Cordyceps militaris EXTRACT INDUCES PROLIFERATION, MIGRATION AND EXPRESSION OF GROWTH FACTORS IN HUMAN DERMAL FIBROBLAST CELLS FOR ENHANCED WOUND HEALING

KANJANA SOODPAKDEE

MASTER OF SCIENCE
IN
BIOLOGICAL SCIENCE

SCHOOL OF SCIENCE
MAE FAH LUANG UNIVERSITY

2024

Cordyceps militaris EXTRACT INDUCES PROLIFERATION, MIGRATION AND EXPRESSION OF GROWTH FACTORS IN HUMAN DERMAL FIBROBLAST CELLS FOR ENHANCED WOUND HEALING

KANJANA SOODPAKDEE

THIS THESIS IS A PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
IN
BIOLOGICAL SCIENCE

SCHOOL OF SCIENCE
MAE FAH LUANG UNIVERSITY
2024

Cordyceps militaris EXTRACT INDUCES PROLIFERATION, MIGRATION AND EXPRESSION OF GROWTH FACTORS IN HUMAN DERMAL FIBROBLAST CELLS FOR ENHANCED WOUND HEALING

KANJANA SOODPAKDEE

THIS THESIS HAS BEEN APPROVED
TO BE A PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE
IN
BIOLOGICAL SCIENCE
2024

EXAMINATION COMMITTEE

.....CHAIRPERSON
(Yin Quan Tang, Ph. D.)

.....ADVISOR
(Asst. Prof. Sunita Chamyuang, Ph. D.)

.....CO-ADVISOR
(Prof. Suttichai Krisanaprakornkit, Ph. D.)

.....CO-ADVISOR
(Anupong Makeudom, Ph. D.)

.....EXAMINER
(Amorn Owatworakit, Ph. D.)

ACKNOWLEDGEMENTS

Firstly, I would like to thank and express my deepest gratitude to my advisor, Asst. Prof. Dr. Sunita Chamyuang and co-supervisor, Prof. Dr. Suttichai Krisanaprakornkit, and Dr. Anupong Makeudom for their valuable guidance, encouragement, and unwavering support throughout my study. I also thank the committee, Dr. Yin-Quan Tang, and Dr. Amorn Owatworakit, for their valuable suggestions.

I would like to thank the Microbial Products and Innovation (MP&I) research group, the Scientific and Technological Instruments Center, as well as the school of Dentistry, Mae Fah Luang University for laboratory facilities. Thanks to the group members of MP and I for sharing, encouraging, and making the nice laboratory. Moreover, I would like to thank Mae Fah Luang university for financial support. Special thanks to Dr. Nanthanit Jaruseranee for her assistance in the primary cell culture part.

For my last note, I would like to say thanks to my family, especially my mom for her constant love, encouragement, and support during my studies. Their unwavering belief in me and my abilities gave me the strength to persevere through the challenges and difficulties of completing the thesis.

Kanjana Soodpakdee

Thesis Title	*Cordyceps militaris* Extract Induces Proliferation, Migration and Expression of Growth Factors in Human Dermal Fibroblast Cells for Enhanced Wound Healing
Author	Kanjana Soodpakdee
Degree	Master of Science (Biological Science)
Advisor	Asst. Prof. Sunita Chamyuang, Ph. D.
Co-Advisor	Prof. Suttichai Krisanaprakornkit, Ph. D. Anupong Makeudom, Ph. D.

ABSTRACT

Angiogenesis plays a crucial role in wound healing by facilitating the delivery of oxygen and nutrients, which are required for cell proliferation and tissue regeneration. *Cordyceps militaris* extract (CME) has found extensive use in both traditional and contemporary medicine due to its diverse therapeutic compounds. However, the specific impact of CME, which contains adenosine and a high amount of cordycepin, on promoting the proliferation of neonatal human dermal fibroblast cells (HDFn) and wound healing has not been clarified. Herein, we first optimize the concentration of CME commercial adenosine, commercial cordycepin and mixture of adenosine and cordycepin (ratio 1:5) which could be used to treat HDFn without toxicity. Then, investigated the effect of CME on HDFn since the cells secrete pro-angiogenic factors related to cell proliferation, migration and angiogenesis that affect healing process. Concentration of CME at 0.156 and 0.313 μM significantly increased (p<0.001) HDFn proliferation around 60% compared to untreated. Furthermore, CME also had migration capacity treatment and upregulated the expression of FGF-2 and VEGF-A which are pro-angiogenic factors in HDFn. Our research indicates that CME

shows potential as a therapeutic agent for wound healing and angiogenesis. Its capacity to boost cell proliferation and facilitate vascular development positions it as a valuable candidate for medical use.

Keywords: *Cordyceps militaris*, Adenosine, Cordycepin, Fibroblast Growth Factor-2, Vascular Endothelial Growth Factor-A

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

Table **Page**

LIST OF FIGURES

LIST OF FIGURES (continued)

Figure **Page**

CHAPTER 1

INTRODUCTION

1.1 Background and Significant of Research

The World Health Organization (WHO) has estimated that approximately 75% of the global population relies on herbs and various traditional medical practices for disease treatment. Nowadays, the advancement of modern chemistry has enabled the extraction of compounds from medical fungi, which have been instrumental as drugs or as precursors for creating numerous significant pharmaceuticals in use today (Das et al., 2010). *Cordyceps militaris* is a traditional medical fungus that has been valued for its multifunctional role as a food supplement and its potential therapeutic applications in modern medicine such as anticancer, (Shrestha et al., 2012) immune system enhancement, aging delay, viral and bacterial infection resistance, energy-boosting, and wound healing properties (Choi et al., 2020; Phull et al., 2022). They also contains important bioactive components including cordycepin (3'-deoxyadenosine), adenosine, pentostatin, γ-Aminobutyric acid (GABA) and polysaccharides (Do Hai Lan et al., 2016). Cordycepin, primarily found in *Cordyceps militaris*, has recently gained attention for its remarkable health and medicinal benefits, notably its potential in cancer treatment (Tuli et al., 2013), also findings underscore the potential of cordycepin as a new class of anti-inflammatory agents (Tan et al., 2021), while adenosine has been observed to encourage the movement (migration) and growth of endothelial cells, which are instrumental in the formation of new blood vessels, a process known as angiogenesis (Clark et al., 2007). Wound healing is an intricate physiological process that leads to the re-establishment of tissue structure, it encompasses four critical stages: hemostasis, inflammation, proliferation, and tissue remodeling (Singh et al., 2017). Adenosine, a powerful endogenous physiological mediator and plays crucial responsibilities in regulating

tissue homeostasis via their receptors (Montesinos et al., 2002). Moreover, adenosine also significantly helps the complex process of new blood vessels formation by interacting with critical growth factors such as fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor-A (VEFG-A) (Auchampach, 2007). In contrast to the pro-angiogenic effects of adenosine, numerous past research has demonstrated the anti-angiogenic properties of cordycepin and the mechanisms behind these effects (Tuli et al., 2014). However, as far as we are aware, the impact of crude extracted from *C. militaris* on promoting wound healing via FGF-2 and VEFG-A has not been explored in normal cells, like primary human dermal fibroblast neonatal cells (HDFn). Fibroblasts, the most common cell type found in connective tissues throughout the body plays crucial roles in various biological processes such as acting as mediators during inflammatory responses, and angiogenesis, and they are frequently used in studies related to wound healing, tissue engineering, and regeneration applications (Kendall & Feghali-Bostwick, 2014). Despite the potential of adenosine and cordycepin from *C. militaris* for cell proliferation and cell migration are still unknown. Thus, this study aimed to investigate the effect of crude extract from *C. militaris* on the expression of growth factor in primary dermal fibroblast human neonatal cells as well as to observe protein expression via the Western blot technique.

1.2 Research Objectives

1.2.1 To investigate the effect of the crude extract from *C. militaris* on the cell viability, cell proliferation and cell migration on primary dermal fibroblast human neonatal cells (HDFn) by comparing with standard adenosine and cordycepin.

1.2.2 To observe interested FGF-2 and VEFG-A proteins expression via Western blot analysis.

1.3 Scope of Research

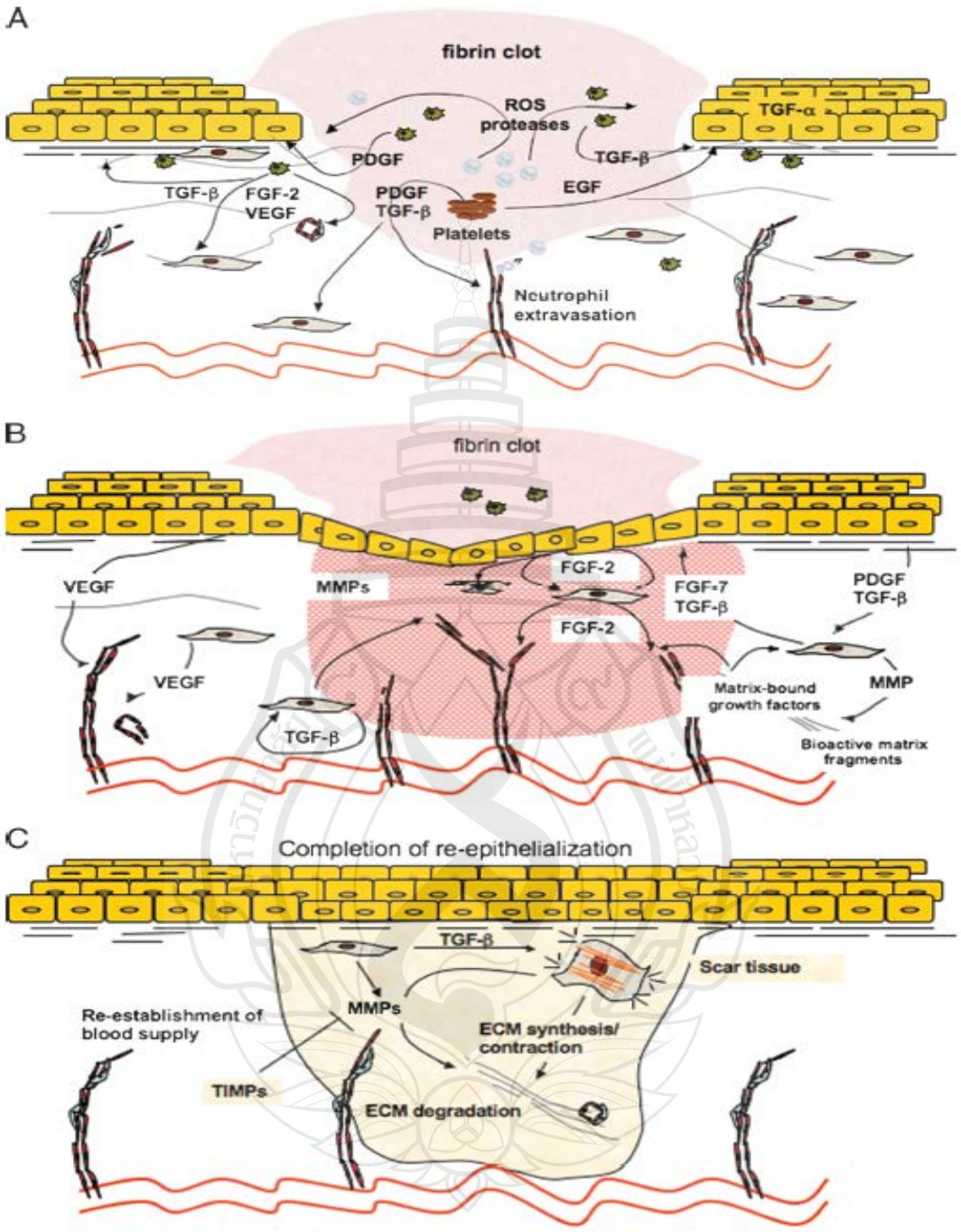
Cordyceps militaris (SH01) mycelium was cultivated in liquid media prior subjected to extraction. Then, crude extract was analyzed for observed adenosine and cordycepin content by using High-performance liquid chromatography (HPLC) technique. The MTT (colorimetric assay) was used to determine cell viability and cell proliferation after tested crude sample on human dermal fibroblast neonatal cell (HDFn) compared with adenosine and cordycepin commercial. Cell migrations were observed under live cell imager before investigated the proteins expression by Western blot assay

