

# Genotoxicity Analysis of Microwave-Extract of *Cordyceps militaris* by Ames Test and In Vitro Mouse Lymphoma tk Assay

林彥君、孫芳君 ; 陳小玲

E-mail: 9901149@mail.dyu.edu.tw

## ABSTRACT

*Cordyceps militaris*, belongs to the Dong-Chong-Xia-Cao group in Chinese herbs, possesses pharmacological activities similar to *Cordyceps sinensis*. Several studies have been demonstrated that *C. militaris* has multiple biological actions, including anti-cancer, anti-infection, anti-inflammatory and immunological stimulating activities. In recent years, *C. militaris* is more popular and cheaper than *C. sinensis* on the market. In this study the cytotoxicity and mutagenicity of extracts from *C. militaris* were determined in the Ames test and in vitro mouse lymphoma tk assay. The Ames test was conducted by the plate incorporation method using five strains of *Salmonella typhimurium*, TA97a, TA98, TA100, TA102 and TA1535. The L5178Y tk+/- cell was used for detecting and assessing the mutability of extracts from *C. militaris* by microwell method. In the cytotoxicity test, water extracts and 50% ethanol extracts from *C. militaris* significantly inhibited the growth of *S. typhimurium*. When the concentration of water extract of fruiting body from *C. militaris* was 5 mg/plate, the survival rate was 41.1% and 52.7% in TA100 and TA98, respectively. However, when the concentration of 50% ethanol extracts of *C. militaris* locus was 0.63 mg/plate, the survival rate of TA100 and TA98 were 40.8% and 49.1%, respectively. In the mutagenesis analysis, the results show that whether the water extract and ethanol extract of fruiting body from *C. militaris* were treated with the S9 mix or not, no significant was shown. In addition, the inhibition of cell proliferation was assessed by XTT. The results showed that the water and ethanol extracts of fruiting body from *C. militaris* effectively inhibited the proliferation of breast cancer (MB-MDA231 cell) with the range of 50% inhibited concentration (IC<sub>50</sub>) of 29 µg/ml and 27 µg/ml, respectively.

Keywords : *Cordyceps militaris*, Microwave Extraction, Ames Test, Mouse Lymphoma Assay

## Table of Contents

封面內頁	
簽名頁	
授權書	iii
中文摘要	iv
英文摘要	vi
誌謝	vii
目錄	viii
圖目錄	xiii
表目錄	xiv
1. 前言	1
2. 文獻回顧	2
2.1 北蟲草	2
2.2 蟲草活性成份	4
2.2.1 蟲草素 (Cordycepin)	4
2.2.2 蟲草多醣 (Cordyceps polysaccharide)	5
2.2.3 蟲草酸 (D-Mannitol)	5
2.2.4 硒 (selenium)	5
3. 微波萃取	7
3.1 微波萃取背景	7
3.2 微波萃取特色	7
4. 基因毒性相關法規	9
4.1 《健康食品管理法》	9
5. Ames Test	12

5.1 背景		12
5.2 食品常見致癌物	12	
5.3 Histidine 生合成路徑		13
5.4 本研究 <i>S. typhimurium</i> 差異		15
6. 顯鼠淋巴瘤 tk+/- 分析法		17
6.1 顯鼠淋巴瘤 tk+/- 分析法試驗背景		17
7. 材料方法		19
7.1 Ames Test		19
7.1.1 藥劑		19
7.1.2 藥品配法		20
7.1.3 製備樣品		23
7.1.4 菌株活化		23
7.1.5 菌株保存		24
7.1.6 平板培養		24
7.1.7 菌株表現型確認		24
7.1.7.1 Histidine 篩檢	25	
7.1.7.2 rfa mutation 檢測		25
7.1.7.3 uvrB mutation 檢測	25	
7.1.7.4 R質體檢測	26	
7.1.8 經各正對照組確認菌株		27
7.1.9 毒性試驗		28
7.1.10 致突變測試		28
7.2 顯鼠淋巴瘤 tk+/- 分析法		30
7.2.1 藥品		30
7.2.2 藥品配置		31
7.2.3 製備樣品		32
7.2.4 細胞培養		32
7.2.5 L5178Y tk-/- 基因型刪除		33
7.2.6 基因毒性測試		33
7.2.6.1 樣品處理及細胞誘導	32	
7.2.6.2 細胞毒性測試 (Cytotoxicity)	34	
7.2.6.3 細胞存活測試 (Survival test)	34	
7.2.6.4 Expression period		35
7.2.6.5 Viability test		35
7.2.6.6 TFT test	36	
7.3 北蟲草抑癌測試		37
7.3.1 藥品		37
7.3.2 細胞培養		38
7.3.3 細胞生長抑制率測試 (XTT assay)		38
7.4 統計分析		39
8. 結果與討論		39
8.1 Ames test 結果		41
8.1.1 菌株確認		41
8.1.2 毒性測試		42
8.1.3 致突變測試		44
8.2 顯鼠淋巴瘤 tk+/- 分析結果		50
8.2.1 細胞毒性評估		50
8.2.2 生長毒性評估		50
8.2.3 基因毒性評估		55
8.3 抑制乳癌細胞測試		58
9. 結論		60
參考文獻		63

