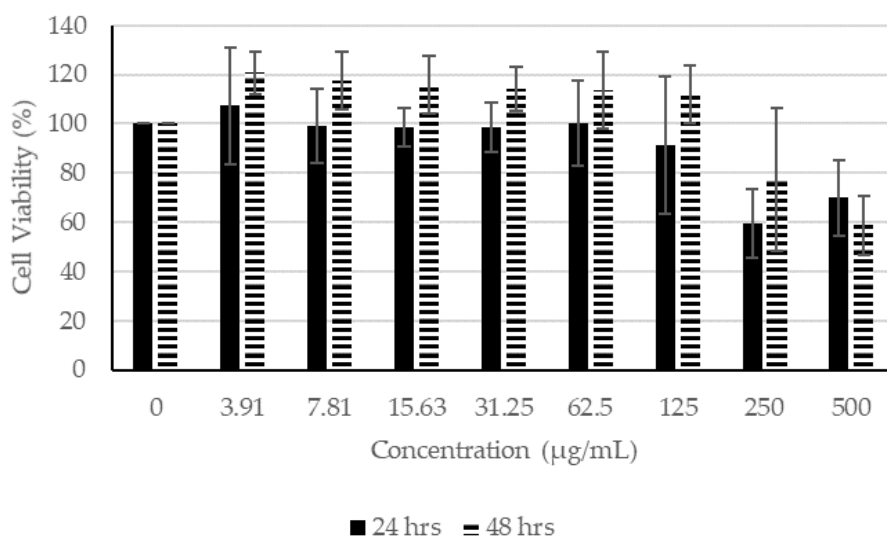


Figure 4.3: Cell Viability Assay of different concentrations of *C. nutans* chloroform extract applied on the normal human skin fibroblast and incubated for 24 and 48 hours respectively.

In the MTT assay, the *S. crispus* ethanol extract at 500 µg/mL proved to be lethal as the fibroblast cell viability dropped to 9 % and 1 % after incubation for 24 and 48 hours respectively (**Table 4.5, Figure 4.4**). However, in 24 hours incubation at concentrations lower than 62.5 µg/mL, the normal fibroblast cells seemed to be thriving, exhibiting a biphasic trend of increasing and decreasing cell viability. In 48 hours samples, the overall cell viability trend decreased with the increase in extract concentration. For *S. crispus* acetone extract (**Table 4.5,**

Figure 4.6), the fibroblasts cell viability remained at 100 % or slightly higher when the concentrations were 62.5 µg/mL and lower after 24 hours incubation. However, despite the biphasic trend observed, at the highest concentration of 500 µg/mL, the cell viability dramatically dropped to below the IC₅₀ line. After 48 hours incubation, the biphasic trends were observed with a decrease and a slight increase in the cell viability before it declined from 31.25 µg/mL at a dose-dependent manner.

Based on **Figure 4.6**, it showed the MTT assay results of different concentrations of *S. crispus* chloroform extract applied on the normal human skin fibroblasts and incubated for 24 and 48 hours, respectively. The overall results from *S. crispus* chloroform extracts were similar to those of acetone extract. The fibroblast cell viability remained higher than 100 % when the concentrations were 62.5 µg/mL or lower after 24 hours incubation. At 125 µg/mL and above, the cell viability decreased in a dose-dependent manner. Similarly, after 48 hours incubation with *S. crispus* chloroform, the cell viability decreased in a dose-dependent manner. At 500 µg/mL, the cell viability dropped to 0 % in the 48 hours samples. Therefore, to maintain cell viability at 100 % for incubation of 48 hours, the *S. crispus* chloroform extract concentration must be lower than 3.91 µg/mL.

Table 4.5: Cell Viability Assay of different concentrations of *S. crispus* extracts applied on the normal human skin fibroblast and incubated for 24 and 48 hours respectively.

Concentration of <i>S. crispus</i> extracts ($\mu\text{g/mL}$)	24 Hours			48 Hours		
	Ethanol	Acetone	Chloroform	Ethanol	Acetone	Chloroform
0	100.0 $\pm$ 0	100.0 $\pm$ 0	100.0 $\pm$ 0	100.0 $\pm$ 0	100.0 $\pm$ 0	100.0 $\pm$ 0
3.91	159.7 $\pm$ 16.3	117.5 $\pm$ 5.2	125.3 $\pm$ 10.2	109.8 $\pm$ 3.5	89.5 $\pm$ 15.0	99.7 $\pm$ 14.3
7.81	157.2 $\pm$ 16.6	94.2 $\pm$ 8.7	117.5 $\pm$ 7.0	89.7 $\pm$ 8.9	88.3 $\pm$ 2.5	99.1 $\pm$ 9.6
15.63	152.2 $\pm$ 11.9	101.7 $\pm$ 6.6	108.4 $\pm$ 8.0	83.0 $\pm$ 6.8	96.3 $\pm$ 9.0	92.4 $\pm$ 17.1
31.25	140.5 $\pm$ 21.4	106.2 $\pm$ 6.6	123.9 $\pm$ 13.8	95.7 $\pm$ 16.1	90.7 $\pm$ 5.4	97.0 $\pm$ 10.4
62.5	135.9 $\pm$ 24.4	109.4 $\pm$ 4.2	110.5 $\pm$ 1.6	82.5 $\pm$ 3.2	79.3 $\pm$ 9.7	86.9 $\pm$ 11.0
125	92.7 $\pm$ 7.1	72.5 $\pm$ 2.8	77.9 $\pm$ 15.0	67.7 $\pm$ 9.1	56.3 $\pm$ 8.5	88.3 $\pm$ 9.4
250	60.1 $\pm$ 23.1	85.4 $\pm$ 4.8	72.9 $\pm$ 11.1	37.3 $\pm$ 6.8	20.0 $\pm$ 7.2	76.5 $\pm$ 16.3
500	9.3 $\pm$ 5.7	25.7 $\pm$ 3.3	20.4 $\pm$ 25.7	1.2 $\pm$ 0.1	2.7 $\pm$ 0.1	1.3 $\pm$ 0.2

Therefore, this study selected a standardised *S. crispus* extract concentration at 70 µg/mL to proceed with co-incubation of normal human fibroblasts with MRSA to study the effects of dose and time from different *S. crispus* extracts, respectively.

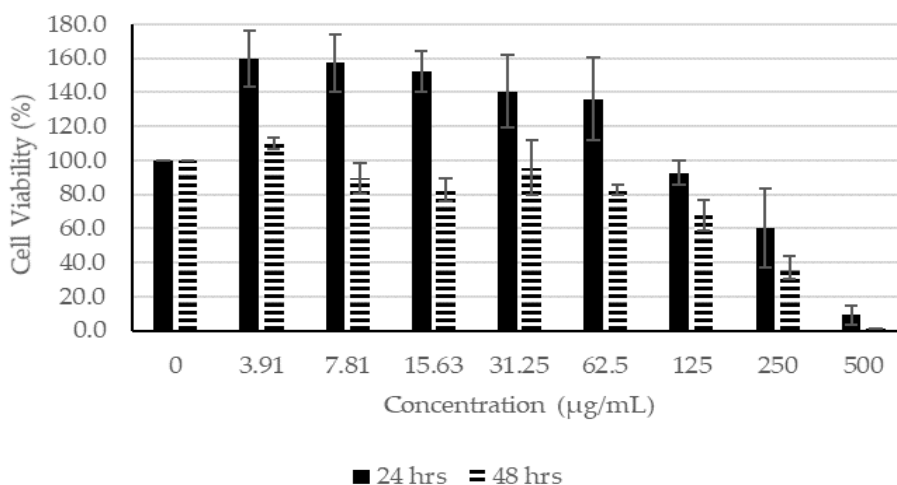


Figure 4.4: Cell Viability Assay of different concentrations of *S. crispus* ethanol extract applied on the normal human skin fibroblast and incubated for 24 and 48 hours respectively.

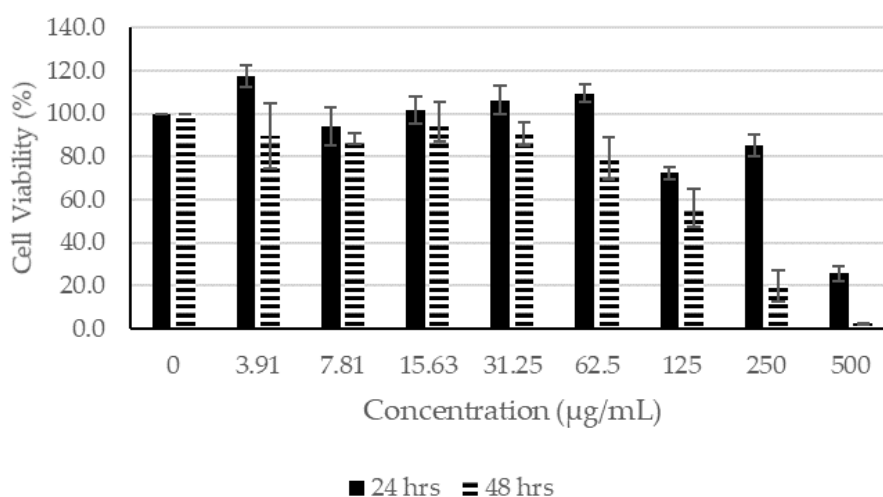


Figure 4.5: Cell Viability Assay of different concentrations of *S. crispus* acetone extract applied on the normal human skin fibroblast and incubated for 24 and 48 hours respectively.

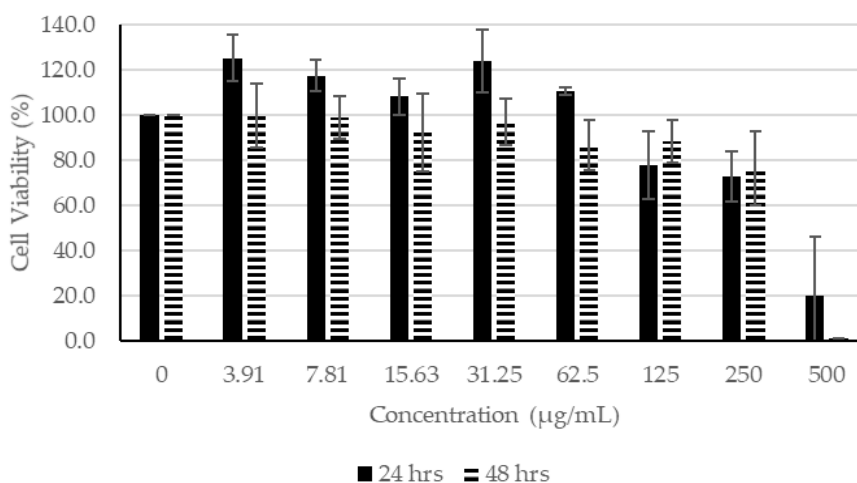


Figure 4.6: Cell Viability Assay of different concentrations of *S. crispus* chloroform extract applied on the normal human skin fibroblast and incubated for 24 and 48 hours respectively.

4.4 Wound Healing Effects of *C. nutans* and *S. crispus* Extracts Treated on Pseudo-wound in the Presence of MRSA

The growth of MRSA over a duration of 24 hours when co-incubated with normal human skin fibroblasts was plotted (**Figure 4.7**). When added more than 1 % of MRSA, the MRSA was able to take over and cover the surface of the flask to achieve 100 % or higher coverage in that time duration. When 0.5 % of MRSA was added, the growth and coverage achieved about 90 %, while 0.25 % MRSA was added to the flask and coverage only reached about 80 %, respectively.

