Metabolic investigation and activity of *Cordyceps militaris* and cordycepin in cancer cell lines

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A thesis submitted for the degree of Doctor of Philosophy

September 2022

Declaration

I declare that except where acknowledged in the text, this thesis is my work and is based on research undertaken at the School of Pharmacy, Faculty of Science, University of Nottingham.

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- Gene Regulation and RNA Biology Laboratory, Molecular Therapeutics and Formulation Division, School of Pharmacy

Acknowledgement

First, I would thank and show my deepest gratefulness to **Dr Cornelia de Moor** for her continuous support, guidance, enthusiasm and immense knowledge. Her motivation and mentoring helped me and enabled me to finish my project successfully. Lia has been an exceptionally patient and encouraging supervisor throughout my PhD and allowed me to design many experiments and decide the track of my research study – while keeping me on track when needed. Writing this thesis was one of the challenging stages of my PhD; without her guidance and support, this study would not have been completed. She constantly showed a strong interest in my personal growth and professional goals. I could not have imagined having a better supervisor.

I thank **Dr Dong Hyun Kim** for his expertise and supervision of metabolomics work by bringing up new ideas and advice. Thanks to Dr Andrej Gregori for providing the Goba Cordycepin and *Cordyceps militaris* samples. Dr Salah Abdelrazig, Dr Cath Ortori and Dr Laudina Safo were great teachers in the operation of the metabolomic instrument. Keith Spriggs expertise in writing improved my thesis writing. Barbara Rampersad was a fantastic technician, cheerful and happy to help. Steven Laurence, Masar Radhi, Bismoy Mazumder, Kathryn Williams, Asta Tranholm, and Jonathan Whitchurch were great company in the lab and made my PhD enjoyable. Thanks to my good friends and student peer for making my PhD memorable - Abdulmohsin Alamoudi and Peter Wellham. Thank you, Dr Ihsan Ul Haq, for guiding me, introducing me to research, and building my passion for it.

Finally, I thank my Dad **Abdul Sattar** and **Mom** for all the hard work and efforts in my upbringing and for unbelievable support, which was impossible without you. Many thanks to my brothers and sisters for the encouragement and for making me able throughout my life to face tough times and especially during my PhD. Finally, I thank my wife for being so supportive, understanding and there for me through all the difficulties.

Abstract

Inflammation is a normal reaction to infection or injury, but if the process is not resolved, it can lead to a variety of diseases, including cancer and arthritis. The available treatments for chronic inflammation are often insufficient or have unacceptable side effects, leading to a continued search for anti-inflammatory medicines with novel mechanisms of action. Natural sources are rich in anti-inflammatory compounds and are re-gaining interest. A common problem with natural medicines and natural compounds is their quality, consistency and purity. In this study, metabolomic analysis of *Cordyceps militaris* (L.) Fr., *Cordyceps militaris* extract was undertaken and thousands of compounds were identified by untargeted metabolomic analysis. Different batches from one producer were similar but had a somewhat variable cordycepin content and large differences in the cordycepin-potentiating compound pentostatin. There were much larger differences between batches from different suppliers. A comparison of the effects of cordycepin and *Cordyceps* extracts on inflammation in a macrophage cell line suggested that the extract contained additional activity. Untargeted metabolomics of commercially purified cordycepin (sold as 98% pure) revealed that six different batches had large numbers of significant contaminants, which were similar between suppliers. Remarkably, the batches of cordycepin differed in their cordycepin content as well as their potency. Some of the contaminants were tested in tissue culture for effects on inflammation, but many were commercially not available or even not fully identifiable. Pentostatin was also detected in cordycepin preparations. These data suggest that the reported biological activity of cordycepin may not be due to the presence of this compound alone.

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LIST OF ABBREVIATIONS

AAM – Amino acid metabolism

ACN – Acetonitrile

Aloha – *C. militaris* product (Aloha)

Adn – Adenine

Adr – Adenosine

BSM – Biosynthesis of secondary metabolism

cDNA – Complementary DNA

***CM* –** *C. militaris* strain (MycoMedica, Slovenia)

***CM12 to 19* –** *C. militaris* strain 12 to 19 (MycoMedica, Slovenia)

CordyvtK – *C. militaris* from Goba (MycoMedica, Slovenia)

Cpn. – Cordycepin

ddH_2O – Deionised distilled water

DEA – Diethylamine

DMEM – Dulbecco's modified Eagle's media

DMSO – Dimethyl sulfoxide

dNTPs – Deoxynucleotide triphosphates

E – Ethanol

EEPF – Endophytic entomopathogenic fungus

EM – Energy metabolism

EI – Electron ionisation

ESI – Electrospray ionisation

EW – Ethanol-water (60%)

FBS – Foetal bovine serum

FDR – False discovery rate

FTIT – Fourier transform ions trap

GC-MS – Gas chromatography-coupled mass spectrometry

HCD – Higher-energy C-trap dissociation

HILIC – Hydrophilic interaction liquid chromatography

HMDB – Human metabolome database

HPLC – High-performance liquid chromatography

IC50 – half maximal inhibitory concentration

IL-1-β – Interleukin-1-beta

IM – Identified metabolites

Ins – Inosine

KEGG – Kyoto Encyclopedia of Genes and Genomes

LC-MS – Liquid chromatography coupled mass spectrometry

LM – Lipids metabolism

LOD – Limit of detection

LPS – Lipo-polysaccharide

Lpr – L-proline

M – Methanol

MAP/MAPK – Mitogen-activated protein/kinase

MCV – Metabolism and cofactor and vitamins

MS/MS – Tandem mass spectrometry

m/z – Mass to charge ratio

NF-κB – Nuclear factor-kappa B

NM – Nucleoside metabolism

NMR – Nuclear magnetic resonance

OPLS-DA – Orthogonal partial least squares-discriminant analysis

PBS – Phosphate-buffered saline

PDTT – Peptides di, tri, tetra

PCA – Principle component analysis

PCR – Polymerase chain reaction

Ptn – Pentostatin

QC – Quality control

QqQ – Triple quadrupole

Q-TOF – Quadrupole-time of flight

RT-qPCR – Reverse transcription quantitative PCR

RP – Reverse phase

Rpl – Ribosomal protein L gene

SIMCA – Soft Independent Modelling of Class Analogy software

SSIII – SuperScript® III First-Strand Synthesis System

TIC – Total ion count

TNF – Tumour necrosis factor

UHPLC – Ultra high-performance liquid chromatography

UIM – Unidentified metabolites

VIP – Variable importance in projection

W – Water

WE – Water and ethanol

XE – Xenobiotics

ZIC-pHILIC – Zwitterionic stationary phase polymeric HILIC column

CHAPTER 1
INTRODUCTION

CHAPTER 1: INTRODUCTION

In this introductory chapter, I will discuss some aspects of inflammation, the currently available treatments and why natural compounds are an excellent potential source of a new therapy. I will then discuss some of the technical advances that make natural compound discovery easier and hurdles that are still common in the field. The end of this introduction will focus on the caterpillar fungus *Cordyceps militaris* and its active metabolite cordycepin as potential anti-inflammatory medicines.

At the beginning of the nineteenth century, inflammation became one of the major areas of biomedical science (Heidland, Klassen, Rutkowski, & Bahner, 2006). Inflammation is the underlying factor of many diseases such as cancer (Leonardi, Accardi, Monastero, Nicoletti, & Libra, 2018), stress, diabetes (Wellen & Hotamisligil, 2005), obesity (U. Das, 2001), rheumatoid arthritis (Choy & Panayi, 2001), atherosclerosis (Lind, 2003), pulmonary fibrosis and coronary heart disease (Young *et al*., 2007). All these diseases are significant causes of death and disability around the globe. Inflammation-associated cancer can develop at sites previously affected by chronic inflammation. Thus, inflammation could also be considered responsible for a large percentage of cancer incidence (Coussens & Werb, 2002). These diseases continue to burden humanity because inflammation is a diverse and complex response that is inadequately controlled by available therapies.

1.1 Inflammation.

Inflammation is the body's defence against trauma, stress or infection. In response to molecular clues such as microbial penetration into the tissue, it initiates and maintains a coordinated response. This includes signalling instructions to dispatch the cells to kill microbes and the infected host cells, liquefaction of the surrounding tissue to avoid microbial metastasis and the healing of damage caused by trauma, infection and immune responses. If the process proceeds to the next step but progress are blocked, the inflammatory process may divert into a holding pattern. In such cases, the result can be fibrosis, pain, tissue degradation or

hyperplasia, leading to the diseases listed previously. (Kumar, Clermont, Vodovotz, & Chow, 2004; Lentsch & Ward, 2000; Nathan, 2002; Sousa, Alessandri, Pinho, & Teixeira, 2013)

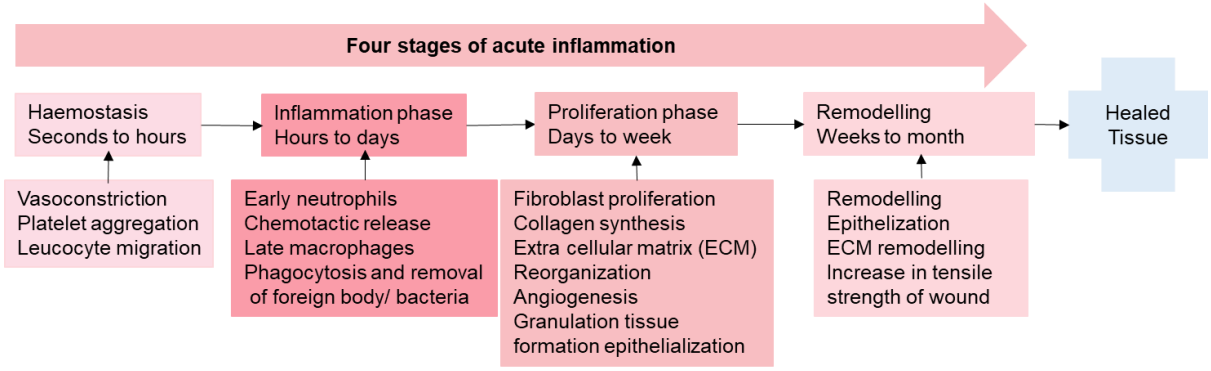


Figure 1.1: Stages of normal acute inflammation**,** adapted from (Thiruvoth, Mohapatra, Kumar, Chittoria, & Nandhagopal, 2015).

In the first stage of most inflammations, microorganisms that infiltrate skin or mucosal membranes and underlying tissue are confronted by specialized cells known as phagocytes, which engulf the invader, such microorganisms and cell particles by phagocytosis. The neutrophils activate macrophages, which are present in the organs and tissues all over the body. Meanwhile, the damage caused to resident cells by the infection discharges substances that activate other processes of inflammation, such as proteins that help blood clotting by repairing the wall of the affected area. This delays the propagation of the microorganisms and their toxic products. The local endothelial cells' lining becomes leaky, allowing the white blood cells to penetrate the wall area. Monocytes arrive more slowly and gradually over days or weeks, developing into macrophages at the infection site. This is in addition to activating macrophages resident in the tissue. These macrophages and neutrophils release substances, including cytokines, metabolites and adhesion proteins, drawing additional neutrophils and monocytes to the area. Meanwhile, macrophages and neutrophils, toxic oxygen-containing compounds and potent degenerative enzymes destroy the phagocytosed microorganisms, causing unavoidable damage to the surrounding tissues (Adamo, Rocha-Resende, Prabhu, & Mann, 2020; McClain, Shedlofsky, Barve, & Hill, 1997). The blood flow increases with blood

cell seepage occurring locally, and fluids contribute to the familiar symptoms of redness, swelling and pain commonly related to acute inflammation.

Macrophages activation plays an essential role in the inflammatory responses origination and propagation by the production of tumour necrosis factor-alpha (TNF- α), granulocytes/macrophages stimulating factor (GM-CSF), cytokines, interleukin-1 beta (IL-1 β), cycloxygenase-2 (COX-2), nitric oxide (NO) and other inflammatory mediators. Gram-negative bacteria cell wall contain lipopolysaccharide (LPS), and human cells commonly have receptors that can detect this pathogen associated molecular pattern. (Rietschel *et al*., 1994). Activating macrophages using LPS increases cytokine production for instance TNF-α, IL-1β, nitric oxide and GM-CSF, which is controlled by the nitric oxide synthase induction or NOS2. The septic shock animals model shows that TNF-α, IFN-γ, and IL-10 were involved in regulating LPS-induced NO discharge (ter Steege, Koster-Kamphuis, van Straaten, Forget, & Buurman, 1998). LPS is a potent activator for mitogen-activated protein kinases (MAPKs), often activated in cellular inflammation (Chan & Riches, 2001).

Macrophages play an important role in mediating responses to foreign bodies by secreting a wide range of pro- and anti-inflammatory cytokines (Anderson, Rodriguez, & Chang, 2008). These pro-inflammatory cytokines are essential for initiating wound healing at the early stages of recovery since they function to draw in healing cells and initiate angiogenesis (Turabelidze & Dipietro, 2011; Willenborg *et al*., 2012). However, macrophages eventually cease producing inflammatory signals and begin secreting cytokines that promote the formation of blood vessels, tissue matrix remodelling, and local immunological balance. A premature or delayed switch-off disrupts the healing process; thus, this switch-off must occur in a timely manner. In addition, resolving inflammation is of special attention, where macrophage activities vary from those under normal conditions (Zhenzhen Wang *et al*., 2021).

The detailed kinetics of each healing phase depends on the level of injury but is also influenced by the age of the individual at injury time, the organ or tissue that was injured, the

individual general health, and many other factors. After a tissue injury, the inflammatory process lasts for one to two days; after one week, the proliferation phase peaks, and remodelling begins during in proliferation phase but could last months (Gurtner, Werner, Barrandon, & Longaker, 2008). The degree of macrophage activation varies between pro-inflammatory, early-stage M1 macrophages and pro-reparative, later-stage M2 macrophages. During the acute reaction to trauma, M1 macrophages are seen, producing a lot of reactive oxygen species (ROS). M1-macrophages support innate immunity by increasing phagocytosis and releasing pro-inflammatory cytokines, which help clear the injury site of foreign microorganisms and wound debris. In addition to having abilities to repair tissue, M2-type macrophages also produce less ROS and fewer pro-inflammatory cytokines.

Additionally, they release chemokines (such as interleukin-10 or IL-10) and chemokines to draw in anti-inflammatory leukocytes, boost phagocytic receptors, and upregulate extra cellular matrix elements and growth factors (e.g., chemokine (C-C motif) ligand17 (CCL17), CCL18, and CCL22) (Galli, Borregaard, & Wynn, 2011; Mosser & Edwards, 2008; Van Assche, Deschacht, da Luz, Maes, & Cos, 2011). M2-type macrophages can regulate inflammatory responses, scavenge waste, and encourage tissue remodelling and repair. In skin and muscle injury cases, this sequential M1-M2 macrophage action results in effective wound healing (Gensel & Zhang, 2015).

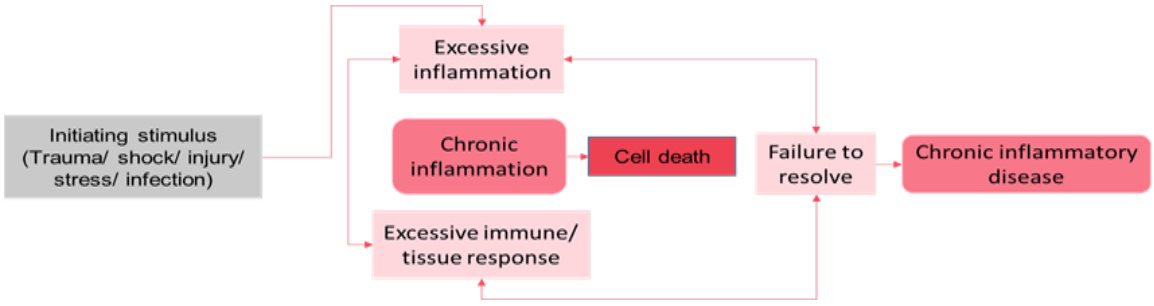


Figure 1.2: Stages of chronic inflammation, adapted from (Anderton, Wicks, & Silke, 2020).

1.1.1 Chronic inflammation

Chronic inflammation is usually associated with the build-up of monocytes and the long-term presence of macrophages accompanied by progressive tissue destruction (Ferrero-Miliani, Nielsen, Andersen, & Girardin, 2007; Huseby, Huseby, Shah, Smith, & Stadinski, 2012). Chronic inflammation can be the result of failure in recovery from acute inflammation. Because of its irritant nature, it is thought to share several characteristics of acute inflammation. However, chronic inflammation has a unique response pattern to the irritant. It can be classified into granulomatous and non-granulomatous. The term granulomatous in chronic inflammation is referred to a localized collection of active macrophages and their product. The host response to acute inflammation involves eliminating the irritant, which is followed by a recovery that involves tissue repair and regeneration. However, in chronic inflammation, the repair appears to be attempted concurrently and goes awry. Repair is usually attained by tissue granulation ingrowth, which comprises macrophages, new blood vessels and fibroblasts (Furman *et al*., 2019; Kilarski, Samolov, Petersson, Kvanta, & Gerwins, 2009). Moreover, the host immune recognises the more persistent irritant as foreign antigens. It may contribute to tissue destruction and chronic inflammation. This is well demonstrated in diseases such as hepatitis and tuberculosis, where the precursor agents carry on in the host and persist in initiating a chronic inflammation response. Another significant distinction between chronic and acute inflammation is the balance between cellular recruitment and the cell types that predominate in an inflammatory response. In chronic inflammatory response, there is usually a less prominent exudative response and enhanced inflammatory cellular recruitment, which might be accompanied by proliferation by local cells.

Many diseases are caused by the overexpression of inflammatory mediators, such as atherosclerosis (Coker & Laurent, 1998), pulmonary fibrosis (M. Chen *et al*., 2012), rheumatoid arthritis and chronic hepatitis (Tilg *et al*., 1992: Lind, 2003). The observation showed severe inflammation in multi-organ failure and shock, conditions with the highest

mortalities. Patients with depression also show signs of inflammation even in the absence of apparent causes of inflammation (Benito, Veale, FitzGerald, Van Den Berg, & Bresnihan, 2005; Hedbom & Häuselmann, 2002; Kop *et al*., 2002; Pitzer, Del Zoppo, & Schmid-Schönbein, 1996; Schmid-Schönbein & Hugli, 2005; Stürmer, Brenner, Koenig, & Günther, 2004; Toker, Shirom, Shapira, Berliner, & Melamed, 2005; Waxman, 1996). Clinical studies also showed that obesity is linked to inflammation. The list of disorders that are connected with the molecular markers of inflammation is long and growing (Calabro, Chang, Willerson, & Yeh, 2005; Nicklas *et al*., 2004).

Prostaglandins are bioactive lipids having potent activity in both physiological (platelet aggregation, protection of gastric mucosa) and pathological (inflammation, pain and fever) events. Cyclooxygenase enzymes convert un-esterified fatty acids, particularly arachidonic acid, into prostaglandins. The anti-inflammatory, analgesic and antipyretic therapeutic effectiveness of the pharmaceuticals as well as their main side effects are due to the aspirin-like medications' ability to suppress the production of prostaglandins (NSAIDs) (inhibition of platelet aggregation and gastric damage). Early suggestions in the literature showed that enzymes may occur in more than one form. When the second isoform of COX2, an aspirin target, was shown to rise in inflammatory tissues. It has been proposed that the constitutive COX1 isozyme is in charge of the physiological effects of prostaglandins on platelets and the stomach.

In contrast, COX2 is the main isoform that is believed to be in charge of inflammation. The majority of NSAIDs formerly in use inhibited both isoforms, which could have influenced both the therapeutic and unfavourable effects of these drugs. A thorough search has been launched to discover a selective COX2 inhibitor. The result was the development of a brand-new family of anti-inflammatory drugs called COX2 selective inhibitors. Other COX subtypes may be responsible for some of the residual variations in NSAID actions (Flower, 2003).

1.1.2 Cytokines

Acute and chronic inflammation is regulated by cytokine-secreted peptides that bind to receptors. Cytokines can be released by several immune cells in response to various stimuli, with numerous effects on various target cells. Along with cells from most other organ systems, immune cells are regulated by cytokines, which control their survival, differentiation, proliferation and function. The basic definition of an anti-inflammatory cytokine: The ability of cytokine to inhibit the synthesis of tumour necrosis factor, IL-1, and other key pro-inflammatory cytokines (Opal & DePalo, 2000). The inflammatory cytokines (principally TNF and IL1β) induce an inflammatory response in the tissue and amplify the inflammatory response beyond the immediate vicinity of the trauma (Verri Jr *et al*., 2006). Chemokines are a family of small secreted proteins. Inflammatory cytokines increase chemokine production from surrounding tissue. Their primary role is to cause leukocyte activation and migration to the inflammation site. They stimulate phagocytes, increase blood coagulation, and stimulate the secretion of more cytokines and chemokines. (Kubo, Hanada, & Yoshimura, 2003; McClain *et al*., 1997; Nicola, 1994).

It has been observed that in innate immunity, the cellular signalling mechanisms activate the NF-κB transcription factors to mediate many of these responses (Hatada, Krappmann, & Scheidereit, 2000). NF-κB regulates numerous different immune system-controlling genes. These NF-κB target genes, such as encoding cytokines, adhesion molecules, chemokines, acute phase proteins, and inducible enzymes, such as iNOS and COX-2. Cytokines include TNF- α, LT α, LTβ, IL-1, IL-2, IL-6, IL-12, and GM-CSF. Adhesion molecules include intercellular adhesion molecules, endothelial leukocytes and vascular cell adhesion molecules (Caamano & Hunter, 2002; Ghosh, May, & Kopp, 1998; May & Ghosh, 1998). Furthermore, recently it has been shown that NF-κB can control several evolutionarily conserved antimicrobial peptides, such as β-defensins (protein) (Diamond, Kaiser, Rhodes, Russell, & Bevins, 2000). These molecules are important part of innate immune response to foreign

pathogens and are necessary for inflammatory and phagocytic cells to migrate to regions where NF-κB has been activated in response to an injury or infection (Q. Li & Verma, 2002).

1.1.3 Inflammation treatment

Slowing down the inflammatory process has turned out to be very important for therapy; NSAIDs are often used. NSAIDs are commonly used to treat many conditions of chronic inflammation worldwide. A USA-based survey on NSAIDs consumption shows that at least once a week, usage of NSAIDs in adults is greater than 37% (Hoffmeister, Chang-Claude, & Brenner, 2007). NSAID drugs act non-selectively preventing COX-1 and COX-2 prostaglandin synthase isozymes by covalent modification of the enzyme or substrate active site competition (Williams, Mann, & DuBois, 1999). Continuous use of NSAIDs has both harmful and beneficial effects, mostly because COX-1 prostaglandin synthesis is reduced. gastrointestinal issues are the primary negative effects, which are reversible and moderate, though some patients can undergo severe intestinal bleeding, peptic ulcer, digestive tract perforation and small-intestine damage. In the UK, 3,500 hospitalisations and about 400 deaths have been reported annually related to NSAIDs due to ulcer bleeding at 60 years of age and above (Hawkey & Langman, 2003).

Glucocorticoids usually referred to as corticosteroids or simply known as steroids, are among the most widely used medications in the world; they are effective in treating various inflammatory and immunological disorders. The main action of corticosteroids is to turn off a range of genes responsible for inflammation that have been active during the chronic inflammatory process -encoding cytokines, adhesion molecules, chemokines, inflammatory receptors, enzymes, and proteins. They also have added impacts on the production of anti-inflammatory proteins and postgenomic effects at greater dosages (Barnes, 2006). Chronic glucocorticoids can cause serious side effects on the immune system, metabolism and tissue regeneration, leading to an increased risk of infection, diabetes and osteoporosis.

TNF is the chief pro-inflammatory cytokine, despite its initial classification as an anti-tumour mediator. It is crucial to the pathophysiology of inflammatory illnesses such as psoriasis, ankylosing spondylitis (AS), inflammatory bowel disease, and rheumatoid arthritis (RA). As a result, anti-TNF therapy has become a mainstream treatment option for autoimmune illnesses. Anti-TNF biologics have been developed in the previous 20 years. The anti-TNF antibodies and soluble TNF receptor 2 fusion proteins are examples of drugs in this class. The autoimmune disorders treatment such as rheumatoid arthritis, psoriatic arthritis, plaque psoriasis and ulcerative colitis has been transformed by the development of anti-TNF treatments (UC). However, anti-TNF therapy is ineffective in about 40% of the patients. Additionally, this medication has several adverse side effects, including an elevated risk of infection and the de novo onset of autoimmune illnesses. Such negative effects of anti-TNF therapy are probably brought about by the widespread suppression of TNF biological processes. Therefore, as suggested by several recent research, targeted TNF receptor inhibition (TNFR1 or TNFR2) may be a more safe and effective treatment (P. Li, Zheng, & Chen, 2017).

1.1.4 Herbal medicines as an anti-inflammatory treatment

WHO estimates that over 75% of people worldwide utilise herbs and other traditional treatments to cure inflammatory and other illnesses. (Inamdar, Edalat, Kotwal, & Pawar, 2008). Natural medicines are widely credited and accepted by most of the world's population due to their availability, low cost, and presence of traditional and local pharmacopoeia. Natural medicines are generally considered safe by the public; however, a lack of recording of adverse effects and no effective quality control hamper their reputation in the medical community and put the patients at risk. Natural medicines have several problems which can be avoided with purified synthetic drugs. For example, herbal medicines are usually a mixture of constituents whose active constituents are undefined in most cases. Another reason is the lack of standardised references available commercially. Furthermore, natural medicines are affected by the variability of the quality and source of raw material (Ernst, 2000; Folashade, Omoregie,

& Ochogu, 2012; Winslow & Kroll, 1998). The two mainstays of anti-inflammatory treatment, NSAIDS and corticosteroids, were first discovered as natural products. Decades of rational drug design have still not produced anything that is as widely applicable. It is time to back to nature for inspiration.

1.2 Natural medicine

Natural medicines; is a broader term used for traditional, folk and herbal medicines. It consists of medicines from plants, animals, microbes and fungi from a large geographical distribution (Holland, 1997; Ji, Larregieu, & Benet, 2016; Verdine, 1996). Almost all living organisms can produce specialised molecules that distinguish species from others and enable them to adapt to the unique challenges they encounter in their environment. These specialised metabolites (known as secondary metabolites) help in various mechanisms, such as chemical warfare, communication, nutrition acquisition, and stress resistance. These metabolites are chemically classified into several types, including peptides, nucleotides, polyketides, flavonoids, saccharides and terpenes. With the vast chemical space and the amazing diversity and dynamic nature of ecological interactions and selection forces, species across the evolutionary tree have produced thousands and thousands of structurally diverse metabolites. Naturally, this affluence has been widely exploited as an excellent resource for drug development. Many antimicrobials, anti-cancer agents, and other medications are either natural compounds or inspired by natural products. Natural products have also been employed as crop protection agents and as components in producing food, dyes, cosmetics, and numerous other products (van Der Hooft *et al*., 2020). Natural product systems known as traditional medicine are found around the world and are developed into organised systems and used by people for centuries.

1.2.1 Systems of traditional medicine around the world

Around the world, many countries have their traditional medicine system consisting of Chinese and, Japanese traditional medicine known as Kampo medicine, and AYUSH system of medicine consisting of Ayurveda, Yoga, Unani, Siddha, and Homeopath which represent Indian

traditional systems of healthcare, Thai herbal medicine, traditional medicine in Bangladesh known as Unani Tibb and Ayurveda, traditional Iranian medicine, Malaysian traditional and complementary medicine, Australian complementary medicine, “Sowa-Rigpa” originated in Tibet and has been commonly practised in Nepal, India, Mongolia, Bhutan, and Russia (Mukherjee, 2019).

1.2.2 Chinese traditional medicine

Traditional Chinese medicine (TCM) originated in ancient China that evolved for more than 1000 years. Practitioners employ herbal medications and various mind-body techniques, including acupuncture therapy or judo, to cure or prevent medical conditions. Acupuncture conducted by skilled practitioners using sterilized needles is considered safe. It was founded on the ancient Taoist idea and dates back over 25 centuries. A number of East and South Asian nations, like Japan and Korea, have traditional medical systems as well. Many of these fields have been inspired by traditional Chinese medicine and are comparable in some aspects, but each has distinctive features (Tang & Eisenbrand, 2013).

Recent research on Chinese herbal medicine quality focuses on isolating and structurally determining substances with pharmacological activity derived from Chinese medicine, followed by determining their therapeutic characteristics. Chinese herbal medicine research focuses on medicinally important drug discovery of Chinese traditional medicine based on standard pharmacological usage and techniques developed. Numerous herbs and preparations used in Chinese medicine therapy are subject to quality monitoring. Since the 1920s, significant efforts have been made to analyse Chinese medical resources, yielding progress. Through National Projects of Science and Technology, the government of China inspired Chinese medication analysis to develop a high-standard system. The extensive usage of Chinese medicine in China and the West requires the development of in-depth research monographs. These are developed by institutions approved by China's Ministry of Public Health's New Drug Analysis Committee. Current regulations are being introduced throughout China as part of the high-

quality assurance programme based on the findings of current research programs. Thin layer chromatography (TLC) is used to compare sample fingerprints and authentication of material required for plant samples for which no alternative active ingredient assays have been developed. The manufacturers must provide documentation for each herbal product's pharmacodynamics, toxicity, quality control technique, efficiency, and safety (Gilhooley, 1989; Mukherjee, 2019).

1.2.3 Drugs derived from traditional medicine

In most nations today, traditional medicine systems and allopathic medicine coexist in a complementary manner. To maximise the usage of botanicals, measures should be made to synchronise the evaluation and quality control process. Complementary and alternative therapy was used by around 70% and 42% of cancer and rheumatoid arthritis patients. Garlic, Ginseng, ginger, Ispaghula, ginkgo, and saw palmetto are a few examples of plants whose use is supported to some extent by research and are prescribed by modern physicians in the US. The first critical step in avoiding admixtures, adulteration or confusion in botanicals would be to ensure herbs' precise identification and authenticity. Plant and fungi identification is critical for ensuring quality assurance since it ensures that the correct plant raw material was used. Combining methodologies such as microscopic and macroscopic analysis, DNA-based analysis and chemical fingerprinting can be used to identify medicinal plants. The effective active ingredients in plants as medicinal preparations are a range of secondary plant metabolites, most of which are specific to certain plant groups or plants (Mukherjee, Sahoo, Narayanan, Kumar, & Ponnusankar, 2009). Some measures are taken to make sure the identification is right and verification of traditional medicinal herbs, detailed in later paragraphs (Mukherjee, 2002). Numerous critical integrated methods must be examined to validate traditional medical systems like Ayurveda. Evidence-based approaches for validating natural medicine show the key elements of evidence-based validation (Mukherjee, Bahadur, Chaudhary, Kar, & Mukherjee, 2015) [figure 1.2].

1.2.4 Ethno-medicine validation

With the worldwide interest in medicinal plant or plant-derived drugs and microbial and fungal treatment, It is crucial to guarantee the effectiveness and safety of natural medications through the use of various scientific methods. The composition of chemicals in the prescribed natural may change depending on the season, sources, drying methods, and other relevant factors. As a result, it is crucial to determine the number of phytochemical constituents of herbal medicine products, with the end objective of ensuring the unwavering quality and reproducibility of pharmacological and clinical research, as well as understanding the bioactivities and potential reactions in terms of improving the characteristics of the herbal medicine products.

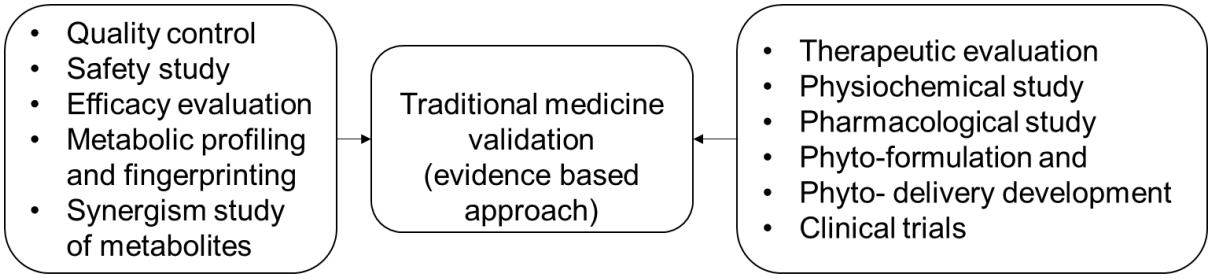


Figure 1.3: Evidence-based approaches for validation of natural medicine, adapted from (Mukherjee, 2019).

Standardised methods for natural medication composition ensure their quality, sufficiency, and safety. The lack of chemical biomarkers remains a significant challenge for the quality control of herbal medicine. Chemical and pharmacological research on chemical markers is generally lacking. Furthermore, there are various unique obstacles in marker development. For instance, Solvent, temperature and light generally tend to deteriorate along with variations in pure components; isomers and adaptation may also cause a change in the markers. In any instance, to build an evidence-based practice of herbal medications, a concept of understanding complex standards of natural medication should be produced using marker profiling and related approaches (Ren *et al.*, 2020).

Chemo-profiling, safety evaluations, quality control, and burdensome administrative regulations for natural prescriptions are among the key challenges to advancing and improving natural medicines. An improved global coordinated effort and governance are required to alter current standards and build new standard procedures for improving natural products and dietary supplements. Through collaborative efforts and engagement across the country, research may contribute significantly to the progress and improvement in overall health care. Different offices in various countries manage and implement the advancement and evaluation of natural and natural derived products. The collection and preparation procedures, however, are still not standardised. As a result, a variety of elements, like the extraction procedure, ripening, or the cultivars utilised, may affect the actual product concentration. As a result, regulatory bodies like the FDA constantly check the production and marketing claims of supplements. (Maroon, Bost, & Maroon, 2010).

1.2.5 Authentication and quality control methods

The word phyto-equivalence refers to the bioequivalence of phyto-medicine compared to another product that may have been the subject of substantial research. In this concept, Pharmaceutical grade equivalent formulation of drug products produced to have the same concentration of the active ingredient in the same dosage form and meet the same compendia and other applicable standards, including quality, purity, strength and identity. However, it may vary in the characteristics such as shape, release mechanisms, scoring configuration, packaging, excipients (including preservatives, Colour and flavours), expiry time and, in certain limits, labelling. Advanced procedures such as spectrophotometry, chromatography and a combination of these approaches, polarography, electrophoresis and molecular biomarker fingerprints, are now being employed in standardising herbal medications. The standardisation of herbal medicine includes specifying the chemical constituents or a combination of substances (Mukherjee & Houghton, 2009).

In Germany, in the mid-1990s, the idea of phyto-equivalence was developed, which signifies that the extract of one herbal product matches or is equivalent to the extract of another herbal product, specifically one of the clinically validated extracts. In Germany, the phyto-equivalence history was defined by previous German Pharmacopeia versions, which defined the exact conditions regarding plant preparations, comprising extraction solvents, time and methods. Several comparative clinical trials demonstrated that phyto pharmaceuticals have full therapeutic equivalency with chemotherapeutics and are free of side effects. Herbal remedies' and their extract formulations' modes of action, which differ from those of synthetic medicines or mono compounds in several ways, can be characterised as polyvalent and seen as an additive or, in some situations, potentiating factor.

Chemical equivalence is formally known as phyto-equivalence. A chemical fingerprint profile of the proven efficacious herbal product should be developed, which can be used as a reference for commercial quality control. Standardization based on one or a small number of chemical compound markers or classes facilitates quality control and batch-to-batch uniformity. Various variables are significant in the formulation of natural goods, including the amount of alcohol in a hydro-alcoholic solvent, extract concentration, plant material homogeneity, and extraction duration and temperature. Phyto-medicines must be directly compared and tested against synthetic drugs. The moment has arrived to create a systematic plan. and well-designed techniques for standardising natural drugs' raw materials and formulations (Mukherjee & Houghton, 2009).

1.2.6 Natural products for novel drug discovery

Although, significant chemical variety results from recent advances in combinatorial technologies and high-throughput screening technologies, natural products and their related structures remain vital components of pharmacopoeias. Due to the wide range of secondary metabolites of plant and microbial species that are easily used, Natural products and their associated structures might

be much more important in the future for the development of enhanced and innovative medications, which composition and genetic variation are now being revealed by DNA sequencing along with related genomics and bioinformatics technologies (Zeilinger, Gruber, Bansal, & Mukherjee, 2016). Until now, approaches for discovering and characterizing secondary metabolite activities have been inefficient and frequently frustrating; however, recent advancements in informatics, genomics, and similar omics innovations in the 21st century are substantially accelerating the pace of medication research and discovery. The metabolomes of cells, tissues, and even living beings may be identified using improved fractionation procedures in conjunction with contemporary spectrometry and spectroscopy methods (Mukherjee, Harwansh, Bahadur, Banerjee, & Kar, 2017).

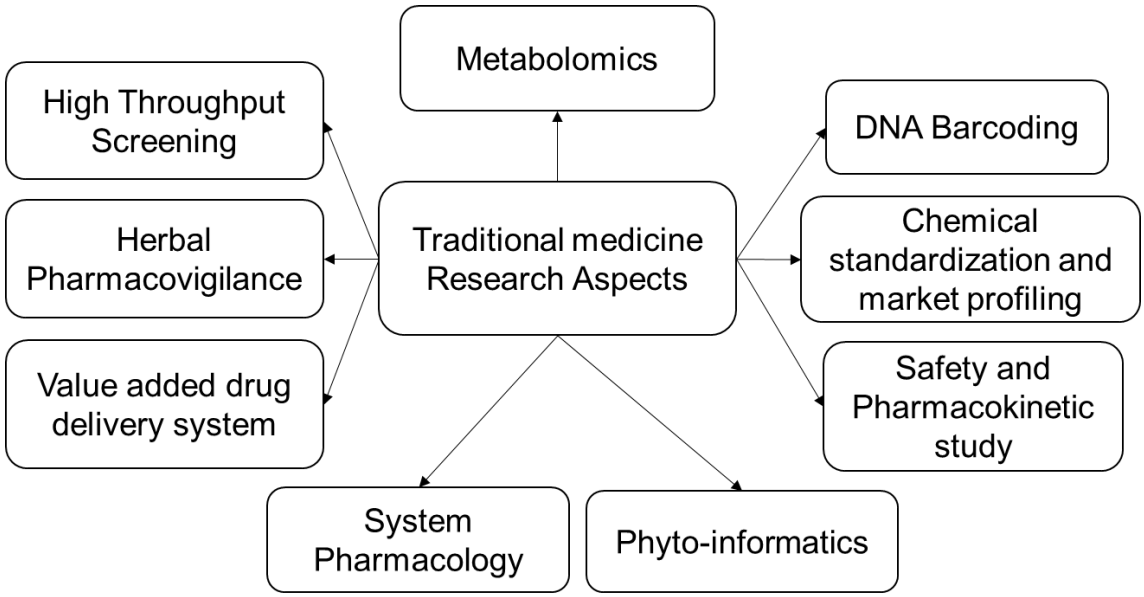


Figure 1.4: Traditional medicine development and research aspects, adapted from (Mukherjee, 2019).