CHAPTER 2

LITERATURE REVIEW

2.1 Skin and Wound Healing

The skin, being the most extensive organ of the human body, is vital for numerous functions including moisture retention, shielding against harmful substances and microorganisms, initiating the production of vitamin D, eliminating waste, and regulating body temperature (Collier & Simon, 2016). Consequently, extensive damage to the skin can pose a significant threat to life. The wound-healing process of the skin is a remarkable display of cellular activity that is unique. This process encompasses the collaborative efforts of various cells, growth factors, and cytokines that work together to seal the wound (Tottoli et al., 2020). Acute wound healing typically unfolds in a systematic and orderly manner, resulting in consistent tissue restoration (Cañedo-Dorantes & Cañedo-Ayala, 2019). Essential contributors to this process include platelets, keratinocytes, cells responsible for immune surveillance, cells of the microvasculature, and fibroblasts, all of which are instrumental in reinstating tissue wholeness (Raziyeva et al., 2021). The repair sequence is categorized into four phases that temporally and spatially intersect: the coagulation phase called hemostasis, the inflammatory phase, the proliferative phase marked by the development of granulation tissue, and the phase of remodeling or scar tissue formation (Figure 2.1) (Demidova-Rice et al., 2012). During each phase of wound healing were required several kinds of growth factor such as epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin like growth factor (IGF), keratinocyte growth factor (KGF), platelet-derived growth factor (PDGF), transforming growth factor (TGF) and vascular endothelial growth factor (VEGF) (Greenhalgh, 1996).



Source Demidova-Ric et al. (2012)

Figure 2.1 Normal mechanism of wound healing

2.1.1 Hemostasis Phase

In the hemostasis phase, a temporary scaffold forms at the site of injury, setting in motion the wound-healing process (cell migration) (Gonzalez et al., 2016). Hemostasis starts when blood leaks out of the body, and blood clot develops to occupy the wound area, acting as a temporary matrix that offers a structure for various cells to move across by following an initial 5 to 10 minutes of blood vessel constriction, the vessels then expand, allowing platelets and white blood cells to move into this temporary framework (Landén et al., 2016). Next, platelets stick together to seal the break in the wall of the blood vessel in fibrin network (Martin, 1997). Moreover, an array of cytokines and growth factors are discharged into the wound at this phase, facilitating the interaction and coordinating the functions of diverse cellular participants to achieve healing (Barrientos et al., 2008).

Furthermore, growth factors like fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF) have a specific affinity for binding to fibrinogen (and fibrin), indicating that fibrinogen's role extends beyond hemostasis to also include homeostasis (Laurens et al., 2006).

2.1.2 Inflammatory Phase

During the inflammatory stage, which primarily involves the activation of the innate immune response, there is a swift influx of neutrophils and monocytes to the site of skin injury (Koh & DiPietro, 2011). In normal skin wound healing, the inflammation usually lasts for 2–5 days, this phase occurs simultaneously with hemostasis and is characterized as the initial phase of the wound-healing process against pathogen invasion and aids in the elimination of necrotic tissue (Turabelidze & Dipietro, 2011). Several elements, such as lipoxins and eicosanoid resulting from the metabolism of arachidonic acid, are believed to possess anti-inflammatory qualities(Esser-von Bieren, 2019). These properties help to mitigate the immune response, paving the way for the subsequent stage of wound healing to commence (Harper et al., 2014).

2.1.3 Proliferation or Granulation Tissue Formation Phase

At proliferation or also known as granulation tissue formation phase, growth factors secreted by the residual inflammatory cells and the migrating cells of the epidermis and dermis function in an autocrine, paracrine, and juxtracrine manner (Nanney, 1990; Werner & Grose, 2003). This activity is essential to stimulate and sustain cell division and movement, which are necessary for the development of granulation tissue and the promotion of epithelialization (Pastar et al., 2014). Concurrently, as the dermal and epidermal cells move and multiply within the wound, there is a clear necessity for a sufficient blood supply to ensure the delivery of nutrients, oxygen, and waste removal (Demidova-Rice et al., 2012). Wound healing angiogenesis begins immediately after injury when local hypoxia, secondary to injury-induced blood vessel disruption, occurs (Lokmic et al., 2012). This event fosters the production of proangiogenic factors such as VEGF, FGF-2, and PDGF, which are initially released by platelets and then by resident cells within the wound bed, are all central mediators of injury-induced angiogenic induction (Demidova-Rice et al., 2012).

2.1.4 Matrix Remodeling or Scar Formation Phase

The matrix remodeling (or scar formation) phase is the final stage of wound healing. During this phase, the new tissue slowly gains strength and flexibility, the period often begins around 2 weeks after injury and can persist for a year or longer (Gurtner et al., 2008). Fibroblast, endothelial, and keratinocyte cells are all of the cells type which play major roles in this phase (Lux, 2022). In 2012, Demidova-Rice et al. reported that inside of the wound, fibroblasts multiply and produce the extracellular matrix (ECM), which leads to the development of granulation tissue infused with new blood vessels (Demidova-Rice et al., 2012). Moreover, in Tracy et al. (2016) demonstrated a diverse array of ECM elements such as collagens, fibrin, fibronectin, proteoglycans, glycosaminoglycans, and matricellular proteins play a crucial role in supporting the life, movement (migration), and metabolic processes of dermal fibroblasts.

Each stage requires several kinds of growth factors which are crucial for proper wound healing, and keeping the wound clean and covered can aid the body's repair process.

2.2 Biological Growth Factors

Growth factors are critical components in the wound healing process, serving as signaling molecules that regulate cellular activities essential for repair and regeneration (Steenfos, 1994). During wound healing, growth factors such as Epidermal Growth Factor (EGF), Fibroblast Growth Factor (FGF), Insulin-like Growth Factor (IGF), Keratinocyte Growth Factor (KGF), and Vascular Endothelial Growth Factor (VEGF) orchestrate a range of processes including cell migration, proliferation, and differentiation (Vaidyanathan, 2021).

In this experiment, the enhancement of the growth factors (FGF-2 and VEGF-A) was investigated to determine its effect on response element-binding protein levels, demonstrating potential for improving the wound healing process.

2.2.1 Fibroblast Growth Factor-2 (FGF-2)

The FGFs family consists of heparin-binding growth factors that are secreted proteins. They are involved in a wide range of cellular activities, including cell proliferation, and angiogenesis in animal model (Nies et al., 2016). The FGF family in mammals has 22 members and seven subfamilies based on sequence homology and phylogenetic analysis (Ornitz & Itoh, 2001). FGF-2, or basic FGF, is one of the most extensively studied members of this family due to its significant role in angiogenesis and its therapeutic potential in wound healing and tissue regeneration (Nakamizo et al., 2012).

In 2016, Song et al. reported FGF-2 has been effective in enhancing the movement of cells in the fibroblast cell line (NIH3T3) and has also been associated with a rise in the levels of JNK phosphorylation (Song et al., 2016). Moreover, previous studies have demonstrated that FGF-2 can induce VEGF-A secretion from cells in a dose-dependent manner, and co-stimulation with both FGF-2 and VEGF-A

can synergistically promote cell migration, an essential step in wound repair (Yanagita et al., 2014).

In 2019, Veith et al. demonstrated the wound-healing process, there is an inverse relationship between the concentrations of VEGF-A and FGF-2 by initially, elevated levels of FGF-2 are crucial for attracting endothelial cells, which is then succeeded by increased VEGF-A levels that help to solidify the newly formed blood vessels and ensure an extended period of angiogenic signaling (Veith et al., 2019).

Thus, this cooperative effect underscores the importance of FGF-2 and VEGF-A in the complex interplay of growth factors that orchestrate the wound-healing process.

2.2.2 Vascular Endothelial Growth Factor- A (VEGF-A)

VEGF is a one of protein types that performs a critical part in the regulation of angiogenesis (Karamysheva, 2008). VEGF family consist of six members including VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PlGF), the angiogenic actions of VEGF family members are mediated by three receptor tyrosine kinases: VEGFR-1, VEGFR-2, and VEGFR-3 (Sullivan & Brekken, 2010). Isoforms A and B primarily contribute to the formation of blood vessels, while isoforms C and D are predominantly involved in developing lymphatic vessels (Sedighi et al., 2023). VEGFR-2 is primarily present in vascular endothelial cells and shows the greatest affinity for VEGF-A, functioning as the main receptor for angiogenesis signaling (Lee et al., 2021). During the wound healing process, VEGF-A is actively involved especially in granulation tissue formation and re-epithelialization (Park et al., 2018). VEGF-A also induces vascular permeability, allowing an influx of inflammatory cells into the injury site, which contributes to the inflammatory phase of wound healing (Vaidyanathan, 2021).

In 2006, Eming et al. reported that VEGF-A is not present in intact skin, but a mechanical injury triggers a marked increase in VEGF-A production, which is closely associated with the timing and location of new blood vessel formation (Eming & Krieg, 2006). In 2022, Shams et al. demonstrated the distribution of VEGF-A protein or mRNA for a shorter duration was less successful in repair, and it has been hypothesized that sustained expression of VEGF-A during the early angiogenic phase

from specific cells may be more helpful for optimal wound healing (Shams et al., 2022).

2.3 *Cordyceps militaris*

Cordyceps militaris is an entomopathogenic fungus that widely utilized as a traditional medicinal substance in East Asian regions (Kontogiannatos et al., 2021). *C. militaris* contains several kinds of bioactive compounds that responsible for the diverse pharmacological actions, which mostly include polysaccharides, adenosine, cordycepin (3'- deoxyadenosine), ergosterol, and myriocin (Zhang et al., 2019). These compounds are thought to have a range of health benefits, such as improving energy levels, boosting immunity, and reducing inflammation, some studies have also suggested anti-cancer properties (Mehra et al., 2017).

However, more research is needed to fully understand the effects of crude extract from *C. militaris* containing adenosine and cordycepin on inducing cell proliferation and migration as well as determine optimal dosages.

2.3.1 Adenosine

Adenosine is ribonucleoside that is composed of a molecule of adenine attached with d-ribose sugar (Figure 2.2 A). Adenosine plays a crucial part in the inflammatory processes that are essential for wound healing and tissue remodeling (Pasquini et al., 2021). Adenosine exerts its effects primarily through interactions with four G protein-coupled receptors, which are known as A1, A2A, A2B, and A3 adenosine receptors and are found in a variety of cells and organs throughout the body (Borea et al., 2018). In 2006, Chan et al. reported that activating adenosine A2A receptors aids in the healing of excisional wounds in both healthy mice and diabetic rats, this improvement in skin wound repair is also associated with a rise in the matrix (collagen) within the wounds (Chan et al., 2006). Particularly, A2A and A2B are essential for proper granulation tissue formation, which is a pivotal step in wound healing, they stimulate critical functions like new matrix production and angiogenesis in endothelial cells as well as other cells that are present in the wound (Feoktistov et al., 2009). Moreover, Burnstock (2020) demonstrated adenosine receptors A2A and

A2B are expressed on endothelial cells, and activation of these receptors can stimulate angiogenesis by inducing the production of angiogenic factors such as VEGF and platelet-derived growth factor (PDGF) (Burnstock, 2020). Furthermore, the activation of the A3 receptor seems to play a role in tissue remodeling in human lung mast cells by upregulating genes like interleukin 8, interleukin 6, and VEGF, which are all key agents in tissue remodeling in wound healing process and angiogenesis (Borea et al., 2016).