## 圖目錄

圖2.2.1. Cordycepin 化學結構圖	4
圖5.3.1. Histidine 主要生成路徑	14
圖7.1.1. 致突變測試流程圖	30
圖7.3.1. 細胞生長抑制率測試 (XTT assay) 流程圖	39
圖8.1.1. 北蟲草子實體水萃取物在無 S9 mix 誘導下之 Ames Test 結果	47
圖8.1.2. 北蟲草子實體水萃取物在有 S9 mix 誘導下之 Ames Test 結果	47
圖8.1.3. 北蟲草基座50%乙醇萃取物在無 S9 mix 誘導下之 Ames Test 結果	49
圖8.1.4. 北蟲草基座50%乙醇萃取物在有 S9 mix 誘導下之 Ames Test 結果	49
圖8.2.1. 北蟲草萃取物在無 S9 mix 誘導下之細胞24小時生長抑制結果	53
圖8.2.2. 北蟲草萃取物在有 S9 mix 誘導下之細胞24小時生長抑制結果	53
圖8.2.3. 北蟲草萃取物在無 S9 mix 誘導下之細胞14天生長抑制結果	54
圖8.2.4. 北蟲草萃取物在有 S9 mix 誘導下之細胞14天生長抑制結果	54
圖8.2.5. 北蟲草子實體水萃取物在無 S9 mix 誘導下致細胞基因突變檢測	56
圖8.2.6. 北蟲草子實體水萃取物在有 S9 mix 誘導下致細胞基因突變檢測	56
圖8.2.7. 北蟲草基座50%乙醇萃取物在無 S9 mix 誘導下致細基因傷害檢測	57
圖8.2.8. 北蟲草基座50%乙醇萃取物在有 S9 mix 誘導下致細胞基因傷害檢測	57
圖8.3.1. 北蟲草子實體水萃取物抑制 MB-MDA231 細胞生長檢測	59
圖8.3.2. 北蟲草基座50%乙醇萃取物抑制 MB-MDA231 細胞生長檢測	59

## 表目錄

表2.1.1. 北蟲草與冬蟲夏草成分分析比較	3
表4.1.1. 健康食品分類	11
表5.4.1. Ames Test 菌株基因型突變差異	16
表7.1.1. Ames Test 菌株在 uvrB mutation 檢測條件	26
表7.1.2. Ames Test 所使用正對照組	28
表8.1.1 Ames Test 菌株表現型確認結果	41
表8.1.2 Ames Test 菌株經各正對組確認結果	42
表8.1.3. 北蟲草子實體水萃取物對 TA100 及 TA98 毒性測試	43
表8.1.4. 北蟲草基座50%乙醇萃取物對 TA100 及 TA98 毒性測試	44
表8.1.5. 北蟲草子實體水萃取物之 Ames Test 結果	46
表8.1.6. 北蟲草基座50%乙醇萃取物之 Ames Test 結果	48
表8.2.1. 北蟲草萃取物對細胞24~48小時及14天生長抑制結果	52