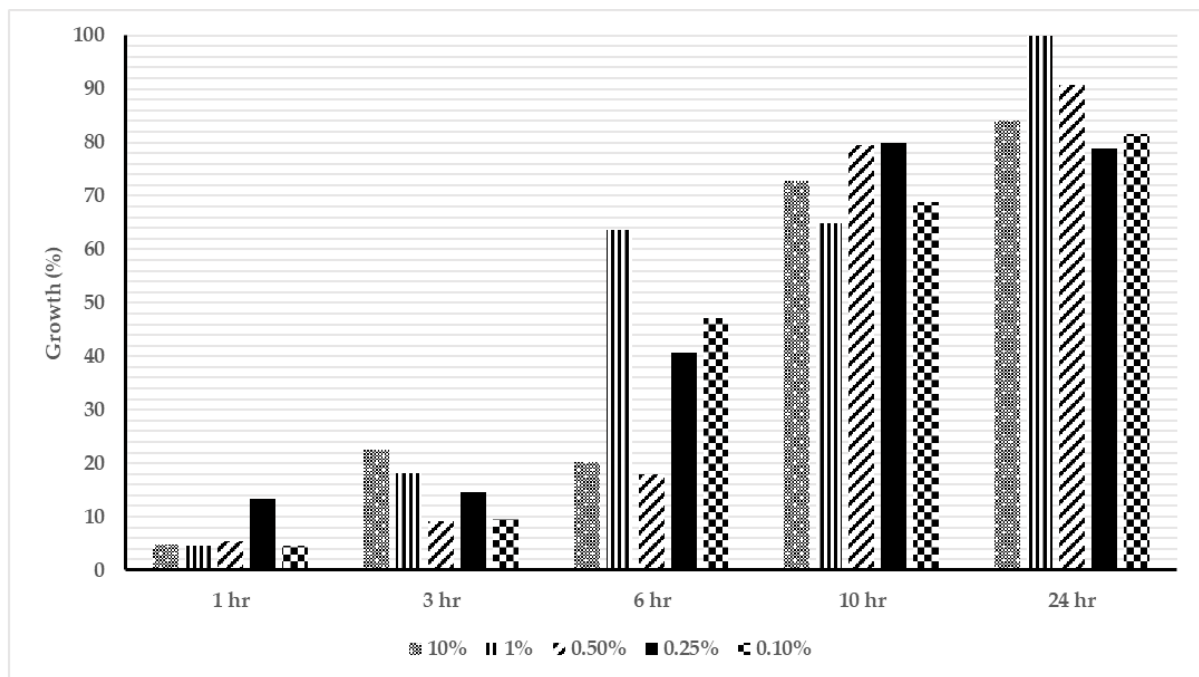


Figure 4.7: Growth and coverage of MRSA over a duration of 24 hours when different concentration of MRSA were co-incubated with normal human skin fibroblasts.

During the *in vitro* wound healing study, a pseudo-wound was created using a sterile pipette tip as described in **3.8**. When the *C. nutans* extracts were treated on the pseudo-wound of the normal human skin fibroblasts, the ethanol and chloroform extracts were capable of closing the pseudo-wound after incubation of 24 hours, respectively, but the acetone extract required 48 hours of incubation to fully close up the pseudo-wound (**Figure 4.8**).

However, when the pseudo-wound was co-incubated with MRSA, the MRSA completely covered the flasks after 3 to 10 hours incubation. The pseudo-wound did not close, indicating no or insignificant wound healing activity (**Figure 4.9**). The skin fibroblast coverages were recorded in percentage of the fibroblast present in the flask and the data were tabulated as shown in **Table 4.6**.

Similarly, based on **Table 4.7**, *S. crispus* extracts did not aid wound healing activity. When *S. crispus* extracts were treated on the pseudo-wound of the skin fibroblasts, the pseudo-wound with ethanol and acetone extracts did not close respectively, while only the *S. crispus* chloroform extracts were capable of fully closing up the pseudo-wound after 48 hours of incubation (**Figure 4.10**).

Interestingly, when co-incubated with MRSA, the *S. crispus* extracts inhibited the MRSA activity, and the fibroblasts were able to regenerate for a short time period. However, the MRSA took over the surface in the flasks after 6 to 10 hours incubation (**Figure 4.11**).

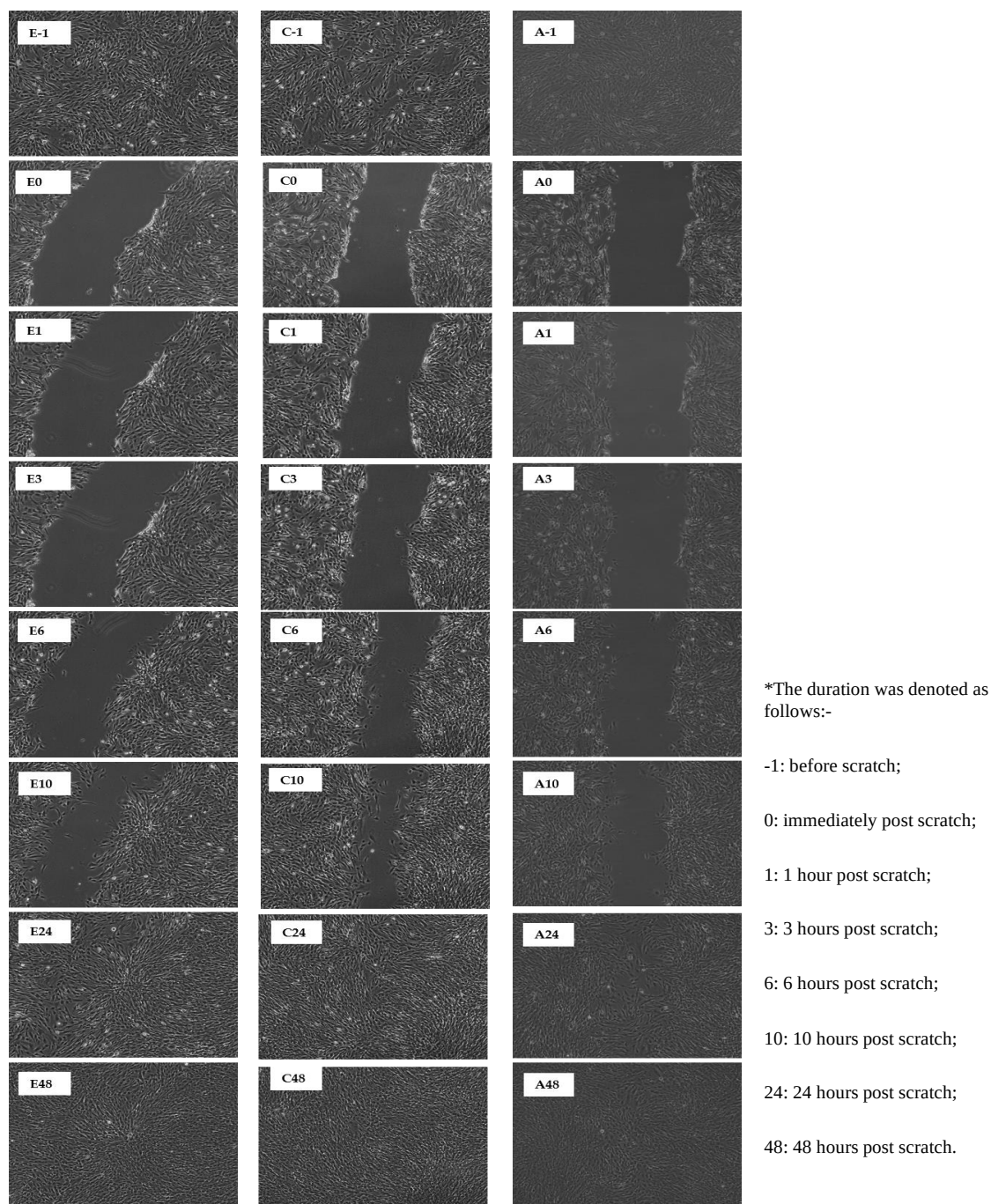


Figure 4.8: Scratch Assay on normal human fibroblasts in the presence of *C. nutans* ethanol (E), chloroform (C) and acetone (A) extracts respectively. An *in vitro* wound healing process was observed in a time-dependent manner on normal human fibroblasts in the presence of ethanol (E), chloroform (C) and acetone (A), respectively.

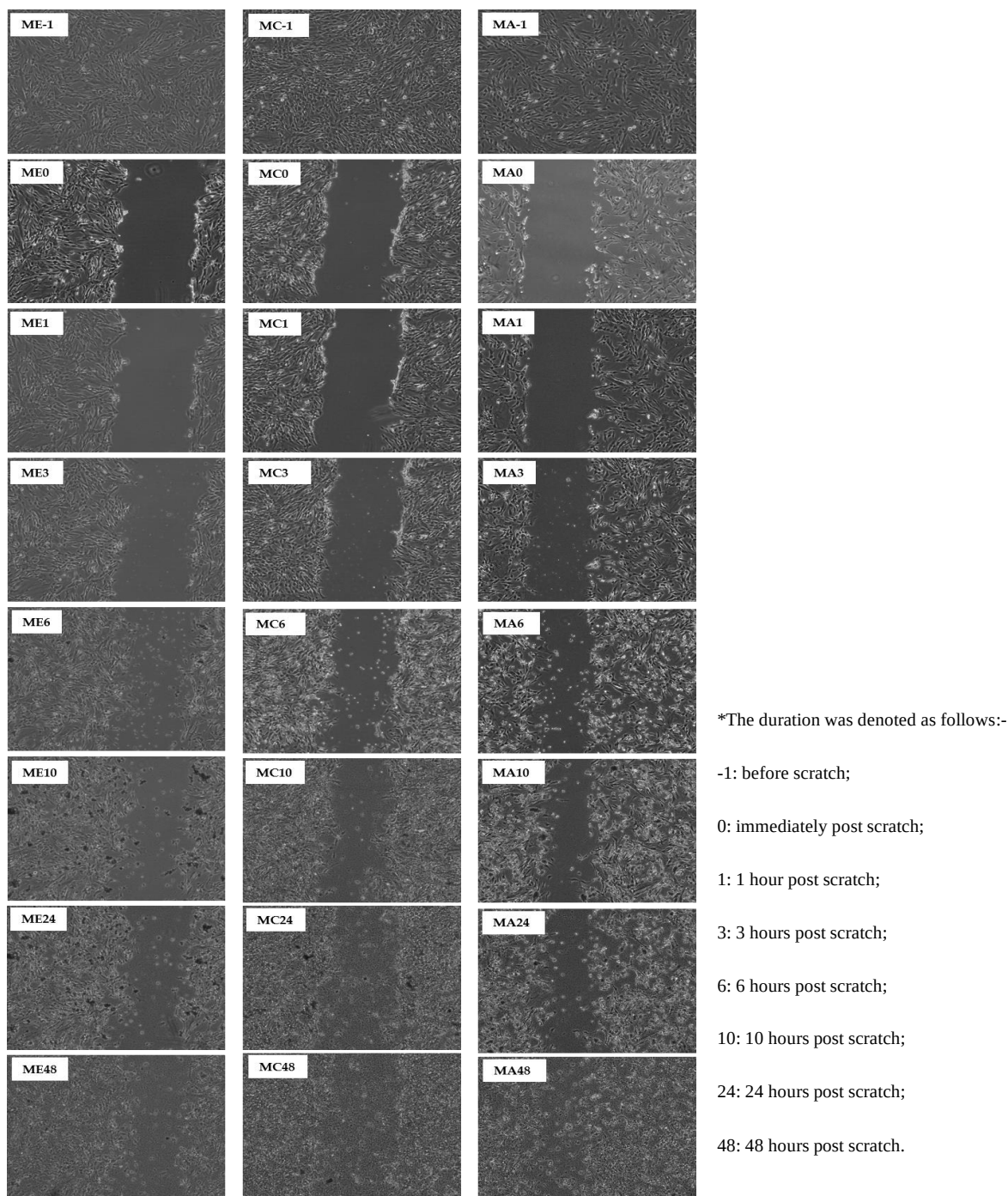


Figure 4.9: Scratch Assay on normal human fibroblasts and MRSA. An *in vitro* wound healing process was observed in a time-dependent manner on human fibroblasts in the presence of ethanol (E), chloroform (C) and acetone (A) *C. nutans* extracts respectively.