2.3.2 Cordycepin (3'-deoxyadenosine)

Cordycepin (3'deoxyadenosine), a nucleoside analogue which lacks 3' hydroxyl group compared to adenosine structures (Figure 2.2 B) that demonstrate a broad spectrum of medical fields such as antibacterial, antifungal, antitumor, an immunoregulatory effect as well as enhance systemic blood circulation (Jiang et al., 2019; Jin et al., 2018; Lee et al., 2019). In 2020, Ashraf et al. reported that cordycepin extracts were shown to alleviate lung fibrosis by suppressing TGF-β1 and enhancing collagen breakdown, indicating its strong potential as an anti-inflammatory agent (Ashraf et al., 2020). Cordycepin mostly reported their potential for cancer cells inhibition, induces apoptosis in cells as well as controls proliferation of cancer cells (Tian et al., 2015). In 2019, Lin et al. demonstrated that cordycepin shown potential to diminish liver cancer cell proliferation, migration, also anti- angiogenesis in animal model (Lin et al., 2019). Cordycepin is extensively studied for its potential in cancer treatment and reducing inflammation, while adenosine is known to encourage the growth of endothelial cells and the formation of new blood vessels via its receptors (Nguyen et al., 2022). Due to its structural resemblance to adenosine, cordycepin is presumed to act as either an agonist or antagonist in adenosine's target pathways, particularly affecting adenosine receptors. It has been observed that cordycepin can trigger the adenosine A2A receptor, influencing spontaneous behavioral changes in the hippocampus (Cao et al., 2018). Kim et al. (2021) reported that both adenosine and cordycepin have been shown to induce cell movement in a laboratory-based wound healing model and to advance the reorganization of the cellular structure, highlighting the increased movement capabilities of dermal fibroblasts (Kim et al., 2021). Nonetheless, research into how specifically and selectively cordycepin interacts with

various adenosine receptor subtypes in skin cells, like dermal fibroblasts, remains scarce.

The combination of adenosine and cordycepin were related to angiogenesis process because adenosine shown potential for enhance the growth of blood vessels from the existing vasculature and cordycepin also shown capacity to improve blood circulation system. From the reason, crude extract from medical fungus (*C. militaris*) that contain both of adenosine and cordycepin might be a novel treatment in medical field in future.

(A) Adenosine ($C_{10}H_{13}N_5O_4$) (B) Cordycepin ($C_{10}H_{13}N_5O_3$)

Figure 2.2 Chemical structure of adenosine and cordycepin

CHAPTER 3

MATERIALS AND METHODS

3.1 Sample Preparation

3.1.1 *Cordyceps militaris* Cultivation

Cordyceps militaris strain SH01 which obtained from Shanghai, China were used in the present experiments. The fungal mycelium was maintained on potato dextrose agar (PDA) at 20°C for 21 days before stored at 4°C as stock culture. The seed culture medium was modified from (Shih et al., 2007), which contain necessary components including 40 g/L glucose, 10 g/L yeast extract, 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4, and 0.5 g/L $MgSO_4 \cdot 7H_2O$, then initial pH was adjusted to 7.0. The seed culture was created by cutting out 5 mm diameter agar discs from a PDA plate using a sterilized cork borer. These discs were then placed into 300 mL of seed culture medium and incubated in darkness at 25°C on a rotary shaker (at 110 rpm) for 7 days and kept in static condition until 14 days. The mycelium part was collected via filter glass vacuum using nylon fabric size 45 micrometer and freeze-drying prior extraction to determine adenosine and cordycepin content.

3.1.2 *Cordyceps militaris* Extraction and Analysis

The dried mycelium was extracted and quantified amount of adenosine and cordycepin followed (ChokeUmnuay & Owatworakit, 2021). One gram of dried weight mycelium was extracted in twenty milliliters of deionized water at 60℃ for 3 hr. before centrifuged at 8000 rpm, at 4°C for 15 mins. The supernatant was filtered using a 0.2-μm filter membrane and then analyzed by the amount of adenosine and cordycepin by using HPLC.

Standard adenosine and cordycepin (from Sigma, USA) were initially prepared as stock solutions at a concentration of 2 mg/mL. Subsequently, these solutions were

further diluted in a range of concentrations from 100 μg/mL to 0.76 μg/mL using a o-fold dilution method. This process was carried out to establish the standard curve for adenosine and cordycepin via HPLC analysis. The crude extract from *C. militaris* (CME) was quantified by creating a plot of peak area against concentration. The 1 μL of the crude extract was then analyzed using a reversed-phase ACE Excel 3 column (manufactured by Water, USA) with specifications of C18, 4.6 × 250 mm, 5-μm. The analysis was conducted at a flow rate of 0.2 mL/min and a temperature of 30°C. The chromatographic condition was isocratic, with a 95:5 ratio of 0.2% formic acid in water (A) to methanol (B). Both adenosine and cordycepin were monitored at a wavelength of 260 nm.

3.2 Neonatal Human Dermal Fibroblast (HDFn) Culture

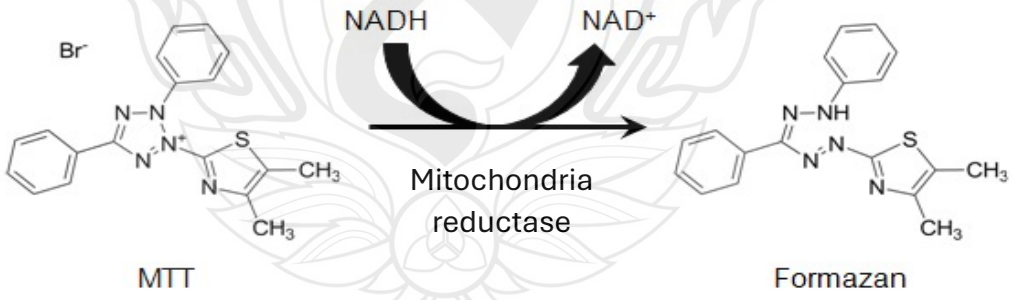
Primary Dermal Fibroblast Normal; Human, Neonatal cell (HDFn) was purchased from American Type Culture Collection (ATCC, PCS-201-010™) and cultured in fibroblast basal medium (ATCC, PCS-201-030™) supplemented with fibroblast growth kit-low serum (ATCC, PCS-201-041™) shown in Table 3.1 and added 1% of antibiotic-antimycotic (100X) solution contains 10,000 units/mL of penicillin, 10,000 μg/mL of streptomycin, and 25 μg/mL of amphotericin (Gibco™, Cat. No. 15240062). HDFn was maintained in a humidified atmosphere with 5 % CO_2 at 37°C and complete media was replaced every 2 days. The cultures were washed with phosphate buffered saline pH 7.4 (PBS, Gibco, Cat. No. 10010023) then passaged with 0.25% Trypsin-EDTA solution (Gibco, Cat. No. 25200072) when the cells confluence reached ~80%. HDFn cells were centrifuged at 150 g for 5 minutes and cells pellet was suspended in complete culture medium. HDFn cells were passaged 2-5 times before used in the following experiment.

Table 3.1 List of components in fibroblast kit-low serum in basal medium

Component	Volume (mL)	Final Concentration
rh FGF basic	0.50	5 ng/mL
Phenol red	0.50	33 μM
L-glutamine	18.75	7.5 mM
Ascorbic acid	0.50	50 mg/mL
Hydrocortison	0.50	1 mg/mL
rh Insulin	0.50	5 mg/mL
Fetal bovine serum	10.00	2 %

3.3 MTT Assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) is a colorimetric assay for assessing cellular metabolic activity (Figure 3.1) were used to determined cell viability and cell proliferation.



Source Riss et al. (2016)

Figure 3.1 MTT is reduced intracellularly to purple formazan granules

3.3.1 MTT Reagent Preparation

The MTT reagent preparation procedure was modified from (Bahuguna et al., 2017). Five hundred milligrams of MTT powder (Sigma–Aldrich, USA) were dissolved in 10 mL of sterile PBS (pH 7.4) and then homogenized before filtering with a 0.2-μm nylon syringe filter membrane. Aliquot (MTT reagent 50 mg/mL) into the sterile container for prepared working solution (MTT reagent 5 mg/mL) and keep it at -20℃ until had an experiment.

3.3.2 Cell Viability

HDFn cells were counted by using a hemocytometer (Lauda-Könlgshofen, Germany) and ten thousand of HDFn cells ($1x10^4$ cell/well) were seeded in each well of 96-well plates (Nunclon^{TM}) and then incubated at 37℃ with 5% CO_2 in humidified atmosphere for 24 hours. Samples: CME, adenosine standard, cordycepin standard, and the mixture of adenosine and cordycepin standard in the same ratio in CME were used. The samples were varied concentration at 0.078, 0.156, 0.313, 0.625, 1.250, 2.50, 5 and 10 μM. A 100 μL of samples were added into 96-well plates after removed old complete medium and then incubated at 37℃ with 5% CO_2 for 24 hours. The solution was discarded before adding 100 μL of MTT solvent (0.5 mg/mL) and incubated at 37℃ with 5 % CO_2 for 3 hours. Removed the solution and added 100 μL of absolute dimethyl sulfoxide (DMSO) in each well for disintegrate formazan crystal as a byproduct and then measured the absorbance at 570 nm. using microplate reader (Envision, PerkinElmer, MA, USA). The results were interpreted and compared with control (untreated). The percentage of cell viability was calculated by following equation 1 below and evaluated minimal non-toxic dose (MNTD) respectively.

$$\% \text{ Cells viability} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100 \quad (1)$$

3.3.3 Cell Proliferation

Ten thousand HDFn cells were seeded in each well of 96-well plates before incubated at 37°C with 5% CO_2 in humidified atmosphere for 24 hours. The concentration of each sample was followed MNTD value which shown the best potential for induced cell proliferation without toxic including commercial adenosine (0.156 μM), commercial cordycepin (0.625 μM), mixture of commercial adenosine and

cordycepin (5 µM) and CME (0.156 and 0.313 µM). HDFn cells was pre-explosion with samples (one hundred microliters) by incubated at 37℃ with 5% CO_2 in humidified atmosphere for 5, 15, 30, 60, 180 mins and 24 hours as a positive control. Soluble was discarded before adding 100 µL of MTT solvent (0.5 mg/mL) and incubated at 37℃ with 5 % CO_2 for 3 hours. Removed the solution and added 100 µL of absolute dimethyl sulfoxide (DMSO) in each well for disintegrate formazan crystal as a byproduct and then measured the absorbance at 570 nm. using microplate reader. The results were interpreted and compared with negative control (untreated). The percentage of cell viability was calculated by following equation 1.

3.4 Cell Migration for Wound Healing Assay

The HDFn migration assay was performed in 24-well plate, flat-bottle sterile (SPL Life Sciences, Pocheon, Korea). Briefly, $1x10^5$ cells were seeded in each well and incubated with 5% CO_2 for 24 h. Discarded complete media and added basal media without necessary supplement into well before incubated at 37℃ with 5% CO_2, 24 hours for cell starvation. The gap was created by scratching the cell in each well with pipette tip (size 10µL) and washed cell using PBS (pH 7.4) twice for removed cell debris. The samples including commercial adenosine (0.156 µM), commercial cordycepin (0.625 µM), a mixture of commercial adenosine and cordycepin (5 µM), and CME (0.156 and 0.313 µM) were prepared with the basal medium before added (2 mL) into wells. The plate was imaged every 2 hours for 24 hours with an IX83 inverted microscope (Olympus) equipped with a UPlanFL-PH 10×/0.3 (Olympus), a DOC CAM-HR CCD camera (Molecular Devices), a Spectra-X0 light engine (Lumencor Inc.), an IX3-ZDC laser-based autofocusing system (Olympus), an electric XY stage (Sigma Koki) and a stage top incubator (Tokai Hit). The cell migration area from the captured images was calculated using ImageJ public domain software (NIH, Bethesda, MD, USA), and the wound healing percentage was demonstrated via equation 2.

$$\% \text{ Wound healing} = \left[\frac{A_0 - A_t}{A_0}\right] \times 100 \quad (2)$$

Where A_0 is the initial wound area and A_t is the wound area after treatment at span hours, scratch, both in μm^2.

