DEVELOPMENT OF EFFECTIVE COSMECEUTICAL AND NUTRACEUTICAL INGREDIENTS FROM *CORDYCEPS MILITARIS* AND SECRETOME EXTRACTS

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A Thesis Submitted in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy in Biotechnology
Suranaree University of Technology
Academic Year 2022

การพัฒนาสารสกัดจากถั่งเช่าและสารสกัดโปรตีนคัดหลั่งจากเซลล์ต้นกำเนิดผิวหนังเพื่อเป็นส่วนประกอบในผลิตภัณฑ์เครื่องสำอางและอาหารเพื่อสุขภาพ

นายพงศกร กันหอม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2565

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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พงศกร กันหอม : การพัฒนาสารสกัดจากถั่งเช่าและสารสกัดโปรตีนคัดหลั่งจากเซลล์ต้นกำเนิดผิวหนังเพื่อเป็นส่วนประกอบในผลิตภัณฑ์เครื่องสำอางและอาหารเพื่อสุขภาพ (DEVELOPMENT OF EFFECTIVE COSMECEUTICAL AND NUTRACEUTICAL INGREDIENTS FROM *CORDYCEPS MILITARIS* AND SECRETOME EXTRACTS) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.ปริญญา น้อยสา, 133 หน้า.

คำสำคัญ: ถั่งเช่าสีทอง/คอร์ไดเซปิน/การเพาะเลี้ยงในอาหารเหลวที่สภาวะคงที่/การดัดแปรทางเอพิเจเนติก/Natural killer cell/มะเร็ง/ระบบภูมิคุ้มกัน/เคราติโนไซต์/HACAT/เซลล์ผิวหนังชนิดไฟโบรบลาสต์/สารสกัดโปรตีนคัดหลั่ง/การฟื้นฟูความสมบูรณ์ของผิวหนัง/การหายของแผล

ปัจจุบันผลิตภัณฑ์เสริมอาหารเพื่อสุขภาพ (nutraceutical) และเวชสำอาง (cosmeceuticals) ถือเป็นกลุ่มผลิตภัณฑ์ใหม่ในอุตสาหกรรมเพื่อดูแลสุขภาพ เพื่อให้ผลิตภัณฑ์ดังกล่าวมีประสิทธิภาพและสร้างข้อได้เปรียบในการแข่งขันในตลาด จึงจำเป็นอย่างยิ่งที่จะเฟ้นหาสารออกฤทธิ์ทางชีวภาพ (bioactive compound) ที่มีความเป็นเอกลักษณ์และมีประสิทธิภาพสูงมาใช้เป็นส่วนผสมในผลิตภัณฑ์ ซึ่งสารออกฤทธิ์ทางชีวภาพที่ผู้วิจัยมุ่งศึกษาและพัฒนาและเป็นหัวใจของงานวิจัยชิ้นนี้คือ สารคอร์ไดเซปิน (cordycepin) และสารสกัดโปรตีนคัดหลั่งจากเซลล์ผิวหนัง (secretome) สารคอร์ไดเซปินเป็นสารออกฤทธิ์ทางชีวภาพที่สามารถสกัดได้จาก ถั่งเช่าสีทอง (*Cordyceps militaris*) วิธีการเพาะเลี้ยงถั่งเช่าสีทองที่ได้รับความนิยมคือ Solid State Fermentation ที่ได้ชีวมวลถั่งเช่าจำนวนน้อยและใช้เวลานานในการเพาะเลี้ยงแล้ว ยังต้องอาศัยกระบวนการสกัด ดังนั้นผู้วิจัยจึงมุ่งพัฒนากระบวนการเพาะเลี้ยงถั่งเช่าที่สามารถผลิตสารคอร์ไดเซปินได้อย่างมีประสิทธิภาพมากขึ้นผ่านกระบวนการการเพาะเลี้ยงในอาหารเหลวที่สภาวะคงที่ (Liquid static culture) ร่วมกับการดัดแปรทางเอพิเจเนติกเพื่อเพิ่มปริมาณสารออกฤทธิ์สำคัญ พบว่าการเติม Valproic acid ที่ความเข้มข้น 50 ไมโครโมลาร์ ให้ปริมาณสารคอร์ไดเซปินเท่ากับ 2,835.32 มิลลิกรัม/ลิตร ที่สัปดาห์ที่แปดของการเพาะเลี้ยง มากกว่ากลุ่มควบคุม 1.38 เท่า ซึ่งเกิดจากยีนที่เกี่ยวข้องกับกระบวนการสังเคราะห์ cordycepin มีการแสดงออกเพิ่มขึ้น และเพื่อเป็นการเน้นย้ำถึงศักยภาพของสารคอร์ไดเซปินในการเป็นส่วนผสมในผลิตภัณฑ์เสริมอาหาร โดยเฉพาะอย่างยิ่งผลิตภัณฑ์ในกลุ่มต้านมะเร็ง ผู้วิจัยจึงทำการศึกษาฤทธิ์ในการต้านมะเร็งของสารคอร์ไดเซปินเพิ่มเติม โดยมุ่งเน้นไปในด้านการเพิ่มประสิทธิภาพของเซลล์เม็ดเลือดขาวชนิด Natural killer cell (NK cell) ในการเข้ากำจัดเซลล์มะเร็งเมื่อกระตุ้น NK-92 cell ด้วยสารคอร์ไดเซปิน ส่งผลทำให้ความสามารถในการยับยั้งการเจริญของ

เซลล์มะเร็ง THP-1 และU-251 เพิ่มมากขึ้น โดยอาจสืบเนื่องมาจากการที่สารคอร์ไดเซปินช่วยกระตุ้นการสังเคราะห์และหลั่ง TNF- α, IFN- γ, IL-2, IL-12 IFN- γ และ granzyme B อีกทั้งยังช่วยส่งเสริมให้ NK cell เข้าสู่สภาที่พร้อมต่อการทำงาน ผ่านการลดการแสดงออกของ CD27 และเพิ่มการแสดงออกของ CD11b, CD16, และ NKG2D สำหรับการพัฒนากระบวนการผลิตสารสกัดโปรตีนคัดหลั่งจากเซลล์ผิวหนัง เซลล์ผิวหนังที่ใช้ในการผลิตคือเซลล์ HaCaT ซึ่งเป็นเซลล์ผิวหนังชนิดเคราติโนไซต์ที่ผ่านกระบวนการทำให้เป็นอมตะ โดยทำการเติมสารคอร์ไดเซปิน ความเข้มข้น 2.5 µM เข้าไปในระบบเพาะเลี้ยงเซลล์ก่อนทำการเก็บสารสกัดโปรตีนคัดหลั่ง ที่เรียกว่า Cordycepin induced HaCaT Secretome (CHS) พบว่าสามารถเพิ่มประสิทธิภาพการออกฤทธิ์ทางชีวภาพเพื่อฟื้นฟูความสมบูรณ์ของผิวหนังในเซลล์ผิวหนังชนิดไฟโบรบลาสต์ ได้แก่ การต้านอนุมูลอิสระ การกระตุ้นการสร้างโครงข่ายเมทริกซ์ในผิวหนัง และการกระตุ้นกระบวนการออโต้ฟาจี้ ซึ่งเกิดจากการเพิ่มขึ้นของปริมาณโปรตีนหลายชนิดในสารคัดหลั่ง ได้แก่ CXCL1, IL-1Ra, IL-8, MIF, และ PAI-1 ดังนั้นการประยุกต์ใช้แนวคิดทางการดัดแปรทางเอพิเจเนติก และการกระตุ้นด้วยสารคอร์ไดเซปินตามลำดับนั้นสามารถปรับปรุงกระบวนการผลิตวัตถุดิบทั้งสองชนิดได้จริงในระดับห้องปฏิบัติการ ซึ่งจะเป็นรากฐานสำคัญในการนำไปประยุกต์ใช้ต่อในระดับอุตสาหกรรม

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา Phanyatorn Kunhong

ปีการศึกษา 2565 ลายมือชื่ออาจารย์ที่ปรึกษา

PHONGSAKORN KUNHORM : DEVELOPMENT OF EFFECTIVE COSMECEUTICAL AND NUTRACEUTICAL INGREDIENTS FROM *CORDYCEPS MILITARIS* AND SECRETOME EXTRACTS. THESIS ADVISOR : ASSOC. PROF. PARINYA NOISA, Ph.D., 133 PP.

Keyword: CORDYCEPS MILITARIS/CORDYCEPIN/STATIC LIQUID CULTURE/EPIGENETIC MODIFICATION/NATURAL KILLER CELL/CANCER/IMMUNE SYSTEM/KERATINOCYTE/ HACAT/DERMAL FIBROBLAST/SECRETOME/SKIN REGENERATION/WOUND HEALING

Effective bioactive ingredients are needed to stay ahead of the competition in the nutraceutical and cosmeceutical industries. In this study, we develop the novel production procedures of cordycepin and secretome extracts and used them as such promising bioactive ingredients. To increase cordycepin production, epigenetic modification was performed to static liquid cultures. *Cordyceps militaris* was grown in a static liquid culture with valproic acid, a histone deacetylase (HDAC) inhibitor, added to change the epigenetic state. Fifty micromolar valproic acid increased cordycepin production up to 41.187% by increasing global histone acetylation and upregulating 5'-nucleotidase, adenylate kinase, phosphorybosyltransferase, Cns1, Cns2, Cns3, and Cns4. Cordycepin production peaked at 2,835.32 mg/L in a 400 mL working volume. Therefore, epigenetic modification may enhance cordycepin production by changing the gene regulatory network of *C. militaris*. Then, we examined the biological effects of cordycepin on NK-92 cell physiology, cell stage development, and cytokine production that could result in the enhancement of cytotoxicity of NK cells against cancer cells. The cordycepin treatment of NK-92 cells improved their cytotoxicity against THP-1 and U-251 cells. In addition, cordycepin greatly stimulated the synthesis of TNF-, IFN-, IL-2, and IL-12 cytokine-related genes and the release of IFN- and granzyme B from NK-92 cells. Cordycepin was shown to increase the maturation of NK-92 cells by lowering the expression of the immature marker CD27 and increasing the expression of the mature markers CD11b, CD16, and NKG2D. Such results encouraged the development of cordycepin as an active ingredient in anti-cancer nutraceutical products. For secretome production, we modulated cytokine components and increase the quality of HaCaT secretome, by means of cordycepin administration. The

secretome produced by such strategy was designated as cordycepin-induced HaCaT secretome (CHS). The results suggested that CHS induced facilitated fibroblast proliferation/migration, ROS scavenging, extracellular matrix formation, and autophagy. These improved bioactivities of CHS may be attributable to an increase in important cytokines, including as CXCL1, IL-1Ra, IL-8, MIF, and PAI-1. Together, cordycepin and CHS might be demonstrated as promising bioactive compounds for nutraceutical and cosmeceutical formulations.

School of Biotechnology

Academic Year 2022

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ACKNOWLEDGEMENT

I'd like to express my gratitude to my thesis adviser, Assoc. Prof. Dr. Parinya Noisa, for his excellent assistance and continual support during the course of this project. I am thankful to him for his instruction and advise on not just research methodology but also many other life methodologies. I would not have gotten this far, and my thesis would not have been done, if it hadn't been for all of his help.

In addition, I am grateful for the lecture at School of Biotechnology: Prof. Dr. Neung Teaumroong, Prof. Dr. Montarop Yamabhai, Assoc. prof. Dr. Mariena Ketudatcairns, Assoc. prof. Dr. Apichat Boontawan, Assoc. Prof. Dr. Kaemwich Jantama, Assoc. prof. Dr. Panlada Tittabutr for teaching and suggestions and all their help.

I would like to express my gratitude to the dissertation committee, Assoc. Prof. Dr. Sasimanas Unajak, Asst. Prof. Dr. Rawiwan Charoensup, Asst. Prof. Dr. Anyanee Kamkaew and Asst. Prof. Dr. Paiboon Jitprasertwong, for their time, knowledge, comments and advices on my work.

I'd like to thank my closest CBAI-Lab friends, Ni, Wi, Bom, Koy, and Ben, for their companionship, understanding, and sharing of difficult times, as well as their assistance and warm hospitality. Furthermore, I am grateful to Suranaree University of Technology for providing me with a scholarship and laboratory facilities while I was a doctoral student there. Finally, I'd want to express my deepest gratitude to my parents for their continuous support during this research.

Phongsakorn Kunhorm

TABLE OF CONTENTS

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

%	=	Percentage
(m, μ) g	=	(milli, micro) Gram
(m, μ) L	=	(milli, micro) Liter
(m, μ) M	=	(milli, micro) Molar
Mm3	=	Cubic millimeter
℃	=	Degree Celsius
2'-C-3'-dA	=	2'-carbonyl-3'-deoxyadenosine
2D	=	Two-dimensional
3'-AMP	=	Adenosine-3'-monophosphate
3'-dADP	=	3'-deoxyadenosine 5'-diphosphate
3'-dAMP	=	3'-deoxyadenosine 5'-phosphate
3'dI	=	3'-deoxyinosine
4NAOC-1	=	Murine cancer cell
5AZA	=	5-azacytidine
ADA	=	Adenosine deaminase
ADCC	=	Antibody-dependent cell cytotoxicity
ADP	=	Adenosine diphosphate
ADSS	=	Adenylosuccinate synthetase
AK	=	Adenylate kinase
Akt	=	protein kinase B
AMP	=	Adenosine monophosphate
APRT	=	Adenine Phosphoribosyltransferase
ATG5	=	Autophagy related 5
ATP	=	Adenosine triphosphate
bp	=	Base pair
Bcl-xL	=	B-cell lymphoma-extra large
$CaCl_2$	=	Calcium chloride
CAT	=	Catalase

LIST OF ABBREVIATIONS (Continued)

Cns1	=	Protein with dehydrogenase oxidoreductase domains
Cns2	=	Protein with metal-dependent phosphohydrolase domain
Cns3	=	Protein with N-terminal nucleoside/nucleotide kinase and C-terminal ATP phosphoribosyltransferase domains
Cns4	=	ATP-binding cassette pentostatin transporter
cAMP	=	Cyclic adenosine monophosphate
CCA	=	Cholangiocarcinoma
CCL2	=	Monocyte chemoattractant protein-1
CD11b	=	α-chain of integrin receptor
CD16	=	Low affinity Fc receptor for IgG
CD27	=	Tumor Necrosis Factor Receptor Superfamily Member 7
CD56	=	Neural cell adhesion molecule
cDNA	=	Complementary deoxyribonucleic acid
CHS	=	Cordycepin-induced HaCaT secretome
COL1A1	=	Collagen type I alpha 1 chain
COL1A2	=	Collagen type I alpha 2 chain
COL3A1	=	Collagen type III alpha 1 chain
COX-2	=	Cyclooxygenase 2
CREB	=	cAMP response element-binding protein
CXCL1	=	Chemokine C-X-C motif ligand 1
DAPI	=	4',6-diamidino-2-phenylindole
DCF	=	2'-7'dichlorofluorescein
DCFH-DA	=	Dichloro-dihydro-fluorescein diacetate
DMSO	=	Dimethyl sulfoxide
DNMT	=	DNA methyltransferases
DNA	=	Deoxyribonucleic acid
DO	=	Dissolved oxygen
ECM	=	Extracellular Matrix
ELISA	=	Enzyme-linked immunosorbent assay

LIST OF ABBREVIATIONS (Continued)

ERK	=	Extracellular signal–regulated kinase
FBS	=	Fetal bovine serum
$FeSO_4$	=	Ferrous Sulfate
FGF (-2)	=	Fibroblast growth factors (2)
G-6-P	=	Glucose-6-phosphate
GAPDH	=	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	=	Ggranulocyte/monocyte colony-stimulating factor
GMP	=	Guanosine monophosphate
GPX	=	Glutathione peroxidase
G-CSF	=	Granulocyte colony-stimulating factor
h	=	Hour
H_2O_2	=	Hydrogen peroxide
HaCaT	=	Nontumorigenic immortalized keratinocyte cell line
HDAC	=	Histone deacetylase
HDF	=	Human dermal fibroblast
IFN-γ	=	Interferon gamma
IGF-2	=	Insulin-like growth factor 2
IL-1β	=	Interleukin 1 beta
IL-1RA	=	Interleukin 1 receptor antagonist
IL-2	=	Interleukin 2
IL-4	=	Interleukin 4
IL-6	=	Interleukin 6
IL-8	=	Interleukin 8
IL-10	=	Interleukin 10
IL-12	=	Interleukin 12
IL-17A	=	Interleukin 17A
IL-17RA	=	Interleukin 17 receptor antagonist
IMP	=	Inosine phosphate
IMPC	=	Inosine phosphate cyclohydrolase

LIST OF ABBREVIATIONS (Continued)

IMPDH	=	Inosine phosphate dehydrogenase
KEGG	=	Kyoto Encyclopedia of Genes and Genomes
K_2HPO_4	=	Dipotassium hydrogen phosphate
KH_2PO_4	=	Monopotassium phosphate
Ki-67	=	Cellular marker for proliferation
LC3	=	Microtubule-associated protein 1 light chain 3
LDH	=	Lactate Dehydrogenase
LPS	=	Lipopolysaccharides
Lx	=	Lux
MCP-1	=	Monocyte chemoattractant protein 1
$MgSo_4$	=	Magnesium sulfate
MIF	=	Macrophage migration inhibitory factor
MITF	=	Microphthalmia-associated transcription factor
MMP (-1,3)	=	Matrix metalloproteinase (1, 3)
mRNA	=	Messenger ribonucleic acid
mTOR	=	Mammalian target of rapamycin
MTT	=	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
NAC	=	N-acetyl cysteine
NH_4^+	=	Ammonium ion
$(NH_4)_2SO_4$	=	Ammonium sulfate
NHS	=	Normal HaCaT secretome
NF-kB	=	Nuclear factor kappa B
NK cell	=	Natural killer cell
NK-92 cell	=	Interleukin-2 dependent natural killer cell line
NKDCC	=	NKG2D-dependent cell cytotoxicity
NKG2D	=	The Natural Killer Group 2D (NKG2D) receptor
nrDNA-ITS	=	Internal transcribed spacer of the ribosomal DNA
NT5E/ 5'NT	=	5'-Nucleotidase
O.D.	=	Optical density

LIST OF ABBREVIATIONS (Continued)

P62	=	Ubiquitin-binding p62 protein
PAI-1	=	Plasminogen activator inhibitor 1
PBS	=	Phosphate buffer saline
PDA	=	Potato dextrose agar
PRAT	=	Phosphoribosylamidotransferase
proIL-1α	=	pro-Interleukin 1 alpha
PRPP	=	Phosphoribosyl pyrophosphate
qPCR	=	Quantitative polymerase chain reaction
R-5-P	=	Ribose-5-phosphate
RNA	=	Ribonucleic acid
RNR	=	Ribonucleotide reductase
ROS	=	Reactive oxygen species
RT-PCR	=	Reverse transcription polymerase chain reaction
SAHA	=	Suberoylanilide hydroxamic acid
SCAR	=	Specie-specific sequence characterized amplified region
SOD	=	Superoxide dismutase
SD	=	Standard deviation
STZ	=	Streptozotocin
THP-1	=	Human monocytic leukaemia cell line
TGF-β	=	Transforming growth factor beta
TNF-α	=	Tumor necrosis factor alpha
U-251	=	Human malignant glioma cells
Ubi	=	Polyubiquitin-binding protein
UV	=	Ultraviolet
UVB	=	Ultraviolet B-rays
VPA	=	Valproic acid
Wnt	=	Wingless-related integration site

CHAPTER 1
INTRODUCTION

1.1 Rational and background

Recently, nutraceuticals and cosmeceuticals become the worldwide trend and serve as another segment of personal health care industry. Nutraceutical is a combination of the term's "nutrition" and "pharmaceutical" to define the products that serves as both nutritional sources and drugs aiming to boost health, postpone the aging process, prevent chronic illnesses, extend life expectancy, or support the body function and integrity (Das et al., 2012; Nasri et al., 2014). Cosmeceuticals are also the combined disciplines between drugs and cosmetics, which intends to promote health benefits of human body, including cleaning, beautifying, enhancing attractiveness or altering appearance by the noticeable and measurable biological activities (Lohani et al., 2014; Oricha, 2010). The biological activities of nutraceutical and cosmeceutical products depend on their bioactive ingredients, obtained from natural sources or chemical synthesis, therefore the desirable attributes of such products could be designed and formulated. To keep ahead the competition in nutraceutical and cosmeceutical industry, the effective bioactive compounds are needed. For this particular study, cordycepin and secretome are focused to be used as such effective bioactive compounds.

Cordycepin is the unique nucleoside analogue that is naturally found in fungi only *in* genus *Cordyceps*. This nucleoside analogue is structurally similar with adenosine, but lacking of 3' hydroxyl group. Even with such simple difference, cordycepin exhibits completely different biological activities from adenosine including purine biosynthesis suppression (Rottman and Guarino, 1964), premature transcription termination (Chen et al., 2008), mTOR signal interfering (Wong et al., 2010), apoptosis induction and metastasis inhibition of cancer cells, platelets aggregation (Cho et al., 2006) and immunoregulatory (Li et al., 2016; Shin et al., 2009; Zhou et al., 2008). Besides being a potent therapeutic agent, cordycepin was also reported to exhibit anti-photoaging

(Lee et al., 2009) and anti-pigmentation activities (Jin et al., 2012) which make it become the potential candidate to be used as a bioactive ingredient cosmeceutical product with whitening and anti-photoaging attributes. In order to use cordycepin in nutraceutical and cosmeceuticals industry, the great amount of cordycepin is required. The natural Cordyceps as the source of cordycepin is impossible to fulfill such a large demand. Therefore, the development of artificial Cordyceps culture for cordycepin enrichment could be the potential way to biologically synthesized great amount of cordycepin under safe, controllable environment. Studies indicated that 2 different basic procedures, solid-state fermentation and liquid culture, were used to produce cordycepin (Shashidhar et al., 2013). Among them, Liquid culture especially static liquid culture is considered to be more suitable procedure for large scale cordycepin production. Cordyceps is allowed to grow in liquid medium as mycelia without fruiting body formation. At certain point, cordycepin is produced and secreted out of Cordyceps mycelium to be accumulated in liquid medium without a need of extraction process. Liquid culture is considered as the better culture procedure to be used in industrial purposes by some researchers because of its potential dominant of shorter time and higher cordycepin production. The operation steps of liquid culture are also simpler which favors time/cost/labor minimization and mistake reduction. The cultivation can carry out in some certain temperature without the need of light/dark treatment and specific relative humidity so that it could be the good alternative for industrial scale optimization with less budget and complex system requirement. There were many studies that focused on static liquid culture aiming to optimize such procedure for cordycepin enrichment. The strategies for enhancement of cordycepin production are centered around the optimization of medium composition, the optimization of culture condition and strain improvement (Das et al., 2010; Das et al., 2009; Hung et al., 2009; Jiapeng et al., 2014; Kang et al., 2014; Masuda et al., 2007; Masuda et al., 2006; Sari et al., 2016; Tuli et al., 2014; Wen et al., 2017). Lately, the fundamental knowledge of epigenetic modifications was applied to the area of fungi culture in order to enhance production of some desirable chemical compounds. Epigenetic modifications are the heritable alterations that are not due to changes in DNA sequence but changes in chromatin structure reversibly between euchromatin and heterochromatin Normally, DNA methylation and histone hypoacetylation lead to

densely packed chromatin called “heterochromatin”. At such structural stage, less genes on DNA exposed to intranuclear environment so transcription process is more difficult to be occurred. However, with some chemicals called epigenetic modifiers, including DNA methyltransferase (DNMT) inhibitors and histone deacetylase (HDAC) inhibitors, heterochromatin could be loosed and became euchromatin so the rate of transcription is increased. Therefore, the epigenetic alteration of chromatin from heterochromatin to euchromatin could randomly increase the opportunities to be expressed for all genes in genome. Suberoylanilide hydroxamic acid (SAHA; HDAC inhibitor) was previously applied to culture media of endophytic fungi isolated from Datura stramonium L. and found that fusaric acid derivatives production were enhanced (Chen et al., 2013). SAHA was also supplemented in Phoma sp. culture for the induction of (10’S)-verruculide B production (Gubiani et al., 2017). And recently, the incorporation of valproic acid (VPA; HDAC inhibitor) to Aspergillus fumigatus (GA-L7) culture broth resulted in 10 times higher fumiquinazoline C production compared to those in basal broth (Magotra et al., 2017). Consequently, the knowledge of epigenetic modification could possibly be incorporated in static liquid culture of C. militaris to enhance cordycepin production to be used in nutraceutical and cosmeceutical industry.

The most renowned pharmacological activity of cordycepin is its anti-cancer potential. To date, there is more than 800 scientific articles worldwide reported about its anti-cancer efficiency against various type of cancer and the underlying mechanisms. Particularly, cordycepin can trigger apoptosis, autophagy, cell cycle arrest, and DNA damage in cancer cells, therefore controlling the proliferation and metastasis of cancer cells. Additionally, cordycepin also possess the immunoregulatory potential, influencing more immune cell to infiltrate into tumors. However, the mechanisms behind such immune-related activities remains obscure and have not yet been fully explored. Natural killer cell (NK cell) is a kind of cytotoxic lymphocyte that plays an essential function in the innate immune system as a cancer/tumor-fighting effector cell. The investigation of elements that improve or enhance NK function might therefore give information on cancer treatment. Since cordycepin exceptionally exhibits both anticancer and immunoregulatory effects, it is

conceivable to improve NK cell capabilities, which might be one of its alternate anti-cancer mechanisms. In this work, we evaluated the biological effects of cordycepin on the physiological parameters, developmental stage, and cytokine-producing capacity of NK cells. In addition, the ability of cordycepin to stimulate NK cell cytotoxicity against cancer cell models, THP-1 and U-251 cells, was evaluated. This work explained the process and confirmed the evidence that cordycepin might stimulate the anti-cancer activity of NK cells through maturation, cytokine synthesis, and granzyme B release to emphasize the pharmacological potential of cordycepin as an active ingredient in the nutraceutical product with anti-cancer activities.

For secretome, this term is described the global study of proteins or even represented the proteins that are secreted by cells, tissues or organisms at any given time or under certain conditions. The secretome extracts includes a wide range of important class of proteins that might be able to control and regulate biological and physiological processes of surrounding cells, tissues and organs including skin. The skin is the first line of defense against all environmental toxins, protecting the body from chemicals, radiation, microorganisms, and mechanical forces. Additionally, it prevents the loss of internal fluids and regulates body temperature (Hwa et al., 2011; Menon, 2015). Therefore, the condition of the skin is crucial to human health and well-being. Histologically, the skin consists of three vertically aligned layers: the epidermis, dermis, and hypodermis. Nonetheless, when skin is damaged and wounded, cutaneous wound healing plays a significant role in skin regeneration, primarily through the dynamic sequences of synergistic interaction between various cell types localized in both the epidermis and dermis layers. In such healing processes, the interaction between keratinocytes and fibroblasts is the most important. Despite being separated by basement membrane and having minimal direct cell-to-cell contact, keratinocyte–fibroblast interaction is predominantly mediated by secretable factors to regulate cell proliferation and extracellular matrix (ECM) remodeling (Sato et al., 1997). There were many studies reporting that the cell-cell interaction between skin cells via secreted proteins could promote the wellness of skin. For example, proIL-1α released from

keratinocytes was reported to induce cyclooxygenase 2 (COX-2) expression and prostaglandin E_2 resulting in the promotion of wound healing (Sato et al., 1997). Collagen type I and III precursors were reported to secreted by dermal fibroblasts to enriched the network of extracellular matrix within dermis layer of skin (Hathout, 2007) which provides the decent foundation for epidermis skin layer. Fibroblast growth factors (FGF) was also secreted by dermal fibroblast to acts as the mitogen for keratinocytes and dermal fibroblasts themselves (Gron et al., 2002). Thus, to use secretome extracts as active ingredient in cosmeceutical products, the secretome extracts from skin cells is reasonable candidate. In addition, cordycepin is planned to be cooperated to skin cell secretome production since cordycepin targets many cellular pathways. Therefore, the proteomic profile of skin cell secretome extracts might be altered and led to the alteration of biological activities.

This study aims to develop the procedures to produce to produce bioactive compounds, cordycepin (in *Cordyceps* culture media form) and secretome extracts using epigenetic modification and cordycepin treatment, respectively to be used as effective cosmeceutical ingredients. Moreover, the biological activities of cordycepin as the NK cell inducer to antagonize cancer will also be investigated.

1.2 Research objectives

The primary objectives of this study were:

I. To develop production procedures of cordycepin by *cordyceps* static liquid culture using epigenetic modification.

II. To investigate the effect of cordycepin on NK cell regarding to the improvement of its anti-cancer activity.

III. To develop production procedure of secretome from HaCaT cells and improve the secretome quality using cordycepin treatment strategy.

1.3 Research hypotheses

- Cordycepin production could be enhanced using epigenetic modification in static liquid culture system via the up-regulation of cordycepin production related genes.

- Cordycepin could stimulate the anti-cancer activity of NK cells through the induction of NK cell maturation, cytokine synthesis, and granzyme B release.

- The proteomic profile of secretome extracts from skin cells could be altered by cordycepin treatments and lead to better skin regeneration beneficial such as antioxidant and promoting wound healing and extracellular matrix components production.

1.4 Scope of this study

The static liquid culture of *Cordyceps militaris* will be supplied with epigenetic modifiers (VPA; HDAC inhibitors or 5AZA; DMNT inhibitors). Then, codycepin production will be investigated to indicate the effects of such epigenetic modifiers. Then, the expression of cordycepin production related genes will be evaluated in the epigenetically modified culture compared with the normal culture to verify the epigenetic modification activities. Then, the biological effects of cordycepin on NK-92 cells will be studied with a focus on physiological features, cell growth stage, and cytokine production capacity. In addition, the potential of cordycepin to enhance NK cell cytotoxicity against THP-1 and U-251 cancer cell models will be evaluated. Secretome extracts will be harvested from HaCaT, immortalized keratinocytse, cell cultures that will be proteomically altered by cordycepin treatment. Several bioactivities of produced secretome extracts, will also be investigated using human dermal fibroblast as cellular platform. The enhancement of cell proliferation, antioxidant activity, would healing property, and enhancement of extracellular matrix production will be used as the key parameters to verify the potential of both cosmeceutical ingredients. The resulting proteomic profile of secretome extracts from skin cells, altered by cordycepin treatments will be evaluated compared to the non-cordycepin treated secretome.

1.5 Expected results

By the treatment of epigenetic modifiers, the expression of genes related in cordycepin biosynthesis in *Cordyceps militaris* could be upregulated resulting in the enhancement of cordycepin production. The enhanced cordycepin production should be not less than 2000 mg/L, comparable or higher that previously reported studies.

For the investigation of codycepin biological influences on NK cells, cordycepin could facilitate the maturation and activation of NK cells resulting in the enhancement of cytokine production. Thus, cordycepin-activated NK cells could exhibit higher cytotoxicity against cancer cell models. For development of secretome production, cordycepin could altered the profile of cytokine/releasable factors of HaCaT secretome. Such cordycepin-induced HaCaT secretome could performed better skin beneficial activities regarding to skin regeneration and wound healing compared to normal HaCaT secretome. Taken together, cordycepin and secretome could be proven to be the potential bioactive compounds to be used as the ingredients of nutraceutical and cosmeceutical products.

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CHAPTER 2
LITERATURE REVIEW

2.1 *Cordyceps* and cordycepin

2..1.1 *Cordyceps* genus

Cordyceps is the genus of entomopathogenic fungi, belonging to the class *Ascomycetes* and naturally parasitizing the arthropods, mainly lepidopteron larvae and pupae. Among over 540 species of *Cordyceps* (Guo, Guo, Huaijun, Bu, and Dong, 2016), *Cordyceps* (*Ophiocordyceps*) *sinensis* and *C. militaris* are best known as the traditional herbal medication in East Asia, and rooted in Chinese history for more than 300 years. Such fungi were firstly introduced to the western country in 17th century, and have been studied and used as the source of bioactive constituents since then (Fan, Wang, and Zhong, 2012; Hong et al., 2010; Tuli, Sandhu, and Sharma, 2014; Yue, Ye, Zhou, Sun, and Lin, 2013; Zheng et al., 2011). Comparing the two well-known species, *C. sinesis* is more famous and expensive, since it is comparatively rare, exclusively collected from specific habitats, such as Tibetan Plateau (Lin et al., 2016), and proven to be difficult to cultivate in artificial conditions (Holliday, 2017; Hong et al., 2010). *C. militaris* is then considered as the potent and versatile candidate for artificial cultivation. There have been a broad range of bioactive constituents extracted from *Cordyceps*, including cordycepin, cordycepic acid (D-mannitol) (Chatterjee, Srinivasan, and Maiti, 1957), polysaccharides (D. T. Wu et al., 2014), sterols (Bok, Lermer, Chilton, Klingeman, and Towers, 1999; Yang, Feng, Zhao, and Li, 2009) and macrolides (Rukachaisirikul, Pramjit, Pakawatchai, Isaka, and Supothina, 2004).