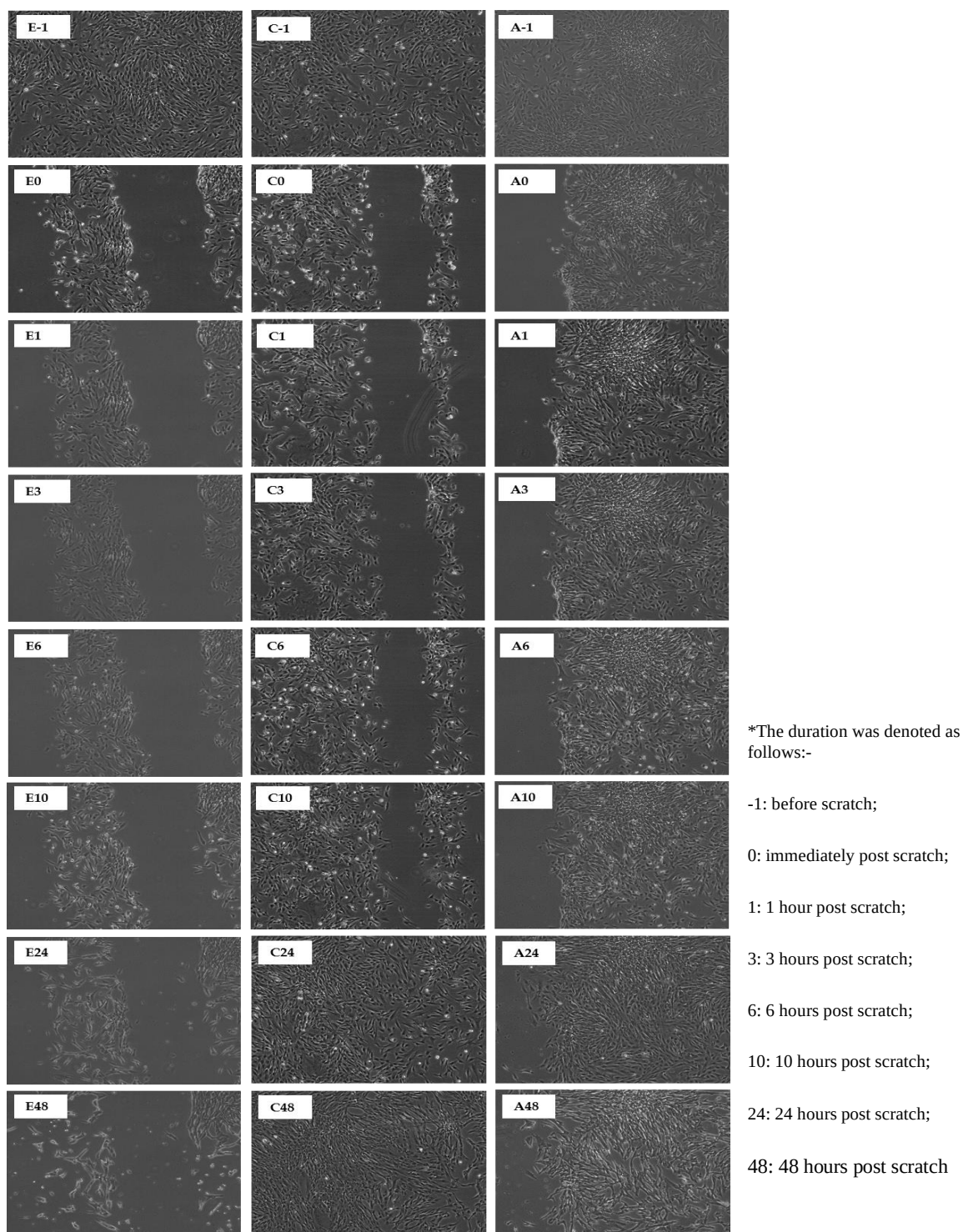


Figure 4.10: Scratch Assay on human fibroblasts in the presence of *S. crispus* extracts. An *in vitro* wound healing process was observed in a time-dependent manner on human fibroblasts in the presence of ethanol (E), chloroform (C) and acetone (A) respectively

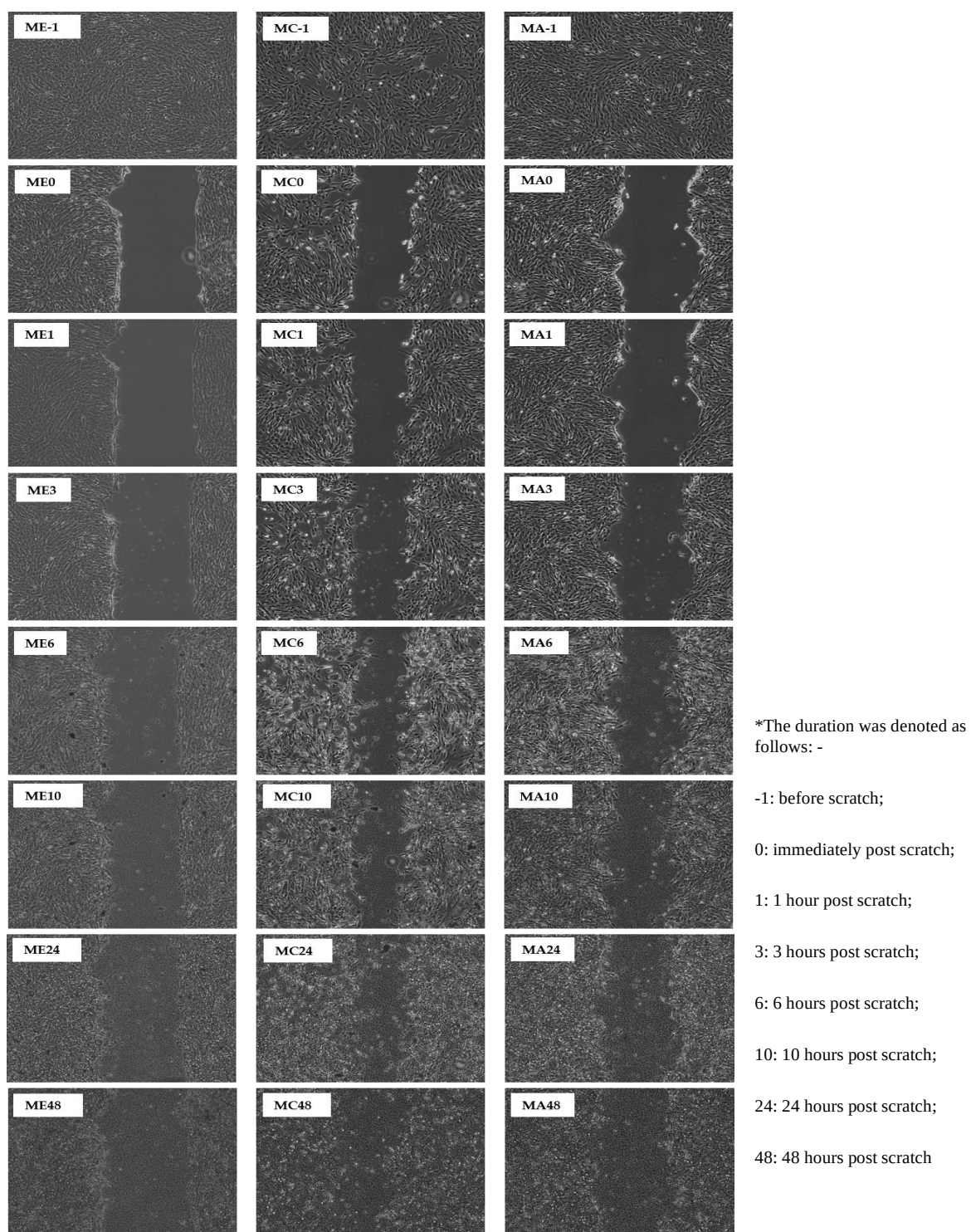


Figure 4.11: Scratch Assay on human fibroblasts in the presence of *S. crispus* extracts and MRSA. An *in vitro* wound healing process was observed in a time-dependent manner on human fibroblasts in the presence of ethanol (E), chloroform (C) and acetone (A) respectively.

Table 4.6: The Result of *in vitro* Scratch Assay with *C. nutans* extracts.

Incubation Time (hr)		0	1	3	6	10	24	48
Extract only	Control	36.8±5.1	40.2±10.1	44.9±8.9	58.0±9.5	70.4±10.3	85.1±25.9	113.4±23.6
	Ethanol	41.2±5.1	47.0±7.1	56.7±2.5	61.8±3.4	71.9±7.6	114.7±23.0	141.3±38.9
	Chloroform	41.7±7.5	52.2±10.1	56.4±10.4	59.5±10.4	74.1±11.7	111.1±25.9	137.1±13.9
	Acetone	22.9±4.0	26.1±10.3	25.1±6.0	30.3±11.9	39.7±11.0	83.6±27.7	100.9±29.7
Co-incubation with extract and MRSA	Ethanol	28.1±4.0	30.9±6.0	38.3±14.4	43.1±17.5	38.0±3.2	39.2±16.8	33.7±9.8
	Chloroform	35.0±4.0	36.3±9.6	37.9±11.5	44.0±11.0	52.4±21.6	47.7±17.4	46.2±22.0
	Acetone	36.3±7.5	38.5±12.4	48.6±10.0	44.0±5.1	41.7±1.6	37.2±0.9	20.8±7.8

Note: Effects of *C. nutans* extracts (7 µg/mL) co-incubated with fibroblasts in the absence of MRSA in dose-dependent and time-dependent assays respectively. The results were expressed in % of coverage by the cells in that area.

Table 4.7: The Result of *in vitro* Scratch Assay with *S. crispus* extracts.

Incubation Time (hr)		0	1	3	6	10	24	48
Extract only	Control	47.4±12.3	54.8±12.4	60.2±12.2	79.8±7.6	94.9±6.5	102.7±15.9	137.9±13.2
	Ethanol	32.9±14.1	35.9±15.9	42.0±18.8	43.5±15.1	42.5±14.8	43.5±14.8	35.7±17.4
	Chloroform	26.0±5.4	26.1±8.4	32.7±10.7	38.6±6.9	52.4±3.8	78.3±16.7	101.0±38.7
	Acetone	39.3±10.8	40.8±11.7	45.3±10.5	51.0±8.4	53.1±6.3	73.0±21.1	62.3±5.0
Co-incubation with extract and MRSA	Ethanol	42.0±8.2	52.9±9.4	59.0±13.2	64.5±13.1	76.1±21.1	58.4±8.8	13.4±12.6
	Chloroform	29.4±4.1	37.3±6.0	43.0±2.3	57.7±10.6	60.2±16.8	42.2±15.5	25.7±11.7
	Acetone	28.7±10.1	34.3±9.2	40.5±13.3	45.8±14.1	31.1±3.7	20.1±7.1	12.5±6.5

Note: Effects of *S. crispus* extracts (70 µg/mL) co-incubated with fibroblasts in the absence of MRSA in dose-dependent and time-dependent assays respectively. The results were expressed in % of coverage by the cells in that area.

