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Identification and Determination of Secondary Metabolites and Amino Acids in Cordyceps

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Abstract - The Cordyceps are used around the world due to its high medical and pharmaceutical value. Advanced biotechnology has revealed that Cordyceps sinensis has a wide range of secondary metabolites and amino acids. The secondary metabolites and amino acids are very important for the prevention and cure of diseases in human. The purpose of this study was to identify and determine of secondary metabolites and amino acids in Cordyceps. The secondary metabolites were qualitatively determined by gas chromatography mass spectrometer (GCMS). And then, the amino acids were determined by high performance liquid chromatography with fluorescence detection (HPLC-FLD). The secondary metabolites in Cordyceps were found to be cordycepin, octadecanoic acid, ethyl ester, hexadecanoic acid, linoleic acid, d-Mannitol, vitamin A, ecosanoic acid, cholesta, L-lysine, L-Glucose, l-Methionine, oxazolidine, phenylquinoline, adenosine, L-lysine, heptasiloxane, oleic acid and propionic acid respectively. The amino acid concentrations in Cordyceps samples were found to range from 127.43±1.74 to 700.23±1.48 µg/g for tyrosine, 2.014±1.01 to 12.56±1.29 µg/g for valine, 8.57±0.69 to 14.35±1.17 µg/g for iso-leucine and 9.35±0.81 to 18.47±0.88 µg/g for leucine, respectively. Cordyceps contain mainly active compound of cordycepin that have many health benefits and improve immunity on human health. Moreover, other secondary metabolites are mostly present in Cordyceps. The most abundant amino acid is tyrosine and leucine in Cordyceps. Therefore, the regular consumption of Cordyceps can help for human health such as improvement of cardiac functions, lung functions, sexual dysfunctions and immunity.

Keywords: Amino acids, Cordyceps, GCMS, HPLC-FLD, secondary metabolites

1.INTRODUCTION

The Cordyceps contain in the family of Clavicipitaceae and the class of Pyrenomycetes in the order Hypocreales of the ascomycetous fungi[1]. The varieties of bioactive compounds are found in Cordyceps. These compounds from Cordyceps can modulate immune responses, inhibit of tumor cell growth, promote hepatic energy and enhance the secretion of adrenal hormones, suppress of the viral replication and it has hypotensive and vasorelaxant actions[2,3,4,5,6].

The Cordyceps contains 18 of the essential amino acids that tend to be quite high (5-10%). The highest amino acids are tyrosine, arginine, glutamate, tryptophan and aspartic acid. Moreover, the vitamins in Cordyceps are vitamins E, K, B1, B2 and B12. The Cordyceps contains not only various monosaccharide, disaccharide and oligosaccharide sugars but also cordycepin, sterols, proteins, nucleosides, wide range of minerals such as potassium, sodium, calcium, magnesium, iron, copper, manganese and zinc[7,8].

The Cordyceps is famous as herbal medicine for increasing physical stamina. Cordyceps is used in the

treatment of immune disorder, kidney, impotence, chronic obstructive pulmonary disease (COPD) and asthma. Cordyceps can increase the cellular bio-energy ATP and therefore, that can cause not only improve internal balance, but also more efficient oxygen utilization in the body. These properties can obtain the extra endurance, anti-fatigue and physical enhancement. The Cordyceps widely used for boosting weaker immune systems and also used as an adjunct to the modern cancer treatments of chemotherapy, surgery and radiation9. Moreover, Cordyceps is very effective for the treatment of liver disease patients, such as hepatitis B, hepatitis C, hepatitis A and liver fibrosis[8].

The secondary metabolites are essential constituents for plant survival and however, they also play an important role in promoting of human health. Humans can obtain many marvelous health benefits from consuming secondary metabolites rich plants. Moreover, the secondary metabolites responsible not only play a role in plant defense, but also support human health. The secondary metabolites and plant compounds are playing an important role in health promotion, disease prevention and aesthetic properties[10]

The amino acid such as leucine, iso-leucine and valine are nutritionally essential and moreover, they cannot be synthesized endogenously by humans. Therefore, they can obtain by the supplement of diet and they are the liver lacks enzymes necessary for their catabolism. In addition, these amino acids are important for structural components of proteins and regulatory control of protein metabolism11. The tyrosine is also amino acid produce from phenylalanine in the body. Tyrosine supplement can increase the important brain chemicals and it affects human mood and stress response[12].