*D*ue to their multiple beneficial bioactivities, including their anti-inflammatory qualities, *Artemisia herba alba* and *Magnolia officinalis* are extensively employed. A reduction in NO and a suppression or elevation of the formation of the cytokines IL-12 and IL-4, respectively, are what cause anti-inflammatory activity, according to Messaoudene et al. (2011). Certain herbal infusions are utilised in Mexican folkloric medicine; recently, research has shown that the *Buddleia scordioides*, *Chamaemelum nobile*, and *Listea glaucescens* had a positive impact on the pro-inflammatory markers, improving oxidative stress and downregulating

cyclooxygenase 2, tumor necrosis factor alpha, nuclear factor kappa B, and Interleukin 8 (Herrera-Carrera *et al*., 2015). Recent research has shown that pomegranate extract consumption reduces in vivo inflammation symptoms. According to Shukla et al. (2008), the anti-inflammatory action is accomplished by inhibiting the generation of prostaglandin E2 and nitric oxide that is triggered by inflammatory cytokines. M ethanolic extracts of Kohlrabi varieties are naturally occurring nitric oxide inhibitors that reduce the generation of inducible nitric oxide synthase and cyclooxygenase-2 to block LPS-induced nitric oxide in a dose-dependent manner (Im, Jung, Choi, Yu, & Jeong, 2014). *Smallanthus sonchifolius* (Yacon) has long been used to cure diabetes. However, recent research showed that its leaves exhibit anti-inflammatory effects due to chlorogenic acid and nitric oxide, as well as the suppression of prostaglandin E2 and tumour necrosis factor alpha. (Oliveira, Braga, & Fernandes, 2013).

The pharmaceutical industry has taken over the quest for natural anti-inflammatory drugs with fewer side effects shifted from research labs. For example the blend of free-B-ring flavonoids from *Scutellaria* and flavones from the *Acacia* species that Unigen Pharmaceuticals, Inc. patents. It has been discovered that the active ingredients function by successfully inhibiting the enzymes cyclooxygenase-2 and arachidonate 5-lipoxygenase. They advise using the new drug to treat joint pain and discomfort brought on by conditions including osteoarthritis, rheumatoid arthritis, and other conditions caused by misuse of the joints. The chemicals specified in the invention demonstrated equal effectiveness in pain relief, superior effectiveness in decreasing stiffness, and improved functional ability when compared to the prescription drug CelebrexTM by Pfizer, Inc (Burnett, Jia, Zhao, & Levy, 2007).

Food has also been used to decrease the occurrence of chronic conditions of diabetes, heart disease, dementia, Parkinson's disease, and inflammation (Mohamed, 2014). Some phenolic substances have demonstrated anti-inflammatory effects. There is an association between consuming a lot of food high in these chemicals and a reduced expression of the inflammatory response, even though the exact mechanisms behind this anti-inflammatory effect are not entirely understood (De La Lastra & Villegas, 2005). The relationship between phenolic

structure and different inflammation targets has been used to identify structural requirements and discuss anti-inflammatory efficacy (Lago *et al*., 2014). Its anti-inflammatory properties have been observed in both proliferative and exudative phases of inflammation. Numerous proposed mechanisms of action explain the anti-inflammatory action of flavonoids. A review by Rathee shows the anti-inflammatory activity and possible mechanisms of action (Rathee *et al*., 2009).

Phenolic compounds' anti-inflammatory effect mechanisms are greatly influenced by their structural makeup. Although the precise mechanisms are still not fully known, there is a link between consuming a lot of foods high in flavones and the inflammatory response downregulation (De La Lastra & Villegas, 2005). Some flavonoids present in food have been shown to regulate the mediators of inflammation such as Interleukin -6. It has been shown that the amounts of Interleukin-6 in blood plasma are directly correlated with the amounts of cocoa and tea flavonols (Stote *et al*., 2012). In contrast, Mathur *et al*. (2002) published no effect on the consumption of cocoa phenolic and inflammation markers (Interleukin-1 beta, Interleukin-6, Tumor necrosis factor); however, a decline in low density lipoprotein oxidation was seen, which could lead to a lowered vascular inflammation, oxidative stress, NO and termination of platelet aggregation, which results in heart diseases prevention. Some studies demonstrate a positive effect on inflammatory markers inhibition (Allgrove & Davison, 2014; Rodrigo, Miranda, & Vergara, 2011).

1.2.7 Fungal anti-Inflammatory treatments

Several anti-inflammatory and anti-cancer drugs are obtained from fungi. For instance, *Taxomyces andreanae*s produce Taxol and other taxanes (Stierle, Strobel, & Stierle, 1993). Furthermore, paclitaxel (Taxol®) was isolated from *Seimatoantlerium nepalense* and *S. tepuiense* (Gangadevi & Muthumary, 2008, 2008a; K. Liu, Ding, Deng, & Chen, 2009). Lignan podophyllotoxin is isolated from *Podophyllum Hexandrum and Podophyllum peltatum (Berberidaceae*) plants. It is a precursor for etoposide and teniposide, a highly valued clinically

used in treating brain tumours (Eyberger, Dondapati, & Porter, 2006; Puri *et al*., 2006). Desoxypodophyllotoxin is isolated from *Aspergillus fumigatus:* an endophytic fungus, which is an anti-inflammatory, anti-allergic, anti-tumour and anti-proliferative compound (Kusari, Lamshöft, & Spiteller, 2009). *Cordyceps militaris* is an endophytic fungus with anti-inflammatory, neuroprotective (Chiu *et al*., 2016; X.-l. Zhang *et al*., 2021), immunostimulating (J. S. Lee & Hong, 2011), anti-cancerous and antioxidant activities (X. Li *et al*., 2021; X. Zhang *et al*., 2021).

1.2.8 Secondary metabolites

The activity of fungi, plants and micro-organisms in nature is performed with the help of chemicals known as metabolites. These metabolites are of two types, known as primary and secondary metabolites. The distinction between primary and secondary metabolism can be vague, and occasionally primary metabolites, such as oxalic acid (de Oliveira Ceita *et al*., 2007; Dutton & Evans, 1996; Schmalenberger *et al*., 2015), act similarly to secondary metabolites. Secondary metabolites might comprise simple compounds derived from primary metabolisms, which are alcohol, sugars and organic acids, based on the perceived importance and functions. The identification and characterisation of quorum-sensing molecules, similar systems in bacteria, and lately, in yeasts, have altered popular perceptions about the role of low molecular weight compounds found in microbes (Albuquerque & Casadevall, 2012). Numerous agricultural chemical compounds are also developed from natural products (Asolkar, Cordova-Kreylos, Himmel, & Marrone, 2013; Rimando & Duke, 2006)

Fungi, bacteria and plants as the main kingdoms of species with highly developed secondary metabolism, perhaps because they are generally not very mobile and have to make the most of where they end up. Approximately five hundred thousand secondary metabolites have already been reported. Around 350,000 are sourced from plants, 100,000 from animals, and 70,000 from microorganisms. Approximately 33,500 pharmacologically active metabolites have been identified from microbial origin. Of these microbial metabolites, around 12.5 percent

(4,200) are bacteria, and cyanobacteria metabolites, 41 percent (13,700) are *Actinomycete* fermentation products, and approximately 47 percent (15,600) are fungal metabolites (Bérdy, 2012; Nett, Ikeda, & Moore, 2009).

Furthermore, in the past twenty years, the novel fungal metabolites discovery has increased significantly in comparison to *Actinomycetes*; filamentous bacteria have historically been the most abundant source of natural microbial compounds (Nett *et al*., 2009). The filamentous Basidiomycota and Ascomycota have a well-developed secondary metabolism that is both diverse and abundant. However, it has been undeveloped in unicellular *Ascomycota, Basidiomycota, Zygomycota, Chytridiomycota* and *Blastocladiomycota*. The biodiversity of fungal species, notably in the Basidiomycota and Ascomycota, as well as the concurrent diversification of biosynthetic gene clusters, and genes, suggests that metabolic variation in fungi has almost infinite potential (Bills & Gloer, 2017).

Indeed, the success of the fungus in the eco-system and colonization of virtually any habitats present on the earth is due to the ability of secondary metabolites production and using it for its propagation and protection. The fact that so many species produce multiple secondary metabolites, their development is regulated by environment and life cycle, and the fact that large sections of their genomes are devoted to digesting and controlling the generation of these products shows how dependent the fungi dependence on the secondary metabolites to colonize range of habitats and maintain their existence within those habitats (Bills & Gloer, 2016).

Fungi are an especially abundant source of beneficial secondary metabolites (Pelaez, 2004) and have made significant and indispensable contributions to human and animal health advances. Secondary metabolites and their derivatives have yielded several therapeutic and promising lead compounds from bioactive natural products (Demain & Vaishnav, 2011). Furthermore, the success of penicillin effectively inspired critical technological improvements in the fields of microbiology, biochemistry, chemistry, and engineering. It made a number of

contributions to the development of the pharmaceutical industry in the modern era. Many more metabolites of fungal with useful bio-activities are included in the table 1.1

Fungi can discharge volatile secondary metabolites from the cells into the environment, incorporate them into the cellular structural elements, or stay inside the cell. The term "extrolites" has been considered that it could be used to describe these sorts of substances, mainly on the assumption that their biological effects are external (Frisvad & Larsen, 2015). However, the classical word "secondary metabolite" is preferred due to the function and cellular biosynthesis sites; many remain unknown because some of them have self-directed activities. The term secondary metabolite is also used for the sake of continuity. Secondary metabolites are biosynthesized by the pathways, which usees primary metabolites as basic building blocks to construct complex molecules, which are terpenoids, polyketides, nuclear-encoded ribosomal peptides, non-ribosomal peptides, and mixed biogenic origin molecules arising from combination pathways. Numerous secondary metabolites have been identified and compiled in various databases and compendia (Barrow, 2016).

1.2.9 Fungal secondary metabolites

Secondary metabolites are not important for the development or survival of the organism. While most species share primary metabolism, secondary metabolites have a biased historical distribution due to many factors that have promoted the extinction and diversity of their gene encoding and gene clusters along with the evolutionary lineages. Genes encoding the key catalytic enzymes enabling metabolites production can frequently be silenced in the laboratory with little impact on sporulation or vegetative growth (Chiang *et al*., 2016; Gaffoor *et al*., 2005; Wilkinson, Ramaswamy, Sim, & Keller, 2004). Many secondary metabolites seem to fall into this group, and secondary metabolism is typically important in context., as colours, siderophores, pheromones and sunscreens, are vital to survival in nature. Their significance to the organisms that generate them is certainly undervalued due to insufficient research and unexplained or misunderstood roles. The abundance and conservation of secondary

metabolites suggest that these are so important to the organisms that they should be considered alongside DNA, RNA, and proteins (Schreiber, 2005).

Many of these have already been discovered and identified due to their link to the biological activity or phenomena discovered outside of a lab setting (For instance, mycotoxins, phytotoxins, and pigments). In a few fungal groups, the fungal metabolic profiles of reproductive structures and cultures were identified and connected to taxonomy and phylogenetic data classification. (Frisvad, Andersen, & Thrane, 2008; Röhrich *et al*., 2014; Schardl *et al*., 2013; Stadler *et al*., 2014; Steglich, 1987). However, extensive screening programmes have contributed much to our knowledge of such chemistry done by the pharmaceutical sector and agricultural businesses and university laboratories in search of low molecular weight compounds with potentially beneficial effects.

Secondary metabolites are frequently isolated from complicated extracts obtained from fermentation using different chromatographic methods. Detected by spectroscopic techniques, such as nuclear-magnetic resonance (NMR), Liquid-chromatography mass-spectrometry (LCMS), and X-ray crystallography. The power to separate and identify compounds as well as detect and quantify them in a variety of materials has significantly increased because of the rapid expansion of the capabilities of these methods and databases storing spectral and other physical metabolite data. It is rare for metabolites to be present in both fungi and plants. Since various categories of eukaryotic species have different primary metabolisms and different evolutionary origins for their respective biosynthetic pathway, variations in secondary metabolism would be predicted between them. These differences can sometimes be attributed to the early stages of biosynthetic processes that have an impact on the number of certain precursors. Plants decarboxylate amino acids more often than fungi resulting in amine precursors that are eventually integrated into numerous kinds of plant alkaloids that are absent in fungi. Another example is the phenyl propanoid pathway, which is more common in plants than in fungi. The differences in secondary metabolites also exist between fungi and other microbes.

Table 1.1: Fungal metabolites lead to commercial pharmaceutical products (Bills & Gloer, 2017).

Metabolites	Source species	Structural classification	Commercial product(s)
Compactin (Mevastatin)	*Penicillum citrinum, Penicillum solitum,* and other *Penicilium* spp.	Polyketide, a product of two highly reducing polyketide synthases	Pravachol, Selektine, Lipostat
Penicillins G and V	*Penicillium rubens, Penicillium chrysogenum*	Nonribosomal peptide	Piperacillin, amoxicillin, and ampicillin
Cephalosporin C	*Acremonium chrysogenum*	Nonribosomal peptide	Cephalothin, cephalexin, cephradine, and cefadroxil
Pleuromutilin	*Clitopilus passeckerianus,* other *Clitopilus* spp	Diterpene	Retapamulin (Altabax) and animal, Antibiotics tiamulin and valnemulin.
Fusidic Acid	*Acremonium fusidioides*	Triterpene	Usidin, Fucidin, Fucicort, Fucibet, and Taksta
Strobilurins, A–D and other Strobilurins and Oudemansins	*Strobilurus tenacellus*	Polyketide with benzoyl CoA starting unit	Azoxystrobin, fenamidone, fluoxastrobin, kresoxim-methyl, and Trifloxystrobin
Griseofulvin	*Penicillium griseofulvum, Penicillium aethiopicum, Penicillium Coprophilum*, and *Penicillium* spp.	Halogenated polyketide	Fulcin, fulsovin, grisovin
Pneumocandin B_0	*Glarea lozoyensis*	Nonribosomal peptide acylated to polyketide	Aaspofungin (cancidas)
FR901379	*Coleophoma cylindrospora*	Nonribosomal peptide acylated to fatty acid	Micafungin (Mycamine, Micafungin (Mycamine, Fungiguard)
Echinocandin B	*Aspergillus pachycristatus* and other *Aspergillus* spp.	Nonribosomal peptide acylated to fatty acid	Anidulifungin (eraxis)
Enfumafungin	*Hormonema carpetanum*	triterpene glycoside	Precursor of the partial chemical Synthesis of experimental drug Scy-078 that is in phase ii clinical trials

Lovastatin (Monacolin K)	*Aspergillus terreus, Monascus, purpureus*, also occurs in *basidiomata* of *Pleurotus ostreatus*	Polyketide, a product of two highly reducing polyketide synthases	Mevacor Simvastatin (Zocor, Simvacor, Simvastin, and other brand names) is synthesised from lovastatin.
Cyclosporin A	*Tolypocladium inflatum*	Nonribosomal peptide	Cyclosporine A (Sandimmune, Neoral, Restasis, Gengraf, and others).
Mycophenolic acid	*Penicillium, brevicompactum* and other *Penicillium* spp.	Meroterpenoid	Used to produce mycophenolate mofetil (Cellcept) and mycophenolate sodium (Myfortic).
Myriocin (ISP-I)	*Isaria sinclairii*	Amino acid	Structural template for synthesis of fingolimod (Gilenya).
Ergotamine	*Claviceps purpurea, Claviceps fusiformis*, and *Claviceps paspali*	Prenylated nonribosomal peptide	Ergotamine tartrate (Ergomar, Migril, and others).
Ergometrine (Ergonovine)	*C. purpurea, C. fusiformis,* and *C. paspali*	Prenylated nonribosomal peptide	Ergometrine maleate (Ergotrate, Ergovin, and others).
Ergocryptine	*C. purpurea, C. fusiformis,* and *C. paspali*	Prenylated nonribosomal peptide	Partial chemical synthesis of bromocriptine (Parlodel).
Mizoribine	*Penicillium brefeldianum*	Imidazole nucleoside	Bredinin
Kojic acid	*Aspergillus oryzae. Aspergillus tamarii, Aspergillus flavus*	Pyrone derived from glucose	Kojic dipalmitate
PF1022A	*Rosellinia sp.*	Nonribosomal peptide	Emodepside (bis- paramorphonyl-derivative of PF1022A)
Fumagillin	*Aspergillus fumigatus*	Meroterpenoid	Bicyclohexyl ammonium fumagillin
Gibberellic acid (GA)	*Fusarium fujikuroi*	Diterpene	Produced by fermentation of F. fujikuroi
A-Zearalanol (zeranol)	*Fusarium culmorum, Fusarium sporotrichioides, Fusarium equiseti,* and *Fusarium semitectum*	Polyketide	Ralgro implants

1.2.10 Re-emergence of natural product drug discovery

The difficulties and decrease in the prominence of natural product drug discovery to reach clinical trials can be attributed to several factors. For example, the discovery of bioactive lead compounds from a natural product is considered a lengthy and time-consuming process compared to the beginning of high-throughput screening against clear molecular targets. It encourages many pharmaceutical companies to transfer towards 'screening friendly' pure chemical synthetic libraries from libraries of natural-product extracts. The development of combinatorial chemistry offers more straightforward and diverse drug-like libraries for drug candidate screening. The advancement in genomics, cellular biology and molecular biology increased the number of molecular targets and shortened the drug discovery timeline. The natural product extract's complexity is also one reason to slow down the lead drug discovery of the natural product (Butler, 2004; Koehn & Carter, 2005).

Newly developing trends, accompanied by the current research and development strategies, are reviving the interest in natural product drug discovery as a chemically diverse and lead generation because these compounds were initially generated by natural selection for bioactivity. While the complexity of extracts of natural compounds is technically challenging, it also is an opportunity to identify compounds that act synergistically. In addition to the screening of extract libraries, bioassay-guided isolation of compounds and their structure elucidation using the latest technology of high throughput screening, nuclear magnetic resonance, mass spectrometry, and high-performance liquid chromatography is making natural product drug discovery faster and less time-consuming (Koehn & Carter, 2005).

Several metabolic profiling tools have been widely used to develop metabolomics studies. The primary metabolomics profiling tools are nuclear magnetic resonance (NMR) and mass spectrometry (Nicholson, Lindon, & Holmes, 1999). NMR is considered a high-resolution and easy sample preparation instrument. In addition to the NMR, mass spectrometry (MS) has also been recognized as useful for metabolomics profiling (Aharoni *et al*., 2002; Madalinski *et al*., 2008).

Mass spectrometry offers metabolite identification and sensitive detection and can be coupled with chromatography. Recently liquid chromatography-mass spectrometry (LC-MS) technique has been widely used for metabolomics profiling (D.-H. Kim, Achcar, Breitling, Burgess, & Barrett, 2015; Surrati, Linforth, Fisk, Sottile, & Kim, 2016). It is a robust, selective and sensitive technique. It also has become popular for qualitative and quantitative analysis. The LC-MS technique can be used for natural product secondary metabolic analysis. It will help to identify the pharmacologically active compounds for pharmaceutical development and quality control of natural products. Mass spectrometry is an efficient technique for standardising and detecting secondary metabolites present in natural products. The compound responsible for the pharmacological activities of natural products can be identified and quantified based on LC-MS results. The increase in the demand for natural products around the world and the growth of the natural products industry have led to an increase in concern about their safety (Gogtay, Bhatt, Dalvi, & Kshirsagar, 2002). LC-MS metabolic profiles could be pretty helpful in the safety testing of the drugs. Once safety is established in natural products, they can play a vital role in treating several diseases due to their wide distribution and easy access.

1.3 *Cordyceps militaris*

Cordyceps militaris (L.) Fr., is a fungus commonly known as the orange caterpillar fungus; *C. militaris* is found worldwide, with several strains available for research. It has a diverse spectrum of insect hosts, predominantly larvae of lepidopterans (moth and butterfly caterpillars) (Shrestha, Zhang, Zhang, & Liu, 2012). There have been from 400 to 500 *Cordyceps* species (*sensu lato*) worldwide (Z.-y. Liu, Liang, Liu, Yao, & YU, 2002) (Webster & Weber, 2007), with 18 species in Europe and 29 species in North America (Humber, 2000). The majority of these are necrotrophic entomopathogens (Webster & Weber, 2007). In literature, the word – *Cordyceps sensu lato* – is used to describe species of the modern *Cordyceps* genus and is closely related to the previous former genus members, such as that

of *OphioCordyceps, Tolypocladium, ElaphoCordyceps, Beauveria*, and others (Shrestha *et al*., 2016).

Figure 1.5: *Cordyceps sinensis*, pictured in situ with insect host and cleaning after collection [Cornelia de Moor, reproduced with permission].

Cordyceps militaris is used as a natural medicine in China and far-east Asia. It contains many active metabolites such as cordycepin, pentostatin, adenosine, ergo-sterol, polysaccharides, mannitol and other important metabolites, which exhibit different physiological activities (Ng & Wang, 2005; J. Zhang, Wen, Duan, Zhang, & Ma, 2019). Cordycepin is a crucial constituent of *C. militaris*; studies reveal its anti-fungal properties and activity against herpes; in some cell lines, it also showed polyadenylation inhibitor effect, stimulating effect on interleukin-10 production as an immune modulator, anti-metastatic, anti- leukemic properties (Kondrashov et al., 2012; C. Park et al., 2005; J. H. Wong et al., 2011). Cordycepin (3′-Deoxyadenosine) is a polyadenylation inhibitor known for its broad spectrum of biological activities such as anti-inflammatory, anti-proliferative and pro-apoptotic effects (W. Chang et al., 2008; H. Kim, Naura, Errami, Ju, & Boulares, 2011; Kondrashov et al., 2012; Hardeep S Tuli, Sharma, Sandhu, & Kashyap, 2013). *Cordyceps militaris* is a major source of cordycepin and other important anti-inflammatory secondary metabolites.

The lack of significant literature on quality control and safety makes *Cordyceps militaris* products unsafe. Quality control is crucial for the authenticity and quality of the *Cordyceps militaris* products. For safety and efficacy and to ultimately test the fungal products in the clinic, product standardization and reproducibility are crucial. Presently the market product efficacy claims are not backed with solid clinical evidence and research data. Building trust and gaining confidence in people standardising the products is an important factor. Testing *Cordyceps* in the laboratory needs sample preparation. In sample preparation through extraction, solvent selection plays an important role in the nature and physiological activity of the extract. It helps to determine the yield and nature of metabolites of the extract obtained from *Cordyceps militaris* powder.

Several methods are adopted in extracting cordycepin from *Cordyceps militaris*. Studies have shown that ultrasonic waves and microwaves increase the cordycepin yield compared to the traditional maceration method. Extraction with the help of microwave aid and radiation, the resultant content cordycepin obtained was about 12mg/g. The extracting volume used for the solution was 10mL with a solid-to-liquid ratio of 1:200, and HPLC is used to detect and determine cordycepin (Min & Lu, 2006). Another technique adopted to extract the maximum quantity of the cordycepin is ultra-sonication. About 7mg/g of cordycepin was obtained with 60 min extraction time with heat and sonication in ethanol solvent (H.-J. Wang, Pan, Chang, Chang, & Hsieh, 2014). Another study shows that a solid-liquid ratio of 1:240 results in a 9mg/g yield. The percent recovery ranged from about 97% to 99% (T. Wang, Li, Chen, Chen, & Diao, 2010). *Cordyceps militaris* is an important fungus due to cordycepin, a nucleoside produced, and a topic of great importance for pharmacological-based research in recent decades. Besides cordycepin, other potentially important metabolites are also present in *Cordyceps militaris* extract.

Cordycepin has been reported to be unstable in animals due to deamination by adenosine deaminases. To bring cordycepin to the clinic, efforts have been made to circumvent this problem, focussing on the formulations, chemical modifications and the co-administration with

the adenosine deaminases (pentostatin) (Dalla Rosa *et al*., 2013; Rodman *et al*., 1997; Vodnala *et al*., 2013; Xia *et al*., 2017(a); Z. Zhao *et al*., 2012). Surprisingly, a recent study by Xia *et al.* (2017) demonstrated that in *C. militaris*, pentostatin is co-produced with cordycepin. The genes essential for both syntheses are found in adjacent loci cns1, cns2, and cns3 (Xia *et al*., 2017(a)). The evolutionary stresses on *C. militaris* likely led to the production of cordycepin coupled with pentostatin. Indeed, cordycepin may suppress the immune system of its insect hosts, which rely almost exclusively on innate immunity. Cordycepin has been identified as the primary factor in insect host death after *C. militaris* infection of insects (Wellham, Kim, Brock, & de Moor, 2019). Therefore, research into how cordycepin, pentostatin, and other secondary metabolites present in *C. militaris* affect insect immunity and fungus infection could result in targeted biological insect pest control.

This evidence on the evolution of cordycepin illustrates why there is a revival in the interest in natural products for drug discovery because these compounds were initially generated by natural selection for bioactivity. The co-production of cordycepin and pentostatin shows that while the complexity of extracts of natural compounds is technically challenging, it also is an opportunity to identify compounds that act synergistically.

1.3.1 Methods for natural product discovery.

In addition to the screening of extract libraries, bioassay-guided isolation of compounds and their structure elucidation using the latest technology of high throughput screening, nuclear magnetic resonance, mass spectrometry, and high-performance liquid chromatography is making natural product drug discovery faster and less time-consuming (Koehn & Carter, 2005).

Recently liquid chromatography-mass spectrometry (LC-MS) technique has been widely used for metabolomics profiling (D.-H. Kim *et al*., 2015; Surrati *et al*., 2016). It is a robust, selective and sensitive technique. It also has become popular for qualitative and quantitative analysis.

The LC-MS technique can be used for natural product secondary metabolic analysis. It will help to identify the pharmacologically active compounds for pharmaceutical development and quality control of natural products. Mass spectrometry is an efficient technique used for the standardization of natural products. LC-MS is used to identify the secondary metabolites present in natural products. The compound responsible for the pharmacological activities of natural products can be identified and quantified based on LC-MS results. The increase in the demand for natural products around the world and the growth of the natural products industry have led to an increase in concern about their safety (Gogtay *et al*., 2002). LC-MS metabolic profiles could be beneficial in the safety testing of the drugs. Once safety is established in natural products, it can play a vital role in treating many diseases due to their wide distribution and easy access.

1.3.2 *Cordyceps militaris* extract metabolite composition

Several studies have been done using various analytical methods, such as liquid chromatography, UV, gas chromatography and mass spectrometry (Guan, Yang, & Li, 2010; F.-Q. Guo, Li, Huang, Liang, & Chen, 2006; L. Li *et al*., 2019). These are some of the frequently used in the analysis of extract. Various LC-MS study shows that the metabolites detected in the extract are given in table 1.2.

Table 1.2: Metabolites and nucleobases detected in *Cordyceps*.

Metabolites	***Cordyceps sp.***	***Mobile phase***	***Column used***	***Detector***	***Reference***
Cordycepin	*C. militaris*	Methanol–water (15:85)	Phenomenex C18	UV 260 nm	(Huichun, Liang, Lao, Zhang, & Ito, 2011)
Cordycepin	Cultured *C. militaris*	Methanol–water (20:80)	YMC-packed C18	UV 260 nm	(Ni, Zhou, Li, & Huang, 2009)
Cordycepin	*C. militaris*	Acetonitrile–water (10:90)	ZORBAX SB-Aq	UV 260 nm	(Mao, Zhang, Zhang, & Chen, 2010)
Cordycepin	*C. militaris*	Methanol–water (17:83)	Alltima C18	UV 260 nm	(X. Ju *et al*., 2009)
Cordycepin	Cultured *C. militaris*	Gradient elution of water and acetonitrile	Eclipse XDB-C18	UV 260 nm	(Song, Liu, Li, & Jin, 2007)
Cordycepin	*C. militaris*	Methanol–20 mM phosphoric acid (15:85)	Mightysil RP-18	UV 260 nm	(Rao, Chou, & Tzeng, 2006)
Cordycepin	Natural and cultured *Cordyceps*	Water–methanol (72:28)	Waters ODS C-18	UV 260 nm	(Meena, Pandey, Negi, & Ahmed, 2010)
Cordycepin, Adenosine	*C. kyushuensis*	Phosphate buffer–methanol (17:3)	Kromasil 100-C18	UV 260 nm	(Ling, Zhang, Lin, Cui, & Zhang, 2009)
Cordycepin, Adenosine	*C. sinensis*, cultured *C. militaris*	Water–methanol (92:8)	Waters Symmetry Shield RP 18	UV 254 nm	(L. Huang, Li, Chen, Wang, & Zhou, 2009)
Uridine, Adenine, Cytosine, Adenosine, Guanosine	*C. militaris*	Acetonitrile–water (87:13) containing 10 mmol/L ammonium formate	Imidazole-functionalized silica columns: Sil-Im-2	UV 254 nm	(Zhu *et al*., 2012)
Sadenosine, Cordycepin, Cytidine, Guanosine, Inosine, Thymidine, Uridine, Adenine, Cytosine, Thymine, Uracil, Hypoxanthine	Cultured *C. militaris*	Gradient elution of 20 mmol/L phosphate buffer and methanol	ZORBAX Eclipse XDB-C18	UV 260 nm	(Yuan, Zhao, Wang, Kuang, & Liu, 2008)

Adenosine, Cordycepin	Cultured *C. cicadae*	Methyl alcohol-water (85:15)	YMC-Pack C8	UV 258 nm	(Y. Wang, Guo, Zhang, & Wu, 2012)
Adenosine, Cordycepin, Cytidine, Guanosine, Inosine, Thymidine, Uridine, Cytosine, Guanine, Thymine, Uracil	Natural and cultured *Cordyceps*	Gradient elution of water and methanol	Zorbax 300SB C18	UV 260 nm MS	(L. Yu *et al*., 2006)
Adenosine, Cordycepin	*Cordyceps*	Methanol–water (7:93)	Eclipse XDB-CN	UV 260 nm	(C. Li, Yan, Cai, & Liu, 2012)
Uracil, Uridine, Hypoxanthine, Inosine, Guanosine, Adenine, Adenosine, Cordycepin	*Cordyceps*	Gradient elution of water and acetonitrile	Zobax SB-AQ	UV 260 nm	(Feng *et al*., 2009)
Adenine, Cytosine, Guanine, Hypoxanthine, Thymine, Uracil	Natural and cultured *Cordyceps*	Gradient elution of 5 mmol/L aqueous TEA and methanol	Zorbax SB-AQ	UV 254 nm	(H. Fan, Yang, & Li, 2007)
Adenine, Adenosine, Cytosine, Cytidine, Hypoxanthine, Uracil, Guanosine, Uridine, Guanine, Inosine, Thymine, Thymidine, 2-Deoxyuridine, Cordycepin	Cultured *Cordyceps*	Gradient elution of 0.5 mM acetic acid and acetonitrile	Acquity UPLC BEH C18	UV 254 nm	(F. Yang, Guan, & Li, 2007)
Uridine, Adenine, Adenosine, Cordycepin	Natural and cultured *Cordyceps*	Gradient elution of acetonitrile and water	Symmetry Shield Rp18	UV 260 nm	(S. P. Li, Li, Dong, & Tsim, 2001)
Adenosine, Cordycepin, 2′-Deoxyadenosine, Guanosine, Uridine	*C. sinensis*, cultured *C. militaris*	Acetonitrile–water (5:95)	Daisopak 120-5-ODS-BP	UV 260 nm	(Ikeda, Nishimura, Sun, Wada, & Nakashima, 2008)
Uridine, Inosine, Guanosine, Adenosine, Cordycepin	*C. sinensis*, cultured *C. militaris*	Gradient elution of 10 mM aqueous TEA and methanol	Zobax SB-AQ	UV 254 nm	(F. Yang & Li, 2008)

1.3.3 Cordycepin production and isolation

Many studies have been done to study the growth of *C. militaris* in artificial environments and the maximisation of cordycepin production from the fungus. The culturing methods and types of media for the production of *Cordyceps militaris* in a laboratory setting are discussed by Das *et al.,* (2010) (S. K. Das, Masuda, Sakurai, & Sakakibara, 2010). Silkworm larvae and cereal grains are traditionally used to effectively culture the mycelium of *Cordyceps militaris* commercial usage (Cleaver, Loomis-Powers, & Patel, 2004). On the surface and submersion (in which biomass of fungus is grown in liquid media under aerobic conditions) are the two methods of fermenting and cultivating mycelium. For the detection of cordycepin, HPLC (high-performance liquid chromatography) – mass spectrometry and other approaches, including UV detection, have been proposed (C.-Y. Chang, Lue, & Pan, 2005).

The primary edible and therapeutic component of *C. militaris* are the fruiting bodies, cordycepin however is also detected in the mycelium, which is only produced during the sexual life cycle. *C. militaris* lack fruiting bodies during the asexual life cycle. When *C. militaris* degenerates, it frequently loses its ability to develop fruiting bodies or has its ability reduced (C.-Y. Chang *et al*., 2005; A. Chen, Wang, Shao, & Huang, 2017; J. Yin, Xin, Weng, & Gui, 2017). Fruiting bodies' deformation can also result from *C. militaris* degeneration. As a result, the fruiting body production is decreased, leading to considerable economic losses. Slower growth, lesser conidia production, fewer primordia (origin of the fruiting body), an extended growth cycle, and decreased secondary metabolites content also occur in degradation (Wellham *et al*., 2021; Xiong, Xia, Zheng, & Wang, 2013; J. Yin *et al*., 2017).

Several studies show the enhancement of the production of cordycepin, pentostatin and adenosine production in *C. militaris* using various substrates. The cultivation of *C. militaris* on oats and Pattern I enhanced the formation of fruiting bodies. Regardless of the type of grain and growing methods, the amount of pentostatin and adenosine in harvested fruiting bodies

was much higher than that of fermented grains. It was found that brown rice and buckwheat had higher levels of pentostatin and adenosine in the fruiting bodies. Additionally, the cultivation of control pattern and coix seeds favoured an increased cordycepin production and showed that *C. militaris* fermented grains and fruiting bodies might contain more cordycepin after being subjected to a temperature treatment during development (C.-Y. Wu, Liang, & Liang, 2022). Some of the culturing methods used are spawn production, sawdust culture, husked rice culture, shaking culture, submerged culture, surface liquid culture, and repeated batch culture. Solid and liquid media are used to culture *C. militaris*. Solid media may be wheat grains, beech wood meal, rice bran, husked rice, wheat bran, and cultured in a bottle or bag (S. K. Das *et al*., 2010). High production of 8.57 g/l of cordycepin was reported from mutant *C. militaris* and added 6 g/l adenosine using the ion beam irradiation method (S. Das, Masuda, Sakurai, & Sakakibara, 2009)

Various extraction methods for cordycepin have been shown, most of which are unsuitable for industrial scale (reviewed by Tuli *et al.,* 2015) (Hardeep Singh Tuli, Kashyap, & Sharma, 2015). Examples consist of evaporation and precipitation followed by filtering (Dowex-I-chloride column) (Kredich & Guarino, 1960); hydrothermal reflux (Yingjuan, Duowei, Yichao, & Tingting, 2005); hexane, and ethyl acetate, butanol solvent extractions from dried material (H. G. Kim *et al*., 2006; Rao, Fang, Wu, & Tzeng, 2010); silica gel /ion-exchange resin chromatography (Jiansheng, 2008); ethanol, water and ultrasonic extraction methods (H. Zhang, Wang, Dong, Xu, & Wang, 2012); and extraction using microwave (C. Li *et al*., 2012). Currently, commercial cordycepin is predominantly purified from *Cordyceps militaris* liquid fermentation media (S. K. Das *et al*., 2010). The cordycepin content varies from strain to strain. The liquid fermentation broth contains more cordycepin and pentostatin content.

Table 1.3: Methods used for isolation and purification of cordycepin.

Strains	Isolation method	Cordy(mg/g)	Purity	Reference
C. militaris	HSCCC and cation-exchange resin	299.7	98.9%	(Huichun *et al*., 2011)
C. militaris (shanghai)	Surface imprinting technology	95.4	98%	(Y. Zhang, Wan, & Cao, 2016)
C. militaris (jiangsu)	Macro-porous resin and HSCCC	31.2	98.5%	(Z. Zhang *et al*., 2016)
C. kyushuensis	supercritical fluid extraction (SFE) and high-speed counter-current chromatography (HSCCC)	22.3	98.9%	(Ling *et al*., 2009)
C. militaris	Silica gel column chromatography and reversed-phase (RPC)	0.4	99%	(H. Zhang *et al*., 2015)

1.4 Analytical methods for detection of cordycepin and other metabolites

Analytical techniques, including liquid chromatography (LC), mass spectrometry (MS) and UV detection, have been employed in studies on metabolites of *C. militaris*. Coupled LC-MS approaches, in particular, have been adopted to detect small compounds, including nucleosides and their homologues, like cordycepin and pentostatin. Further detail on these analytical methods is discussed in methods chapter 2.

1.4.1 Untargeted metabolomics studies of *Cordyceps*

Untargeted metabolomics was used to compare various *Cordyceps militaris* samples – Such as analysing the amount and number of metabolites present in the extract extracted with water, ethanol and water-ethanol mixture (He *et al*., 2019) and confirming the fungal species identity of the extracted sample (J. Zhang *et al*., 2015). To analyse the metabolomic results of samples, multivariate statistical approaches such as principal component analysis (PCA) were utilised. Following metabolic pathway analysis from GC-MS (gas chromatography-coupled mass spectrometry) data, Oh *et al.* (2019) found that cordycepin production was enhanced in

senesced stromata of *C. militaris* and that this was simultaneous with stimulation of the glutamine and glutamic acid pathway (Oh, Yoon, Shrestha, Choi, & Sung, 2019). The authors used electron ionisation and identified compounds using retention time and accurate mass. An LC-MS (electrospray ionisation) based method comparing three samples (*C. militaris* grown on brown rice and tussah moth caterpillar and a *Cordyceps sinensis* collected from the wild) (L. Chen, Liu, Guo, Zheng, & Zhang, 2018), An advanced method with the LC-MS/MS (tandem mass spectrometry) was employed by Zhang *et al.,* (2015a) to differentiate between genuine and the counterfeit *O. sinensis* samples using multivariate analysis (PCA) and reported a higher number of metabolite in positive mode than in negative. γ-glutamyl-glutamine, agmatine, 2′-deoxyadenosine, 3′-deoxyadenosine, and 5′-deoxyadenosine were reported and confirmed in the study. Tandem mass spectrometry, which provides the highest level of fragmentation identification, is now regarded as the gold standard for untargeted metabolomics research (J. Zhang *et al*., 2015).