CHAPTER 5

DISCUSSION

5.1 Phytochemical

Flavonoids are found predominantly in fruits and vegetables. These phytochemicals are reported or purported to be high in phenolic acids, potent with antioxidants and associated with antimicrobial, and antiviral properties. Therefore, assays were formulated and designed to study these phytochemicals. Qualitative assays based on the colour intensity commonly provide the preliminary results and the estimation of the total flavonoids present.

Dragendorff's reagent is commonly used for routine detection of alkaloids present in the samples. When alkaloids were present, these would react with the Dragendorff's reagent to form orange red precipitate. The higher the content of alkaloids, the more precipitates were formed (Raal et al., 2020). Based on **Table 4.1**, the ethanol and acetone extracts from both *C. nutans* and *S. crispus* showed positive results of 4+ for the alkaloids tests while no precipitates were observed in the chloroform extracts. Chloroform has a pH value of 4.5, an acidic solvent compared with ethanol and acetone which were neutral. Therefore, it was likely that the alkaloids extracted in chloroform would be lower or absent. The findings are supported by Sreevidya and Mehrotra (2003), the pH value is a crucial factor for the formation of precipitation. If the mixture has a lower pH, there was a high possibility the precipitates were dissolved in the excess acid.

In the *C. nutans* extracts results tabulated in **Table 4.1**, the ethanol extract showed the strongest colour intensity with an intense yellowish colouration followed by acetone extract and chloroform extract, respectively. For *S. crispus* extracts, the acetone extract

displayed the highest intensity, followed by chloroform extract and lastly, ethanol extract. The results obtained for *C. nutans* and *S. crispus* extracts showed different trends. The *S. crispus* extracts have stronger green colour intensity compared to *C. nutans* extracts when these were still in the crude form. Therefore, quantitative tests would need to be carried out to determine the flavonoids present in the extracts accurately.

Epidemiology studies have been conducted on the health benefits of saponin. Saponin are polar molecules, may play an important role in protection against cancer, hypocholesterolemic effect, antioxidative, antiviral and other beneficial properties (Mohan et al., 2016). Therefore, slightly polar acetone relative to water at 0.355 and non-polar chloroform extracted slightly polar or non-polar viscous compounds from the crude extracts. When performing the saponin test, all the acetone and chloroform extracts could not dissolve in distilled water, while only the ethanol extracts dissolved in distilled water. The ethanol extract of *C. nutans* showed a 10 mm foam layer while *S. crispus* showed a 5 mm foam layer, indicating that the *C. nutans* ethanolic extract contained a higher saponin level than the *S. crispus* ethanolic extract.

From the phytochemical screening tests, antioxidants such as flavonoids and phenolics were present within the *C. nutans* and *S. crispus* extracts. Therefore, the bioassays for antioxidant activity, Total Flavonoids Content and Total Phenolic Content were performed.

5.2 Total Phenolic Content and Total Flavonoid Content of *Clinacanthus nutans* (*C. nutans*) and *Strobilanthes crispus* (*S. crispus*)

Despite phenolics and flavonoids being the most common antioxidant components present in plant samples, the available amount may vary due to its natural properties, or the

extraction solvent involved. Therefore, the total phenolic and flavonoid contents of *C. nutans* and *S. crispus* extracts were performed to determine the contents and effects of antioxidant components present on chemically generated free radicals.

Phenol is a type of antioxidant that is commonly present in plants. It has a lower polarity due to its phenyl group. Thus, phenols were most likely to be extracted with semi-polar to non-polar solvent (Hattenschwiler & Vitousek, 2000). Folin-Ciocalteu Reagent Assay was used to determine the Total Phenolic Content (TPC) present in the extracts. Gallic acid was selected to act as the standard and the results were presented as mg Gallic Acid Equivalent (GAE) /g defatted material.

From **Table 4.1**, the *C. nutans* acetone extract displayed the highest TPC at 4.335 mg GAE/g defatted material, followed by chloroform extract with 2.934 mg GAE/g defatted material, which was close to half of that in acetone extract. However, the ethanol extracts only contained about 0.258 mg GAE/g defatted material, almost 11-fold less than that of chloroform extract. Based on the results above, the acetone extract had the highest TPC compared to that of ethanol extract. It might be due to the natural properties of ethanol with its high polarity and the additional hydroxyl (OH-) group resulting in higher polarity compared to acetone.

However, for the *S. crispus* extracts, the ethanol extract obtained the highest TPC value of 1.878 mg GAE/g defatted material. The TPC obtained from the chloroform and acetone extracts were almost similar; 0.207 and 0.165 mg GAE/g defatted material, respectively. Based on the visual observation, ethanol extract exhibited a higher green colour intensity (most likely rich in chlorophyll) than to the other two extracts, affecting the results.

According to Marcheafave et al. (2019), chlorophylls and carotenoids were easier extracted with ethanol. Thus, the result obtained for *S. crispus* ethanol extract was affected.

The aluminium calorimetric method was conducted for Total Flavonoid Content (TFC), and rutin acted as the standard. The results were presented in mg Rutin Equivalent (RE)/g defatted material. The *C. nutans* ethanol extract showed the highest TFC of 0.472 mg RE/g defatted material. The TFC of *C. nutans* acetone and chloroform extracts was much lower than ethanol extract; 0.190 mg RE/g defatted material and 0.080 mg RE/g defatted material, respectively. Based on the TFC results, there were distinctive differences when used different polarity extraction solvent. This demonstrated that most of the flavonoids present in *C. nutans* belong to flavonoids with relative polarity, and very few non-polar flavonoids were available (Panche et al., 2016).

However, for *S. crispus* extracts, the differences between the three different extracts were comparatively similar. They were 0.252 mg RE/g defatted material, 0.278 mg RE/g defatted material, and 0.313 mg RE/g defatted material for ethanol, acetone, and chloroform, respectively. The findings showed that the types of flavonoids present in *S. crispus* were almost similar.

5.3 Antioxidant Properties

The antioxidant content was assessed through DPPH radical scavenging ability, and the IC₅₀ of ascorbic acid recorded from the standard curve was 5.5 µg/mL. It was used to calculate the Ascorbic Acid Equivalent Antioxidant Content (AAEAC) with the formulae below (Equation 5.1):

$$\text{Antioxidant Content} = \frac{\text{IC}_{50} \text{ of Standard}}{\text{IC}_{50} \text{ of Sample}} \times 100\% \quad \text{Equation 5.1}$$

Based on the results shown in **Table 4.2** for *C. nutans* extracts, the acetone extract demonstrated the highest antioxidant potential at 25 % AAEAC, comparable with those of ethanol and chloroform extracts which were 17.74 % and 11.96 % AAEAC, respectively.

It was noted that the acetone extract showed approximately 2-fold higher antioxidant capacity than the chloroform extract. This finding was supported by Shimamura et al. (2014). While polar acetone extract obtained the highest antioxidant content, ethanol with the higher polarity did not show a better result, indicative that most of the antioxidant components present in the *C. nutans* were semi-polar or non-polar molecules. When extracted with semi-polar solvents such as acetone, the solvent extracted higher antioxidant content than ethanol and chloroform as extraction solvents. As the type of antioxidants present within the extracts may differ, further tests were required to identify them (Barchan et al., 2014).

The antioxidant results obtained from the *S. crispus* extracts differed from those of *C. nutans*. The *S. crispus* extracts exhibited a higher green colour intensity compared to *C. nutans* extracts. The extracts were filtered with a 0.45 µm syringe filter to remove particles though this did not improve the situation. Therefore, the optical density of the extracts was not available. As such, no IC₅₀ was recorded. However, Tan et al. (2019) showed that *S. crispus* was a stronger reactive oxygen species scavenger than *C. nutans*. In this study, starting with a lower concentration of 20 µg/mL, a steep increasing curve was observed, indicative of a high possibility that the AAEAC value might be higher than 55 % for all *S. crispus* extracts if unmasked from the high chlorophyll background noise.

The findings of this study clearly showed that the total antioxidants content present in *C. nutans* included phenols and polar flavonoids (**Table 4.2**). Besides, ethanol and acetone were proven to be excellent extraction solvent. However, both solvent extracts contained different TPC and TFC values. The acetone extract *C. nutans* contained the highest antioxidant activity reported and the highest TPC but lower in TFC. Its total phenolic content was about 2-fold higher than that of ethanol extract. On the other hand, the antioxidant activity of ethanol extract was slightly lower than that of acetone extract but obtained the highest TFC of 0.472 mg RE/g defatted material. The TFC of acetone extract was reported to be about 2-fold lesser than that of the ethanol extract. These results indicated that the antioxidant components present in the ethanol extract mainly were polar flavonoids and the antioxidant components present in acetone extract were mostly phenolic compounds.

The total antioxidant content present in the *C. nutans* extracts was between 11.96 % to 25 % AAEAC, while the average total antioxidant content present in the *S. crispus* extracts mainly were higher than 55 %. Therefore, the total antioxidant content of *S. crispus* was higher than that of *C. nutans*. However, based on TPC and TFC, the highest value of TPC recorded was 4.335 mg GAE/g defatted material from *C. nutans* acetone extract, while 0.472 mg RE/g defatted material is the highest value recorded for TFC from *C. nutans* ethanol extract.

S. crispus extracts may have a higher total antioxidant content than *C. nutans* extract, but still high *S. crispus*'s green colour intensity (most likely due to chlorophyll) was a crucial factor affecting the results. Even though the microfiltration of the extracts removed minute particles and the colour intensity was also slightly reduced. Therefore, it was also a possibility that the microparticles filtered out antioxidant compounds.

As the high green colour intensity affected the absorbance measurement, it was highly recommended to determine the antioxidant compounds of *S. crispus* with fluorescence decay measurements, such as oxygen radical absorbance capacity (ORAC), photochemiluminescence assay, or others (Gupta, 2015).

5.4 Antimicrobial Properties

According to the results shown in **Table 4.3**, only *Ps. aeruginosa* showed zones of inhibition in the disc diffusion assay. When *C. nutans* ethanol, acetone, and chloroform extracts were applied on *Ps. aeruginosa* bacterial lawn, zones of inhibition ranging between 14 mm to 16 mm in diameter. When *S. crispus* extracts were applied onto the *Ps. aeruginosa* bacterial lawn, clear distinct zones of inhibition were observed as well. Ethanol extract showed the largest zones of inhibition between 13 mm to 15 mm in diameter, followed by acetone extract at 12 mm and chloroform extract at 11 mm in diameter, respectively.

According to Ong et al. (2020), the ethanol and chloroform extracts of *C. nutans* exhibited a minimum inhibitory concentration (MIC) of 0.63 mg/mL when *Ps. aeruginosa* was applied to it. This showed that *C. nutans* extracts have antimicrobial properties against *Ps. aeruginosa*, but the effects were not significant due to the low concentration administered. A fractionated, purified extract may present a more efficacious effect.

However, based on previous studies, *C. nutans* showed positive results when it was against *B. cereus*, *Staph. aureus*, *E. coli*, and *K. pneumoniae* (Ong et al., 2020). Zones of inhibition were also observed in *S. crispus* ethanol extract at 200 mg/mL when the extract was applied onto *Staph. aureus* and *Strep. pneumonia* bacterial lawns, respectively, but no visible inhibition was detected when treated on *Ps. aeruginosa*.

Based on the results in **Table 4.3**, clearly showed that *C. nutans* and *S. crispus* exhibited antimicrobial properties, which were most effective against Gram-negative bacteria, specifically *Ps. aeruginosa*. It may only affect Gram-positive bacteria when a higher concentration is administered.

Based on another study by Heesterbeek et al. (2019), most of the newly developed antibiotics targeted Gram-positive bacteria due to the impermeable outer membrane of Gram-negative bacteria. In this study, the antimicrobial properties of *C. nutans* and *S. crispus* extracts were found only active against Gram-negative bacteria. These results provided exciting insight and potential for development into an antibacterial agent against Gram-negative bacteria, incredibly opportunistic pathogens such as *Ps. aeruginosa*. The components present in *C. nutans* and *S. crispus* might target certain pathways or the DNA replication enzyme of the bacteria (Sanyal & Doig, 2012).