2.1.2 Cordycepin and its biosynthesis pathway

Cordycepin, in particular, is the unique nucleoside analogue that is naturally found in many, but not all, *Cordyceps* species, including *C. sinensis, C. militaris, C. cicadae (Y. Wang, Guo, Zhang, and Wu, 2012),* and *C. kyushuensis (Zhao, Zhang, Li, and Ling, 2019).* Some other species, such as *C. bassiana* (or *Beauveria*

bassiana), is capable of producing N^6-(2-Hydroxyethyl)-adenosine, another bioactive nucleoside analogue, but not cordycepin (Liu, Wang, Wang, and Dong, 2017). Further investigation is required to complete the list of *Cordyceps* species that are able to produce cordycepin. Cordycepin is structurally similar with adenosine, but lacking of 3' hydroxyl group (Figure 2.1). Even with such simple difference, cordycepin exhibits completely different biological activity from adenosine, and is reported to interfere many molecular and cellular processes within cells. For instance, cordycepin could target purine biosynthesis (Rottman and Guarino, 1964), mTOR signaling(Wong et al., 2010), apoptosis (Chaicharoenaudomrung, Jaroonwitchawan, and Noisa, 2018; Liao et al., 2015), metastasis (Nakamura, Shinozuka, and Yoshikawa, 2015; A. Sato et al., 2013), platelets aggregation (H. J. Cho, Cho, Rhee, Lim, and Park, 2006), immunoregulatory (Zhou et al., 2008), and inflammation pathway (Li et al., 2016; Shin et al., 2009).

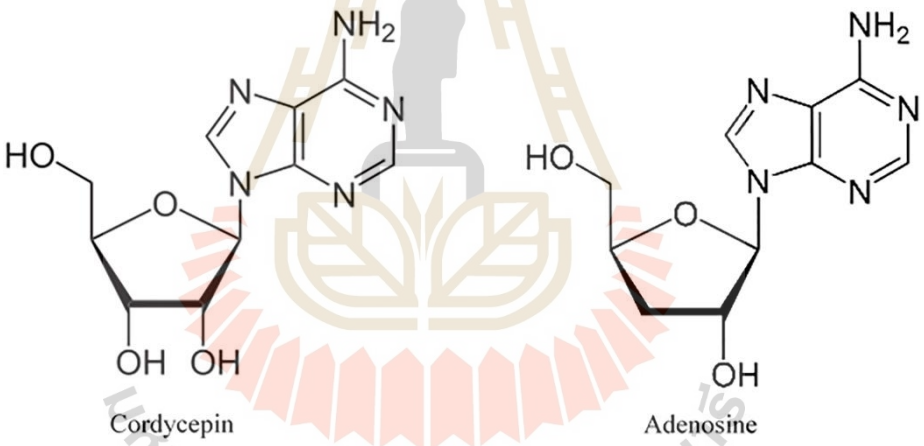

Figure 2.1 The structural difference between cordycepin and adenosine.

Recently, nutraceuticals and cosmeceuticals become the worldwide trend and serve as another segment of personal health care industry. Nutraceutical is an integration of the terms "nutrition" and "pharmaceutical" that describes substances that act as both nutritional supplies and pharmaceuticals to improve health, delay the aging process, prevent chronic diseases, increase life expectancy, or maintain the body wellness (L. Das, Bhaumik, Raychaudhuri, and Chakraborty, 2012; Nasri, Baradaran, Shirzad, and Rafieian-Kopaei, 2014). Similarly, Cosmeceuticals are a discipline that combines pharmaceuticals with cosmetics and aims to provide health advantages to

the human body, such as cleansing, beautifying, improving attractiveness, or modifying appearance by means of observable and quantifiable biological effects. (Lohani, Verma, Joshi, Yadav, and Karki, 2014; Oricha, 2010). The biological activities of nutraceutical and cosmeceutical products are dependent on their bioactive substances, which are derived from natural sources or chemical synthesis; hence, the desirable characteristics of such products may be created and produced. To compete in nutraceutical and cosmeceutical sectors, the effective bioactive compounds are required. In addition to being a powerful therapeutic agent with a broad spectrum of bioactivities, cordycepin was shown to display anti-photoaging (Y. R. Lee et al., 2009) and anti-pigmentation pigmentation (Jin et al., 2012), making it an interesting bioactive component for cosmeceutical products. To apply cordycepin to the nutraceutical and cosmeceutical industries, a large volume of cordycepin is required, and naturally grown *Cordyceps* as a source of cordycepin is unsuitable for this purpose. Therefore, the establishment of an artificial Cordyceps culture for cordycepin enrichment may be a viable strategy for biologically synthesizing a large quantity of cordycepin in a controlled setting. Earlier studies indicated that the two different basic procedures, solid- state fermentation and liquid culture, were used to produce cordycepin (Shashidhar, Giridhar, Sankar, and Manohar, 2013).

The biosynthetic pathway of cordycepin by *Cordyceps militaris* was firstly investigated *in vivo* since 1976, using [U-^{14}C] adenosine and [3-^{3}H] ribose for metabolic tracking based on purine metabolic pathway. The ^{3}H: ^{14}C ratio of radioactive cordycepin and the intermediates of purine pathway was compared, and implied that the cordycepin might be formed from adenosine without cleavage of the adenine-ribose bond. The formation of cordycepin might proceed by a reductive mechanism similar to the formation of 2'-deoxynucleotides by ribonucleotide reductase. It is also suggested that *Cordyceps militaris* did not begin the production of 3'-deoxyadenosine until DNA synthesis was terminated. Therefore, ribonucleotide reductase might be modified to produce cordycepin when it is not necessary to synthesize 2'-deoxynucleotides for DNA synthesis (Lennon and Suhadolnik, 1976). To further model the biosynthesis pathway of cordycepin, the *de novo* purine metabolic pathway in *Cordyceps* was constructed based on the KEGG (Kyoto Encyclopedia of Genes and Genomes) annotations for both *C. sinensis* and *C. militaris* (Lin et al., 2016; Zheng et

al., 2011). The serial conversion of *Hirsutella sinensis, anamorph of C. sinensis* to biosynthesize cordycepin was described as followed. Glucose is firstly converted to glucose-6-phosphate (G-6-P), and then transformed into ribose-5-phosphate (R-5-P) by pentose phosphate pathway. R-5-P is the primary substrate of the *de novo* purine nucleotide pathway, which is then sequentially converted into phosphoribosyl pyrophosphate (PRPP), IMP, and finally AMP/GMP. Glutamine and glycine involve in the serial conversions as precursors of nitrogenous base residue (Q. Zhang and Liu, 2016). AMP is converted to ADP by adenylate kinase (AK), ADP is converted to 3'-deoxyadenosine 5'-diphosphate (3'-dADP) by ribonucleotide reductase (RNR), and 3'-dADP is converted to 3'-deoxyadenosine 5'-phosphate (3'-dAMP) by adenylate kinase. In such biosynthesis model, 3'-dAMP and adenosine could be the potential intermediates to be converted to cordycepin, in which 3'-dAMP was hypothetically converted to cordycepin by 5'nucleotidase (Lin et al., 2016). However, the mechanism of adenosine-cordycepin conversion remains unknown. Cordycepin biosynthetic pathway of *C. miltaris* was also proposed in the similar manner except the involvement of ribonucleotide reductase (Zheng et al., 2011). In addition, the regulatory enzymes of purine metabolic pathway have been hypothetically established (Fan et al., 2012). Phosphoribosylamidotransferase (PRAT) is the rate-limiting enzyme of the purine metabolic pathway, and IMP cyclohydrolase (IMPC) catalyzes the last step, specifically to produce IMP (Wolan, Cheong, Greasley, and Wilson, 2004). IMP dehydrogenase (IMPDH) regulates oxidation step of IMP towards GMP biosynthesis (Shimura, Okada, Shiraki, and Nakagawa, 1983). Adenylosuccinate synthetase (ADSS) controls AMP biosynthesis from IMP (Poland et al., 1993).

Even with over 4 decade-long historical investigations of cordycepin biosynthetic pathway, there was no clear experimental evidence to elucidate whether "*how cordycepin is produced*" and "which genes are directly responsible for cordycepin production" in *Cordyceps*. Until in 2017, the gene cluster responsible for the biosynthesis of cordycepin was identified and verified by Xia *et al* (Xia et al., 2017), based on the previously established genome information of *C. miltrais* (Zheng et al., 2011). The genome-wide reciprocal analyses compared over 5,800 orthologous proteins between C. *militaris* and *Aspergillus nidulan*s, a fungi in the same class that was capable of producing cordycepin (Kaczka, Dulaney, Gitterman, Boyd Woodruff,

and Folkers, 1964). The 4-highly conserved genes were revealed and designated as *cns1-cns4*. Those genes were physically linked as a gene cluster and encoded proteins with different conserved domains, which differently mediated cordycepin metabolism. Firstly, Cns1 contains oxidoreductase/dehydrogenase domain, while Cns2 possesses the HDc family of metal-dependent phosphohydrolase domain. Cns3 contains two functional domains: an N-terminal nucleoside/nucleotide kinase (NK) and a C-terminal HisG family of ATP phosphoribosyltransferases, and, lastly, Cns4 is identified as a member of ATP-binding cassette (ABC) transporters. Based on the authors' finding, cordycepin biosynthesis pathway was further fulfilled. Starting from adenosine, the hydroxyl phosphorylation is catalyzed by nucleoside/nucleotide kinase domain of Cns3 at 3'-OH position to yield adenosine-3'-monophosphate (3'-AMP). 3'-AMP is then dephosphorylated to 2'-carbonyl-3'-deoxyadenosine (2'-C-3'-dA) by phosphohydrolase activity of Cns2. Cordycepin is finally produced from the 2'-C-3'-dA by oxidoreduction reactions mediated by Cns1. Pentostatin, a purine analogue with the activity of anticancer and adenosine deaminase inhibition (Kane, Kuhn, and Roush, 1992), was noteworthy produced in coupling with cordycepin by phosphoribosyltransferase domain of Cns3. This process was to maintain the stability of cordycepin; not to be converted to 3'-deoxyinosine (3'dI) by deaminase via the protector-protégé strategy (P. Wu et al., 2017). Such pentostatin is then pumped out of the cell by Cns4 transporter when cordycepin accumulation reaches cytotoxic levels. This process neutralizes cordycepin to nontoxic 3'dI via the deamination reaction. In addition, Cns1 and Cns2 were proved to be critically essential, and needed to interact directly with each other in order to produce cordycepin, since the deletion of either *cns 1* or *cns2* caused the absolute deficiency of cordycepin production in *C. militaris*. The schematic representation of cordycepin biosynthetic pathway was illustrated in Figure 2.2.

The understanding of cordycepin biosynthesic pathway could be translated toward the artificial *Cordyceps* culture, relied on the basis of metabolic and genetic engineering, or the modification of culture systems.

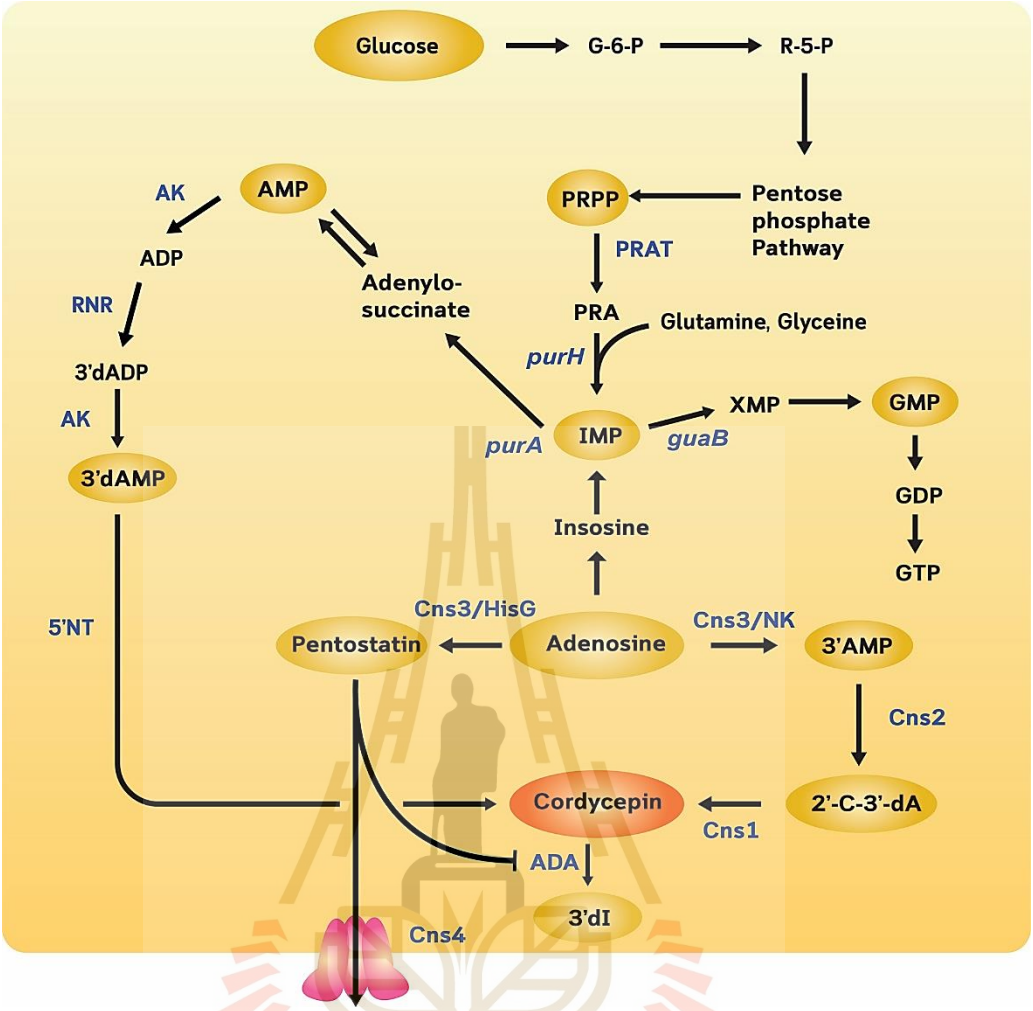

Figure 2.2 The biosynthesis pathway of cordycepin based on *de novo* purine metabolic pathway(Fan et al., 2012; Lin et al., 2016; Q. Zhang and Liu, 2016) and coupled biosynthetic pathway of cordycepin and pentostatin(Xia et al., 2017) (PRAT: Phosphoribosylamidotransferase, purH: Bifunctional purine biosynthesis protein, purA: purine rich element binding protein A, guaB: Inosine-5'-monophosphate dehydrogenase, RNR: Ribonucleotide reductase, 5'NT: 5'-nucleotidase, AK: Adenylate kinase, Cns1: protein with oxidoreductase/dehydrogenase domain, Cns2: protein with the HDc family of metal-dependent phosphohydrolase domain, Cns3; protein with an N-terminal nucleoside/nucleotide kinase (NK) and a C-terminal HisG family of ATP phosphoribosyltransferases, Cns4: a member of ATP-binding cassette transporters and ADA: adenosine deaminase).

2.2 Artificial culture of *Cordyceps*

According to the large demands of cordycepin to be used as the therapeutic and cosmeceutical composition, the up- scale production of cordycepin has drawn attention of scientists and business sectors. To fulfill such demand- supply gap and prevent the extinction of natural *Cordyceps* resulting from over harvesting, artificial culture of *Cordyceps* was developed and optimized.

2.2.1 Solid substrate fermentation

Solid substrate fermentation is the cultivation procedure, in which *Cordyceps* is grown on solid substrate comprising of grains or cereals. The grain is usually rice, wheat, or rye (Adnan, Ashraf, Khan, Alshammari, and Awadelkareem, 2017; Chiang, Liang, Wang, and Liang, 2017; Gregori, 2014; N. Kang, Lee, Park, and Seo, 2017; Kim, Shrestha, Sung, Han, and Sung, 2010; Liang, Liang, and Wu, 2014; Lim, Lee, and Chang, 2012; Wen et al., 2016; Wen, Li, Kang, Kang, and Hyde, 2014). Arthropod larvae are sometimes mixed in solid substrate to mimic natural condition (Jian and Li, 2017). There are three main steps for overall solid substrate fermentation, including mycelium cultivation, fruiting body formation, and cordycepin accumulation. Each step requires specific condition to be controlled. Mycelium cultivation step is the period after the inoculation of seed culture, which the *Cordyceps* mycelium is allowed to grow and penetrate throughout the entire solid substrate at 19- 21 ℃ for 7- 14 days without illumination. After the whole substrate is covered with mycelia, the condition is adjusted for fruiting body formation. In such step, the light/ dark treatment is provided to mimic daytime and nighttime of the nature in order to stimulate fruiting body formation. In light phase, light source with the intensity of 500 lx – 1000 lx is supplied for 8-12 hours per day. Temperature and relative humidity are also controlled at 16-23 ℃ and 70-95 % in this light/dark treatment, respectively. After the noticeable fruiting body formation, some studies adjusted the culture condition again to promote fruiting body elongation and cordycepin accumulation. In the cordycepin accumulation step, the light- dark treatment is still required, but temperature and relative humidity could be slightly different depending on the optimization of each study (Adnan et al., 2017; Chiang et al., 2017; Gregori, 2014; Jian and Li, 2017; N. Kang et al., 2017; Kim et al., 2010; Liang et al., 2014; Lim et al., 2012; Wen et al., 2016; Wen et al., 2014). The illustration of solid substrate fermentation was showed in Figure 2.3.

The solid-state fermentation was studied and optimized since 1968 (Basith and Madelin, 1968) until the modern 20th century (Table 2.1). The effect of different solid substrates on cordycepin production was the main issue to be explored. It was indicated that cordycepin content depended on the nutrient composition of solid substrate. Lim *et al*. cultured *Cordyceps* in different organic substance and found that soybean was the best choice for enhancing cordycepin production (Lim et al., 2012). Wen *et al*. also studied the effects of different solid substrates, in combination with the variations of carbon, nitrogen, mineral salts, growth factors, and pH, on the production cordycepin by using either one-factor-at-a-time or orthogonal layout methods. It was concluded that the combination of brown rice, peptone, K_2HPO_4, $MgSo_4$, and non-essential amino acids at pH 5-5.6 was the best medium composition. They also monitored the correlation between cordycepin and adenine content, and found that adenosine started to increase to the maximum prior to decrease and fluctuate, while cordycepin increased continuously over time. This might due to the fact that adenosine was used in DNA synthesis for proliferation, fruiting body formation, and cordycepin synthesis (Wen et al., 2014). Similar optimization experiments of solid-substrate fermentation were carried and focused on the effects of different solid media composition. Monosodium glutamate and histidine were found to be beneficial for cordycepin production, yet the mechanism remained unknown (Liang et al., 2014; Wen et al., 2016). Adenine was found to be a potent nucleoside additive, since it is one of the possible intermediates to be converted to cordycepin (Wen et al., 2016).

Besides the optimization of solid medium composition, Chiang *et al*. studied the effect of different light emitting diode condition on the mycelium biomass and cordycepin production. It was found that the combination of blue and green light was ideal for cordycepin production, while red light could be used to enhance biomass production (Chiang et al., 2017). Kang *et al*. developed high cordycepin-producing *C. militaris* strains by mating-based sexual reproduction. The spores with opposite mating type idiomorph, MAT 1-1 and MAT 1-2, were used for mating experiments. With such mating technique, *C. militaris* mated strain KSP8 was established and improved cordycepin production by 35%, compared with the wild type. The spore mating might lead to genetic recombination of genes related to cordycepin synthesis (N. Kang et al., 2017). Lately, various plant growth regulators, for example, triacontanol, presented an

effect on cordycepin production (Jian and Li, 2017). In summary, solid- state fermentation of *Cordyceps* could be developed and optimized by modifying the solid medium compositions. Types and concentrations of carbon sources, nitrogen source, and additives were considered as the key parameters. Moreover, the strain development of *Cordyceps* was also the crucial factor for cordycepin enrichment.

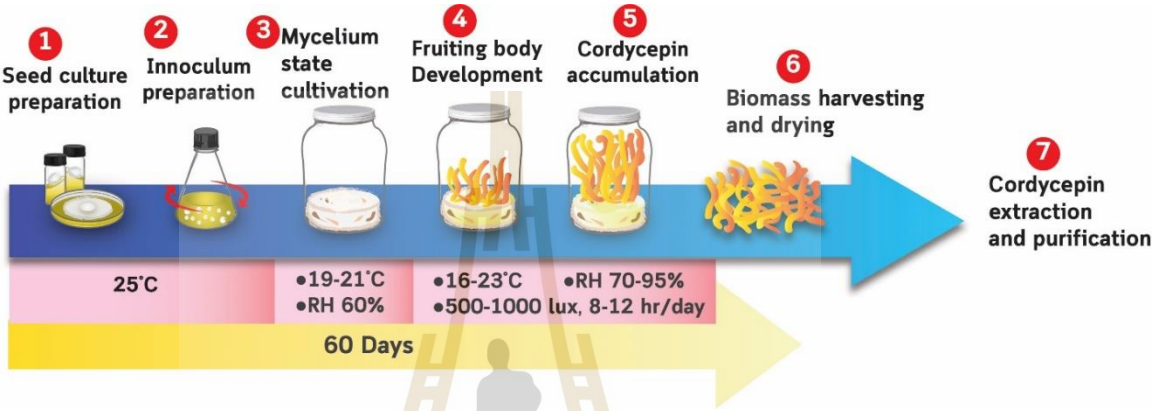

Figure 2.3 The illustration of solid substrate fermentation of *Cordyceps*.

Table 2.1 Cordycepin production by solid-state fermentation of *Cordyceps* in different studies.

No.	Strain	Solid substrates	Carbon sources (g/L)	Nitrogen sources (g/L)	Micro-nutrients	Additives/ Strategies	Cultivation time (day)	Biomass (g/unit)	Cordycepin (mg/g)	References
1	*C. militaris*	Soy bean	Glucose/ 8	Yeast extract/ 8	KH_2PO_4 K_2HPO_4 $MgSO_4$	-	40	?	8.621	Lin *et al*., 2012
2	*C. militaris* CGMCC2459	Brown Rice	Glucose/ 10	Peptone 10	K_2HPO_4 $MgSO_4$	+Non-essential amino acids	60	Fruiting body/ 1.73	9.17	Wen *et al*., 2014
3	*C. militaris* Strain H	Wheat	-	Monosodium glutamate/ 0.3	-	-	60	Fruiting body/ 25.16	25.07	Liang *et al*., 2014
4	*C. militaris* CM016	Rice (*Oryza sativa*)	Glucose/ 26.25	Peptone/ 26.25	KH_2PO_4 $MgSO_4$	+Adenine + Histidine	39	?	18.92	Wen *et al*., 2016
5	*C. militaris* 101	Brown rice	-	-	-	Red light: blue light = 3:3	15	?	6.1	Chiang *et al*., 2017
6	*C. militaris* mated strain KSP8	Silk worm pupae	-	-	-	Strain improvement by mating	50	?	> 8	Kang *et al*., 2017
7	*C. militaris* CM-16	Wheat	-	-	-	+Triacontanol	?	?	6.13	Jian and Li, 2017

2.2.2 Liquid culture

Liquid culture is the alternative procedure of *Cordyceps* cultivation. *Cordyceps* is allowed to grow in liquid as mycelia without the formation of fruiting body. At certain time point, cordycepin is produced and secreted out of *Cordyceps* mycelium, and accumulated in the liquid medium without a need of extraction process. Liquid culture is considered as the better culture procedure to be used in industrial purposes because of the shorter cultivation time and the higher cordycepin production yield. The liquid culture can be carried without the need of light/dark treatment and specific relative humidity; thus it could be the good choice for industrial scale application with less cost and complex system requirement (S. K. Das, Masuda, Hatashita, Sakurai, and Sakakibara, 2010; S. K. Das, Masuda, Sakurai, and Sakakibara, 2009; Fan et al., 2012; Hung, Keawsompong, Hanh, Sivichai, and Hywel-Jones, 2009; C. Kang et al., 2014; Mao, Eksriwong, Chauvatcharin, and Zhong, 2005; Mao and Zhong, 2004, 2006; Masuda, Urabe, Honda, Sakurai, and Sakakibara, 2007; Masuda, Urabe, Sakurai, and Sakakibara, 2006; Shih, Tsai, and Hsieh, 2007; Tang, Qian, and Zhu, 2015; Wen, Long, Kang, Wang, and Zeng, 2017). The liquid culture could be classified into three different methods, including submerged culture, surface/static cultured, and two-step shaking static culture (Figure 2.4).

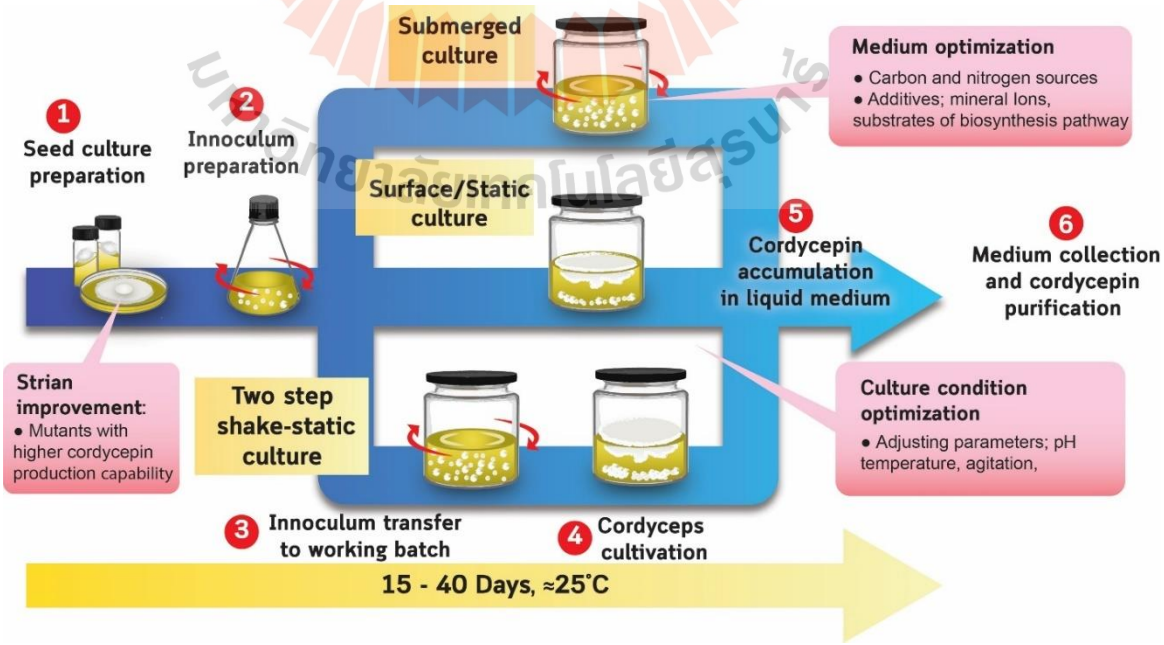

Figure 2.4 The illustration of liquid culture of *Cordyceps*.

2.2.2.1 Submerged culture

Submerged culture is one of the liquid culture methods, in which shaking or agitating is provided to dissolve oxygen and nutrients in liquid medium. Submerged culture was widely used in both experimental and industrial cultivation of many microorganisms, including yeast (Vlaev et al., 2013), bacteria (Gullo, Verzelloni, and Canonico, 2014), and other fungi (Vieira, Liebl, Tavares, Paulert, and Smânia Júnior, 2008). The strategy of submerged culture was based on the optimization of medium compositions. Types and concentrations of different carbon sources of culture medium were optimized by using central composite design and response surface analysis. Glucose was proved as the best carbon source for cordycepin production with the concentration of 42 g/L (Mao and Zhong, 2004). Peptone was the most beneficial complex nitrogen source, and it was found that NH_4^+ was the chemically defined nitrogen source in peptone, promoting cordycepin production. Besides the role as nitrogen sources, $NH4^+$ was also related to cellular energy metabolism (synthesis of ATP and ADP) and the metabolism of adenosine, adenylate, and ADP (Mao and Zhong, 2006). Metal salts were used as an additive in the submerged culture and found that $FeSO_4$ presented the best induction of cordycepin accumulation. Under $FeSO_4$ supplied condition, the expression of purine nucleotide synthetic genes, *purA*, was highly expressed, indicating that *purA* should be a key gene of cordycepin biosynthesis triggered by ferrous ion (Fan et al., 2012). Dissolved oxygen (DO) was also an important factor for the submerged culture. The control of DO at 60% from the beginning of cultivation, and then reduced to 30% afterward could promote cordycepin production. This suggested that the DO required for cell growth and cordycepin production was dynamic (Mao and Zhong, 2004).

2.2.2.2 Surface/static culture

Surface culture, or known as static culture, is method system that allows *Cordyceps* to be grown in a static condition after seeding of the inoculum. During the cultivation, the biofilm of *Cordyceps* was formed on the surface of medium, and some mycelia precipitated at the bottom of culture containers, while cordycepin was secreted and accumulated in the medium. Surface/static culture was the most studied and optimized method out of the three methods decried in this review.

The strategies to optimize such method for cordycepin enrichment included the optimization of medium compositions, the adjustment of culture conditions, and the improvement strain. The ratio between carbon source and nitrogen source at 2:1 was found to be the most efficient for cordycepin production, and the ratio 1:3 of peptone and yeast extract was the optimal combination of nitrogen source (Masuda et al., 2006). Nucleosides and amino acids are the major compounds, related to purine biosynthetic pathway. They could enhance cordycepin production, since nucleosides and amino acids function as backbone precursors of cordycepin. Among them, adding glycine and adenine yielded the highest cordycepin production (Masuda et al., 2007). Recently, vegetable oil was also used as a secondary carbon source in cultivation system in order to favor the mycelium growth and avoid carbon catabolite repression caused by glucose. Among various types of vegetable oil, the supplementation of peanut oil was found to upregulate the glyoxylate pathway, pentose phosphate pathway, and cordycepin biosynthesis pathway, in which resulted in enhancing cordycepin production (Tang, Qian, and Wu, 2018).

The optimization of culture condition was also studied. Since surface/static culture is carried out without agitation, the depth of medium was found to affect cordycepin production. Masuda *et al*. found that the time required to reach the constant value increased with the medium depth indicating that the utilization efficiency of the surfaced biofilm of *Cordyceps* decreases with an increase in the medium depth (Masuda et al., 2006). The effect of temperature on mycelial growth and cordycepin production was also investigated. Hung *et al*. found that 15-20 °C was beneficial for mycelial growth and 25 °C was ideal for cordycepin production (Hung et al., 2009). The strain improvement was found to be the most potential strategy to yield the highest cordycepin. Das *et. al* established 5 Azaguanine resistant mutants G81-3 via the mutagenesis of *C. militaris* using proton beam irradiation. The cordycepin production by such mutant dramatically increased to more than 6 g/L which has been reported to be the highest cordycepin production from artificial cultivation to date. Generally, the levels of the end products from the biosynthesis are regulated by the feedback inhibition and thus the removal of the feedback cycle could lead to an accumulation of the end products. The guanine- related compounds (guanine, guanosine, GMP) might be accumulated in the 8-Azaguanine resistant mutant that

could increase the cordycepin production, because the production of cordycepin and guanine is related based on purine *de novo* pathway (S. K. Das et al., 2010).

2.2.3 Two-step shaking-static culture

The two-step shaking-static culture is the method, combining submerge culture and surface/static culture together. After seeding, *Cordyceps* is allowed to grow in shaking condition which is found to promote biomass production for a short period. Then, *Cordyceps* is further cultivated in static condition to promote cordycepin accumulation. Tang *et al*. observed the morphology of Cordyceps biomass in both step of two step shaking-static culture using scanning electron microscope found that the mycelia were thick and smooth, having many articulate branches under shaking condition. On the contrary, the mycelia under static condition were long and thin, owning little branches, having rough surface with many wrinkles under static state and possessed the typical characteristics of aerial hyphae and conidiophores (Tang et al., 2015). Shih *et al.* optimized the culture condition of two step shaking-static culture considering the initial pH of the medium, the concentration of nitrogen source, and cultivation time of shaking and static culture as factors using A Box– Behnken experimental design. They found that culture system with 45 g/L yeast extract, initial pH 6 and 8.0 day of the shake culture followed by 16 days of the static culture was the optimal condition for cordycepin production (Shih et al., 2007).