## REFERENCES

1. 呂青。2000。北蟲草(*Cordyceps militaris*) 產多糖條件的篩選及多糖的生物活性研究。中國海洋大學。
2. 汪太初、李瑞雪、賈鴻英及胡飛。2009。蟲草素的研究應用進展。現代農業科技。332-333。
3. 胡昆。2006。硯對蛹蟲草[*Cordyceps militaris* (Fr.) Link.]的生物學功效及降脂抗氧化功能的影響。沈陽農業大學。
4. 夏敏及溫魯。2006。微波法提取蟲草素研究。食品科學。27:248-251。
5. 曹慶穗、嚴俊文、褚芳及李超。2008。均勻設計優選蟲草素微波輔助提取工藝研究。江蘇農業科學。
6. 陳登科。2004。超臨界二氧化碳萃取蛹蟲草蟲草素之探討。朝陽科技大學應用化學系。
7. 楊杰及陳順志。2008。蟲草素研究進展。中國生化藥物雜誌。414-417。
8. 陳美璇。2007。台灣產之不同筋骨草屬蛻皮甾酮，總黃酮及抗氧化能力的分析比較。大葉大學生物產業科技學系。
9. 溫魯、翁梁、朱明偉及劉森琴。2008。不同林區蛹蟲草活性成分含量的比較。林農科學。
10. 劉波及鄧中平。2005。tk 基因突變的分子機制研究。癌變畸變突變。17:190-192。
11. 劉彥威、劉娜及劉利強。2004。冬蟲夏草有效成分的研究進展。動物醫學進展。51-53。
12. 盧翠文。2007。北冬蟲夏草液體培養條件的優化與誘變育種。廣西師範大學。
13. Amacher, D. E., Paillet, S. C., Turner, G. N., Ray, V. A., and Salsburg, D. S. (1980). Point mutations at the thymidine kinase locus in L5178Y mouse lymphoma cells. II. Test validation and interpretation. *Mutat Res* 72, 447-474.
14. Ames, B. N., McCann, J., and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. *Mutat Res* 31, 347-364.
15. Caldwell, J. (1993). Perspective on the usefulness of the mouse lymphoma assay as an indicator of a genotoxic carcinogen: ten compounds which are positive in the mouse lymphoma assay but are not genotoxic carcinogens. *Teratog Carcinog Mutagen* 13, 185-190.
16. Chen, Y. J., Shiao, M. S., Lee, S. S., and Wang, S. Y. (1997). Effect of *Cordyceps sinensis* on the proliferation and differentiation of human leukemic U937 cells. *Life Sci* 60, 2349-2359.
17. Cheung, J. K., Li, J., Cheung, A. W., Zhu, Y., Zheng, K. Y., Bi, C. W.,