In Myanmar, the Cordyceps are found in Puta-O Township, Kachin State. The Cordyceps has a long history of use in Myanmar people for tonic and various medicinal purposes. In modern medical research, it is necessary to test and understand why and how these natural medicines work. Moreover, Cordyceps may have the variety of secondary metabolites and amino acids (leucine, isoleucine, valine and tyrosine) that required for human health. Therefore, the present study identified and determined the secondary metabolites and amino acids in Cordyceps in Myanmar.

2.OBJECTIVES

2.1 General objective

To identify and determine of the secondary metabolites and amino acids in Cordyceps.

2.2 Specific objectives

1. To identify the secondary metabolites of Cordyceps by Gas Chromatography Mass Spectrometry (GCMS).

2. To determine the tyrosine, valine, iso-leucine and leucine by High Performance Liquid Chromatography with Fluorescence Detection (HPLC-FLD) method.

3. RESEARCH METHODOLOGY

Laboratory based descriptive study was used. The Cordyceps samples were collected from producers and retail shops in Puta-O and Pyinmana Township. This study was done at Department of Pharmacology Research, DSMRC, Naypyitaw. The study period was from January 2022 to May 2022. Three different

Cordyceps samples were studied. And, the convenience sampling method was used. The inclusion criteria was all samples having good storage condition, good appearance and not expired.The exclusion criteria was any samples that have not good storage condition and good appearance and package damage were excluded.

3.1 MATERIALS AND METHODS

3.1.1 Chemicals and apparatus

The chemicals used during this study were reference standards of amino acids (tyrosine, valine, iso-leucine and leucine), acetone, isopropyl alcohol, acetonitrile (99.99%), methanol (99.99%), de-ionized water and apparatus were such as Whatman filter paper, conical flask, syringe filter (0.22 µm), volumetric flask, beakers, oven, GCMS and HPLC.

3.1.2 METHODS

3.1.2.1 Qualitative determination of secondary metabolites in Cordyceps by Gas Chromatography Mass Spectrometry (GCMS)

The 100 g of Cordyceps powder was added to extraction flask and then 500 mL of ethanol (99.99 %) was added. Then, the extraction flask was shaken on the orbital shaker at 150 rpm for 6 hours and it stand for 18 hours at in a dark place. The shaking and standing process was done for 4 days. After 4 days, the mixture of Cordyceps powder and ethanol (99.99 %) was filtered with Whatman filter paper. The filtrate solution was concentrated on rotatory evaporator and the concentrated extract was dried on petri dish at room temperature. The 1 g of dry and sticky Cordyceps extract was added into a test tube containing the 10 mL of chloroform and methanol (9:1, v/v) mixture. Then, the test tube was shaken on vortex mixer and then, this solution was filtered by syringe filter (0.22 µm). Finally, the filtrate solution was injected into the GCMS system that already optimized method for Cordyceps.

3.1.2.2 Determination of tyrosine, valine, iso-leucine and leucine in Cordyceps by High Performance Liquid Chromatography (HPLC)

3.1.2.2.1. O-Phthalaldehyde (OPA) solution preparation

The O-phthaladehyde (OPA) derivatization reagent solution was prepared in a 50 mL amber bottle by dissolving 50 mg of O-phthaladehyde (OPA) powder in 1 mL methanol. And then, made up to mark (5 mL) with 400 mM borate buffer (pH 10.2). Then, added 125 µL of 2-mercaptoethanol into this solution and left 6 hours at 4°C in the refrigerator before using it.

3.1.2.2.2 Standard solutions preparation

Standard solutions (100 µg/mL) of amino acids (tyrosine, valine, iso-leucine and leucine) were prepared from their stock solutions to desired concentrations by the serial dilution process. For fluorescence derivatization, the 10 µL of O-phthaladehyde (OPA) solution was added into each standard solution.

3.1.2.2.3 Calibration curves preparation

The calibration curves were prepared peak areas versus difference concentration of the amino acid standards (tyrosine, valine, iso-leucine, leucine) were plotted. The concentrations of amino acids (tyrosine, valine, iso-leucine, leucine) from Cordyceps samples were calculated by using the regression equation of the best line of fit.