Based on the results obtained, *C. nutans* and *S. crispus* extracts exhibited the antioxidant and antimicrobial properties, as shown in the previous studies. Even though for the antimicrobial properties only showed positive result with *Ps. aeruginosa*, since the MIC for *Ps. aeruginosa* was 0.63 mg/mL (Ong et al., 2020), but it was sufficient to represent the antimicrobial properties of *C. nutans* and *S. crispus* extracts.

5.5 Wound Healing Properties

The main purpose of the co-incubation fibroblasts with MRSA was to imitate the wound healing process where invaders involved. *C. nutans* and *S. crispus* extracts were treated on the pseudo-wound to observe the wound healing activities.

Based on the scratch assay on normal human fibroblast in the absence of both extracts and bacteria, the pseudo-wound achieved 80 % coverage in 24 hours of incubation. Therefore, the results for the 24 hours observation from the MTT assay and bacterial selection were used to select the concentration of the extracts and bacteria for the co-incubation scratch assay.

According to **Figure 4.1**, the cell viability of fibroblast remained high at 100 % when concentrations lower than 62.5 µg/mL *C. nutans* ethanol extract were exposed to it for 24 hours, indicating that it was not toxic at the concentrations to the cells. Even after incubation for 48 hours, at concentrations lower than 125 µg/mL, the cell viability remained at 100 % or slightly higher. For *C. nutans* acetone extract, the fibroblast cell viability at 100 % was achieved at concentrations lower than 7.81 µg/mL for 24 hours incubation, and lower than 31.25 µg/mL for 48 hours incubation. Concentrations of extract higher than these were proven to be detrimental to the cell line. Based on **Figure 4.3**, the effects of *C. nutans* chloroform extract were similar to ethanol extract *C. nutans* as the concentration of chloroform allowed for the fibroblast cell viability to remain at 100 % was 62.50 µg/mL for 24 incubation and 125 µg/mL for 48 hours of incubation, respectively.

Based on the results from **Figure 4.4** to **Figure 4.6**, *S. crispus* ethanol extract at concentrations lower than 62.50 µg/mL was selected as it was able to maintain the fibroblast cell viability at 100 % after 24 hours of incubation. However, after incubation for 48 hours,

the ethanol extract was detrimental to the fibroblast, with the exception of the concentration at 3.91 µg/mL or lower. For acetone and chloroform extracts, the concentration that enabled the cell viability to maintain at 100 % for 24 hours incubation needed to be lower than 62.5 µg/mL. *S. crispus* chloroform extract at concentrations lower than 3.91 µg/mL was selected as it was able to maintain the fibroblast cell viability at 100 % after 48 hours of incubation. On the other hand, at *S. crispus* acetone extract concentration of 3.91 µg/mL with 48 hours incubation, the fibroblast cell viability had dropped to 90 %. This demonstrated that despite the concentration being maintained at 3.91 µg/mL, a longer duration of exposure to *S. crispus* acetone extract proved to be detrimental to the fibroblast. Based on the MTT assay, *S. crispus* extracts were shown to be more cytotoxic than *C. nutans* extracts when incubated for longer than 24 hours. From **Figure 4.4** to **Figure 4.6**, when the concentration of *S. crispus* extracts was at 500 µg/mL, the fibroblast cell viability was lower than 26 % when incubated for 24 hours and lower than 3 % if incubated for 48 hours.

On the contrary, this trend was not observed in **Figure 4.1** to **Figure 4.3** when *C. nutans* extracts were administered. Most of the fibroblast cell viability were higher than 50 % with *C. nutans* extracts, even at a 500 µg/mL concentration. The lowest fibroblast cell viability recorded was *C. nutans* acetone extract after incubation of 48 hours. Moreover, according to **Figure 4.1** and **Figure 4.5**, both plants were extracted with acetone. Their fibroblast cell viability at the lowest concentration of 3.91 µg/mL was lower than the other extracts from their respective plants. Therefore, this study showed that acetone extracts from plants were harmful to normal human fibroblasts compared to ethanol and chloroform extracts. In order to standardize the concentration for the same plant for comparison, this study selected a concentration of 7 µg/mL for all *C. nutans* extracts and 70 µg/mL was

selected for all *S. crispus* extracts as the fibroblast cell viability for the extracts remained 100 % at these concentrations after incubation of 24 hours.

The growth rate of MRSA is a critical variable for the co-incubation assay as it should not fully ‘contaminated’ the flask before the wound healing activity is initiated. In addition, MRSA has been reported to form biofilm in overnight culture (Oyama et al., 2016, Akbari-Ayezloy et al., 2017). It was evidenced in studies that the biofilm is one of the critical hindrances against drug entry and prevents direct contact with the bacteria. The growth of MRSA in the absence of extracts was determined and shown in **Figure 4.7**. Before the MRSA was introduced into the culture media, the OD₆₀₀ value was recorded. The readings were between 0.271 to 0.504. The MRSA and fibroblasts were observed at 1 hour, 3 hours, 6 hours, 10 hours, 24 hours, and 48 hours. However, observation at 48 hours was not showed in **Figure 4.7** because the MRSA had fully covered the flasks and formed clumps. The highest density of bacteria recorded was 1 % MRSA at 24 hours observation. This was fixed as the 100 % growth of MRSA. From **Figure 4.7**, 1 % MRSA achieved the highest growth at 24 hours and was denoted as 100 % coverage of MRSA tested. Interestingly, When 10 % MRSA was introduced, its growth and coverage were 84.01 % and lower than the percentage of 1 % MRSA inoculum at 24 hours of observation. There was a high possibility that 10 % MRSA inoculum had quickly overgrown and covered the flask to the extent of forming small clumps which the ImageJ software identified as cells.

The growth and coverage of 0.25 % and 0.1 % MRSA inoculant at 24 hours observation were 78.84 % and 81.55 % respectively. Even though the 0.1 % MRSA inoculum achieved slightly higher growth than the 0.25 % MRSA inoculum, but the difference was not as pronounced as with the 0.5 % MRSA inoculum. Despite the growth

and coverage of 0.25 % and 0.1 % MRSA inoculant was similar and suitable to be used in the co-incubation assay, but 0.1 % MRSA was selected to observe a more obvious antimicrobial effect.

Based on **Table 4.6**, the pseudo-wound of the negative control flasks was fully covered up after being incubated for 24 hours. Similarly, when the *C. nutans* ethanol and chloroform extracts were administered to the pseudo-wound, the wound covered up after 24 hours of incubation. Contrarily, the *C. nutans* acetone extract only fully covered up the pseudo-wound after 48 hours of incubation. For *S. crispus* extracts in **Table 4.7**, the pseudo-wound of negative control flasks was fully covered up after 24 hours of incubation. When the *S. crispus* chloroform extract was applied to the pseudo-wound, the pseudo-wound only fully covered up after 48 hours of incubation. Unlike the chloroform extract, the *S. crispus* ethanol and acetone extracts did not aid in the wound healing activity but slowed down the wound healing activity and became cytotoxic to the fibroblasts after 48 hours incubation.

The overall wound healing activity dropped when the MRSA was introduced for the co-incubation with the fibroblasts. This fully represented the condition of an open wound when MRSA invaded (Wang et al., 2021, Lam et al., 2020). All the co-incubation assay results showed a peak period of wound healing activity of closing up between 3, 6, or 10 hours of incubation before the level of activity based on the % of coverage and width of the wound started to drop, an indication of the MRSA invasion on the fibroblasts (Wu et al., 2021). The highest peak of wound healing activity based on the % of wound width was recorded for the *C. nutans* extracts with chloroform extract at 52.4 %, followed by acetone extract at 48.6 % and ethanol extract at 43.1 % respectively. For *S. crispus* extracts, the highest peak of wound healing activity based on the % of wound width was recorded at 76.1

% from ethanol extract, followed by chloroform and acetone extracts at 60.2 % and 45.8 % respectively.

According to the results summarised in **Table 4.3**, *C. nutans* and *S. crispus* extracts did not inhibit MRSA on MHA. Correspondingly, the *C. nutans* and *S. crispus* extracts did not deter the growth of MRSA in the flasks. Yet, *C. nutans* extracts aided in the wound healing activity of closing up and reducing the wound width. Unlike *S. crispus*, it had low toxicity, which resulted in the easy ‘take over’ and coverage of inoculum MRSA in the flasks. This was quantified by the highest wound healing activity peak recorded at a mere 52.4 %. Based on **Table 4.7**, *S. crispus* extracts did not aid in the wound healing activity, but it also contained higher cytotoxicity than *C. nutans* extracts as showed by the MTT assay. It is highly probably due to *S. crispus* extracts high cytotoxicity, it inhibited the MRSA activity slightly while the more complex pseudo-wound regenerated. Over time, when the density of MRSA increased, it ‘took over’ and covered the surface of the flasks. Therefore, the highest wound healing activity peaked and was recorded for the co-incubation at 76.1 % from *S. crispus* ethanol extract, followed by 60.2 % from *S. crispus* chloroform extract, respectively. Both *S. crispus* extracts were much higher than the peak recorded for *C. nutans* extracts.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study which aimed to investigate wound healing activity of *Clinacanthus nutans* (*C. nutans*) and *Strobilanthes crispus* (*S. crispus*) extracts when harmful bacteria present, is being reported for the first time. In addition, the phytochemical contents, antioxidant properties and antimicrobial activities were also assessed to understand their correlations further to wound healing properties. The antioxidants, TPC, and TFC for *C. nutans* and *S. crispus* extracts were determined, with *S. crispus* exhibiting higher antioxidant content than *C. nutans* while *C. nutans* contained more phenolics and flavonoids than *S. crispus*. However, more studies need to be conducted on *S. crispus* as the AAEAC showed it might have higher than 55 % indicating its antioxidant potency. The effects of different extraction solvents used were evident in this study. When used solvents with different polarity for extracting the *C. nutans* plant, the results for antioxidant content, TPC, and TFC present varied. Still, it did not show distinctive differences during disc diffusion assay. On the other hand, when different polarity solvents were for extraction of *S. crispus*, the antimicrobial properties were distinctively different, with ethanol extract of *S. crispus* exhibiting a larger inhibition zone than those of acetone and chloroform extracts. Overall, *C. nutans* was superior in antimicrobial properties to *S. crispus* extracts as the inhibition zones were more prominent in diameter. During the scratch assay, *C. nutans* extracts performed better than *S. crispus* in aiding the wound healing activity in terms of wound coverage post scratched. However, when MRSA was introduced and co-incubated with the normal human fibroblasts in the scratch assay, *S. crispus* better aided the wound healing activity. In conclusion, *C.*

nutans exhibited wound healing properties and performed better than *S. crispus*. However, when MRSA was introduced at the pseudo-wound, *S. crispus* performed better at resisting the growth of MRSA and allowed the wound to heal better before the MRSA took over. The findings from this study provided evidence-based support for understanding the antimicrobial efficacy of *C. nutans* and *S. crispus* in relation to wound healing and addressing the emerging antimicrobial resistance. It may contribute to the Government's efforts and aspirations as outlined in the Malaysian Action Plan on antimicrobial resistant, the UN Global Development Goals (SGD) and the 10-10 MYSTIE Framework on medical and health care and environment and biodiversity.

6.2 Recommendations

The findings demonstrated these studied plants could resist the growth of MRSA and enable the wound to close up and heal. Hence, it is suggested to extend this work using Gram-negative bacteria such as *Ps. aeruginosa* in the co-incubation *in-vitro* assay for future research with *C. nutans* and *S. crispus* extracts. It is also recommended to perform future *in-vivo* assays to validate its effectiveness. It has an excellent commercialisation potential to develop into natural resource-based health care and medical products.