Comparing between 3 methods of liquid culture, studies indicate that surface/static culture and two step shaking-static culture could be the potent method to be applied to industrial cultivation since higher cordycepin production could be obtained (Table 2.2). In summary, liquid culture is reasonably considered as the alternative procedure to be the good foundation for industrial scaled cultivation. With well-optimized medium composition and culture condition and strain improvement, high cordycepin production could be achieved to be used for commercialization purpose.

Table 2.2 Cordycepin production by liquid culture of *Cordyceps* in different studies.

No.	Strain	Culture method	Carbon source (g/L)	Nitrogen source (g/L)	Micro-nutrients	Additives/ Strategies	Cultivation Time (day)	Working Volume	Biomass (g/L)	Cordycepin (mg/L)	References
1	*C. militaris*	Submerged culture	Glucose/ 40	Peptone/ 10	KH_2PO_4 K_2HPO_4 $MgSO_4$	Two-Stage DO Control in bioreactor	17	5 L	?	201.1	Mao and Zhong, 2004
2	*C. militaris*	Submerged culture	Glucose/ 42	Peptone/ 15.8	KH_2PO_4 K_2HPO_4 $MgSO_4$	-	18	50 mL	19.2	345.4	Mao *et al*., 2005
3	*C. militaris*	Submerged culture	Glycose/ 10	Peptone/ 10	KH_2PO_4 K_2HPO_4 $MgSO_4$ $CaCl_2$ $FeSO_4$	Fed-bacth with NH_4^+	17	3.51 L	19.5	346.1	Mao and Zhong, 2006
4	*C. militaris*	Submerged culture	Glucose/ 40	Peptone/ 10	KH_2PO_4 K_2HPO_4 $MgSO_4$ $(NH_4)_2SO_4$	+ $FeSO_4$	20	?	?	596.59	Fan *et al*., 2012

Table 2.2 (continued).

No.	Strain	Culture method	Carbon source (g/L)	Nitrogen source (g/L)	Micro-nutrients	Additives/ Strategies	Cultivation Time (day)	Working Volume	Biomass (g/L)	Cordycepin (mg/L)	References
5	*C. militaris* NBRC 9787	Surface/static culture	Glucose/ 20	Peptone/ 2.5 Yeast extract/ 7.5	Mixed trace elements	-	27	100 mL	?	640	Masuda *et al.*,2006
6	*C. militaris* NBRC 9787	Surface/static culture	Glucose/ 20	Peptone/ 2.5 Yeast extract/ 7.5	Mixed trace elements	+ Adenine + Glycine	33	100 mL	9.8	2370	Masuda et al.,2007
7	*C. militaris* NBRC 9787	Surface/static culture	Glucose/ 86.2	Yeast Extract/ 93.8	Mixed trace elements	8-azaguanine resistant mutant	36	100 mL	?	6840	Das *et al.*, 2010
8	*C. militaris* CGMCC2459	Surface/static culture	Sucrose/ 24.7	Peptone/ 20	K_2HPO_4 $MgSO_4$	+Vitamin B_1 +Hypoxanthine +L-alanine	35	700 mL	?	2008.48	Kang *et al.*,2014
9	*C. militaris* GACP08Y5	Surface/static culture	Sucrose/ 20	Peptone/ 20	K_2HPO_4 $MgSO_4$	+Adenine +Glycine	40	2 L	?	3005.83	Wen *et al*, 2017

Table 2.2 (continued).

No.	Strain	Culture method	Carbon source (g/L)	Nitrogen source (g/L)	Micro-nutrients	Additives/ Strategies	Cultivation Time (day)	Working Volume	Biomass (g/L)	Cordycepin (mg/L)	References
10	*C. militaris CICC 14014*	Surface/static culture	Glucose/ 86.2	Tryptone/ 6 Yeast extract/ 0.5	$MgSO4{\cdot}7H_2O$, $K_2HPO_4{\cdot}3H_2O$ KH_2PO_4	+Peanut oil (30g/L)	20	100 mL	≈ 28g/L	5290	Tang *et al*, 2018
11	*C. militaris* CCRC 32219	Two step culture	Glucose/ 40	Yeast Extract/ 45	KH_2PO_4 K_2HPO_4 $MgSO_4$ $FeSO_4$	-	Shaking/ 8 Static/ 16	100 mL	?	2214.5	Shih *et al*.,2007
12	*C. militaris* 14014	Two step culture	Glucose/ 40	Tryptone/ 10 Yeast extract/ 6	KH_2PO_4 K_2HPO_4 $MgSO_4$	-	Shaking/ 4 Static/ 25	50 mL	?	2620	Tang et al.,2015

2.3 Future perspective for nutraceuticals applications regarding to anticancer activity

As previously described, cordycepin possesses a variety of biological activities mostly interfering and regulating a number of biochemical and molecular processes including purine biosynthesis, mTOR signaling, apoptosis, metastasis, platelets aggregation, immunoregulatory, and inflammation pathway. Such activities make it a promising agent against cancer. More than 800 research studies have been published on its anti-cancer efficacy against different types of cancer with some proposed underlying processes. Most of anti-cancer activities of cordycepin was reported to be mediated through apoptosis pathway. Lee et al. (2013), Chaicharoenaudomrung et al. (2018) and Y. Zhang et al. (2018) reported that cordycepin inhibited prostate carcinoma cells (PC-3), glioblastoma cells (U-251) and human pancreatic cancer cells (MIAPaCa-2 and Capan-1) proliferation respectively by the intrinsic apoptosis pathway with the amplifying Bax/Bcl-2 ratio and the downregulation of inhibitor of apoptosis protein (IAP) family members. Y. Wang et al. (2017) proposed that the viability of THP-1, human leukemia monocytic cell was inhibited by cordycepin through downregulation of Bcl-2, Akt1, Akt2 and Akt3 and the upregulation of Bax, hence apoptosis pathway was induced. The proliferation of 4NAOC-1 cells, murine oral cancer model, were also inhibited by cordycepin via apoptosis pathway with the involvement of IL-17A/IL17RA suppression (Hsu et al., 2017). The viability of cisplatin-resistant lung cancer cell (A549CR cells) was decreased by cordycepin treatment via both intrinsic and extrinsic apoptosis along with cell cycle arrest at sub G1 stage (S. H. Cho and Kang, 2018). Although cordycepin also exhibit immunoregulatory potential, the immune-mediated anticancer activity of codycepin remains unclear and have not yet been fully explored.

2.4 Future perspective for cosmeceutical applications

Skin Aging, hyperpigmentation and darken skin are the main concerns for cosmetic and cosmeceutical industry. Aging is the degenerative changes of physiology over time, occurring in all organisms, including human. This process is the result of the naturally genetic programming and the accumulation of cellular damages that is caused by both internal and external factors (Park and Yeo, 2013). Additionally, the term “ *skin aging*” mainly refers to the loss of skin integrity resulting from the loss of

skin cells or the degradation of skin extracellular matrix(Robert, Labat-Robert, and Robert, 2012). Reactive oxygen species (ROS), by-products of cellular metabolism, is the most concerned one among internal factors that cause damages to cellular components and finally lead to skin deterioration (Poljsak and Dahmane, 2012; Rinnerthaler, Bischof, Streubel, Trost, and Richter, 2015). Cordycepin was reported to exhibit antioxidant properties by scavenging hydroxyl radicals in vitro and the scavenging capacity of hydroxyl radicals could reach to 50% at 1.8 mg/ml of cordycepin (He, Zhang, Xie, Xu, and Li, 2013). Cordycepin was also reported to exhibit anti-photoaging and anti-pigmentation activities in 2D cell culture platform (Figure 2.5). Extrinsic aging is caused by external stimuli including UV radiation which induced the production of matrix metalloproteinase of dermal fibroblast to degrade the network of extracellular matrix in dermis layer of skin. Studies proposed that UV radiation activated the function of NF-kB transcription factor to translocate to nucleus and up-regulate matrix metalloproteinase genes. With cordycepin treatment to UVB exposed dermal fibroblasts; cordycepin could suppress the activation and translocation of NF-kB to nucleus. Therefore, the expression of matrix metalloproteinase genes was decreased (Y. R. Lee et al., 2009). Melanogenesis, the biosynthesis pathway to produce melanin in melanocyte, could be induced by external stimuli such as UV radiation and some chemicals. With such stimuli, the signal transduction cascade mediated by cAMP response element-binding protein (CREB) is activated resulting in the activation of microphthalmia-associated transcription factor (MITF). MITF then up-regulate melanogenesis involving genes. From the experiment done in mouse melanoma, cordycepin could somehow activate protein kinase B (Akt) and extracellular signal–regulated kinase (ERK) dependent mechanisms which then inhibit MITF activity. Therefore, the expression of melanogenesis involving genes was not up-regulated (Jin et al., 2012). Therefore, studies convinced that cordycepin could be used as bioactive ingredient of cosmeceutical products to emphasize the uniqueness and provide the effective anti-photoaging and anti-pigmentation attribute. In addition, cordycepin could be applied to the treatment of various dermatologic hyperpigmentation disorders, such as freckles and melisma.

To produce sufficient amount of cordycepin for nutraceutical and cosmeceutical applications, cordycepin could be biologically synthesized via 2 different *Cordyceps* artificial cultivation procedures including solid substrate fermentation and liquid culture. To maximize cordycepin production, strain improvement medium optimization and condition optimization strategy could be applied. Solid state fermentation requires long cultivation time and complex cultivation system. Therefore, the liquid culture is considered to be the alternative method for large- scaled cordycepin production for large-scaled cordycepin production and possibly maximized cordycepin production by strain improvement medium optimization and condition optimization strategy.

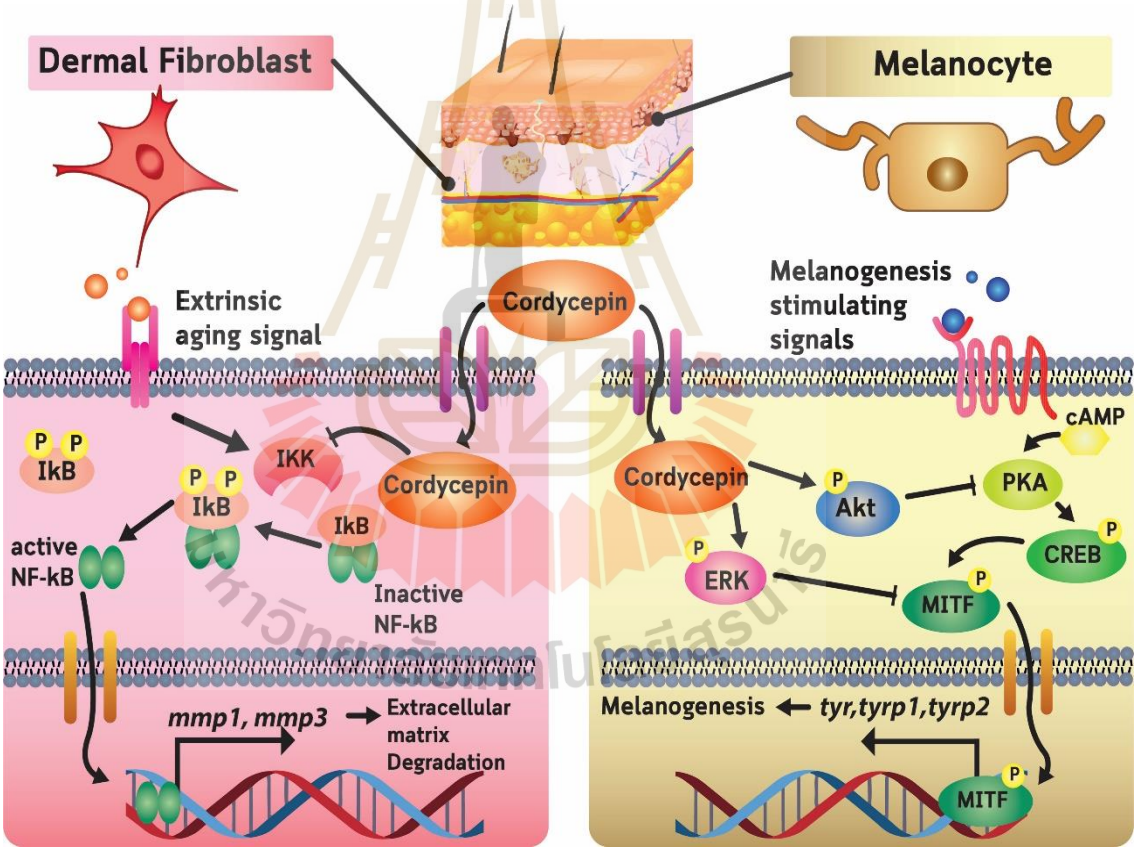

Figure 2.5 Schematic representation of cordycepin as the anti- photoaging and hypopigmentation agent.

2.5 Epigenetic modification

The term, “epigenetics,” was originally refer to the complex interactions between the genome and the environment that are involved in development and differentiation in higher organisms. Nowadays, this term is used to refer to heritable alterations that are not due to changes in DNA sequence. Rather, epigenetic modifications, such as DNA methylation and histone modification, alter DNA accessibility and chromatin structure reversely between euchromatin and heterochromatin, thereby regulating patterns of gene expression (Figure 6).

2.5.1 DNA methylation

The methylation in DNA is normally found as the covalent attachment of a methyl group to the C5 position of cytosine residues in CpG dinucleotide sequences (CpG methylation) via the activity of DNA methyltransferases (DNMTs). Transcriptional gene silencing by CpG methylation also restricts the expression of some tissue-specific genes during development and differentiation by repressing some genes in non-expressing cells. CpG methylation can suppress transcription by several mechanisms. First, the presence of the methyl group at a specific CpG possibly block DNA recognition and binding by some transcription factors. Alternatively, other factors may preferentially bind to methylated DNA, blocking transcription factor access and recruit of histone-modifying proteins, such as histone deacetylases (HDAC) (Handy, Castro, and Loscalzo, 2011). The DNA methylation could be inhibited by some chemicals called DNA methyltransferase (DNMT) inhibitors such as 5-azacytidine (Poirier et al., 2014) and 5-aza-2’-deoxycytidine (Zych et al., 2013).

2.5.2 Histone modification

Histone modifications are other key epigenetic processes that control chromatin structure and gene transcription via the modification of function group of histone protein, several forms of histone modifications are known including. acetylation, methylation, phosphorylation, and ubiquitination Among them, histone acetylation is the best-studied and most essential in terms of chromatin re-structure and transcriptional regulation. The deacetylation of histone protein in chromosome processes that the lysine residues within the N-terminal tail at the histone core of the nucleosome become deacetylated to regulate gene expression governed by histone

deacetylase (HDAC) allowing the histones to wrap the DNA more tightly resulting in less gene expression. Histone deacetylation inhibited by some chemicals called histone deacetylase (HDAC) inhibitors such as trichostatin A (Zych et al., 2013), trapoxin B (Lal and Bromberg, 2009) and valproic acid (Chen, Dzitoyeva, and Manev, 2012).

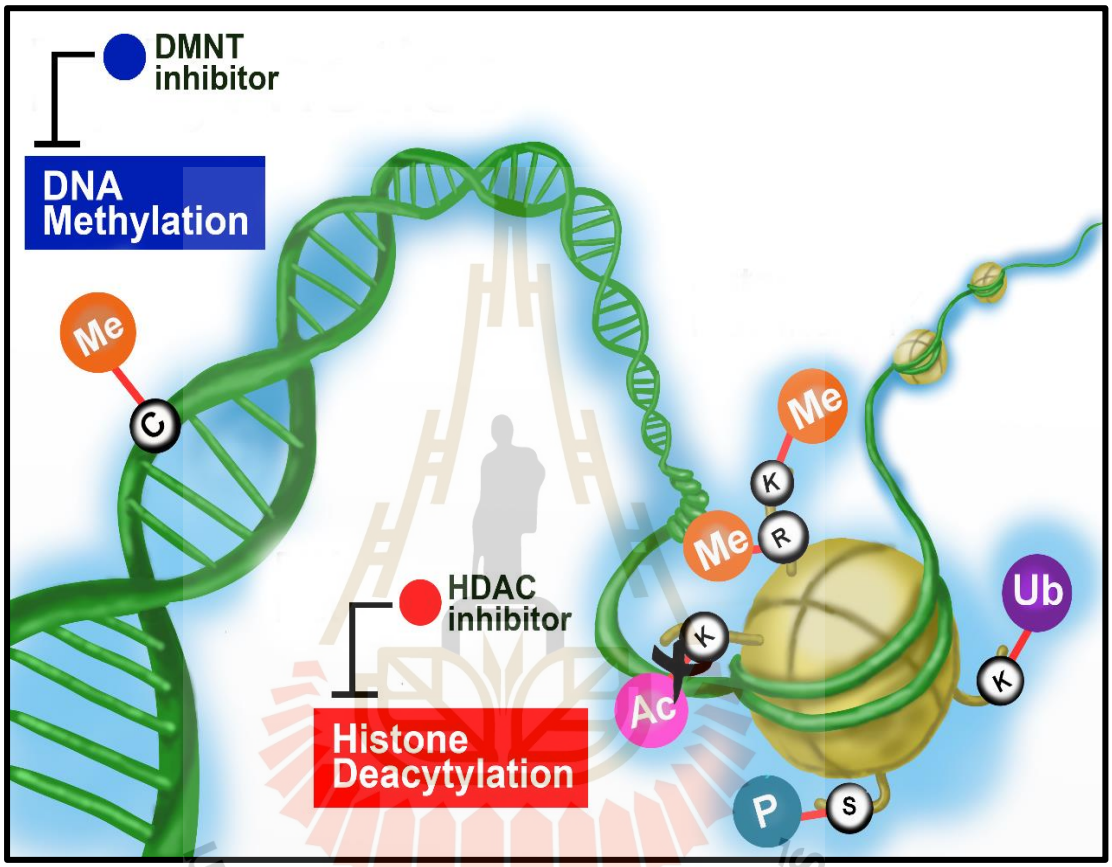

Figure 2.6 Epigenetic modification and inhibitors.

2.5.3 Epigenetic modification applied to microorganism cultures

Recently, the fundamental understanding of epigenetic modifications has been used to the field of fungal culture in order to increase the production of a desirable chemical compound that, in certain cases, is generated by infrequently expressed or inactive secondary metabolic pathways. With DNMT inhibitors and HDAC inhibitors, heterochromatin could be loosed and became euchromatin so the rate of transcription is increased. Therefore, the epigenetic alteration of chromatin from heterochromatin to euchramatin could randomly increase the opportunities to be

expressed for all genes in genome leading to the production of new natural products and/or enhanced accumulation of constitutive secondary metabolites (Williams, Henrikson, Hoover, Lee, and Cichewicz, 2008). Suberoylanilide hydroxamic acid (SAHA) was added to culture media of endophytic fungi isolated from *Datura stramonium* L. and found that fusaric acid derivatives production were enhanced (Chen, Awakawa, Sun, Wakimoto, and Abe, 2013). SAHA was also applied to *Phoma sp*. culture for the induction of (10'S)-verruculide B production (Gubiani et al., 2017). And recently, valproic acid was incorperated to *Aspergillus fumigatus* (GA-L7) culture broth resulting in 10 times higher fumiquinazoline C production compared to those in basal broth (Magotra et al., 2017). Consequently, the knowledge of epigenetic modification could possibly be incorporated in static liquid culture of *C. militaris* to enhance cordycepin production to be used in cosmeceutical industry.

2.6 Natural killer cell

Natural killer cell (NK cell) is a kind of cytotoxic lymphocyte that plays an important function in the innate immune system as a protective effector cell against cancer/tumor and microbial infection without the need for prior vaccination. (Abel, Yang, Thakar, and Malarkannan, 2018). NK cells are capable of targeting and destroying malignant cells using cytolytic and cytokine-producing abilities (R. Wang, Jaw, Stutzman, Zou, and Sun, 2012). To induce cancer cell death, activated NK cells produce cytolytic granules containing perforin, a pore-forming glycoprotein, and granzyme, a group of serine proteases (Pardo, Balkow, Anel, and Simon, 2002) Under the influence of immunoregulatory interleukin, NK cells can additionally release cytokines such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) to further control adaptive immunity (Vivier, Tomasello, Baratin, Walzer, and Ugolini, 2008; R. Wang et al., 2012). Human NK cell maturation and functional phases can be recognized and discriminated by changes in the relative expression of particular surface markers under certain conditions (Abel et al., 2018). The transition between two functionally separate subpopulations was discovered to be predicated on the level of CD56, a neural cell adhesion molecule, and CD16, a low affinity Fc receptor for IgG, on the surface of NK cells $CD56^{bright}CD16^{dim}$/NK cell has been shown to be a potent cytokine generator, whilst $CD56^{dim}CD^{16+}$ has been shown to directly engage with cancer

cells and alter cytolytic activity via both antibody-dependent cell cytotoxicity (ADCC) and NKG2D-dependent cell cytotoxicity (NKDCC) (Jaime et al., 2017; Poli et al., 2009). The density of CD27 and CD11b were also found to further functionally separate the NK subsets and utilized to suggest NK cell insufficiency in humans, similar to mouse NK cells (Q. F. Zhang et al., 2017). Some cases of NK cell functional changes and limitations have been observed. Despite an increase in the total NK cell population with age due to the consolidation of long-standing NK cells, CD56bright NK cell population was considerably reduced in over 50-year-old seniors, indicating poorer cytokine generating efficacy and lytic potential (Chidrawar, Khan, Chan, Nayak, and Moss, 2006; Gounder et al., 2018) NKG2D downregulation was discovered in cancer patients as a result of TGF-1 production by cancer cells, which resulted in cancer immune evasion (J. C. Lee, Lee, Kim, and Heo, 2004; Saito, Osaki, and Ikeguchi, 2012). As a result, investigating factors that improve or enhance NK function may give information on cancer therapy.

2.7 Skin and its major cell types regarding cosmeceutical approaches

The skin, the biggest organ in the human body, has a surface area of around 2 m^2 and is considered the body's first line of defense against the environment, protecting it from toxins, radiation, microbial infection, and mechanical force. Skin also plays a crucial part in maintaining the water and electrolyte balance. (Hwa, Bauer, and Cohen, 2011; Menon, 2015). Skin consists of 3 main layer, epidermis, dermis and hypodermis respectively to the depth from skin surface. In a cosmeceutical approach, the epidermis and dermis layers of the skin are nourished. Keratinocytes and dermal fibroblasts are hence cell models for cosmeceutical research. Keratinocytes, the most prevalent neuroendocrine cells in the skin, are the major component of the epidermal tissue (more than 95%). Only those keratinocytes located at the basal layer of the epidermis are able to maintain a stem cell-like property to supporting a continuous cell division and cell turnover. The main role of keratinocytes is keratin producing to preserve skin against microbial, viral, fungal and parasitic invasion and to protect against UV radiation and to minimize heat, solute and water loss. Due to their important roles in skin biology, keratinocytes have served as a potent model for the assessment of potential toxicities of various chemicals used in cosmetics.

Human dermal fibroblasts. Human dermal fibroblasts (HDF) are major cell components with the dermis layer of skin which appear as spindle-shaped cells with an oval flat nucleus in cell 2D culture. Human dermal fibroblasts are responsible for producing the extracellular matrix and forming the connective tissue of the skin, and play a crucial role during wound healing (Stunova and Vistejnova, 2018).

2.8 Skin regeneration and the involvement of autophagy

Since skin serves vital roles in a number of life-sustaining activities, including hydration, protection against toxins and viruses, and regulation of body temperature, serious skin injury might be potentially fatal (Kamolz, Griffith, Finnerty, and Kasper, 2015). When skin is injured and wounded, cutaneous wound healing plays a significant role in skin regeneration, primarily through the dynamic sequences of synergistic interaction between diverse cell types located within both the epidermis and dermis layers such as keratinocyte, dermal fibroblast, immune cells and thrombocyte. Inflammation, blood clotting, cellular proliferation, and extracellular matrix (ECM) remodeling are the processes that are involved in wound healing, which is a process that has been passed down from species to species throughout evolution (Takeo, Lee, and Ito, 2015). Among all cell types, the relationship between keratinocytes and fibroblasts is the most important factor in such healing processes. Despite being separated by basement membrane and having limited direct cell-to-cell contact, keratinocyte–fibroblast interaction is predominantly mediated through secretable factors to govern cell proliferation and extracellular matrix (ECM) remodeling (T. Sato, Kirimura, and Mori, 1997). Autophagy, a lysosome-dependent recycling system, plays a crucial role in wound healing, particularly in dermal fibroblasts. In general, autophagy plays a function in the aging process and skin homeostasis in response to damaging environmental stimuli in order to repair cellular machinery (Jeong, Qomaladewi, Lee, Park, and Cho, 2020). Autophagy is also triggered during the proliferative and remodelling stages of dermal fibroblasts, where it aids in the prevention of oxidative stress, the promotion of survival, and the maintenance of normal activities. (Ren, Zhao, Zhang, Huang, and Wang, 2022) Autophagy has also been linked to the regulation of extracellular matrix (ECM) and matrix metalloproteinase (MMP) processes. The major autophagic marker, microtubule-associated protein 1 light chain 3 (LC3), was found to

be increased in vivo and substantially accumulated around the wound margin during the healing processes of rat models (Asai, Yamamoto, Ueda, and Waguri, 2018). Through the production of matrix metalloproteinases 1 (MMP-1) and matrix metalloproteinases 3, it was discovered that defective fibroblast autophagy induces the breakdown of extracellular matrix components (MMP-3)) (Ren et al., 2022; Y. Wang et al., 2019)

2.9 Skin cell secretome with special reference to keratinocyte secretome

Secretome is described as the global study of proteins or even represented the proteins that are secreted by cells, tissues or organisms at any given time or under certain conditions. The secretome extracts includes a wide range of important class of proteins that might be able to control and regulate biological and physiological processes of surrounding cells, tissues or organisms. The secretome of humans account for 13–20% of the entire proteome and include growth factors, chemokines, cytokines, adhesion molecules, proteases and shed receptors. There were many studies reporting that the cell-cell interaction between skin cells via secreted proteins could promote the wellness of skin. For example, proIL- 1α released from keratinocytes was reported to induce cyclooxygenase 2 (COX- 2) expression and prostaglandin E2 resulting in the promotion of wound healing (T. Sato et al., 1997) Collagen type I and III precursors were reported to secreted by dermal fibroblasts to enriched the network of extracellular matrix within dermis layer of skin (Hathout, 2007) which provides the decent foundation for epidermis skin layer. Fibroblast growth factors (FGF) was also secreted by dermal fibroblast to acts as the mitogen for keratinocytes and dermal fibroblasts themselves (Gron, Stoltze, Andersson, and Dabelsteen, 2002). Thus, to use secretome extracts as active ingredient in cosmeceutical products, the secretome extracts from skin cells such as dermal fibroblasts and keratinocytes is reasonable candidate. Keratinocytes were also discovered to control the expression of dermal fibroblast genes via soluble substances generated by keratinocytes and dissolved in its secretome (Bukowska et al., 2018; Ghaffari, Kilani, and Ghahary, 2009; Ghaffari et al., 2006; T. Sato et al., 1997). Therefore, the keratinocyte secretome is mainly focused in this study. The study of the effects of keratinocyte secretome and its containing factors on dermal fibroblasts should be

beneficial for the development of novel therapies to maintain skin integrity. Due to their short lifespan and limited working passageways, the utilization of primary keratinocytes as a source of secretome might be a burden in both research and large-scale manufacturing. HaCaT, the spontaneously immortalized human keratinocytes, are a well-known alternative to primary keratinocytes in skin research (Choi and Lee, 2015). They are nontumorigenic monoclonal cell lines that not only have the ability to continue proliferating, but also have almost identical genotype and phenotype to primary keratinocytes, including contact inhibition and anchorage-dependent growth in cell culture, as well as the ability to form well-constructed epidermis in mouse model transplantation and the ability to produce keratinocyte-derived soluble factors/cytokines (Breitkreutz et al., 1998; Maas-Szabowski, Starker, and Fusenig, 2003). Using HaCaT also alleviates some of the concerns associated with using primary keratinocytes, such as donor-to-donor variability in growth characteristics and in vitro responses, varying plating efficiencies, a short lifetime in culture, and changes in proliferation and differentiation characteristics with increasing number of passages, which may complicate experimental data interpretation (Colombo et al., 2017). .

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CHAPTER 3
ENHANCEMENT OF CORDYCEPIN PRODUCTION FROM CORDYCEPS CULTURE BY EPIGENETIC MODIFICATION

3.1 Abstract

Cordycepin (3'-deoxyadenosine), a nucleoside analogue, is biosynthesised by *Cordyceps militaris*, an entomopathogenic fungus. In this study, epigenetic modification was applied to static liquid cultures to enhance cordycepin production. *C. militaris* was cultured in a static liquid culture, and valproic acid, a histone deacetylase (HDAC) inhibitor, was supplemented to modify the epigenetic status. Gene regulatory network was also explored to understand the molecular mechanisms underlying cordycepin production. Fifty micromolar valproic acid could enhance cordycepin production by 41.187% via upregulation of 5'-nucleotidase, adenylate kinase, phosphorybosyltransferase, Cns1, Cns2, Cns3 and Cns4 of *C. militaris* for up to 2 days after valproic acid treatment. The maximum production of cordycepin obtained was 2,835.32 ± 34.35 mg/L in 400 mL working volume. A scaled-up culture was established with a working volume of 10 L, which led to the decrease of cordycepin production. This might due to an uneven dispersion of valproic acid in the culture system, which was needed further optimization. The modification of epigenetic status by valproic acid could enhance cordycepin production by altering gene regulatory network of *C. militaris*. The strategy used in this study might be applicable to other static liquid culture systems to improve the production of bioactive compounds.

Keyword: *Cordyceps militaris*/Cordycepin/Static liquid culture/Epigenetic modification

3.2 Introduction

Cordyceps spp. is entomopathogenic fungus that mainly occurs in the Northern Hemisphere, including China, and has been used as a nutraceutical in traditional Chinese medicine (Fan et al., 2012; Guo et al., 2016). The species *C. militaris* can perform sexual reproduction on artificial media without a mating partner, although it is sexually heterothallic (Zheng et al., 2011), making it a versatile species for commercial and industrial applications. It produces and accumulates various bioactive compounds including cordycepin, mannitol (Guo et al., 2016), polysaccharides (Wu et al., 2014), sterols (Yang et al., 2009) and macrolides (Rukachaisirikul et al., 2004), of which cordycepin is a unique and potent bioactive compounds with a wide array of bioactivities.

Cordycepin, or 3'-deoxyadenosine, is a nucleoside analogue and can influence and interfere with various molecular and cellular processes, especially in cancer cells. For example, it can target purine biosynthesis, mammalian target of rapamycin (mTOR) signalling, apoptosis, metastasis (Nakamura et al., 2015), platelet aggregation (Cho et al., 2006), immunoregulatory (Zhou et al., 2008) and inflammation pathways (Li et al., 2016; Shin et al., 2009), making it a potent anti-cancer agent. Besides being a potent therapeutic agent, cordycepin was also reported to exhibit anti-photoaging (Lee et al., 2009) and anti-pigmentation (Jin et al., 2012). To use cordycepin as an anti-cancer agent in patients or as an active ingredient in healthcare products, large amounts of this substance are needed. Therefore, *C. militaris* cultivation for cordycepin enrichment could be a potential method to biologically synthesise sufficient amounts of this compound.

Static liquid culture is a simple yet effective way to produce cordycepin. It is considered an appropriate procedure to be used in industrial purposes because of the production of large amounts of cordycepin in a short period (Das et al., 2008; Wen et al., 2017). Cultivation can be performed at a mostly 25 °C without the need for specific equipment, enabling industrial-scale optimisation. The strategies for the enhancement of cordycepin production based on static liquid culture are centred around the optimisation of medium composition (Kang et al., 2014; Wen et al., 2017) and culture condition (Hung et al., 2009; Jiapeng et al., 2014) as well as strain improvement (Das et al., 2008).