3.1.2.2.4 Sample preparation for amino acid analysis

Firstly, weighted 200 mg of Cordyceps sample powder and then added 2 mL of methanol: water (50:50, % v/v) solution. Then, this Cordyceps sample was shaken 3 min on vortex mixer and then placed on centrifuge with 10,000 rpm for 5 minutes. And then, the supernatant was collected and added 50 µL of O-phthaladehyde (OPA) solution. Finally, this solution was filtered with syringe filter (0.45 µm) and the filtrate solution was injected into High Performance Liquid Chromatography with fluorescence detection (HPLC-FLD, Shimadzu).

3.2 Ethical consideration

Ethical approval was obtained from the Institutional Review Board (IRB) of Defence Services Medical Research Centre (DSMRC).

4. RESULTS

The most containing secondary metabolites in the Cordyceps were identified by Gas Chromatography Mass Spectrometry (GCMS) as shown in Table 1.

Table -1: Molecular identification of secondary metabolites in Cordyceps by Gas Chromatography Mass Spectrometry (GCMS)

Compound Name	Molecular Structure	Molecular weight	Base Peak (m/z)	Pharmacological Activity
Octadecanoic acid	$C_{19}H_{36}O_3$	312	55	Antimicrobial
Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284	88	Antimicrobial
Linoleic acid, ethyl esterd	$C_{20}H_{36}O_2$	308	67	Antimicrobial
d-Mannitol	$C_{16}H_{34}O_7S$	370	207	Immune-support
Vitamin A	$C_{36}H_{60}O_2$	524	73	Vitamin Supplement
Ecosanoic acid	$C_{20}H_{40}O_2$	312	57	Antimicrobial
Cordycepin	$C_{10}H_{13}N_5O_3$	251	49	Help for Heart Health
Cholesta	$C_{27}H_{44}$	368	105	Lipid lowering
L-lysine	$C_{18}H_{46}N_2O$	450	148	Amino acid supplement

L-Glucose	$C_6H_{12}O_6$	180	73	Glucose supplement
I-Methionine	$C_{16}H_{34}O_7S$	370	207	Amino acid supplement
Oxazolidine	$C_9H_{11}NO$	149	149	Antimicrobial
Phenylquinoline	$C_{16}H_{11}NO$	233	232	Antimicrobial
Adenosine	$C_{19}H_{19}N_7O$	441	60	Immunomodulation
L-lysine	$C_{18}H_{46}N_2O$	450	148	Amino acid supplement
Heptasiloxane	$C_{16}H_{48}O_6Si$	532	74	Antioxidant
Oleic acid	$C_{18}H_{34}O_2$	282	55	Antioxidant
Propionic acid	$C_8H_{18}O_2Si$	174	75	Antioxidant

The amino acid standards (100 µg/mL) of tyrosine, valine, iso-leucine and leucine were diluted by five serial dilutions with methanol and derivatized with OPA solution. Then, these standard solutions were filtered by syringe filter (0.45 µm) and then injected into the HPLC-FLD system. The obtain chromatograms is shown in the following figure,