1.4.2 Targeted metabolomics with cordycepin and related small molecules

Research on small molecule targets using mass spectrometry analysis of *C. sinensis,* and *C. militaris* samples have been reported, all of which used dried, commonly mycelial, or extracts. Metabolites of particular interest, include nucleosides, nucleobases, and analogues and derivatives such as cordycepin. The reported standard techniques in LC-MS analysis have improved recently, shown in the table, with an increasing focus on the identification confidence of metabolites by fragmentation – it is achieved by employing either quadrupole and time of flight (Q-TOF) or triple quadrupole (QqQ) mass analysers. The detection limits (LOD) were also enhanced.

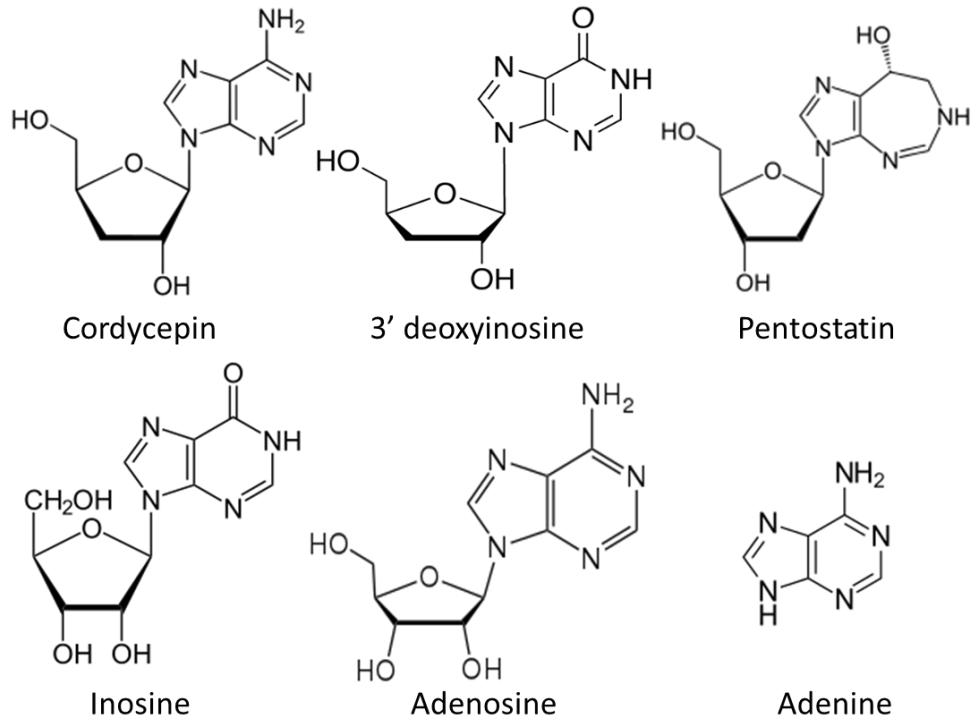


Figure 1.6: Structures of various nucleobases, nucleosides, derivatives, and analogues of interest.

Table 1.4: Published studies using mass spectrometry analyses of the dried mycelial *C. militaris* and sinensis extracts, targeting nucleobases and nucleosides.

			Extraction	
Reference	**Separation method**	***Cordyceps sp.***	**Solvent**	**Method**
Yang *et al.*, 2010	Ion-pairing reversed-phase liquid chromatography	*C. sinensis, C. militaris*	Water	Heating (95°C)
Yang *et al.*, 2009	Capillary electrophoresis	*C. sinensis, C. militaris*	Water	Heating (95°C), sonication
Guo *et al.*, 2006	HPLC	*C. sinensis*	Water, methanol (reconstitution)	Drying (35°C), sonication
Huang *et al.*, 2003	HPLC	*C. sinensis, C. militaris*	Water	Sonication
Xie *et al.*, 2010	HPLC	*C. sinensis*	Water	Sonication
Zong *et al.*, 2015	UHPLC	*C. sinensis*	Methanol (10%)	Sonication
Zhao *et al.*, 2013	HPLC (HILIC)	*Cordyceps sp.*	Methanol (60%)	Sonication
Fan *et al.*, 2006	HPLC	*C. sinensis, C. militaris*	Methanol	Heating (160°C)
Huang *et al.*, 2004	HPLC	*C. sinensis*	Methanol, water (reconstitution)	Drying by vacuum

Table 1.5: List of nucleosides and metabolites studies using mass spectrometry.

Reference	Mass spectrometry method		Metabolites detected
	Instrument	**Cordycepin LOD**	
Yang *et al.*, 2010	Agilent LC/MSD Trap	0.04µg/mL	Adenine, adenosine, adenosine-5'-monophosphate, cordycepin, cytosine, cytidine, guanine, guanine-5'-monophosphate, guanosine, hypoxanthine, inosine, thymine, thymidine, uracil, uridine, uridine-5'-monophosphate
Yang *et al.*, 2009	Agilent Trap XCT	0.12µg/mL	Adenine, adenosine, cordycepin, cytosine, 5-chlorocytosine, cytidine, guanine, guanosine, hypoxanthine, inosine, thymidine, uridine, 2'-deoxyuridine, arabinoside
Guo *et al.*, 2006	Shimadzu LCMS-2010	0.1µg/mL	Adenine, adenosine, 2'-chloroadenosine, cordycepin, guanine, hypoxanthine, thymine, uracil, uridine
Huang *et al.*, 2003	Shimadzu LCMS-2010	0.1µg/mL	Adenine, adenosine, cordycepin, hypoxanthine
Xie *et al.*, 2010	Shimadzu LCMS-2010	0.1µg/mL	Adenine, adenosine, cordycepin, thymine
Zong *et al.*, 2015	Agilent 6460 Triple Quadrupole Tandem MS	0.04ng/mL	Adenine, adenosine, 2'-chloroadenosine, cordycepin, cytosine, guanine, guanosine, hypoxanthine, inosine, thymine, thymidine, uracil, uridine, 2'-deoxyuridine
Zhao *et al.*, 2013	Agilent G6520 Q-TOF MS, Agilent G6420A MS	0.21ng/mL	Adenine, adenosine, 2'-chloroadenosine, cordycepin, cytosine, cytidine, guanine, guanosine, xanthine, hypoxanthine, inosine, thymine, thymidine, uracil, uridine, 2'-deoxyuridine
Fan *et al.*, 2006	Agilent 1100 LC/MSD Trap	1.16µg/mL	Adenine, adenosine, cordycepin, cytosine, cytidine, guanine, guanosine, hypoxanthine, inosine, thymine, thymidine, uracil, uridine, 2'-deoxyuridine
Huang *et al.*, 2004	Shimadzu LCMS-2010		Adenine, adenosine, uridine

1.4.3 Modern advanced metabolomics studies and “omics”

Recent high-impact advanced metabolomics applications include the use of metabolomics to identify bioactive compounds, cancer research, and the discovery of novel metabolic pathways (Pareek, Tian, Winograd, & Benkovic, 2020; Rinschen, Ivanisevic, Giera, & Siuzdak, 2019). The integration of high-throughput datasets or "omics" to acquire a better knowledge of biological impacts and processes is a newly emerging field known as integrated omics.

It has been suggested that metabolomics may be the omics method that best reflects the phenotype. In a process called metabolomics activity screening, metabolomics linked with genomics, proteomics, and other high throughput datasets may help extract metabolites of interest for further investigation (Guijas, Montenegro-Burke, Warth, Spilker, & Siuzdak, 2018). As a result, metabolomic regulation of processes in organisms including macrophage immunological activity, oligodendrocyte maturation, and cancer development, may be determined (Guijas *et al*., 2018; Rinschen *et al*., 2019). Metabolite activity screening is an extension of metabolites bioactivity study indicators, which can be employed as a diagnostic tool for malignancies (C. Sun *et al*., 2019). Screening of metabolite activity has been used in treating disorders such as immune-metabolism, arthritis, asthma, and ageing research in model organisms (Rinschen *et al*., 2019).

1.5 Cordycepin

Cordycepin was isolated and characterized for the first time from *Cordyceps militaris* in 1950 (Cunningham, Manson, Spring, & Hutchinson, 1950). At the beginning of molecular biology research, it was frequently utilized as a tool to examine polyadenylation, the final step in RNA processing, especially once it became commercially accessible in the early 1970s (Brawerman & Diez, 1975; Diez & Brawerman, 1974; Penman, Rosbash, & Penman, 1970; Philipson, Wall, Glickman, & Darnell, 1971; A. Wu, Ting, Paran, & Gallo, 1972). This chemical application declined in the 1980s when small molecule inhibitors of biological

processes became less popular because they were found to be insufficiently specific for most applications. This led to fewer cordycepin publications, resulting in only five during 1997. Ever since, research articles on cordycepin have increased again, with remarkably rapid growth in the recent decade. The interest in cordycepin as an active ingredient of traditional medicine is obvious with a majority of articles published from Asia, particularly China, with 60-70 papers a year in recent years (Radhi *et al*., 2021).

1.5.1 Cell Death, Survival and Division

To examine the effects of various cytotoxic drugs on the cell lines, 50% inhibitory concentrations (IC_{50}) are often used. Cordycepin IC_{50} values for diverse cell lines range from 15μM to 2mM, with incubation times typically ranging from 24 to 48 hours. It was discovered that the average IC_{50} for 126 tests from 74 articles is 194μM, with a standard error of 250 μM, showing huge variability. However, most IC_{50} values cluster around the median 135μM, with a small number of nearly resistant cell lines misinterpreting the distribution data. A limitation of this study is that if a type of cell does not achieve the IC_{50} inside the dosage range tested, it was not included in the analysis; hence these results were more likely to be accurate for cell types and situations that do display cordycepin sensitivity (Radhi *et al*., 2021).

1.5.2 Effects on the Inflammatory Response

The inflammatory response is a well-defined array of gene expression processes initiated by signals showing tissue injury or infection (Mitchell & Carmody, 2018). The mediated inflammatory genes include cytokines (-for example, TNFα, TGFβ and IL1β), prostaglandin synthases (– for example, COX-2 and PTGES), nitric oxide synthase (iNOS), and genes associated with tissue remodelling and cell migration, namely the metalloproteinase (e.g., MMP9) and cell adhesion molecule VCAM-1. Recently, we have shown in a review that 36 out of 38 papers investigating the effect of cordycepin on inflammation inhibited the expression of inflammatory gene products (Radhi *et al*., 2021). Several studies shows show that cordycepin may reduce TGFβ activity by lowering activation and preventing the cellular response

(Finnson, Chi, Bou-Gharios, Leask, & Philip, 2012; Khandia & Munjal, 2020; Z.-G. Ma, Yuan, Wu, Zhang, & Tang, 2018; Meng, Nikolic-Paterson, & Lan, 2016).

NFκB is a transcription factor, which plays a vital function in gene activation during inflammation and wound repair (Mitchell & Carmody, 2018). When inflammatory signals cascade leads to NFκB translocation from cytoplasm to nucleus and binds to DNA. Many studies show that cordycepin affects NFκB-mediated transcriptional activation (S. Ashraf *et al*., 2019; Cheng & Zhu, 2019; Y. H. Choi, Kim, & Lee, 2014; Cui *et al*., 2018; Z. Guo, Chen, Dai, & Huang, 2020; Jeong *et al*., 2010; H. G. Kim *et al*., 2006; Peng, Wang, Ge, Qu, & Jin, 2015; Yan *et al*., 2017; Jianping Yang *et al*., 2015; Ying *et al*., 2014). Moreover, a wealth of papers indicate that cordycepin suppresses inflammatory processes in a range of diseased animal models (S. Ashraf *et al*., 2019; M. Chen *et al*., 2012; Cheng & Zhu, 2019; X. Li *et al*., 2017; Radhi *et al*., 2021; Jing Yang, Zhou, & Shi, 2020). The literature, therefore, indicates that cordycepin has a potent anti-inflammatory effect in both cell cultures and animals.

1.5.3 Cordycepin mechanism of action

The mechanism whereby cordycepin suppresses inflammation is not entirely understood; however, the effects on signalling pathways appear to play a role. Cordycepin inhibits the PI3K/mTOR/AKT pathway and activates AMPK (S. Ashraf *et al*., 2019). mTOR/AKT and AMPK are known to play a role in the control of inflammation and are linked to metabolic alterations in macrophages (Gershon, Galiani, & Dekel, 2006; Hara *et al*., 2005; Traverso, Donnay, & Lequarre, 2005; D. X. Zhang, Cui, & Kim, 2009). Furthermore, many of the reported diseases attempt to replicate age-related disorders such as rheumatoid arthritis, diabetes (type II), heart problems, and neurological impairment, all of which are connected to chronic inflammation (Wyss-Coray, 2016). Surprisingly, some mTOR inhibitors (rapamycin/ sirolimus) have also been shown to enhance longevity (and hence age) in small doses (Smith, Sharma, & Mair, 2020). Similarly, additional AMPK activators (e.g., metformin) have been shown to enhance metabolic health in the elderly (Smith *et al*., 2020).

Cordycepin literature is overall of modest quality; nonetheless, the amount of research demonstrating promising biological results is already so vast that the number of repetitions compensates for unwanted noise. Cordycepin has anti-proliferative and anti-inflammatory properties, activates AMPK, represses phosphorylation of AKT by mTOR, and frequently lowers phosphorylation of ERK by MEK, according to our meta-analysis (Radhi *et al*., 2021). While many animal models employed do not precisely duplicate human disease, there is no question that cordycepin has obvious beneficial effects in several animals with a range of disease-related symptoms.

The lack of a characterized cordycepin-binding target molecule and a mechanism of action that links this binding to therapeutic effects is a key ongoing challenge impeding the development of cordycepin as a lead chemical. Many targets, including poly (A) polymerases, adenosine receptors AMPK and PARP, have all been proposed as binding targets (Qi *et al*., 2019; Thomadaki, Tsiapalis, & Scorilas, 2008; Wei *et al*., 2019). Since cordycepin is the result of evolution, it is also possible that it has several targets that work synergistically to create biological effects (Wellham *et al*., 2019).

The wide range of systems and commonalities in the responses outlined here suggest that cordycepin primary target(s) cannot be very cell or tissue specific. Research has shown that cordycepin is readily converted into cordycepin triphosphate and trapped in cells. It has been demonstrated that inhibiting cordycepin import and phosphorylation reduce its effects, implying that intra-cellular and phosphorylated cordycepin is likely the active metabolite (Cuenda & Rousseau, 2007; Gu et al., 2013; Hiraoka, Tanabe, Kuwabara, & Sato, 1988; D. Ju et al., 2020; Kadomatsu et al., 2012; Kefford, Taylor, & Fox, 1983; Kitamura et al., 2011; Kondrashov et al., 2012; Overgaard-Hansen, 1964; Takahashi et al., 2012; Y. Y. Wong et al., 2010; Zieve, Feeney, & Roemer, 1987).

Cordycepin demonstrated biological action in animal studies when administered orally, intraperitoneal or intravenously. It is readily deaminated by the adenosine deaminase in blood or tissue culture conditions, producing 3' deoxyinosine (Hawley *et al*., 2020; Kodama, McCaffrey, Yusa, & Mitsuya, 2000; J. B. Lee *et al*., 2019; G. Li, Nakagome, Hirono, Itoh, & Fujiwara, 2015; Y.-J. Tsai, Lin, & Tsai, 2010; Vodnala *et al*., 2013). Even sensitive assays cannot identify cordycepin in the blood after oral administration, raising questions about the cordycepin active metabolite. However, it was recently shown in research that at least certain cell types could be converted from 3' deoxyinosine into cordycepin triphosphate (J. B. Lee *et al*., 2019). This suggests that cordycepin may target specific tissues throughout the body, not due to a tissue-specific molecular target but because of the tissue-specific conversion of the 3' deoxyinosine into cordycepin triphosphate. Furthermore, if the cordycepin circulates as 3' deoxyinosine, the cordycepin may avoid harmful effects produced by adenosine-like molecule build-up (Hirschhorn, 1990).

Another aspect limiting cordycepin research is the unavailability of commercially highly pure and/or synthetic preparations. The most extensively used Sigma preparation (now known as Merck) is obtained from *Cordyceps militaris* and is listed as 98 percent pure. Because cordycepin is often utilised in micro-molar amounts, it is plausible that active contaminants in the sub-micro molar contribute to cordycepin biological activity. Since similar effects have been reported across time and with different suppliers implies that if there are significant bioactive contaminants, they should be present quite consistently. Fortunately, in some cases, purer and synthetic preparations of cordycepin were observed to have effects similar to standard preparations (E. J. Lee, Kim, & Moon, 2010; Y. Li *et al*., 2018; Niu *et al*., 2010). Purer cordycepin preparations would be highly beneficial to the field if they were available commercially as analytical standards for activity comparison. In the absence of adenosine deaminase inhibitors, the toxic effects of cordycepin have been reported to be minimal in animal studies (Aramwit, Porasuphatana, Srichana, & Nakpheng, 2015; J. Gao, Lian, Zhu, & Zhu, 2011; Rodman *et al*., 1997; Williamson & Scott-Finnigan, 1978). However, no dose

progression investigations of cordycepin to several times the therapeutic dose for intravenous or oral administration are available. This is to be expected for intravenous dosages, which are restricted by the solubility of cordycepin in basic formulations (J. B. Lee *et al*., 2017). Intraperitoneal delivery gave different results, with one study reporting no adverse effects at 900mg/kg, another 50% mortality at 400mg/kg and a third one several deaths after three daily doses of 150mg/kg (Jagger, Kredich, & Guarino, 1961; L. Ma, Zhang, & Du, 2015).

Cordycepin appears to have very little or no mutagenic activity according to the Ames test (Aramwit *et al*., 2015; Inoue, Murakami, & Fujii, 1986). Knowing the effect of cordycepin on the mTOR pathway, the concern is that, like the mTOR inhibitor rapamycin (sirolimus), it might inhibit the immune system or impact wound healing, although cordycepin has been shown to enhance healing so far (M.-j. Wang, Peng, Lian, & Zhu, 2019). Another source of concern is that cordycepin-reported effects on the meiotic cell cycle and early embryogenesis may have an impact on female fertility in particular. To determine the safety of cordycepin, complete dosage escalation tests and careful investigation of the long-term effects of the therapeutic doses, particularly on immunity, wound repair, and fertility will be required (Mannick *et al*., 2018).

Therapeutic effects in animal models frequently do not translate into effective medicines for human patients, and it will have to wait until clinical study results to learn whether cordycepin, as well as its derivatives, are bioactive in humans (e.g., ChiCTR-INR-17014074, NCT00709215, NCT00003005, and NCT03829254). A preliminary report on a cordycepin trial in conjunction with an adenosine deaminase inhibitor known as pentostatin for acute lymphocytic leukaemia was published in 2000 (Foss, 2000); however, the study has yet to publish a final report. Treatment of a 40% pure cordycepin formulation for individuals with chronic renal disease showed promising results regarding kidney function (Liang *et al*., 2017). A preliminary report on a phase 1 trial of a cordycepin analogue for cancer indicates it is well tolerated (Schwenzer *et al*., 2021).

In summary, cordycepin shows demonstrable bioactivities in many diseased animal models. It inhibits the PI3K/AKT/mTOR signalling pathway while activating AMPK. Most cordycepin therapeutic actions are consistent with being mediated by these activities on signal transduction. Furthermore, the broad spectrum of therapeutic effects in animal models is similar to that seen with other mTOR inhibitors and AMPK activators. The unclear mode of cordycepin action, the lack of the availability of commercial cordycepin with high purity, and an incomplete understanding of bio-distribution are the major obstacles. Nonetheless, cordycepin looks to be a very promising lead for drug development, which can lead to the treatment of a variety of common disorders and demands further investigation.

1.6 Project Aims and outcome

My PhD project's primary purpose was to characterise the anti-inflammatory effect of *C. militaris* extracts and compare the composition and potency of different cordycepin preparations. Initially, extraction from the fungus was standardised, and extracts were subjected to metabolomics analysis and tested for inflammatory and anti-inflammatory activities. Then I turned to investigating the anti-inflammatory potency of purified cordycepin preparations and examining the contaminants of these preparations. The results are described in the rest of this thesis.

CHAPTER 2
MATERIAL AND METHODS

CHAPTER 2: MATERIALS AND METHODS

The work in this thesis includes the application of analytical chemistry to understand the biological metabolome, which implicates the use of analytical instrumentation techniques along with a range of different experimental methods, including molecular biology techniques and cell culture experiments. All the materials, reagents, and chemicals used in the molecular biology and tissue culture experiment are listed in tables 2.1 to 2.3, and all the materials, reagents, chemicals and software used in the analytical chemistry experiment are in tables 2.2 to 2.4.

Table 2.1: Materials, reagents and chemicals used in molecular biology and tissue culture experiment.

Material/Chemical/ Reagent	Cat no	Supplier
RAW 264.7 cells (ATCC® TIB-71™)	93150	ATCC
Six well plates (TPP)	92006	Merck
Dulbecco's Modified Eagle media	RNBK7347	Merck
Foetal bovine serum	2440086H	Merck
Phosphate buffered saline	BR0014G	ThermoFisher
Lipopolysaccharide	L6761	Merck
Reliaprep® RNA Cell Miniprep System	Z6010	Promega
SuperScript® III First-Strand Synthesis System	2291369	ThermoFisher
GoTaq® G2 polymerase	0000516159	Promega
GoTaq® qPCR Master Mix	0000516159	Promega

Table 2.2: Materials and chemicals used in molecular biology, tissue culture experiment, and liquid chromatography-mass spectrometry.

Material/Chemical	Cat no	Supplier
Cordycepin	ND029301501	Carbosynth
Cordycepin	0000111585	Merck (Sigma)
Cordycepin Phyproof ®	113233095	PhytoLab
Cordycepin	3A/226117	Tocris
Cordycepin	J65366	Alfa Aesar
Cordycepin		Goba
Pentostatin	1C/99117	Merck (Sigma)
3’ deoxyinosine	13146-72-0	Santa Cruz Biotec
Adenine	LRAC2871	Merck (Sigma)
Adenosine	A4036	Merck (Sigma)
Inosine	SLCH8567	Merck (Sigma)
L-proline	BCBZ5040	Merck (Sigma)
Diethyamine	10224332	Merck (Sigma)

Table 2.3: Materials, Chemicals and reagents used in liquid chromatography and mass spectrometry experiments.

Material/Chemical/ Reagent	Experiment	Supplier
Methanol (LC-MS grade)	LC-MS (sample preparation; solvent)	VWR
Ethanol (HPLC grade)	DNA extraction	VWR
Isopropanol (HPLC grade)	DNA and RNA extraction	VWR
Acetonitrile (LC-MS grade)	LC-MS (mobile phase)	VWR
Ammonium Carbonate	LC-MS (mobile phase)	VWR
Pierce LTQ ESI Positive Ion Calibration Solution (modified)	LC-MS (calibration)	ThermoFisher
Pierce ESI Negative Ion Calibration Solution (modified)	LC-MS (calibration)	ThermoFisher

Table 2.4: Software used.

Software name	Application
Microsoft Office Excel® 2016	Data analysis and calculations
GraphPad Prism® 9	Data figures and statistical analysis
R version 4.0.0	Used in IDEOM analysis
Exactive Tune® Thermo Scientific	Mass spectrometry operation
TOXID	Extracting data from LC-MS raw data files
ProteoWizard 3.0.1	Convert RAW LC-MS files to readable formats
Chromeleon® Thermo Scientific	Overview of LC-MS on the (Q-)Exactive mass spectrometer; operation of liquid chromatography method
Thermo XCalibur®	Overview of LC-MS on the (Q-)Exactive mass spectrometer; operation of liquid chromatography method
XCMS	Processing analysis of LC-MS raw data files
MzMatch	Processing analysis of LC-MS raw data files
IDEOM_v19	An excel-based platform for LC-MS high-throughput metabolomics data
Thermo Compound Discoverer®	Identification of metabolites by mass spectra following LC-MS/MS
Thermo TraceFinder®	Identification of metabolites using standards following LC-MS
Sartorius SIMCA-P® 4	Statistical analysis of metabolomics data – PCA and OPLS-DA

2.1 *Cordyceps militaris* extraction

Wild *Cordyceps militaris* stromata collected in Slovenia, grown and processed in the laboratory under controlled conditions by Andrej Gregori at MycoMedica. 1 gm of powdered *Cordyceps militaris* (CM) extracted with 20mL of extraction solvent. The solvent was selected based on the compound of interest, cordycepin, which is a polar compound and therefore readily soluble in polar solvents. The aim was to obtain a product with determined safety, efficacy, and economics which can be consumed orally with a reproducible biological activity. Ethanol and water were selected for extraction based on their relative safety for human consumption

compared to other solvents. Solvent mixtures used for extraction included 100% ethanol (E), 100% water (W) and 60% ethanol in water (EW). The extraction efficiency by the dual extraction method was also checked, extracting with water, dried, and then 1mL of the dried extract was re-extracted with 1mL ethanol (WE). A previous study by Wang, Pan *et al.,* (2014) shows that heating to 60 ºC, sonication (56 kHz) and maceration time (1 hr and 24 hr) maximise the extraction of cordycepin; the method was adopted for extraction with some changes to optimise the extraction of cordycepin and pentostatin (H.-J. Wang *et al*., 2014). The macerated samples were centrifuged at 15000 RPM for 15 min, and then the supernatant was pipetted 1mL in each Eppendorf and dried using Jouan concentrator centrifugal evaporator system (RC10 22); the dried samples were stored at -20ºC. Samples were prepared in a laminar flow hood by dissolving the 1mL extract dried in eppendorf was dissolved in 10µl tissue culture grade DMSO for tissue culture use. The highest solubility of dried extract in DMSO was E, followed by EW, WE and the least solubility was of the W sample.

2.2 Tissue culture experiments

The RAW 264.7 macrophages were used to determine the anti-inflammatory activity of cordycepin and other significant metabolites present in *Cordyceps militaris* extract. The literature shows that macrophages are sensitive to cordycepin (H. G. Kim *et al*., 2006; Shin *et al*., 2009). The RAW 264.7 cells used in the study were maintained in DMEM, added with 10% foetal bovine serum (FBS) in an incubator with a humid environment of 95% air and 5% CO2 at 37°C. The cells were split at a confluence of about 80%. The passage range of 07 to 42 was used for all cell-based experiments.

2.2.1 Stimulation of RAW264.7 cells and activity of extracts and compounds

The RAW264.7 cells were seeded into 6-well plates TPP® at seeding densities of 2×10^3 to 6×10^4 cells/cm^2. Lipopolysaccharide (LPS) was used to stimulate the inflammatory response in RAW264.7 cells. The cells were treated with LPS at a final concentration of 1µg/mL. Cells were treated with a range of bioactive compounds or extracts for one hour before stimulation with LPS. Cordycepin was purchased from Merck, Carbosynth, Alfa Aesar, PhytoLab and Tocris. Goba cordycepin was gifted by Andrej Gregori our collaborator from Goba MycoMedica. The cordycepin for treatment was first dissolved in cells culture safe dimethyl sulfoxide (DMSO) at the required concentration of 100µM and then diluted to 50µM, 25µM, 12.5µM and 6.26µM. In all the cell experiments, cells were seeded in 10% FBS supplemented DMEM media on the first day. After 24 hours, the cells were washed with sterile PBS, and the media was changed by 0.5% serum-supplemented media added for 24 hours before treatment. The RAW264.7 cells were treated with samples 1 hour before LPS treatment at mentioned concentrations by adding 3µl of the sample per well containing cells and 3 mL of DMEM media. Then cells were treated with 3µl of LPS for 1 hour, the media was discarded, and cells were washed with sterile PBS. The cells were lysed by adding RNA lysis buffer supplemented with 10% β-mercaptoethanol purchased from Promega, ReliaPrep™ RNA Cell Miniprep System.

2.2.2 RNA isolation, reverse transcription and quantitative real-time polymerase chain reaction (RT-qPCR)

RNA extraction from the cells followed the manufacturer's instructions (Promega, ReliaPrep™ RNA Cell Miniprep System), except that the DNase treatment step was done for 1 hour instead of 15 minutes. NanoDrop 1000 spectrometer (Thermo Scientific) was used for RNA concentration and quality determination, using A260/A280 ratios. 1µL of RNA was used for nanodrop to determine the concentration and purity of the isolated RNA.

500ng of isolated RNA was used for cDNA synthesis in a 20µl reaction containing 1µl of 10 mM dNTPs (supplied separately from Thermo), 60ng of random hexamers, 4µl of 5×First Strand 68 Buffer (Invitrogen) and 1µl of 100mM DTT (Invitrogen) was used to synthesize cDNA, using 0.5µl of SuperScript® III (Thermofisher) for 5 min left at room temperature after mixing then placed for 1 hour at 50 °C temperature then transferred to 70 °C for 10 min. The reaction was diluted five times before using it in quantitative PCR.

Relative mRNA levels were measured through qPCR (Qiagen Rotor-Gene Q qPCR machine) using GoTaq® qPCR Master Mix (Promega), containing SYBR Green dye and gene-specific primers. Merck prepared primers according to designs requested by our group [figure 2.5] and diluted them to 20mM concentration. For each sample, the RT-qPCR mix was prepared with 0.5µl of each forward and reverse primers, 5µl of qPCR Master-mix (Promega) and 2µl of water, and then 2µl of cDNA was added to make a final volume of 10µl. The programme setting used for the thermo-cycler (Qiagen Rotor-Gene Q series software) was 95º C for 5 minutes, 40 cycles at 95º C for 30 seconds, 58º C for 30 seconds and 72º C for 60 seconds.

2.2.3 Analysis of real-time PCR data

Three biological replicates were used for the treatment, and three technical replicates were used for RT-qPCR. For statistical analysis, technical replicates averages constitute individual biological replicates. Ct values obtained from the qPCR runs for the gene of interest were compared with a reference gene. Experimental levels were normalized to Rpl28 and calculated to the mean value for each gene. All data were analysed using the 2^-ΔΔCT method (Schmittgen & Livak, 2008).

Table 2.5: Sequence of forward primers and reverse primers used for RT-qPCR.

Gene	Sequence of forward primer	Sequence of reverse primer
Rpl28	TACAGCACGGAGCCAAATAA	ACGGTCTTGCGGTGAATTAG
Tnf	CTATGGCCCAGACCCTCACA	CCACTTGGTGGTTTGCTACGA

2.3 Liquid chromatography-mass spectrometry (LC-MS)

Figure 2.1: The Exactive Orbitrap Mass Spectrometer and Q Exactive Orbitrap Mass Spectrometer, setup used for LC-MS analysis.

The liquid chromatography-mass spectrometry (LC-MS) method was used for the chemical analysis of samples with metabolite identification by retention time and m/z. LC-MS is a more selective approach than NMR for small molecule identification, often resulting in the data output with less background noise from solvent. Given the sample's chemical diversity, this

study was carried out with particular interest in the targeted analysis of the compound of interest, cordycepin, pentostatin and 3' deoxyinosine and to study the overall metabolic profile by an untargeted and high-throughput screening of samples cordycepin and *Cordyceps militaris* extract. A hydrophilic interaction liquid chromatography (HILIC) column and mass spectrometer (Thermo Exactive® Orbitrap and Thermo Q Exactive® Orbitrap) were used in this study [figure 2.1], which are present at the Central for Analytical Biosciences (CAB), as this system has high selectivity and resolution, particularly for polar metabolites.

2.3.1 Sample preparation

Samples were prepared for LC-MS by dissolving 1mL of dried extract in eppendorf in 1mL of ethanol and water (60%). The sample was mixed well before being centrifuged to remove any insoluble debris; 120µL of supernatant was transferred into the LC-MS vial with a slit lid. A blank sample was prepared by using solvent only, QC (quality control) sample was prepared by pooling all the samples in equal volume and was included in the LC-MS run in between the samples, and the spiked QC was prepared by adding standards to the QC with known concentration.

Standards of the compound of interest, cordycepin, pentostatin and 3' deoxyinosine, were prepared for targeted analysis with a known concentration of 100µM, 50µM, 25µM, 12.5µM, 6.25µM and 3.1µM. The mixture of these authentic standards at each concentration allows the targeted analysis by quantifying cordycepin, pentostatin and 3' deoxyinosine concentration in the samples by all the standards with the same concentration with serial dilution, which were used for fitting the calibration curve. In untargeted analysis, a mixture of 250 authentic standards was used, as previously studied by Surrati *et al.,* 2016 (Surrati *et al*., 2016). In this method, the identification of metabolites was done based on retention time and *m/z* of an extensive list of metabolites in the LC-MS run.

2.3.2 Liquid Chromatography

Liquid chromatography was employed using the hydrophilic interaction technique for compound separation before the sample was introduced to the mass spectrometer. Two mobile phases were used; acetonitrile (100%) and aqueous buffer ammonium carbonate (20mM), which allowed the compound to separate based on the electrostatic interactions (polar compounds) and hydrophilic interaction of the compounds between the stationary and mobile phases. Elution of the compounds depends on the interaction with the column stationary phase. SeQuant® ZIC-pHILIC 5µm, 4.6 x 150mm column (Merck).

The manufacturer's instructions for the column assembly in the Accela® auto-sampler (ThermoFisher Scientific) and the LC pump system were followed. Compound separation was done by gradient system; A: ammonium carbonate (20mM) at a ratio of 20:80 with pH 9.1 was used with 100% acetonitrile (B) at a flow rate of 300µL/min and a maintained temperature of 45ºC in the column oven. A 24 min per sample method was used with a specific ratio of A: B (Schatschneider *et al*., 2018), as shown in figure 2.2 and table 2.6.

Time (min)	A (%)	B (%)
0	20	80
15	95	5
17	20	80
24	100	0

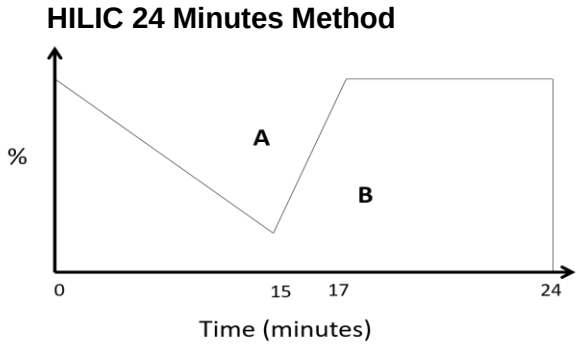


Figure 2.2 and Table 2.6: 24-minute LC method, through the ZIC®-*p*HILIC column.

2.3.3 Mass spectrometry

The Orbitrap Exactive® mass spectrometer (Thermo Fisher Scientific, USA) couples with the Accela system used in most untargeted and targeted analyses in this study, along with ESI running in both the positive and negative ionisation. To analyse *C. militaris* extract and

cordycepin using untargeted and targeted analysis. Thermo Q Exactive® mass spectrometer was opted for analysis of cordycepin samples to achieve a high level of confidence in identifying the compounds using tandem mass spectrometry (MS/MS). The MS/MS was performed on the QC samples analysed by Exactive and cordycepin samples. The mass spectrometer was calibrated before each run with modified Pierce ESI positive and negative solutions from ThermoFisher. The mass spectrometry process in detail is presented in figure 2.3.

After the liquid chromatography and separation of the compounds in the sample through the SeQuant® ZIC-pHILIC column, the flow from the column was fed into the MS. In both instruments, ESI was used for ionisation. Q-Exative MS was used for tandem mass spectrometry (MSMS) to identify metabolites of relevance with greater certainty by utilising fragmentation patterns in mass spectra. The **Q-Exactive** has two mass analyzers: one (MS1) is a quadrupole mass filter with four electrical current and radio frequency-conducting rods, and the other (MS2) is an Orbitrap mass analyzer. The Orbitrap mass analyzer played a different function in the Q-Exactive than it did in the Exactive that only precursor ions were chosen in MS1 and sent through it following collection in a C trap paired with an argon-containing HCD collision cell. Ion fragmentation occurred in the collision cell as a result of collision-induced dissociation. **Exactive MS** Ions resulting from the charged droplets generated by ESI were captured by a C trap®, which were then injected into a mass analyser orbitrap in high-speed pulses. Orbitrap is a Fourier transform ions trap (FTIT) mass analyser in which the ions oscillate and orbit around the inner electrode. This process generates a complex ion path that projects an image charge to outer detectors, which are processed to give m/z values.

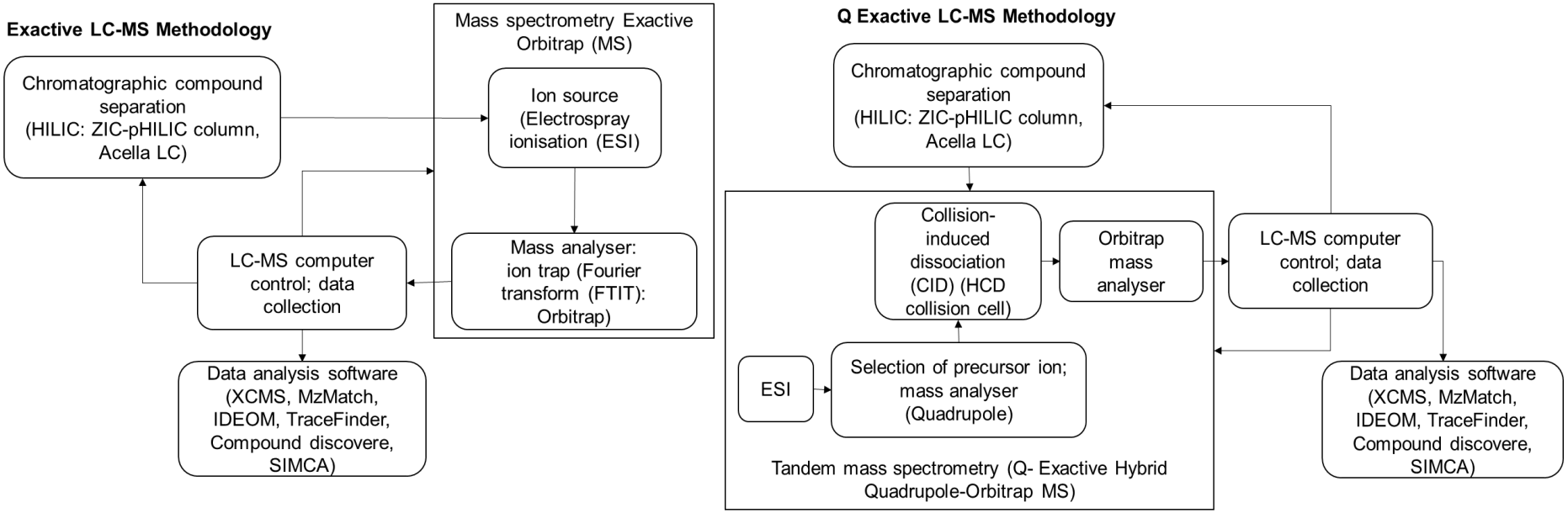


Figure 2.3: Diagrammatic illustration of LC-MS methods.

2.3.4 LC-MS steps and setting using ZIC-pHILIC column and Exactive MS

The LC-MS equipment was electronically controlled by an attached desktop computer running ThermoFisher Xcalibur® and Thermo Tune® software. The Exactive MS and Accela HPLC systems were used for most of the runs. For cordycepin runs, Q-Exactive MS and UltiMate 3000 HPLC system setup were used, and Chomeleon® software by ThermoFisher controlled the HPLC system.