Duan, R., Choi, R. C., Lau, D. T., Dong, T. T., et al. (2009). Cordysinocan, a polysaccharide isolated from cultured *Cordyceps*, activates immune responses in cultured T-lymphocytes and macrophages: signaling cascade and induction of cytokines. *J Ethnopharmacol* 124, 61-68.18. Clements, J. (2000). The mouse lymphoma assay. *Mutat Res* 455, 97-110.19. Clive, D., Johnson, K. O., Spector, J. F., Batson, A. G., and Brown, M. M. (1979). Validation and characterization of the L5178Y/TK+/- mouse lymphoma mutagen assay system. *Mutat Res* 59, 61-108.20. Cunningham, K. G., Manson, W., Spring, F. S., and Hutchinson, S. A. (1950). Cordycepin, a metabolic product isolated from cultures of *Cordyceps militaris* (Linn.) Link. *Nature* 166, 949.21. Fani, R., Brilli, M., Fondi, M., and Lio, P. (2007). The role of gene fusions in the evolution of metabolic pathways: the histidine biosynthesis case. *BMC Evol Biol* 7 Suppl 2, S4.22. Forster, M., Laabs, V., Lamshoft, M., Putz, T., and Amelung, W. (2008). Analysis of aged sulfadiazine residues in soils using microwave extraction and liquid chromatography tandem mass spectrometry. *Anal Bioanal Chem* 391, 1029-1038.23. Hartman, P. E., Ames, B. N., Roth, J. R., Barnes, W. M., and Levin, D. E. (1986). Target sequences for mutagenesis in *Salmonella* histidine-requiring mutants. *Environ Mutagen* 8, 631-641.24. Hozier, J., Scalzi, J., Sawyer, J., Carley, N., Applegate, M., Clive, D., and Moore, M. M. (1991). Localization of the mouse thymidine kinase gene to the distal portion of chromosome 11. *Genomics* 10, 827-830.25. Huang, B. M., Chuang, Y. M., Chen, C. F., and Leu, S. F. (2000). Effects of extracted *Cordyceps sinensis* on steroidogenesis in MA-10 mouse Leydig tumor cells. *Biol Pharm Bull* 23, 1532-1535.26. Isono, K., and Yourno, J. (1974). Chemical carcinogens as frameshift mutagens: *Salmonella* DNA sequence sensitive to mutagenesis by polycyclic carcinogens. *Proc Natl Acad Sci U S A* 71, 1612-1617.27. Jagerstad, M., Olsson, K., Grivas, S., Negishi, C., Wakabayashi, K., Tsuda, M., Sato, S., and Sugimura, T. (1984). Formation of 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline in a model system by heating creatinine, glycine and glucose. *Mutat Res* 126, 239-244.28. Jin, C. Y., Kim, G. Y., and Choi, Y. H. (2008). Induction of apoptosis by aqueous extract of *Cordyceps militaris* through activation of caspases and inactivation of Akt in human breast cancer MDA-MB-231 Cells. *J Microbiol Biotechnol* 18, 1997-2003.29. Johnston, H. M., and Roth, J. R. (1979). Histidine mutants requiring adenine: selection of mutants with reduced hisG expression in *Salmonella typhimurium*. *Genetics* 92, 1-15.30. Jost, L. M., Kirkwood, J. M., and Whiteside, T. L. (1992). Improved short- and long-term XTT-based colorimetric cellular cytotoxicity assay for melanoma and other tumor cells. *J Immunol Methods* 147, 153-165.31. Kjellstrom, A., Brantlind, M., and Eldsater, C. (2008). Optimized microwave extraction for trace detection of 2,4,6-trinitrotoluene in soil samples. *Chemosphere* 71, 1701-1708.32. Koh, J. H., Kim, J. M., Chang, U. J., and Suh, H. J. (2003). Hypocholesterolemic effect of hot-water extract from mycelia of *Cordyceps sinensis*. *Biol Pharm Bull* 26, 84-87.33. Kuo, Y. C., Lin, C. Y., Tsai, W. J., Wu, C. L., Chen, C. F., and Shiao, M. S. (1994). Growth inhibitors against tumor cells in *Cordyceps sinensis* other than cordycepin and polysaccharides. *Cancer Invest* 12, 611-615.34. Kylin, H., H. H. J. B., and Wittig, R. (1994). Environmental Monitoring of Polychlorinated Biphenyls Using Pine Needles as Passive Samplers. *Environ Sci*, 1320-1324.35. Lee, S. J., Kim, S. K., Choi, W. S., Kim, W. J., and Moon, S. K. (2009). Cordycepin causes p21WAF1-mediated G2/M cell-cycle arrest by regulating c-Jun N-terminal kinase activation in human bladder cancer cells. *Arch Biochem Biophys* 490, 103-109.36. Levin, D. E., Marnett, L. J., and Ames, B. N. (1984). Spontaneous and mutagen-induced deletions: mechanistic studies in *Salmonella* tester strain TA102. *Proc Natl Acad Sci U S A* 81, 4457-4461.37. Levin, D. E., Marnett, L. J., and Ames, B. N. (1984). Spontaneous and mutagen-induced deletions: mechanistic studies in *Salmonella* tester strain TA102. *Proc Natl Acad Sci U S A* 81, 4457-4461.38. Li, S. P., Li, P., Dong, T. T., and Tsim, K. W. (2001). Anti-oxidation activity of different types of natural *Cordyceps sinensis* and cultured *Cordyceps* mycelia. *Phytomedicine* 8, 207-212.39. Li, S. P., Yang, F. Q., and Tsim, K. W. (2006). Quality control of *Cordyceps sinensis*, a valued traditional Chinese medicine. *J Pharm Biomed Anal* 41, 1571-1584.40. Li, S. P., Zhao, K. J., Ji, Z. N., Song, Z. H., Dong, T. T., Lo, C. K., Cheung, J. K., Zhu, S. Q., and Tsim, K. W. (2003). A polysaccharide isolated from *Cordyceps sinensis*, a traditional Chinese medicine, protects PC12 cells against hydrogen peroxide-induced injury. *Life Sci* 73, 2503-2513.41. Lo, H. C., Tu, S. T., Lin, K. C., and Lin, S. C. (2004). The anti-hyperglycemic activity of the fruiting body of *Cordyceps* in diabetic rats induced by nicotinamide and streptozotocin. *Life Sci* 74, 2897-2908.42. Lin, Y.-W., and Chiang, B.-H. (2007). Anti-tumor activity of the fermentation broth of *Cordyceps militaris* cultured in the medium of *Radix astragali*. *Food Science and Technology*, 244-250.43. Lv, Z. M., Jiang, Y. T., Wu, L. J., and Liu, K. (2008). [Chemical constituents from dried sorophore of cultured *Cordyceps militaris*]. *Zhongguo Zhong Yao Za Zhi* 33, 2914-2917.44. MacPhee, D. G. (1973). *Salmonella typhimurium* hisG46 (R-Utrecht): possible use in screening mutagens and carcinogens. *Appl Microbiol* 26, 1004-1005.45. Maron, D. M., and Ames, B. N. (1983). Revised methods for the *Salmonella* mutagenicity test. *Mutat Res* 113, 173-215.46. MARTIN., R. G. (1962). The First Enzyme in Histidine Biosynthesis: The Nature of Feedback Inhibition by Histidine. *Received for publication* 238, 257-268.47. Ohmori, T., Tamura, K., Ohgane, N., Nakamura, T., Kawanishi, G., Yamada, H., and Nomoto, K. (1989). The correlation between molecular weight and antitumor activity of galactosaminoglycan (CO-N) from *Cordyceps ophioglossoides*. *Chem Pharm Bull (Tokyo)* 37, 1337-1340.48. Pare, J. (1991). Microwave-assisted natural products extraction. united states patent.49. Park, B. T., Na, K. H., Jung, E. C., Park, J. W., and Kim, H. H. (2009). Antifungal and Anticancer Activities of a Protein from the Mushroom *Cordyceps militaris*. *Korean J Physiol Pharmacol* 13, 49-54.50. Puck, T. T., Cieciura, S. J., and Robinson, A. (1958). Genetics of somatic mammalian cells. III. Long-term cultivation of euploid cells from human and animal subjects. *J Exp Med* 108, 945-956.51. Qing-jun, J., Sheng-xue, L., and Jin-yi, L. (2004). studies of tk gene mutation by hydroxylammonium nitrate. third military medical university, chingqing, 365-369.52. Riela, S., Bruno, M., Formisano, C., Rigano, D., Rosselli, S., Saladino, M. L., and Senatore, F. (2008). Effects of solvent-free microwave extraction on the chemical composition of essential oil of *Calamintha nepeta* (L.) Savi compared with the conventional production method. *J Sep Sci* 31, 1110-1117.53. Shen, Y. D., Shao, X. T., Ni, Y. D., Xu, H., and Tong, X. M. (2009). [Cordyceps sinensis polysaccharide enhances apoptosis of HL-60 cells induced by triptolide]. *Zhejiang Da Xue Xue Bao Yi Xue Ban* 38,

158-162.54. Sugar, A. M., and McCaffrey, R. P. (1998). Antifungal activity of 3'-deoxyadenosine (cordycepin). *Antimicrob Agents Chemother* 42, 1424-1427.55. Sugimura, T., Nagao, M., and Wakabayashi, K. (1996). Carcinogenicity of food mutagens. *Environ Health Perspect* 104 Suppl 3, 429-433.56. Yoshikawa, N., Kunitomo, M., Kagota, S., Shinozuka, K., and Nakamura, K. (2009). Inhibitory effect of cordycepin on hematogenic metastasis of B16-F1 mouse melanoma cells accelerated by adenosine-5'-diphosphate. *Anticancer Res* 29, 3857-3860.57. Yu, R., Yang, W., and Song, L. (2007). Structural characterization and antioxidant activity of a polysaccharide from the fruiting bodies of cultured *Cordyceps militaris*. *Carbohydrate Polymers*, 430-436.58. Zill, E. H., Abert Vian, M., Maingonnat, J. F., and Chemat, F. (2009). Clean recovery of antioxidant flavonoids from onions: optimising solvent free microwave extraction method. *J Chromatogr A* 1216, 7700-7707.