3.5 Protein Extraction and Quantification

The HDFn was seeded in 6 well plates (2 x 10^5 cell/well) and incubated at 37℃ with 5% CO_2 for 24 h. Applied each sample including commercial adenosine (0.156 µM), commercial cordycepin (0.625 µM), a mixture of commercial adenosine and cordycepin (5 µM), and CME (0.156 and 0.313 µM) with complete medium into the well before incubated at 37℃ with 5% CO_2 for 24 h. PRO-PREP™ Protein Extraction Solution (iNtRON Biotechnology, Korea) was used as the alternative protein extraction. After 24 hours, treatments were removed and before added hundred microliters of PRO-PREP™ solution for cell lysate (on ice for 30 mins). Transferred cells into new sterile tube and separated by using centrifugation at 13,000 rpm, 4 °C for 5 minutes and kept supernatant. Total protein concentrations were assayed using BCA assay (Cat. No. 23227, (Pierce Biotechnology, Rockford, IL, USA).) and stored at -80 °C prior to analysis.

3.6 SDS-PAGE and Western Blot Analysis

Equal amounts of protein (40 µg) from each sample were homogenized with sample buffer and 6X loading dye and boiled at 95℃ for 5 mins and then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% (w/v in water) resolving gel. Run the gel for 2 h 15 mins at 100 V. at room temperature for separated protein. Proteins in gel were transferred to nitrocellulose membrane (Bio-Rad) assembled with cassette ice pack under before transferred into transfer tank that already contain ice pack under the electrical current at 0.1 A., 4℃ for 12 h. for protein transferred. The membrane was collected and rinsed with 1X Tris-Buffered Saline (TBS; 50 mM Tris–HCl (pH 7.5), 150 mM, NaCl, 2.5 mM KCl),) three times

before blocked the membrane with 5% (v/w) non-fat dry milk in TBST (TBS containing 0.1% Tween 20) and incubated for 1 h at 25℃ (darkness). The membranes were probed overnight at 4℃ with antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:10,000, AF7021, Affinity Biosciences), VEGF-A (1:1000, AF7021, Affinity Biosciences) or FGF-2 (1:1000, DF6038, Affinity Biosciences), and biotinylated protein ladder was used as marker (Cell Signaling Technology, Beverly, MA, USA) The membranes were washed with TBST and incubated with peroxidase conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) (1:200,000, Jackson Immuno-Research) at room temperature for 1 h. followed by another three-time washing with TBST. The proteins were reacted with chemiluminescence (ECL) reagents substrate (LumiGLO Chemiluminescent Substrate) and seen under Chemi-Doc MP Imaging System machine (Bio-Rad). The gray value of the protein band was analyzed using the Image-J public domain software (NIH, Bethesda, MD). The ratio of the gray value of the target protein band to the gray value of the GAPDH band was the relative expression of the target protein.

3.7 Statistical Analysis

All data are presented as the mean + standard deviation and were performed using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by post-hoc multiple comparison Duncan test was carried out for determination the significantly Differences with $p < 0.05$, and $p < 0.001$ were considered to indicate significance differences.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 High-Performance Liquid Chromatography (HPLC) Determines Amount of Adenosine and Cordycepin in *C. militaris* Extract

The analysis of adenosine and cordycepin levels in *Cordyceps militaris* extract (CME). The process involved extracting the dried mycelium with distilled water at 60°C for 3 hours. Subsequent High-Performance Liquid Chromatography (HPLC) analysis revealed that the concentrations of adenosine and cordycepin in CME were 1.382±0.161 mg/g and 6.544±1.109 mg/g respectively. Notably, the CME exhibited a cordycepin content that was approximately 4.7 times greater than that of adenosine. *Cordyceps militaris* is known to have a significantly higher cordycepin concentration compared to other species, particularly under static culture conditions, as noted by Shih et al. (2007). With the quantification of these compounds in the crude extract complete, we proceeded to evaluate their efficacy in promoting cell proliferation in human skin cells.

4.2 Optimizing the Concentration of CME via Viability of Human Dermal Fibroblast Neonatal Cells

This section aims to determine the optimal concentration of *Cordyceps militaris* extract (CME) to effectively promote proliferation in human dermal fibroblast neonatal cells (HDFn). The objective is to ascertain the Minimal Non-Toxic Dosage (MNTD) that can be administered to cells without significantly compromising their viability. Various CME concentrations—0.078, 0.156, 0.313, 0.625, 1.250, 2.50, 5, and 10 µM—were examined, as shown in Table 4.1, alongside controls including

standard adenosine, standard cordycepin, and a mixture of both in the same ratio as found in CME (1:5), over a period of 24 h. As illustrated in Figure 4.1, HDFn cell viability increased by up to 60% when treated with 0.313 and 0.156 µM of CME, compared to untreated cells. Enhanced HDFn confluence post-CME treatment was also observed under an inverted optical microscope (10X), as shown in Figure 4.2. Additional treatments involving adenosine (0.156 µM), cordycepin (0.625 µM), and a standard mixture (1:5 ratio at 5 µM), yielded increases in HDFn viability by approximately 54%, 53%, and 56%, respectively, relative to the negative control.

Corroborating studies, such as those by Park et al. (2014), suggest that *C. militaris* extract may offer protective benefits against premature aging due to oxidative stress and may enhance cell proliferation (Park et al., 2014). With these results, we established the MNTD for each sample within the tested range, which proved to be conducive for HDFn treatment, as they did not significantly reduce cell viability while also demonstrating the highest potential for cell proliferation.

Table 4.1 The amount of adenosine and cordycepin in CME

The concentration of CME (µM)	**Adenosine content (µg/mL)**	**Cordycepin content (µg/mL)**
10	0.466	2.191
5	0.233	1.096
2.5	0.116	0.548
1.25	0.058	0.274
0.625	0.029	0.137
0.313	0.014	0.068
0.156	0.007	0.034
0.078	0.004	0.017

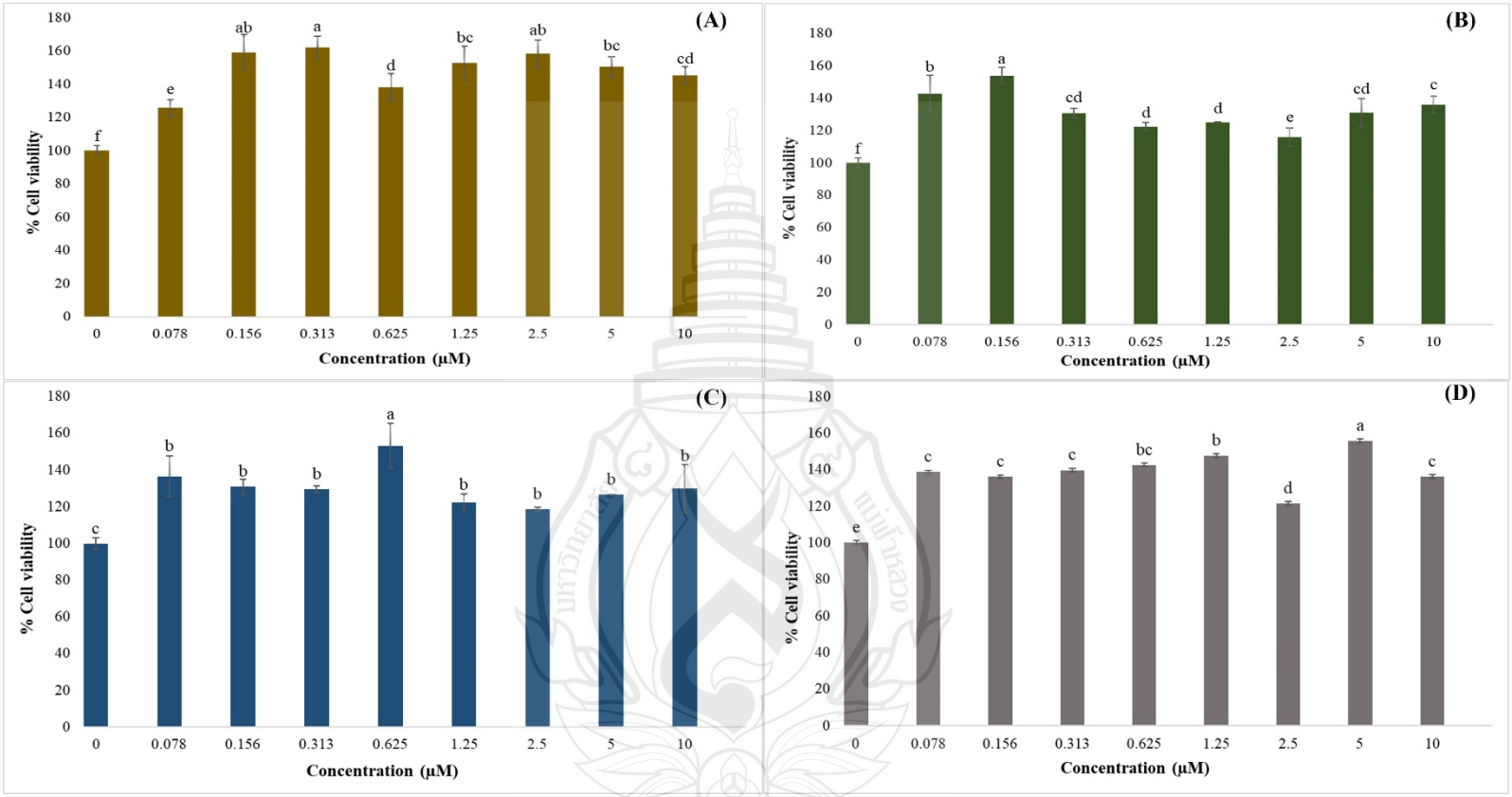


Figure 4.1 The percentage of HDFn cells viability was determined through MTT assay. The effects of various concentrations of CME (4.1A), adenosine standard (4.1B), cordycepin standard (4.1C) and mixture of adenosine and cordycepin standard in ratio 1:5 (4.1D) on HDFn cell viability were examined by compared with untreated. Duncan's multiple range tests, $p < 0.001$

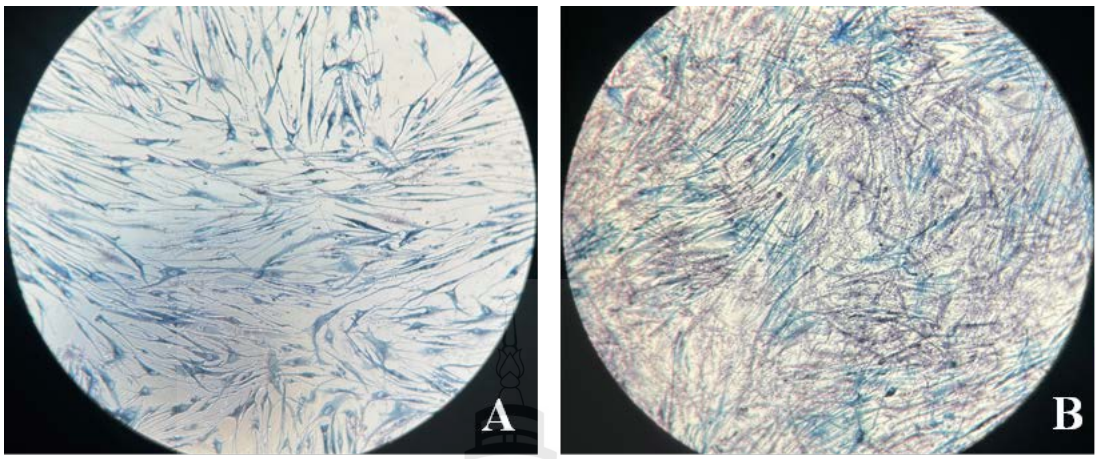


Figure 4.2 HDFn cell confluency of untreated (A) and treatment with CME (B) under an inverted optical microscope (magnification ×100) after 24 h prior measuring cell viability via MTT assay

4.3 HDFn Cell Proliferation after Treated with CME

Subsequent to identifying the Minimal Non-Toxic Dosage (MNTD), the MTT assay was employed to assess the proliferation potential of human dermal fibroblast neonatal cells (HDFn) post-treatment with each sample's MNTD, in comparison to the untreated control group. Remarkably, within fifteen minutes, all treatments facilitated more than 50% proliferation in HDFn cells relative to the control (untreated experiment). Notably, a CME concentration of 0.156 µM propelled HDFn cell proliferation to 80%, outperforming both adenosine and cordycepin standards, as depicted in Table 4.2.