Sample preparation for the LC-MS run; Methanol (LC-MS grade) was diluted 50% in ddH2O, which was used for all the LC-MS sample preparation. The standard operating protocol for operating the LC-MS system consists of a Thermo Accela® LC system with SeQuant® ZIC-pHILIC column (Merck) and Thermo ExactiveTM mass spectrometer separated into five major processes. These are (i) sample and phase preparation; (ii) mass spectrometer calibration; (iii) HPLC system settings and purging; (iv) conditioning the column; and (v) creating a run sequence and initiating the run.

(i) Sample and phase preparation. The sample preparation is described in the methods chapter in the extraction section earlier. The sample was stored at -80º C before the LC-MS run. The mobile phases for HPLC were 20mM of ammonium carbonate (pH 9.1) (phase A) and 100% acetonitrile (phase A). Phase A was used for washing the system.

(ii) Mass spectrometer calibration. Mass-spectrometer was calibrated using a modified version of Thermos Fisher solution Pierce ESI positive and negative. Contaminants with small masses were incorporated into the mixture to cover low masses metabolites in the calibration mass range; they were C2H6NO2 with m/z 76.0393 for positive mode and C3H5O3 with m/z = 89.0244 for negative mode. Calibrations were carried out in positive and negative modes using a calibration tune file on the Thermo Tune software. A Hamilton syringe (500µL) was used to pump ddH2O 10µL-20µL per minute into a mass spectrometer. Calibration was started when the total ion count (TIC) fell below 5%.

(iii) HPLC system setting and purging. The parameters for the Accela^{TM} HPLC system were configured using the Xcalibur software. The oven and auto-sampler tray temperatures were adjusted to 45°C and 4°C, respectively. Before the run started, the syringe and needle were cleaned. The system was purged (bubbles were removed).

(iv) Column conditioning. After the system purging, the SeQuant® ZIC-pHILIC column was fitted and conditioned with the solvent for at least 30 min before the beginning of the LC-MS run, with a flow rate of 300µL per min and a running ratio of both phases A and B- 20 percent A and 80 percent B with a flow rate of 300µL per minute.

(v) **Creating a run sequence and initiating the run.** The last step in the standard operation procedure of LC-MS setup for generating the sequence of samples with the Xcalibur software. Across all runs, QC samples (quality control) were utilised for both spiked and un-spiked with standards, and in targeted LC-MS runs, a standard sample containing the metabolites of interest cordycepin and pentostatin, 3' deoxyinosine was used at 3.1µM, 6.25µM, 12.5µM, 25µM, 50µM, 100µM, and 200µM. For an untargeted run, five combinations labelled as A, B, C, D and E consisting of 250 standards were employed, as a previous study by Surrati *et al.,* (2016). The sample injection sequence followed the guidelines of Want *et al.,* (2010), as shown in figure 2.4. Five randomised treatment group samples were interspersed with QC samples, and repeated QC samples were injected at the beginning of the run.

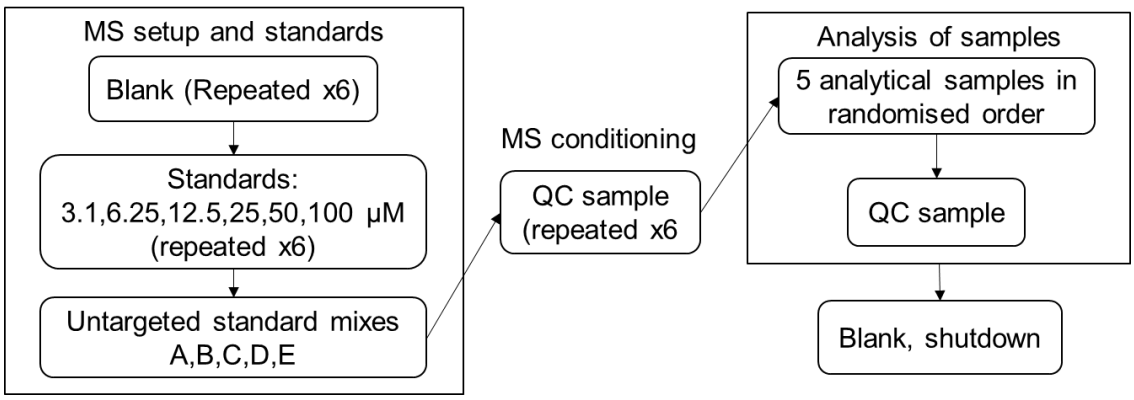


Figure 2.4 Sequence of the samples used in LC-MS run (Want *et al*., 2010).

2.4 LC-MS data analysis

2.4.1 Levels of metabolites identification.

Metabolite identification was based on accurate masses derived from retention time and accurate mass of (A, B, C, D and E) standards, where present in standard samples in the same LC-MS run using metabolomics standards initiative Level 1 identification based on the accurate mass matching, MS/MS fragmentation and retention time of metabolites peaks detected with 250 authentic standards (analysed under similar experimental conditions). While, other metabolites present in the sample were identified with a lower level of certainty (putative identification) based on predicted retention time and accurate mass matching with the detected metabolites peaks of authentic standards, as performed by IDEOM v19 software under default parameters (metabolomics standards initiative Level 2 identification) (Abuawad, Mbadugha, Ghaemmaghami, & Kim, 2020; Sumner *et al*., 2007; Sumner *et al*., 2014).

IDEOM also did noise filtering of the sample, the XCMS software (Tautenhahn, Böttcher, & Neumann, 2008) and MzMatch (Scheltema, Jankevics, Jansen, Swertz, & Breitling, 2011) were used after the untargeted LC-MS run after picking the peaks and annotation and matching of peaks, respectively, as done in the previous studies [e.g. Kim *et al.,* 2015]. The maximum level (level 1) of metabolites identification was obtained following a tandem MS (MS/MS) run using Q-Exactive MS. Several metabolites of interest were identified using mass spectra of fragmentation products using the Compound Discoverer® software (Thermo Fisher).

2.4.2 Metabolites quantification.

After the targeted run, the standard compounds of interest, such as cordycepin, pentostatin and 3' deoxyinosine, were used for quantification of the levels of these compounds present in the analytical sample. TraceFinder® software was used for the targeted analysis, and the calibration curve was fitted from standard samples. Only the calibration curve with the R

square value of 0.9 or greater was considered acceptable for use –and the repeats of the standard sample were included for a well-fitted curve. Care was taken during the standard sample preparation to achieve the best-fitted straight curve, such as dilution to the predicted concentration of 3.1µM to 200µM or 3.1µM to 100µM. This was done to avoid the issue of detector saturation at higher concentrations. The example is shown in figure 2.5. By adopting this method, the peak area of the sample in the chromatogram at a retention time matching the compound present in the standard was used, and the values were converted to concentrations.

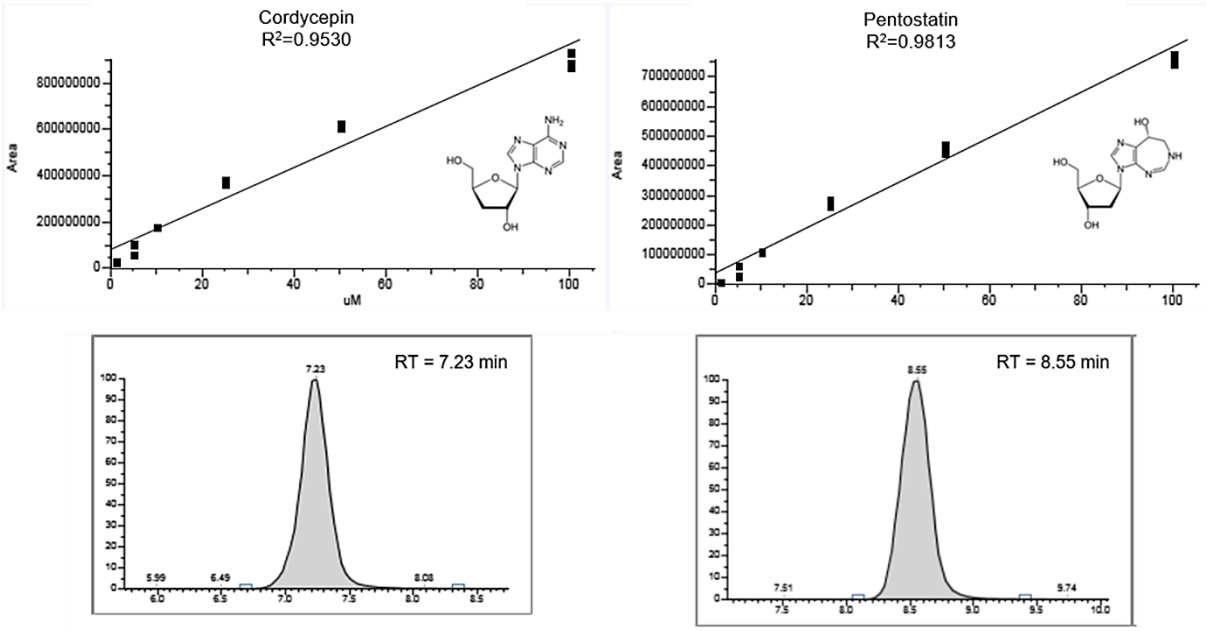


Figure 2.5: The calibration line fitted and chromatogram peaks showing the area and retention time for the cordycepin and pentostatin.

The compound identification was based on the m/z and retention time in the untargeted LC-MS run, using the sample output obtained from five mixtures (A, B, C, D, E) of standards after the untargeted run using Exactive MS. Furthermore, the relative concentration of the putatively identified metabolite was calculated using IDEOM v19 software.

2.4.3 Untargeted metabolomic data presentation

The untargeted LC-MS run data was processed, and the processed data was presented in pictorial form constructed by GraphPad prism® 9, Simca P® ver4 and QIAGEN Ingenuity Pathway Analysis software. This includes the heatmaps, pie charts, graphs and multivariate analysis as principal component analysis PCA and the orthogonal partial least squares discriminant analysis known as OPLS-DA. Significant differences between the levels of the metabolites were determined using t-tests with false discovery rate (FDR) correction and with VIP values >1 following the OPLS-DA using the SIMCA-P software.

CHAPTER 3
RESULTS

CHAPTER 3: DEVELOPMENT AND OPTIMISATION OF THE EXTRACTION METHOD

Cordyceps militaris is an insect infecting fungus. It produces cordycepin and pentostatin as two important metabolites responsible for its anti-inflammatory activity (H. G. Kim et al., 2006; Kondrashov et al., 2012; Won & Park, 2005; Xia et al., 2017(a)). To choose the most effective extraction technique, extracts of dried and powdered *C. militaris* mycelium were tested with different solvents. A bioassay for inflammation in the RAW 264.7cells line was used to determine the inflammatory and anti-inflammatory activity of these extracts (Y. H. Choi *et al*., 2014).

The choice of solvent is based on the metabolites of interest, as no solvent will extract all compounds. In some cases, the particular activity can be due to the synergism of more than one compound, requiring optimal extraction of multiple products. It is usually too laborious to use all the possible solvents, and even then, insoluble polymers and volatile compounds are usually lost. Solvents are often selected based on solvent polarity (Tir, Dutta, & Badjah-Hadj-Ahmed, 2012). A polar solvent such as ethanol or methanol can be used for extraction; a more polar solvent usually extracts more compounds in comparison to a non-polar solvent. A non-polar solvent only extracts non-polar solvents leaving out the majority of the polar solvents. In addition, the solvent can also influence the saleability of the extract as a food supplement. The solvent selection and extraction method are indeed suggested as a crucial factor in the anti-inflammatory activity of *Cordyceps militaris* extracts (H.-J. Wang *et al*., 2014; F. Yang & Li, 2008).

The goal of this study was to achieve a food-grade product that can be tested for anti-inflammatory activity and is suitable for consumption as a food supplement produced by our collaborators at MycoMedica/GOBA. To obtain a food-grade product, the best options for solvents were water and ethanol. Water has the highest polarity, and ethanol is the second most suitable polar solvent for our extraction to obtain a food-grade product with proven bioactivity that can be consumed directly (Ni *et al*., 2009). After selecting the solvent,

metabolomics and cell study were done on the sample extracted with water, ethanol and their combination. The untargeted metabolomics analysis was performed to detect the number of metabolites in the extract, and tissue culture experiments were carried out to test the anti-inflammatory activity. Based on the results obtained from metabolomics and macrophage experiments, a solvent method was selected.

Metabolomics analysis was performed on fungal samples gifted by Goba MycoMedica to estimate the extraction ability of solvent and the number of putative metabolites in the extract. Samples were analysed by un-targeted protocols using the Exactive Orbitrap MS instrument. The extracted m/z data from the detector was exported to text file data, which was further analysed by MzMatch and IDEOM (Creek, Jankevics, Burgess, Breitling, & Barrett, 2012; Scheltema *et al*., 2011; Wellham *et al*., 2021). The macrophage derived RAW264.7 cells, which respond readily to LPS, were used to investigate the inflammatory and anti-inflammatory activity of the samples (S. Ashraf *et al*., 2019; Wellham *et al*., 2019).

3.1 Solvent extraction

To select the solvent which extracts the highest number of metabolites, a series of extractions were carried out. Samples were prepared from powdered fungus material by maceration. The dried extract was suspended in methanol-water 50%, and samples were analysed using LC-MS. The metabolites were identified based on the accurate masses (calculated from mass: charge ratios (*m/z* values)) and the retention times of standards. The total number of detected putative metabolites by the LC-MS in the extracts with E, W and EW vary due to polarity differences. The objective of this study was to maximise the number of metabolites extracted, as the results show that EW extracted the highest number of metabolites 1623, followed by W 1379 and E 1231.

To identify the nature of metabolites detected with LC-MS, samples were extracted with different solvents, and all the putative metabolites were classed based on IDEOM analysis. Each extract varies in the number of metabolites detected from each group. These putatively

identified metabolites groups were lipids, amino acids, peptides, carbohydrates, cofactors and vitamins, nucleotides, xenobiotics, biosynthesis of secondary metabolites and unidentified metabolites. The highest number of metabolites in the extract were unidentified by the human metabolome database (HMDB) due to the limitation of the database [figure 3.1]

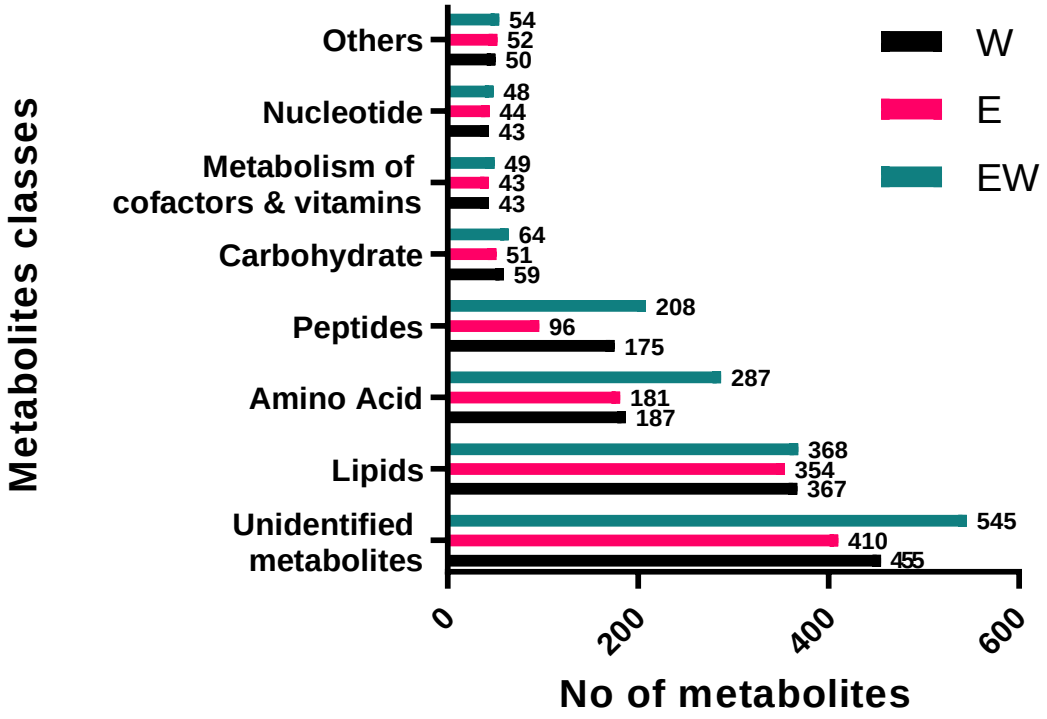


Figure 3.1: Comparison of identified putative metabolites from various classes using untargeted LC-MS profiling of *C. militaris* (MycoMedica) using W (Water extract), E (ethanol extract), EW (ethanol Water, 4:6), metabolites level 2 identification based on predicted retention time and accurate mass matching with the detected metabolites peaks of 250 authentic standards, as performed by IDEOM v19 software under default parameters extracts were analysed using Exactive Orbitrap MS instrument, processed by ToxID and untargeted metabolic profiling by MzMatch and IDEOM_v19 analysis, figure created in GraphPad Prism v9.

The results show EW as a superior solvent system for extraction. The number of metabolites in most of the groups extracted by EW was higher in comparison to that of E and W extracts. The only group of metabolites in which ethanol showed the better result was xenobiotics, 15 metabolites were extracted by ethanol while EW extracted 12 metabolites. On the other hand, EW extracted the highest number of metabolites in lipids, amino acids, peptides,

carbohydrates, cofactors and vitamins, nucleotides, and biosynthesis of secondary metabolites.

3.2 Anti-inflammatory activity of extracts

The focus of our study was to identify a suitable extraction method to achieve an anti-inflammatory activity in extracts. The data obtained from the LC-MS results [figure 3.1] suggested that EW is a better extract in comparison to E and W. To further investigate the point of the most potent extract, the macrophage cell line (RAW294.7) was used to study the anti-inflammatory activity of the extracts. RAW 264.7 cells show a robust inflammatory response to lipopolysaccharide (LPS), and cordycepin is known to inhibit the induction of inflammation in this system, and the effects on inflammatory gene expression can be determined by isolating RNA and doing RT-qPCR (S. Ashraf *et al*., 2019). Cordycepin indeed repressed the mRNA encoding the inflammatory cytokine TNF, and pentostatin potentiated this effect [figure 3.2a and 3.2b]. EW extract has the highest anti-inflammatory effect, followed by water and pure ethanol [figure 3.2a]. These results correlate to the LC-MS data, which indicated that EW was a superior extract. Notably, pentostatin had no anti-inflammatory activity on its own in these cells [figure 3.2b].

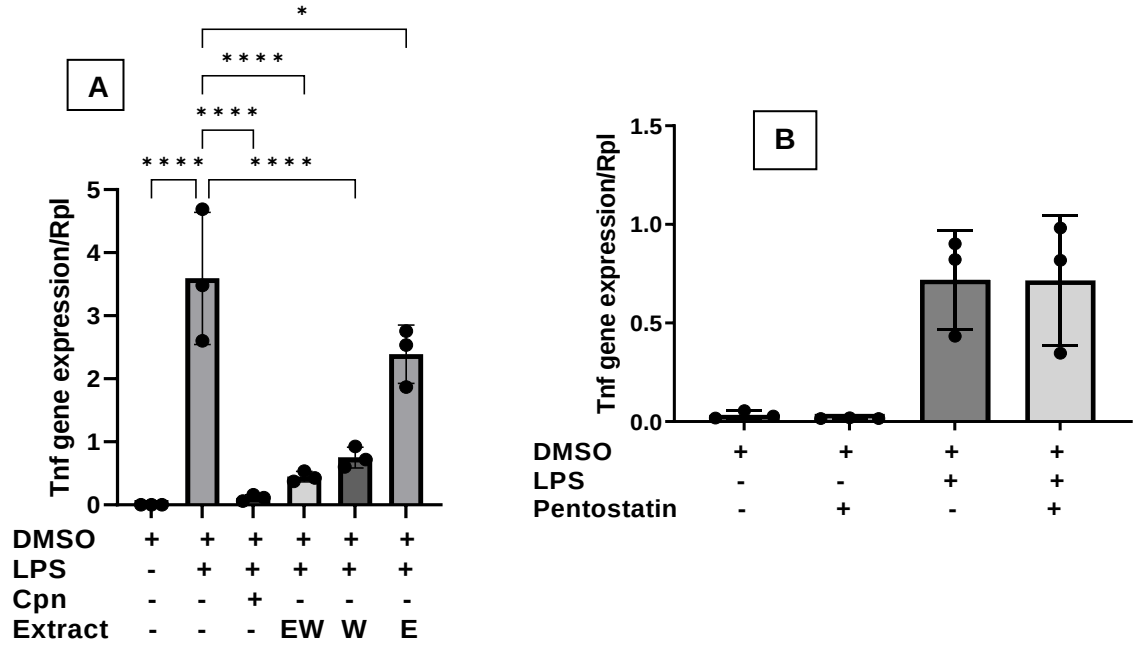


Figure 3.2: *C. militaris* (MycoMedica) extracts replicating the anti-inflammatory effect of cordycepin. RAW 264.7 cells treated with DMSO or extracts or cordycepin or pentostatin for an hour before treating with LPS, samples of W (water), E (ethanol), EW (ethanol-water 6:4) and pentostatin were used, 16.6mg dried *C. militaris* weight used for all the extraction samples based on LC-MS quantification. RT-qPCR was performed, error bars represent standard deviation across three RT-qPCR replicates, ****p<0.0001 ***p<0.001 ** p<0.01, * p<0.5, ^{ns} p<0.1. P values were obtained by using ordinary one-way ANOVA with multiple comparisons using GraphPad Prism v9.

3.3 Inflammatory effects of extracts

Extracts consist of numerous metabolites, which may also include inflammatory compounds, which can negate anti-inflammatory activity. Thus to evaluate the inflammatory activity of extracts, the cells were treated with extracts without activating the cells with LPS. The results obtained from the RT-qPCR were analysed and graphed, as shown in Figure 3.3. The result shows DMSO as a control and the inflammatory effect of the extract on the RAW264.7 cells. W extract exhibits the highest inflammatory response, followed by E and then EW. As we are interested in the extract with maximum anti-inflammatory activity so the inflammatory activity of the extract will reduce the efficacy of the extract, so it proves that the EW extract is better in comparison to E and W extracts.

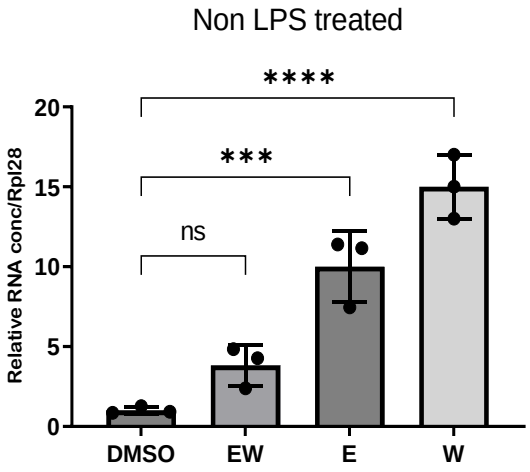


Figure 3.3: inflammatory effect of *C. militaris* (MycoMedica) extracts, RAW 264.7 cells treated with DMSO or extract for an hour before treating with LPS, W (water), E (ethanol), EW (ethanol-water 6:4), 16mg dried *C. militaris* weight used for all the extraction samples based on LC-MS quantification, RT-qPCR was performed, error bars represent standard deviation across three RT-qPCR replicates. ****p<0.0001 ***p<0.001 ** p<0.01, * p<0.5, ^{ns} p<0.1. P values were obtained by using ordinary one-way ANOVA with multiple comparisons using GraphPad Prism v9.

3.4 Effect of extraction parameters on cordycepin, pentostatin and 3' deoxyinosine concentration

The composition and activity of the extracts are affected by several parameters besides solvent; the other parameter includes heating, sonication and maceration time of the extraction. The effect of these parameters was investigated to test their effect on the concentration of cordycepin, pentostatin and 3' deoxyinosine. The cordycepin exhibits anti-inflammatory activity, and the pentostatin enhances its activity by safeguarding the cordycepin from deamination. 3' deoxyinosine is the deaminated metabolite of cordycepin (Xia *et al*., 2017(a)). Extraction was carried out by varying these parameters; these extracts were tested by a targeted analysis comparing to authentic standards using TraceFinder software (Figure 3.4).

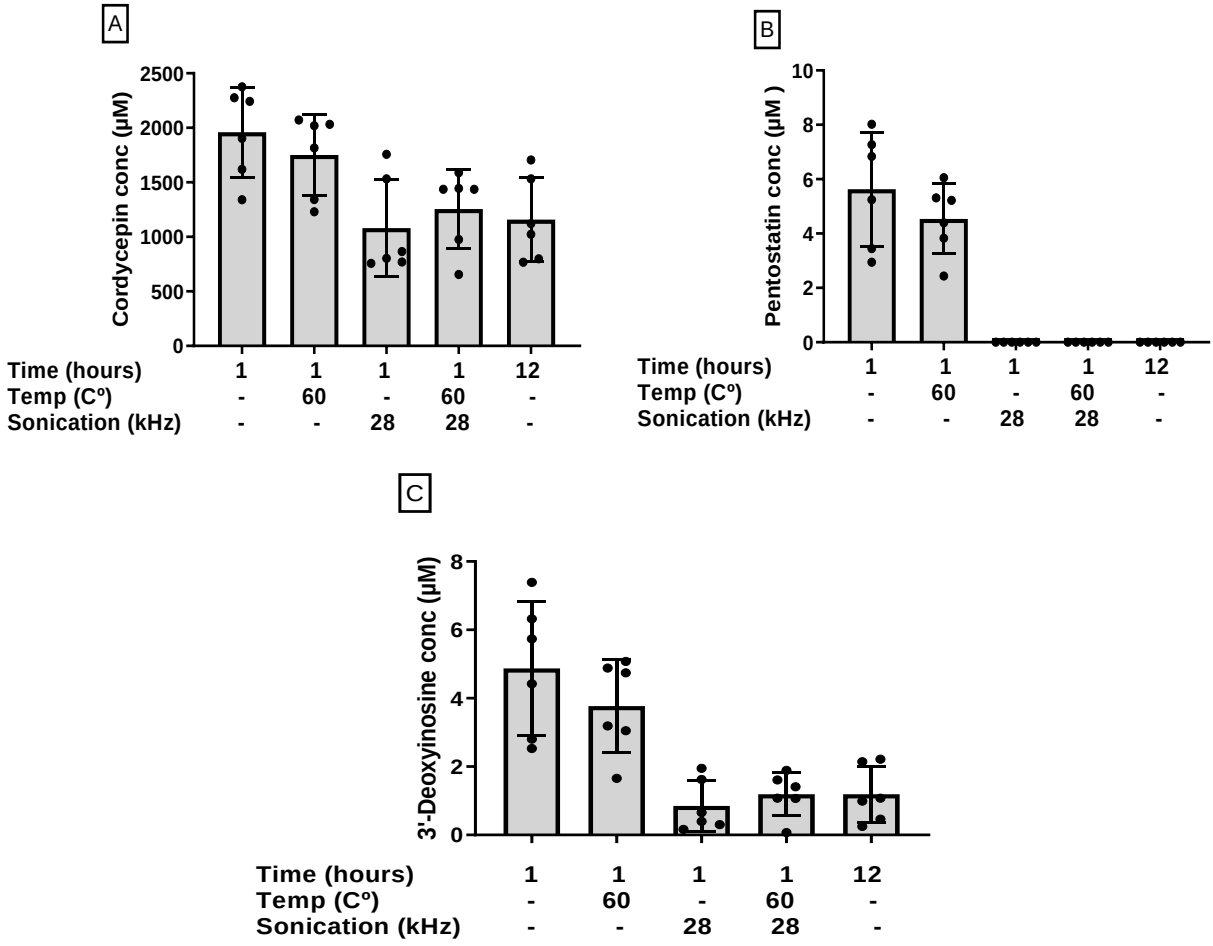


Figure 3.4: Effect of extraction parameters on (a.) cordycepin, (b.) pentostatin and (c.) 3' deoxyinosine concentration using heating (60ºC), sonication (28 kHz), 1 hr and 12 hr maceration used for extraction of *C. militaris* (MycoMedica), cordycepin, pentostatin and 3' deoxyinosine standard were used to calculate their concentration in the extract, and standard deviation indicates six instrumental replicas. Figures were obtained using GraphPad Prism v9.

The results show the effect of heating, sonication and maceration time. The concentration of cordycepin, pentostatin and 3'deoxyadenosisne was determined by targeted LC-MS analysis. These metabolites are important in the extract as they are associated with the anti-inflammatory effect of the extract. It is clear from the results that maceration time does not enhance the concentration of cordycepin and 3' deoxyinosine, but it decreases the concentration of pentostatin. Heating also affected the concentration of pentostatin adversely, but the sonication effect was the most adverse as the concentration was decreased to

undetectable levels. So only maceration for an hour at room temperature in the absence of sonication is the most efficient method of extraction to avoid the degradation of pentostatin.

3.5 Discussion

This study aimed to determine the best extraction method to maximise the number of metabolites and anti-inflammatory activity, while also minimising the inflammatory activity of the extract. These activities were verified by testing ethanol, water and ethanol 60% extracts in the cell line. To standardise the extract, various extraction methods were used to select the most efficient solvent and extraction parameters and to obtain an anti-inflammatory product that is safe and can be consumed directly by humans; based on these criteria, the solvent choice was limited, and only water and ethanol could be used. As cordycepin is the main reported metabolite in *C. militaris* with numerous activities and is readily soluble in polar solvents. 60% ethanol is the best option for extraction because the components are widely available in food grade, and this mixture maximises the cordycepin extraction(Kwon *et al*., 2018; Won & Park, 2005).

The LC-MS untargeted results show that the highest number of metabolites were detected in EW extract, and all solvents extract a wide variety of compounds, with EW extracting more compounds in nearly every category. Interestingly, the largest group in all *C. militaris* extracts are the “unidentified metabolites”, suggesting that there are many unknown molecules in this mixture as well. The bioassay for inflammation in RAW 264.7 showed EW extract had the highest repression of the inflammatory gene expression, followed by the W and E sample with the least repression (Figure 3.3). The samples were also tested for any inflammatory response, which shows that EW with the least inflammatory response, followed by E and then W with the highest inflammatory activity. This could be the reason that W and E samples have inflammatory activity, decreasing their anti-inflammatory activity in comparison to the EW sample. Based on all these results (figures 3.1 to 3.3), EW is a superior anti-inflammatory extract in comparison to W and E.

The targeted LC-MS analysis results show the effect of extraction parameters such as maceration time, heat and sonication on the concentration of cordycepin, pentostatin and 3' deoxyinosine. The concentrations of the three metabolites with simple maceration for an hour resulted in the highest level of these metabolites in comparison to sonication, heat and extended maceration. Pentostatin is a vital metabolite which helps safeguard cordycepin; it enhances its anti-inflammatory activity even at very low concentrations (Wellham *et al*., 2021). Pentostatin concentration was significantly affected by the parameters, and it dropped to undetectable levels; it indicates that pentostatin is unstable and disintegrates by the application of heat, sonication and extended maceration time during extraction. A study was done to test these parameters' effect on the cordycepin concentration by using heating, sonication and extended maceration. It states that it enhances the extraction concentration of cordycepin, but pentostatin was not included in this study, which is very important in enhancing the cordycepin anti-inflammatory activity (Chamyuang, Owatworakit, & Honda, 2019; H.-J. Wang *et al*., 2014; Xia *et al*., 2017(a)).

This study concludes that EW is superior to E and W extract by extracting the highest number of metabolites in the metabolomic study and exhibiting higher anti-inflammatory activity and lowest inflammatory activity in the cell culture assay. Several concentrations of extracts were used but concentrations below 33mg/ml (dried powdered *C. militaris* weight) did not show any anti-inflammatory activity, those results were not included in this thesis. The effect of heating, sonication and extending maceration was adverse to the metabolic profile of the *Cordyceps militaris* extract by decreasing pentostatin levels. A decrease in pentostatin concentration or its elimination from the extract is likely to result in a decrease in the anti-inflammatory activity of cordycepin. The use of these parameters is also less cost-effective in comparison to one-hour maceration at room temperature, as it can be scaled up for industrial extraction.

CHAPTER 4
RESULTS

CHAPTER 4: ANTI-INFLAMMATORY METABOLITES IN THE EXTRACTS BASED ON LC-MS ANALYSIS

Cordyceps militaris is a fungus used in Chinese medicine for its anti-inflammatory activity (S. K. Das *et al*., 2010; H.-B. Fan *et al*., 2021). It is commercially available under various brand names. This work was done in collaboration with Goba MycoMedica, a fungal and fungal extract producer from Slovenia. The very large scale production of *Cordyceps* is found in China (C. Dong, Guo, Wang, & Liu, 2015). The metabolites present in *Cordyceps militaris* are profoundly affected its cultivation and processing (Kang *et al*., 2014; Wellham *et al*., 2021), but how reproducible the content of cordycepin, pentostatin and 3' deoxyinosine is in commercial preparations has not been addressed.

Analytical techniques, including liquid chromatography (LC), mass spectrometry (MS) and UV detection, have been employed in studies on metabolites of *C. militaris*. Liquid chromatography coupled with mass spectrometry (LC-MS) approach, in particular, has been adopted for the detection of small compounds, including nucleosides and their homologues like cordycepin and pentostatin and other metabolites classes. Further detail on these analytical methods is discussed in the methods (chapter 2). As metabolites are chemically diverse, no one analytical approach can inclusively cover the whole metabolome (complete set of small chemical molecules) of any given organism or sample (J. Zhang *et al*., 2015).

This study compared ten different batches of dried powdered *Cordyceps militaris,* different batches of Cordyvit®K Plus (commercial product) gifted by Andrej Gregori (MycoMedica), and commercially available Aloha (Aloha medicinal). It was used for the majority of the crude extract experiments. In this study, two methods were adopted for metabolite study and their effects on the extract. First, the metabolic profile was studied by LC-MS (targeted and untargeted analysis) and second, RAW264.7 cell culture experiments were performed to investigate the anti-inflammatory activity of the extracts.

4.1 Metabolites distribution based on molecular mass and retention time

Metabolic profiling of *Cordyceps militaris* extract using untargeted LC-MS analysis was performed to observe the nature and number of metabolites present in the extract. The optimisation of extraction was done in Chapter 3. Extraction was done by macerating dried powdered *Cordyceps militaris* in ethanol (E), water (W) and ethanol-water (60%) (EW) and then dried; the extract was re-dissolved in methanol (50%) for LC-MS run, and seven instrumental replicates were used. Data obtained from the instrument was processed by using ToxID, XCMS, MzMatch, and analysed by IDEOM_v19 (Creek *et al*., 2013). The accurate mass and retention time were plotted, which showed the nature of the metabolites present in the extract. Most of the compounds were eluted roughly between 4 to 12.5 min, and m/z ranged from 100 to 450 [figure 4.1]. The metabolite's mass and retention time data were compared to the authentic standards used for level 1 identification by predicted retention time and mass based on authentic standards, and level 2 identification was achieved by predicted retention time without authentic standards resulting in putative identification (Creek *et al*., 2014; Kodra *et al*., 2021). It showed the effect of solvent on the metabolites extracted in the extracts. The total detected metabolites in the extract were 1909, and these metabolites belong to various metabolites groups, of which 43.48% were identified metabolites (IM) due to the limitation of the metabolome database used for identification as these compounds are not well documented in metabolites biological databases (Creek *et al*., 2012; Srivastava & Creek, 2020). Other groups of metabolites detected were 19.28% lipid metabolism (LM), 14.35% peptides di, tri, tetra (PDTT), 10.90% amino acid metabolism (AAM), and among the minor number of metabolites, nucleotide metabolism was 2.15% [figure 4.2].

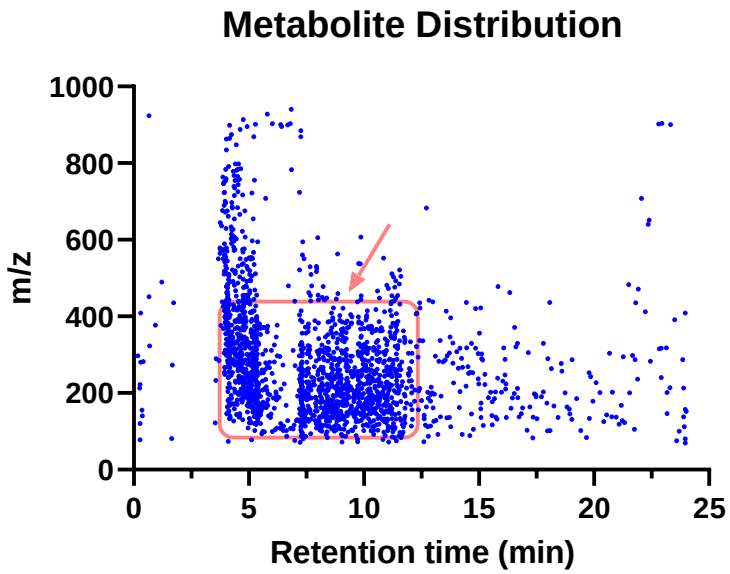


Figure 4.1: Metabolites distribution in ethanol-water (60%) extract (MycoMedica) based on their accurate mass and retention time detected by untargeted metabolic profiling. Each point represents a single metabolite, using Exactive Orbitrap MS instrument, analysed by untargeted metabolic profiling using MzMatch and IDEOM_v19 analysis. Six LC-MS replicates were used and the red box shows the area with major metabolites detected.

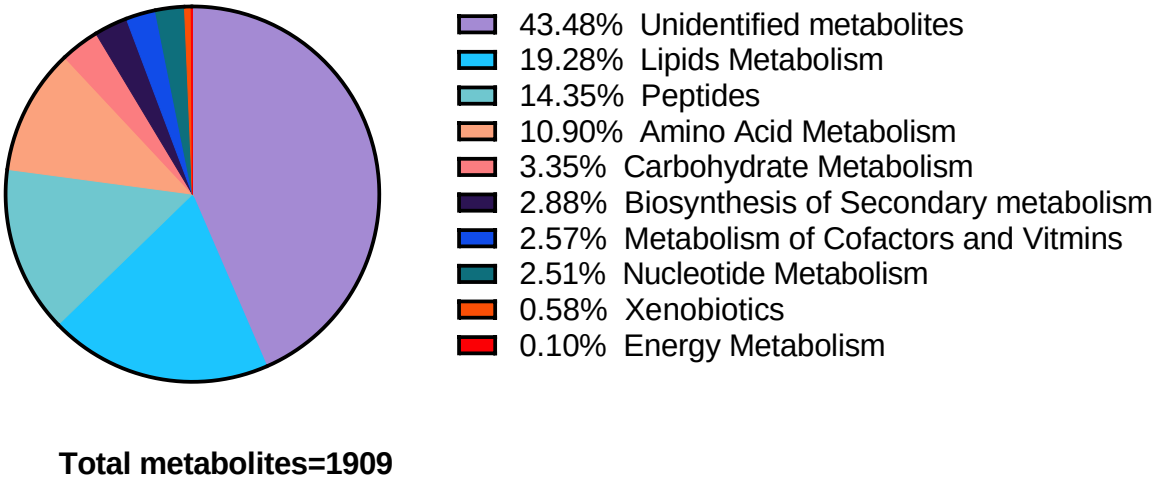


Figure 4.2: Metabolites detected in ethanol-water (60%) extract (MycoMedica) by untargeted LC-MS. Metabolites were detected using powdered fungal material extract. Untargeted data were processed by using ToxID, MzMatch, and analysed by IDEOM_v19. Putative identification (Level 2) of the metabolites was obtained by using the accurate mass and predicted retention times based on authentic standards.