REFERENCES

- Abd Hamid H. A., & Yahaya I. H. (2016). Cytotoxicity of *Clinacanthus nutans* extracts on human hepatoma (HepG2) cell line. *International Journal of Pharmacy and Pharmaceutical Sciences*, 8(10), 8-10.
- Abou Muamar, A. F. H. (1999). *Isolation, Identification and Evaluation of Antibacterial Activity of the Semi-purified Compound from Strobilanthes crispus (L. bremek)* (Doctoral dissertation, Universiti Putra Malaysia).
- Abu Bakar, M. F., Teh, A. H., Rahmat, A., Hashim, N., Othman, F., & Fakurazi, S. (2006). Antiproliferative properties and antioxidant activity of various types of *Strobilanthes crispus* tea. *International Journal of Cancer Research*, 2(2), 152-158.
- Agustina S. (2011). Isolation of flavonoids as antioxidant from *Dandang Gendis* (*Clinacanthus nutans*) leaves. *Bogor Agricultural University*.
- Akbari-Ayezloy, E., Hosseini-Jazani, N., Yousefi, S., & Habibi, N. (2017). Eradication of methicillin resistant *S. aureus* biofilm by the combined use of fosfomycin and β -chloro-L-alanine. *Iranian Journal of Microbiology*, 9(1), 1.
- Akova, M. (2016). Epidemiology of antimicrobial resistance in bloodstream infections. *Virulence*, 7(3), 252-266.
- Alam, A., Ferdosh, S., Ghafoor, K., Hakim, A., Juraimi, A. S., Khatib, A., & Sarker, Z. I. (2016). *Clinacanthus nutans*: A review of the medicinal uses, pharmacology and phytochemistry. *Asian Pacific journal of tropical medicine*, 9(4), 402-409.

Al-Henhena, N., Mahmood, A. A., Al-Magrami, A., Syuhada, A. N., Zahra, A. A., Summaya, M. D., Suzi, M.S., & Salmah, I. (2011). Histological study of wound healing potential by ethanol leaf extract of *Strobilanthes crispus* in rats. *Journal of Medicinal Plants Research*, 5(16), 3660-3666.

Arullappan, S., Rajamanickam, P., Thevar, N., & Kodimani, C. C. (2014). *In vitro* screening of cytotoxic, antimicrobial and antioxidant activities of *Clinacanthus nutans* (Acanthaceae) leaf extracts. *Tropical Journal of Pharmaceutical Research*, 13(9), 1455-1461.

Baba, S. A., & Malik, S. A. (2015). Determination of total phenolic and flavonoid content, antimicrobial and antioxidant activity of a root extract of *Arisaema jacquemontii* Blume. *Journal of Taibah University for Science*, 9(4), 449-454.

Barchan, A., Bakkali, M., Arakrak, A., Pagán, R., & Laglaoui, A. (2014). The effects of solvents polarity on the phenolic contents and antioxidant activity of three *Mentha* species extracts. *International Journal of Current Microbiologyl and Applied Science*, 3(11), 399-412.

Burkill, I. H. (1935). *A dictionary of the economic products of the Malay Peninsula*, 2nd ed., pp. 2086-2087. London: Crown Agents for the Colonies.

Chan, K. W., Khong, N. M., Iqbal, S., & Ismail, M. (2012). Simulated gastrointestinal pH condition improves antioxidant properties of wheat and rice flours. *International Journal of Molecular Sciences*, 13(6), 7496-7507.

Chelbyn, J. L., Omar, M. H., Mohd Yousof, N. S. A., Ranggasamy, R., Wasiman, M. I., & Ismail, Z. (2014). Analysis of flavone C-glycosides in the leaves of *Clinacanthus nutans*

(Burm. f.) Lindau by HPTLC and HPLC-UV/DAD. *The Scientific World Journal*, 2014, 724267.

Chhibber, S., Kaur, J., & Kaur, S. (2018). Liposome entrapment of bacteriophages improves wound healing in a diabetic mouse MRSA infection. *Frontiers in Microbiology*, 9, 561.

Cho, O. H., Baek, E. H., Bak, M. H., Suh, Y. S., Park, K. H., Kim, S., Bae, I. G., & Lee, S. H. (2016). The effect of targeted decolonization on methicillin-resistant *Staphylococcus aureus* colonization or infection in a surgical intensive care unit. *American Journal of Infection Control*, 44(5), 533-538.

Chomnawang, M. T., Surassmo, S., Nukoolkarn, V. S., & Gritsanapan, W. (2005). Antimicrobial effects of Thai medicinal plants against acne-inducing bacteria. *Journal of Ethnopharmacology*, 101(1-3), 330-333.

Chomnawang, M. T., Surassmo, S., Wongsariya, K., & Bunyaphrathatsara, N. (2009). Antibacterial activity of Thai medicinal plants against methicillin-resistant *Staphylococcus aureus*. *Fitoterapia*, 80(2), 102-104.

Chong, H. Z., Rahmat, A., Yeap, S. K., Md Akim, A., Alitheen, N. B., Othman, F., & Gwendoline-Ee, C. L. (2012). *In vitro* cytotoxicity of *Strobilanthes crispus* ethanol extract on hormone dependent human breast adenocarcinoma MCF-7 cell. *BMC Complementary and Alternative Medicine*, 12(1), 1-10.

Chua, L. Y. W., Chua, B. L., Figiel, A., Chong, C. H., Wojdyło, A., Szumny, A., & Choong, T. S. Y. (2019). Antioxidant activity, and volatile and phytosterol contents of *Strobilanthes crispus* dehydrated using conventional and vacuum microwave drying methods. *Molecules*, 24(7), 1397.

- Da Costa, E., Silva, J., Mendonça, S. H., Abreu, M. H., & Domingues, M. R. (2016). Lipidomic approaches towards deciphering glycolipids from microalgae as a reservoir of bioactive lipids. *Marine Drugs*, 14(5), 101.
- Dan, P. N. M. (2014). *Evaluation of biological activities of clinacanthus nutans (Burm. F.) Lindau* (Doctoral dissertation, International University, Vietnam).
- Endrini, S., Rahmat, A., Ismail, P., & Taufiq-Yap, Y. H. (2007). Comparing of the cytotoxicity properties and mechanism of *Lawsonia inermis* and *Strobilanthes crispus* extract against several cancer cell lines. *Journal of Medical Science*, 7(7), 1098-1102.
- Esmaili, K., Alsuede, F., Abdalrahim, A., Shafaei, A., & Ismail, Z. (2013). Preliminary phytochemical analysis and cytotoxicity studies of *Clinacanthus nutans* (Sabah snake grass). In *The Open Conference Proceedings Journal*, 4(1).
- Fong, S. Y. (2015). *Genetic, phytochemical and bioactivity studies of Clinacanthus nutans (Burm. f.) Lindau (Acanthaceae)* (Doctoral dissertation, RMIT University).
- Fong, S. Y., Piva, T., Dekiwadia, C., Urban, S., & Huynh, T. (2016). Comparison of cytotoxicity between extracts of *Clinacanthus nutans* (Burm. f.) Lindau leaves from different locations and the induction of apoptosis by the crude methanol leaf extract in D24 human melanoma cells. *BMC Complementary and Alternative Medicine*, 16(1), 1-12.
- Fortune Business Insight. (2022). Market Research Report 2021. Fortune Business Insight, India.
- Frykberg, R. G., & Banks, J. (2015). Challenges in the Treatment of Chronic Wounds. *Advances in Wound Care*, 4(9), 560–582.

Gambhire, M. N., Wankhede, S. S., & Juvekar, A. R. (2009). Antiinflammatory activity of aqueous extract of *Barleria cristata* leaves. *Journal of Young Pharmacists*, 1(3), 220.

Ghasemzadeh, A., Jaafar, H. Z., & Rahmat, A. (2015). Phytochemical constituents and biological activities of different extracts of *Strobilanthes crispus* (L.) Bremek leaves grown in different locations of Malaysia. *BMC Complementary and Alternative Medicine*, 15(1), 1-10.

Ghasemzadeh, A., Nasiri, A., Jaafar, H. Z., Baghdadi, A., & Ahmad, I. (2014). Changes in phytochemical synthesis, chalcone synthase activity and pharmaceutical qualities of Sabah snake grass (*Clinacanthus nutans* L.) in relation to plant age. *Molecules*, 19(11), 17632-17648.

Grada, A., Otero-Vinas, M., Prieto-Castrillo, F., Obagi, Z., & Falanga, V. (2017). Research techniques made simple: analysis of collective cell migration using the wound healing assay. *Journal of Investigative Dermatology*, 137(2), e11-e16.

Grice, E. A., & Segre, J. A. (2011). The skin microbiome. *Nature Reviews Microbiology*, 9(4), 244-253.

Gul, M. T., Dheyab, A. S., Shaker, E. K., Muhammad, N., & Pauzi, A. N. (2020). *In vitro* evaluation of anti-urolithiatic properties of *Strobilanthes crispus* extracted using different solvents. *Research Journal of Chemistry and Environment*, 24(1).

Gul, R., Jan, S. U., Faridullah, S., Sherani, S., & Jahan, N. (2017). Preliminary phytochemical screening, quantitative analysis of alkaloids, and antioxidant activity of crude plant extracts from *Ephedra intermedia* indigenous to Balochistan. *The Scientific World Journal*, 2017.

- Gupta, D. (2015). Methods for determination of antioxidant capacity: A review. *International Journal of Pharmaceutical Sciences and Research*, 6(2), 546.
- Hättenschwiler, S., & Vitousek, P. M. (2000). The role of polyphenols in terrestrial ecosystem nutrient cycling. *Trends in Ecology & Evolution*, 15(6), 238-243.
- Heesterbeek, D. A. C., Martin, N. I., Velthuisen, A., Duijst, M., Ruyken, M., Wubbolts, R., Rooijakkers, S. H. M. & Bardoel, B. W. (2019). Complement-dependent outer membrane perturbation sensitizes Gram-negative bacteria to Gram-positive specific antibiotics. *Scientific Reports*, 9(1), 1-10.
- Hufbauer, M., & Akgül, B. (2017). Molecular mechanisms of human papillomavirus induced skin carcinogenesis. *Viruses*, 9(7), 187.
- Hussain, M., & Ghani, A. (2008). Herbal remedies used for gastrointestinal disorders in Kaghan valley, NWFP, Pakistan. *Pakistan Journal of Weed Science Research*, 14(3-4), 169-200.
- Ismail, M., Bagalkotkar, G., Iqbal, S., & Adamu, H. A. (2012). Anticancer properties and phenolic contents of sequentially prepared extracts from different parts of selected medicinal plants indigenous to Malaysia. *Molecules*, 17(5), 5745-5756.
- Itam, A., Wati, M. S., Agustin, V., Sabri, N., Jumanah, R. A., & Efendi, M. (2021). Comparative Study of Phytochemical, Antioxidant, and Cytotoxic Activities and Phenolic Content of *Syzygium aqueum* (Burm. f. Alston f.) Extracts Growing in West Sumatera Indonesia. *The Scientific World Journal*, 2021.

Kavitha, K., Sujatha, K., & Manoharan, S. (2016). Antidiabetic Potential of Acanthaceae Family. *International Journal of Pharmaceutical Science Review and Research*, 36(1), 30-37.

Khan, I., Jan, S. A., Shinwari, Z. K., Ali, M., Khan, Y., & Kumar, T. (2017). Ethnobotany and medicinal uses of folklore medicinal plants belonging to family Acanthaceae: An updated review. *MOJ Biology and Medicine*, 1(2), 34-38.