Recently, epigenetic modifications have been applied in the area of fungus cultivation to enhance the production of chemical compounds. Epigenetic modifications are the heritable alterations due to reversible changes in chromatin structure between euchromatin, a lightly packed form, and heterochromatin, a tightly packed form. With epigenetic modifiers, including DNA methyltransferase (DNMT) inhibitors and histone deacetylase (HDAC) inhibitors, heterochromatin could be loosened and can become euchromatin, increasing the rate of the transcription of genes in the genome. Chen *et al*. (2013) added suberoylanilide hydroxamic acid (SAHA; HDAC inhibitor) to culture media of endophytic fungi isolated from *Datura stramonium* L. and found an enhanced fusaric acid derivate production (Chen et al., 2013). Recently, Magotra et al. (2017) incorporated valproic acid (VPA; HDAC inhibitor) to an *Aspergillus fumigatus* (GA- L7) culture broth, resulting in 10 times higher fumiquinazoline C production (Magotra et al., 2017). The co-cultivation of *Cochliobolus lunatus* with 5-azacytidine (5AZA; DMNT inhibitor) harnessed its metabolic pathways to yield the new types of α- pyrone metabolites differing its naturally produced resorcylic acid lactones (Wu et al., 2019). Consequently, a deeper knowledge of epigenetic modification could possibly be incorporated in the static liquid culture of *C. militaris* to enhance cordycepin production.

In this study, the effects of epigenetic modifiers were explored, with the aim to enhance the cordycepin production by a static liquid culture of *C. militaris* at different scales. Besides, the expression of cordycepin production-related genes was also investigated.

3.3 Materials and methods

3.3.1 Microorganisms and seed culture

Cordyceps militaris (strain SIP2) used in this study was obtained from Science Innovative Products Limited Company, Nakhon Ratchasima, Thailand, and stored on potato dextrose agar (PDA) slant at 4°C. To be prepared for the experiment, the mycelia were transferred to new PDA slants and cultured at 25°C for 7 days for microorganism reactivation and then to PDA plates for further culturing at 25°C for 14 days. Subsequently, 5 mL of sterilised deionised water was added into the plates and spread all over the mycelia, using a sterilised spreader, to squeeze out the spores.

The spore solution was collected and filtered through sterilised filter paper no. 1 (Whatman, Kent, UK). The liquid filtrate containing 1.5 x 10^7 spores (counted using Thoma's haematocytometer) was added to seed culture medium modified from Kang et al. (2014) (24.7 g/L of sucrose; 20 g/L of peptone; 1.11 g/L of KH_2PO_4; 0.9 g/L of MgSO4·7H_2O and 10 mg/L of vitamin B1; the pH was not adjusted). Each seed culture was prepared in a 250-mL Erlenmayer flask (DURAN, Mitterteich, Germany) containing 100 mL of seed culture medium. Seed cultures were incubated in a rotary shaker (Innova 43, Eppendorf, New York, USA) at 150 rpm, 25°C, for 5 days.

3.3.2 Detection and verification of authentic *Cordyceps militaris*

The *Cordyceps militaris* mycelia from the stock culture were collected. Genomic DNA was extracted from mycelia using DNA GF-1 Nucleic Acid Extraction Kit (Vivantis, Darul Ehsan, Malaysia) according to the manufacturer's protocol. Then, specie-specific sequence characterized amplified region (SCAR) markers particularly found in *C. militaris* were detected by PCR technique in a BioRad/C1000 Touch Thermocycle (Bio-Rad Laboratories, California, USA) using specific primers (Table 3.1) and 2x Taq Master Mix (Vivantis Technologies, Selangor, Malaysia). The amplification conditions were as follows: initial denaturation at 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 63 °C for 30 s, and 72 °C for 30 s, and a final extension step at 72 °C for 5 min. PCR products were resolved by agarose gel electrophoresis and visualized under UV after staining with RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology, Gyeonggi-do, Korea). The specific size of DNA fragments at 339 bp and 102 bp was determined with 100 bp DNA ladder (Bio-Rad Laboratories, California, USA).

Table 3.1 Specific primers tageting specie-specific sequence characterized amplified region (SCAR) markers used for the detection and verification of authentic *Cordyceps militaris* (Moon et al., 2018).

Marker	Primers	Sequence (5'-3')
C. militaris-specific Sequence Characterized Amplified Region Marker 1	CM SCAR 1 Forward	5'- GGCCCCAAACAGTGTATCTAC -3'
	CM SCAR 1 Reverse	5'- CCGGTGCGAGTTGGCGTACTA -3'
C. militaris-specific Sequence Characterized Amplified Region Marker 2	CM SCAR 2 Forward	5'- CAACCCTTTGTGAACATACCT -3'
	CM SCAR 2 Reverse	5'- GTAGATACACTGTTTGGGGCC -3'

3.3.3 Static liquid culture and epigenetic modification strategy

In order to produce cordycepin from artificial *C. militaris* culture, static liquid culture was frequently preferable over submerged culture (Kang et al., 2014; Wen et al., 2017). A submerged culture provides better aeration and nutrient circulation so such culture system is more favourable for the fungi to produce primary metabolites, focus more on growth and biomass production and lead to the rapid nutrient depletion (Shih et al., 2007). However, the simultaneously limited conditions occurred within static liquid culture provide a decent environment for fungi to gradually grow, enter its stationary growth phase and then better produce cordycepin, which is naturally considered as a secondary metabolite(Sari et al., 2016; Suparmin et al., 2017). Therefore, A static liquid culture was chosen to be used in this particular study.

After 5 days of incubation, 100 mL of seed culture was inoculated into a 1,000-mL Erlenmayer flask (DURAN, Mitterteich, Germany) containing 300 mL of basal medium (same formula as seed culture medium), resulting in a working volume of 400 mL. The cultures were incubated at 25°C at static conditions for at least 4 weeks. A 1-mL portion of the medium from each flak was sampled each week for later

cordycepin content determination. The initial number of *C. militaris* spores added into the seed culture was also varied (0.75, 1.5, 2.25, 3.0 x 10^7 spores per seed culture, respectively) to optimise the spore number for later experiments.

Valproic acid (VPA) and 5-azacytidine (5AZA) (Sigma-Aldrich, Missouri, USA), epigenetic modifiers, were added into the culture medium as additives to stimulate epigenetic modification. Every 7 days of culturing, VPA or 5AZA or both were added to culture medium to obtain a final concentration of 25 μM. The epigenetic modifier that yielded the highest cordycepin level, compared to the basal medium, was VPA, whose concentration was further varied at 25, 50, 100 and 200 μM in the culture medium to determine the optimal concentration for cordycepin production. The cultivation period for these experiments was 56 days.

3.3.4 Scale-up of the static liquid culture

Static liquid cultures of *C. militaris* with the optimal VPA concentration were carried out following the same procedure, but the working volume was increased to 10 L in 20-L autoclavable plastic containers (Nalgene™ Autoclavable Carboy, Thermo Fisher Scientific, Massachusetts, USA). The cordycepin production of the scale-up static liquid culture was compared to that of the original working volume of 400 mL (0.4 L).

To expand the working volume of the liquid static culture, the seed cultures were initially prepared from the volume of 100 mL and then scaled up to 625 mL, 1.25 L and 2.5 L, respectively. Additionally, the seed cultures were incubated in a rotary shaker at 150 rpm, 25°C, for 5 days per each scaling-up step.

3.3.5 Measurement of biomass and cordycepin

At the end of culturing, cultures were filtered through pre-weighed filter paper (Qualitative filter paper no. 1, Whatman, Kent, UK) for separating culture broth and mycelia, and subsequently, both surfaced and precipitated mycelia were dried at 60°C for 24 h and weighed using a 4-digit weighing balance (Sartorius, Göttingen, Germany).

For cordycepin measurement, cordycepin (MW, 251.2; product no. C3394) was purchased from Sigma-Aldrich, Missouri, USA, and dissolved in deionised water at various concentrations for standard calibration. Culture media were filtered through cellulose acetate filter paper with a 0.2-μm pore size (Whatman, Kent, UK), and the

cordycepin concentration was determined using high-performance liquid chromatography (HPLC) (Agilent 1200 series equipped with a 1260 DAD Detector, Agilent Technologies, California, USA). A Zorbax C18 column (Agilent Technologies, California, USA) with 4.6 x 150 mm was used for chemical separation. The mobile phase consisted of methanol and deionised water (2/98, v/v); the flow rate was 1.0 mL/min and the column temperature 35°C. The chromatogram was monitored by UV absorbance at 260 nm (Huang et al., 2009).

3.3.6 Histone extraction and global histone H3 acetylation assay

The *Cordyceps militaris* mycelia of 3 different conditions (treated with 0, 25 and 50 µM VPA weekly) grown in liquid static culture were collected at day 28 and 35 days of cultivation. The mycelia were then cut into a small pieces (1-2 $mm^{3)}$ prior histone extraction and histone acetylation detection using an EpiQuik™ Global Histone H3 Acetylation Assay Kit (Epigentek, New York, USA). The histone H3 acetylation level of C. *militaris* culture treated with 25 and 50 VPA weekly was relatively compared with those without VPA to demonstrate the histone deacetylase inhibition activity occurring in culture system that influenced the increase of cordycepin production.

3.3.7 RNA isolation and quantitative RT-PCR (qRT-PCR)

The *Cordyceps militaris* mycelia grown in liquid static culture were collected at day 28 of cultivation since this was at the logarithmic phase of cordycepin production. Total RNA was extracted from mycelia using a Plant/Fungi RNA Purification Kit (Norgen Biotek Corp., Thorold, Canada) according to the manufacturer's protocol. Subsequently, 1 µg of RNA was converted to complementary DNA (cDNA) in a BioRad/C1000 Touch Thermocycle (Bio-Rad Laboratories, California, USA) using a 2-step RT-PCR Kit (Vivantis Technologies Sdn. Bhd, Selangor, Malaysia). Quantitative RT-PCR (qRT-PCR) was carried out in a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, Massachusetts, USA) using SYBR® Green Master Mix (Thermo Fisher Scientific, Massachusetts, USA) with specific primers (Table 3.2). The relative expression level of a target gene was quantified by normalisation with the internal control polyubiquitin-binding protein (*ubi*) gene (Lian et al., 2014).

Tables 3.2 Primer sets used for Quantitative RT-PCR for expression analysis of cordycepin production-related genes (Xia et al., 2017).

Genes	Primers	Sequence (5'-3')
Polyubiquitin binding protein (House keeping gene)	*ubi* Forward	5'-GGTACATGGGCTACGGCTAC-3'
	ubi Reverse	5'-TCCGACACAAACTCGTCCAG-3'
5'-nucleotidase	*nt5e* Forward	5'-TGGACCTCACCATTCTGCAC-3'
	nt5e Reverse	5'-AGCTAGGCCGAAAAATCCCA-3'
Adenylate kinase	*ak* Forward	5'-TCGTATGCTGCTCATCGGAC-3'
	ak Reverse	5'-AGGCGTTAGCCACCAATCAA-3'
Adenine Phosphoribosyltransferase	*aprt* Forward	5'-CAGCCGCCCAAGATGCCTCG-3'
	aprt Reverse	5'-TTGTCGCGGCCGTTGAGACC-3'
Cns1: Protein with Dehydrogenase Oxidoreductase domains	*cns1* Forward	5'-TCACGACCGCCGCACAATCC-3'
	cns1 Reverse	5'-CCAAGCCTGCTGGCACGGAG-3'
Cns2: Protein with Metal-dependent phosphohydrolase domain	*cns2* Forward	5'-GGTATCTCAAGACGACGCGG-3'
	cns2 Reverse	5'-CCAGACATCCTTGTCCGTCG-3'
Cns3: Protein with N-terminal nucleoside/nucleotide kinase and C-terminal ATP phosphoribosyltransferase domains	*cns3* Forward	5'-GGGACTGGCTGTACAAGGTTAG-3'
	cns3 Reverse	5'-AGCGTTCTTGCCCACAGATGCC-3'
Cns4: ATP-binding cassette pentostatin transporter	*cns4* Forward	5'-GTATGACGGCCTTGTTTCGT-3'
	cns4 Reverse	5'-CAGCGAATTCTTCCTTTTCG-3'

3.3.8 Statistical analysis

All experiments were performed for five replicates, and data were expressed as mean ± standard deviation (SD). Significant differences among treatments were determined by one-way analysis of variance (ANOVA), followed by Tukey's test at $P < 0.05$. All statistical analyses were performed using SPSS (version 16.0, SPSS Inc., USA).

3.4 Results

3.4.1 Authenticity verification of used *Cordyceps militaris*

To verify the authenticity of *C. militaris* used in the experiments, the specie-specific sequence characterized amplified region (SCAR) markers previously reported by Moon et al. (2018) were detected. Such SCAR markers located on internal transcribed spacer of the ribosomal DNA (nrDNA-ITS) gene in the fungal nuclear genome. As a result, the SCAR markers with the specific size of 339 bp and 102 bp could be detected and amplified out of our fungal genomic DNA indicating that the fungi used in the experiment were authentically *C. militaris* (Figure 3.1).

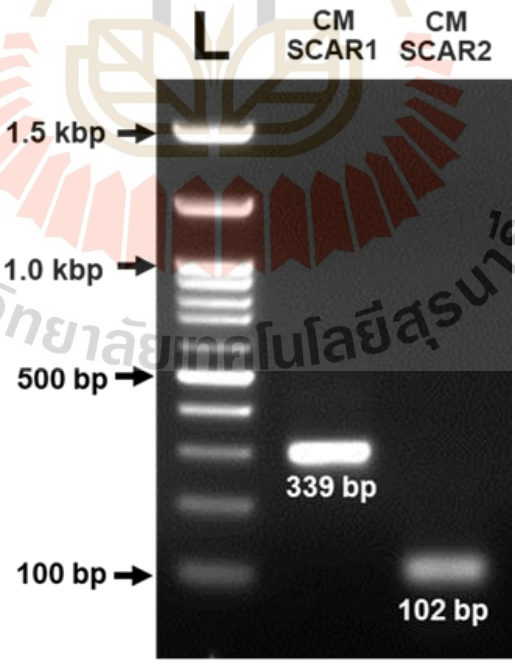

Figure 3.1 Verification of specie-specific sequence characterized amplified region (SCAR) marker located on internal transcribed spacer of the ribosomal DNA (nrDNA-ITS) gene of *C. militaris.*

3.4.2 Spore number optimisation

The seed culture were prepared with 4 different initial spore numbers ($7.5x\ 10^6$, $1.5x\ 10^7$, 2.25×10^7 and 3.0×10^7 spores/ 100 mL inoculum) and inoculated to the culture media to determine the optimized spore number to be used in *C. militaris* culture for cordycepin production. At week 4 of culturing, the inoculation of 1.5×10^7 spores into the seed culture yielded the highest cordycepin production (472.21 ± 3.54 mg/L; Figure 3.2a) hence it was potentially an optimized spore number for seed culture preparation. However, the development and the final biomass at the surface of the culture media of each variable was not significantly different (Figure 3.2b and 3.2c). To further determine the cordycepin production profile, the static liquid cultures with 1.5×10^7 initial spores were continuously cultivated for 2 months. Cordycepin production and accumulation in culture media increased linearly ($y = 19.783x$, $R^2 = 0.9863$; Figure 3.2d).

3.4.3 Enhancement of cordycepin production by epigenetic modification

To investigate the effects of an epigenetic modifier on cordycepin production in a static liquid culture system, 25 μM of VPA or 5AZA or both of them were added to the culture media weekly; both chemicals have a short half-life, ranging from 5 -20 hours. The addition of VPA alone resulted in the highest cordycepin production and accumulation (Figure 3.3a and 3.3b), although the differences were not statistically significant. Subsequently, the concentration of VPA was varied to determine the optimal concentration. The results of the experiment indicated that 50 μM of VPA resulted in a 41.187% enhancement of cordycepin production compared with that in the basal medium. The final cordycepin concentration of *C. militaris* static liquid cultures treated with 50 μM at week 8 of culturing was 2,835.32 ± 34.35 mg/L (Figure 3.4a and 3.4b). Therefore, VPA could be used as an epigenetic modifier to potentially enhance cordycepin production. In addition, the cordycepin production decreased when the cultures were treated with 100 and 200 μM of VPA due to the toxicity of VPA to *C. militaris*, based on the dramatic decrease in biomass (Figure 3.4c and 4d). Therefore, other epigenetic modifiers with less negative effects on *Cordyceps* need to be identified and verified to obtain more effective static liquid culture procedures.

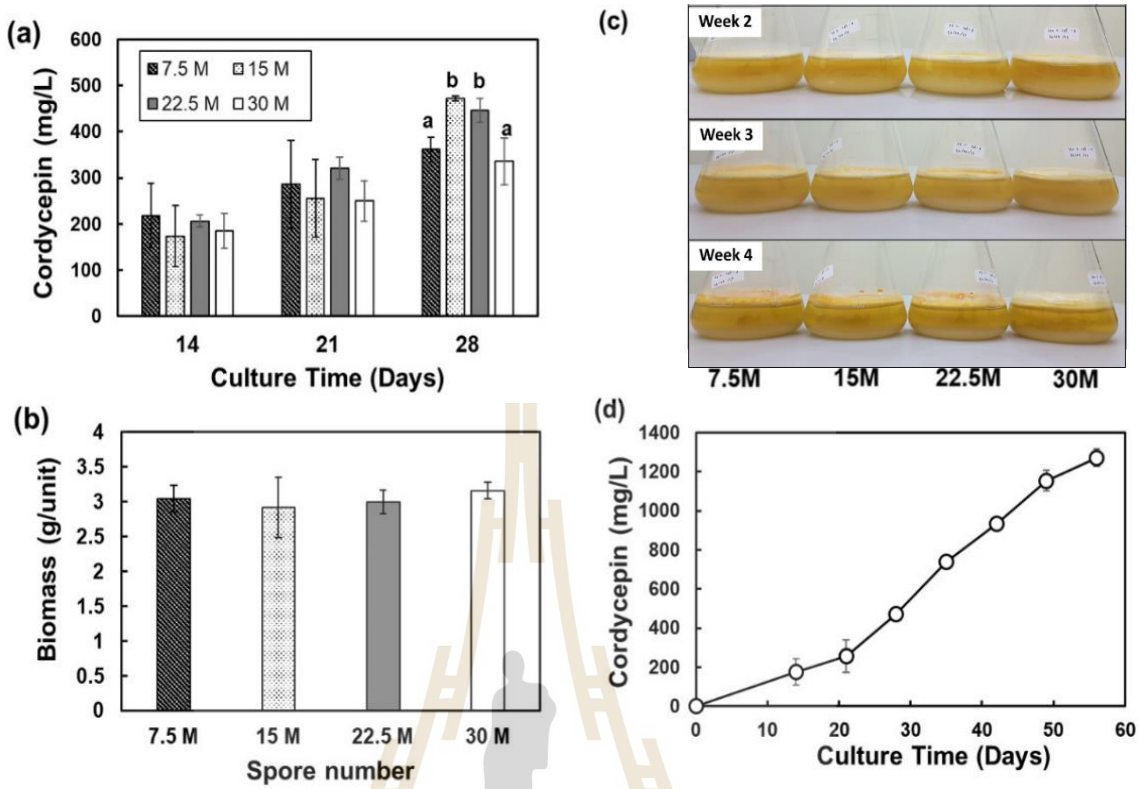

Figure 3.2 The effect of initial spore number of *C. militaris* on static liquid culture. (a) The initial spore number of 1.5 x10^7 spores used in the seed culture preparation yielded the highest cordycepin production. (b-c) The varied initial spore number did not affect biomass production due to the confinement of surface area. (d) Time course of cordycepin production by static liquid culture using 1. 5x10^7 spores for seed culture preparation appeared to be linear up to 8 weeks of cultivation.

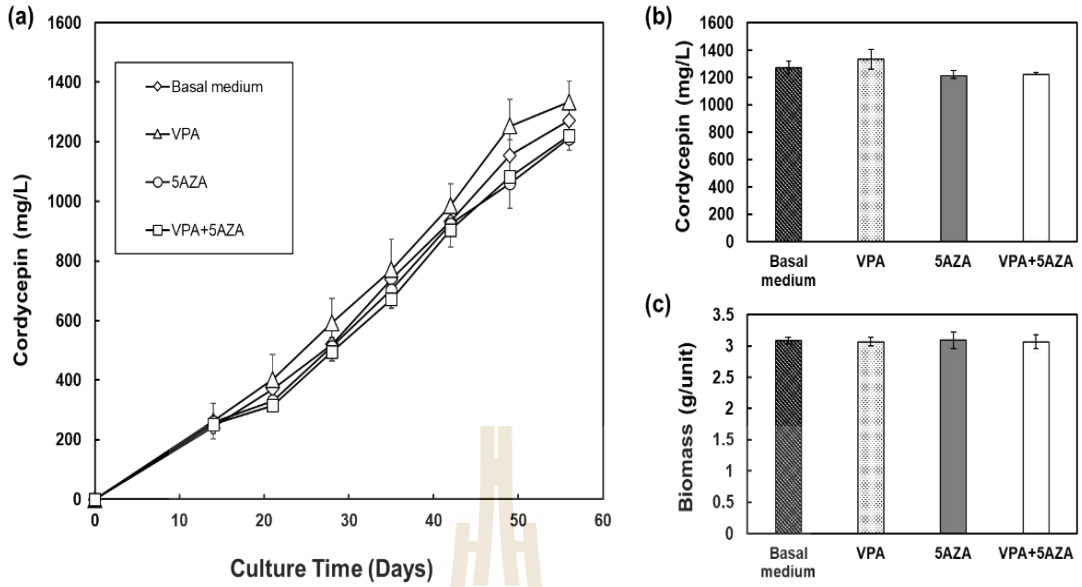

Figure 3.3 The effect of epigenetic modifiers on cordycepin production. (a) VPA weekly treatment resulted in the noticeably enhancement of cordycepin production starting from week 3 of *C. militaris* cultivation (b) VPA weekly treatment yielded the highest cordycepin production week 7 of culturing. (c) The treatment of different epigenetic modifiers did not affect biomass production due to the confinement of surface area.

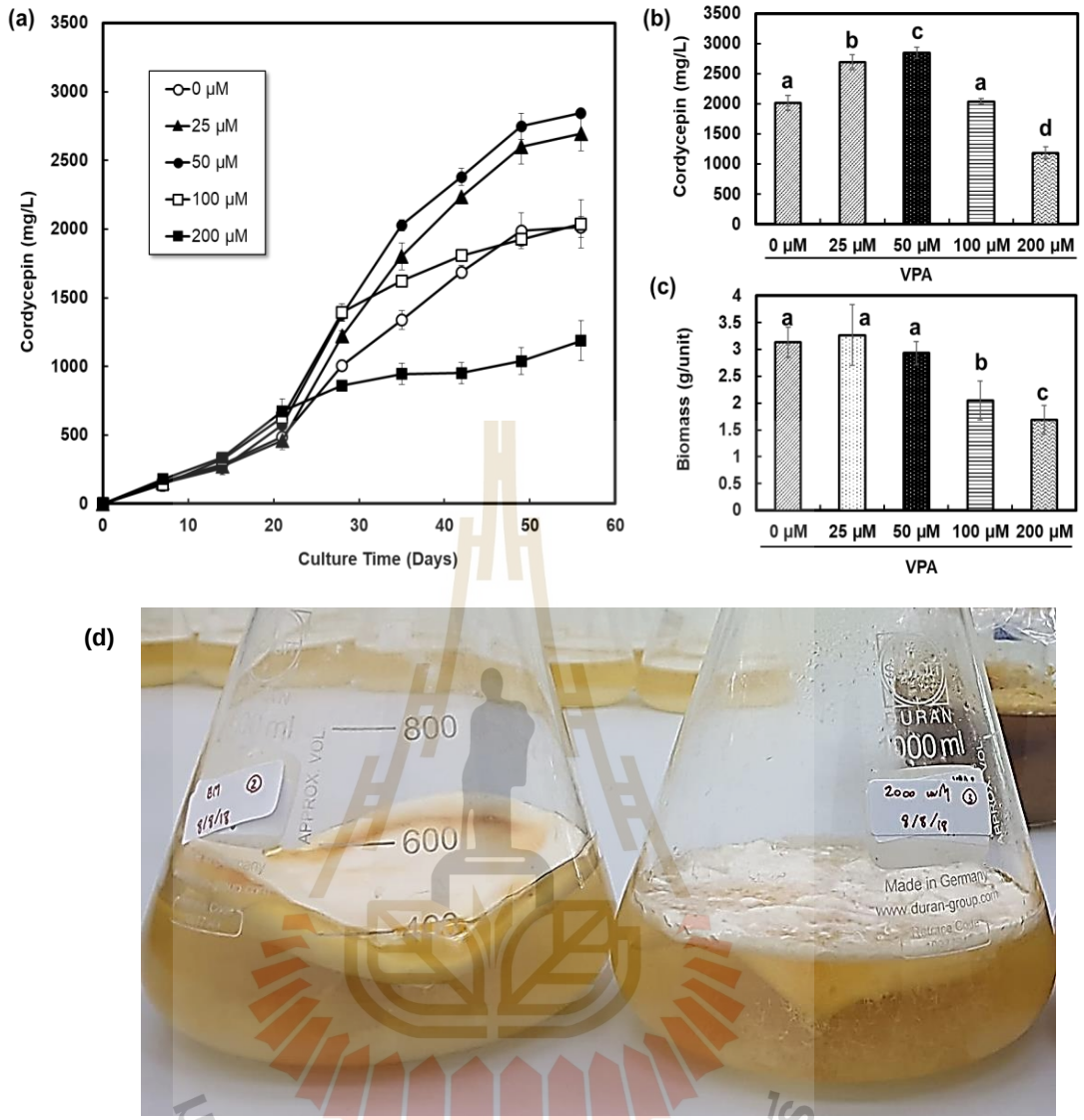

Figure 3.4 The effect of VPA concentration on cordycepin production a) 50 µM VPA weekly treatment drastically enhanced cordycepin production especially from week 5 of cultivation. b) 50 µM VPA weekly treatment yielded the highest cordycepin production at week 7 of culturing (2,835.32 ±34.35 mg/L / 41.187 % enhancement) **c**)100 µM and 200 µM VPA treatment possessed lethal effect over *C. militaris* which caused the decrease of biomass and cordycepin production. d) Lethal toxicity of valproic acid at high concentration (left: Control group without VPA, right: Treatment group with 200 µM VPA).

3.4.4 Scale-up of the static liquid culture affected cordycepin production

Cultures of *C. militaris* were prepared at the working volumes of 400 mL and 10 L and supplied with 50 μM of VPA to compare cordycepin production efficiencies with those of cultures without VPA of the same respective working volume. As shown in Figure 3.5a and 3.5b, VPA supplement increased the cordycepin production in both working volumes (2,027.849 ± 41.21 mg/L and 1,527.90 ± 59.35 mg/L, with a 51.77 and 86.12% enhancement of cordycepin production in the cultures with 400 mL and 10 mL of working volume, respectively). However, the cordycepin production gradually decreased with increasing working volume. The scale-up of the static liquid culture caused a 72% decrease in cordycepin production in the VPA-supplied culture at week 5 of cultivation.

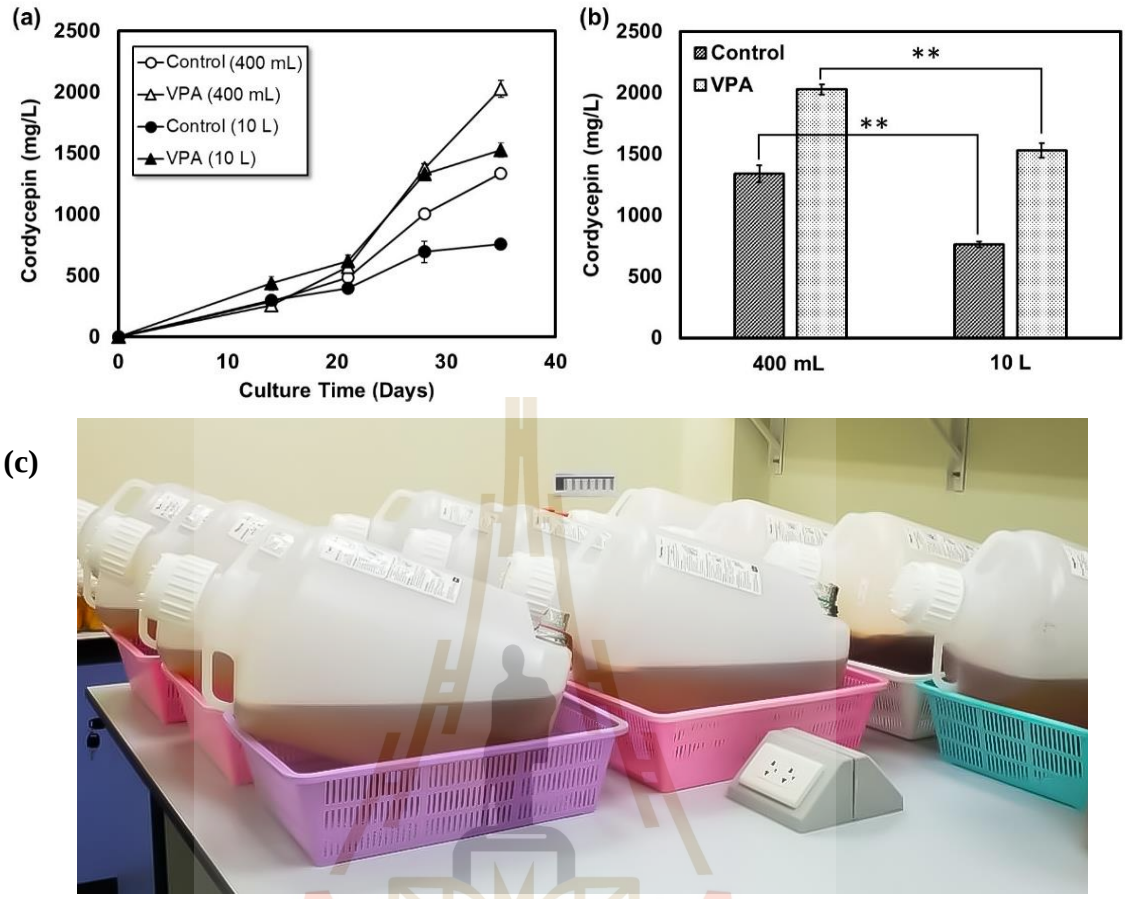

Figure 3.5 The effect of scale-up working volume on cordycepin production (a) The increase of *C. militaris* culture working volume to be 10 L relatively burdened cordycepin production in both VPA treated and non- treated culture possibly caused by the alteration of dissolved oxygen level and nutrient circulation. b) VPA could still be proved to enhance the cordycepin production (1527.90± 59.35/ 86.12% enhancement) even with the negative effect of scale-up culture. c) The setup of 10 L cultivation container.

3.4.5 Epigenetic modification subsequently affected the expression of cordycepin production-related genes

In order to monitor and relatively compare the histone acetylation status of *C. militaris* in each VPA-treated condition (0, 25 and 50 μM VPA weekly), the global histone acetylation H3 was measured using ELISA-based Global Histone H3 Acetylation Assay Kit at day 28 and 35 of culturing. The relative global histone H3 acetylation was found to be drastically elevated in the VPA-treated cultures in the dose-dependent manner at both time point of culturing (Figure 3.6b). The expression of genes encoding the seven enzymes including 5'-nucleotidase, adenylate kinase, adenine phosphoribosyltransferase, adenine phosphoribosyltransferase, Cns1 protein with dehydrogenase oxidoreductase domains, Cns2 protein with metal-dependent phosphohydrolase domain, Cns3 protein with N-terminal nucleoside/nucleotide kinase and C-terminal ATP phosphoribosyltransferase domains and Cns4 ATP-binding cassette pentostatin transporter was also investigated using quantitative RT-PCR to observe the consequences of the epigenetic modification . As shown in Figure 6a, the expression of all genes was upregulated for at least 2 days after treatment with VPA compared to the culture without VPA, which finally resulted in the enhancement of cordycepin production.