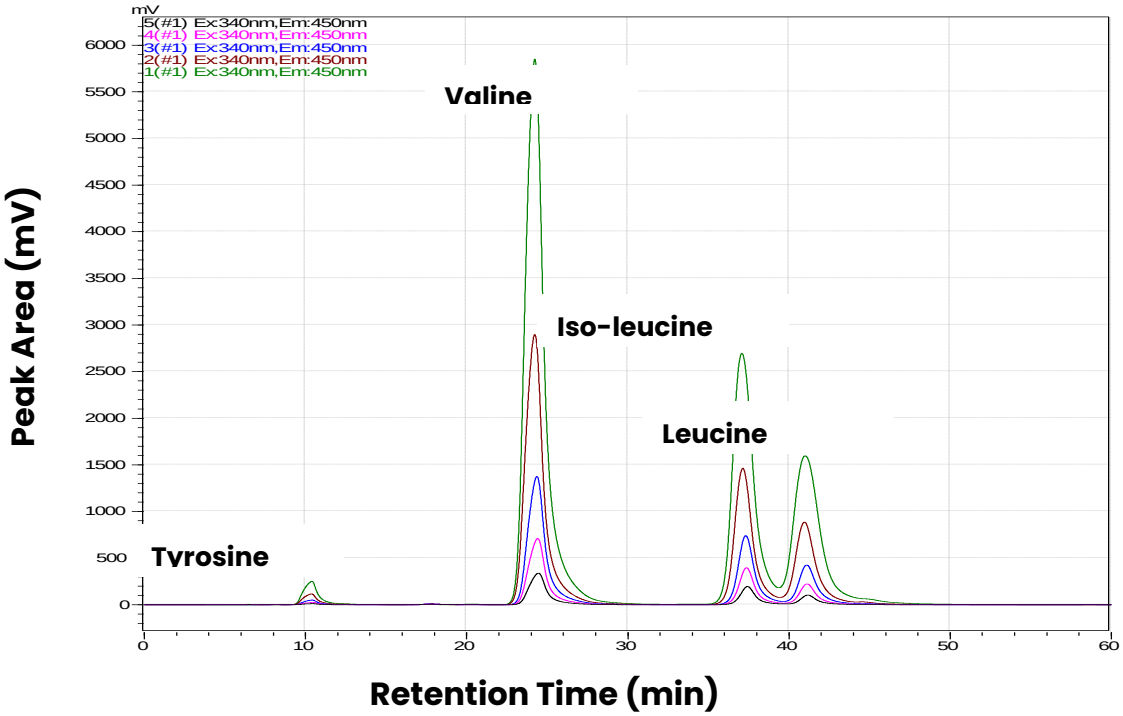

Fig -1: Chromatograms of amino acids overlapping with difference standard concentrations

The obtained High Performance Liquid Chromatography (HPLC) chromatogram of amino acids was shown in the following figure,

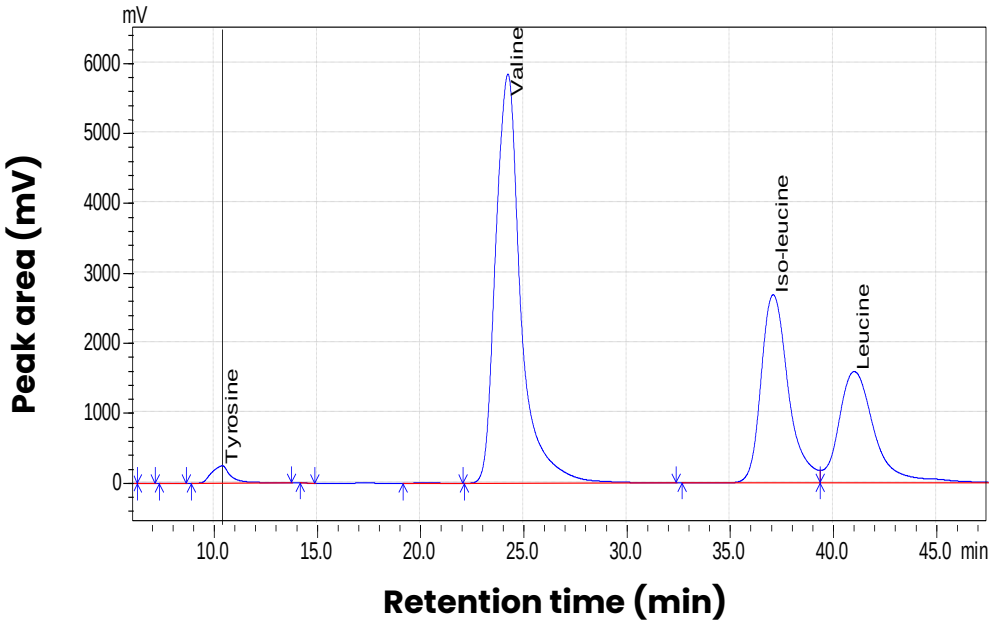

Fig -1: HPLC Chromatograms of Tyrosine, Valine, Isoleucine and Leucine in Cordyceps

Table -2: The amino acids (tyrosine, valine, isoleucine and leucine) concentrations in Cordyceps samples

Cordyceps Sample	Tyrosine µg/g Mean±SD n=3	Valine µg/g Mean±SD n=3	Iso-leucine µg/g Mean±SD n=3	Leucine µg/g Mean±SD n=3
C-1	700.23±1.48	12.56±1.29	10.68±0.74	10.61±1.12
C-2	127.43±1.74	2.014±1.01	8.57±0.69	9.35±0.81
C-3	326.89±2.01	10.88±1.04	14.35±1.17	18.47±0.88

5. DISCUSSION

The secondary metabolites are very important for the prevention and cure diseases on human health. The Cordyceps contain the variety of bioactive compounds and have the difference medicinal and pharmaceutical activities according to the other studies. Moreover, the Cordyceps is famous as a tonic and immune-stimulant medicinal herb. However, the scientific researches of Cordyceps related to bioactive compounds and secondary metabolites are still limited.