Khoo, L. W., Audrey Kow, S., Lee, M. T., Tan, C. P., Shaari, K., Tham, C. L., & Abas, F. (2018). A comprehensive review on Phytochemistry and pharmacological activities of *Clinacanthus nutans* (Burm. F.) Lindau. *Evidence-Based Complementary and Alternative Medicine*, 2018.

Kirchner, S., Lei, V., & MacLeod, A. S. (2020). The cutaneous wound innate immunological microenvironment. *International Journal of Molecular Sciences*, 21(22), 8748.

Lam, A. K., Moen, E. L., Pusavat, J., Wouters, C. L., Panlilio, H., Ferrell, M. J., Houck, M. B., Glatzhofer, D. T., & Rice, C. V. (2020). PEGylation of Polyethylenimine Lowers Acute Toxicity while Retaining Anti-Biofilm and β -Lactam Potentiation Properties against Antibiotic-Resistant Pathogens. *ACS Omega*, 5(40), 26262-26270.

Lanau-Roig, A., Fabrellas, N., Sáez-Rubio, G., & Wilson, K. (2017). Time of Chronic wound healing, as part of a prevalence and incidence study. *Enfermería Global*, 16(2), 445-453.

Lee, S. Y., Mediani, A., Nur Ashikin, A. H., Azliana, A. B. S., & Abas, F. (2014). Antioxidant and α -glucosidase inhibitory activities of the leaf and stem of selected traditional medicinal plants. *International Food Research Journal*, 21(1), 379-386.

- Liew, S. Y., Stanbridge, E. J., Yusoff, K., & Shafee, N. (2012). Hypoxia affects cellular responses to plant extracts. *Journal of Ethnopharmacology*, 144(2), 453-456.
- Lim, V., Yap, C. S., Chong, H. W., Shukkoor, M. S. A., & Priya, M. (2013). Antimicrobial evaluation and GC-MS analysis of *Strobilanthes crispus* ethanolic leaf extract. *European Journal of Medicinal Plants*, 10(4), 1-8.
- Manzuoerh, R., Farahpour, M. R., Oryan, A., & Sonboli, A. (2019). Effectiveness of topical administration of *Anethum graveolens* essential oil on MRSA-infected wounds. *Biomedicine & Pharmacotherapy*, 109, 1650-1658.
- Marcheafave, G. G., Tormena, C. D., Pauli, E. D., Rakocevic, M., Bruns, R. E., & Scarminio, I. S. (2019). Experimental mixture design solvent effects on pigment extraction and antioxidant activity from *Coffea arabica* L. leaves. *Microchemical Journal*, 146, 713-721.
- Melissa W. (2020) *Wallin Biovisuals: Stages of Wound Healing* [online] Available at : <https://www.mcwallin.com/wound-healing/472rsn2vbchx2ts6tscz739v8di0mo> [Assessed on 28 February 2022]
- Mohan, V. R., Tresina, P. S., & Daffodil, E. D. (2016). Antinutritional factors in legume seeds: characteristics and determination. *Encyclopedia of Food and Health*, pp. 211-220. Oxford: Academic Press.
- Muflihah, Y. M., Gollavelli, G., & Ling, Y. C. (2021). Correlation Study of Antioxidant Activity with Phenolic and Flavonoid Compounds in 12 Indonesian Indigenous Herbs. *Antioxidants*, 10(10), 1530.

- Muskhazli M., Dirnahayu M., Nor Azwady A. A., Nurhafiza Y., Nor Dalilah E., & Che Ku Nurshaira C. K. N. (2009). Antibacterial activity of methanolic crude extracts from selected plant against *Bacillus cereus*. *Pertanika Journal of Tropical Agriculture Science*, 32(2), 175-183.
- Muslim, N. S., Ng, K. W., Itam, A., Nassar, Z. D., Ismail, Z., & Majid, A. A. (2010). Evaluation of cytotoxic, anti-angiogenic and antioxidant properties of standardized extracts of *Strobilanthes crispus* leaves. *International Journal of Pharmacology*, 6(5), 591-599.
- Nurraihana, H., & Norfarizan-Hanoon, N. A. (2013). Phytochemistry, pharmacology and toxicology properties of *Strobilanthes crispus*. *International Food Research Journal*, 20(5), 2045.
- Nyawai, T. N., Asaruddin, M. R., Rosli, M. F. A., Romli, A. M., Radhakrishnan, S. E., & Ahmad, M. N. (2017). Pharmacophore Modelling of Phytochemicals from *Clinacanthus Nutans* for Antimicrobial Activity. *Transactions on Science and Technology*, 4(4), 498-503.
- O'Meara, S., Cullum, N., Majid, M., & Sheldon, T. (2000). Systematic reviews of wound care management: (3) antimicrobial agents for chronic wounds; (4) diabetic foot ulceration. *Health Technology Assessment (Winchester, England)*, 4(21), 1-237.
- Ong C. W., Chan Y. S., Chan S. M., Chan M. W., Teh E. L., Soh C. L. D., Khoo K. S., Ong H. C., & Sit N. W. (2020). Antifungal, antibacterial and cytotoxicity activities of non-indigenous medicinal plants naturalised in Malaysia. *Farmacia*, 68(4), 687-696.
- Oyama, T., Miyazaki, M., Yoshimura, M., Takata, T., Ohjimi, H., & Jimi, S. (2016). Biofilm-forming methicillin-resistant *Staphylococcus aureus* survive in Kupffer cells and exhibit high virulence in mice. *Toxins*, 8(7), 198.

P'ng, X. W., Akowuah, G. A., & Chin, J. H. (2012). Acute oral toxicity study of *Clinacanthus nutans* in mice. *International Journal of Pharmaceutical Sciences and Research*, 3(11), 4202.

Pannangpetch, P., Laupattarakasem, P., Kukongviriyapan, V., Kukongviriyapan, U., Kongyingyoes, B., & Aromdee, C. (2007). Antioxidant activity and protective effect against oxidative hemolysis of *Clinacanthus nutans* (Burm. f) Lindau. *Songklanakarin Journal of Science and Technology*, 29(Suppl 1), 1-9.

Panche, A. N., Diwan, A. D., & Chandra, S. R. (2016). Flavonoids: an overview. *Journal of Nutritional Science*, 5.

Peng, T. W., Han, C. J., & Akowuah, G. A. (2014). Effect of methanol extract of *Clinacanthus nutans* on serum biochemical parameters in rats. *Journal of Applied Pharmacy*, 6, 77-86.

Pongmuangmul, S., Phumiamorn, S., Sanguansermisri, P., Wongkattiya, N., Fraser, I. H., & Sanguansermisri, D. (2016). Anti-herpes simplex virus activities of monogalactosyl diglyceride and digalactosyl diglyceride from *Clinacanthus nutans*, a traditional Thai herbal medicine. *Asian Pacific Journal of Tropical Biomedicine*, 6(3), 192-197.

Prasad, M. P. (2014). Studies on phytochemical analysis and antimicrobial activity of Acanthaceae species. *International Journal of Current Research*, 6(9), 8630-8637.

Qader, S. W., Abdulla, M. A., Chua, L. S., Najim, N., Zain, M. M., & Hamdan, S. (2011). Antioxidant, total phenolic content and cytotoxicity evaluation of selected Malaysian plants. *Molecules*, 16(4), 3433-3443.

Raal, A., Meos, A., Hinrikus, T., Heinämäki, J., Romāne, E., Gudienė, V., Koshovyi, O., Kovaleva, A., Fursenco, C., Chiru, T. & Nguyen, H. T. (2020). Dragendorff's reagent: Historical perspectives and current status of a versatile reagent introduced over 150 years ago at the University of Dorpat, Tartu, Estonia. *Die Pharmazie-An International Journal of Pharmaceutical Sciences*, 75(7), 299-306.

Rahmat, A., Edrini, S., Akim, A. M., Ismail, P., Yap, T. Y., & Fadzelly, A. M. (2006). Anticarcinogenic properties of *Strobilanthes crispus* extracts and its compounds *in vitro*. *International Journal of Cancer Research*, 2(1), 47-49.

Rodrigues, M., Kosaric, N., Bonham, C. A., & Gurtner, G. C. (2019). Wound healing: a cellular perspective. *Physiological Reviews*, 99(1), 665-706.

Ruffin, M., & Brochiero, E. (2019). Repair process impairment by *Pseudomonas aeruginosa* in epithelial tissues: major features and potential therapeutic avenues. *Frontiers in Cellular and Infection Microbiology*, 9, 182.

Samuel, A. J. S. J., Kalusalingam, A., Chellappan, D. K., Gopinath, R., Radhamani, S., Husain, H. A., Muruganandham, V., & Promwichit, P., (2010). Ethnomedical survey of plants used by the Orang Asli in Kampung Bawong, Perak, west Malaysia. *Journal of Ethnobiology and Ethnomedicine*, 6(1), 1-6, 20137098.

Sanyal, G., & Doig, P. (2012). Bacterial DNA replication enzymes as targets for antibacterial drug discovery. *Expert Opinion on Drug Discovery*, 7(4), 327-339.

Sekar, M., & Rashid, N. A. (2016). Formulation, evaluation and antibacterial properties of herbal ointment containing methanolic extract of *Clinacanthus nutans* leaves. *International Journal of Pharmaceutical and Clinical Research*, 8(8), 1170-1174.

Shackira, A. M., Puthur, J. T., & Nabeesa Salim, E. (2017). *Acanthus ilicifolius* L. a promising candidate for phytostabilization of zinc. *Environmental Monitoring and Assessment*, 189(6), 1-13.

Shah, M. D., & Hossain, M. A. (2014). Total flavonoids content and biochemical screening of the leaves of tropical endemic medicinal plant *Merremia borneensis*. *Arabian Journal of Chemistry*, 7(6), 1034-1038.

Shim, S. Y., Aziana, I., & Khoo, B. Y. (2013). Perspective and insight on *Clinacanthus nutans* Lindau in traditional medicine. *International Journal of Integrative Biology*, 14(1), 7-9.

Shimamura, T., Sumikura, Y., Yamazaki, T., Tada, A., Kashiwagi, T., Ishikawa, H., Matsui, T., Sugimoto, N., Akiyama, H., & Ukeda, H. (2014). Applicability of the DPPH assay for evaluating the antioxidant capacity of food additives—inter-laboratory evaluation study—. *Analytical Sciences*, 30(7), 717-721.

Shiuan, J. M. P. Y., Wang, H. L. J., Lin, C. C., & Liang, J. Y. (2012). Effects of *Clinacanthus nutans* (Burm. f) Lindau leaf extracts on protection of plasmid DNA from riboflavin photoreaction. *MC-Transaction on Biotechnology*, 4(1), e5.

Singh, D., & Aeri, V. (2013). Phytochemical and pharmacological potential of *Acanthus ilicifolius*. *Journal of Pharmacy & Bioallied Sciences*, 5(1), 17.

Sreevidya, N., & Mehrotra, S. (2003). Spectrophotometric method for estimation of alkaloids precipitable with Dragendorff's reagent in plant materials. *Journal of AOAC International*, 86(6), 1124-1127.

Tan, S. A., Lim, S. Y., Law, C. S., Yue, C. S., Poh, T. V., Saad, W. Z., Ismail, S., Yusoff, K. M., & Loke, C. F., (2019). Antioxidative and photocytotoxic effects of standardized *Clinacanthus nutans* and *Strobilanthes crispus* extracts toward HepG2 liver cells. *Pharmacognosy Magazine*, 15(65), 613.

Teoh, P. L., Cheng, A. Y. F., Liao, M., Lem, F. F., Kaling, G. P., Chua, F. N., & Cheong, B. E. (2017). Chemical composition and cytotoxic properties of *Clinacanthus nutans* root extracts. *Pharmaceutical Biology*, 55(1), 394-401.