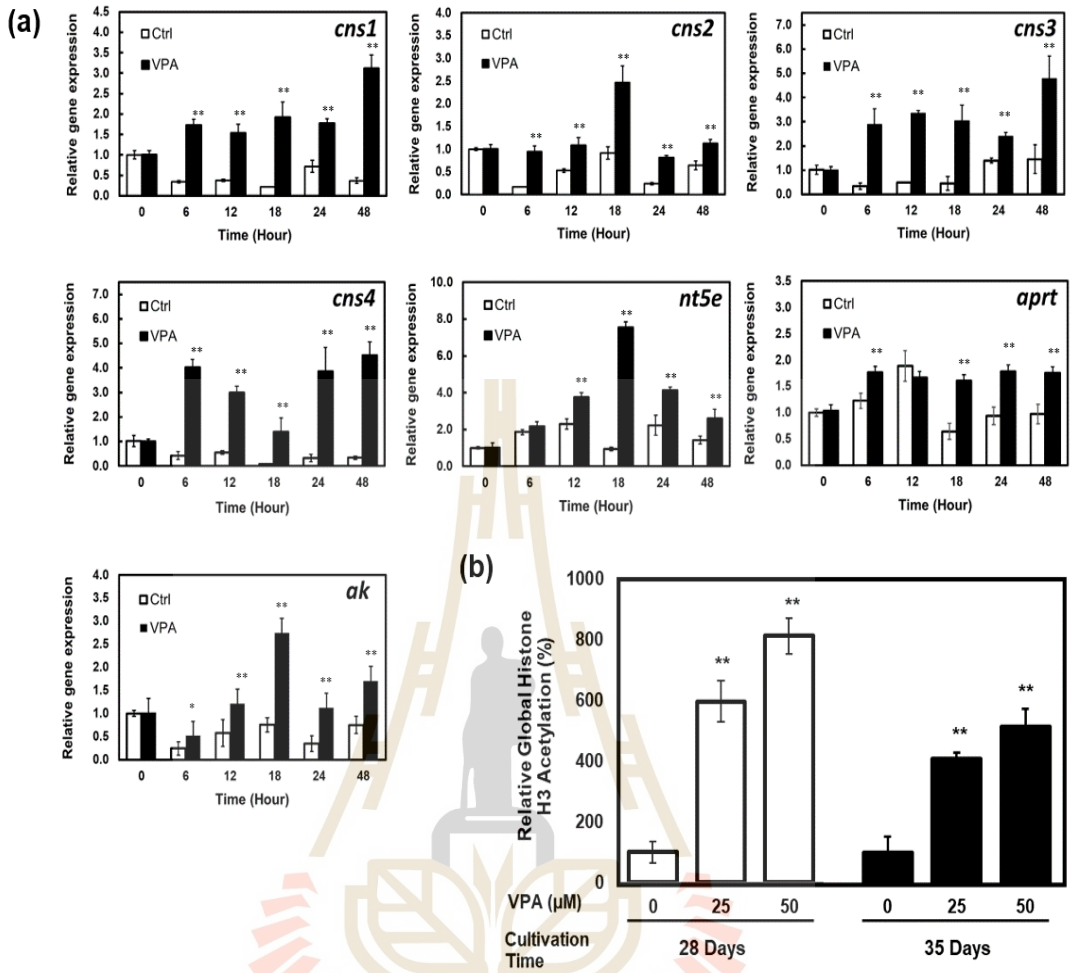

Figure 3.6 The effects of VPA treatment at Transcriptional and epigenetic levels during *Cordyceps militates* cultivation (a) Comparison of relative expression of cordycepin production related genes demonstrated the up-regulation of such genes in 50 μM VPA-treated condition which lasted up to 2 days after the treatment and possibly lead to the enhancement of cordycepin production. (b) The effect of VPA on Cordyceps global histone H3 acetylation activity. Histone H3 acetylation was increased by VPA treatment in a dose-dependent manner at 28 and 35 days of cultivation.

3.5 Discussion

To maintain the consistency of seed cultures and to optimise spore number added to the seed cultures, which might affect cordycepin production, the initial number of *C. militaris* was varied. In the first three weeks of culturing, the cordycepin production with four different spore numbers did not differ significantly, most likely because *C. militaris* used the metabolic flux mainly in the production of primary metabolites to increase its biomass (Suparmin et al., 2017). The concentration of cordycepin accumulated in the culture media was significantly different in the fourth week of culturing, indicating that metabolite production was altered (Oh et al., 2019). Based on this experiment, inoculation of 1.5×10^7 spores into the seed culture is optimal because of the high cordycepin production at week 4 of culturing (472.21 ± 3.54 mg/L; Figure 3.1a). Considering biomass production, the development of biomass at the surface of the culture media of each variable was not noticeably different (Figure 3.1c), and the final biomass weight was not significantly different (Figure 3.1b), most likely because of the limited surface area. This phenomenon has previously been mentioned by Kang et al. (2014). Therefore, cordycepin production was not directly dependent on biomass production.

To further assess the profile of cordycepin production, static liquid cultures with 1.5×10^7 starting spores were continually grown for two months. Cordycepin production and accumulation in culture media increased linearly ($y = 19.783x$, $R^2 = 0.9863$; Fig. 1d), in contrast to previous studies in which the cordycepin concentration became constant after 15–30 days of culturing (Das et al., 2010; Masuda et al., 2007; Sari et al., 2016). This might be due to the differences in initial spore number among studies. High initial spore numbers could lead to rapid growth, with enhanced cordycepin production and carbon source depletion in a shorter time, which affects the cordycepin production profile.

In order to investigate the effects of an epigenetic modifiers on cordycepin production in a static liquid culture system, 25 M of VPA or 5AZA or both of these chemicals were applied to the culture media on a weekly basis since both of these chemicals have a relatively short half-life, ranging from 5 to 20 hours. The maximum cordycepin production and accumulation occurred as a direct consequence of the addition of VPA alone. (Figure 3.2a and 3.2b), although the differences were not

statistically significant. Subsequently, the concentration of VPA was varied to determine the optimal concentration. The results of the experiment indicated that 50 μM of VPA resulted in a 41.187% enhancement of cordycepin production compared with that in the basal medium. The final cordycepin concentration of *C. militaris* static liquid cultures treated with 50 μM at week 8 of culturing was 2,835.32 ± 34.35 mg/L (Fig. 3a, 3b). Therefore, VPA could be used as an epigenetic modifier to potentially enhance cordycepin production.

Based on previous reports, there is no information about the use of DNA methyltransferase (DNMT) inhibitors to enhance biological compound production. So far, all studies incorporated only histone deacetylase (HDAC) inhibitors (Chen et al., 2013; Gubiani et al., 2017; Magotra et al., 2017). Although the underlying mechanisms are still unclear, HDAC governs the expression of genes involved in both chromatin structure alteration and DNA-transcription factor affinity aspects, whereas DMNT mainly influences the accessibility of transcription components to DNA, with a minor effect on DNA-histone binding (El-Osta and Wolffe, 2000). Therefore, the inhibition of HDAC leads to the drastic changes in gene expression superior to the inhibition of DMNT.

Suberoylanilide hydroxamic acid (SAHA) could to be the potential alternative of valproic acid and even was stated to function at the relatively low concentration in mammalian cell studies (Zhu et al., 2016). However, there was a study reporting some concerns about the HDAC inhibition property of SAHA. In primary human cell platform, SAHA-treated cells were found to possess an extensive histone deacetylation instead of expected histone acetylation while the level of elevated and reduced histone acetylation in VPA-treated cells was balanced (Lunke et al., 2021). Valproic acid has also been found to maintain the loosely packed chromatin structure that last longer due to its minor effect on DNA methylation (Lunke et al., 2021; Milutinovic et al., 2007). Additionally, using VPA in the microbial culture is considered to be more cost-effective due to its 20–100 time cheaper price depending on the quality.

Cultures of *C. militaris* were prepared at the working volumes of 500 mL and 10 L and supplied with 50 μM of VPA to compare cordycepin production efficiencies with those of cultures without VPA of the same respective working volume. As shown in Figure 3.4a and 3.4b, VPA supplement increased the cordycepin production in both working volumes (2,027.849 ± 41.21 mg/L and 1,527.90 ± 59.35 mg/L, with a 51.77

and 86.12% enhancement of cordycepin production in the cultures with 400 mL and 10 mL of working volume, respectively). However, the cordycepin production gradually decreased with increasing working volume. The scale-up of the static liquid culture caused a 72% decrease in cordycepin production in the VPA-supplied culture at week 5 of cultivation. The increase in the working volume increased the depth of the culture medium, which might have resulted in an uneven dispersion of VPA in the static liquid culture system since no agitation was applied. Therefore, *C. militaris* filaments might not have been exposed to VPA evenly, resulting in a lower cordycepin production. Moreover, an increased depth of the culture medium affects the dissolved oxygen (DO) concentration, mainly at the deeper levels. Dissolved oxygen concentration is a key factor for not only for cell growth but also for metabolite biosynthesis (Mao and Zhong, 2006). Therefore, our results suggest that medium depth in the bioreactor is a critical factor for large-scale cordycepin production by a static liquid culture.

Valproic acid (VPA) is widely reported to inhibit class I and II histone deacetylases (HDACs) which led to the hyperacetylation of histones, especially at the lysine residues of histone H3 (Hezroni et al., 2011). The higher level of lysine acetylation of histones neutralizes their positively-charged tails and lessen the interaction between them and the natural negatively-charged DNA resulting in the loosely packed chromatin (Marks, 2010). Therefore, transcription promoters are more accessible and the expression of various genes are upregulated (Mello, 2021). In order to monitor and relatively compare the histone acetylation status of *C. militaris* in each VPA-treated condition (0, 25 and 50 μM VPA weekly), the global histone acetylation H3 was measured using ELISA-based Global Histone H3 Acetylation Assay Kit at day 28 and 35 of culturing. The relative global histone H3 acetylation was found to be drastically elevated in the VPA-treated cultures in the dose-dependent manner at both time point of culturing (Figure 3.4b). However, the elevation of acetylated histone level was found to be fluctuated and slightly reduced at the latter time point. Since epigenetic modification always occurs responsively to the both intrinsic and extrinsic factors beyond genetic influence, it is reasonable that histones of *C. militaris* in VPA-free condition were also simultaneously acetylated in such stationary phase of growth to alternate the metabolic pathway and produce more secondary metabolites. Considering the reversible nature of histone acetylation/deacetylation processes, the acetylated

histone level may be saturated at day 28 of culturing and was balanced out to maintain the viability. Additionally, the study focusing on the elucidation of histone modification pattern reported that epigenome is tended to undergo histone deacetylation at genomic regions that were previously acetylated (Lunke et al., 2021).

The expression of genes encoding the seven enzymes including 5'-nucleotidase, adenylate kinase, adenine phosphoribosyltransferase, adenine phosphoribosyltransferase, Cns1 protein with dehydrogenase oxidoreductase domains, Cns2 protein with metal-dependent phosphohydrolase domain, Cns3 protein with N-terminal nucleoside/nucleotide kinase and C-terminal ATP phosphoribosyltransferase domains and Cns4 ATP-binding cassette pentostatin transporter was also investigated using quantitative RT- PCR to observe the consequences of the epigenetic modification . As shown in Figure 5a, the expression of all genes was upregulated for at least 2 days after treatment with VPA compared to the culture without VPA, which finally resulted in the enhancement of cordycepin production. The highest relative upregulation of most genes was found at 18 h of treatment. Since VPA is a potent epigenetic modifier, its inhibition activity against histone deacetylation processes of *C. militaris* caused the reconstruction of DNA to the euchromatin structure (Milutinovic et al., 2007). Euchromatin, a lightly packed form of chromatin, allows transcriptional components, such as RNA polymerase and other transcription factors, to easily access the gene regions, leading to the upregulation of global genes or, at least, some subsets of genes (Monti et al., 2009). According to the cordycepin biosynthesis pathway, there are seven enzymes that possibly govern cordycepin production is a (Figure 3.7). The dramatic upregulation of 5'-nucleotidase (*nt5e*) promoted the conversion of 3'-deoxyadenosine 5'-phosphate to cordycepin. Adenylate kinase (*ak*) was also upregulated to increasingly convert adenosine monophosphate to 3'-deoxyadenosine-5'-phosphate, the intermediate of cordycepin production. Adeneine phosphoribosyltransferase (aprt), which catalyses the phosphoribosyl pyrophosphate (PRPP) conversion for IMP production (Xia et al., 2017), was upregulated to further increase the intermediate flux of cordycepin production. If the process starts from adenosine, hydroxyl phosphorylation is catalysed by the nucleoside/nucleotide kinase domain of Cns3 at 3’-OH position to yield adenosine-3’-monophosphate (3’ - AMP), which is then dephosphorylated to 2’ - carbonyl-

3’-deoxyadenosine (2’-C-3’-dA) by the phosphohydrolase activity of Cns2. Cordycepin is finally produced in the 2’-C-3’-dA by oxidoreduction reactions mediated by Cns1. The expression levels of Cns1, Cns 2 and Cns3 were significantly upregulated, leading to the enrichment of cordycepin. Notably, pentostatin, a purine analogue with anticancer and adenosine deaminase inhibition (Kane et al., 1992), was produced in coupling with cordycepin by the phosphoribosyltransferase domain of Cns3. This process maintained the stability of cordycepin, which was not converted to 3’-deoxyinosine (3’dI) by deaminase via the protector-protégé strategy (Wu et al., 2017). Subsequently, the pentostatin is pumped out of the cell by a Cns4 transporter when the cordycepin accumulation reaches cytotoxic levels. The upregulation of Cns4 might be due to the need for pentostatin removal to detoxify the enriched cordycepin. However, the upregulation of cordycepin production- related genes was not permanent. The expression of some genes slightly decreased after 48 h of VPA treatment, most likely because of the degradation of VPA itself; therefore, euchromatin started to be reconstructed back to heterochromatin.

Comparing to other studies, our study showed promising result on cordycepin production (Table 3.3) even without additional optimization on medium compositions and complex mode of operation required. The simplicity of epigenetic modification strategy could be beneficial on the translation and optimization to the practical industrial cordycepin production.

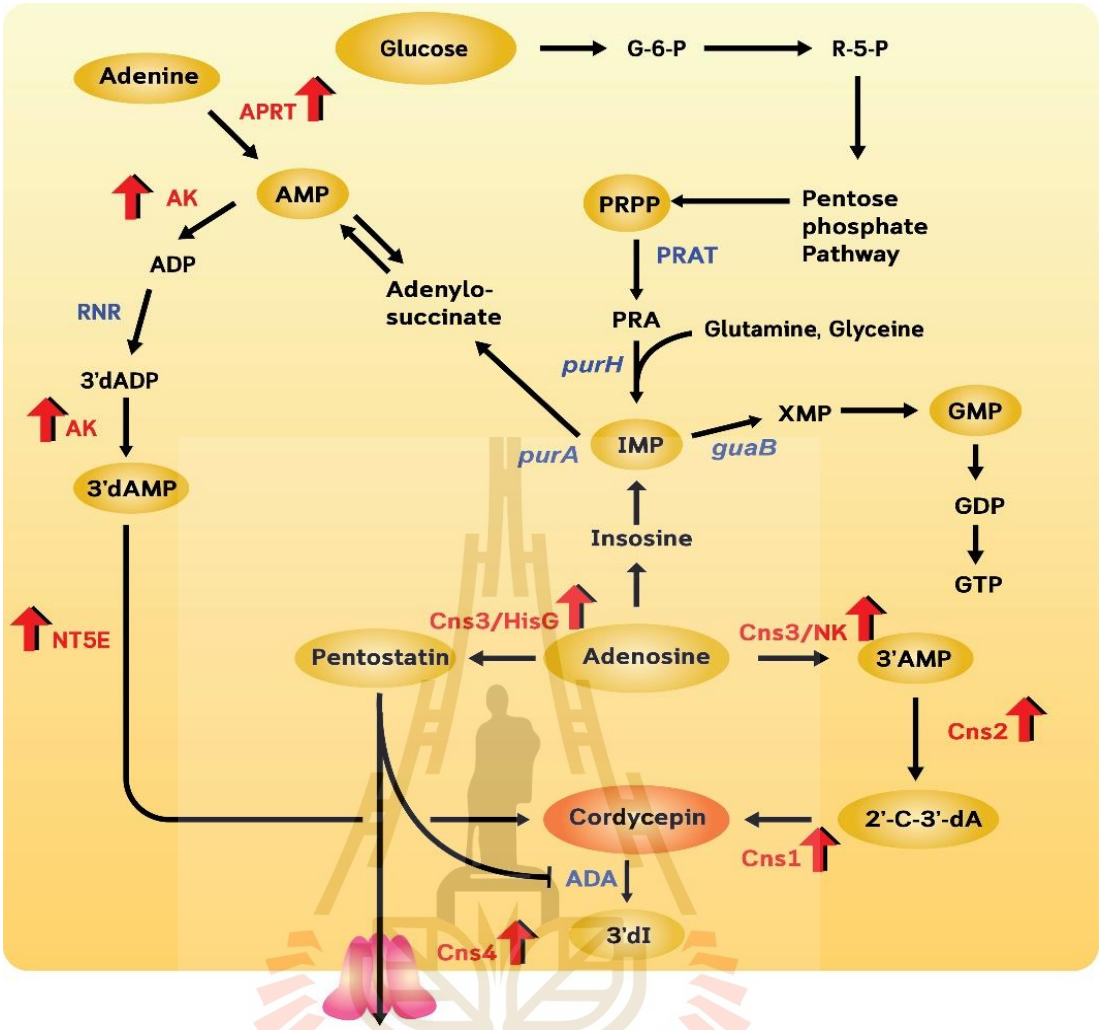

Figure 3.7 Schematic representation of the cordycepin biosynthesis pathway influenced by VPA supplementation.

Table 3.3 The comparison of cordycepin production by liquid culture of *Cordyceps* in this study and other studies.

	Strain	Culture method	Carbon source (g/L)	Nitrogen source (g/L)	Micro-nutrients	Additives/ Strategies	Cultivation Time (day)	Working Volume	Biomass (g/L)	Cordycepin (mg/L)	References
1	*C. militaris*	Submerged culture	Glucose/ 40	Peptone/ 10	KH_2PO_4 K_2HPO_4 $MgSO_4$	Two-Stage DO Control in bioreactor	17	5 L	?	201.1	Mao and Zhong (2004)
2	*C. militaris*	Submerged culture	Glucose/ 42	Peptone/ 15.8	KH_2PO_4 K_2HPO_4 $MgSO_4$	-	18	50 mL	19.2	345.4	Mao et al. (2005)
3	*C. militaris*	Submerged culture	Glycose/ 10	Peptone/ 10	KH_2PO_4 K_2HPO_4 $MgSO_4$ $CaCl_2$ $FeSO_4$	Fed-bacth with NH_4^+	17	3.51 L	19.5	346.1	Mao and Zhong (2006)
4	*C. militaris*	Submerged culture	Glucose/ 40	Peptone/ 10	KH_2PO_4 K_2HPO_4 $MgSO_4$ $(NH_4)_2SO_4$	+ $FeSO_4$	20	?	?	596.59	Fan et al. (2012)

Table 3.3 (continued).

	Strain	Culture method	Carbon source (g/L)	Nitrogen source (g/L)	Micro-nutrients	Additives/ Strategies	Cultivation Time (day)	Working Volume	Biomass (g/L)	Cordycepin (mg/L)	References
5	*C. militaris* NBRC 9787	Surface/static culture	Glucose/ 20	Peptone/ 2.5 Yeast extract/ 7.5	Mixed trace elements	-	27	100 mL	?	640	Masuda et al. (2006)
6	*C. militaris* NBRC 9787	Surface/static culture	Glucose/ 20	Peptone/ 2.5 Yeast extract/ 7.5	Mixed trace elements	+ Adenine + Glycine	33	100 mL	9.8	2370	Masuda et al. (2007)
7	*C. militaris* BCC2819	Surface/static culture	Glycose/ 40	Peptone/ 15	KH_2PO_4 K_2HPO_4 $MgSO_4$	-	15	45 mL	10.19	587.68	Hung et al. (2009)
8	*C. militaris* NBRC 9787	Surface/static culture	Glucose/ 86.2	Yeast Extract/ 93.8	Mixed trace elements	8-azaguanine resistant mutant	36	100 mL	?	6840	Das et al. (2010)

Table 3.3 (continued).

	Strain	Culture method	Carbon source (g/L)	Nitrogen source (g/L)	Micro-nutrients	Additives/ Strategies	Cultivation Time (day)	Working Volume	Biomass (g/L)	Cordycepin (mg/L)	References
9	*C. militaris* CGMCC2459	Surface/static culture	Sucrose/ 24.7	Peptone/ 20	K_2HPO_4 $MgSO_4$	+Vitamin B_1 +Hypoxanthine +L-alanine	35	700 mL	?	2008.48	Kang et al. (2014)
11	*C. militaris* CCRC 32219	Two step culture	Glucose/ 40	Yeast Extract/ 45	KH_2PO_4 K_2HPO_4 $MgSO_4$ $FeSO_4$	-	Shaking/ 8 Static/ 16	100 mL	?	2214.5	Shih et al. (2007)
12	*C. militaris* 14014	Two step culture	Glucose/ 40	Tryptone/ 10 Yeast extract/ 6	KH_2PO_4 K_2HPO_4 $MgSO_4$	-	Shaking/ 4 Static/ 25	50 mL	?	2620	Tang et al. (2015)
13	*C. militaris CM-1*	Surface/static culture	Sucrose/ 24.7	Peptone/ 20	K_2HPO_4 $MgSO_4$	+Vitamin B_1 + VPA	49	400 mL	4.27	2,835	This study

3.6 Conclusion

This study investigated the enhancement of cordycepin production by a static liquid culture of *C. militaris* with epigenetic modification (Figure 3.8). In this culture system, 1.5 x 10^7 initial spores were optimal for seed culture preparation. Using 50 μM of VPA as epigenetic modifier yielded a 41. 187% enhancement of cordycepin production, which consequently resulted from the up-regulation of 5'-nucleotidase, adenylate kinase, phosphorybosyltransferase, *Cns1*, *Cns2*, *Cns3,* and *Cns4*. An increase in the working volume resulted in a decreased cordycepin production, which could due to the uneven dispersion of valproic acid in the culture systems. The highest cordycepin production obtained was 2,835. 32 ± 34. 35 mg/ L. The epigenetic modification strategies used in this study could find a wide application in other static liquid culture processes.

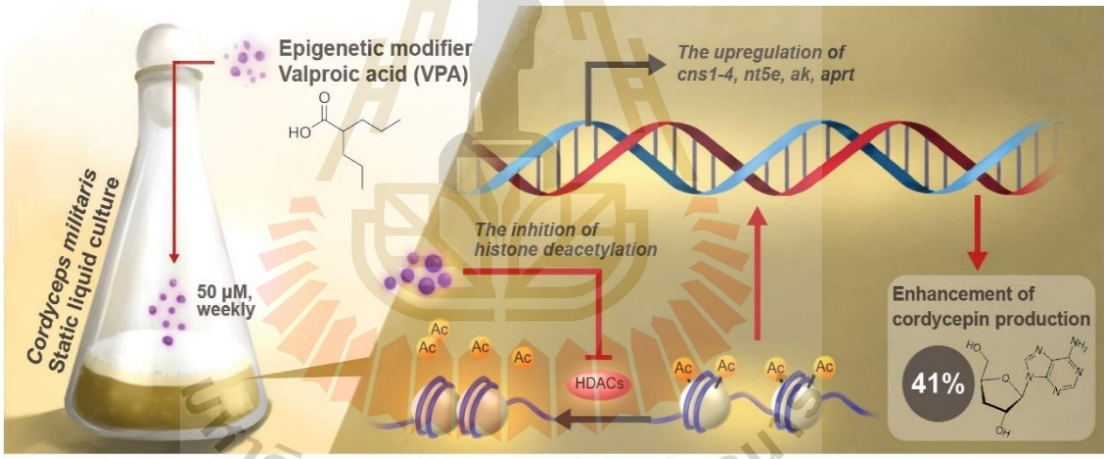

Figure 3.8 Schematic illustration of the possible biological activity of VPA on cordycepin production.

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CHAPTER 4
ENHANCEMENT OF CORDYCEPIN PRODUCTION FROM CORDYCEPS CULTURE BY EPIGENETIC MODIFICATION

4.1 Abstract

Immunotherapy is a promising strategy for cancer treatment. Cancer sensitization is one approach to enhance cancer cell susceptibility to immune cell cytotoxicity that can be used in combination with immunotherapy to achieve therapeutic efficiency in the form of medicine or nutraceuticals. Cordycepin, a bioactive compound from *Cordyceps spp*., has been reported to effectively inhibit tumor growth; however, the mechanism of its tumor sensitization activity is unknown. In this study, we investigated the biological influences of cordycepin on NK-92 cell physiological properties, cell stage development, and cytokine producing efficacy. We also determined cordycepin–activated NK cell cytotoxicity against THP-1 and U-251 cancerous cell lines. The treatment of NK-92 cells with cordycepin increased cytotoxicity against THP-1 and U-251 cells. Moreover, cordycepin remarkably increased the production of *TNF-**α**, IFN-**γ**, IL-2*, and *IL-12* cytokine-related genes, and significantly enhanced the secretion of IFN-**γ** and granzyme B from NK-92 cells. It was noted that cordycepin could induce the maturation of NK-92 cells by decreasing the expression of immature marker, CD27, and increasing expression of mature markers, CD11b, CD16, and NKG2D. In conclusion, this was the first report on the enhancement cytotoxicity of NK cells against cancerous cells, THP-1 and U-251, by cordycepin, which supported the application of cordycepin for further development as an alternative immunoactivity agent against cancer cells.

Keywords: Cordycepin/Natural killer cell/Cancer/Immune system

4.2 Introduction

Natural killer cell (NK cell) is a type of cytotoxic lymphocyte playing crucial role in the innate immune system as a defensive effector cell against cancer/tumor and microbial infection without the prior immunization required (Abel, Yang, Thakar, and Malarkannan, 2018), NK cells are able to specifically target and diminish cancerous cells with both cytolytic and cytokine-producing activity (R. Wang, Jaw, Stutzman, Zou, and Sun, 2012). Activated NK cells release cytolytic granules containing perforin, a pore forming glycoprotein and granzyme, a group of serine proteases to mediate cancer cell apoptosis (Pardo, Balkow, Anel, and Simon, 2002). NK cells can also secrete cytokines such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) under the stimulation of immunoregulatory interleukin to further manipulate adaptive immunity (E. Vivier, E. Tomasello, M. Baratin, T. Walzer, and S. Ugolini, 2008; R. Wang et al., 2012). The maturation and functional stages of human NK cell can be identified and distinguished by the alteration of the relative expression of its specific surface markers under certain conditions (Abel et al., 2018). The transition between two functionally distinct subpopulations was found to be based on the level of CD56 and CD16. $CD56^{bright}CD16^{dim/-}$ NK cell was reported to be a potent cytokine producer, while $CD56^{dim}CD16^{+}$ directly interact with cancer cell and manipulate cytolytic activity via both antibody-dependent cell cytotoxicity (ADCC) and NKG2D-dependent cell cytotoxicity (Jaime et al., 2017; Poli et al., 2009). Resembling in murine NK cells, the density of CD27 and CD11b were reported to further functionally distinguish the NK subsets and used to indicate NK cell deficiency in patients (Zhang et al., 2017). Some circumstances of NK cell functional alterations and impairments were reported. In older people, despite of the increase of total NK cell population upon aging due to the accumulation of long-standing NK cells, $CD56^{bright}$ NK cell population was significantly decreased in over 50 years-old elders, indicating the lower cytokine producing efficacy and lytic capacity (Chidrawar, Khan, Chan, Nayak, and Moss, 2006; Gounder et al., 2018). NKG2D downregulation could be found in cancer patients due to the effect of TGF-β1 secretion by cancer cells, and caused cancer immune evasion (Lee, Lee, Kim, and Heo, 2004; Saito, Osaki, and Ikeguchi, 2012). Therefore, the exploration of factors improving or enhancing NK activity could shed the light on cancer therapy.

Cordycepin, 3'-deoxyadenosine, is the renowned natural compounds isolated from *Cordyceps militaris* (Tuli, Sharma, Sandhu, and Kashyap, 2013; Xia et al., 2017). This unique nucleoside derivative possesses a wide-range of biological activities, including immunoregulatory and apoptosis-based anticancer (Chaicharoenaudomrung, Jaroonwitchawan, and Noisa, 2018; Y. Wang et al., 2017). However, the mechanisms underlying such bioactivities is elusive, and not yet fully elucidated. Since cordycepin can exhibit the remarkable influences in both immunity and anticancer aspects, it is possible to enhance NK cell functions, and could be one of alternative routes of its anticancer mechanisms.

In this study, we investigated the biological influences of cordycepin on NK-92 cell physiological properties, cell development stage, and cytokine producing efficacy. Besides, the capacity of cordycepin in activating NK cell cytotoxicity against cancerous cells, THP-1 and U-251 cells, was also examined. This study, for the first time, elucidated the mechanism and verified the evidence that cordycepin could trigger the anti-cancer activity of NK cells via the maturation, cytokine production, and granzyme B secretion.

4.3 Materials and methods

4.3.1 Cell line and cell culture

U-251 cells were purchased from the European Collection of Authenticated Cell Cultures (Salisbury, UK), and cultured in Dulbecco's modified Eagle's medium (DMEM) media containing 10% fetal bovine serum (FBS), and 1% (v/v) penicillin-streptomycin (HyClone, Logan, UT, USA). THP-1 (ATCC TIB-202) were purchased from the American Type Culture Collection (ATCC), and cultured in RPMI 1640 (HyClone, Logan, UT, USA) containing 10% FBS and 1% (v/v) penicillin-streptomycin. NK-92 cell line (CRL2408TM) was also purchased from ATCC, and cultured in Eagle's minimum essential medium (α-MEM) media containing 1.5 mg/ml sodium bicarbonate (Sigma, M4655), 2 mM L-glutamine, 0.2 mM inositol (Sigma, St. Louis, MO), 0.1 mM 2-mercaptoethanol (Gibco, CA, USA), 0.02 mM folic acid (Sigma, St. Louis, MO), 12.5% horse serum (Sigma, St. Louis, MO) and 12.5% FBS (HyClone, Logan, UT, USA). All cells were maintained in a humidified incubator with 5% CO_2 environment at 37 °C.

4.3.2 NK-92 cell viability and cytotoxic activity tests (LDH release-based)

NK-92 cells were seeded on 96-well microplates at a density of 2×10^4 cells/ml, and exposed to cordycepin at varying concentrations (0, 5, 10, 20, 40, 80, 160, 320, 640 and 1280 μM). After 24 hours of NK-92 cells treatment microplates were centrifuged at 300×g, 10 minutes, at room temperature. The culture supernatants were then collected in new 96- well microplates, which were used to perform LDH assay following the manufacturer's instruction (LDH cytotoxic assay kit, ab65393; Abcam). Absorbance was recorded on a microplate reader SPECTROstar Nano (BMG Labtech) at 450 nm wavelength. NK-92 cells cytotoxicity against tumor cells (THP-1 and U-251) was analysed using a lactate dehydrogenase (LDH) release assay. Target cells 2,500 cells were plated, and on the next day NK-92 cells were added at various ratios (1:1, 1:5, and 1:15, target cells: effector cells) (all samples in triplicate). After 24 hours of co-culture, an aliquot of 50 μl media was used in LDH cytotoxic assay using the LDH cytotoxic assay kit. The value of corrected experimental LDH release was calculated by subtracting the value of spontaneous LDH release from effector cells at corresponding dilutions. NK cytotoxicity was defined as % Cytotoxicity = (Experimental value – Effector Cells Spontaneous Control – Target Cells Spontaneous Control)/(Target Cell Maximum Control – Target Cells Spontaneous Control) × 100.

4.3.3 RNA isolation and PCR analysis

NK-92 cells were treated with cordycepin at varying concentrations (0, 5, 10, 20, 40, 80 μM). for 24 h. mRNA were isolated using the commercial using NucleoSpin RNA kit (Macherey-Nagel, Dueren, Germany) according to the manufacturer's instructions. Total RNA was converted into a complementary DNA (cDNA) using cDNA synthesis kits (Toyobo Co., Ltd., Osaka, Japan). Quantitative PCR was done by using specific primers and cDNA in 2X Taq Master Mix (Vivantis, Selangor Darul Ehsan, Malaysia), according to the manufacturer's instructions. RT-PCR was performed using the Biorad/C1000Touch Thermocycle (Biorad, USA). The amplified cDNA products were electrophoretically separated on 1.5 % agarose gel, and visualized by RedSafe Nucleic Acid staining (iNtRON Biotechnology, Inc., Korea). The relative expression level of a target gene was quantified by normalization with the internal control *GAPDH* gene. Primers used in the experiment were showed in Table 4.1.

Tables 4.1 Primer sets used for Quantitative RT-PCR of cytokine genes.