Furthermore, a standard mixture of adenosine and cordycepin at a 1:5 ratio demonstrated a proliferative effect nearly on par with that of the CME treatment and commercial adenosine. It was also observed that HDFn cells sustained their proliferation potential consistently up to the one-hour point. At this one hour and three hours, however, cordycepin alone was found to significantly diminish the proliferation rate of HDFn cells.

Table 4.2 Proliferation percentage of HDFn treated with the MNTD value of various treatment in different times as evaluated with MTT assay

Treatments	Pre-explosion time					
	5 mins	**15 mins**	**30 mins**	**60 mins**	**180 mins**	**24 hrs.**
Adenosine	177.78 ± 6.97^{d}	242.32 ± 10.92^{bc}	293.30 ± 8.91^{a}	297.86 ± 8.46^{a}	223.48 ± 5.76^{c}	260.68 ± 14.70^{b}
Cordycepin	249.85 ± 6.60^{a}	271.37 ± 13.25^{a}	258.12 ± 11.19^{a}	211.27 ± 16.19^{b}	183.79 ± 1.64^{c}	250.85 ± 11.90^{a}
Mixture 1:5	208.12 ± 7.42^{d}	248.52 ± 5.72^{c}	294.06 ± 10.49^{a}	299.95 ± 7.35^{a}	242.49 ± 4.41^{c}	265.21 ± 7.13^{b}
CME (0.156μM)	221.00 ± 5.68^{c}	263.25 ± 8.78^{b}	286.97 ± 6.62^{a}	284.41 ± 1.08^{a}	221.00 ± 8.07^{c}	278.94 ± 17.6^{ab}
CME (0.313μM)	215.47 ± 3.58^{c}	254.71 ± 6.97^{c}	249.15 ± 3.19^{c}	282.65 ± 2.44^{a}	245.81 ± 4.61^{c}	269.00 ± 8.91^{b}

Note Data represents the mean ± standard deviation (n=3). The different superscript letters (a–c) indicate not significant differences of each treatment in difference pre-explosion time ($p > 0.05$).

The findings corroborate with the study by Radhi et al. (2021), which demonstrated that cordycepin consistently inhibits cell proliferation, migration, and inflammation, particularly in cancerous cells (Radhi et al., 2021). Notably, a decline in cell proliferation across all treatments was observed at the 3-hour mark. This suggests that HDFn cells may undergo cell cycle arrest and prepare for the transition to mitosis (G2/M phase) post-treatment. During the G2 phase, cells continue to grow, synthesize proteins and organelles, and reorganize their contents in anticipation of mitosis—a process lasting approximately 3-4 hours as described by Israels & Israels (Israels & Israels, 2000). Consequently, the observed reduction in HDFn proliferation could be attributed to disruptions during the medium change process. Although direct evidence for this specific effect is lacking, the collective body of research implies that *Cordyceps militaris* extract could potentially initiate and augment fibroblast cell proliferation.

4.4 Angiogenic Potential of CME-treated HDFn

Fibroblasts are pivotal in wound healing, as they synthesize key extracellular matrix components, including collagen, facilitating tissue repair and regeneration (Bainbridge, 2013). Moreover, angiogenesis is essential during wound healing, as it establishes a functional vascular network critical for the process (Guerra et al., 2018). In monolayer cell scratch experiments, the potential of CME and other treatments followed MNTD value to the HDFn under starved condition settings to investigate whether it could promote angiogenesis**.** In our study, human dermal fibroblasts (HDFn) from passage 4 were cultured in basal growth medium as an untreated control, while 30% FBS served as the positive control. The HDFn cells were treated with various samples, including CME at concentrations of 0.156 µM and 0.313 µM, commercial adenosine at 0.156 µM, commercial cordycepin at 0.625 µM, and a 1:5 mixture of adenosine and cordycepin at 5 µM. The outcomes of cell migration and wound healing were depicted in Figures 4.3 and 4.4, respectively. At the 12-hour mark post-treatment, HDFn cells treated with CME exhibited migration rates of 72.83% ± 18.38 at 0.156 µM and 72.22% ± 17.32 at 0.313 µM. These rates were significantly higher than those of the untreated cells (47% ± 5.10) and the positive control (60.97% ± 9.35). Notably, adenosine also demonstrated a potential to enhance cell migration rates to over 70% at 12 hours which similar to CME extract, outperforming both cordycepin and the adenosine-cordycepin mixture. Previous studies, such as Valls et al. (2009), have indicated that adenosine stimulates essential functions in granulation tissue formation, including matrix production and angiogenesis, mediated by A2A and A2B adenosine receptors (Valls et al., 2009). Recent studies also reported that a new intracellular adenosine triphosphate (ATP) delivery system enhanced granular tissue, epithelialization, and the higher expression of growth factor such a VEGF during the wound healing process in an animal model (Benjamin et al., 2007). By 24 hours, all treatments had augmented HDFn migration to exceed 90%. The findings suggest that the synergistic effect of adenosine and cordycepin may promote tissue remodeling processes in HDFn cells.

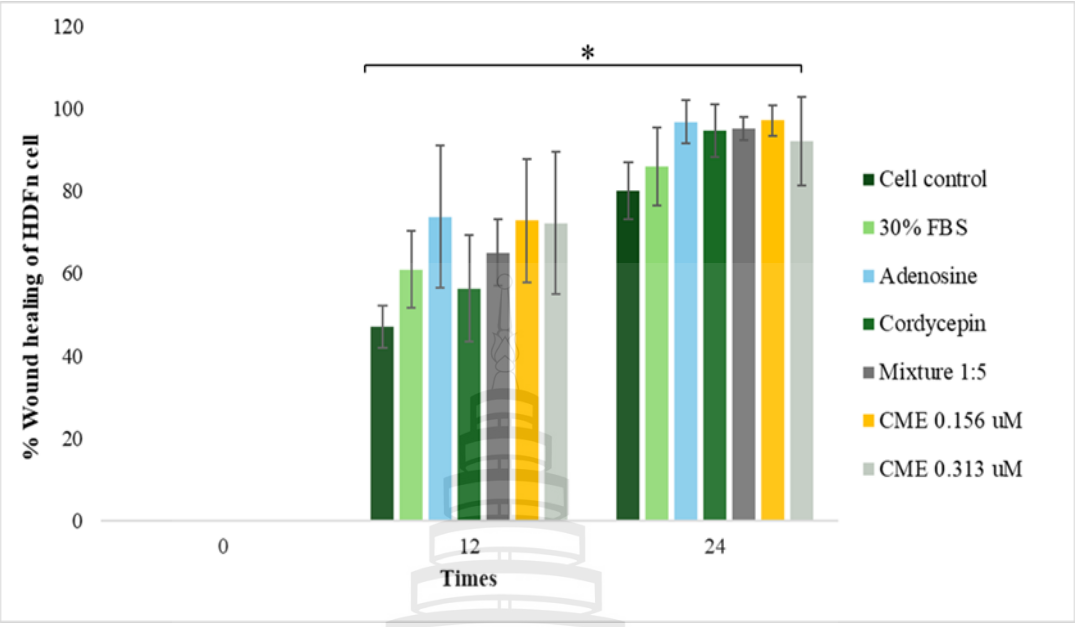


Figure 4.3 Quantitative wound closer percentage after treatment at time point using ImageJ software. Asterisks (*) indicated no statistically significant differences ($p > 0.05$) in wound healing percentage of HDFn between treatment groups at any time point

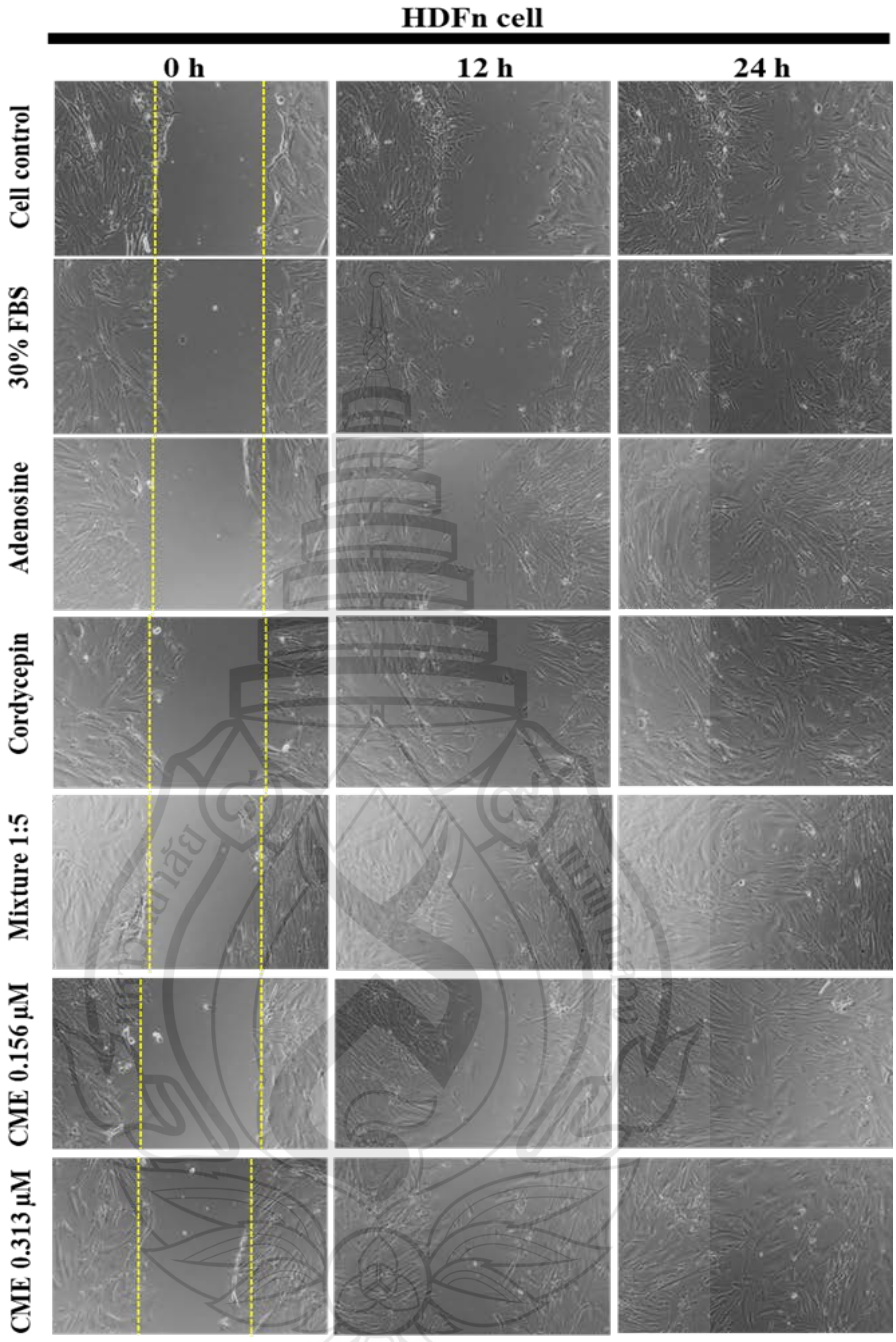


Figure 4.4 The migration rate of HDFn cells were photo-documented at the beginning until twenty-four hours after treatment compared with untreated cells and 30% FBS was used as positive control