Thammavongsa, V., Kim, H. K., Missiakas, D., & Schneewind, O. (2015). Staphylococcal manipulation of host immune responses. *Nature Reviews Microbiology*, 13(9), 529-543.

Tinh, T. D. D. (2014). *Biological activities of Clinacanthus nutans (Burm. F) Lindau extracts* (Doctoral dissertation, International University HCMC, Vietnam).

Uwamahoro, N., Verma-Gaur, J., Shen, H. H., Qu, Y., Lewis, R., Lu, J., Bamberg, K., Masters, S. L., Vince, J. E., Naderer, T., & Traven, A. (2014). The pathogen *Candida albicans* hijacks pyroptosis for escape from macrophages. *mBio*, 5(2), e00003-14.

Velmani, S., Perumal, B., Santhosh, C., Vetrivel, C., & Maruthupandian, A. (2016). Phytochemical and Traditional uses on *Acanthus ilicifolius* (L). *Journal of Advanced Applied Scientific Research*, 1(3), 43-48.

Vora, J., Srivastava, A., & Modi, H. (2018). Antibacterial and antioxidant strategies for acne treatment through plant extracts. *Informatics in Medicine Unlocked*, 13, 128-132.

Wang, M., Yang, Y., Chi, G., Yuan, K., Zhou, F., Dong, L., Liu, H., Zhou, Q., Gong, W., Yang, S., & Tang, T. (2021). A 3D printed Ga containing scaffold with both anti-infection

and bone homeostasis-regulating properties for the treatment of infected bone defects. *Journal of Materials Chemistry B*, 9(23), 4735-4745.

Wanikiat, P., Panthong, A., Sujayanon, P., Yoosook, C., Rossi, A. G., & Reutrakul, V. (2008). The anti-inflammatory effects and the inhibition of neutrophil responsiveness by *Barleria lupulina* and *Clinacanthus nutans* extracts. *Journal of Ethnopharmacology*, 116(2), 234-244..

Weisel, J. W., & Litvinov, R. I. (2017). Fibrin formation, structure and properties. *Fibrous Proteins: Structures and Mechanisms*, 405-456.

Wong, F. C., Yong, A. L., Ong, H. C., & Chai, T. T. (2013). Evaluation of the antibacterial activities of selected medicinal plants and determination of their phenolic constituents. *ScienceAsia*, 39(6), 591-595.

Wong, F. C., Yong, A. L., Ting, E. P. S., Khoo, S. C., Ong, H. C., & Chai, T. T. (2014). Antioxidant, metal chelating, anti-glucosidase activities and phytochemical analysis of selected tropical medicinal plants. *Iranian Journal of Pharmaceutical Research*, 13(4), 1409.

Wu, D., Wei, D., Du, M., Ming, S., Ding, Q., & Tan, R. (2021). Targeting antibacterial effect and promoting of skin wound healing after infected with methicillin-resistant *Staphylococcus aureus* for the novel polyvinyl alcohol nanoparticles. *International Journal of Nanomedicine*, 16, 4031.

Yang, H. S., Peng, T. W., Madhavan, P., Abdul Shukkoor, M., & Akowuah, G. (2013). Phytochemical analysis and antibacterial activity of methanolic extract of *Clinacanthus nutans* leaf. *International Journal of Drug Development and Research*, 5(3), 349-355.

Yong, Y. K., Tan, J. J., Teh, S. S., Mah, S. H., Ee, G. C. L., Chiong, H. S., & Ahmad, Z. (2013). *Clinacanthus nutans* extracts are antioxidant with antiproliferative effect on cultured human cancer cell lines. *Evidence-Based Complementary and Alternative Medicine*, 2013.

APPENDICES

Oral Presentation at InnoSTRE



Journal Publication:

1. Ban W.K., Fong I. L., Khong H. Y. & Phung J. H. Y. (2022) Wound healing, antimicrobial and antioxidant properties of *Clinacanthus nutans* (Burm. f.) Lindau and *Strobilanthes crispus* (L.) Blume extracts. *Molecules*, 27, 1722.



Article

Wound Healing, Antimicrobial and Antioxidant Properties of *Clinacanthus nutans* (Burm.f.) Lindau and *Strobilanthes crispus* (L.) Blume Extracts

Weng Kit Ban ¹, Isabel Lim Fong ^{1,*}, Heng Yen Khong ² and Joyce Hui Yie Phung ¹

¹ Department of Paraclinical Sciences, Faculty of Medicine and Health Sciences, Universiti Malaysia Sarawak, Kota Samarahan 94300, Malaysia; wengkitban14@gmail.com (W.K.B.); joycephy24@gmail.com (J.H.Y.P.)

² School of Chemistry and Environment, Faculty of Applied Sciences, Universiti Teknologi MARA, Sarawak Branch, Kota Samarahan 94300, Malaysia; khonghy@uitm.edu.my

* Correspondence: ifisabel@uitm.edu.my; Tel: +60-82-267500

Abstract: *Clinacanthus nutans* is known to be an anticancer and antiviral agent, and *Strobilanthes crispus* has proven to be an antidiuretic and antidiabetic agent. However, there is a high possibility that these plants possess multiple beneficial properties, such as antimicrobial and wound healing properties. This study aims to assess the wound healing, antioxidant, and antimicrobial properties of *Clinacanthus nutans* and *Strobilanthes crispus*. The *Clinacanthus nutans* and *Strobilanthes crispus* leaves were dried, ground, and extracted with ethanol, acetone, and chloroform through cold maceration. In a modified scratch assay with co-incubation of skin fibroblast and Methicillin-resistant *Staphylococcus aureus*, *Clinacanthus nutans* and *Strobilanthes crispus* extracts were assessed for their wound healing potential, and the antimicrobial activities of *Clinacanthus nutans* and *Strobilanthes crispus* extracts were performed on a panel of Gram-positive and Gram-negative bacteria on Mueller–Hinton agar based on a disc diffusion assay. To assess for antioxidant potential, 2,2-diphenyl-1-picrylhydrazyl (DPPH), total phenolic and total flavonoid assays were conducted. In the modified scratch assay, *Clinacanthus nutans* extracts aided in the wound healing activity while in the presence of MRSA, and *Strobilanthes crispus* extracts were superior in antimicrobial and wound healing activities. In addition, *Strobilanthes crispus* extracts were superior to *Clinacanthus nutans* extracts against *Pseudomonas aeruginosa* on Mueller–Hinton agar. Acetone-extracted *Clinacanthus nutans* contained the highest level of antioxidant in comparison with other *Clinacanthus nutans* extracts.

Keywords: *Clinacanthus nutans*; *Strobilanthes crispus*; wound healing; methicillin-resistant *Staphylococcus aureus*; *Pseudomonas aeruginosa*; scratch assay; antimicrobial; antioxidant



Citation: Ban, W.K.; Fong, I.L.; Khong, H.Y.; Phung, J.H.Y. Wound Healing, Antimicrobial and Antioxidant Properties of *Clinacanthus nutans* (Burm.f.) Lindau and *Strobilanthes crispus* (L.) Blume Extracts. *Molecules* **2022**, *27*, 1722. <https://doi.org/10.3390/molecules27051722>

Academic Editors: Silvia Fialová and Pavel Matěj

Received: 31 December 2021

Accepted: 1 March 2022

Published: 6 March 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Wound healing, a skin repairing process, is one of the most complicated processes in the human body. To close an open wound, multiple cells and stages are intrinsically involved, including haemostasis, inflammation, angiogenesis, growth, re-epithelialization, and re-modelling. However, harmful substances or opportunistic microorganisms may invade the body and hamper this process.

According to [1], commensals, such as *Propionibacterium acnes*, *Streptococcus pyogenes*, and *Staphylococcus aureus*, can be easily isolated on the surface of the skin, whereas opportunists *Enterococcus* spp. and *Pseudomonas aeruginosa* can be isolated in the absence of healing and in the presence of persistent inflammation, eventually leading to chronic wounds. The existence of methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most challenging issues when dealing with chronic wounds, as colonization and replication of MRSA on the wound further delays the wound healing process [2]. With the discovery of antibiotics, bacterial infections were alleviated but at a dire cost with the emergence of

2. Phung J. H. Y., Fong I. L., Khong H. Y. & Ban W. K. (2021) *In-vitro* antioxidant and anti-cancer activities of *Clinacanthus nutans* extracts. *Science, Engineering and Health Studies Journal*, 15, 21050014.

***In vitro* antioxidant and anticancer activities of *Clinacanthus nutans* extracts**

Joyce Hui Yie Phung¹, Isabel Lim Fong^{1*}, Heng Yen Khong² and Weng Kit Ban¹

¹ Faculty of Medicine and Health Sciences, Universiti Malaysia Sarawak, Kota Samarahan 94300, Sarawak, Malaysia

² Faculty of Applied Sciences, Universiti Teknologi MARA, Kota Samarahan 94300, Sarawak, Malaysia

ABSTRACT

*Corresponding author:
Isabel Lim Fong
lisabel@umimas.my

Received: 28 September 2020

Revised: 25 January 2021

Accepted: 5 February 2021

Published: 27 December 2021

Citation

Phung, J. H. Y., Fong, I. L.,
Khong, H. Y., and Ban, W. K.
(2021). *In vitro* antioxidant and
anticancer activities of
Clinacanthus nutans extracts.
*Science, Engineering and
Health Studies*, 15, 21050014.

Clinacanthus nutans (CN), from the Acanthaceae family is a medicinal plant widely used in Thailand and Malaysia. CN is commonly used as a treatment of inflammation, cancer, and herpes virus infection. This study aimed to determine the antioxidant activity and anticancer properties of CN leaves extracts on human colorectal cancer cell lines, HCT 116 and HT-29. In this study, CN leaves powders were extracted in methanol, chloroform, and acetone at different durations. The crude extracts were assessed for total phenolic content (TPC), total flavonoid contents (TFC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. The extract using acetone showed with the highest TPC. The highest total flavonoid content was the methanol extract. The DPPH radical scavenging activity, IC₅₀ of methanol extract was 19.67 µg/mL and exhibited the most efficacious antioxidant property among the others. The 24-hour methanol extract showed the most promising results on MTT assay. Therefore, CN methanol extract is a promising candidate to proceed in other anticancer studies such as cell cycle arrest and apoptosis assays.

Keywords: *Clinacanthus nutans*; colorectal cancer; anticancer; antioxidants; phenolic; flavonoid

1. INTRODUCTION

Cancer causes the second highest mortality in the world. In 2018, it was estimated to result in 9.6 million deaths (WHO, 2019). Colorectal cancer is the third most fatal cancer and the fourth most diagnosed cancer worldwide (Rawla et al., 2019). According to the Malaysian national cancer registry report 2012-2016, colorectal cancer was the most common cancer in male and the second most common in female (Azizah et al., 2019). It is possible to prevent colorectal cancer progression if early polyps were detected. Nonetheless, early detection of polyps is not routinely performed unless the patient has underlying hereditary history of colorectal cancer. This is commonly treated with surgery and in the advanced stage, a single

chemotherapeutic drug, 5-fluorouracil (5-FU) would be routinely used or combined with adjuvants like avastin and oxaliplatin (Sheikh et al., 2017a; Cutsem et al., 2015). Undetected polyps usually lead to formation of adenomas and carcinomas before the late stage of metastasis leading to high mortality risk (Sheikh et al., 2017a).

Many studies have reported that increased levels of intracellular free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) triggered cancer formation (Yong et al., 2013). ROS and RNS are normally found in the human body as they are by-products of metabolism in normal cells (Yong et al., 2013). These ROS and RNS damage the normal cells, especially DNA, resulting in genomic instability and induce cancer initiation (Yong et al., 2013). Many medicinal plants have been reported to