Table 4.1: *Cordyceps* metabolites classes with reported activity in literature. The table consists of classes with reported metabolites from *Cordyceps*. Putative metabolites classes: lipid metabolism (LM), peptides di, tri, tetra (PDTT), carbohydrate metabolism (CM), Nucleotide metabolism (NM).

Activity	Class	Reference
Reduction of nitric oxide	LM	(Jo *et al*., 2010; Nallathamby, Abd Malek, Vidyadaran, Phan, & Sabaratnam, 2020; M. Yu *et al*., 2021)
Anti-hyperlipidemia	LM	(S. B. Kim *et al*., 2014; L. Wang *et al*., 2015; W.-Q. Yu *et al*., 2021)
Liposome oxidation	LM	(H. M. Yu, Wang, Huang, & Duh, 2006)
Hepatoprotective	LM	(L. Wang *et al*., 2015)
Anti-inflammatory	LM	(Jo *et al*., 2010; Lin *et al*., 2021; Qian, Pan, & Guo, 2012)
Anti-nociceptive activity	LM	(Qian *et al*., 2012)
Anti-atherosclerotic	LM	(Lin *et al*., 2021)
Anti-fungal	PDTT	(B. T. Park, Na, Jung, Park, & Kim, 2009; J. H. Wong *et al*., 2011)
Anti-inflammatory	PDTT	(J. Wang *et al*., 2012)
Anti-cancer	PDTT	(B. T. Park *et al*., 2009)
Acetylcholinesterase	PDTT	(C.-H. Tsai, Yen, & Yang, 2015)
Anti-oxidant	PDTT	(J. Wang *et al*., 2012)
Anti-microbial	PDTT	(Jędrejko, Lazur, & Muszyńska, 2021; M. Zhang *et al*., 2018)
Anticoagulant activity, inhibitory activity of TPA induced O2 generation (a tumour promotor)	PDTT	(B.-J. Wang, Won, Yu, & Su, 2005)
Anti-complimentary	PDTT	(J. N. Choi *et al*., 2010)
Intestinal system immune-modulatory activity	PDTT	(K.-W. Yu *et al*., 2001).
Anti-oxidant	PDTT	(B.-J. Wang *et al*., 2005)
Hypoglycemic	PDTT	(Shang *et al*., 2020)
Antidiabetic	CM	(Y. Dong *et al*., 2014; J.-y. Liu *et al*., 2016)
Anti-hyperglycemic	CM	(Deng *et al*., 2020; H.-S. Kim, Ro, & Choe, 2005; L. Ma *et al*., 2015)
Hepatoprotective	CM	(D. J. Kim *et al*., 2017)
Hyperlipidemia	CM	(Deng *et al*., 2020)
Anti-hyperlipidemic /hepative protective	CM	(L. Wang *et al*., 2015)
Anti-atherosclerotic	CM	(Lin *et al*., 2021)
Anti-nephritic activity	CM	(J.-y. Liu *et al*., 2016)
Anti-inflammatory	CM	(Chiu *et al*., 2016)
Anti-tumour	CM	(S. Yang, Jin, Ren, Lu, & Meng, 2014)
Anti-ageing/ effect on mitochondria	CM	(X.-T. Li, Li, Li, Dou, & Gao, 2010)
Immunostimulant	CM	(J. S. Lee & Hong, 2011; M. Wang *et al*., 2012)
Anti-oxidant	CM	(M. Wang *et al*., 2012)
Obesity	NM	(S. B. Kim *et al*., 2014)
Anti-inflammatory	NM	(Chiu *et al*., 2016)

4.2 Targeted metabolomics; quantification of the compound of interest

In the targeted LC-MS run, standards of cordycepin and pentostatin (compounds of interest) were used for their quantification and to find out their concentration in the sample. TraceFinder® software and calibration line curve fitting were used with standards (cordycepin and pentostatin). The calibration curve with an R square value of 0.99 or above was considered acceptable to use – and the standard sample replicates were used to achieve a well-fitted curve, such as using dilution to the predicted concentrations of 3.5µM - 200µM [figure 4.3a and 4.4b]. To avoid the issue of incorrect quantification caused by the saturation of the detector at higher concentrations, a serial dilution method was used to test the limit of detection of the instrument [figure 4.3-4.8].

The results showed that the highest cordycepin concentration of 245µM was detected at 50mg/mL of extract. On further dilution, the concentration of cordycepin remains high at 25mg/mL (217µM) and 12.25mg/mL (172µM). However, when sample concertation reaches 6.25mg/mL (90µM) it becomes linear., which indicates that the best concentration for cordycepin detection is around 100µM, demonstrating that extracts should be diluted to be close to this concentration for reliable detection on Exactive LC-MS instrument [figure 4.3]. However, the pentostatin result shows that 50mg/mL of extract contains 6.5µM of pentostatin. On further dilution to 25mg/mL (5.2µM), 12.5mg/mL (4.7µM), and 6.25mg/mL (4.3µM), the pentostatin concentration of remains almost the same. This indicates that the concentration of pentostatin was low even at 50mg/mL. These results show that cordycepin concentration is very high in comparison to pentostatin concentration in the EW sample. The same dilution could not be used for both cordycepin and pentostatin quantification [figure 4.4].

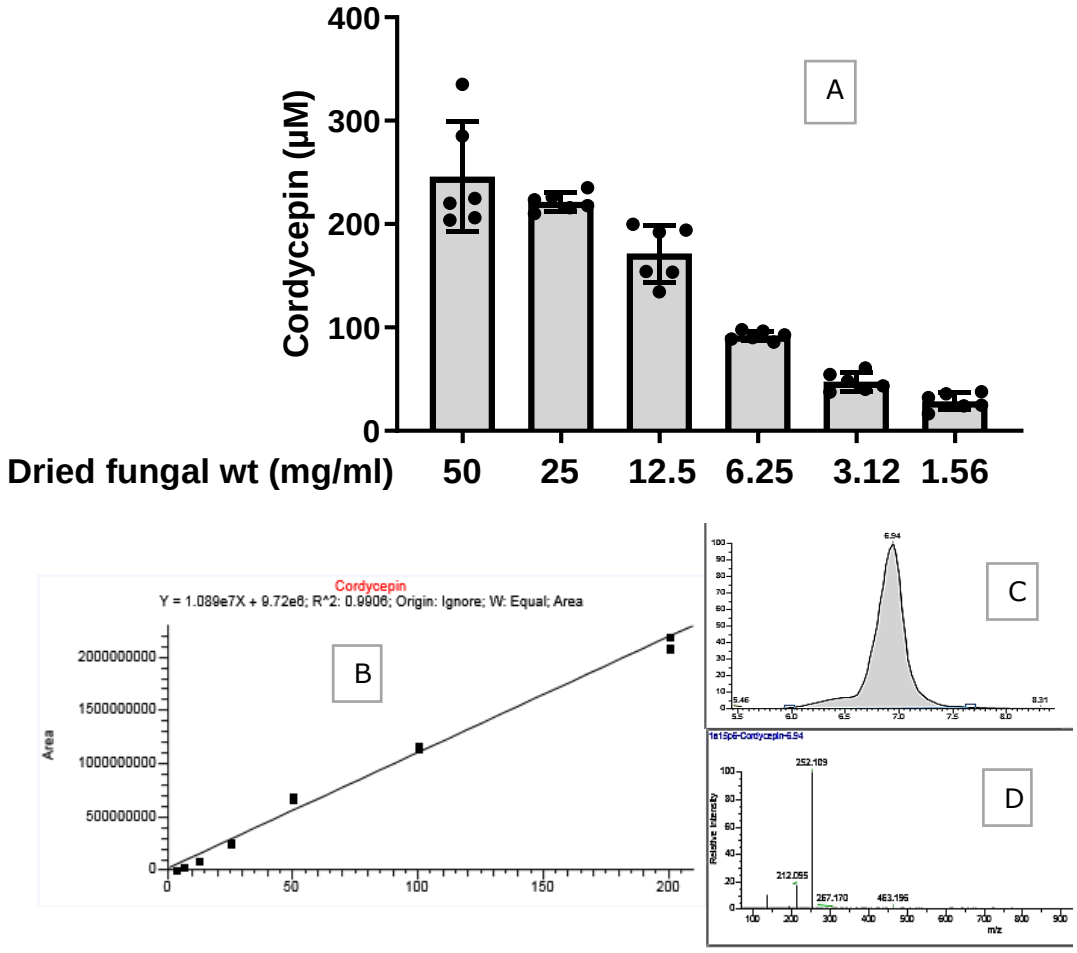


Figure 4.3: To identify the optimum concentration for quantification of cordycepin in extract (MycoMedica). (A.) Cordycepin concentration in extract, (B.) Calibration curve with dilution for 200µM, 100µM, 50µM, 25µM, 12.5µM, 6.25µM and 3.1µM, n=6, (C.) cordycepin peak, (D.) mass spectra showing the m/z and intensity, the data was obtained from Exactive Orbitrap MS instrument and analysed by TraceFinder® software for cordycepin targeted analysis.

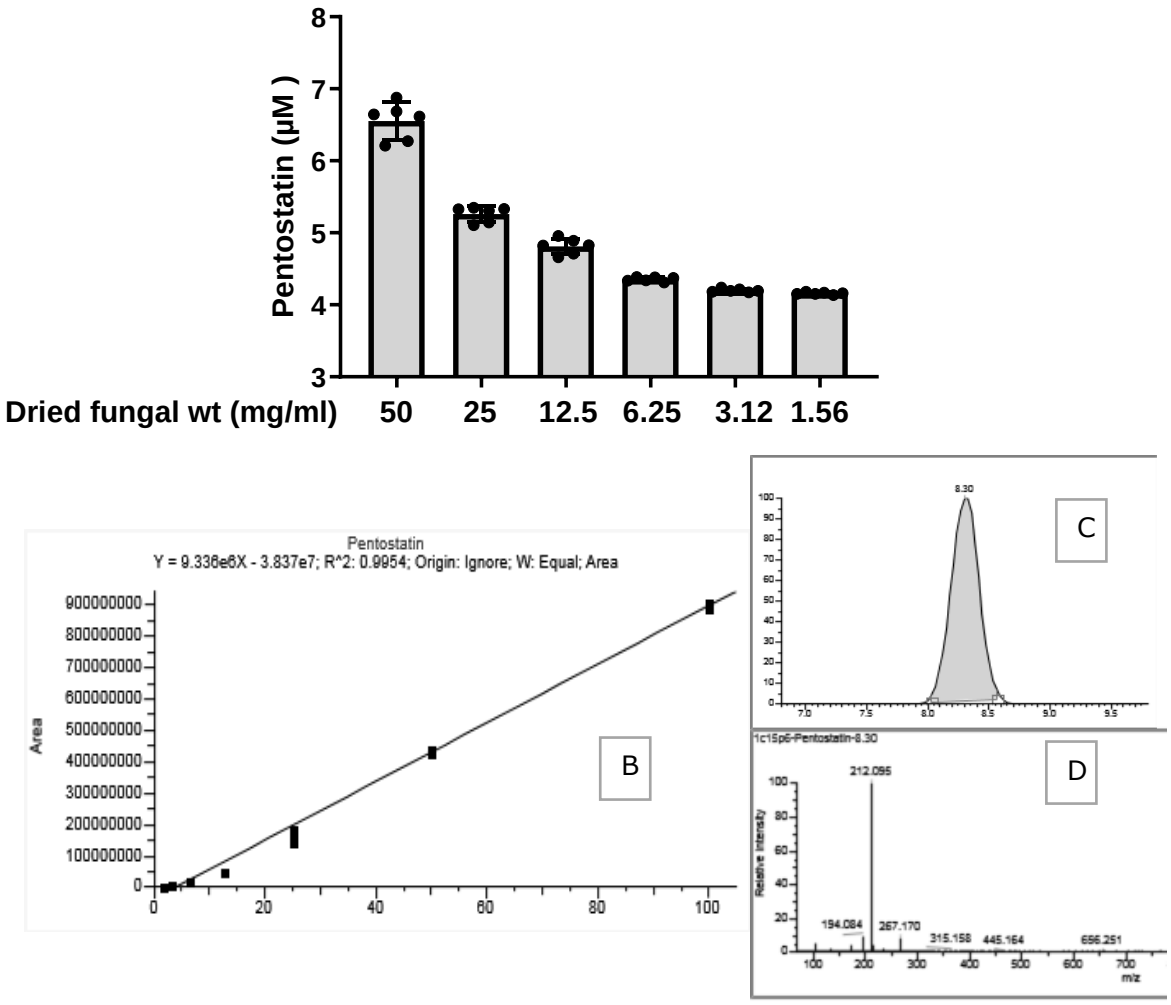


Figure 4.4: To identify the optimum concentration for quantification of pentostatin in extract (MycoMedica). (A.) Pentostatin concentration in extract, (B.) Calibration curve with serial dilution for 100µM, 50µM, 25µM, 12.5µM, 6.25µM and 3.1µM, n=6, (C.) Pentostatin peak, (D.) Mass spectra showing the m/z and intensity, the data was obtained from Exactive Orbitrap MS instrument and analysed by TraceFinder® software for pentostatin targeted analysis.

4.3 Comparative study of different *Cordyceps militaris* batches and commercial products

In targeted LC-MS of the ten batches gifted by Goba MycoMedica and the purchased Aloha (Aloha Medicinal) and CordvitK (Goba MycoMedica) of *Cordyceps militaris*, authentic standards were used for quantification of compounds of interest (cordycepin, pentostatin and 3' deoxyinosine) in the sample. TraceFinder® software and calibration line curve fitting were

used for authentic standards. The calibration curve with an R square value of 0.99 or above was considered acceptable to use – and the standard sample replicates were used to achieve a well-fitted curve, – such as by using dilution to the predicted concentrations of 3.5µM - 100µM. To avoid the issue of errors in quantification caused by the saturation of the detector at high concentrations, a serial dilution of the extract was used [figure 4.6-4.8]. The results showed variation in the concentration of cordycepin in the different batches. The concentrated extract CordVit®K Plus (MycoMedica) did not have a higher concentration than the raw powder from Goba (MycoMedica), perhaps due to the processing method of raw material and cultivation being done under the same conditions. Surprisingly, no cordycepin was detected in Aloha, which is supposedly a similar dried mycelial product [figure 4.6]. The targeted analysis showed that the 3' deoxyinosine concentration was low in all the CM, CM12-CM19 batches. About 4.5µM was detected in CordVit®K Plus, but no 3' deoxyinosine was found in Aloha [figure 4.7]. Pentostatin is an important compound for enhancing the activity of cordycepin. Pentostatin was also detected in EW, More than 5µM was detected in CM12, CM14 and CM18 but low levels of pentostatin were detected in CM9, CM13, CM15 – CM17 and CM19. The lowest concentration was detected in CM, but no pentostatin was detected in Cordyvt®k Plus and Aloha [figure 4.7].

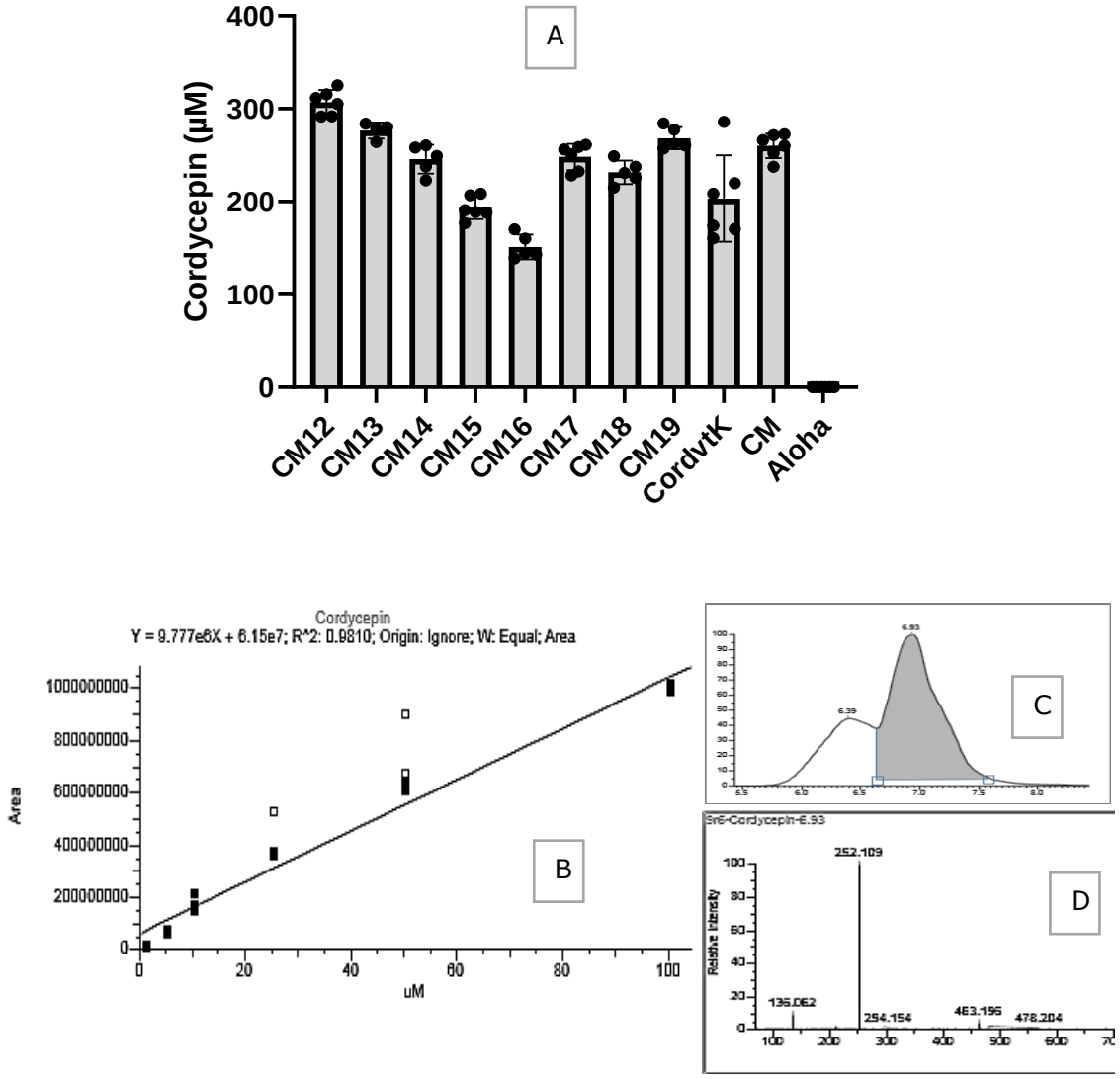


Figure 4.5: (A.) Cordycepin concentration detected in different batches from MycoMedica, commercial Aloha and cordvitK samples (B.) Calibration curve with serial dilution of 100µM, 50 µM, 25µM, 12.5µM, 6.25µM and 3.1µM, n=6, (C.) Cordycepin peak, (D.) Mass ion showing intensity and m/z. Data obtained from the Exactive Orbitrap MS instrument and analysed by Xcalibur™ Software for pentostatin targeted analysis, Mass ion graph showing the m/z and intensity and cordycepin peak detected. CM, CM12- CM19 are the batches of *Cordyceps* obtained from MycoMedica. The commercial *Cordyceps* product Aloha and CordvtK (Cordyvit®K Plus) from MycoMedica.

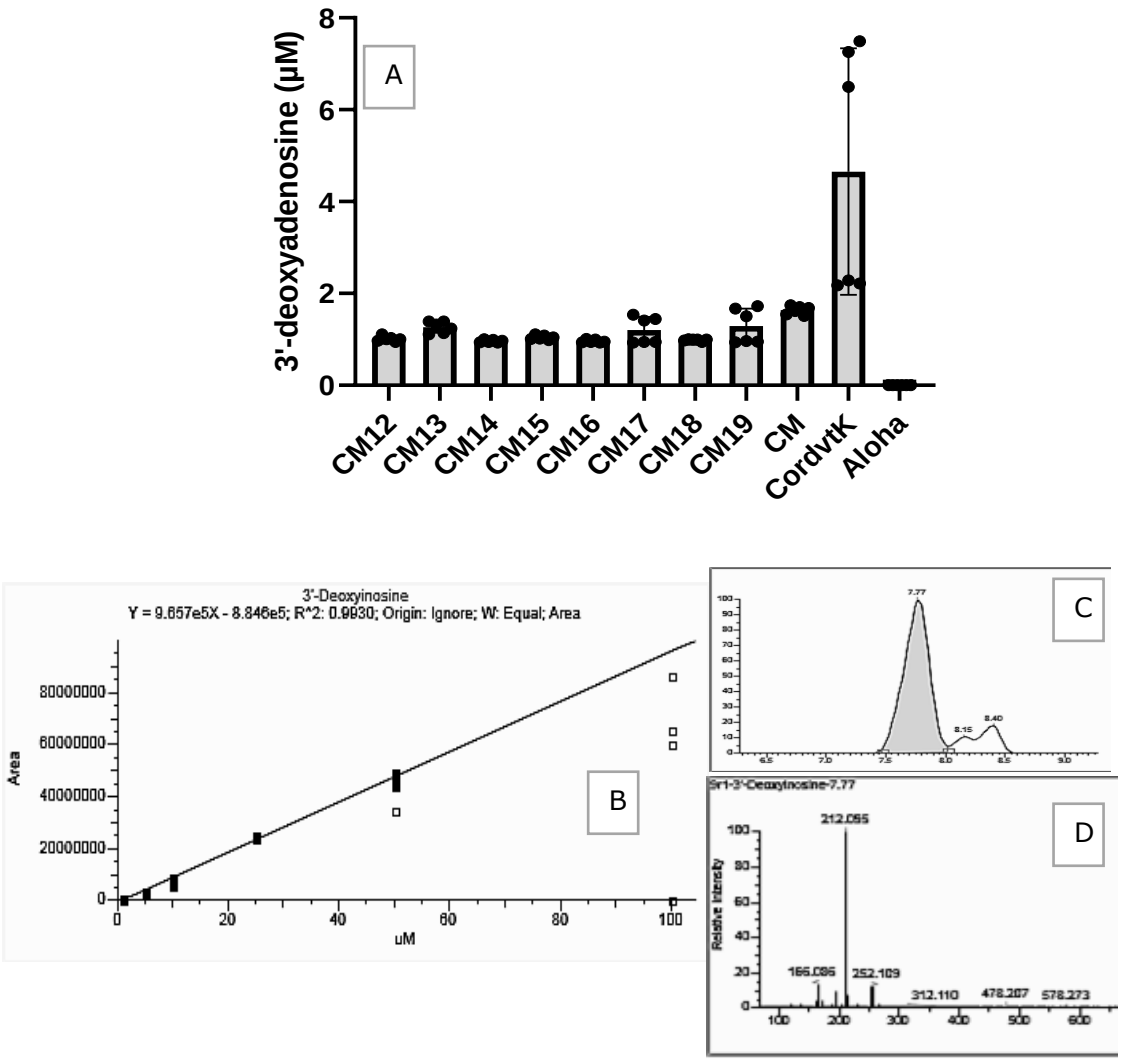
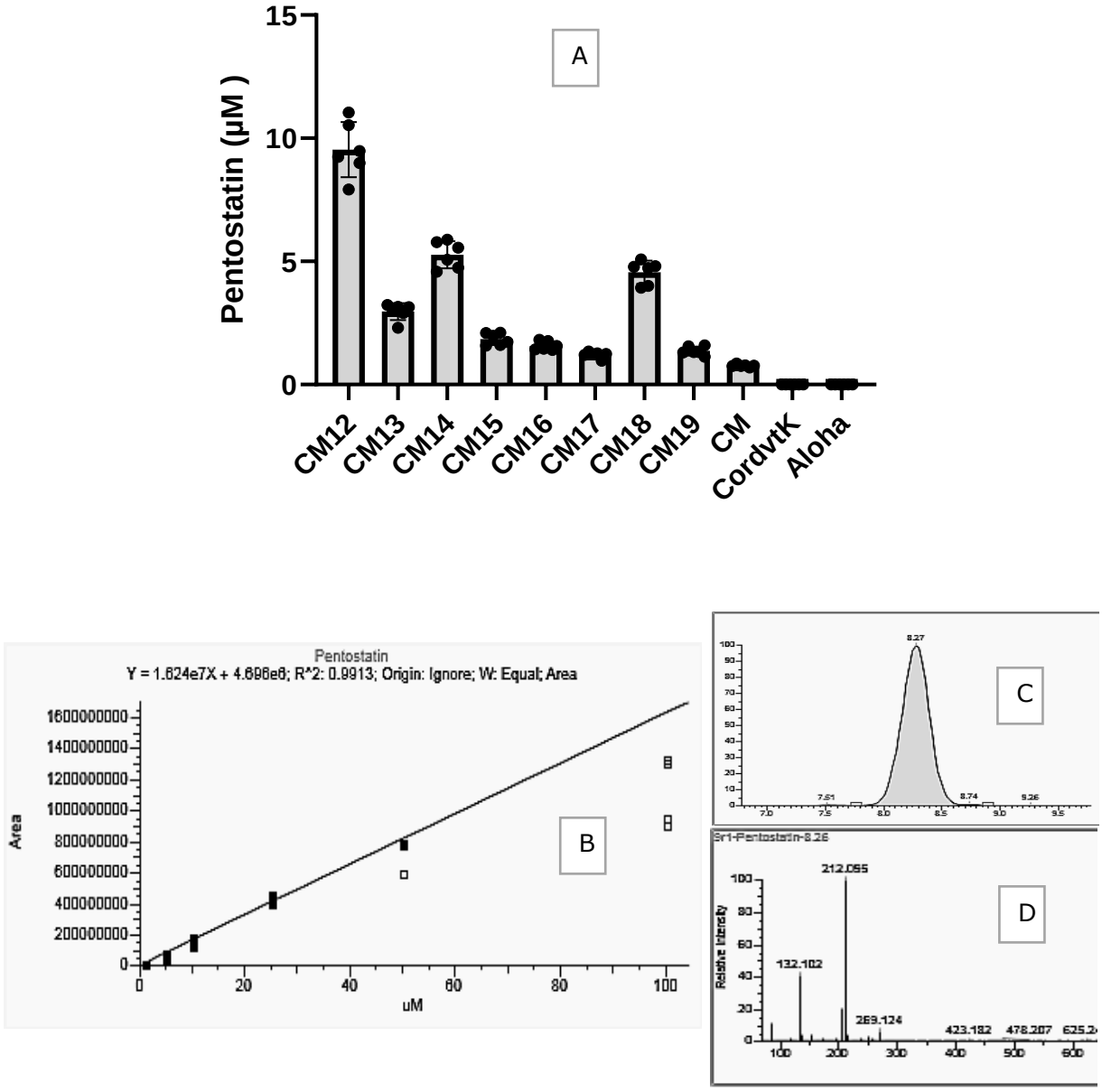


Figure 4.6: (A.) 3’ deoxyinosine concentration detected in *Cordyceps militaris* different batches from MycoMedica, commercial Aloha and CordvtK (Cordyvit®K Plus), (B) Calibration curve with serial dilution of 100µM, 50µM, 25µM, 12.5µM, 6.25µM and 3.1µM, n=6, (C) Cordycepin peak, (D) Mass ion showing intensity and m/z. Data obtained from the Exactive Orbitrap MS instrument and analysed by Xcalibur™ Software for pentostatin targeted analysis, Mass ion graph showing the m/z and intensity and cordycepin peak detected. CM, CM12- CM19 are the batches of *Cordyceps* obtained from MycoMedica. The commercial *Cordyceps* product Aloha and CordvtK (Cordyvit®K Plus) from MycoMedica.



Figur4.7: (A.) Pentostatin concentration detected in *Cordyceps militaris* different batches from MycoMedica, commercial Aloha and CordvtK, (B) Calibration curve with serial dilution of 100µM, 50µM, 25µM, 12.5µM, 6.25µM and 3.1µM, n=6, (C) Cordycepin peak, (D) Mass ion showing intensity and m/z. Data obtained from the Exactive Orbitrap MS instrument and analysed by Xcalibur™ Software for pentostatin targeted analysis, Mass ion graph showing the m/z and intensity and cordycepin peak detected. CM, CM12- CM19 are the batches of *Cordyceps* obtained from MycoMedica. The commercial *Cordyceps* product Aloha and CordvtK (Cordyvit®K Plus) from MycoMedica.

4.4 *Cordyceps militaris* extract suppresses the inflammatory gene expression

The anti-inflammatory activity of EW was assessed, and RAW 264.7 cells were incubated and treated with DMSO, or cordycepin, or EW before being treated with LPS to stimulate the inflammatory response. The cordycepin concentration in EW (5mg/mL) (100µM equivalent cordycepin) was first determined by targeted LC-MS analysis as shown in [figure 4.3], then cells were treated with isolated standard cordycepin and EW containing an equivalent cordycepin concentration (100µM). The result shows no significant inhibition of Tnf inflammatory gene expression [figure 4.8A). To assess the anti-inflammatory activity, the cells were also treated with a high concentration of EW, such as 50mg/mL, 100mg/mL and 150mg/mL. All concentrations show significant repression [figure 3.8B]. The EW exhibited an inflammatory effect, as shown in the standardisation of extract chapter 3. The EW at higher concentrations of more than 150mg/mL was also assessed. The cells were treated with only extract at 50mg/mL, 100mg/mL and 150mg/mL, only DMSO and no LPS treatment [figure 4.8C].

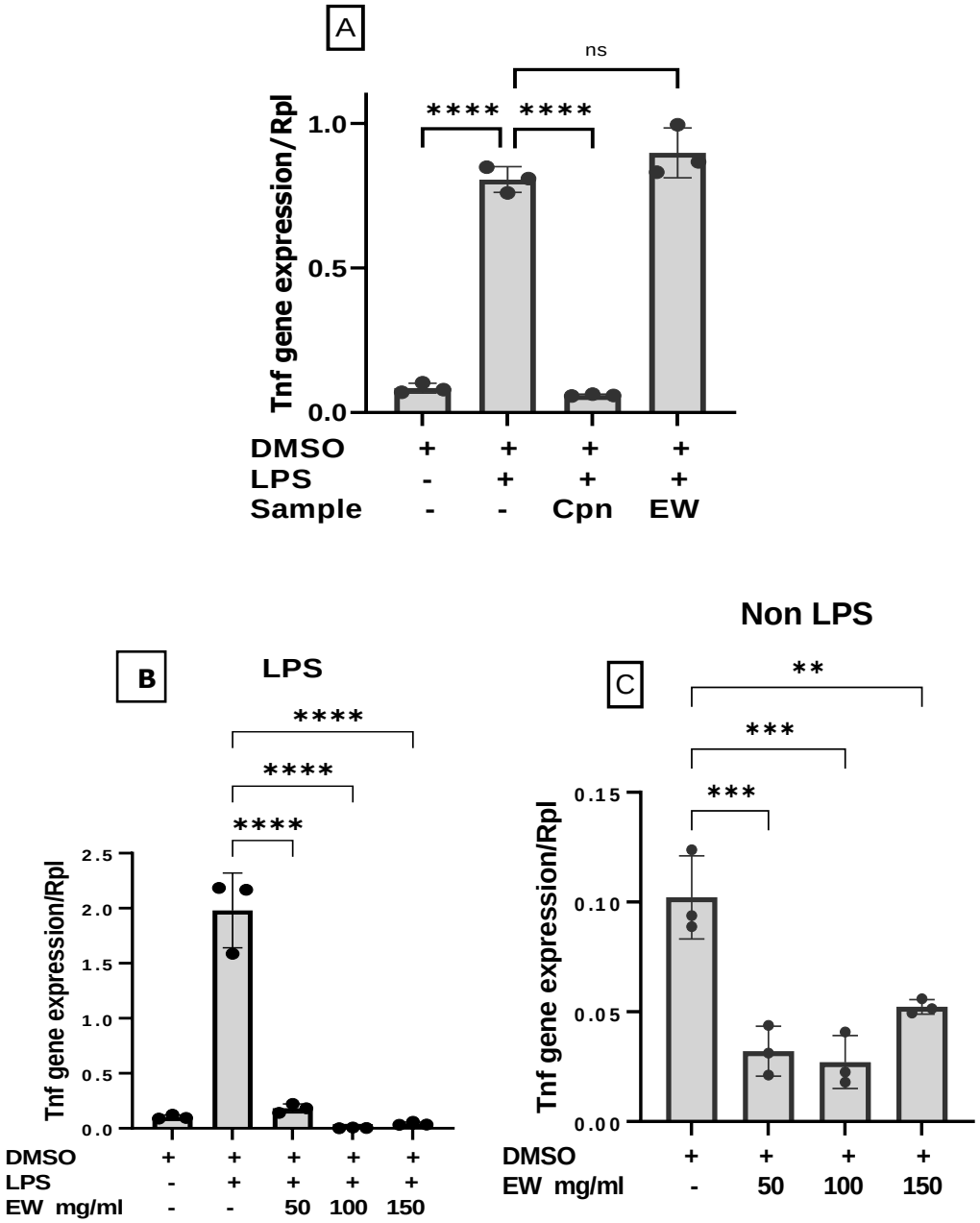


Figure 4.8: *Cordyceps militaris* extract (MycoMedica) anti-inflammatory activity. **A**; RAW 264.7 cells treated with DMSO or cordycepin (100µM) for an hour before treating with LPS, EW (ethanol-water 60%) 3mg/ml extract with equivalent cordycepin (100µM) concentration quantified with LC-MS, **B**; cells treated with a higher concentration of extract 50mg/mL, 100mg/mL and 150mg/mL before treating with LPS to activate the inflammatory response, **C**; cells only treated with DMSO and EW without LPS treatment, inflammatory gene expression was determined by RT-qPCR, error bars represent standard deviation across three RT-qPCR replicates, ****p<0.0001 ***p<0.001 ** p<0.01, * p<0.5, ns p<0.1. P values were obtained by using ordinary one-way ANOVA with multiple comparisons using GraphPad Prism v9.

4.5 Discussion

The literature showed that *Cordyceps militaris* (CM) has the potential to inhibit inflammation (Chiu *et al*., 2016; S. K. Das *et al*., 2010; Jo *et al*., 2010; Liao *et al*., 2022; Rao *et al*., 2010; Y. Yu *et al*., 2022). In addition to the anti-inflammatory activity of CM, it has been shown in numerous studies that it also exhibits biological and pharmacological activity in immunomodulation (H.-B. Fan *et al*., 2021; J.-y. Liu *et al*., 2016; Y. Yu *et al*., 2022), renal, hepatic, and cardiovascular system (Y. Jin *et al*., 2018; Lin *et al*., 2021; L. Ma *et al*., 2015; S.-H. Yu *et al*., 2016), antioxidant activity (W.-T. Wu, Hsu, & Lo, 2021; L. Yin *et al*., 2021) and anti-cancer activity (Chantawannakul *et al*., 2021). This study shows that the metabolic profile and anti-inflammatory activity of ethanol-water (60%) LC-MS is an effective analytical tool for identifying metabolites present in the extract. Two approaches were adopted to characterise the metabolic profile of ethanol water (60%). Untargeted analysis using IDEOM v19 to study all the possible metabolites present, and targeted analysis to quantify the concentration of metabolites of interest (cordycepin, pentostatin and 3' deoxyinosine).

A hydrophilic interaction liquid chromatography column (ZIC-pHILIC) was used for liquid chromatography and the Exactive MS system. Several studies have suggested that the use of polar solvents for CM extraction results in bioactive extract (J. N. Choi *et al*., 2010; Y.-L. Gao, Yu, & Li, 2022; Jo *et al*., 2010; S. Y. Yoon, Park, & Park, 2019). In EW, a total of 1909 metabolites were identified putatively from different metabolite classes. The highest number of metabolites were unidentified metabolites 830 (43.48%). The human metabolome database HMDB was used for the identification of metabolites. HMDB is considered a standard source of metabolic resources for the study of human metabolites. Over the last decade, HMDB has been improving significantly. It contains about 90 separate data fields on average, including names, synonyms, comprehensive compound description, physiochemical data, structural data, reference MS and NMR spectra, disease associations, enzyme data, pathway information, and gene sequence data, which are some of the data fields used for identification of metabolites (Wishart *et al*., 2022). A significant number of 43.48% of

metabolites were unidentified metabolites detected in EW, which could not be identified due to the limitation of the metabolomics method. The significant number indicates that there are still a lot of undiscovered metabolites which could be potentially biologically active metabolites [figure 4.2]. Furthermore, EW was also analysed for the quantification of cordycepin and pentostatin, which play an essential role in the bioactivity of EW. A high level of cordycepin was detected in ethanol-water (60%) extract. A serial dilution-targeted analysis was performed to determine the concentration of cordycepin in the extracts. The cordycepin results show that the highest concentration of 245µM was detected at 50mg (dried fungus)/mL. On further dilution, the concentration was still high and could be saturating the detector, which might be resulting in inaccurate results. To quantify and enhance the accuracy of results. The range of concentrations at which the MS signals were linear with the concentration was about 90µM at 6.25mg/mL, therefore, all samples need to be diluted to be within this range of 100µM concentration [figure 4.3].

The same serial dilution strategy was adopted for pentostatin quantification. The results show that even at the maximum concentration of the extract 50mg/mL, 6.5µM concentration was detected, which indicates that the concentration of pentostatin in EW is very low in comparison to cordycepin. At further dilution, the concentration of pentostatin did not drop respective to dilution [figure 4.4]. These results show that cordycepin concentration is very high in the extract in comparison to pentostatin, and using the same sample for quantifying both is not a suitable strategy. Because diluting the sample reduces the concentration of pentostatin to undetectable levels and results may be inaccurate.

Several commercial products are available on the market. To know their quality, it is crucial to identify and quantify essential metabolites in the extract, such as cordycepin, pentostatin, and 3’ deoxyinosine. The literature shows that changes in substrate, temperature, PH and incubation time affect the level of cordycepin in CM (Adnan, Ashraf, Khan, Alshammari, & Awadelkareem, 2017; S. K. Lee *et al*., 2019; Shih, Tsai, & Hsieh, 2007). The concentration of cordycepin, pentostatin and 3’ deoxyinosine EW (ethanol 60%) extract was analysed to detect

and quantify the concentration of metabolites of interest. The results show that a high level of cordycepin was detected in all the batches CM12- CM19, with the lowest concentration being detected in CM16. However, the commercial products Cordyvit®K Plus (MycoMedica) show a high concentration of cordycepin, but in Aloha (Aloha medicinal), no cordycepin was detected [figure 4.6].