Gene set	Primers	Sequence (5'-3')
Glyceraldehyde 3-phosphate dehydrogenase (House keeping gene)	*gapdh* Forward	5'- ACCTGACCTGCCGTCTAGAA -3'
	gapdh Reverse	5'- TCCACCACCCTGTTGCTGTA -3'
Tumor necrosis factor alpha (TNF-α)	*tnfa* Forward	5'- GAGCACTGAAAGCATGATCC -3'
	tnfa Reverse	5'- CGAGAAGATGATCTGACTGCC -3'
Interferon gamma (IFN-γ)	*infg* Forward	5'-TCGTATGCTGCTCATCGGAC-3'
	infg Reverse	5'-AGGCGTTAGCCACCAATCAA-3'
Interleukin 2 (IL-2)	*il2* Forward	5'- AGAACTCAAACCTCTGGAGGAAG -3'
	il2 Reverse	5'- GCTGTCTCATCAGCATATTCACAC -3'
Interleukin 12 (IL-12)	*il12* Forward	5'-ACCACTCCCAAAACCTGC-3'
	il12 Reverse	5'- CCAGGCAACTCCCATTAG -3'

4.3.4 IFN-γ and Granzyme B assay

NK-92 cells were cultured with α-MEM complete medium or 2.5 µg/ml cordycepin in α-MEM complete medium at 37 °C for 24 hours prior to co-incubation with target THP-1 acute myeloid leukemia cells and U-251 glioblastoma cells at target-effector ratios (1:10), and the mixture was then incubated for 24 hours in the presence of 5% CO_2. Supernatants were collected after centrifugation at 2,000 x g for 10 minutes, and were assayed for both IFN-γ and Granzyme B using ELISA kits (ab 174443 IFN-γ and ab235635 for Granzyme B, Abcam, Shanghai, China), according to the manufacturer's instructions.

4.3.5 Flow Cytometry

NK-92 cells were cultured with α-MEM complete medium or 2.5 and 5 ug/ml cordycepin in α-MEM complete medium at 37 °C for 24 hours. Cells were washed twice with PBS and stained with antibody mixes in 100 µl PBS or staining buffer (PBS, 1% FCS) for 15 to 30 minutes at room temperature (RT) in the dark. The following

antibodies were used at concentrations of 1:100: PE anti-Human CD27 (Biolegend, 356406), FITC anti-Human CD11b (BioLegend 301330), FITC anti-human CD16 (BioLegend Cat. No. 302006), PE/Cyanine5 anti-human CD56 (BioLegend 362516), PE anti-human CD314 (NKG2D) (BioLegend Cat. No. 320806). After two washes, cells were suspended in 50-100 µl PBS or staining buffer and analyzed on a Cytomics FC500 Flow Cytometer (Beckman Coulter, Indianapolis, IN, USA).

4.3.6 Statistical analysis

IBM SPSS Statistics, version 16.0, was used for statistical analysis (SPSS Inc., Chicago, USA). All experiments were run three times, and data were reported as mean standard deviation (SD). ANOVA, followed by Tukey's test at $p < 0.05$, was used to evaluate whether there were significant changes between treatments.

4.4 Results

4.4.1 Effects of cordycepin on NK-92 cell viability and cytotoxic activity

In order to assess the effects of cordycepin on NK-92 cell viability, NK-92 cell were exposed to a wide range of cordycepin concentrations for 24 hours prior to determining the cell viability by using the LDH assay. As shown in Fig. 4.1a, the treatment of 5-80 µM cordycepin had no effect on the cell viability of NK-92 cells. Treatment with higher than 40 µg/ml cordycepin dose-dependently declined cell viability of NK-92 cells compared with the control group (*p<0.05, **p<0.01). We investigated the effects of cordycepin on NK-92 cytotoxic activity against THP-1 acute myeloid leukemia cells and U-251 glioblastoma cells. For these experiments, THP-1 and U-251 used as target cancer cells were cultured with effectors NK-92 cells at different target - effector ratios (1:1, 1:5 and 1:10). Notably, NK cell cytotoxic activity increased as a function of the target-effector ratio (Figure 4.1b and 4.1c). Noticeably, we observed that the pre-treatment NK-92 cells with 10 µM cordycepin enhanced the cytotoxic activity. These results suggest that cordycepin enhanced the NK-92 cell cytotoxic activity against THP-1 acute myeloid leukemia cells and U-251 glioblastoma cells.

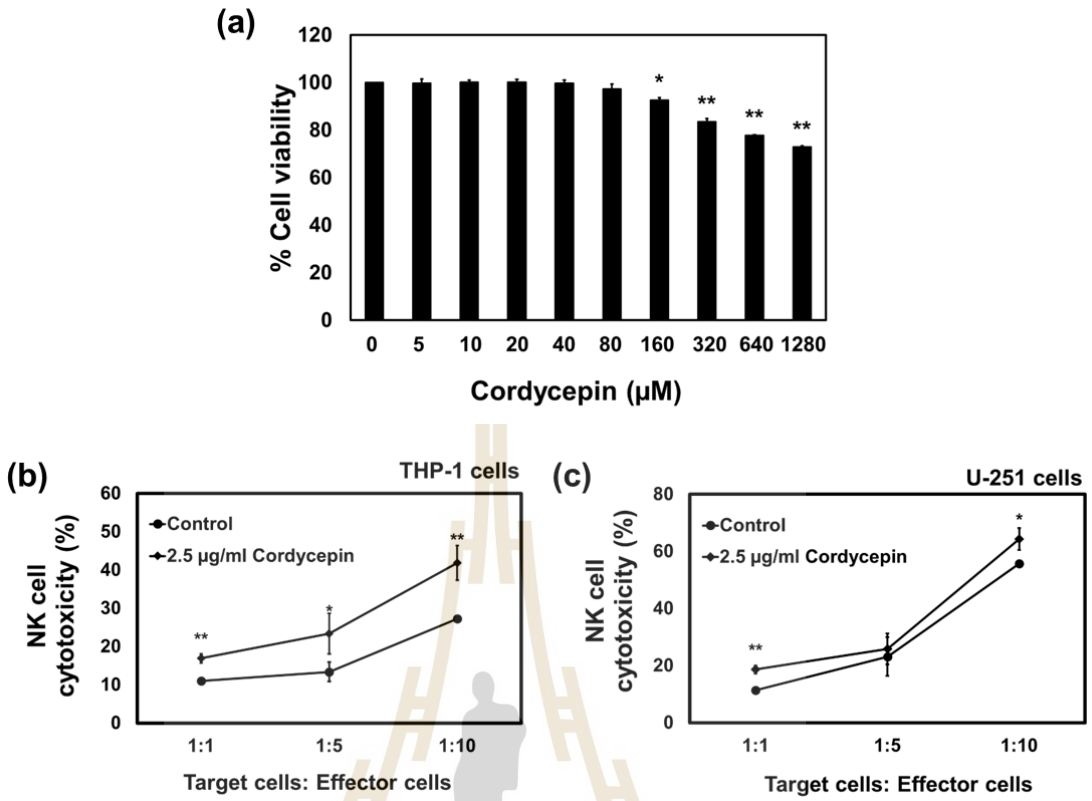

Figure 4.1 Effects of cordycepin on NK-92 cell viability and cytotoxic activity. (a) Effects of cordycepin on NK-92 cell viability. NK-92 cells were treated with different concentrations of cordycepin for 24 hours and cell viability was assessed using the LDH assay. Effector NK-92 cells were cultured with 10 μM of cordycepin for 24 hours prior to incubation with (b) target THP-1 acute myeloid leukemia cells, and (c) U-251 glioblastoma cells at various target-effector ratios (1:1, 1:5, and 1:10) and the mixture was then incubated for 24 hours. Cell killing was measured using LDH assay, based on the release of lactate dehydrogenase. Data are expressed as cell death percentages (%). Data were expressed as % NK cell cytotoxicity against the THP-1 and U-251 cells. The experiments shown in this study were performed in triplicate (*p<0.05; **p<0.01).

4.4.2 Effect of cordycepin on cytokines mRNA expression of NK-92 cells.

To determine whether the cordycepin enhanced the NK-92 cell cytotoxic activity against THP-1 and U-251 cells was due to cytokine regulation, cytokines mRNA expression was measured by RT-PCR. After the treatment of cordycepin at various concentrations (5, 10, 20, 40, and 80 μM) for 24 hours, the expression cytokine-related genes, including tumor necrosis factor alpha (*TNF-**α***), interferon gamma (*IFN-**γ***), and interleukin 2 (*IL-2*), were upregulated in the cordycepin treatment group compared to the control group (Figure 4.2a-d). Moreover, at low concentration of cordycepin (5, 10, and 20 μM), the expression of *IL-12* gene was upregulated, while the expression of *IL-12* gene was downregulated at high concentration of cordycepin (40 and 80 μM). The results revealed that cordycepin enhanced the NK-92 cell cytotoxic activity against THP-1 and U-251 cells by induced the expression of *TNF-**α**, IFN-**γ**, IL-2,* and *IL-12* cytokine-related genes.

4.4.3 Effect of cordycepin on cytokine IFN-γ and granzyme B production by NK-92 cells

Since production of cytokines by activated NK-92 cells is considered essential to the early phases of the immune response (Eric Vivier, Elena Tomasello, Myriam Baratin, Thierry Walzer, and Sophie Ugolini, 2008), the effects of cordycepin on IFN-**γ** secretion by NK cells was determined. At 10 μM cordycepin alone and pre-treatment prior to co-incubation with target THP-1 and U-251 cells at target-effector ratios (1:10) caused statistically significant enhancement in IFN-**γ** production (Figure 4.3a). The stimulatory effects of treatment with cordycepin at 10 μM, prior to co-incubation with THP-1 and U-251 cells in the release of IFN-**γ** were 211.74±6.91, 242.83±27.63, 332.64±31.09 pg/mL, respectively, relative to production levels by untreated cells was 184.11±10.36 pg/mL. Moreover, the release of granzyme B was used to monitor the exocytosis of cytolytic enzymes from NK-92 cells. Figure 4.3b shows the effect of cordycepin on release of granzyme B from NK-92 cells in the presence of 10 μM cordycepin after 24 hours and pre-treatment prior to co-incubation with target THP-1 and U-251 cells at target-effector ratios (1:10). It can be seen that, at all treatment groups caused a statistically significant increase in the release of granzyme B. The treatment of cordycepin, prior to co-incubation with target THP-1 and U-251 cells increase in the release of granzyme B were 201.67±14.14, 223.33±7.07,

288.33±51.85 pg/mL, respectively, relative to production levels by untreated cells was 161.67±14.14 pg/mL. The results demonstrated that cordycepin enhance the IFN-γ secretion and release granzyme B from NK-92 cells after the treatment.

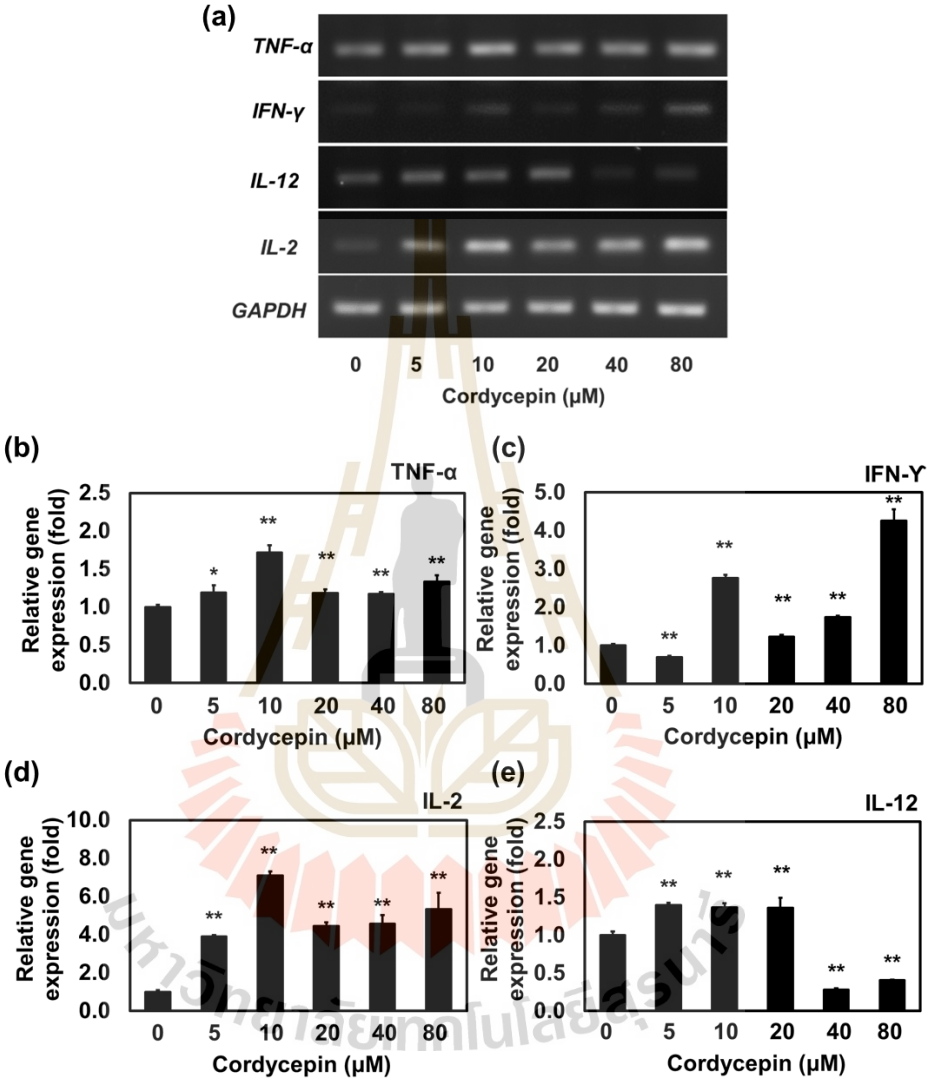

Figure 4.2 Effect of cordycepin on cytokines mRNA expression.in NK-92 cells. NK-92 cells were cultured different concentrations of cordycepin for 24 h, followed by measurement of (a) mRNA levels of *TNF-α, IFN-γ*, *IL-12,* and *IL-2* genes. (b-e) The relative expression of *TNF-α, IFN-γ*, *IL-12,* and *IL-2* were determined by using *GAPDH* as a reference gene. Values were expressed as mean ± SD (n =3). **p* < 0.05 and ***p* < 0.01 versus control cells.

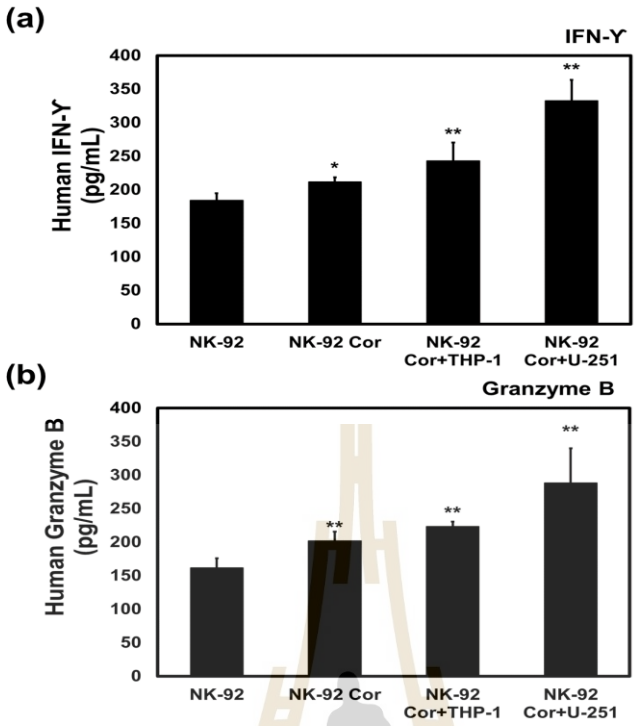

Figure 4.3 Effect of cordycepin on IFN-Ɣ and Granzyme B production by NK-92 cells. NK cells were incubated with 10 μM cordycepin for 24 h prior to co-incubation with target THP-1 acute myeloid leukemia cells and U-251 glioblastoma cells at target-effector ratios (1:10) and the mixture was then incubated for 24 hours. (a) IFN-γ and (b) Granzyme B in culture supernatant were then determined by ELISA. The experiments shown in this study were performed in triplicate (*p<0.05; **p<0.01).

4.4.4 Effect of cordycepin on the characterization of NK cells for expression of surface markers and NK-cell receptors

NK-92 cells that have reached terminal maturation are fully functional; however, evidence suggests that their capabilities with regards to anti-tumor cytotoxicity and inflammatory cytokine production may not be acquired equally. The effects of cordycepin on distinct developmental stages of immature NK-92 cells to mature NK-92 cells, which levels of NK-92 cell surface receptors were monitored using flow cytometry. Treatment of NK-92 cells with 10 and 20 μM cordycepin decreased expression immature marker of CD27 and increased expression mature marker of CD11b in a dose-dependent manner (Figure 4.4a and 4.4b). In human, NK-92 cells can

be divided into $CD56^{bright}$ and $CD56^{dim}$ subsets. Our results showed that treatment of NK-92 cells with 10 and 20 μM cordycepin increased expression of CD16 were 72.3% and 72.1%, respectively. While CD56 expression did not show alterations. Moreover, NKG2D is expressed on NK cells and some T cell subsets, where it functions as an activating receptor to trigger cytolytic activity and cytokine secretion. Results presented here show that the expression of NKG2D can be increased by 10 and 20 μM cordycepin in NK-92 cells. These results indicate that the cordycepin induce maturation from immature to mature NK-92 cells.

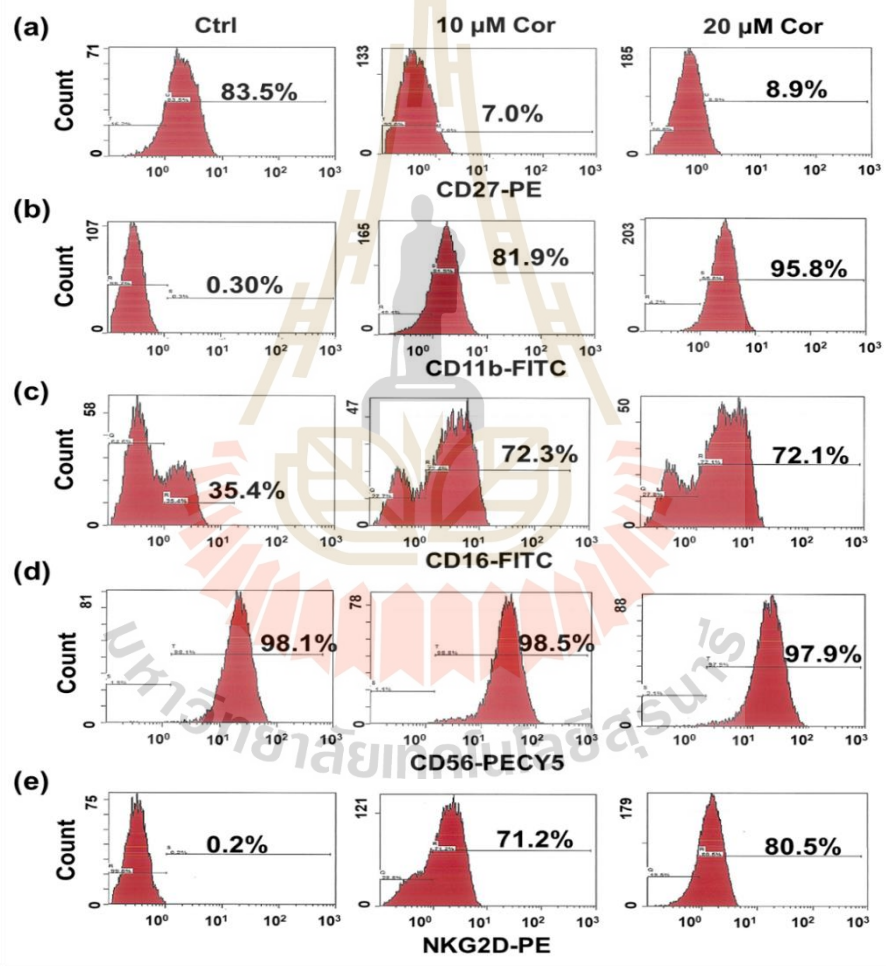

Figure 4.4 Effect of cordycepin (Cor) on the characterization of NK cells for expression of surface markers and NK-cell receptors. Representative flow cytometry analyses for the expression of (a) CD27, (b) CD11b, (c) CD16, (d) CD56, and (e) NKG2D obtained from NK cells treated with cordycepin at 0, 10 and 20 μM, respectively.

4.5 Discussion

NK-92 cells are essential elements in the immune defense against tumor cells and pathogens by modulating both innate and adaptive immune responses. They are directly cytotoxic for the tumor-derived and virus-infected cells. Many strategies are investigated to enhance NK-92 cells activity in a way to combat tumor growth. To assess the influence of cordycepin on *in vitro* immune cell stimulation, NK-92 cell viability was measured after *in vitro* incubation of the cells with various concentrations cordycepin (Figure 4.1a). When we measured the in vitro NK-92 cell viability, 1.25-20 μg/ml cordycepin treatment had no effect on the cell viability of NK-92 cells compared with the control group. We next determined whether *in vitro* treatment of cells with cordycepin could result in the stimulation of NK-92 cell cytotoxic activity. Incubation of NK-92 cells with 10 μM cordycepin for 24 hours resulted in significantly (p<0.01) enhanced cytotoxicity against THP-1 acute myeloid leukemia, and U-251 glioblastoma target cells (Figure 4.2b and 4.2c). Results presented here show that cordycepin pre-treatment stimulates the anti-cancer activity of NK-92 cells. The activation appears to be a result of activation of the major NK cell-activating receptors NKG2D, CD16, CD11b, leading to the up-regulation of TNF-α, IFN-Ɣ, IL-2, IL-12 cytokine, which, in turn, Granzyme B -mediated cytotoxicity. Previously, cordycepin has been reported as able to increase cancer cell death when combined with other treatments. The reported that treatment with cordycepin prior to and during co-culturing with NK-92 cells significantly increased cell death of a lethal cancer, cholangiocarcinoma (CCA) as compared to solitary cordycepin or NK treatment. Moreover, sensitization activity was also observed in the combination of NK-92 cells and *C. militaris* extract that contained cordycepin as a major component. The cordycepin treatment remarkably caused an increase in TRAIL receptor (DR4 and DR5) expression in cholangiocarcinoma, suggesting the possible involvement of TRAIL signaling in cholangiocarcinoma cells (Panwong et al., 2021).

Although, two major mechanisms of NK cells are used to cytotoxic effects against target cell and induce target cell apoptosis, granule exocytosis and death receptor stimulation (Smyth et al., 2005). Granule exocytosis involves the release of perforin and granzymes (Trapani and Smyth, 2002), which cytotoxic granules contain perforin, pore-forming protein, and granzymes. Perforin generates pores in the target cell

membrane allowing granzymes to enter the cell and initiate the apoptosis by caspase-dependent and independent pathways (Smyth et al., 2005). In death receptor-mediated cytotoxicity pathway, the death ligands produced by NK cells and attached to the death receptors expressed on the target cell surface, thereby activating the caspase cascade leading to the death of the recognized objects. Fas ligand, tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand (TRAIL), and lymphotoxin alpha (LTα), have been identified as death receptor ligands (Screpanti, Wallin, Grandien, and Ljunggren, 2005; Smyth et al., 2005). NK cells can also secrete many different cytokines (*e.g.* IFN- γ, TNF-α and GM-CSF (granulocyte/monocyte colony-stimulating factor) as well as chemokines, at least some of which have a direct effect on tumors. The best studied cytokine in this respect is IFN-Ɣ, a cytokine which regulates various aspects of immune system responses, including NK cell actions, by forming a positive feedback loop (Schroder, Hertzog, Ravasi, and Hume, 2004), decreasing proliferation, enhancing autophagy, limiting metabolic activity of tumor cells, and inhibiting angiogenesis (Hayakawa et al., 2002). The cytolytic function of NK cells is controlled by a number of activating and inhibitory receptors, as well as the adhesion molecules (Long, 2008; Moretta et al., 2002), and the target cell recognition by activating receptors, such as NKG2D, also leads to the production of IFN- γ. IFN- γ and TNF-α have been shown to be essential in viral and tumor clearance (Balkwill, 2009; Ikeda, Old, and Schreiber, 2002). Previously, it was reported that cytolytic $CD56^{dim}CD16^{+}$NK cells can produce IFN- γ over the duration of their cytolytic activity (De Maria, Bozzano, Cantoni, and Moretta, 2011; Fauriat, Long, Ljunggren, and Bryceson, 2010).

The intensity and the quality of NK cell cytotoxic and cytokine responses depend on the cytokine microenvironment, as well as on interactions with other cells of the immune system, such as T cells, dendritic cells and macrophages (Long, 2007). Type I IFN, IL-12, IL-18 and IL-15 are potent activators of NK cell effector function (Walzer, Dalod, Robbins, Zitvogel, and Vivier, 2005). Interleukin 2 (IL-2) is one of the immune system's signaling molecules, and was initially named T cell growth factor (TCGF). It is also well known that IL-2 promotes NK cell proliferation, cytotoxicity and, to some extent, cytokine secretion (Liu et al., 2021). Interleukin (IL)-12 is a heterodimeric cytokine, consisting of p35 and p40 subunits. It was originally identified as natural killer

(NK) cell stimulatory factor (NKSF), (Kobayashi et al., 1989), and is produced mainly by antigen-presenting cells. IL-12 plays an important role in modulating both innate and adaptive immune responses. It induces the production of interferon (IFN)-**γ** from human NK cells and T cells, and also plays a key role in promoting the development of the Th1-type immune response (Trinchieri, 1998). In addition, IL-12 enhances the cytotoxicity of NK cells and cytotoxic T lymphocytes (CTL). NK cells activated by IL-12 exhibit enhanced cytotoxic activity against NK cell sensitive and resistant target cells, both *in vitro* and *in vivo* (Zwirner and Ziblat, 2017). Moreover, *In vitro* studies demonstrate that the combination of IL-2 and IL-12 has a synergistic effect in promoting IFN-**γ** production by T and NK cells, and the cytotoxic activity of NK cells (K. S. Wang, Frank, and Ritz, 2000).

4.6 Conclusion

In conclusion, this study investigated the potency of cordycepin to enhance cytotoxicity of NK cells against cancerous cells, THP-1 and U-251 cancer cells (Figure 4.5). It was found that cordycepin exhibited potent NK cell activator through the activation of TNF-**α**, IFN-**γ**, IL-2, and IL-12 production, and the enhancement of exocytosis granzyme B secretion. Importantly, cordycepin could induce the maturation of NK-92 cells by decreasing the expression of immature marker, CD27, and increasing expression of mature markers CD11b, CD16, and NKG2D. This evidence supported the further development of cordycepin as anti-cancer agent by elevating NK cell activity.

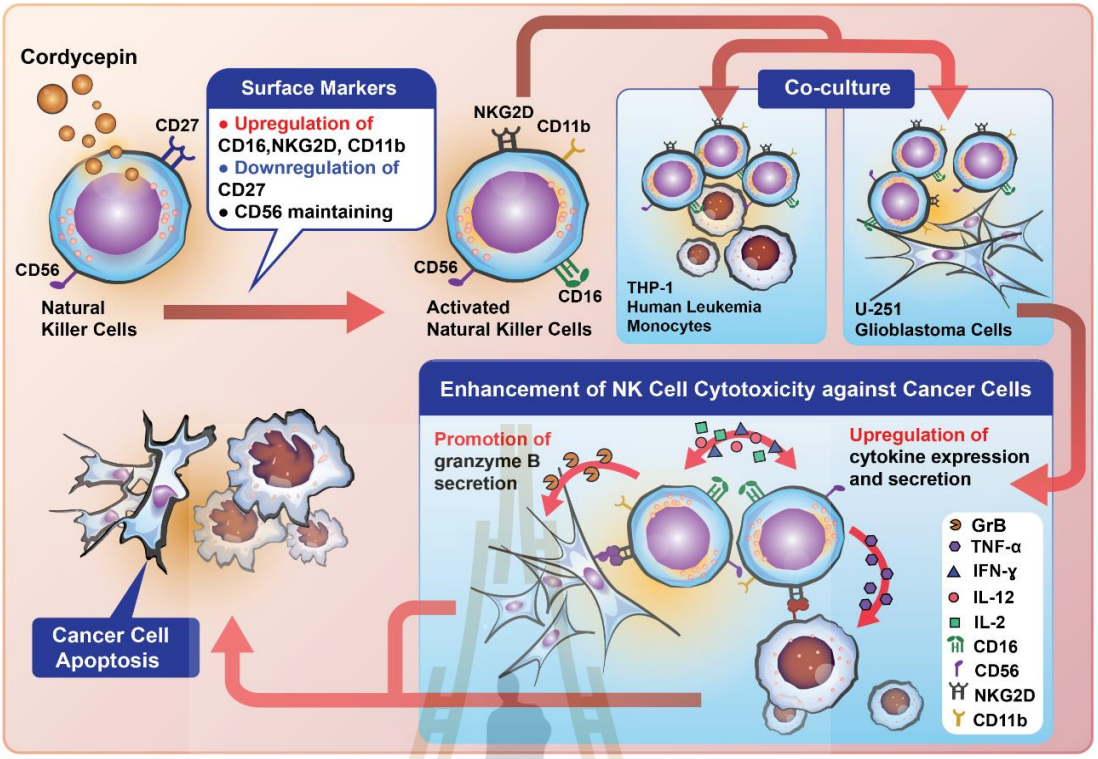

Figure 4.5 Schematic illustration of the possible biological activity of cordycepin on the enhancement of NK cell cytotoxicity against cancer cells.

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CHAPTER 5
CORDYCEPIN-INDUCED KERATINOCYTE SECRETOME PROMOTES SKIN CELL REGENERATION

5.1 Abstract

Skin regeneration is the intrinsic ability to repair damaged area of skin tissues in order to regaining skin well-being. Orchestral processes of wound healing, a major part of skin regeneration, are contributed by various types of cells, including keratinocytes and dermal fibroblasts, through their autocrine/paracrine signals. The releasable factors from keratinocytes were reported to influence dermal fibroblasts behaviors during wound healing processes. Here, we developed a strategy to modulate cytokine components and improve the secretome quality of HaCaT cells, a nontumorigenic immortalized keratinocyte cell line, via the treatment of cordycepin, and designated as cordycepin-induced HaCaT secretome (CHS). The results indicated that CHS exhibited the induction property of fibroblast proliferation/migration, ROS scavenging, extracellular matrix synthesis regulation, and autophagy activation. Such enhanced bioactivities of CHS could be related to the increase of some key cytokines, including CXCL1, IL-1Ra, IL-8, MIF, and PAI-1. This finding highlighted the implications of cordycepin to alter cytokine profile of HaCaT secretome, which offers a novel biosubstance for the development of wound healing and skin regeneration products.

Keywords: Cordycepin/Keratinocyte/Dermal Fibroblast/Secretome/Skin regeneration

5.2 Introduction

Skin serves as the body's first barrier against all harmful substances from environment, protecting body from chemicals, radiation, microbes, and mechanical forces. Besides, it also prevents the internal fluid loss and importantly regulate body temperature (Hwa, Bauer, and Cohen, 2011; Menon, 2015). Thus, the condition of skin is critically important for human's well-being. Histologically, skin could be divided into 3 vertically aligned layers; the epidermis, the dermis, and the hypodermis. However, when skin was damaged and wounded, the cutaneous wound healing take major part in skin regeneration, mainly processed through the dynamic sequences of synergistic interaction of various cell types localized within both epidermis and dermis layers. Among them, the interaction between keratinocytes and fibroblasts plays the most crucial role in such healing processes. Despite being separated by basement membrane with minimal direct cell-to-cell contact, keratinocyte–fibroblast interaction is mostly carried on by releasable factors in an autocrine/paracrine manner to regulate cell proliferation and extracellular matrix (ECM) remodeling (Sato, Kirimura, and Mori, 1997). Keratinocytes were also widely reported to influence and regulate the expression of dermal fibroblast genes via keratinocyte-derived soluble factors dissolved in its secretome (Bukowska et al., 2018; Ghaffari, Kilani, and Ghahary, 2009; Ghaffari et al., 2006; Sato et al., 1997). Therefore, the study of the effects of keratinocyte secretome and its containing factors on dermal fibroblasts should be beneficial for the development of novel therapies to maintain skin integrity.