In this study, the secondary metabolites in Cordyceps were evaluated by Gas Chromatography Mass Spectrometry (GCMS). According to Table (1), the numbers of 18 secondary metabolites were mostly found with the molecular structure, molecular weight and mass fragment ion (m/z). According to other studies, these secondary metabolites have the antimicrobial action, immunomodulation action, vitamin supplement and antioxidant action. Moreover, the mainly secondary metabolites or bioactive

compounds such as cordycepin, d-Mannitol and adenosine were present in all Cordyceps samples. Therefore, Myanmar Cordyceps also contains the valuable secondary metabolites as other countries grown Cordyceps. Therefore, the consumption of locally produce Cordyceps can obtain many health benefits of human.

The amino acids are not detected by HPLC with UV detection and so, the HPLC with fluorescence detection (HPLC-FLD) was used in this study. For the determination of amino acids, the different combination of mobile solvents (methanol, acetonitrile, sodium di-hydrogen phosphate buffer) and different buffer concentrations (10 mM, 25 mM, 40 mM) and different fluorescence wavelengths (excitation and emission wavelengths) and different flow rate (1 mL/min, 0.5 mL/min). Finally, the optimized HPLC-FLD conditions were achieved on mobile phase was methanol : 25 mM sodium di-hydrogen phosphate (55:45,% v/v), flow rate was 0.5 mL/min, column oven temperature was 37 °C and detection wavelengths were excitation at 340 nm and emission at 450 nm. After optimization the amino acid standards of tyrosine (100 µg/mL), valine (100 µg/mL), iso-leucine (100 µg/mL) and leucine (100 µg/mL) were diluted with methanol and derivatized with OPA solution. Finally, these standard solutions were filtered by syringe filter (0.45 µm) and injected into High Performance Liquid Chromatography (HPLC) system.

The Cordyceps contain high amount of amino acids such as tyrosine, valine, iso-leucine and leucine. Among them, the tyrosine was found of ranged from 127.43±1.74 to 700.23±1.48 µg/g, leucine were found ranged from 9.35±0.81 to 18.47±0.88 µg/g, iso-leucine were found ranged from 8.57±0.69 to 14.35±1.17 µg/g and valine were found ranged from 2.014±1.01 to 12.56±1.29 µg/g.

Jianhua L. et al. 1999 studied the amino acid components between the cultivation of Cordyceps militaris and wild Cordyceps militaris. The results revealed that there are 17 kinds of amino acids in both Cordyceps militaris. The amino acids contain the oxygen, hydrogen, nitrogen and carbon[13]. The amino acids have many health benefits such as providing energy for the body, support the immune system, producing neurotransmitters, stimulate the growth of healthy skin, hair and nail, hormone production, enhance the musculoskeletal system and maintain healthy weight[14].

The valine isoleucine and leucine are essential nutrients and they are found in dairy products, meat, and legumes. They stimulate the protein building in muscle and reduce the muscle breakdown. They are used to reduce brain function of people with advanced liver diseases and for the movement disorders often caused by antipsychotic drugs. They are commonly used to improve athletic performance, reduce muscle breakdown and prevent fatigue[15]. Moreover, the L-tyrosine works as a precursor of essential hormones like noradrenaline, dopamine and adrenaline. They work as the capacity of brain chemicals called neurotransmitters for the optimization of brain functions. These hormones are known as catecholamines and are produced from the adrenal glands. Under the physical or emotional stress, the adrenal glands of the body release catecholamines into the blood. The providing of raw materials for catecholamine synthesis and therefore, the optimal amounts of tyrosine those help to maintain healthy brain chemistry[16].

In the present study, only the four amino acids in Cordyceps were analyzed for the quantitative determination. However, other amino acids and secondary metabolites were determined by the GCMS. Therefore, the Cordyceps contains colorful health benefit compounds that are essential for human health.

6. CONCLUSION

The Cordyceps contain many bioactive compounds and amino acids were contained with high amount

that help for human health such as improvement of cardiac functions, lung functions, sexual dysfunctions and improvement of immunity.

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