Pentostatin, another important compound with immune suppressive activity (Delost, Smith, Anderson, & Njardarson, 2018), was approved by FDA for cell leukaemia in 1991 (K.-W. Kim, Roh, Wee, & Kim, 2016) and reported to enhance the cordycepin activity by safeguarding it (Wellham *et al*., 2019). The highest levels of pentostatin were detected in CM12 (9.4µM), followed by CM9 and CM12-CM19, and the lowest concentration of pentostatin was detected in CM (0.7µM), but no pentostatin was detected in CordvtK and Aloha.

A study by our group showed that orally administered cordycepin was rapidly metabolised to 3’ deoxyinosine, and this was the only metabolite found in the circulation (J. B. Lee *et al*., 2019). 3’ deoxyinosine and cordycepin, therefore, have a common active metabolite, cordycepin 5′-triphosphate. The level of both metabolites contributes to intracellular cordycepin triphosphate levels, and they are expected to have similar biological effects. The concentration of 3’deoxyinosine was calculated, showing that the variation of 3'deoxyinosine between the batches was negligible, which indicates cordycepin is stable in these preparations. The highest level was detected in the commercial product CordvtK, but no 3’ deoxyinosine was detected in Aloha.

The anti-inflammatory effect of EW (ethanol-water 60%) extract on the Tnf inflammatory gene was also investigated. A cell-based assay was performed using RAW264.7 cells. Ethanol-water (60%) (100µM cordycepin determined by LC-MS), and cordycepin (100µM). The results show significant inhibition of the Tnf inflammatory gene by cordycepin but no inhibition was caused by EW even though the concentration of purified cordycepin and cordycepin in EW was equivalent while treating the cells [figure 4.8A]. As in chapter 3, it was clearly shown that EW exhibits an inflammatory response due to the presence of inflammatory metabolites, which

might be interfering with the anti-inflammatory effect of cordycepin present in EW extract. Polymyxin B column (absorbs endotoxin) was used to remove the inflammatory metabolites, but no significant difference was found in the EW, and after treating with polymyxin B column, the data was not included in this chapter. Literature shows that if RAW 264.7 are treated with a high concentration of cordycepin 40µg/mL (159µM), the cells changes caused by the LPS treatment are reversed to their inactivated form; however, these changes do not occur at a low concentration of cordycepin (Shin *et al*., 2009). The cells were treated with a higher concentration of EW (ethanol-water 60%) (60mg/mL, 100mg/mL, and 150mg/mL); the repression caused by a higher concentration of EW was more than purified cordycepin which showed almost complete inhibition of inflammatory response [figure 4.8B]. The inhibition was almost similar to the non-LPS treated EW samples [figure 4.8C]. The higher concentration of extract could cause cell death, but it was considered to keep the concentration to the level to avoid cell death. A concentration of more than 150mg/mL or about 400mg/mL caused cell death resulting in very low or no RNA after extraction. Two reasons could be causing the cell death; one could be the PH change of media due to high extract concentration, which was observed by the colour change of media from red to yellow at a very high concentration of cordycepin and metabolites.

CHAPTER 5
RESULTS

CHAPTER 5: METABOLOMICS OF MCF-7 CELLS TREATMENT WITH CORDYCEPIN

Despite much research and improvements in treatment, breast cancer is still the most common cancer in women globally (Collaborators, 2003; Jacques Ferlay *et al*., 2015; Siegel, Miller, & Jemal, 2015). it was reported in 2014 that in the UK and Europe, the number of new cases was 915,674, with 162,259 deaths in 2012 (Jaques Ferlay *et al*., 2013). Breast cancer is a curable disease when diagnosed in its early stages (Coughlin & Ekwueme, 2009; Fowble, Solin, Schultz, & Goodman, 1991; Nemoto *et al*., 1980), but the option available for treatment is limited for more advanced cases. The available treatment regimens also cause severe side effects (Bodai & Tuso, 2015; Hassett, O'Malley, Pakes, Newhouse, & Earle, 2006; Shapiro & Recht, 2001). Using the chemotherapeutic drug for the treatment of breast cancer causes side effects such as hair loss, feeling sick, anorexia (loss of appetite), increased risk of infection due to low blood count, and mouth ulcers are the most common side effects caused by the drug used for chemotherapy in breast cancer (Aslam *et al*., 2014; Azim Jr, De Azambuja, Colozza, Bines, & Piccart, 2011; Kayl & Meyers, 2006); thus, targeted, more effective therapeutics and novel drug discovery is required for better treatment options.

Cordycepin also targets mammalian rapamycin (mTOR) discussed in the introduction chapter (Kondrashov et al., 2012) and therefore demonstrates a great potential candidate for the prevention and treatment of cancer. Due to its potential for cancer treatment, investigating its efficacy against several cancer cells has become promising as a new anti-cancer drug discovery candidate. Several studies suggest that cordycepin kills and/or reduces the proliferation of breast cancer cells (D. Lee *et al*., 2019).

The metabolite's concentration and identity provide a biochemical signature to monitor the physiological effects of the drug (J. Sun, 2012; Surrati *et al*., 2016). The amount of energy metabolism is high, having high levels of phosphate compounds metabolites, and enhances enzyme activity, cell-based metabolomic is a powerful method employed to investigate further diagnosis and treatment of cancer (García-Cañaveras, Castell, Donato, & Lahoz, 2016; J.

Sun, 2012). The objective of this study was to use cell-based metabolomics to investigate the intracellular metabolome changes caused by cordycepin in the MCF7 breast cancer cell line. These data will give an insight into the effect of cordycepin on cellular metabolism as well as provide information on the metabolism of cordycepin itself and treatment with cordycepin.

Untargeted metabolic profiling was used to detect changes induced by cordycepin in MCF-7 cellular metabolism, the experiment work was performed with Surachai (Centre of analytical sciences group, School of Pharmacy, University of Nottingham). The data were reanalysed in this study to identify and investigate the differential effect of cordycepin on the intracellular metabolites as well as the effects involved in the metabolomic pathway responsible for MCF7 cell death (Wada *et al*., 2017). In this study, cell base metabolomics was used to investigate changes in MCF-7 metabolites exposed to cordycepin at various intervals of time. Moreover, to identify the metabolic pathways affected by cordycepin within the MCF-7 cells. To study the effect of cordycepin on MCF-7, cells were treated with DMSO as control and cordycepin for 15, 30, 60 and 120 min. After treatment samples were prepared and analysed by LC-MS. After processing, the sample results were obtained, which were then analysed by IDEOM v19.

5.1 LC-MS-based metabolomics changes in intracellular metabolites in MCF-7cells.

To study the effect of cordycepin on MCF7 cells, the MCF7 cells were treated with cordycepin (50µM) for 15, 30, 60 and 120 min. The data was processed and then analysed by IDEOM_v19 software; the number of putative metabolites in the cell extract was 219 in total (positive and negative mode). These identified metabolites consist of cordycepin and its metabolism compounds, amino acid metabolism, lipids metabolism, carbohydrate metabolism, peptides, nucleotide metabolism, Metabolism of cofactors and vitamins, energy metabolism and biosynthesis of secondary metabolites [figure 5.1]

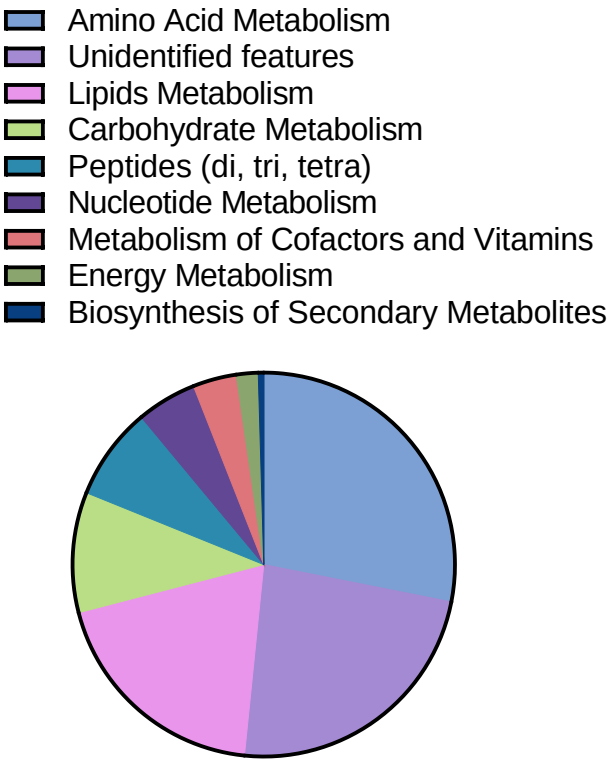


Figure 5.1: Classes of metabolites detected in MCF7 cells treated with cordycepin. Untargeted LC-MS was used following XCMS, MzMatch, and IDEOM analysis of data. Six LC-MS replicates were used for each sample. Level 2 putative identification of metabolites was achieved by accurate mass and predicted retention times using authentic standards performed by IDEOM v19 software under default parameters. The figure was constructed by GraphPad Prism v9.

5.2 Metabolites distribution of cordycepin-treated MCF-7 cells and cordycepin.

The untargeted analysis was used to study the metabolic profile of the MCF-7 cells and the total number of metabolites present. Data obtained from the instrument was processed by ToxID, XCMS, MzMatch and IDEOM_v19 analysis. The m/z was plotted against the retention time of the metabolites detected. The m/z and retention time of metabolites were obtained by comparing them to the authentic standards used for level 2 identification (Abuawad *et al*., 2020; Creek *et al*., 2014; Kodra *et al*., 2021). Most of the metabolite's mass was 50 to 350

m/z and at a retention time of 3 to 11 min, for comparison, the cordycepin m/z and retention time were 100 to 450 m/z and a retention time of 4 to 12 minutes [figure 5.2].

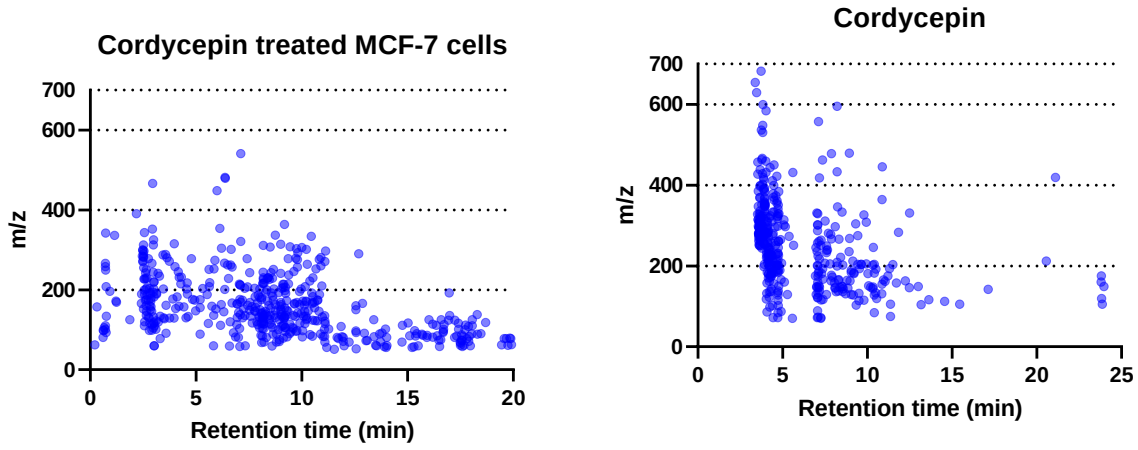


Figure 5.2: Distribution of metabolites based on their m/z and retention time detected in MCF7 cells and cordycepin by untargeted metabolic profile. MCF7 cells were treated with cordycepin for 15 min, 30 min, 60 min and 120 min. The analysis was based on metabolites masses and retention time following XCMS, MzMatch, and IDEOM analysis. Six LC-MS replicates were used. The figures were constructed in GraphPad Prism v9.

5.3 Multivariate statistical analysis of metabolomics data of MCF7 cells treated with cordycepin.

The Orthogonal partial least squares discriminant analysis (OPLS-DA) was used to visualise general trends, clustering or outline and to observe the set spectral data obtained from MCF-7 cells after analysing the samples, based on the t-test with false discovery rate correction (FDR) and OPLSDA model with > 1 VIP value [figure 5.3]. DMSO was used as a control, and cordycepin treatment was analysed at various intervals of time. The samples cluster nicely in the same group (biological replicates) but have a clear separation from another group. It can be observed that the sample at 15 min and 30 min samples are not as far apart with a time difference of only 15 min between the samples, but as the time interval between the samples increases, the distance between the samples also increases, which shows that time has an apparent effect on the composition of these samples [figure 5.3]

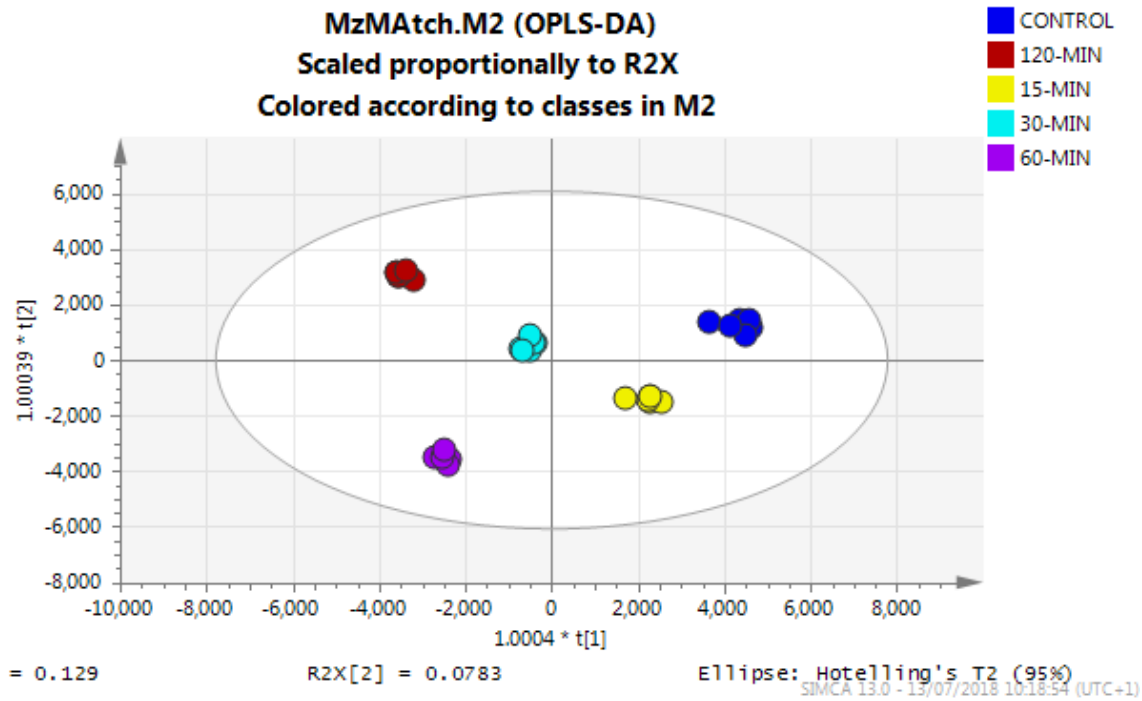
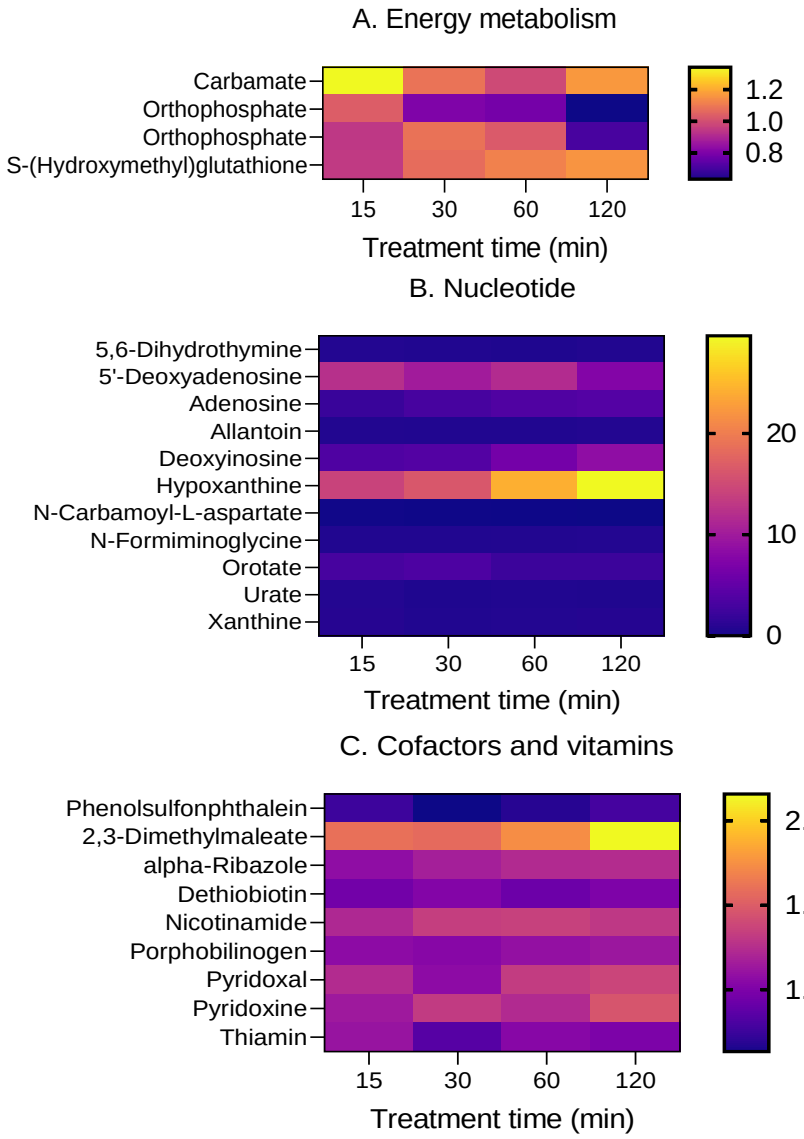


Figure 5.3: Multivariate analysis score plots of detected metabolites (Levels 1 and 2 identification) MCF7 cell treated using untargeted LC-MS. Orthogonal partial least squares-discriminant analysis OPLS-DA, Treatment of MCF7 cells with cordycepin for 15 min, 30 min, 60 min and 120 min, n=6, total detected ions followed by XCMS, MzMatch, and IDEOM analysis of data. Significant difference of metabolites was determined by false discovery rate corrections, t-tests and having VIP values >1 followed by OPLS-DA using SIMCA-P v4 software.

5.4 Cordycepin-treated MCF7 cells fold changes in metabolites and classes.

The untargeted analysis of metabolites after treating the MCF-7 cells for 15, 30, 60 and 120 min. The fold change shows the changes in the levels of detected metabolites. The upregulated or downregulated metabolites were selected after analysing by IDEOM_v19. The heatmap of each class of metabolites was detected with fold changes. The most changed metabolites were in energy metabolism, metabolism of cofactors and vitamins, and carbohydrate metabolism, followed by peptides, lipids metabolism and amino acids metabolism. The heatmap shows the fold changes in each metabolite group; the most significantly changed metabolites were nucleotide metabolism, followed by lipid metabolism, amino acid metabolism, carbohydrate metabolism, cofactors and vitamin metabolism. However, the least change was energy metabolism [figure 5.5].


A. Energy metabolism
Carbamate
Orthophosphate
Orthophosphate
S-(Hydroxymethyl)glutathione
15
30
60
120
Treatment time (min)
1.2
1.0
0.8
B. Nucleotide
5,6-Dihydrothymine
5'-Deoxyadenosine
Adenosine
Allantoin
Deoxyinosine
Hypoxanthine
N-Carbamoyl-L-aspartate
N-Formiminoglycine
Orotate
Urate
Xanthine
15
30
60
120
Treatment time (min)
20
10
0
C. Cofactors and vitamins
Phenolsulfonphthalein
2,3-Dimethylmaleate
alpha-Ribazole
Dethiobiotin
Nicotinamide
Porphobilinogen
Pyridoxal
Pyridoxine
Thiamin
15
30
60
120
Treatment time (min)
2.0
1.5
1.0
D. Peptides
Ala-Pro
Aspartyl-L-proline
Glu-Pro
Glycylproline
Lys-Pro
Asp-Cys-Ser-Tyr
15
30
60
120
Treatment time (min)
3
2
1
E. Carbohydrates
Proclavaminic acid
(R)-Malate
(S)-Malate
2,3,4-trihydroxy-butanoic acid
2,5-Dioxopentanoate
2-Acetolactate
2-Oxoglutarate
3-Butynoate
cis-Aconitate
Citrate
D-Glucose 6-phosphate
Diacetyl
D-Sorbitol
D-Xylulose
Fumarate
Isocitrate
Itaconate
L-Erythrulose
Maleic acid
Succinate
Xylitol
15
30
60
120
Treatment time (min)
3
2
1

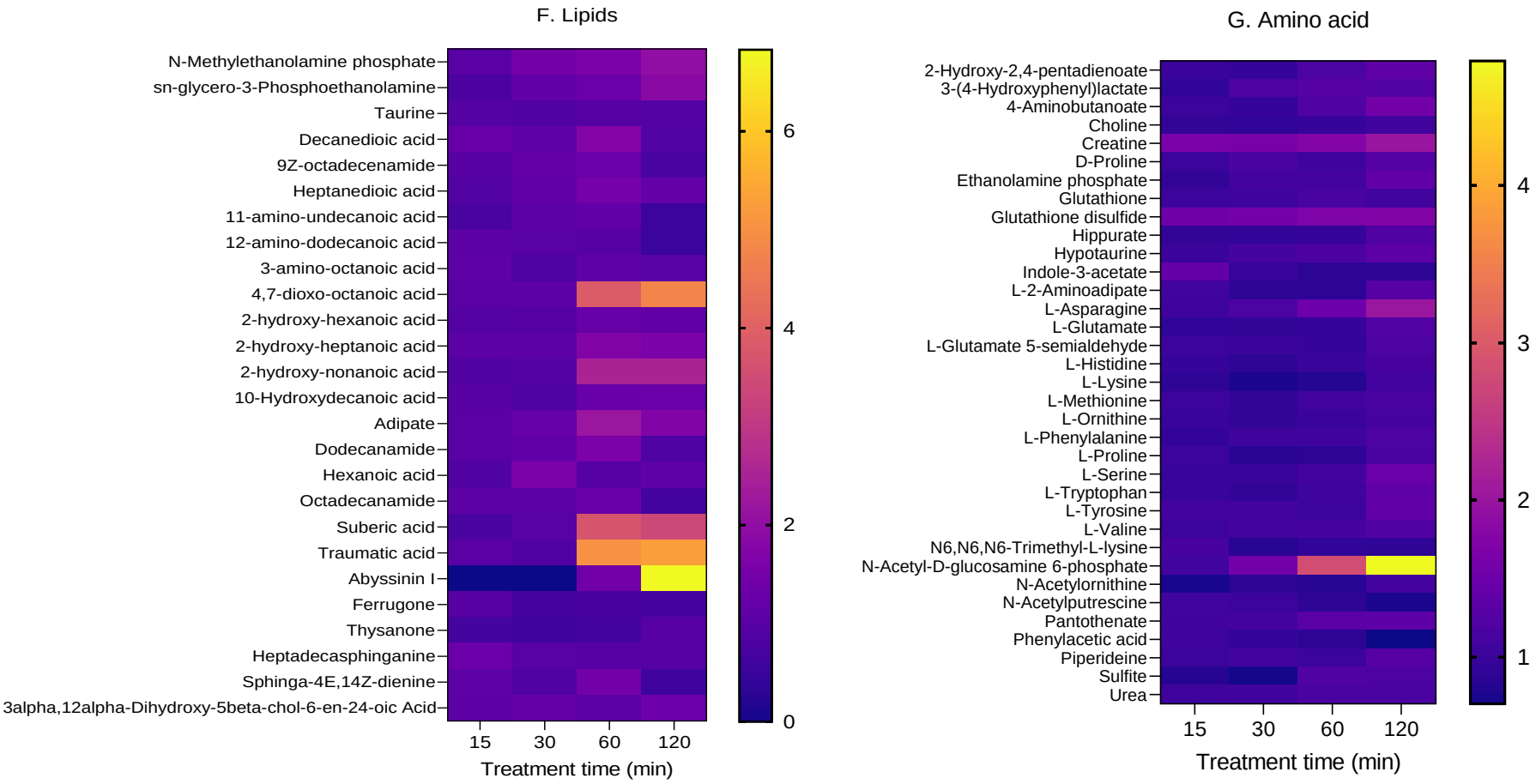


Figure 5.4: Heatmap featuring fold change of putatively identified metabolites in untargeted LC-MS analysis of MCF7 cells treated with cordycepin for 15 min, 30 min, 60 min and 120 min time intervals. LC-MS run was performed following MzMatch, and IDEOM analysis of data. n=6, the heat-maps were constructed in GraphPad Prism v9.

5.5 Biological pathways analysis of MCF-7 cells treated with cordycepin

Table 5.1: Biological pathways analysis of metabolites in an untargeted LC-MS run of MCF-7 cells treated with cordycepin. IDEOM analysis was performed to obtain the fold changes and putative metabolites. Putative identification of metabolites were obtained by level 1 metabolomics standards initiative using accurate mass, MS/MS fragmentation and predicted retention time with authentic standards and level 2 identification was achieved by predicted retention time without standards and accurate mass. Significant difference of metabolites was determined by false discovery rate corrections, t-tests and having VIP values >1 followed by OPLS-DA using SIMCA-P v4 software. The table was produced using QIAGEN Ingenuity Pathway Analysis. Analysis. Absolute values ≥2 of z-score is considered significant, with an up-regulator z-score is ≥2 and inhibited z-score is ≤−2.

Biological Pathways – For 30 to 120 min timepoints

	30 Mins	60 Mins	120 Mins
Insulin Secretion Signaling Pathway	1.633	1.633	1.633
tRNA Charging	0.775	-0.775	-3.357
Arginine Degradation VI (Arginase 2 Pathway)	1	1	1
Proline Biosynthesis II (from Arginine)	1	1	1
Superpathway of Citrulline Metabolism	0.707	0.707	-1.414
Citrulline Biosynthesis	0.816	0.816	-1.633
Arginine Biosynthesis IV	-1	-1	0
L-carnitine Biosynthesis	1	1	1
Cysteine Biosynthesis III (mammalia)	0	-1	-1
Purine Nucleotides De Novo Biosynthesis II	0	0	-1
Purine Ribonucleosides Degradation to Ribose-1-phosphate	0	0	1
Tyrosine Degradation I	-1	0	0
Urea Cycle	0	0	0
Leucine Degradation I	-1	0	0
Superpathway of Methionine Degradation	0	0	0

Activation z-score

Table 5.2: Disease and biological function analysis of metabolites in an untargeted LC-MS run of MCF-7 cells treated with cordycepin. IDEOM analysis was performed to obtain the fold changes and putative metabolites. Putative identification of metabolites was obtained by level 1 metabolomics standards initiative using accurate mass, MS/MS fragmentation and predicted retention time with authentic standards and level 2 identification was achieved by predicted retention time without standards and accurate mass. Significant difference of metabolites was determined by false discovery rate corrections, t-tests and having VIP values >1 followed by OPLS-DA using SIMCA-P v4 software. The table was produced using QIAGEN Ingenuity Pathway Analysis. Absolute values ≥2 of z-score is considered significant, with an upregulator z-score is ≥2 and inhibited z-score is ≤−2.

Disease and Biological Functions – 30 to 120 min timepoints

Biological Functions

	30 Mins	60 Mins	120 Mins
Biosynthesis of hydrogen peroxide	2.213	2.213	2.213
Hyperpolarization	1.334	2.378	2.378
Metabolism of hydrogen peroxide	2.621	1.937	1.937
Concentration of Ca2+	1.947	1.947	1.947
Synthesis of purine nucleotide	1.977	1.977	1.977
Production of hydrogen peroxide	1.91	1.91	1.91
Glucose metabolism disorder	2.51	2.234	1.352
Accumulation of lipid	1.883	1.39	2.375
Concentration of fatty acid	1.766	2.081	1.766
Flow of blood	1.582	1.582	2.379

Activation z-score

5.6 Discussion.

Cell-based metabolomics using an LC-MS technique offers a robust analytical tool for the detection of changes in cellular pathways and potential drug mechanisms. In this study, critical metabolites impacted pathways, and metabolite changes for cordycepin response in the MCF-7 cells were investigated. The number of metabolites detected from various classes of metabolites such as Amino acid metabolism (61) with the highest number of metabolites followed by carbohydrate metabolism (22) and smaller numbers from other classes, notably nucleotide metabolism (11) [figure 5.1]. The metabolites detect mainly range from 50 to 350 m/z with a retention time of 3 to 11 min and in cordycepin were 100 to 450 m/z and a retention

time of 4 to 12 min [figure 5.2], both the data are from two different, but clearly show the variation in the metabolites after cordycepin treatment of MCF-7, which indicates that metabolic profile of cordycepin changed after cordycepin treatment. The PCA and OPLS-DA based on cellular metabolites show that DMSO (control and vehicle), clustered closely inside each group and distinctly from one another, which indicates that different metabolic pattern was present among the samples at different time points [figure 5.3A and B]. The fold changes of the detected metabolites were also studied, showing no notable fold changes of metabolites from 15 to 30 min, the effects of cordycepin are relatively slow, possibly because it needs to be converted to cordycepin triphosphate. Hypoxanthine, cordycepin, deoxyinosine, adenosine, vinyl chloroacetate and prothioconazole-desthio are some of the metabolites with significant fold change observed at time intervals of 15, 30, 60 and 120 min. The fold changes of each class group were also studied, showing nucleosides to have the most significant fold change, followed by lipids and carbohydrates. In nucleotides, hypoxanthine showed the highest and signified fold change at all four-time intervals [figure 5.5]. The biological pathways analysis shows that insulin secretion signalling is consistently upregulated and unchanged with cordycepin treatment compared to control, Glucose-6-phosphate is involved in this pathway, as is oxidative phosphorylation. In MCF-7 cells, it shows a reflection of a shift in carbohydrate metabolism which indicates the Warburg effect. The cordycepin progressively decreases tRNA charging over time, it is well known that cordycepin represses protein synthesis, which is consistent with reduced tRNA charging (Y. Y. Wong *et al*., 2010). As cordycepin is a purine nucleoside, which results in a decrease in purine nucleotide biosynthesis over time. Citrulline metabolism and biosynthesis also decrease over time, it has been suggested to be a cancer biomarker and it is both a degradation product as well as a potential precursor for arginine, which enables tumour cells to recycle (J. H. Kim *et al*., 2007; C. Y. Yoon *et al*., 2007), however, phosphorylation of purine nucleoside increases ribose-1-phosphate increases and as citrulline is a precursor of arginine, it converts over time and results in arginine biosynthesis steady increase [table 5.2].

CHAPTER 6
RESULTS

CHAPTER 6: ANTI-INFLAMMATORY ACTIVITY AND CONTAMINANTS OF CORDYCEPIN PREPARATIONS

The difficulties in the purification of natural products can lead to impurities in the isolated compounds. As single source material for a natural product typically contains hundreds to thousands of secondary metabolites (compounds), there are nearly always other compounds with similar physicochemical properties that are hard to separate from the compound of interest (S. A. Ashraf *et al*., 2020). Often the complex purification process may introduce undesirable "tracer" components into the purified natural product, such as laboratory contaminants, sorbents, residual solvents, and or other chemicals that can evade detection by the standard analytical techniques used (Datta & Henry, 2006; Pauli, Chen, Friesen, McAlpine, & Jaki, 2012). The *Cordyceps militaris* extract exhibits an anti-inflammatory effect that is at least in part due to the presence of cordycepin in a high concentration. However, there are also other metabolites present in the extract which could affect the cordycepin anti-inflammatory activity, such as pentostatin, 3' deoxyinosine, cerebrosides (ceramide derivatives) and polysaccharide (Chiu *et al*., 2016; Du *et al*., 2021; C.-Y. Wu *et al*., 2022; J. Zhang *et al*., 2019).

Inflammation is the innate immune system's reaction to infection and tissue trauma. The term inflammation refers to a complicated biological process, the goal of which is to protect the host by removing invading pathogens, healing damaged tissue, and establishing homeostasis. During the initial stages of inflammation, monocytes in the bloodstream exit the circulation and infiltrate into tissues, where they develop into macrophages after conditioning by localized growth factors and inflammatory cytokines (Sherwood & Toliver-Kinsky, 2004). Lipopolysaccharide (LPS) is an archetypal pathogen-associated molecular pattern found on the external membrane of a gram-negative bacterial organism. It activates the innate immune system by activating Toll-like receptor 4 (TLR4). LPS-induced macrophage activation leads to the production of pro-inflammatory cytokines, including tumour necrosis factor-alpha (TNF-α)

and interleukin-1 beta (IL1) (Fang *et al*., 2018). These two prototypical inflammatory cytokines were linked to the pathology of numerous inflammatory diseases (Ifrim *et al*., 2014; Leal, Casabona, Puntel, & Pitossi, 2013).

In this study, the anti-inflammatory activity of cordycepin from six different suppliers and serial dilution was tested. Given the drug's nucleoside-like structure, the focus of this study was primarily on low micro-molar doses in this investigation to avoid overpowering intracellular molecular processes. Cordycepin is mainly isolated from *Cordyceps militaris* as there is no synthetic alternative available commercially. It is usually sold as 98% pure, but as the active concentrations of cordycepin are in the micromolar range (Qian *et al*., 2012), contaminants may promote or hinder the therapeutic effects of cordycepin and cause variation in the activity of different commercial preparations In this chapter. Presenting data on the activity and purity of 5 cordycepin preparations from a variety of commercial sources and cordycepin purified from a liquid culture by a collaborator at MycoMedica. To assess this possibility a serial dilution of various commercially purified cordycepin was used in the LPS-treated RAW 264.7 cell assay. The inflammatory gene expression at various concentrations was then measured by RT-qPCR. The inflammatory gene expression of the Tnf gene was assessed and was found to be repressed by cordycepin [figure 6.1]. Cordycepin from various suppliers was assessed based on their concentrations.

6.1 Cordycepin suppresses the inflammatory gene expression in cell culture

To assess the relative anti-inflammatory of cordycepin, six different cordycepin were obtained, and their activity was compared using RAW 264.7 cell lines. Purified cordycepin was purchased from Aesar, Carbosynth, Goba, Merck, Phyproof ® reference substance (Reference), and Tocris. Goba cordycepin was gifted by MycoMedica. The cells were treated with DMSO or cordycepin (in DMSO) for an hour before stimulating the inflammatory gene with LPS for an hour. The cells were lysed, followed by RNA extraction, cDNA and RT-qPCR. The results obtained from the RT-qPCR were analysed using the method described in

(material methods chapter 2). Cordycepin was used at a serial dilution of 100µM, 50µM, 25µM, 12.5µM, and 6.25µM. The relative activity of cordycepin is shown [figure 6.1 and table 6.1].

A IC50 of cordycepin

Sample	Alfa Aesar	Carbosynth	Goba	Merck	Reference	Tocris
IC50	8.237	11.53	11.72	9.724	11.22	4.930

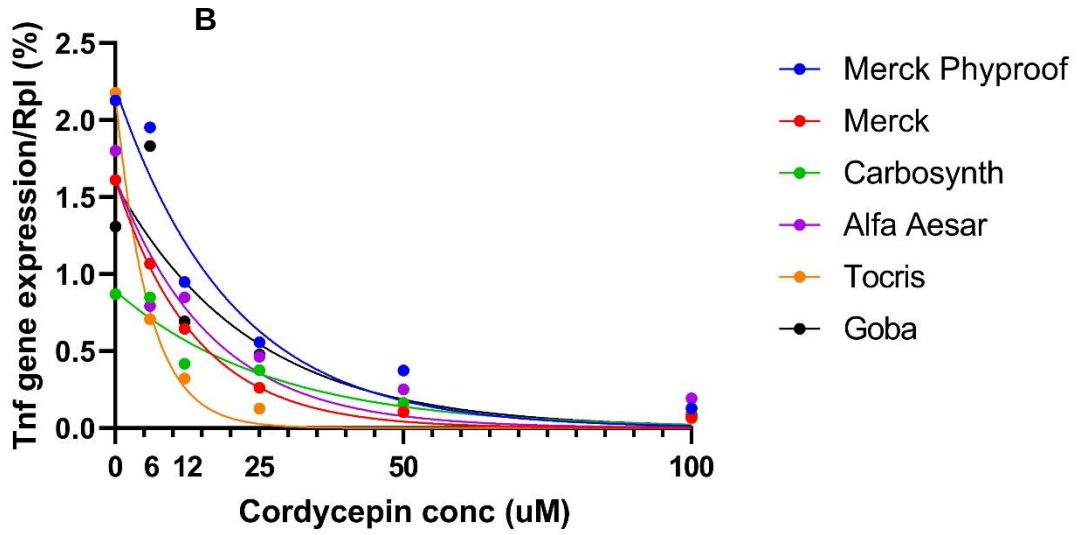


Figure 6.1: (A) IC50 of cordycepin on Tnf gene expression, (B) Anti-inflammatory activity of six cordycepin obtained from Alfa Aesar, Carbosynth, Goba, Merck, Phyproof ® reference substance (Reference), Tocris. Goba cordycepin was gifted by MycoMedica/Goba ® and is not commercially available. RAW 264.7 cells treated with DMSO or cordycepin for an hour with serial dilution of 100µM, 50µM, 25µM, 12.5µM, and 6.25µM before treatment with LPS followed by RNA extraction, reverse transcription and RT-qPCR, Tnf inflammatory gene and Rpl 28 reference gene was used, each point representing three biological replicates. Data was normalised with cordycepin and DMSO sample, log IC50 was calculated by GraphPad Prism v9.

The result shows that at 100µM, all the cordycepin showed maximum inhibition of the Tnf inflammatory gene, but at 50µM, the least activity was shown by Phyproof ® reference substance (Reference) and the highest inhibition was shown by Merck. At the minimum concentration of 6.25µM used in this experiment, Phyproof ® reference substance and Goba showed no activity while Tocris showed significant activity, followed by Alfa Aesar, Carbosynth and Merck [figure 6.1]. IC50 values were similar for most batches of cordycepin, in the range of 8µM - 12µM; in contrast, the Tocris cordycepin batch had lower IC50 values indicating a higher biological activity [figure 6.1A]. This difference was evident throughout the dose-

response curve, as visible [figure 6.1B]. These data suggest that either the amount or the composition of cordycepin preparations is variable, leading to differences in the biological response.