4.5 Effect of CME-treatment on the Cellular Response of HDFn Cells

HDFn cells are key mediators in wound healing and angiogenesis, primarily through the expression of growth factors FGF-2 and VEGF-A (Cao et al., 2004). Adenosine is known to promote cell proliferation and angiogenesis by activating the A2B adenosine receptor, thereby accelerating the wound healing process and influencing fibroblast behavior (Valls et al., 2009). This experiment focused on the secretion of these pro-angiogenic factors after treatment with CME and other agents. The results indicated an upregulation of FGF-2 and VEGF-A expression in HDFn cells within the CME group, particularly at a concentration of 0.156 µM as shown in Figure 4.6. Treatment with CME at 0.156 µM resulted in a 2.8-fold increase in FGF-2 and a 26.7-fold increase in VEGF-A expression, compared to untreated cells. In contrast, adenosine treatment led to a 0.43-fold and 2.4-fold increase in FGF-2 and VEGF-A expression, respectively. Recently studies reported adenosine can modulate VEGF-A expression through its interaction with adenosine receptors, which are coupled to G-proteins, this interaction can lead to the activation of the cAMP/PKA pathway, resulting in increased expression of VEGF-A in fibroblast cell (*in vitro*) (Kim et al., 2021). Despondent expression of VEGF-A protein was also found especially after treatment with cordycepin. The result was consistent with previously report, cordycepin (a derivative of adenosine) has been shown to inhibit VEGF-A expression by down-regulating VEGF and phosphatidylinositol-3-kinase/AKT (PI3K/AKT) signaling pathway which are crucial to process for cell proliferation, survival, metabolism, motility and angiogenesis in cancer cells (Liao et al., 2020). Furthermore, comparing CME at 0.156 µM with other concentrations revealed a 1.4-fold and 2-fold increase in the relative expression of FGF-2 and VEGF-A proteins in HDFn cells. These findings underscore the potential of elevated expression of both pro-angiogenic factors as targets to enhance revascularization in ischemic tissues and promote tissue repair. Seghezzi et al. (1998) demonstrated that FGF-2 was enhanced VEGF expression in vascular endothelial cells (ECs) via autocrine and paracrine signaling, and recombinant FGF-2 to ECs cells or up-regulation of endogenous FGF-2 also lead to increased VEGF expression (Seghezzi et al., 1998). Their synergistic

effect is instrumental for effective angiogenesis and vascular development (Jia et al., 2021).

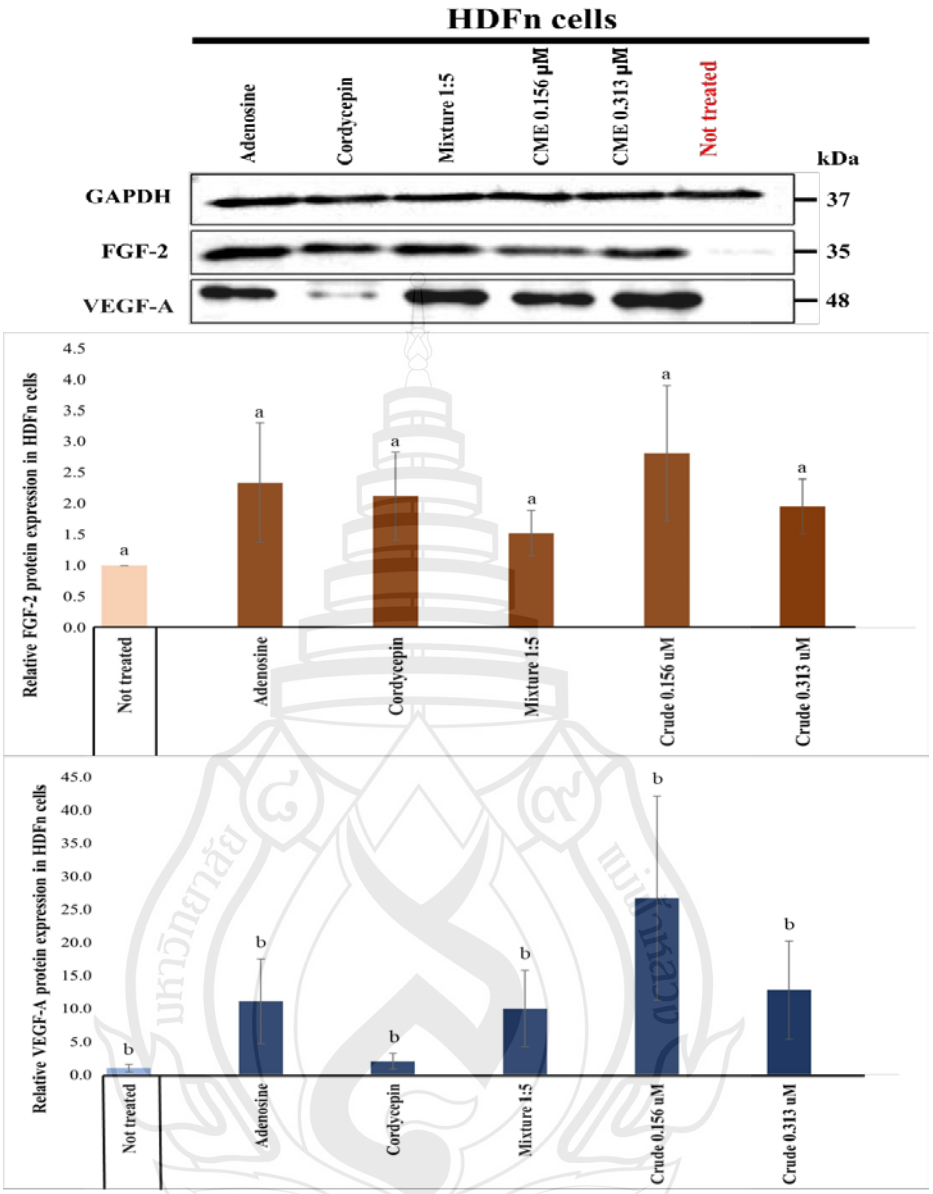


Figure 4.5 The level of GAPDH, FGF-2, and VEGF-A were further investigated by Western blotting after 24 h treatment with adenosine, cordycepin, mixture 1:5, and CME (0.156 µM, 0.313 µM) (4.6A). The relative expression of FGF-2 in HDFn after treatment (4.6B). The relative expression of VEGF-A in HDFn cells after treatment (4.6C). The different superscript letters (a-b) indicate not significant differences of each treatment in difference pre-explosion time ($p > 0.05$)

CHAPTER 5

CONCLUSION

In conclusion, the research successfully cultivated and extracted *Cordyceps militaris* (SH01), which is rich in adenosine and cordycepin, demonstrating significant therapeutic potential in modulating cell proliferation, tissue repair, and angiogenesis. The crude extract (CME) underwent rigorous quantification of its active constituents using HPLC techniques. Subsequent application of CME to Primary Dermal Fibroblast Normal; Human, Neonatal cells (HDFn)—key mediators in tissue repair and angiogenesis—revealed its efficacy in comparison to standard adenosine, standard cordycepin, and their mixture in a 1:5 ratio, as found in CME. Around 60% increase of HDFn cell viability after treatment with CME was determined through MTT assay, which also helped establish the minimal non-toxic dose (MNTD). Following the MNTD value, CME was induced to the increasing of HDFn proliferation rate more than sixty percents during 30 mins to 1 h. was also observed. Additionally, the migration of HDFn and the expression of pro-angiogenic proteins, namely FGF-2 and VEGF-A, were monitored via Western blotting analysis, underscoring their role in angiogenesis. The study's findings indicate that CME, with its high cordycepin content, is not only non-toxic but also significantly enhances HDFn proliferation better than pure standard commercial and mixture of commercial in ratio 1:5. It further demonstrates the capability to promote angiogenesis by encouraging HDFn migration and the secretion of pro-angiogenic factors in dermal fibroblast cells. Collectively, these results position CME as a promising therapeutic agent, offering substantial benefits for wound healing and angiogenesis, and highlighting its potential for medical applications in enhancing cell proliferation and vascular development.

REFERENCES

REFERENCES

Ashraf, S. A., Elkhalifa, A. E. O., Siddiqui, A. J., Patel, M., Awadelkareem, A. M., Snoussi, M., . . . Hadi, S. (2020). Cordycepin for health and wellbeing: A potent bioactive metabolite of an entomopathogenic medicinal fungus Cordyceps with its nutraceutical and therapeutic potential. *Molecules, 25*(12), 2735

Auchampach, J. A. (2007). Adenosine receptors and angiogenesis. *Circ Res, 101*(11), 1075-1077. https://doi.org/10.1161/circresaha.107.165761

Bahuguna, A., Khan, I., Bajpai, V. K., & Kang, S. C. (2017). MTT assay to evaluate the cytotoxic potential of a drug. *Bangladesh Journal of Pharmacology, 12*(2),115-118.

Bainbridge, P. (2013). Wound healing and the role of fibroblasts. *Journal of Wound Care, 22*(8).

Barrientos, S., Stojadinovic, O., Golinko, M. S., Brem, H., & Tomic-Canic, M. (2008). Growth factors and cytokines in wound healing. *Wound Repair and Regeneration, 16*(5), 585-601.

Benjamin, C., Eric, E., William, E., Sidney, M., Mary, A., & Ming, L. (2007). Enhancing skin wound healing by direct intracellular ATP delivery. *Am J Surg, 193*(2), 213-218.

Borea, P. A., Gessi, S., Merighi, S., & Varani, K. (2016). Adenosine as a multi-signalling guardian angel in human diseases: When, where and how does it exert its protective effects. *Trends in pharmacological sciences, 37*(6), 419-434.

Borea, P. A., Gessi, S., Merighi, S., Vincenzi, F., & Varani, K. (2018). Pharmacology of adenosine receptors: The state of the art. *Physiological Reviews*, *98*(3), 1591-1625.

Burnstock, G. (2020). Introduction to purinergic signalling in the brain. *Glioma Signaling*, 1-12.

Cañedo-Dorantes, L., & Cañedo-Ayala, M. (2019). Skin acute wound healing: A comprehensive review. *International Journal of Inflammation*, *2019*(1), 3706315.

Cao, R., Eriksson, A., Kubo, H., Alitalo, K., Cao, Y., & Thyberg, J. (2004). Comparative evaluation of FGF-2–, VEGF-A–, and VEGF-C–induced angiogenesis, lymphangiogenesis, vascular fenestrations, and permeability. *Circulation Research*, *94*(5), 664-670.

Cao, Z.-P., Dai, D., Wei, P.-J., Han, Y.-Y., Guan, Y.-Q., Li, H.-H., . . . Li, C.-H. (2018). Effects of cordycepin on spontaneous alternation behavior and adenosine receptors expression in hippocampus. *Physiology & Behavior*, *184*, 135-142.

Chan, E., Fernandez, P., Merchant, A., Montesinos, M., Trzaska, S., Desai, A., . . . Reiss, A. (2006). Adenosine A2A receptors in diffuse dermal fibrosis: Pathogenic role in human dermal fibroblasts and in a murine model of scleroderma. *Arthritis & Rheumatism: Official Journal of the American College of Rheumatology*, *54*(8), 2632-2642.

Choi, E., Oh, J., & Sung, G.-H. (2020). Beneficial effect of Cordyceps militaris on exercise performance via promoting cellular energy production. *Mycobiology*, *48*(6), 512-517.

ChokeUmnuay, N., & Owatworakit, A. (2021). Effect of successive subculture of Cordyceps militaris on growth, metabolites production and stability of Rhf1 gene. *Agriculture and Natural Resources*, *55*(4), 537–546.