Autophagy, a lysosome-dependent recycling mechanism, plays an important role in wound healing processes, especially in dermal fibroblasts. In general, autophagy plays a role in the aging process and skin homeostasis under harmful conditions generated by external stimuli in order to repair cellular machineries (D. Jeong, Qomaladewi, Lee, Park, and Cho, 2020). Autophagy is also activated during proliferative and remodeling phases of dermal fibroblasts, and helps preventing oxidative stress, promoting their survival and maintaining their normal functions (Ren, Zhao, Zhang, Huang, and Wang, 2022). Besides, autophagy has been reported to regulate extracellular matrix (ECM) and matrix metalloproteinase (MMP) mechanisms. Microtubule-associated protein 1 light chain 3 (LC3), the key autophagic marker, was found to be upregulated *in vivo*, and highly accumulated at the margin of the wound

during the healing processes of rat models (Asai, Yamamoto, Ueda, and Waguri, 2018). Defective autophagic activity of fibroblasts was found to induce the degradation of extracellular matrix components via the expression of matrix metalloproteinases 1 (MMP-1) and matrix metalloproteinases 3 (MMP-3) (Ren et al., 2022; Y. Wang et al., 2019). Thus, autophagy might be the relevant cellular mechanism for skin regeneration, and it may be regulated by keratinocyte secretome factors.

However, the use of primary keratinocytes as a source of secretome could be a burden in both research and large-scale production due to their short lifespan and limited workable passages. HaCaT, the spontaneously immortalized human keratinocytes, are renowned substitute for primary keratinocytes in skin research fields (Choi and Lee, 2015). They are nontumorigenic monoclonal cell line that are not only capable of continuing proliferation, but also exhibit almost identical genotype and phenotype of primary keratinocytes, including the contact inhibition and anchorage-dependent growth in cell culture, the capability to form well-constructed epidermis in mouse model transplantation (Breitkreutz et al., 1998; Maas-Szabowski, Starker, and Fusenig, 2003), and the ability to produce keratinocyte-derived soluble factors/cytokines (Zampetti et al., 2009). Colombo et al. (2017) found that HaCaT was capable of producing cytokines as expected in primary keratinocytes with some slightly differences in CC family of chemokines. Despite such differences, the secretome of HaCaT is sufficient to be used in studies in the substitution for primary keratinocyte secretome. The additional induction/stimulation protocol should be developed and applied to enhance the factors/cytokines production, which could improve the quality of HaCaT secretome.

Cordycepin or 3'-deoxyadenosine, the unique nucleoside analogue that is naturally found in *Cordyceps militaris*, has been reported to exhibit a wide-range of biological activities, in particular the regulatory effects on human cytokines production and release, such as eotaxin, FGF-2, IGF-2, IL-12, and IFN-γ (Y. Sun et al., 2014). Cordycepin also increased the expression of Th2 cytokines, IL-4, and IL-10 in mouse splenocytes (M. H. Jeong et al., 2012). IL-10, IL-1ra, TGF-β could be upregulated by cordycepin treatment in LPS-activated macrophages (Shin et al., 2009). Therefore, the cytokine profile of HaCaT secretome might be altered and improved its quality by cordycepin treatment.

Taken together, we hypothesized that HaCaT cells may be influenced by cordycepin treatment during the cultivation, and yield the secretome with different factor/cytokine compositions that consequently exhibits distinct biological influence regarding to skin regeneration and wound healing on human dermal fibroblasts (HDF). Therefore, in this study, we collected cordycepin-induced HaCaT secretome (CHS), and explored its biological effects on HDF, including antioxidant activity, extracellular matrix mechanisms, autophagy activation, and wound healing capacity. We also further investigated composition changes of CHS, which could be responsible for such different biological effects compared to the normal HaCaT secretome.

5.3 Materials and methods

5.3.1 Cell line and cell culture

Human skin cells, human immortalized keratinocytes (HaCaT), and human dermal fibroblasts (HDF), were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in DMEM medium, containing 10% (v/v) heat-inactivated FBS, 1% (v/v) nonessential amino acids, 1% (v/v) L-glutamine, 1% (v/v) penicillin-streptomycin were used for cell culture. Cell culture was incubated in a humidified incubator with 5% CO_2 atmosphere and 37 °C for 2 days or until cell reached 80% confluence.

5.3.2 Collection of normal HaCaT secretome (NHS) and cordycepin-induced HaCaT secretome (CHS)

The effect of and cordycepin on immortalized keratinocytes (HaCAT) cell toxicity was determined by the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) colorimetric method. HaCAT cells were cultured in a 96-well plate treated with different concentrations of cordycepin (1.25, 2.5, 5, 10, 20 and 50 μM) for 24 hours. Then, MTT solution was added to cell culture media at 0.5 mg/ml final concentration, and incubated for 3 hours at 37 °C in the dark. The media was removed, and the formazan crystal was solubilized in dimethyl sulfoxide (DMSO). The absorbance was measured at 570 nm, using a microplate reader (BMG Labtech, Ortenberg, Germany). The O.D. 570 nm in control condition (no cordycepin added) was taken as 100% viability. The concentration of cordycepin that did not decrease HaCaT cell viability was used for the collection of CHS.

HaCaT cells were cultured in T-175 flask until reaching 80% confluence. The cells were treated with the sub-toxic dose of cordycepin (2.5 μM) for 24 hours. The old media was discarded, washed twice with phosphate buffered saline (PBS), substituted with DMEM medium without FBS and phenol red, and incubated overnight in to allow the cells to secrete extracellular factors/cytokines to the medium, and so-called cordycepin-induced HaCaT secretome (CHS). To collect normal HaCaT secretome (NHS), the previously described protocol was followed without the cordycepin treatment step. Both NHS and CHS were collected by centrifugation at 12,000 rpm for 10 minutes to remove the residue HaCaT cells and kept in -80 °C.

5.3.3 Treatment of HDF with CHS

The effect of NHS and CHS on dermal fibroblast cell viability was determined by the MTT colorimetric method. HDFs were cultured in a 96-well plate, and treated with different concentrations of NHS and CHS (0, 6.25, 12.5, 25, 50 and 100%) for 24 hours. Then, MTT solution was added to cell culture media at 0.5 mg/ml final concentration, and incubated for 3 hours at 37 °C in the dark. The media was removed, and the formazan crystal was solubilized in dimethyl sulfoxide (DMSO). The absorbance was measured at 570 nm, using a microplate reader (BMG Labtech, Ortenberg, Germany). The O.D. 570 nm in control condition (0% of NHS or CHS) was taken as 100% viability.

5.3.4 Detection of reactive oxygen species in HDF by DCFH-DA assays

HDFs were cultured in a black 96-well plate, and treated with different concentrations of either NHS or CHS (6.25, 12.5 and 50%) and 5 mM N-acetyl cysteine (NAC; positive control) for 24 hours. Then, the cells were washed with PBS and incubated with 10 μM DCFH-DA at 37 °C for 1 hour in the dark, and washed twice with PBS again. The cells were then challenged with 1mM of hydrogen peroxide (H_2O_2) at 37 °C for 10-30 minutes. The fluorescence intensity of DCF was measured using a Varioskan™ LUX multimode microplate reader (Thermo Scientific, Massachusetts, USA) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Results were expressed as percentage of controls (0% CHS) (Wolfe and Liu, 2007).

5.3.5 Wound healing assays in HDF

HDFs were cultured in a 6-well plate until reaching 90% confluence of cell monolayer. Wound gap was created by directly scratching cell monolayer with a sterile 200 µL autopipette tip, washed with PBS twice to get rid of excess cells. Then cells were treated with 12.5% of either NHS or CHS to be compared with the control condition without secretome. The images of the cell monolayer were taken under a microscope with the same configurations at 0 -, 24 - and 48 - hour time points. The wound healing efficiency was quantitatively assessed by measuring the number of migrating dermal fibroblasts into the scratching area (Stunova and Vistejnova, 2018). The experiment was performed in triplicate and 3 images of each condition were analyzed.

5.3.6 Reverse transcription polymerase chain reaction (RT-PCR)

HDFs were treated with different concentrations of CHS (0, 6.25, 12.5, 25, 50 and 100%), and collected. Total RNA was extracted using NucleoSpin RNA kit (Macherey-Nagel, Dueren Germany), according to the manufacturer's protocol. Then, 1 µg of RNA was converted to complementary DNA (cDNA), using ReverTra Ace™ qPCR RT Kit (Toyobo. Osaka, Japan). Reverse transcription polymerase chain reaction (RT-PCR) was carried out in C1000 Touch Thermocycle (Biorad, California, USA) for 35-40 cycles using 2x Taq Master Mix (Vivantis Technologies, Selangor, Malaysia) with specific primers as listed in Table 5.1. PCR products were separated by agarose gel electrophoresis and visualized under UV after staining with RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology, Gyeonggi-do, Korea). The relative expression level of a target gene was quantified by normalization with the internal control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene using NIH Image J 2.0 software.

5.3.7 Immunofluorescent microscopy

HDFs were cultured on coverslips, and treated with 12.5 % of NHS and CHS for 24 hours. The cells were then washed twice in PBS, fixed with 4% paraformaldehyde in PBS at room temperature for 30 minutes, washed 3 times for 5 minutes each, and permeabilized with 0.2% Triton X-100 in PBS for 20 minutes. After blocking with 10% FBS in PBS for 1 hour, the cells were incubated with C1A1 or LC3 antibody (Merck, Darmstadt, USA) overnight at 4 °C, washed three times in PBS, and

incubated in FITC-conjugated anti-rabbit IgG (Merck, Darmstadt, USA) for 1 hour. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, California, USA), and the stained cells were observed under ZOE Fluorescent Cell Imager (Biorad, California, USA). Images were analyzed using using NIH Image J 2.0 software (G. Y. Liu et al., 2017).

5.3.8 NHS and CHS composition determined by cytokine array

The presence of cytokines within NHS and CHS was detected using Proteome Profiler™ Array (Catalog No. ARY005B, USA R&D Systems, Minnesota, USA), according to the manufacturer's protocol with adjusted 15-minute exposure. The dot densities both between and among arrays were measured, subtracted with the background signals, and normalized with the referenced spots using NIH Image J 2.0 software.

5.3.9 Statistical analysis

Statistical analyses were performed using the IBM SPSS Statistics, version 16.0 (SPSS Inc., Chicago, USA). All experiments were performed for three replicates, and data were expressed as mean ± standard deviation (SD). Significant differences among treatments were determined by one-way analysis of variance (ANOVA), followed by Tukey's test at $p < 0.05$.

Table 5.1 The comparison of cordycepin production by liquid culture of *Cordyceps* in this study and other studies.

Gene set	Genes	Primers	Sequence (5'-3')
House keeping gene	Glyceraldehyde 3-phosphate dehydrogenase	*gapdh* Forward	5'- CTCTGCTCCTCCTGTTCGAC-3'
		gapdh Reverse	5'- TTAAAAGCAGCCCTGGTGAC-3'
Antioxidant genes	Superoxide dismutase	*sod* Forward	5'-CTAGCGAGTTATGGCGAC-3'
		sod Reverse	5'-CATTGCCCAAGTCTCCAAC-3'
	Catalase	*cat* Forward	5'-TCCGGGATCTTTTTAACGCCATTG-3'
		cat Reverse	5'-TCGAGCACGGTAGGGACAGTTCAC-3'
	Glutathione peroxidase	*gpx* Forward	5'-CGCCAAGAACGAAGAGATTC-3'
		gpx Reverse	5'-CAACATCGTTGCGACACAC-3'
Autophagy related genes	Ubiquitin-binding p62 protein	*p62* Forward	5'-GGTGCAAGAAGCCATTTAGG-3'
		p62 Reverse	5'-GCCATTAGGCAAGCTATGTG-3'
	Autophagy related 5	*atg5* Forward	5'-AGCTCTGGATGGGACTG-3'
		atg5 Reverse	5'- CTCCGTCGCGTGGTCTGAT-3'
	Beclin-1 protein	*becn1* Forward	5'-GTGGACAGTTTGGCACAAT -3'
		becn1 Reverse	5'-TGCCTCCTGTGTCTTCAAT-3'
	Microtubule-associated proteins 1A/1B light chain 3B (LC3)	*1c3* Forward	5'- CCCACCAAGATCCCAGTGAT-3'
		1c3 Reverse	5'- CCAGGAACTTGGCTTGTCCA-3'

Table 5.1 (continued).

Gene set	Genes	Primers	Sequence (5'-3')
Proliferation	Marker Of Proliferation Ki-67	*mki67* Forward	5'- TGTATCCTTTGGTGGTCGTCTA-3'
		mki67 Reverse	5'- GCTGGAGTGTGAGTGGTGAG-3'
Extracellular matrix-related genes	Collagen type I alpha 1 chain	*col1a1* Forward	5'-GGGCAAGACAGTGATTGAATA-3'
		col1a1 Reverse	5'-ACGTCGAAGCCGAATTCCT-3'
	Collagen type I alpha 2 chain	*Col1a2* Forward	5'-TCTGGATGGATTGAAGGGACA-3'
		Col1a2 Reverse	5'-CCAACACGTCCTCTCTCACC-3'
	Collagen type III alpha 1 chain	*col3a1* Forward	5'-AGGTCCTGCGGGTAACACT-3'
		col3a1 Reverse	5'-ACTTTCACCCTTGACACCCTG-3'
	Elastin	*eln* Forward	5'-CCGCTAAGGCAGCCAAGTATGGA-3'
		eln Reverse	5'- AGCTCCAACCCCGTAAGTAGGAAT -3'
	Matrix metalloproteinase 1	*mmp1* Forward	5'-GCTCTTGGCAAATCTGGCCTGTAA-3'
		mmp1 Reverse	5'-AGTGACTGGGAAACCAGATGCTGA-3'
	Matrix metalloproteinase 3	*mmp3* Forward	5'-CCTGCTTTGTCCTTTGATGC-3'
		mmp3 Reverse	5'-TGAGTCAATCCCTGGAAAGTC-3'

5.4 Results

5.4.1 The effects of NHS and CHS on human dermal fibroblast viability

HaCaT cells were cultured with different concentrations of cordycepin (varied from 1.25 μM- 50 μM), and cell toxicity of cordycepin were then assessed by MTT. 2.5 μM cordycepin turned out to be to the optimal sub-toxic concentration to be used in CHS preparation since cell proliferation was increased and no significant change in cell morphology was observed (Figure 5.1a). After NHS and CHS preparation by previously described protocols, both types of HaCaT secretome with different concentrations (0-100% by volume) were applied to HDF cultures to evaluate their effects of each type of secretome on HDF cell viability. While HDF cultured in different concentrations of NHS showed no difference in cell viability, the proliferation of HDF cultured in different concentrations of CHS contrarily increased in dose-dependent manner (Figure 5.1b) reflecting the beneficial effects of CHS on HDF. The expression of Ki67, the marker of proliferation, was also observed. HDF cultured with CHS showed higher mRNA and protein expression levels of Ki67 than those with NHS and without any secretome (Figure 5.1c and 5.1d) which was conformed with previously monitored cell viability. Hence, CHS was obviously more beneficial for HDF and had been evaluated its skin rejuvenation in further experiments.

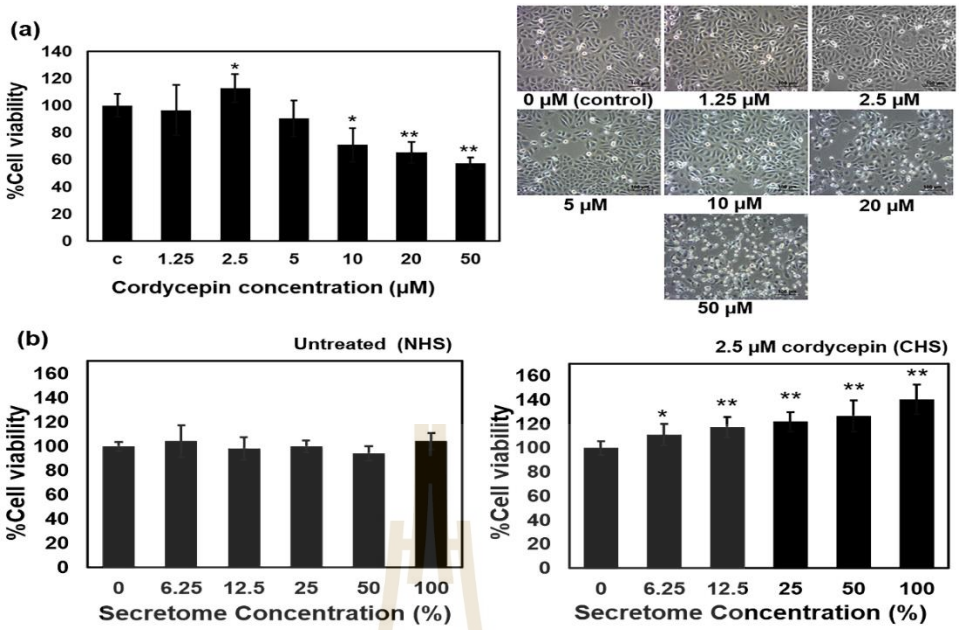

Figure 5.1 The determination of cordycepin optimal concentration used in CHS production and the primarily observation of CHS effect on HDF proliferation. (a) MTT cell viability assay and microscopic images of HaCaT cells in responding to cordycepin. 2.5 μM cordycepin was the optimal concentration since it promoted HaCaT proliferation without any morphological alteration. Scale bar = 200 μm (*p < 0.05, **p < 0.01). (b) MTT cell viability assay of HDF in responding to NHS and CHS. While NHS had no impact on HDF proliferation, CHS was found to promote HDF proliferation in dose-dependent manner (*p < 0.05, **p < 0.01). (c) Densitometry analysis for Ki67 mRNA expression. Such proliferation marker was found to be highly upregulate in NHS- and CHS-treated HDF compared to those and without any secretome. Notably, CHS-treated HDF showed the highest ki67 expression CHS-treated HDF also showed significantly higher Ki67 expression than NHS-treated HDF (d) Representative fluorescence-microscopy images showing the highest fluorescent intensity of Ki67 presented in CHS-treated HDF. Scale bar = 100 μm. The asterisks indicate significant differences relative to the control, non-treated HDF (**p < 0.01). Hashes indicate significant differences between NHS- and CHS-treated HDF (##p <0.01). The experiments were done in triplicate. Scale bar = 100 μm.

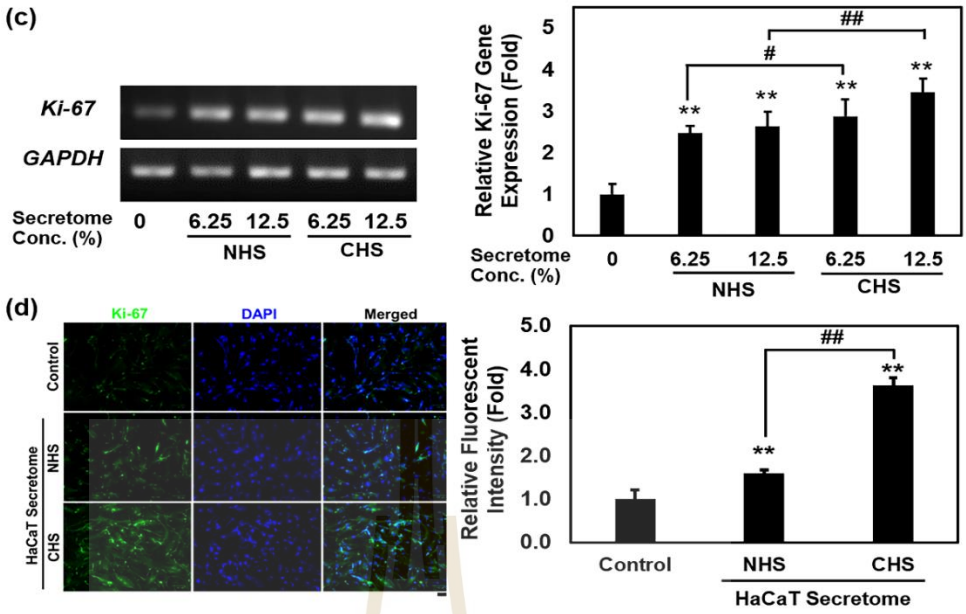

Figure 5.1 (continued).

5.4.2 CHS exhibited superior ROS scavenging property

The over-production and accumulation of reactive oxygen species (ROS) referred as free radicals or oxidants could lead to oxidative that can cause skin damage and aging (J. Chen, Liu, Zhao, and Qiu, 2021). To evaluate the effectiveness of CHS as an antioxidant, HDF were cultured with different concentrations of NHS or CHS (12.5, 25 and 50 μM) and 5mM of NAC, the conventional ROS scavenger, and then challenged with 1 mM H_2O_2 to stimulate mitochondrial ROS generation. The antioxidant efficiency of CHS was presented by scavenging the •OH visualized by DCF relative fluorescent intensity compared to NAC. Prior H_2O_2 challenging, CHS was found not to increase DCF relative fluorescent intensity indicating how it was not causing any oxidative stress to HDF. After Challenging with H_2O_2, CHS was also found to significantly decrease DCF relative fluorescent intensity in the comparable level to NAC while no statistical significance was found with NHS-treated HDF (Figure 5.2a). The gene expression levels of a number of key genes involving in intracellular ROS scavenging mechanisms were also measured. The mRNA expression levels of superoxide dismutase (SOD), superoxide glutathione peroxidase (GPX) and catalase (CAT) were found to be elevated in HDF cultured with different concentrations of CHS and also in dose-dependent manner (Figure 5.2b).

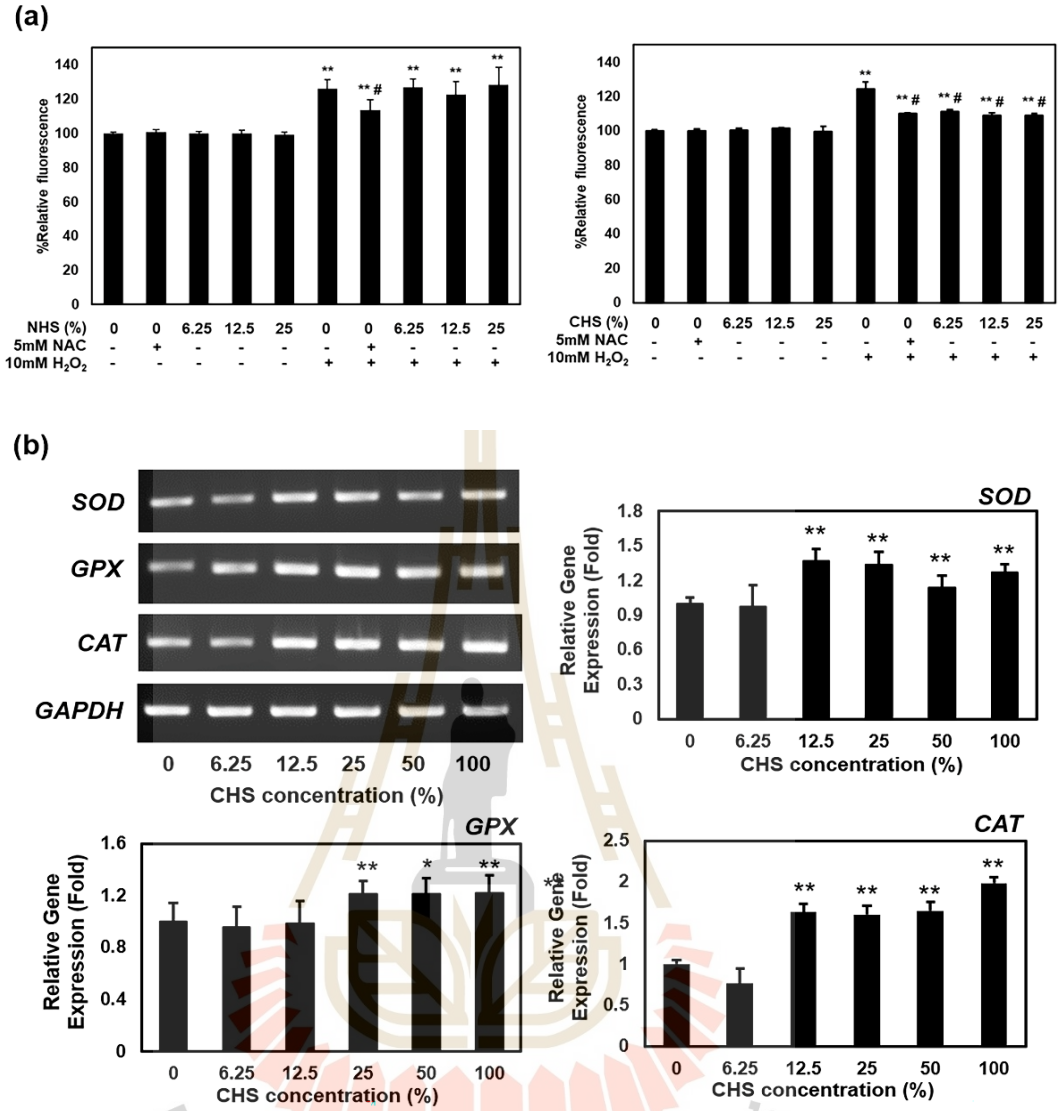

Figure 5.2 CHS exhibited ROS scavenging property through the upregulation of intracellular ROS scavenging pathways (a) The Quantitative analysis of DCFH-DA assay of NHS- and CHS- treated HDF showing the comparable ROS scavenging capability in the comparable level of N-acetyl cysteine (NAC). The asterisks indicate significant differences relative to non-treated HDF (**p < 0.01). Hashes indicate significant differences relative to non-treated HDF with H_2O_2 stimulation (##p <0.01). (b) Densitometry analysis for the expression of genes involving in intracellular ROS scavenging pathways. CHS promoted the expression of superoxide dismutase (*SOD*), superoxide glutathione peroxidase (*GPX*) and catalase (*CAT*). (**p < 0.01). The experiments were done in triplicate.

5.4.3 CHS effectively promoted wound healing in HDF

Skin regeneration was associated with the restoration of dermis caused by migration and proliferation of fibroblasts (Takeo, Lee, and Ito, 2015). The *in vitro* wound-healing assay was performed. The monolayer of HDF were scratched with sterile 200 µL autopipette tip to mimic the cutaneous skin damage and later treated with 12.5 % by volume of NHS and CHS. The images of HDF movement and migration into the scratch area were captured at 0, 24 and 48 hours post scratching. The number of cells in the scratch area was counted and compared to the control condition without any secretome treatment. Both NHS and CHS treating conditions was found to exhibit faster cell migration than the control condition. As expected, HDF treated with CHS exhibited the fastest cell migration implied to its highest wound healing potential (Figure 5.3).

5.4.4 CHS regulated extracellular matrix components in HDF

HDF produces several extracellular matrix proteins to nourish and support the foundation of skin. Collagen and other ECM deposition are required to maintain skin condition and facilitate wound repair. To monitor whether CHS affected on the production and degradation of extracellular matrix (ECM) components, the mRNA expression of genes encoding three ECM components including collagen type I alpha 1 chain (COL1A1*),* collagen type I alpha 2 chain (COL1A2), collagen type 3 alpha 1 chain (COL3A1) and elastin and genes encoding two enzymes including matrix metalloproteinase 1 (MMP-1) and matrix metalloproteinase 3 (MMP-3) was investigated in HDF cultured with CHS. As shown in Figure 4a, the expression of all ECM component genes was upregulated while the expression of both types of MMP, respectively responsible for the degradation of collagen and elastin, were downregulated (Figure 5.4a) indicating that CHS posessed the capability to promote the ECM production and also decrase the tendency of ECM degradation. Additionally, most of these genes exhibited a dose-dependent responsive manner to the concentration of CHS. To further confirm the ECM regulation effect in protein level, the expression of COL1A1 protein were comparatively of observed in HDF with 12.5% by volume of NHS, CHS and without any secretome via immuno-fluorescent microscopy. The significantly highest production of COL1A1 were showed in HDF cultured with CHS as expected (Figure 5.4b).

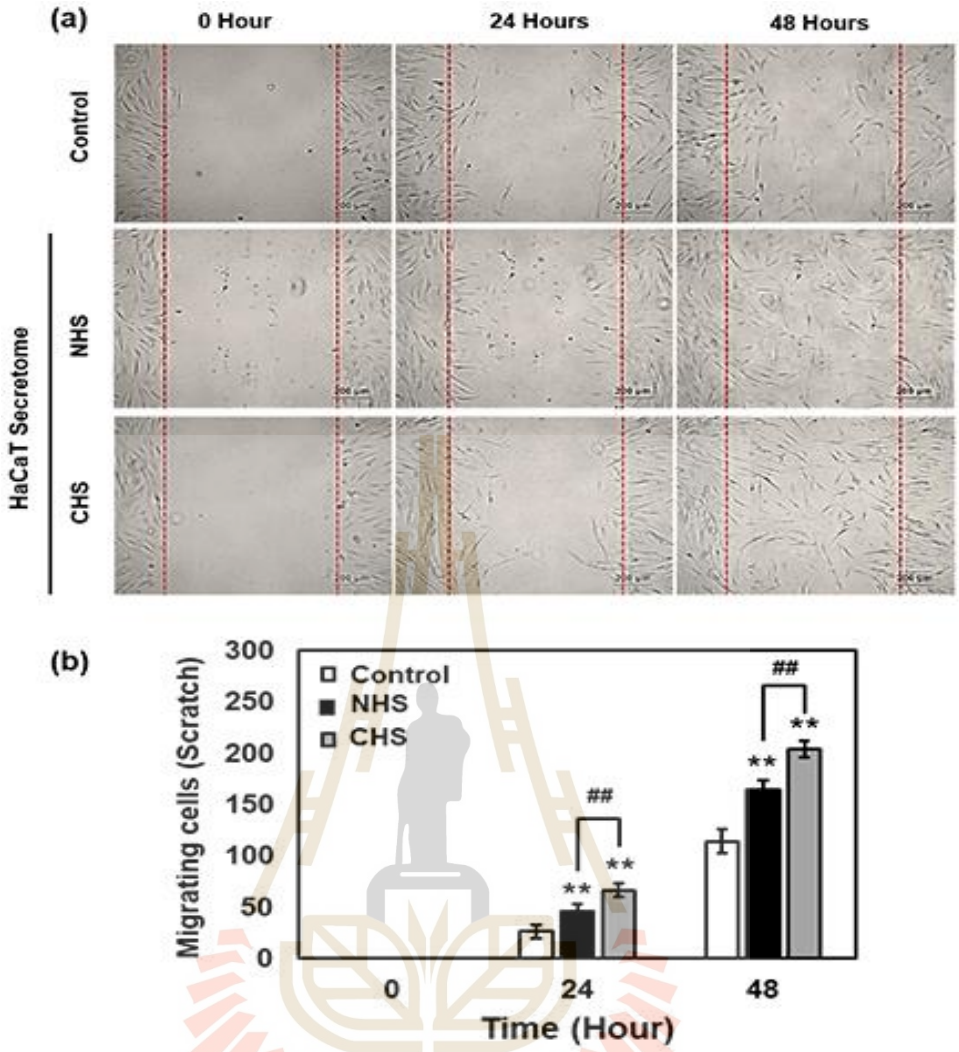

Figure 5.3 CHS promotes wound healing in dermal fibroblasts compared to NHS and control without any secretome. (a) Representative images of wound healing assay and (b) Quantitative analysis of migrating HDF cells in the scratch area in responding to NHS and CHS treatment after at 0, 24 and 48 hours post scratch. The asterisks indicate significant differences relative to the control, non-treated HDF (**p < 0.01). Hashes indicate significant differences between NHS- and CHS- treated HDF (##p <0.01). The experiments were done in triplicate. Scale bar = 200 μm.