6.2 Cordycepin chromatograms

To assess the purity of cordycepin, an untargeted and targeted metabolomic analysis was carried out using all the preparations. The chromatogram of QC (quality control sample prepared using mixtures of equal volumes from all cordycepin samples) obtained from the Thermo Q-Exactive® MS/MS was used for higher-level identification of metabolites by tandem MS (MS/MS) using SeQuant® ZIC®-pHILIC (Merck). The data was processed by Thermos Fisher XcaliburTM software to obtain a chromatogram. Run time of 24min, PH 9.1, Flow rate 300µL, Temp 45ºC in the column oven, Mobile phase (Ammonium carbonate 20mM and Acetonitrile). The output from the instrument was analysed using Compound Discoverer and IDEOM analysis software for untargeted and Trace finder for targeted analysis, which will be discussed later in this chapter. The data obtained from Thermo Q-Exactive® MS, as shown in the chromatogram, several peaks were detected in positive and negative modes [figure 6.2], indicating that significant contaminants are detectable. To compare my analysis with the standard methods of determining purity used by the industry, we commissioned Reach Separations to asses purity using Water UHPLC H-Class instrument. The chromatogram was obtained by Reach Separations (Bio-city Nottingham, UK), the chromatogram from Water UHPLC H-Class instrument using Waters PDAm Waters QDa and Waters ELSD detectors, column Acquity CSH C18 (150mm x 2.1mm, 1.7µm), Mobile phase ((Water phase (0.1% TFA, 0.1% formic, 0.2% NH3) 98-2 gradient) and Acetonitrile 2-98 gradient. This only showed a cordycepin peak, without significant contaminants [figure 6.3].

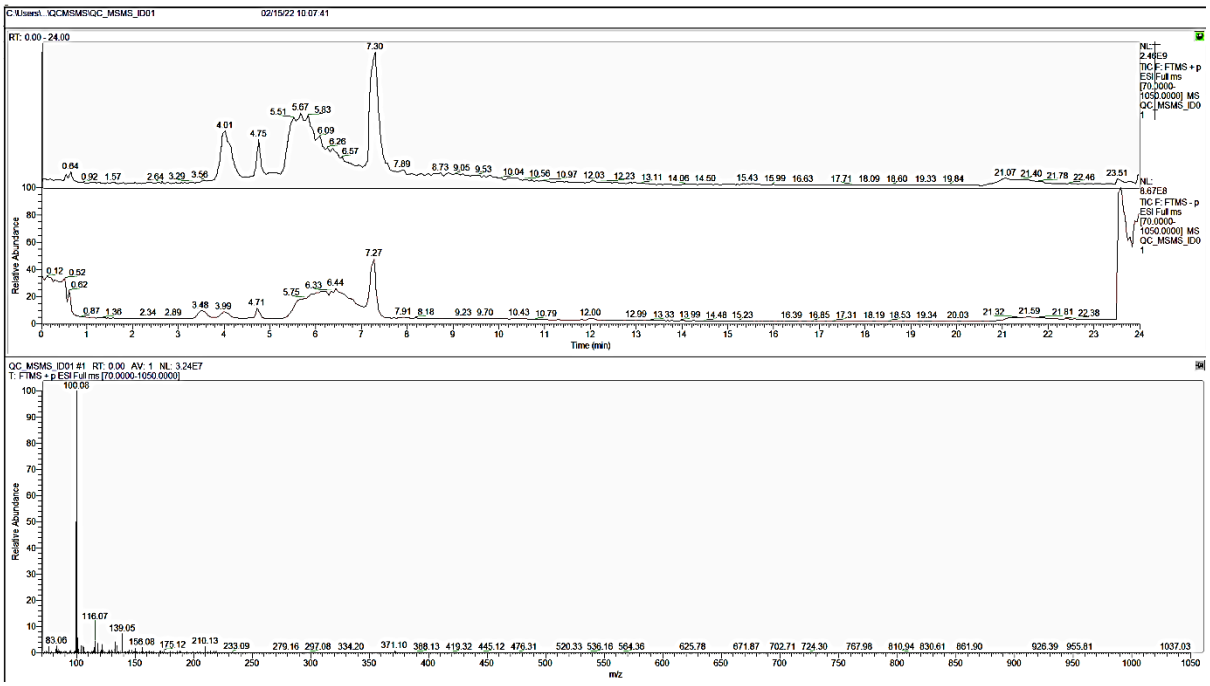
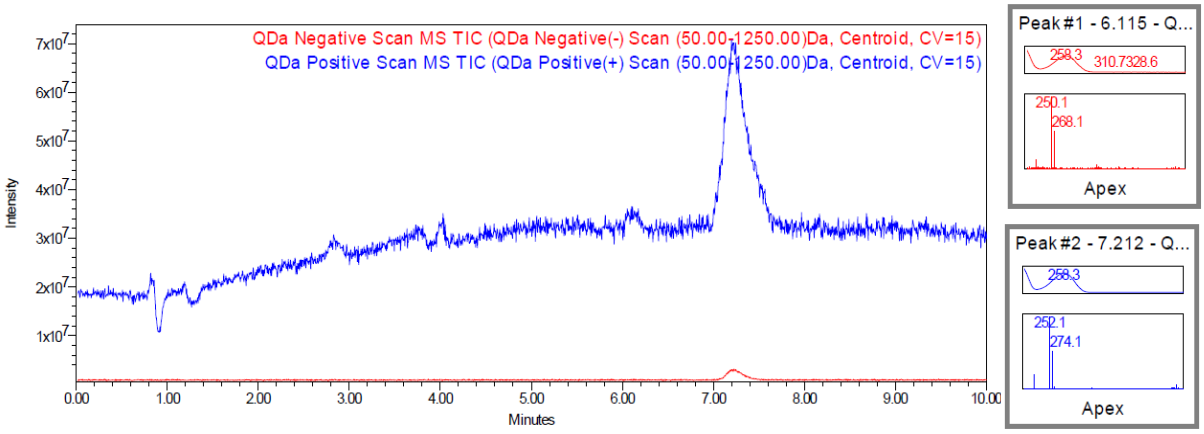
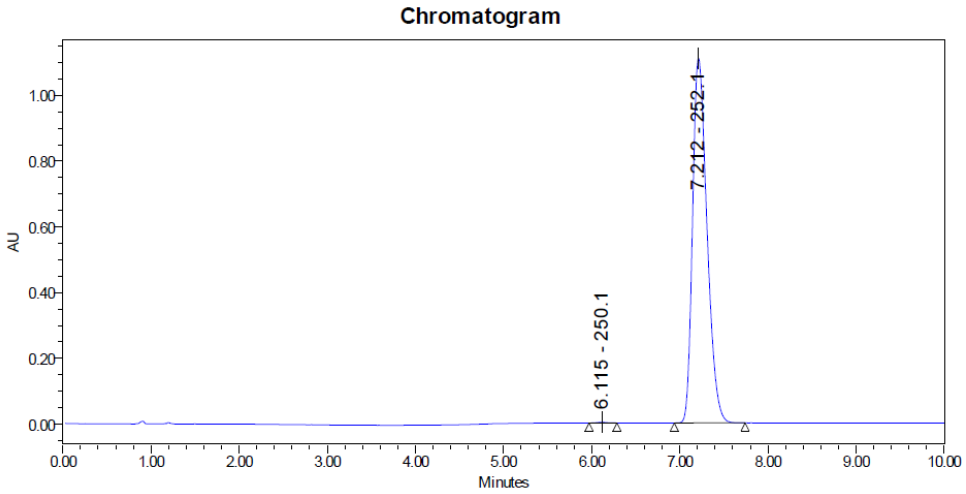


Figure 6.2: Chromatogram of cordycepin QC LC-MS/MS, The chromatogram of QC (quality control sample prepared using mixtures of equal volumes from all cordycepin samples) using Alfa Aesar, Carbosynth, Goba, Merck, Phyproof ® reference substance (Reference), Tocris. It is obtained from Thermo Q-Exactive® MS/MS for higher-level identification of metabolites by tandem MS (MS/MS) using SeQuant® ZIC®-pHILIC (Merck) column. Run time of 24 min, the top chromatogram in positive mood, the middle chromatogram in negative mood and bottom is the mass ion chromatogram.





Peak Results

	Retention Time (min)	Area (µV*sec)	% Area	Width @ 50%
1	6.11	16240	0.1	0.11545
2	7.21	12605981	99.9	0.17586

Figure 6.3: Chromatogram of Carbosynth cordycepin the chromatogram was obtained by Reach Separations (Bio-city Nottingham, UK), the chromatogram from Water UHPLC H-Class instrument, run time 10 min, The top chromatogram of whole run, middle peak detected (cordycepin), bottom table of detected peaks area.

6.3 Distribution of metabolites on the basis of masses and retention time

These six different batches of purified cordycepin were subjected to an untargeted metabolomics analysis to determine the nature and the total number of metabolites present using a Thermo Exactive mass spectrometer and Z-pHILIC column. Seven replicates were performed for each sample. Data obtained from the instrument was pre-processed by ToxID, XCMS, MzMatch and then analysed by IDEOM_v19. The m/z was plotted against the retention time of the metabolites detected in each sample of cordycepin. The data of metabolites were obtained from level 1 identification and level 2 identification was achieved as described in the methods chapter (Creek *et al*., 2014; Kodra *et al*., 2021). Most of the metabolites were 100 to 450 m/z and had a retention time of 4 to 12 minutes, and the composition of the six preparations can be seen to be broadly similar [figure 6.4], suggesting they are prepared by similar methods from similar cultures

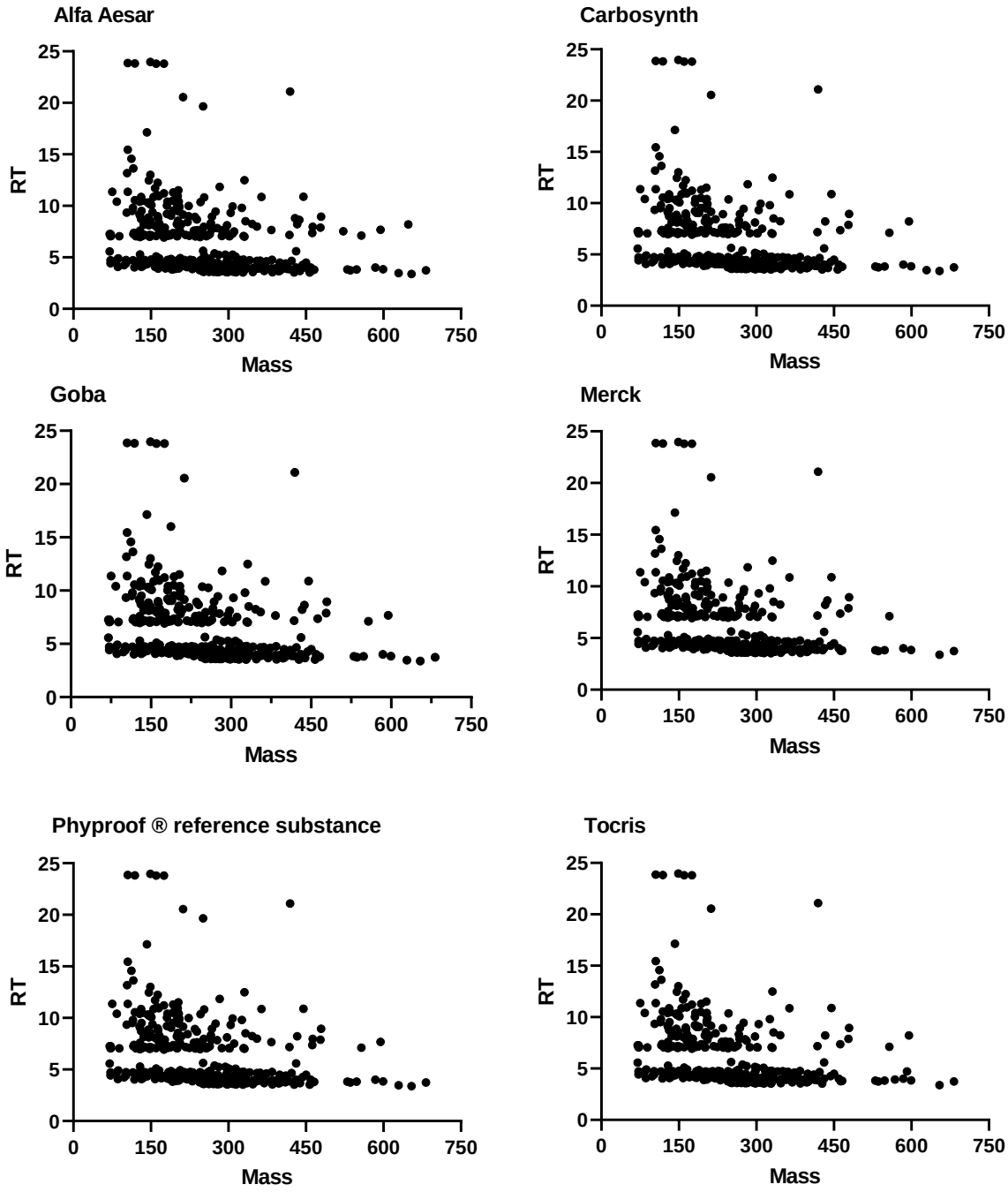


Figure 6.4: Distribution of detected metabolites on the basis of their m/z and retention times in six cordycepin obtained from Alfa Aesar, Carbosynth, Goba, Merck, Phyproof ® reference substance (Reference), Tocris. The analysis was based on metabolites m/z and retention time obtained from IDEOM analysis of data. Putative identification of metabolites was obtained by level 1 metabolomics standards initiative using accurate mass, MS/MS fragmentation and predicted retention time with authentic standards and level 2 identification was achieved by predicted retention time without standards and accurate mass. Six LC-MS replicates were used. The figures were constructed in GraphPad Prism v9.

6.4 Distribution of metabolites and features detected in cordycepin based on classes

The number of metabolites and features detected in Alfa aesar 427, Carbosynth 388, Goba 393, Merck 385, Phyproof ® reference substance (Reference) 400, Tocris 374 [figure 6.3]. The highest number of putative metabolites were lipid metabolism and amino acids. Minor groups of metabolites included peptide metabolism, biosynthesis of secondary metabolism, carbohydrate metabolism followed by the metabolism of cofactors and vitamins, nucleotide metabolism and xenobiotics [figure 6.5 and table 6.1].

Table 6.1: Distribution of metabolites on the basis of their Classes detected in six cordycepin obtained from Alfa Aesar (A), Carbosynth (B), Goba(C), Merck (D), Phyproof ® reference substance (Reference)(E), Tocris (F). The analysis was based on metabolites masses and retention time following XCMS, MzMatch, and IDEOM analysis of data. n=6. Putative identification of metabolites was obtained by level 1 metabolomics standards initiative using accurate mass, MS/MS fragmentation and predicted retention time with authentic standards and level 2 identification was achieved by predicted retention time without standards and accurate mass. Significantly different metabolites obtained using t-tests with false discovery rate corrections and having VIP values > 1 following OPLS-DA. SIMCA-P v4 software. The figures were constructed in GraphPad Prism v9.

Metabolites Classes	Colour	Alfa Aesar	Carbosynth	Goba	Merck	Reference	Tocris
Unidentified metabolites (UIM)		39.81%	37.89%	38.93%	38.44%	38%	39.04%
Lipid metabolism (LM)		32.08%	34.54%	33.59%	34.03%	33.75%	33.69%
Amino Acid Metabolism (AAM)		10.77%	11.34%	11.20%	11.43%	10.75%	11.50%
Peptide (di, tri, tetra) (PDTT)		5.39%	4.64%	4.83%	4.68%	5.25%	4.55%
Biosynthesis of Secondary Metabolism		4.45%	4.38%	4.07%	4.16%	4.50%	4.28%
Carbohydrate metabolism (CM)		2.58%	2.84%	2.80%	2.86%	2.75%	2.94%
Metabolism of Cofactors and Vitamins (MCV)		2.11%	1.80%	2.04%	1.82%	2.25%	1.87%
Nucleotide Metabolism (NM)		2.11%	1.80%	1.78%	1.82%	2.00%	1.60%
Xenobiotic (XEB)		0.70%	0.77%	0.76%	0.78%	0.75%	0.53

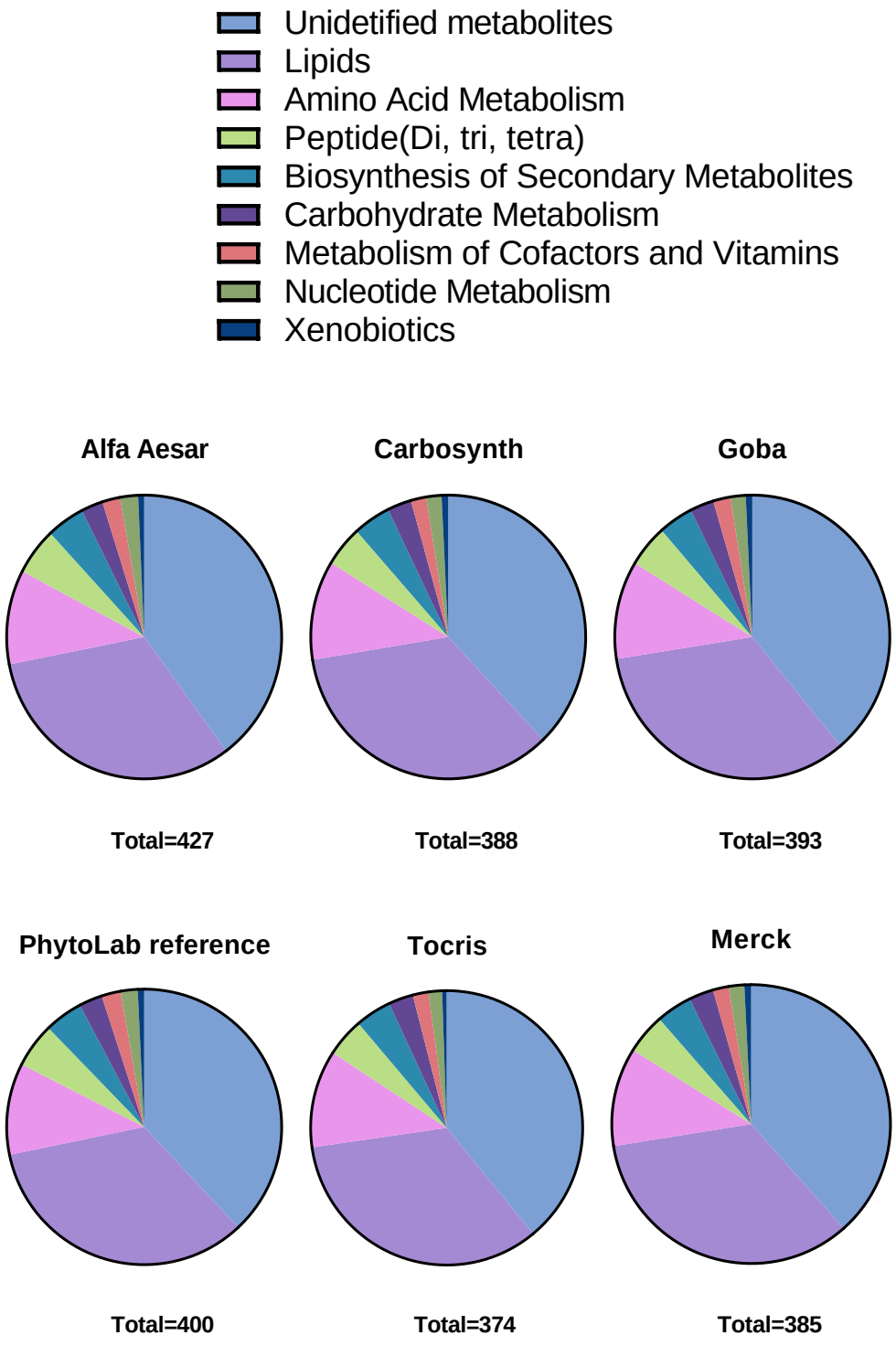
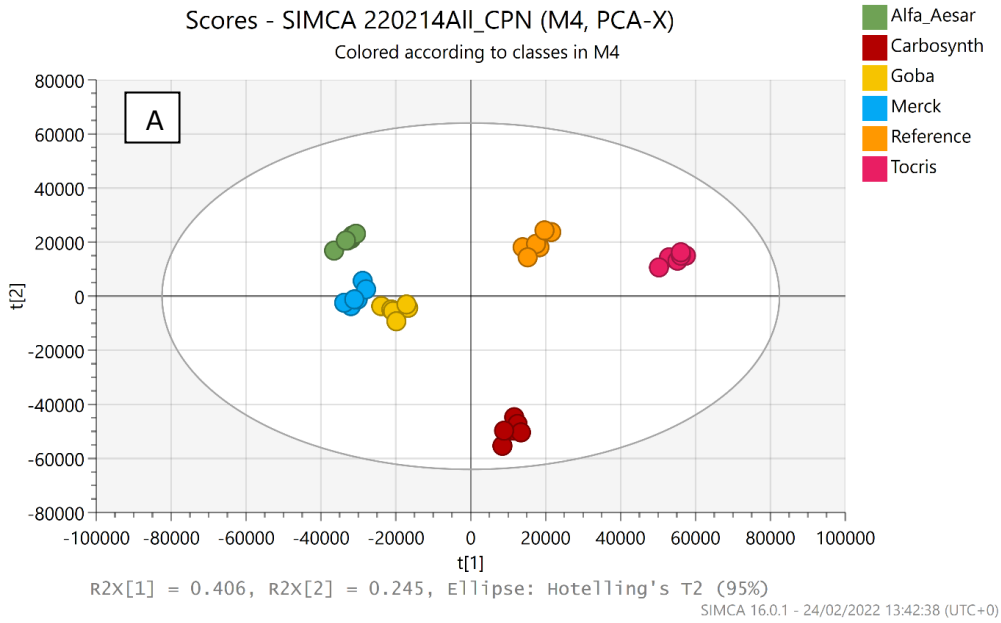


Figure 6.5: Distribution of metabolites based on classes of putatively identified metabolites detected in untargeted LC-MS analysis of six cordycepin, q-Exactive LC-MS was used following XCMS, MzMatch, and IDEOM analysis of data. Six LC-MS replicates were used for each sample. Putative identification of metabolites was obtained by level 1 metabolomics standards initiative using accurate mass, MS/MS fragmentation and predicted retention time with authentic standards and level 2 identification was achieved by predicted retention time without standards and accurate mass. The figures were constructed in GraphPad Prism v9.



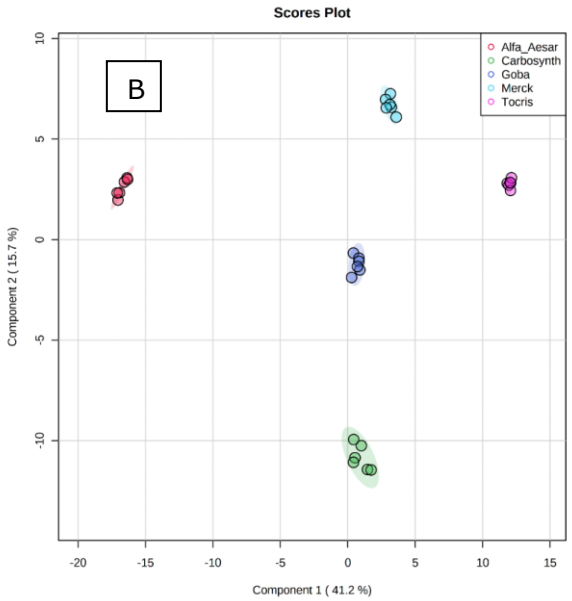


Figure 6.6: Multivariate analysis score plots of detected mass ions in six different cordycepin by untargeted LC-MS Principal component analysis (PCA, figure 6.3a) and orthogonal partial least squares-discriminant analysis (OPLS-DA, figure 6.3b) plots. Six cordycepin were used obtained from Alfa Aesar, Carbosynth, Goba, Merck, Phyproof ® reference substance (Reference), and Tocris. n=6, these multivariate analyses are based on relative concentrations of all putatively identified and identified metabolites followed by XCMS, MzMatch, and IDEOM analysis of data. Putative identification of metabolites was obtained by level 1 metabolomics standards initiative using accurate mass, MS/MS fragmentation and predicted retention time with authentic standards and level 2 identification was achieved by predicted retention time without standards and accurate mass. Significantly different metabolites obtained using t-tests with false discovery rate corrections and having VIP values > 1 following OPLS-DA. SIMCA-P v4 software was used to obtain these figures.

6.5 Most abundant metabolites detected in cordycepin

An untargeted analysis of cordycepin was carried out to estimate the relative concentration of metabolites in cordycepin. Untargeted analysis LC-MS analysis was used on six instrumental replicates of Alfa Aesar (A), Carbosynth (B), Goba(C), Merck (D), Phyproof ® reference substance (Reference)(E), Tocris (F). The data was obtained from the feature in total database data based on the accurate mass and retention time compared to the authentic standards metabolomic standards initiative level 1 identification and level 2 identification also known as putative identification (Creek *et al*., 2014; Sumner *et al*., 2007; Sumner *et al*., 2014). The top 15 most abundant metabolites with relative concentration and abundance rank are shown [table 6.4]. These most abundant metabolites were also reported in the literature and previously isolated from *Cordyceps sp* [table 6.5], suggesting they have been co-purified.

The relative concentration of the identified metabolites in six commercial cordycepin samples shown in the heatmap, the highest concentration of cordycepin was detected in all the samples, followed by dodecanoic acid, which was at the highest concentration in A and F followed by B. E, C and D, the third most abundant metabolite detected was N-(4-Guanidinobutyl)-4-hydroxycinnamide and the fourth was 5'-Deoxyadenosine top 25 most abundant metabolites were selected to be included in the heatmap [figure 6.7].

Table 6.2*:* List of top 15 most abundant metabolites based on relative abundance detected in six cordycepin obtained from Alfa Aesar (A), Carbosynth (B), Goba(C), Merck (D), Phyproof ® reference substance (Reference)(E), Tocris (F). q-Exactive LC-MS was used following XCMS, MzMatch, and IDEOM analysis. n=6. Putative identification of metabolites was obtained by level 1 metabolomics standards initiative using accurate mass, MS/MS fragmentation and predicted retention time with authentic standards and level 2 identification was achieved by predicted retention time without standards and accurate mass. Significantly different metabolites obtained using t-tests with false discovery rate corrections and having VIP values > 1 following OPLS-DA. SIMCA-P v4 software was used to obtain these figures.

Relative abundance rank							
Alfa	**Carbo**	**Goba**	**Merck**	**Ref**	**Tocris**	**Mass**	**Metabolites names**
1	1	1	1	1	1	251.10	Cordycepin
2	2	2	2	2	2	217.20	Dodecnoic acid
3	4	3	3	4	4	251.10	5'-Deoxyadenosine
4	3	4	4	3	3	298.14	N-(4-Guanidinobutyl)-4-hydroxycinnamide
5	NA	NA	NA	NA	NA	416.18	Erioflorin methacrylate
6	5	5	5	5	6	317.29	4-hydroxysphinganine
7	NA	NA	NA	NA	NA	252.09	Deoxyinosine
8	6	6	8	6	5	261.23	2S-Hydroxytetradecanoic acid
9	8	7	6	8	8	462.19	Ala-Met-Asn-Gln
10	9	9	9	7	10	392.28	9'-Carboxy-gama-chromanol
11	11	10	10	10	11	268.08	Inosine
12	NA	NA	NA	NA	NA	311.12	N2-N2-Dimethylguanosine
13	7	8	12	9	7	361.32	[FA hydroxy(20:0)] 11,12-dihydroxy-eicosanoic acid
14	12	11	11	11	13	478.18	Asn-Met-Gln-Ser
NA	NA	12	7	15	NA	266.11	5'-amino-5'-deoxyadenosine
NA	10	NA	13	12	9	280.13	Phaseic acid
NA	14	13	14	14	14	245.24	Tetradecanoic acid
NA	13	NA	NA	NA	12	249.09	S-Acetyldihydrolipoamide
NA	15	14	15	13	NA	329.33	2S-dimethylaminooctadecane-1,3R-diol
15	NA	NA	NA	NA	NA	192.08	Carpacin
NA	NA	NA	NA	NA	15	351.15	O-beta-D-Xylosylzeatin
NA	NA	15	NA	NA	NA	267.10	Adenosine

Table 6.3 Reported metabolites in *Cordyceps sp.* Top 15 most abundant detected metabolites in cordycepin based on relative abundance in six cordycepin Alfa Aesar, Carbosynth , Goba, Merck, Phyproof ® reference substance (Reference), Tocris , see legend table 6.2.

Metabolites	*Cordyceps sp.*	Literature
Cordycepin	*C. militaris*	(Zhibing Wang *et al*., 2013; F. Yang *et al*., 2007; J. Zhang *et al*., 2015)
	C. sinensis	(F.-Q. Guo *et al*., 2006; Shashidhar, Giridhar, Sankar, & Manohar, 2013)
Dodecanoic acid	*C.heteropoda* (Decanoic acid)	(Krasnoff, Reátegui, Wagenaar, Gloer, & Gibson, 2005)
	C. javanica	(Shah *et al*., 2020)
	C. militaris	(X. f. Wu, Zhang, Bhandari, & Li, 2018; F. Yang, Feng, Zhao, & Li, 2009)
	C. sinensis, C. liangshanensis, C. gunnii	(F. Yang *et al*., 2009)
5'-Deoxyadenosine	*OphioCordyceps sinensis,*	(J. Zhang *et al*., 2015)
4-hydroxy-sphinganine	*C. militaris*	(L. Chen *et al*., 2018; Chiu *et al*., 2016)
	C. sinensis	(R. Wu *et al*., 2022)
Deoxyinosine	*C. militaris*	(Y. Liu, Xiao, Wang, Wang, & Xu, 2021)
2S-Hydroxy-tetradecanoic acid	*OphioCordyceps communis*	(Haritakun, Sappan, Suvannakad, Tasanathai, & Isaka, 2010)
Inosine	*C. militaris*	(Gong, Li, Li, Liu, & Wang, 2004; F. Yang & Li, 2008; J. Zhao, Xie, Wang, & Li, 2014)
	C. sinensis	(Gong *et al*., 2004; F. Yang & Li, 2008; L. Yu *et al*., 2006)
	OphioCordyceps sinensis	(J. Zhang *et al*., 2015)
N2-N2-Dimethylguanosine	*C. militaris*	(W. Fan *et al*., 2020; Jiang *et al*., 2011; C. Liu *et al*., 2020)
	C. nidus	(Durán-Aranguren *et al*., 2020)
5'-amino-5'-deoxyadenosine	*C. militaris*	(Qin, Gu, & Guo, 2015)
Tetradecanoic acid	*C. militaris*	(Bian *et al*., 2017; Wen *et al*., 2019; X. f. Wu *et al*., 2018; F. Yang *et al*., 2009)
Adenosine	*C. militaris*	(W. Fan *et al*., 2020; Gong *et al*., 2004; L. Huang *et al*., 2009; Jiang *et al*., 2011; Y. Liu *et al*., 2021; Lu, Zhi, Miyakawa, & Tanokura, 2019; Shih *et al*., 2007; J. Zhao *et al*., 2014)
	C. sinensis	(Gong *et al*., 2004; L. Huang *et al*., 2009; S. P. Li *et al*., 2001; Y.-J. Tsai *et al*., 2010; L. Yu *et al*., 2006)
	C. kyushuensis	(Ling *et al*., 2009)
	C. cicadae	(Olatunji *et al*., 2016; X. Wang *et al*., 2019)

Table 6.4: Reported activity profile of the most abundant metabolites detected in untargeted LC-MS analysis of Alfa Aesar, Carbosynth, Goba, Merck, Phyproof ® reference substance (Reference), Tocris. Top 25 most abundant putative metabolites, n=6, putative metabolites detected bydata obtained by from IDEOM analysis.

.**Putative metabolite**	**Activity**	**Literature**
Cordycepin	Anti-inflammatory Anti-cancerous	(Radhi *et al*., 2021)
Dodecanoic acid	Anti-cancerous activity Plant anti-pathogenic fungi Against inflammatory acne Antimicrobial activity	(Fauser, Matthews, Cummins, & Howarth, 2013; Lappano *et al*., 2017; Miko & Devinsky, 1992; Verma *et al*., 2019; P. Zhang *et al*., 2019) (Walters, Walker, & Walker, 2003) (W.-C. Huang *et al*., 2014; Nakatsuji *et al*., 2009; Simonart, 2012) (Borrelli *et al*., 2021; Dayrit, 2015; Devinsky, Lacko, Mlynarcik, & Krasnec, 1983; Y. G. Kim, Lee, Park, Kim, & Lee, 2021; Nitbani, Siswanta, & Solikhah, 2016)
N-(4-Guanidinobutyl)-4-hydroxycinnamide	Anti-pathogenic (plants) Antimicrobial	(Dobritzsch *et al*., 2016; Muroi *et al*., 2009) (S. Jin, Yoshida, Nakajima, & Murai, 2003)
5'-Deoxyadenosine	Antimicrobial Herbicide	(Namanja-Magliano, Evans, Harijan, Tyler, & Schramm, 2017; Namanja-Magliano, Stratton, & Schramm, 2016) (Rapp *et al*., 2021)
4-hydroxysphinganine	Anti-allergic Protect skin barrier	(Ryu, Lee, Lee, Oh, & Kim, 2010) (Takagi *et al*., 2003)
2S-Hydroxy-tetradecanoic acid	Anti-viral Anti-inflammatory	(Harper, Gilbert, Blunt, & McIlhinney, 1993; M. Perez, Greenwald, & de La Torre, 2004; Tan, Hong, & Chu, 2016) (Nadler, Harrison, Ashendel, Cassady, & Geahlen, 1993)
Inosine	Anti-inflammatory Anti-oxidant	(Roth, Levanon, & Eisenberg, 2019) (Gudkov, Shtarkman, Smirnova, Chernikov, & Bruskov, 2006)
S-Acetyldihydrolipoamide	Pyruvate dehydrogenase complex intermediate product	(Škerlová, Berndtsson, Nolte, Ott, & Stenmark, 2021)

Tetradecanoic acid	Mitogenicity and TNF-inducing activity, with very low pyrogenicity Larvicidal and repellent activity Anti-fungal activity Anti-viral	(KANEGASAKI *et al*., 1986) (Sivakumar, Jebanesan, Govindarajan, & Rajasekar, 2011) (Gajbhiye & Kapadnis, 2021) (Juárez-Rodríguez *et al*., 2021)
9,10-dihydroxy-octadecanoic acid	Anti-bacterial activity	(Silva *et al*., 2018)
Aeruginosin 98-b	Protease inhibitor	(Radau, Schermuly, & Fritsche, 2003)
Hexadecasphinganine	Anti-bacterial Alzheimer's disease	(Nischitha & Shivanna, 2022) (L. Yu *et al*., 2021)
Vomitoxin	Cytotoxic	(Rogers & Héroux-Metcalf, 1983)
Adenosine	Immunoregulatory activity Neuronal activity	(Muller-Haegele, Muller, & Whiteside, 2014) (Brundege & Dunwiddie, 1997)
L-Rhamnulose	Agglutination activity and regulates inflammation	(Mu *et al*., 2022)
Carpacin	Cancer chemo-preventive activity	(Tseng, Tsheng, Lee, & Hsu, 2000)
Erioflorin methacrylate	Insect antifeedant activity	(Tsunaki & Morimoto, 2020)
Bistratamide B	Moderate activity against the human colon tumour	(L. J. Perez & Faulkner, 2003)
2-hydroxy-15-tetracosenoic acid	Anti-inflammatory, analgesic and antimicrobial activity	(Bhalla, Kumar, & Bhatia, 2014)
Euparotin acetate	Tumour inhabitor	(Kupchan, 2013; Kupchan *et al*., 1967)
N2-N2-Dimethyl-guanosine	RNA structure and stability	(Pallan, Kreutz, Bosio, Micura, & Egli, 2008)
Deoxyinosine	Enhances anti-tumour activity	(Ciccolini *et al*., 2001)
Allopurinol	Renal dysfunction Chronic kidney disease progression and cardiovascular risk Gout	(Murrell & Rapeport, 1986) (Goicoechea *et al*., 2010) (Rundles, Metz, & Silberman, 1966)
D-Mycinose	Antibacterial activity	(Iizaka *et al*., 2014)
5'-amino-5'-deoxyadenosine	Anti-proliferative	(Peterson, Oliveira, & Christiansen, 2009)

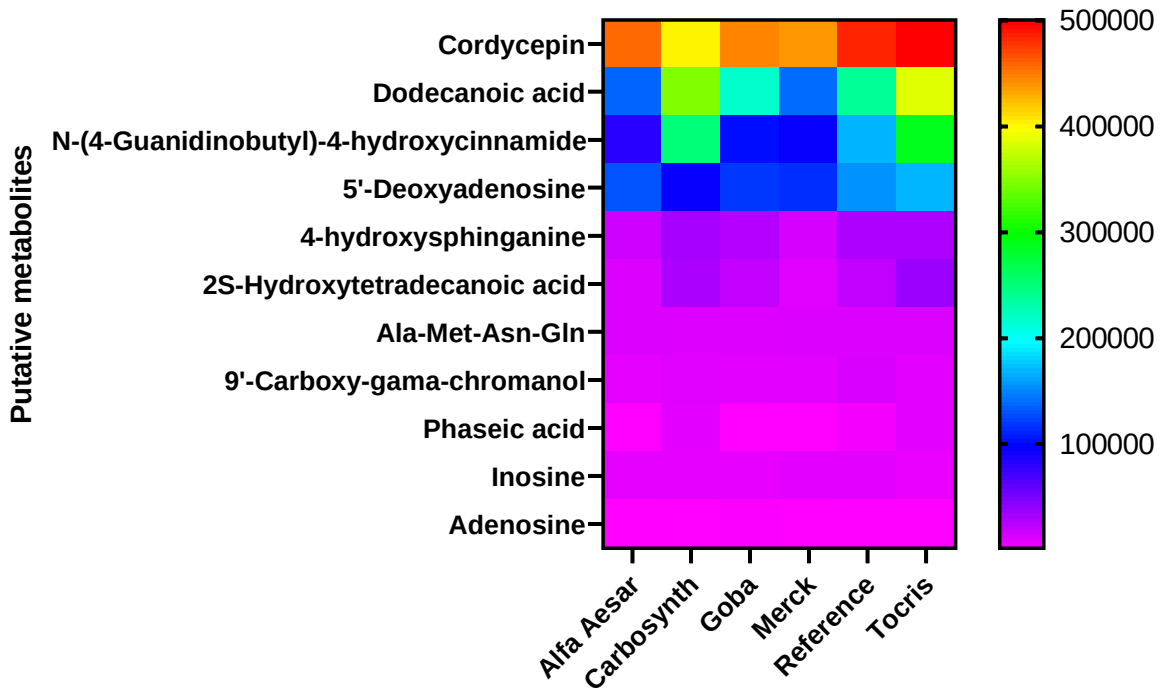


Figure 6.7: Heatmap featuring 11 most abundant putatively identified metabolites in untargeted LC-MS analysis of six cordycepin obtained from Alfa Aesar, Carbosynth, Goba, Merck, Phyproof ® reference substance (Reference), Tocris. Top 11 most abundant metabolites detected in cordycepin. Six LC-MS replicates were used for each sample. The value on the right X-axis denotes that relative metabolites concentration identified. Putative identification of metabolites was obtained by level 1 metabolomics standards initiative using accurate mass, MS/MS fragmentation and predicted retention time with authentic standards and level 2 identification was achieved by predicted retention time without standards and accurate mass. Significantly different metabolites obtained using t-tests with false discovery rate corrections and having VIP values > 1 following OPLS-DA using SIMCA-P v4 and XCMS, MzMatch, and IDEOM analysis of data. Relative concentrations were used to construct a heatmap of the top 11 relative most abundant metabolites detected in each cordycepin using GraphPad Prism v9.