Clark, A. N., Youkey, R., Liu, X., Jia, L., Blatt, R., Day, Y.-J., . . . Tucker, A. L. (2007). A1 adenosine receptor activation promotes angiogenesis and release of VEGF from monocytes. *Circulation Research, 101*(11), 1130-1138.

Collier, M., & Simon, D. (2016). Protecting vulnerable skin from moisture-associated skin damage. *British Journal of Nursing, 25*(20), S26-S32.

Das, S. K., Masuda, M., Sakurai, A., & Sakakibara, M. (2010). Medicinal uses of the mushroom Cordyceps militaris: Current state and prospects. *Fitoterapia, 81*(8), 961-968. https://doi.org/10.1016/j.fitote.2010.07.010

Demidova-Rice, T. N., Hamblin, M. R., & Herman, I. M. (2012). Acute and impaired wound healing: Pathophysiology and current methods for drug delivery, part 1: Normal and chronic wounds: Biology, causes, and approaches to care. *Advances In Skin & Wound Care, 25*(7), 304-314.

Do Hai Lan, D. H. L., Nguyen Thi Kim Thu, N. T. K. T., Pham Thi Lan, P. T. L., Pham Van Nha, P. V. N., & Bui Thanh Tung, B. T. T. (2016). Cordyceps militaris (L.) Link: Chemical bioactive compounds and pharmacological activities. *Journal of Pharmacy and Nutrition Sciences, 6*(4), 153-159.

Eming, S. A., & Krieg, T. (2006). Molecular mechanisms of VEGF-A action during tissue repair. In *Journal of Investigative Dermatology Symposium Proceedings* (Vol. 11, No. 1, pp. 79-86). Elsevier.

Esser-von Bieren, J. (2019). Eicosanoids in tissue repair. *Immunology and Cell Biology, 97*(3), 279-288.

Feoktistov, I., Biaggioni, I., & Cronstein, B. N. (2009). Adenosine receptors in wound healing, fibrosis and angiogenesis. *Adenosine Receptors in Health and Disease*, 383-397.

Gonzalez, A. C. d. O., Costa, T. F., Andrade, Z. d. A., & Medrado, A. R. A. P. (2016). Wound healing-A literature review. *Anais Brasileiros De Dermatologia, 91*, 614-620.

Greenhalgh, D. G. (1996). The role of growth factors in wound healing. *Journal of Trauma and Acute Care Surgery*, *41*(1), 159-167.

Guerra, A., Belinha, J., & Jorge, R. N. (2018). Modelling skin wound healing angiogenesis: A review. *Journal of Theoretical Biology*, *459*, 1-17.

Gurtner, G. C., Werner, S., Barrandon, Y., & Longaker, M. T. (2008). Wound repair and regeneration. *Nature*, *453*(7193), 314-321.

Harper, D., Young, A., & McNaught, C.-E. (2014). The physiology of wound healing. *Surgery (Oxford)*, *32*(9), 445-450.

Israels, E., & Israels, L. (2000). The cell cycle. *The Oncologist*, *5*(6), 510-513.

Jia, T., Jacquet, T., Dalonneau, F., Coudert, P., Vaganay, E., Exbrayat-Héritier, C., . . . Coll, J.-L. (2021). FGF-2 promotes angiogenesis through a SRSF1/SRSF3/SRPK1-dependent axis that controls VEGFR1 splicing in endothelial cells. *BMC Biology*, *19*, 1-26.

Jiang, Q., Lou, Z., Wang, H., & Chen, C. (2019). Antimicrobial effect and proposed action mechanism of cordycepin against Escherichia coli and Bacillus subtilis. *Journal of Microbiology*, *57*, 288-297.

Jin, Y., Meng, X., Qiu, Z., Su, Y., Yu, P., & Qu, P. (2018). Anti-tumor and anti-metastatic roles of cordycepin, one bioactive compound of Cordyceps militaris. *Saudi Journal of Biological Sciences*, *25*(5), 991-995.

Karamysheva, A. (2008). Mechanisms of angiogenesis. *Biochemistry (Moscow)*, *73*, 751-762.

Kendall, R. T., & Feghali-Bostwick, C. A. (2014). Fibroblasts in fibrosis: Novel roles and mediators. *Frontiers in Pharmacology*, *5*, 91491.

Kim, J., Shin, J. Y., Choi, Y.-H., Lee, S. Y., Jin, M. H., Kim, C. D., . . . Lee, S. (2021). Adenosine and cordycepin accelerate tissue remodeling process through adenosine receptor mediated Wnt/β-catenin pathway stimulation by regulating GSK3b activity. *International Journal of Molecular Sciences*, *22*(11), 5571.

Koh, T. J., & DiPietro, L. A. (2011). Inflammation and wound healing: The role of the macrophage. *Expert Reviews in Molecular Medicine*, *13*, e23.

Kontogiannatos, D., Koutrotsios, G., Xekalaki, S., & Zervakis, G. I. (2021). Biomass and cordycepin production by the medicinal mushroom Cordyceps militaris—A review of various aspects and recent trends towards the exploitation of a valuable fungus. *Journal of Fungi*, *7*(11), 986.

Landén, N. X., Li, D., & Ståhle, M. (2016). Transition from inflammation to proliferation: A critical step during wound healing. *Cellular and Molecular Life Sciences*, *73*, 3861-3885.

Laurens, N., Koolwijk, P., & De Maat, M. (2006). Fibrin structure and wound healing. *Journal of Thrombosis and Haemostasis*, *4*(5), 932-939.

Lee, H. J., Hong, Y. J., & Kim, M. (2021). Angiogenesis in chronic inflammatory skin disorders. *International Journal of Molecular Sciences*, *22*(21), 12035.

Lee, J. B., Radhi, M., Cipolla, E., Gandhi, R. D., Sarmad, S., Zgair, A., . . . Adrower, C. (2019). A novel nucleoside rescue metabolic pathway may be responsible for therapeutic effect of orally administered cordycepin. *Scientific Reports*, *9*(1), 1-12.

Liao, X., Tao, L., Guo, W., Wu, Z.-X., Du, H., Wang, J., . . . Lin, L. (2020). Combination of cordycepin and apatinib synergistically inhibits NSCLC cells by down-regulating VEGF/PI3K/Akt signaling pathway. *Frontiers in Oncology*, *10*, 1732.

Lin, Y.-T., Liang, S.-M., Wu, Y.-J., Wu, Y.-J., Lu, Y.-J., Jan, Y.-J., . . . Kuo, C.-C. (2019). Cordycepin suppresses endothelial cell proliferation, migration, angiogenesis, and tumor growth by regulating focal adhesion kinase and p53. *Cancers*, *11*(2), 168.

Lokmic, Z., Musyoka, J., Hewitson, T. D., & Darby, I. A. (2012). Hypoxia and hypoxia signaling in tissue repair and fibrosis. *International Review of Cell and Molecular Biology*, *296*, 139-185.

Lux, C. N. (2022). Wound healing in animals: A review of physiology and clinical evaluation. *Veterinary Dermatology*, *33*(1), 91-e27.

Martin, P. (1997). Wound healing--aiming for perfect skin regeneration. *Science*, *276*(5309), 75-81.

Mehra, A., Zaidi, K. U., Mani, A., & Thawani, V. (2017). The health benefits of Cordyceps militaris—A review. *Kavaka*, *48*(1), 27-32.

Montesinos, M. C., Desai, A., Chen, J.-F., Yee, H., Schwarzschild, M. A., Fink, J. S., . . . Cronstein, B. N. (2002). Adenosine promotes wound healing and mediates angiogenesis in response to tissue injury via occupancy of A2A receptors. *The American Journal of Pathology*, *160*(6), 2009-2018.

Nakamizo, S., Egawa, G., Natsuaki, Y., Miyachi, Y., & Kabashima, K. (2012). Topical treatment with basic fibroblast growth factor promotes wound healing and barrier recovery induced by skin abrasion. *Skin Pharmacology and Physiology*, *26*(1), 22-29.

Nanney, L. B. (1990). Epidermal and dermal effects of epidermal growth factor during wound repair. *Journal of Investigative Dermatology*, *94*(5), 624-629.

Nguyen, L.-T. T., Vo, T.-H., Nguyen, D.-K., Nguyen, M.-C., & Le, U.-C. N. (2022). Effects of Cordyceps militaris extract and its mixture with silica nanoparticles on burn wound healing on mouse model. *Journal of Drug Delivery Science and Technology*, *67*, 102901.

Nies, V. J., Sancar, G., Liu, W., Van Zutphen, T., Struik, D., Yu, R. T., . . . Downes, M. R. (2016). Fibroblast growth factor signaling in metabolic regulation. *Frontiers in Endocrinology*, *6*, 193.

Ornitz, D. M., & Itoh, N. (2001). Fibroblast growth factors. *Genome Biology*, *2*, 1-12.

Park, J. M., Lee, J. S., Lee, K. R., Ha, S.-J., & Hong, E. K. (2014). Cordyceps militaris extract protects human dermal fibroblasts against oxidative stress-induced apoptosis and premature senescence. *Nutrients*, *6*(9), 3711-3726.

Park, K., Amano, H., Ito, Y., Mastui, Y., Kamata, M., Yamazaki, Y., . . . Majima, M. (2018). Vascular endothelial growth factor receptor 1 (VEGFR1) tyrosine kinase signaling facilitates granulation tissue formation with recruitment of VEGFR1+ cells from bone marrow. *Anatomical Science International*, *93*(3), 372-383.

Pasquini, S., Contri, C., Borea, P. A., Vincenzi, F., & Varani, K. (2021). Adenosine and inflammation: Here, there and everywhere. *International Journal of Molecular Sciences*, *22*(14), 7685.

Pastar, I., Stojadinovic, O., Yin, N. C., Ramirez, H., Nusbaum, A., . . . Tomic-Canic, M. (2014). Epithelialization in wound healing: A comprehensive review. *Advances in Wound Care*, *3*(7), 445-464.

Phull, A. R., Ahmed, M., & Park, H. J. (2022). Cordyceps militaris as a Bio Functional Food Source: Pharmacological Potential, Anti-Inflammatory Actions and Related Molecular Mechanisms. *Microorganisms*, *10*(2). https://doi.org/10.3390/microorganisms10020405

Radhi, M., Ashraf, S., Lawrence, S., Tranholm, A. A., Wellham, P. A. D., Hafeez, A., . . . De Moor, C. H. (2021). A systematic review of the biological effects of cordycepin. *Molecules*, *26*(19), 5886.

Raziyeva, K., Kim, Y., Zharkinbekov, Z., Kassymbek, K., Jimi, S., & Saparov, A. (2021). Immunology of acute and chronic wound healing. *Biomolecules, 11*(5), 700.

Riss, T. L., Moravec, R. A., Niles, A. L., Duellman, S., Benink, H. A., Worzella, T. J., . . . Minor, L. (2016). *Cell viability assays*. https://www.ncbi.nlm.nih.gov/books/NBK574243/?report=reader

Sedighi, M., Namdari, M., & Mahmoudi, P. (2023). An overview of angiogenesis and chemical and physiological angiogenic factors: Short review. *Journal of Chemical Health Risks, 13*(3), 411-422.

Seghezzi, G., Patel, S., Ren, C. J., Gualandris, A., Pintucci, G., Robbins, E. S., . . . Mignatti, P. (1998). Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: An autocrine mechanism contributing to angiogenesis. *The Journal of Cell Biology, 141*(7), 1659-1673.

Shams, F., Moravvej, H., Hosseinzadeh, S., Mostafavi, E., Bayat, H., Kazemi, B., . . . Moosavian, H. (2022). Overexpression of VEGF in dermal fibroblast cells accelerates the angiogenesis and wound healing function: in vitro and in vivo studies. *Scientific Reports, 12*(1), 18529.

Shih, L., Tsai, K.-L., & Hsieh, C. (2007). Effects of culture conditions on the mycelial growth and bioactive metabolite production in submerged culture of Cordyceps militaris. *Biochemical Engineering Journal, 33*, 193-201.