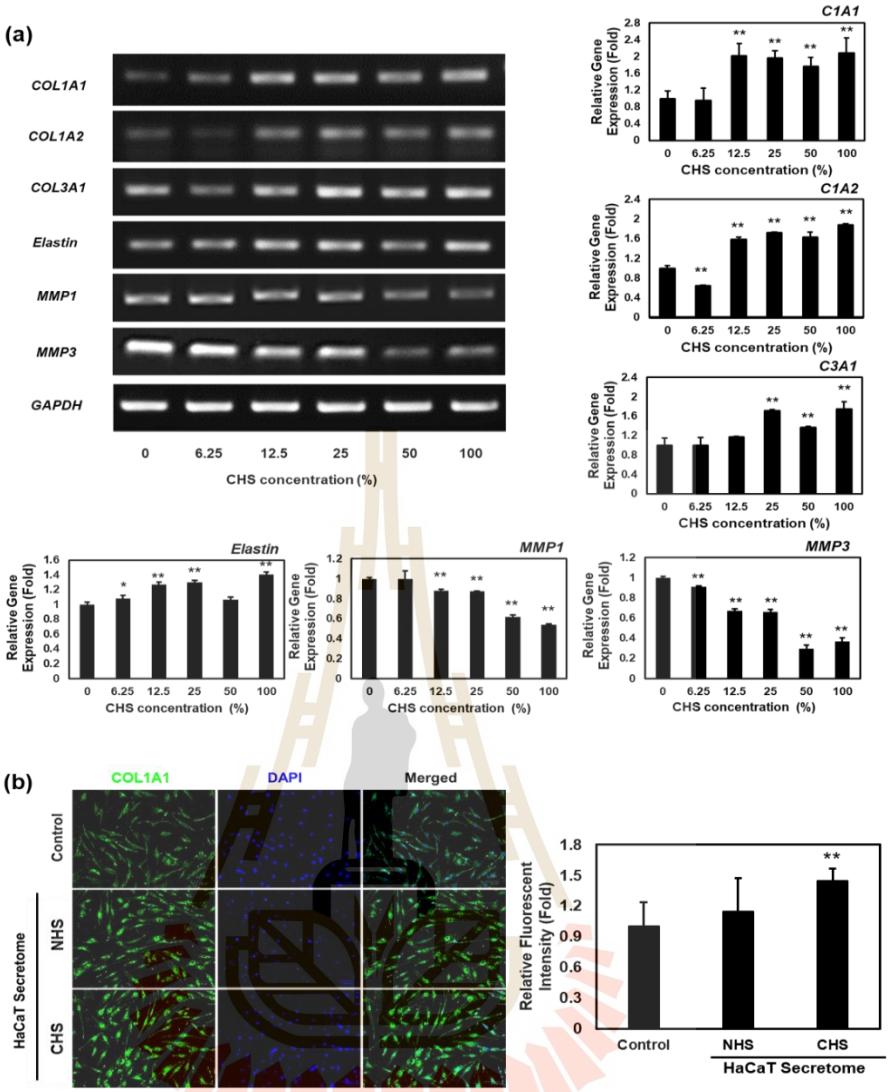

Figure 5.4 CHS regulated the expression of extracellular matrix in both transcriptional and translational level. (a) Densitometry analysis for mRNA expression of ECM-related genes. The mRNA expression of *COL1A1, COL1A2, COL3A1* and *elastin* were upregulated. Contrarily, the mRNA expression of matrix metalloproteinases, *MMP1* and *MMP3* was downregulated. Most all of expression alteration is in dose-dependent manner (*$p < 0.05$, **$p < 0.01$). (b) Representative fluorescence-microscopy images showing the highest fluorescent intensity of COL1A1 protein presented in CHS-treated HDF (**$p < 0.01$) However, the differences between NHS- and CHS- treated HDF was not statistically significant. The experiments were done in triplicate. Scale bar = 100 μm.

5.4.5 CHS activated autophagy in HDF

Autophagy, an intrinsic process of cellular self-digestion, plays important role in the skin response under stress conditions to maintaining cellular homeostasis (H. S. Kim, Park, Moon, Lee, and Kim, 2018). In order to demonstrate how CHS modulates autophagy in HDF, the mRNA expression of genes encoding several crucial proteins functioned along autophagy processes were investigated in CHS-treated HDF. The expression of ubiquitin-binding p62 protein (p62), autophagy related 5 (ATG5), Beclin-1 and Microtubule-associated proteins 1A/1B light chain 3B (LC3) showed a rising tendency along with the increase of CHS concentration. Among them, the mRNA expression of LC3 was drastically upregulated in dose-dependent manner (Figure 5.5a). The highly expressed LC3I/II protein was correspondingly found in CHS-treated HDF compared to the conditions with NHS and without any secretome using immuno-fluorescent microscopy(Figure 5.5b). Both transcriptional and translational evidences emphasized the autophagy activation property of CHS in HDF.

5.4.6 Cordycepin altered the composition of HaCaT secretome, and primed to the superior biological effects of CHS on HDF

Based on biological activity observation of HaCaT secretome in HDF in this study, cordycepin-induced HaCaT secretome (CHS) exhibited predominant skin benefits in many aspects. The determination of the composition differences between CHS and NHS helped shed light on such superior skin benefits. The comparative level of cytokine presented within NHS and CHS was detected using Proteome Profiler™ Array. There were 6 types of cytokines found in HaCaT secretome, both NHS and CHS, including monocyte chemoattractant protein-1 (CCL2), chemokine C-X-C motif ligand 1 (CXCL1), interleukin 1 receptor antagonist (IL1-RA), interleukin 6 (IL-6), interleukin 8 (IL-8), macrophage migration inhibitory factor (MIF) and plasminogen activator inhibitor 1 (PAI-1). Among them, the level of CXCL1 and IL1-RA was drastically higher in CHS. IL-8, MIF and PAI-1 were also found to be slightly increased by the cordycepin induction Additionally, CCL2 and IL-6 were found in minimal level compared to other cytokines in both NHS and CHS (Figure 5.6).

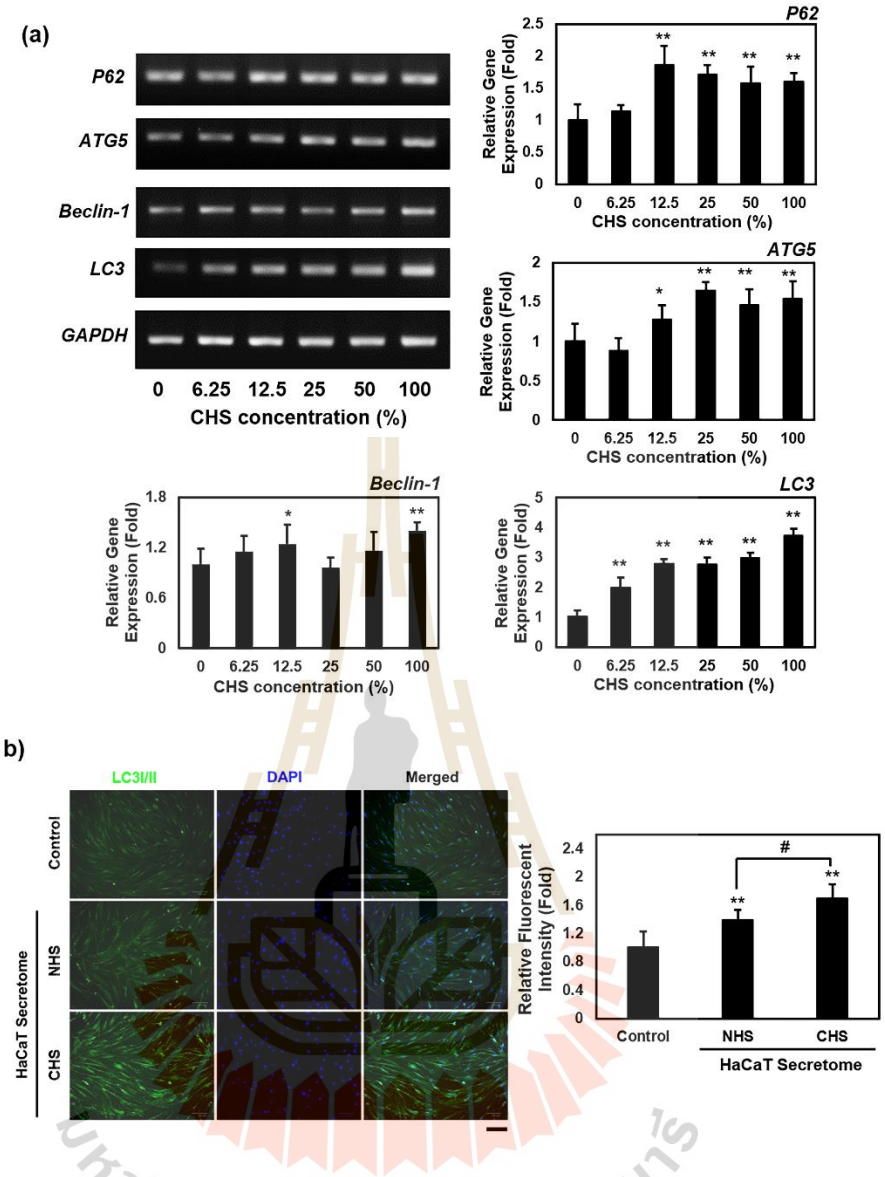

Figure 5.5 CHS activated the expression of autophagy-related genes in both transcriptional and translational level. (a) Densitometry analysis for mRNA expression of autophagy-related genes indicating the upregulation of *P62, ATG5, Beclin-1* and *LC3*. The most obvious upregulation was found in LC3 expression (*p < 0.05, **p < 0.01). (b) Representative fluorescence-microscopy images showing the highest fluorescent intensity of LC3I/II protein presented in CHS-treated HDF. The asterisks indicate significant differences relative to the control, non-treated HDF (**p < 0.01). Hashes indicate significant differences between NHS- and CHS- treated HDF (#p <0.05). The experiments were done in triplicate. Scale bar = 100 μm.

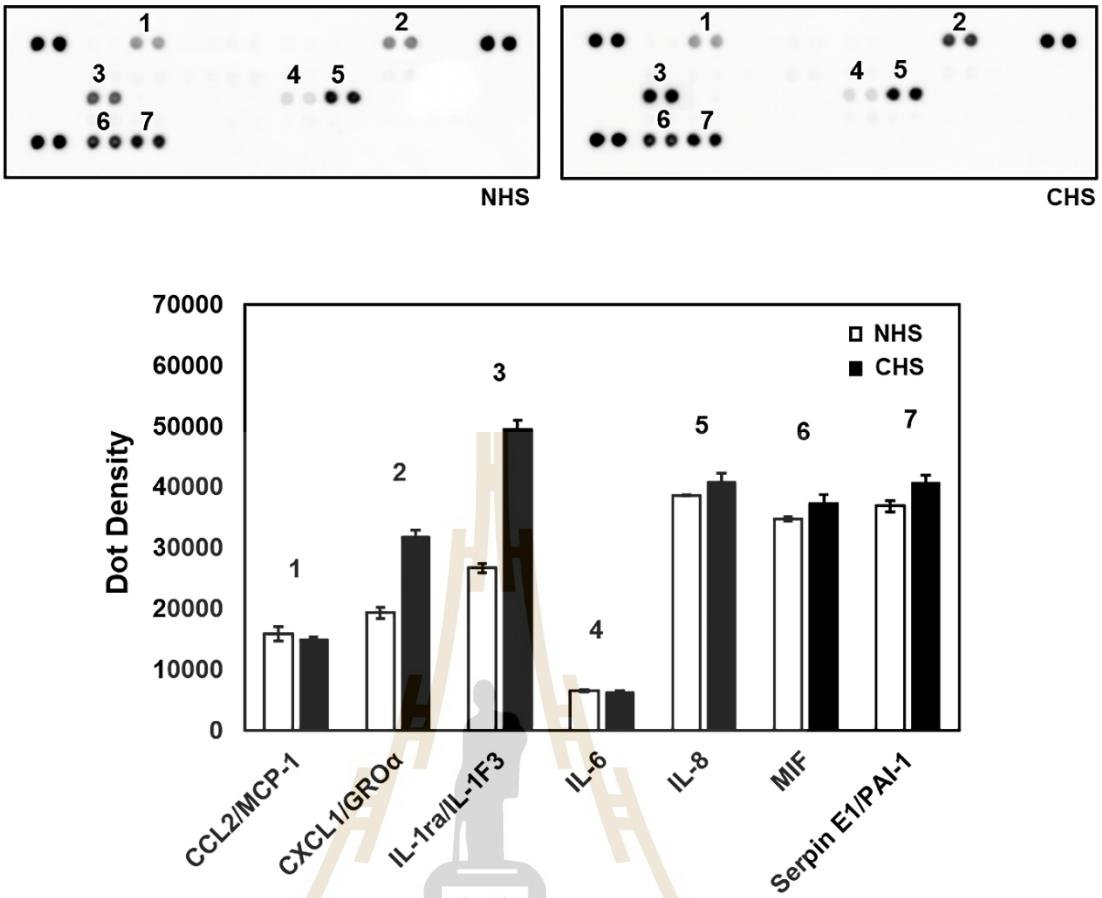

Figure 5.6 Densitometry analysis for the determination and comparison of cytokine components of NHS and CHS. Monocyte chemoattractant protein-1 (CCL2), chemokine C-X-C motif ligand 1 (CXCL1), interleukin 1 receptor antagonist (IL1-RA), interleukin 6 (IL-6), interleukin 8 (IL-8), Macrophage migration inhibitory factor (MIF) and Plasminogen Activator Inhibitor-1 (PAI-1) were found in both NHS and CHS. Among them, CXCL1, IL-1Ra, IL-8, MIF and PAI-1 were Found to be higher in CHS.

5.5 Discussion

Skin regeneration is the innate ability of skin to recover damaged compartments of skin tissue to maintain skin function and integrity (Takeo et al., 2015). Wound healing, a vital process of skin regeneration, requires the collaborative actions of many cell types including the phases of haemostasis, inflammation, re-epithelialization, and contraction/tissue remodelling which are governed by cytokines and matrix signals presented at a wound site (Martin, 1997). Among all cell lineage, the interaction

between skin's resident cells during the inflammatory phase of the skin repair/regeneration process, primarily keratinocytes and dermal fibroblasts, is required in order to proliferate and mature to assist wound healing (Wojtowicz et al., 2014). In addition, keratinocytes and fibroblasts coordinate their activity to restore normal tissue homeostasis after injury through double paracrine signalling loops known as cross talk or dynamic reciprocity, mostly mediated by secretable substances. Several studies have revealed the regulatory involvement of keratinocyte- releasable factors in the expression of genes in dermal fibroblasts. The co-cultures of fibroblasts and keratinocytes was reported to upregulate 243 genes and downregulate 100 genes in dermal fibroblasts compared to the independent culture (Nowinski et al., 2004) including the genes responsible for ECM synthesis (Ghaffari et al., 2009; Harrison et al., 2006). Thus, the investigation of the impact of the keratinocyte secretome and its constituent components on dermal fibroblasts could aid in the development of innovative therapies to maintain skin integrity. However, the variation effects of keratinocyte releasable factors were also reported in different studies depending on the sources and origins of keratinocytes. Sato et al. (1997) found that keratinocyte secretome greatly enhanced the production of lipid mediators Prostaglandin E2 (PGE2) involved in the wound healing response in fibroblasts due to the induction of COX-2 mRNA expression by pro il-1α released from keratinocytes [3]. Ghaffari et al. (2009) found that pro IL-1 in keratinocyte secretome reduced pro-a1(I) collagen expression and increased MMP-1 expression in dermal fibroblasts at the level of mRNA and protein to prevent fibrotic conditions. Bukowska et al. (2018) reported that viability of dermal fibroblast was stimulated by keratinocyte secretome collected from Foxn-1 active keratinocytes. Therefore, the possibility to improve the quality of keratinocyte secretome via some adding regulators is worthy to be explore and may prove to be beneficial for further application.

In this study, we used HaCaT cells as the source of secretome to relieve the concerns associated with using primary keratinocytes, such as donor-to-donor variability in growth characteristics and in vitro responses, different plating efficiencies, the short lifetime in culture, and changes in proliferation and differentiation characteristics with increasing number of passages, which may complicate experimental data interpretation (Colombo et al., 2017). We applied cordycepin to

HaCaT culture aiming to alter the compositions of releasable cytokines containing in the secretome. The results suggested that cordycepin treatment could alleviate the biological effects of HaCaT secretome in many aspects. CHS significantly induced the proliferation and migration of dermal fibroblast indicating how CHS potentially facilitated the dermis to restore through migration and proliferation of fibroblasts to close the wound site and reestablish the skin's barrier function. Accordingly, the increase in proliferation and migration are agreeable with literatures that it influences the success of tissue healing during the skin regeneration process. (Addis et al., 2020; Z. Sun and Williams, 2016; Takeo et al., 2015). Beside the increase of cell viability, CHS possessed the regulation capability on extracellular matrix synthesis in HDF. The upregulated expression of collagen type1 and type 3 and also elastin was reported to support the production of such ECM components to deposit in the wound in a precise reticular pattern, resulting in better extracellular matrix restoration (King, Balaji, Le, Crombleholme, and Keswani, 2014; Rognoni et al., 2018). The presence of CCL2, also referred as monocyte chemoattractant protein 1 (MCP-1) and the upregulation of both chemokine CXCL1 and MIF in the CHS could be responsible for such increase of cell viability, migration and ECM production. It has been observed that CCL2, a powerful macrophage chemoattractant, improves wound healing in mice with diabetes caused by streptozotocin (STZ) through the generating growth factors that promote cell proliferation and protein synthesis. CCL2 therapy boosted the generation of endothelial progenitor cells at wound sites and accelerated wound closure rates, neovascularization and ECM synthesis, which ultimately aided diabetic cutaneous wound healing (Ishida et al., 2019). The CCL-2/MCP-1 was also previously reported to promote pulmonary fibroblast proliferation and migration (Liu et al., 2015). CXC chemokines, such as CXCL1, are recognized for their ability to directly induce angiogenesis, allow cell migration into the wound, and provide the necessary metabolic support for the rapidly multiplying wound cells (Ridiandries, Tan, and Bursill, 2018). Interestingly, cordycepin itself was also reported to bind and activate adenosine receptor which subsequently induce dermal fibroblast migration and promote wound healing *in vitro* via the stimulation of Wnt/β-catenin signaling (J. Kim et al., 2021). Such compatibility of cordycepin to bind and activate adenosine receptor could possibly lead to the modulation of HaCaT secretome since the activation of adenosine A2

receptor was found to enhance the expression of CXCL1 in keratinocyte (Uribe-Herranz, Lian, Hooper, Milora, and Jensen, 2013). MIF, a key pro-inflammatory cytokine that integrates the immune, neuronal and endocrine systems is known to regulate the synthesis and release of other cytokines and interferons during wound healing processes (Calandra and Roger, 2003). Keratinocyte MIF production *in vivo* and *in vitro* was induced by certain uncommon environments such as ultraviolet exposing to cope and response with the responding conditions in order to promote dermal fibroblast migration and artificial wound closure *in* vitro (Gilliver, Emmerson, Bernhagen, and Hardman, 2011). MIF has also been proven to enhance the proliferation and migration of HDF (Dewor et al., 2007). The expression of matrix metalloproteinases, MMP-1 and MMP-3, was contrarily downregulated by CHS administration to even more promote the availability of ECM during wound healing processes. Since MMP-1 and MMP-3 production was reported to be induced by interleukin-1 beta (IL-1β) (M. S. Chen et al., 2018; Stewart et al., 2007), the enhancement of IL1-RA level in CHS could lead to the down-regulation of MMPs. IL1-RA, a cytokine belonging to the interleukin 1 family, is a naturally occurring inhibitor of the pro-inflammatory impact of IL-1β by preventing the binding of IL-1β to its receptor so its bioactivities are minimized, (The induction of MMP production, in this case) of but does not cause any intracellular responses (Arend, Malyak, Guthridge, and Gabay, 1998; Dayer, Oliviero, and Punzi, 2017).

Reactive oxygen species (ROS) have a contribution in the wound-healing process. When ROS levels are low, they help to prevent infections and speed up wound healing by producing cell-survival signalling, but when levels are high, they cause oxidative stress, which damages cells and promotes an inflammatory state (Dunnill et al., 2017). If redox imbalance occurs due to the levels of ROS exceed the capacity of endogenous antioxidants to scavenge them, the healing processes are interrupted so the focus on management of ROS and antioxidant levels to control redox equilibrium is essential (Comino-Sanz, Lopez-Franco, Castro, and Pancorbo-Hidalgo, 2021). Here, we reported the antioxidant potential of CHS to be comparable to NAC which was regulated trough the upregulation of genes involving in intracellular ROS scavenging mechanisms including SOD, GPX and CAT. From literatures, IL-1β has been responsible for •NO and $O•_2$ radical production and disturbed the antioxidant enzyme system in bovine chondrocytes (Mathy-Hartert et al., 2008) lead to a delayed increased of GPX and a

decrease of CAT activity resulting in an accumulation of H_2O_2 in mitochondria. Therefore, the highly presented IL-Ra in CHS could possibly inhibit the ROS production and accumulation in HDF caused by intracellular IL-1β.

As one of the vital mechanisms in both fibroblast proliferation and extracellular matrix remodelling, autophagy also plays a significant role in wound healing. We found that CHS upregulated the gene involving in autophagy including LC3, P62, ATG5 and Beclin-1 indicated that acceleration of wound healing partially involved with autophagy modulation which could be responsible by CXCL1 and MIF. According to reports, CXCL1 is crucial in controlling metastasis and autophagy. With CXCL1 overexpression, the expression levels of autophagy-related proteins were found to be noticeably increased in breast cancer cells, which subsequently promote autophagy and cell migration (N. Wang et al., 2019; Yang et al., 2021). Additionally, MIF was stated to activate autophagy via the AMPK/mTOR signalling pathway, consequently increasing the expression of proteins associated with autophagy (such as LC3BI/LC3BII, Beclin-1, and autophagy protein 5) to help protecting mesenchymal stem cells from apoptosis (Xia and Hou, 2016). The increase of autophagic flux also promote the extracellular matrix production and inhibit matrix metalloproteinase expression. Beside the wound healing perspectives, considering to their lengthy lifespan, human dermal fibroblasts are vulnerable to both intrinsic and extrinsic harm. Skin damage and aging have been linked to changes in fibroblast autophagic flux (Eckhart, Tschachler, and Gruber, 2019). Additionally, the microenvironment may be impacted by autophagy and its age-related dysfunction in chronologic, UV-induced, and premature aging phenotypes (Rubinsztein, Marino, and Kroemer, 2011). Dysregulation of the proteasome and autophagy aging skin is thought to be responsible for the loss of proteostasis and cause skin deterioration and aging. Tashiro et al. (2014) reported that the fibroblast treatment with pepstatin A (leu/pep), a significant lysosomal protease inhibitor, caused autophagy disruption and resulted in decreased levels of type I procollagen, hyaluronan, and elastin as well as elevated levels of MMP-1 which could potentially lead to the loss of dermal integrity. According to genes involved in autophagy were upregulated by CHS treatment, it demonstrated CHS anti-aging properties and suggested that CHS may help solving impaired autophagic flux, reduction of ECM components and collapse of dermal structure. Although autophagy process is potent

to skin regeneration and wound healing, it is still debatable if autophagy processes is advantageous for the treatment of skin problems because autophagic imbalance could give rise to some undesirable skin concerns. Hypertrophic scar (HS), a serious skin fibrotic disease characterized by hypercellularity and excessive extracellular matrix (ECM) component deposition resulting in irregularly shaped nodules, congestive appearances, and an uneven skin surface, accompanied by paraesthesia sensations such as itching and pain (Lingzhi, Meirong, and Xiaobing, 2020), was reported to involve with excessive autophagy activation. Shi et al. (2017) found that autophagosome as well as LC3 protein were elevated in HS tissues with the involvement of B-cell lymphoma-extra large (Bcl-xL) and the knockdown of LC3 protein could improve the appearance of fibrotic scar in rabbit. Recently, Y. Liu et al. (2021) investigated dynamic alteration of autophagy during HS development and reported that autophagic activity increased in the initial and stabilized stage of HS formation. In addition, treatment of autophagy inhibitors could potentially prevent the development of HS in rabbit model, whereas autophagy inducers caused the reverse effect. However, it should be noted that mechanisms of autophagy could work differentially in HS and normal skin and the numbers of scientific literatures pointed out how essential autophagy is to skin wellness. Therefore, the development of novel strategy to indicate and maintain the well-balanced autophagic activity in skin is the key for autophagy-related therapeutic application

Considering other enhanced components in CHS, IL-8, and PAI-1 could be contributed in the superior effects of CHS in skin regeneration. IL-8 or chemokine (C-X-C motif) ligand 8 is a chemoattractant cytokine that was reported to influence dermal fibroblast phenotypic behaviours. IL-8 induced HDF to have a migratory phenotype with the lack of focal adhesion (Dunlevy and Couchman, 1995). Thus, IL-8 mediated the migration oi of fibroblasts and facilitates wound healing (Dobreva, Waeber, James, and Widmann, 2006) Lastly, PAI-1 is the important regulator of the pericellular proteolytic and fibrinolytic cascades in wound healing processes. Although, there was no obvious evidence how this cytokine assists fibroblast in wound healing processes, PAI-1 took part in keratinocytes adhesion and migration during wound healing (Providence et al., 2008).

Notably, the co-culture of human epidermal keratinocyte and dermal fibroblast was previously reported to induce prostaglandin E2 production and cyclooxygenase 2 activity due to the mediation of proIL-1α released from keratinocyte (Sato et al., 1997). Such cell-cell interaction was proposed to involve in wound healing and ECM organization after injury. Contrarily, proIL-1α was not detected in CHS in this study which possibly due to the absence of the dermal fibroblast in the culture environment along the way of CHS collection. Therefore, HaCaT could independently produce the secretome with different cytokine profile from the co-culture scenario. The effect of keratinocyte secretome on collagen synthesis of dermal fibroblast was found to be controversially inconsistent. Granulocyte colony-stimulating factor (G-CSF) inhibited collagen expression in dermal fibroblasts was also found and identified in a co-culture system (Carr, Li, Rezakhanlou, and Ghahary, 2017). The absence of G-CSF in stand-alone HaCaT cultivation is due to the lack of HDF stimulation signals to indicate the excessive collagen production.

Although the findings of this study elucidated the possible biological influences of CHS on HDF to assist skin regeneration and wound healing, some future perspective should be mentioned. To illustrate a clearer regulatory network of CHS, the effects of such secretome on other cell types involved in wound healing such as immune cells and endothelial cells are still needed to be evaluated. Specific study focusing on particular cytokines that were upregulated by codycepin such as CXCL1 and IL-1Ra could also be beneficial to fulfill the big picture of the system. Ultimately, further i*n vivo* study, is necessary to bridge the gap of uncorrelation between *in vitro* and clinical situations of and make the translation of this finding to therapeutic application becoming more practical.

5.6 Conclusion

By the induction of cordycepin, cordycepin-induced secretome (CHS) collected from HaCaT cultured possessed the capability to assist skin regeneration and wound healing through its bioactivities on dermal fibroblasts including induction of cell proliferation and migration, ROS scavenging, regulatory effects on extracellular matrix production and autophagy modulation. The exceptional bioactivity of CHS could be related to the positively alteration of some secretome components including CXCL1, IL-1Ra, IL-8, MIF and PAI-1. Therefore, the cordycepin treatment strategy was efficient

to improve the quality of HaCaT secretome to be the potential ingredient in cosmeceutical product and proven to be beneficial implications towards the further improvement of skin regeneration in advanced novel therapies (Figure 5.7).

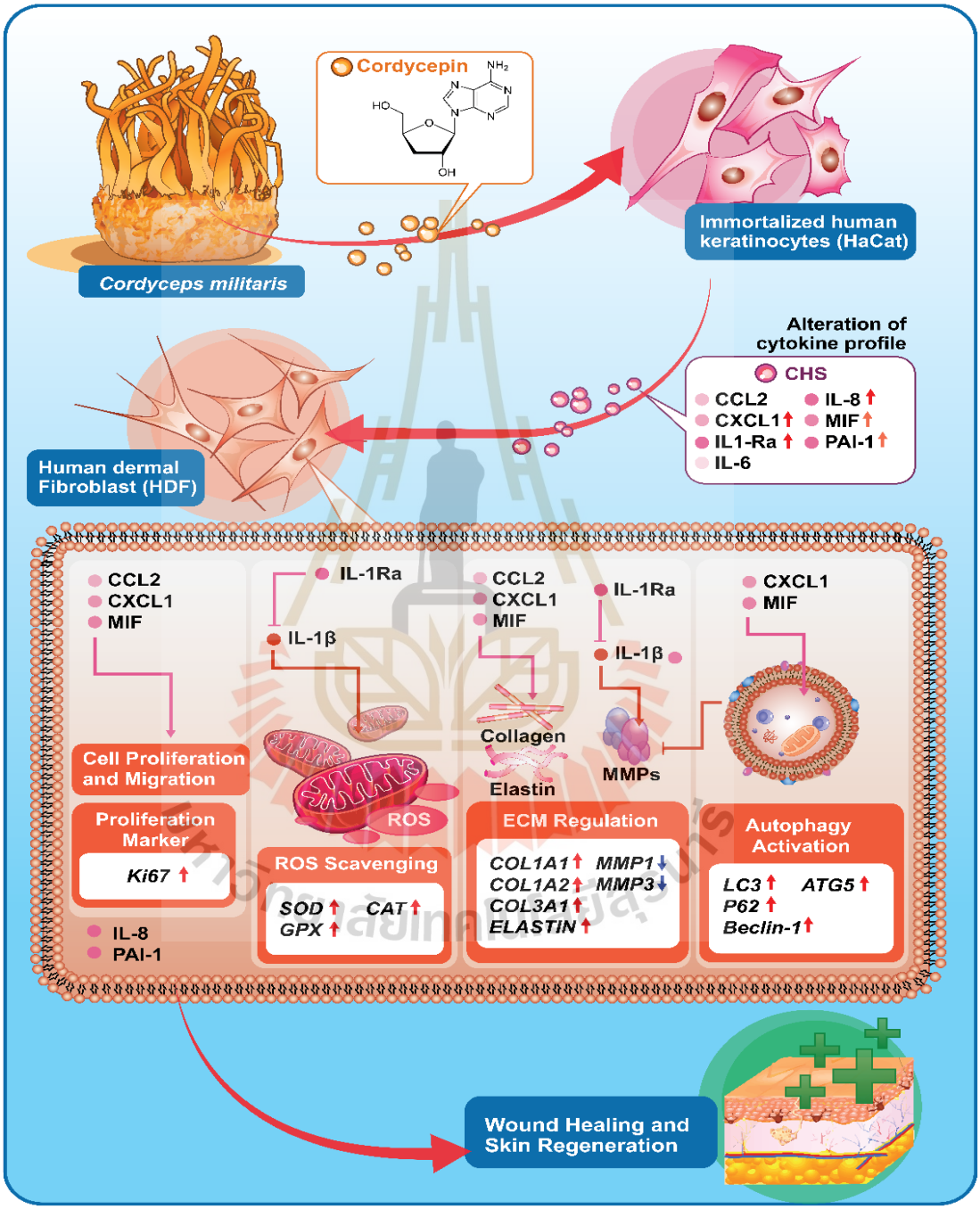

Figure 5.7 Schematic illustration hypothesized the possible biological activity of cordycepin-induced HaCaT secretome (CHS) on promoting skin regeneration.

5.7 References

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CHAPTER 6

SUMMARY

This study investigated the effect of epigenetic modification on the increase of cordycepin synthesis in a static liquid culture of *C. militaris*. In this culture system, seed culture preparation required 1.5×10^7 initial spores. Using 50 M VPA as an epigenetic modifier resulted in a 41.187% increase in cordycepin synthesis, which was caused by an increase in global histone acetylation and the overexpression of 5'-nucleotidase, adenylate kinase, phosphorybosyltransferase, Cns1, Cns2, Cns3, and Cns4. However, due to the unequal distribution of valproic acid throughout larger culture systems with increase in the working volume resulted in a reduction in cordycepin production. The maximum cordycepin yield measured was 2,835.32±34.35 mg/L. The epigenetic alteration approach employed in this work might be translated to the industrial scale cordycepin production and broadly applicable to other static liquid culture procedures. Then, to emphasize the potential of cordycepin as an active ingredient in anti-cancer nutraceutical products, cordycepin's biological functions as an NK cell inducer to combat cancer were explored. Cordycepin was discovered to be a strong promising NK cell activator via the stimulation of TNF-, IFN-, IL-2, and IL-12 production, as well as the augmentation of exocytosis granzyme B release. Cordycepin might enhance NK-92 cell maturation by lowering the expression of the immature marker CD27 and boosting the expression of mature markers CD11b, CD16, and NKG2D. This finding supports cordycepin's continuing development as an anti-cancer agent by increasing NK cell activity. For the development of secretome production, cordycepin-induced secretome (CHS) collected from HaCaT cultured possessed the capability of assisting skin regeneration and wound healing through its bioactivities on dermal fibroblasts including induction of cell proliferation and migration, ROS scavenging, regulatory effects on extracellular matrix production, and autophagy modulation. The remarkable bioactivity of CHS might be attributed to the positive modification of several secretome components such as CXCL1, IL-1Ra, IL-8, MIF, and PAI-1. As a result, the cordycepin

treatment technique was effective in improving the quality of the HaCaT secretome to be an effective component in cosmeceutical products and has been shown to have favourable implications for the future enhancement of skin regeneration in advanced new treatments.

VITAE

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