6.6 Cordycepin purity

The targeted analysis of cordycepin; is to find cordycepin purity and metabolites. It was not fully established as no pure or synthetic cordycepin was available on the market. Relative cordycepin purity was calculated relative to Phyproof reference cordycepin by Phytolab with 95% purity claimed by the supplier.

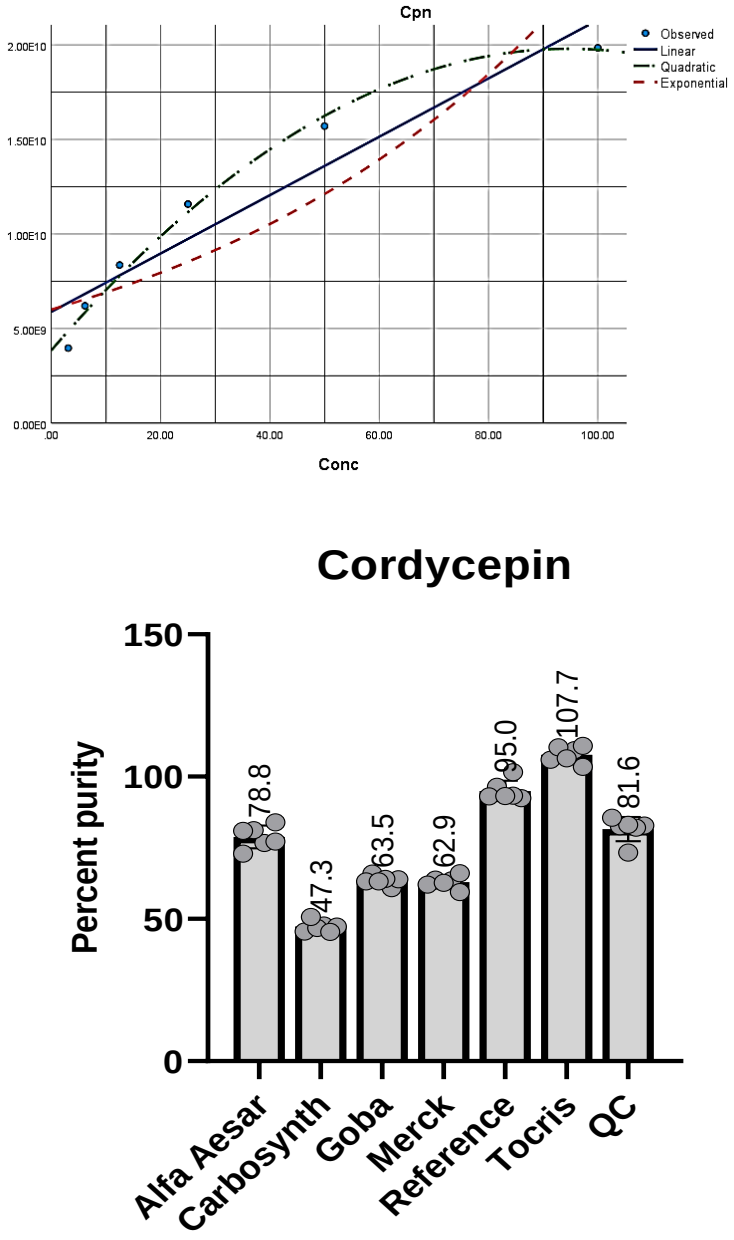


Figure 6.8: Cordycepin purity across six cordycepin samples from different suppliers. Top graph regression line of standard (reference), y axis showing peak area, quadratic fit best with R^2=996, n=6, bottom graph percent purity of cordycepin, data obtained from TraceFinder targeted analysis, GraphPad prime v9 used to generate the graph.

The data obtained after targeted analysis show that the highest purity of cordycepin was detected in Tocris followed by Reference (Phytolab cordycepin), Alpha, Goba, Merck, and the least purity in Carbosynth. The pentostatin concentration show about 1µM across all samples, 3' deoxyinosine was absent in Carbosynth, Merck and Tocris samples, and the detected peak

of all the metabolites tested by targeted analysis in cordycepin samples are included in figure 6.9.

6.7 Metabolites detected in cordycepin

Some of the most abundant and important metabolites were selected and detected in all cordycepin in several analyses and were used as a standard to quantify cordycepin using TraceFinder analysis. The compound used for targeted analysis were cordycepin, 3' deoxyinosine, pentostatin, adenosine, adenine, l-proline, diethylamine and inosine. Some detected peaks with poor baseline separation and symmetry were only used for confirmation of the metabolites and were not used for quantification. The peaks obtained from TraceFinder show that all these metabolites were detected in cordycepin and confirmed with a standard.

These metabolites were also tested for any anti-inflammatory activity using Tnf inflammatory gene in RAW 264.7. The RT-qPCR results are shown in figure 6.10. None of these metabolites showed any anti-inflammatory activity on their own except adenosine, the mixture showed some anti-inflammatory activity, which indicates that they might not have any anti-inflammatory activity on their own but could be having synergistic effects resulting in anti-inflammatory activity.

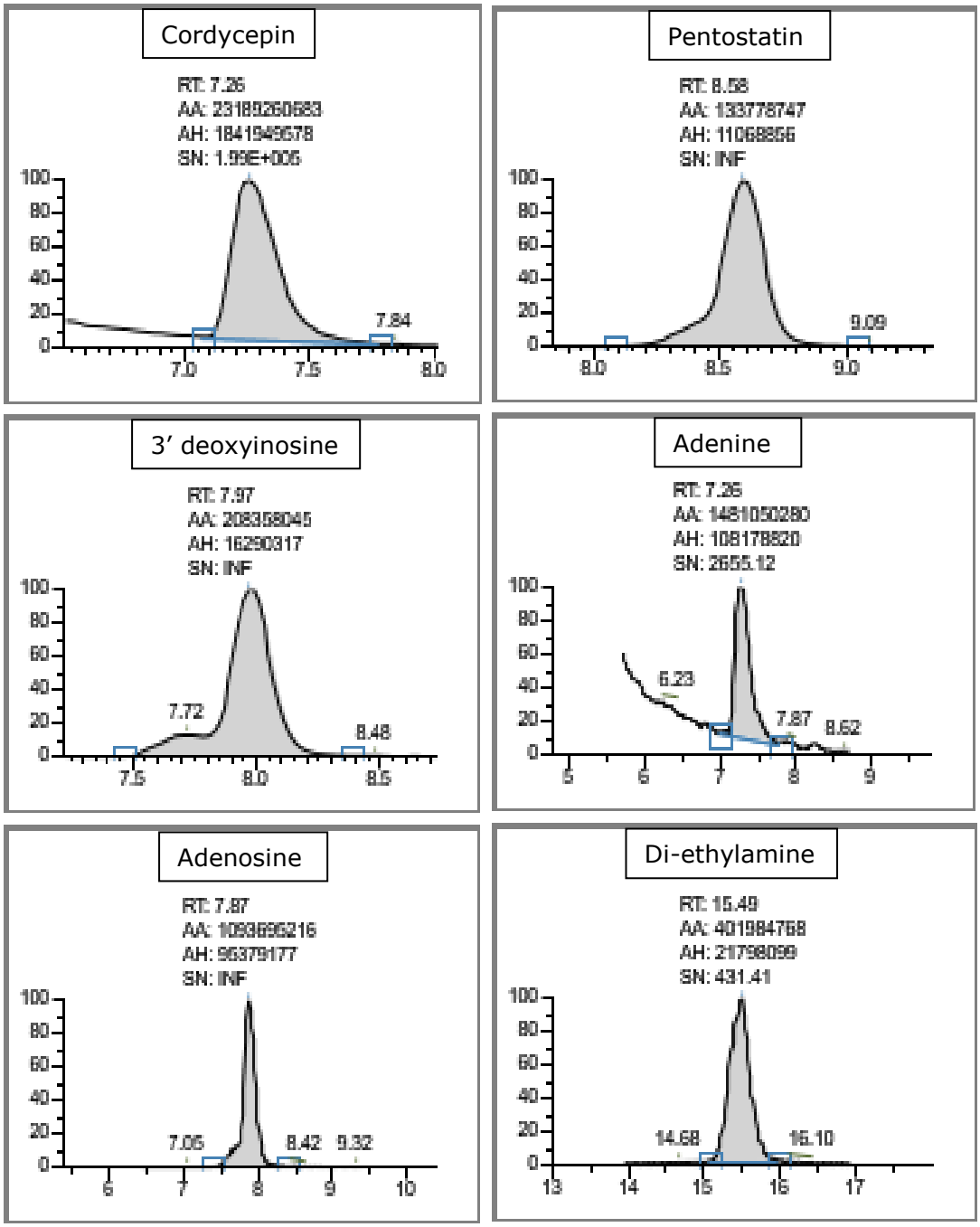


Figure 6.9: Metabolites detected in cordycepin using targeted analysis. Detected peaks in cordycepin based on identified metabolites using authentic standards followed by XCMS, MzMatch. TraceFinder™ software was used to obtain these figures. Putative identification of metabolites was obtained by level 1 metabolomics standards initiative using accurate mass, MS/MS fragmentation and predicted retention time with authentic standards and level 2 identification was achieved by predicted retention time without standards and accurate mass. Significantly different metabolites obtained using t-tests with false discovery rate corrections and having VIP values > 1 following OPLS-DA using SIMCA-P v4

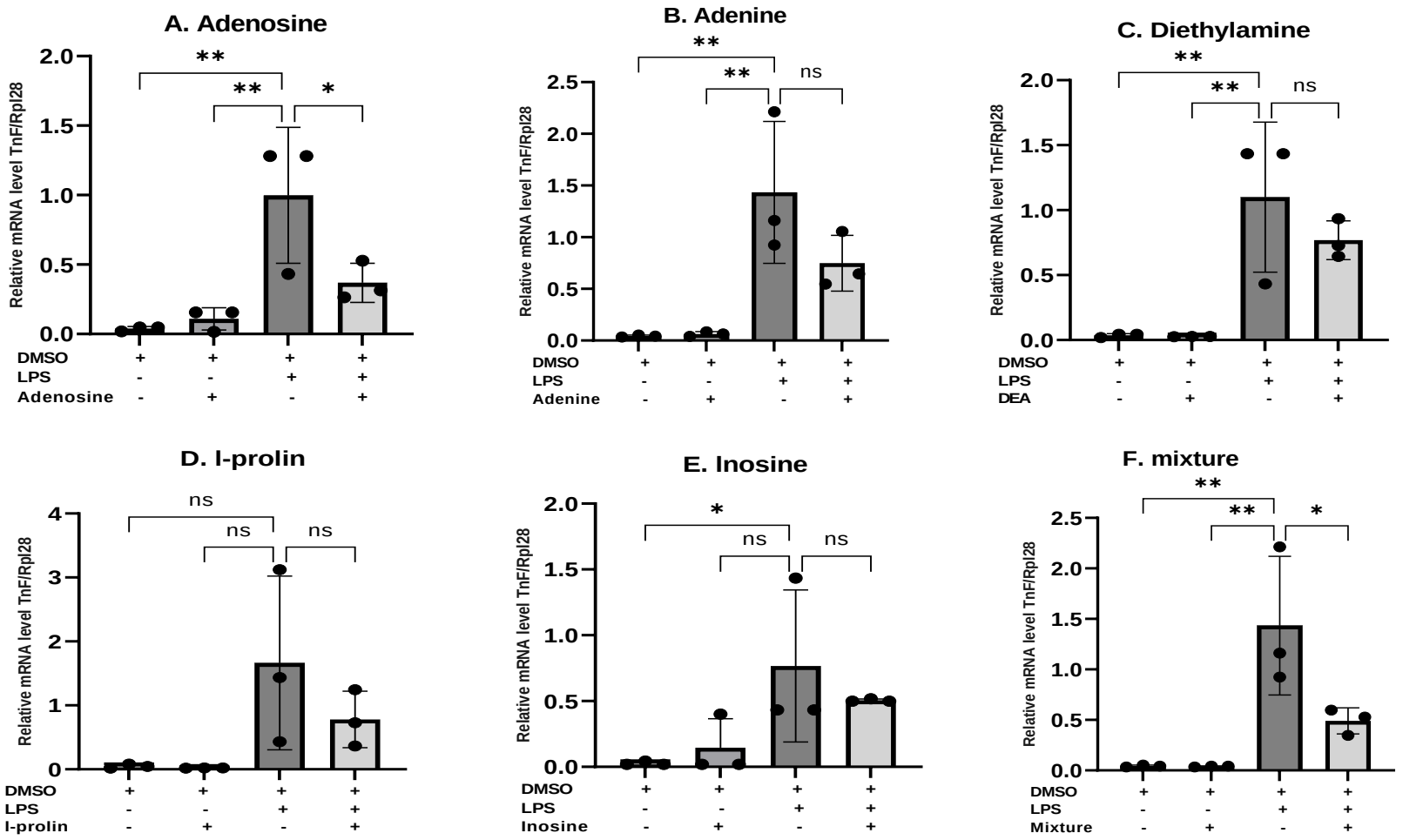


Figure 6.10: Metabolites detected in cordycepin RAW 264.7 cells treated with DMSO or adenosine or adenine or diethylamine or l-proline or inosine or a combination of all (A, B, C, D and E) for an hour before treating with LPS, RT-qPCR was performed, n=3, ****p<0.0001 ***p<0.001 ** p<0.01, * p<0.5, ns p<0.1. P values were obtained by using ordinary one-way ANOVA with multiple comparisons using GraphPad Prism 9.

6.8 Discussion

Commercially isolated cordycepin has been used as a standard in thousands of research studies published on cordycepin (Radhi *et al*., 2021). This study shows the metabolic profile and the anti-inflammatory activity of six preparations of cordycepin, five purchased from different suppliers and one gifted by our collaborator at MycoMedica Goba. The data obtained from LC-MS was analysed for untargeted analysis by IDEOM_v19 and compound discoverer to study all the possible metabolites present in cordycepin (Creek *et al*., 2012).

The anti-inflammatory activity assay indicates that all cordycepin preparations do not have the same activity. RAW 264.7 cell line was used to test the anti-inflammatory activity. The data obtained from the assay were normalised, and the IC50 was calculated; Tocris with IC50 4.930 exhibited the highest inflammatory gene repression, followed by Alfa Aesar, Merck, Phyproof reference substance (Reference), Carbosynth and the lowest was Goba with IC50 11.72 [figure 6.1]. Cells were also treated with cordycepin and pentostatin which resulted in maximum inhibition of inflammatory gene expression even at the lowest concentration of 6.25µM, the figures are included in appendix A at which cordycepin showed not activity when cells treated with only cordycepin, which shows that pentostatin enhances the activity of cordycepin significantly (Xia *et al*., 2017(a)).

The untargeted analysis indicates that cordycepin preparations contain similar contaminants [figures 6.4 and 6.5], indicating they are isolated from similar starting materials and purified in a similar manner. It is puzzling that the contaminants do not show up in the standard purity determination performed by Reach Separations [figure 6.3], but the fact that the contaminants are commonly found in *Cordyceps* extracts, and show up in experiments conducted at different times suggests the samples were not contaminated in the laboratory.

Of the 25 most abundant contaminants, most of the metabolites have known biological activity shown in table 6.5, of which dodecanoic acid, inosine, 2S-Hydroxytetradecanoic acid, L-Rhamnulose, and 2-hydroxy-15-tetracosenoic acid are anti-inflammatory. This suggests that

the contaminants of cordycepin may contribute to its activity. Indeed, adenosine has shown anti-inflammatory activity in the bioassay [figure 6.10]. Contamination with pentostatin may potentiate cordycepin. The concentration is very low (about 1µM), but it is much more potent than cordycepin. The dose is certainly not optimal for stabilisation, as further addition of pentostatin increases the effect of cordycepin [figure 6.9].

The common and most abundant contaminants adenosine, adenine, di-ethylamine l-proline and inosine metabolites were used as an authentic standard for targeted analysis, all were detected, but the quantification could not be completed due to the very low concentration detected in cordycepin. The cordycepin concentration detected in each cordycepin sample shows a different concentration of cordycepin [figure 6.8]. Different preparations of cordycepin have remarkably similar contaminants, which means they are probably all grown and isolated in similar ways. This means that there may be common contaminants of cordycepin that contribute to its activity. The number of metabolites detected in the isolated cordycepin shows that these metabolites are found naturally in the sample. All these samples lack purity due to the isolation of other metabolites besides cordycepin. As HPLC is used for the purity determination method of isolated cordycepin due to its availability, easy to use and economical but due to its limitation of low sensitivity it could not detect all the metabolites present in the sample (Ling et al., 2009; Zhou, Cai, He, & Tong, 2016). It is vital for the development of the cordycepin to test preparations that are purer and/ or with entirely different contaminants (e.g. from a different source or chemically synthesised).

CHAPTER 7
DISCUSSION AND CONCLUSION

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In this project, the metabolic profile of *Cordyceps militaris* and cordycepin was investigated in light of anti-inflammatory activity in RAW 264.7 cells. I hypothesised that many secondary metabolites from *Cordyceps* fungi will have evolved to target the insect immune system. Some of these secondary metabolites are known synergistically, and the potential problem is that *Cordyceps militaris* also contain inflammatory compounds that are usually removed by digestion. The second hypothesis was that cordycepin activity is different between various suppliers due to the variation in the metabolic profile.

7.1 Standardisation of *Cordyceps militaris* extract.

In chapter 3, the aim was to obtain an extract which can be used orally with proven efficacy and safety. The anti-inflammatory activity and the effect of solvents on the metabolic profile and the concentration of metabolites of interest such as cordycepin, pentostatin and 3' deoxyinosine in the extract were studied. The anti-inflammatory activity has been reported in numerous studies (Radhi *et al*., 2021). Chapter 3 results suggest that the anti-inflammatory activity of the extract varies depending on the solvent used, and parameters such as heating, sonication and maceration time also affect the anti-inflammatory activity of the extract (H.-J. Wang *et al*., 2014). These studies only focused on the cordycepin yield in the extract, but other essential metabolites, which can affect the activity of cordycepin significantly were ignored, such as pentostatin and 3' deoxyinosine. By using these parameters, the LC-MS studies show that sonication, heating and extended maceration time have an adverse effect on the pentostatin concentration in the extract. These extracts also showed a decreased anti-inflammatory activity in RAW 264.7 cells. The solvent selection is also an essential factor affecting the extract efficacy. A study by Wang *et al.,* 2014, the best combination of ethanol and water with the highest capacity of cordycepin extraction. Ethanol 60% extracted the highest concentration of cordycepin with sonication, heat and extended maceration time but effects on pentostatin were not studied, which is an imported metabolite co-produced with

cordycepin in *C. militaris* (H.-J. Wang *et al*., 2014). Sonication, heat and extended maceration affect pentostatin adversely and pentostatin levels decreased to undetectable levels. The extract was further studied in cell lines and observed that ethanol exhibited lower anti-inflammatory activity than water and ethanol at 60%, but the water exhibited the highest inflammatory activity due to which the best solvent was ethanol and water combination at 60%, with the highest anti-inflammatory activity and lowest inflammatory response.

After the extract standardisation, the anti-inflammatory activity and metabolic profile of the extracts were studied in chapter 4; the extract metabolomic study shows high cordycepin concentration in comparison to 3' deoxyinosine and pentostatin. The same sample cannot be used for the quantification of all these metabolites, a diluted sample is required for cordycepin, while a more concentrated sample is needed for pentostatin and 3' deoxyinosine quantification. Several batches of *Cordyceps militaris* extract (MycoMedica) and commercial products were compared in order to find the concentration of metabolites of interest (cordycepin, pentostatin and 3' deoxyinosine). The MycoMedica preparations varied somewhat but were generally quite similar, showing it is possible to produce a consistent product, but the Aloha preparation showed that there are some quite poor products on the market. The anti-inflammatory activity in comparison to extract was studies showing that an equivalent concentration of cordycepin in the extract (quantified by LC-MS) and to that of purified cordycepin did not result in the same anti-inflammatory activity, which indicates that there could be other metabolites acting synergistically or having their anti-inflammatory activity.

The insight gained from the successful experiments described earlier, there were also challenges and experiments yielding negative results. Efforts were made to standardise *Cordyceps militaris* extract and quantify the purity of cordycepin. These efforts involve the selection of solvent and parameter in order to achieve anti-inflammatory activity of the extract and economical for scaling up for commercial use. An extract with good anti-inflammatory

activity was achieved, and cordycepin, 3' deoxyinosine and pentostatin are some of the metabolites which could be used for the standardisation and quality control of the extracts.

7.2 Cordycepin purity

The commercial cordycepin is claimed to be 95 % to 99.5 % purity according to the monograph of the supplier, but when the anti-inflammatory activity was test showed that there are differences in activity. Six different cordycepin were tested to assess their purity and activity of cordycepin. The highest anti-inflammatory activity was exhibited by Tocris, followed by Alfa Aesar, Merck, Carbosynth, Phyproof® reference and Goba. The metabolic profile was investigated using untargeted analysis and targeted analysis. Numerous metabolites were detected, as shown in figures 6.4 and 6.5; all the cordycepin have a similar metabolic profile but are distinct from each other. Cordycepin was the most abundant metabolite, but other metabolites with significant concentrations were also detected. These metabolites are isolated beside cordycepin and are naturally found in the *Cordyceps sp*, starting material of cordycepin isolation. These metabolites could be affecting the activity even at a lower concentration, acting synergistically or decreasing the activity of cordycepin. Several available metabolites detected in these samples were ordered and tested in targeted analysis in which pentostatin, 3' deoxyinosine, adenosine, adenine and di-ethylamine were detected except L-proline. These metabolites could not be quantified due to very low concentration. The highest concentration of cordycepin was detected in Tocris, which also showed the highest anti-inflammatory activity in RAW 264.7 cells followed by Alfa aesar but cordycepin concentration was lower than reference which indicates that other metabolites might be affecting its activity [figure 6.1 and 6.7]. Adenosine, adenine and diethyl-amine, inosine and L-proline were investigated for anti-inflammatory and inflammatory activity in RAW 264.7 cells using RT-qPCR, and it was found that only adenosine exhibits anti-inflammatory activity in this assay [figure 6.10]. The presence of cordycepin definitely could affect the potency of cordycepin preparations in tissue culture, but the degradation of pentostatin by stomach acid means it will not have major effects upon

oral administration. As many animal studies show oral activity, not all properties of cordycepin can be dependent on pentostatin contamination.

It is puzzling that such different results on purity were obtained with a less powerful but similar instrument by Reach Separations. We lent the company our chromatographic column, but this did not make the data much more alike. Reach Separations system is very similar to the industry standard but appears to have failed in this case. This raises a worry about the quality control of many other drug preparations and not just those of natural compounds but compounds in general. It seems urgent that this discrepancy is addressed in the near future, to reassess how drug purity and composition of extracts are determined.

7.3 Future work and conclusion

Cordycepin is isolated by resin binding of liquid culture medium from *Cordyceps militaris*, followed by crystallisation (MycoMedica, personal communication). It will be important to improve the purity of cordycepin, either by further purification, e.g. by HPLC or by synthesising the compound chemically. Further testing of the biological activity of the contaminants of cordycepin, alone or in combination, may be desirable if a purer compound is shown to have reduced activity.

In conclusion, this study has provided insight into the biological efficacy of *Cordyceps militaris* extract in inflammation and the compounds of interest for standardisation and quality control in determining the variation between batches and products. Generally, batches of *Cordyceps militaris* mycelium produced by MycoMedica and cordycepin preparations from different suppliers are quite similar, suggesting that it will be possible to establish equivalency criteria and test them as potential medicines for inflammatory diseases.

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APPENDICES

Appendix A (Chapter 6)

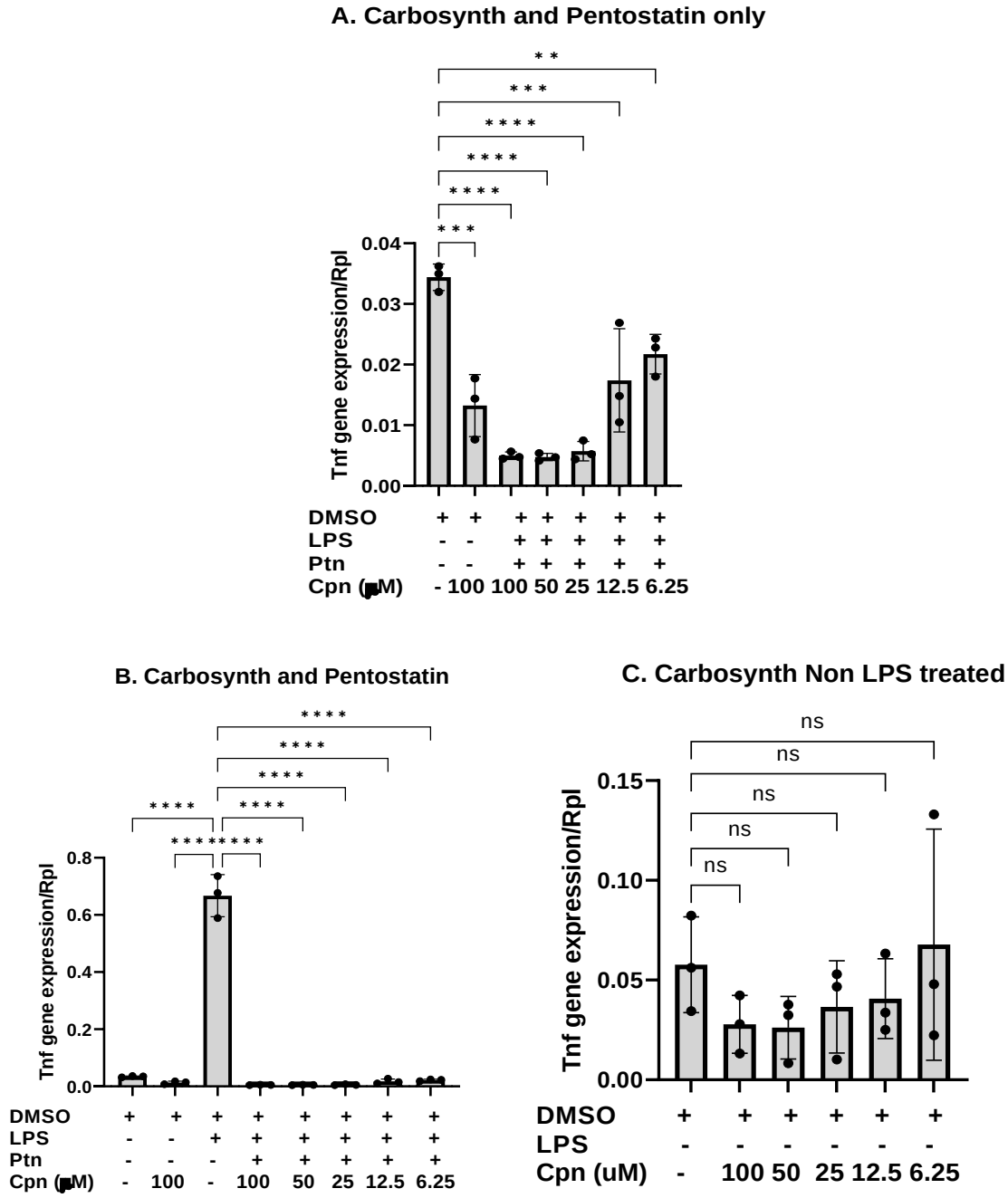
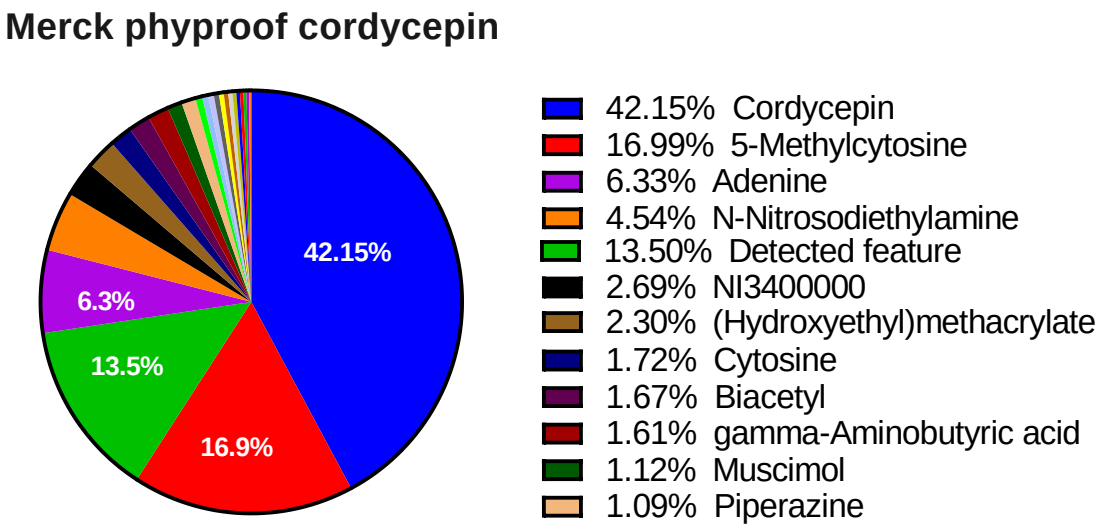


Figure: Anti-inflammatory activity of cordycepin and cordycepin obtained from Carbosynth, RAW 264.7 cells treated with DMSO or cordycepin and pentostatin for an hour with serial dilution of 100µM, 50µM, 25µM, 12.5µM, and 6.25µM before treatment with LPS followed by RNA extraction, reverse transcription and RT-qPCR, Tnf inflammatory gene and Rpl 28 reference gene was used, each point representing three RT-qPCR replicates. Data was normalised with cordycepin and DMSO sample, log IC50 was calculated by GraphPad Prism v9.

Appendix B (Chapter 6)

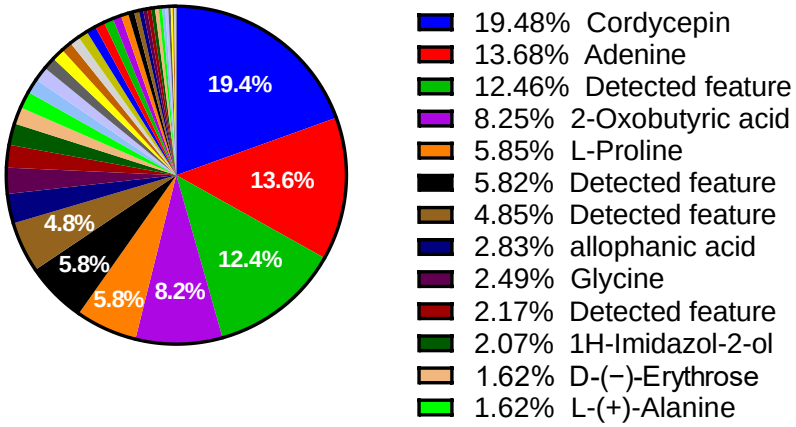


PhytoLab Phyproof cordycepin (LC-MS/MS

Metabolites	Peak Area
Cordycepin	2.487582873e+009
5-Methylcytosine	1.00260486e+009
Detected metabolites	7.96431459e+008
Adenine	3.73815651e+008
N-Nitrosodiethylamine	2.67937512e+008
NI3400000	1.58931325e+008
(Hydroxyethyl)methacrylate	1.35509859e+008
Cytosine	1.01379357e+008
Biacetyl	9.8793193e+007
gamma-Aminobutyric acid	9.5228486e+007
Muscimol	6.6244114e+007
Piperazine	6.4580497e+007
Ethyl acetate	3.0485559e+007
1-nonanoic acid	2.7598096e+007
Furaneol	2.5422213e+007
Sulfuric acid	2.3470366e+007
Vigabatrin	2.1686093e+007
laurilsulfate	1.997774e+007
n-heptanoic acid	1.8985454e+007
3-Methylbutanoic acid	1.7774396e+007
DL-Lactic Acid	1.6156173e+007
Benzoic acid	1.556372e+007
1,2-Cyclohexanediol	1.3464672e+007
Diethyl malonate	1.2094941e+007
Glutaric acid	9897214

Figure: Detected metabolites in Merck Phyproof cordycepin (PhtoLab), putatively identified metabolites in untargeted LC-MS analysis using compound discoverer Six LC-MS replicates were used. Putative identification of metabolites were obtained by level 1 metabolomics standards initiative using accurate mass, MS/MS fragmentation and predicted retention time with authentic standards and level 2 identification was achieved by predicted retention time without standards and accurate mass, the figure was constructed in GraphPad Prism v9.

Goba cordycepin



Goba cordycepin (LC-MS/MS)

Metabolites	Peak area
Cordycepin	8.528315573e+008
Adenine	5.990485595e+008
Detected feature	5.456405186e+008
2-Oxobutyric acid	3.6133789e+008
L-Proline	2.562236974e+008
Detected feature	2.548908816e+008
Detected feature	2.12587743e+008
allophanic acid	1.238428106e+008
Glycine	1.090759886e+008
Detected feature	9.491496818e+007
1H-Imidazol-2-ol	9.048991672e+007
D-(−)-Erythrose	7.100351276e+007
L-(+)-Alanine	7.099522841e+007
Aceturic acid	6.069674564e+007
Carbamoylphosphate	5.991555156e+007
azidamfenicol	5.197556571e+007
Hexitol	5.070337126e+007
Tricine	4.411093507e+007
Succinic acid	4.12316888e+007
L-Pyroglutamic acid	4.067877065e+007
gamma-Aminobutyric acid	4.019327179e+007
Detected feature	4.016296023e+007
Xylitol	3.962665629e+007
Tris(hydroxymethyl)aminomethane	3.532631684e+007
N-Methylpyrrolidone	3.034026344e+007
Glutaric acid	2.519155576e+007
Piperazine	2.035450334e+007
Pyrazolidine	1.774383078e+007
2-morpholinoacetic acid	1.677201791e+007
L-(-)-Threonine	1.653724407e+007
Detected feature	1.585828684e+007
lactide	1.561444123e+007
NPYR	1.399552356e+007
oxazolidinone	1.331723334e+007
Hydantoin	1.243272861e+007
Biacetyl	9814870.986
Benzoic acid	8217723.963
(Hydroxyethyl)methacrylate	6097947.791
2-methylcitric acid	5594273.574
Ethyl acetate	3437631.686

Figure: Detected metabolites in Giba cordycepin (Mycomedica), putatively identified metabolites in untargeted LC-MS analysis using compound discoverer Six LC-MS replicates were used. Putative identification of metabolites were obtained by level 1 metabolomics standards initiative using accurate mass, MS/MS fragmentation and predicted retention time with authentic standards and level 2 identification was achieved by predicted retention time without standards and accurate mass, the figure was constructed in GraphPad Prism v9.

Appendix C (Chapter 6)

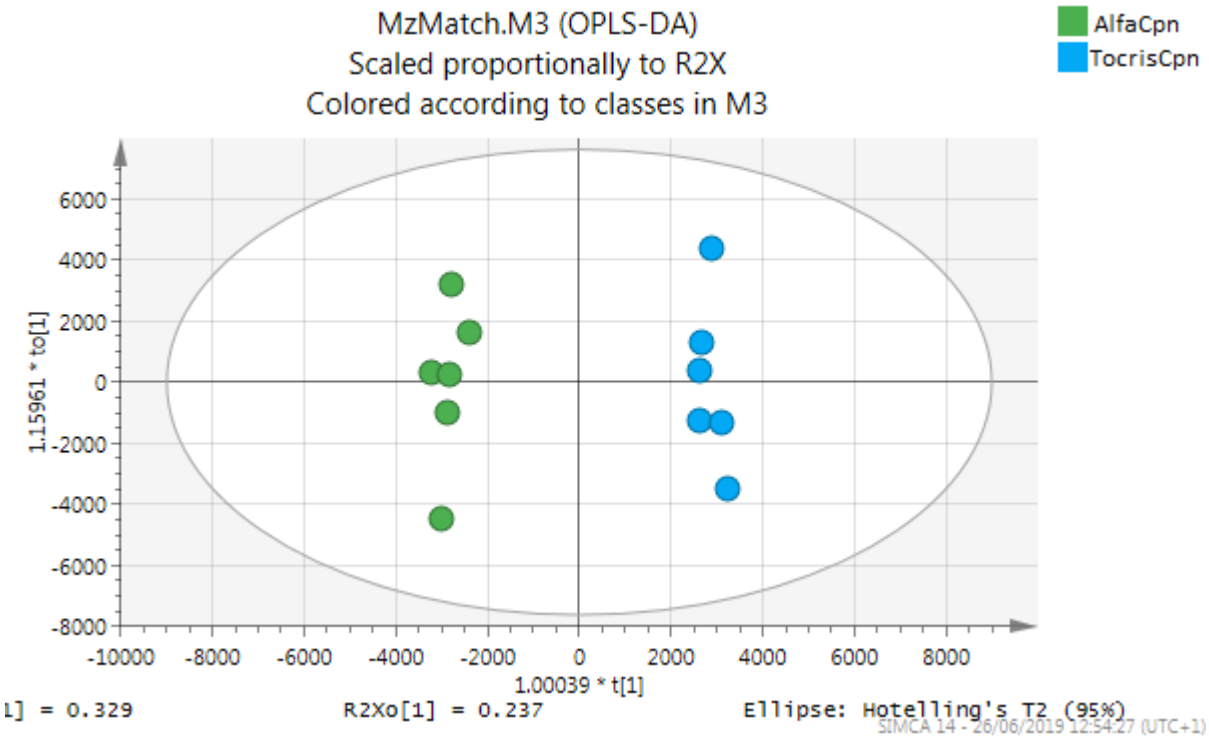


Figure: Multivariate analysis score plots of Alfa aesar and Tocris cordycepin by untargeted LC-MS, orthogonal partial least squares-discriminant analysis (OPLS-DA) plots. n=6, multivariate analyses are based on relative concentrations of all putatively identified and identified metabolites followed by XCMS, MzMatch, and IDEOM analysis of data. Putative identification of metabolites were obtained by level 1 metabolomics standards initiative using accurate mass, MS/MS fragmentation and predicted retention time with authentic standards and level 2 identification was achieved by predicted retention time without standards and accurate mass. Significantly different metabolites obtained using t-tests with false discovery rate corrections and having VIP values > 1 following OPLS-DA. SIMCA-P v4 software was used to obtain these figures.