Shrestha, B., Zhang, W., Zhang, Y., & Liu, X. (2012). The medicinal fungus Cordyceps militaris: research and development. *Mycological Progress, 11*, 599-614.

Singh, S., Young, A., & McNaught, C.-E. (2017). The physiology of wound healing. *Surgery (Oxford), 35*(9), 473-477.

Song, Y. H., Zhu, Y. T., Ding, J., Zhou, F. Y., Xue, J. X., Jung, J. H., . . . Gao, W. Y. (2016). Distribution of fibroblast growth factors and their roles in skin fibroblast cell migration. *Molecular Medicine Reports*, *14*(4), 3336-3342.

Steenfos, H. H. (1994). Growth factors and wound healing. *Scandinavian Journal of Plastic and Reconstructive Surgery and Hand Surgery*, *28*(2), 95-105.

Sullivan, L. A., & Brekken, R. A. (2010). The VEGF family in cancer and antibody-based strategies for their inhibition. Mabs. *Taylor and Francis*,*2*(2),165.

Tan, L., Song, X., Ren, Y., Wang, M., Guo, C., Guo, D., . . . Deng, Y. (2021). Anti-inflammatory effects of cordycepin: A review. *Phytotherapy Research*, *35*(3), 1284-1297.

Tian, X., Li, Y., Shen, Y., Li, Q., Wang, Q., & Feng, L. (2015). Apoptosis and inhibition of proliferation of cancer cells induced by cordycepin. *Oncology Letters*, *10*(2), 595-599.

Tottoli, E. M., Dorati, R., Genta, I., Chiesa, E., Pisani, S., & Conti, B. (2020). Skin wound healing process and new emerging technologies for skin wound care and regeneration. *Pharmaceutics*, *12*(8), 735.

Tuli, H. S., Sandhu, S., Sharma, A. K., & Gandhi, P. (2014). Anti-angiogenic activity of the extracted fermentation broth of an entomopathogenic fungus, Cordyceps militaris 3936. *Int J Pharm Pharm Sci*, *6*(7), 581-583.

Tuli, H. S., Sharma, A. K., Sandhu, S. S., & Kashyap, D. (2013). Cordycepin: A bioactive metabolite with therapeutic potential. *Life Sciences*, *93*(23), 863-869.

Turabelidze, A., & Dipietro, L. A. (2011). Inflammation and wound healing. *Endodontic Topics*, *24*(1), 26-38.

Vaidyanathan, L. (2021). Growth factors in wound healing—a review. *Biomed. Pharmacol. J*, *14*, 1469-1481.

Valls, M. D., Cronstein, B. N., & Montesinos, M. C. (2009). Adenosine receptor agonists for promotion of dermal wound healing. *Biochemical Pharmacology*, *77*(7), 1117-1124.

Veith, A. P., Henderson, K., Spencer, A., Sligar, A. D., & Baker, A. B. (2019). Therapeutic strategies for enhancing angiogenesis in wound healing. *Advanced Drug Delivery Reviews*, *146*, 97-125.

Werner, S., & Grose, R. (2003). Regulation of wound healing by growth factors and cytokines. *Physiological Reviews*, *83*(3), 835-870.

Yanagita, M., Kojima, Y., Kubota, M., Mori, K., Yamashita, M., Yamada, S., . . . Murakami, S. (2014). Cooperative effects of FGF-2 and VEGF-A in periodontal ligament cells. *Journal of Dental Research*, *93*(1), 89-95.

Zhang, J., Wen, C., Duan, Y., Zhang, H., & Ma, H. (2019). Advance in Cordyceps militaris (Linn) Link polysaccharides: Isolation, structure, and bioactivities: A review. *International Journal of Biological Macromolecules*, *132*, 906-914.

APPENDICES

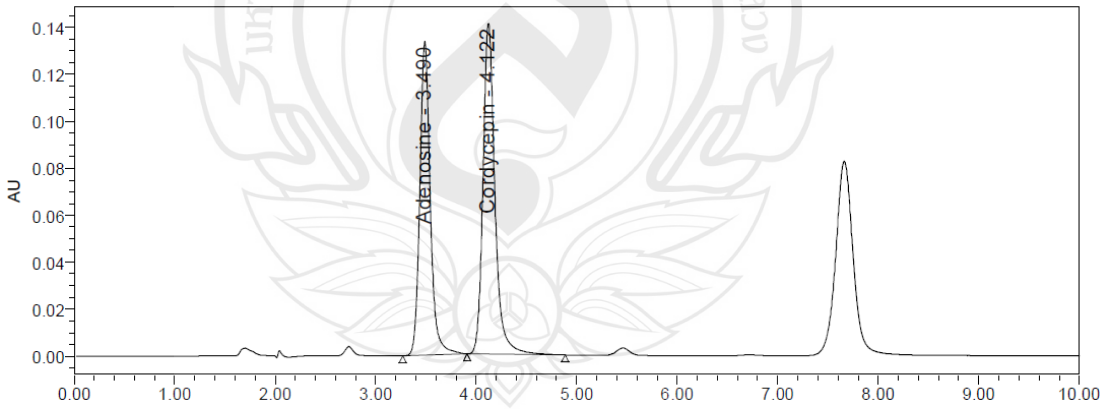
APPENDIX A

QUANTIFICATION OF ADENOSINE AND CORDYCEPIN IN *C. militaris*

Table A1 Adenosine and cordycepin productivity in dry mycelia

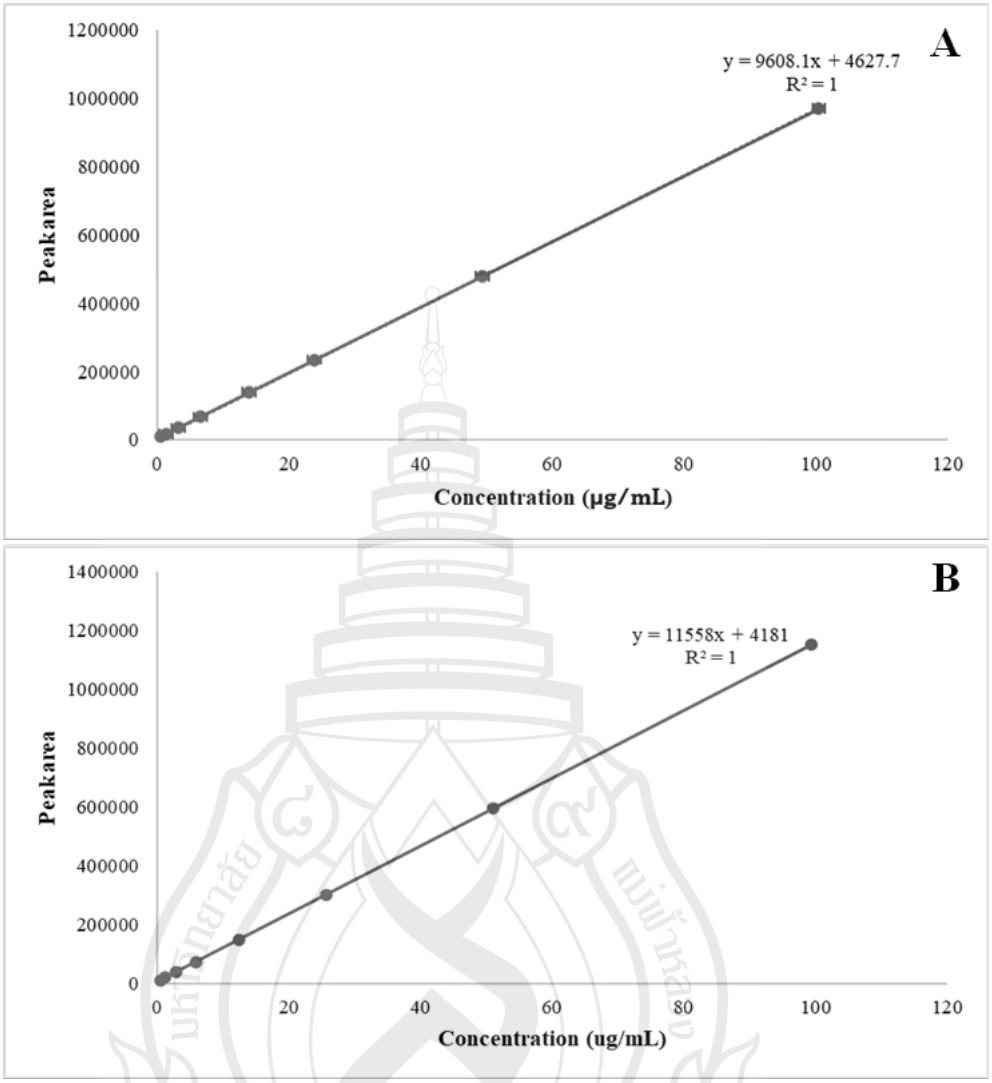
Replication	Adenosine			Cordycepin (mg/g)		
	Reported (µg/mL)	Amount (mg/g)	Sum (mg/g)	Reported (µg/mL)	Amount (mg/g)	Sum (mg/g)
1	9.04	1.21		39.49	5.26	
2	9.12	1.42	1.382±0.161	45.85	7.132	6.544±1.109
3	9.78	1.52		46.51	7.2352	

Note Dried mycelium 4.5 g in 90 mL solvent per replication.



Note Retention time of adenosine = 3.490; cordycepin = 4.122

Figure A1 HPLC Chromatogram of standard adenosine and cordycepin



Note (A) adenosine (B) cordycepin.

Figure A2 Calibration curve of standard adenosine and cordycepin generated by HPLC analysis

APPENDIX B

COMPARE THE AMOUNT OF ADENOSINE AND CORDYCEPIN CONTENT IN EACH TREATMENT

Table B1 Declaration adenosine content in commercial adenosine treatment

Concentration of working solution (µM)	Amount of compound in treatment (µg/mL)	
	Adenosine	Cordycepin
10	2.674	NC
5	1.337	NC
2.5	0.670	NC
1.25	0.335	NC
0.625	0.168	NC
0.313	0.084	NC
0.156	0.042	NC
0.078	0.021	NC

Note NC means do not have a compound in the treatment

Table B2 Declaration cordycepin content in commercial cordycepin treatment

Concentration of working solution (µM)	Amount of compound in treatment (µg/mL)	
	Adenosine	**Cordycepin**
10	NC	2.513
5	NC	0.126
2.5	NC	0.630
1.25	NC	0.314
0.625	NC	0.157
0.313	NC	0.079
0.156	NC	0.039
0.078	NC	0.019

Note NC means do not have a compound in the treatment

Table B3 Declaration bioactive compounds content in mixture of commercial adenosine and cordycepin (ratio 1:5) treatment

Concentration of working solution (µM)	Amount of compound in treatment (µg/mL)	
	Adenosine	**Cordycepin**
10	0.450	2.250
5	0.225	1.125
2.5	0.113	0.562
1.25	0.056	0.281
0.625	0.028	0.141
0.313	0.015	0.070
0.156	0.007	0.035
0.078	0.003	0.018

Table B4 Declaration bioactive compounds content in *Cordyceps militaris* extracted (CME) treatment

Concentration of working solution (µM)	Amount of compound in treatment (µg/mL)	
	Adenosine	Cordycepin
10	0.466	2.191
5	0.233	1.096
2.5	0.116	0.548
1.25	0.058	0.274
0.625	0.029	0.137
0.313	0.014	0.068
0.156	0.007	0.034
0.078	0.004	0.017

CURRICULUM VITAE

CURRICULUM VITAE

NAME Kanjana Soodpakdee

EDUCATIONAL BACKGROUND

2020 Bachelor of Science
Major in Biological Science
School of Science Mae Fah Luang University, Thailand

WORK EXPERIENCE

2023 Teacher-assistant (Sciences and Mathematics All Around)
Mae Fah Luang University

2022 Teacher-assistant (Sciences and Mathematics All Around)
Mae Fah Luang University

2021 Teacher-assistant (Mathematics 1)
Mae Fah Luang University