ISSN (Print) 1225-9918
ISSN (Online) 2287-3406
Journal of Life Science 2016 Vol. 26. No. 2. 259~264 DOI : http://dx.doi.org/10.5352/JLS.2016.26.2.259

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Anti-metastatic Effect of Garlic Hexane Extract on Lung Metastasis Induced by Melanoma B16F10 Cells in Mice

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Received February 1, 2016 / Revised February 14, 2016 / Accepted February 19, 2016

Metastatic cancer is one of the main causes of cancer-related death since they rarely respond to available treatments. There is epidemiologic evidence that high garlic consumption decreases the incidence of cancer. Recent studies of our laboratory have revealed that a garlic-extracts is effective in suppressing metastasis. For experimental metastasis, C57BL/6 mice were injected intravenously with melanoma B16F10 cells in the tail vein, and were orally administered various concentrations (0, 50, 100 or 200 mg/kg body weight) of garlic hexane extract (GHE) for 21 days. The incidence and the area of the melanoma cell colony occupied by the poorly differentiated carcinoma were significantly lower in dose-dependent in 50, 100 and 200 mg/kg BW GHE - treated mice compared with control mice. In conclusion, the results of the present study show that GHE administration prevents lung metastasis in C57BL/6 mice.

Key words : C57BL/6 mice, garlic hexane extract, melanoma B16F10, metastasis

Introduction

Cancer metastasis consists of a complex cascade of events, which ultimately allow for tumor cell escape and seeding of ectopic environments [28]. Metastasis is a sequential process in which tumor cells detach from the primary growth, invade through the surrounding host tissue into the circulation and subsequently disseminate to distant organs, where they arrest, extravasate and proliferate to form metastatic foci [1]. Melanoma is the most aggressive form of skin cancer with high metastatic potential and extraordinary resistance to cytotoxic agents [24, 26]. Despite recent advances, the results of chemotherapy for patients with metastatic melanoma remain inadequate because of the relative drug resistance of metastatic cells.

Garlic (*Allium sativum*) has been used as a spice and an ingredient in folk medicine since ancient times [22, 25]. There have been many literature reports of epidemiological studies indicating that a garlic-rich diet decreases risk of some cancers such as gastric, stomach, and colon *in vitro* [6, 29] and the growth of transitional cell carcinoma xenografts [14, 19]. Recent studies in our team have revealed that a garlic-extracts or derived compounds are effective in suppressing metastasis [9, 16, 20], proliferation of cancer cells [8, 10, 21], sepsis [13] and inflammation [11, 12, 17]. Several individual compounds have been isolated from garlic and two major groups of compounds that show active anticancer effects have been identified [5, 8, 24]. One group is the lipid-soluble allyl sulfur compounds, and the other one is the water-soluble compounds [5, 24]. It is still unclear which compounds in garlic has positive role in the prevention of cancers.

Despite the abundant *in vitro* evidence for anti-cancer and anti-metastatic effect of garlic extract or its compounds is not well studied. In this study, we provide novel anti-metastatic activity from hexane fractions of dried garlic.

Materials and Methods

Animals

C57BL/6 female mice, 6-7 weeks old were purchased from Samtako (Osan-shi, Kyunggi-do, South Korea) and housed in polycarbonate cages under laboratory conditions

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with a 12 hr day-night cycle, room temperature of 22±2℃ and humidity of 50-60%. They were fed standard pelleted rats chow and had free access to water during the experiment. The animal experiments were approved by the Pusan National University, Institutional Animal Care and Use Committee (PNU-2010-000102).

Tumor cell line

B16F10 cells were obtained from Korean Cell Line Bank in Seoul, South Korea, maintained in RPMI-1640 medium supplemented with 10% FBS, antibiotics (100 U/ml penicillin G, 100 μg/ml streptomycin), in an incubator at 37℃ with a humidified atmosphere at 5% CO_2 concentration. When needed for experiments the cells were harvested with trypsin:EDTA (0.05:0.03[w/v]) solution, and then washed in phosphate-buffered saline (PBS, pH 7.4). For the animal experiments, the recovered cells were adjusted to 1×106 cells/ml in PBS and then 100 μl of the suspension was injected into the tail-vein.

Anti-metastasis study of GHE in B16F10 cell implanted C57BL/6 mice

GHE was suspended in olive oil to the desired concentration (50, 100, 200 mg/kg body weight/2 interval day). For the studies, the GHE was administered orally (OA) starting on the day the B16F10 tumor cells were injected (1×10^6cells in 0.1 ml) by tail vein and there after the GHE was given for 21 days by OA.

Experimental groups for metastasis

C57BL/6 mice were divided into 6 groups of 10 mice each to carry out the level of GHE present in lung tumor bearing mice model. B16F10 cell line was injected (1×10^6 cells in 0.1 ml) by tail vein to groups **III**, IV, V and VI. GHE was given for respective group at a dose of 50, 100 and 200 mg/kg/body weight/day for 21 days after cell line implantation.

Group I : Controls animal (Olive oil alone administrated orally for 10 alternative days).

Group II: Drug control (GHE-200 mg/Kg in olive oil administrated orally for 10 alternative days).

Group III: Tumor alone (Olive oil alone administrated orally for 10 alternative days).

Group IV: Tumor induced+GHE-50 mg/Kg in olive oil administrated orally for 10 alternative days.

Group V: Tumor induced+GHE-100 mg/Kg in olive oil administrated orally for 10 alternative days.

Group VI: Tumor induced+GHE-200 mg/Kg in olive oil administrated orally for 10 alternative days.

Pulmonary colonization assay

For *in vivo* experimental pulmonary metastasis assays, mice were injected with 1×10^6 B16F10 melanoma cells through the lateral tail vein. One day after tumor induction, GHE in olive oil at different concentrations were orally administrated for 10 alternative days. Whereas the tumor control mice were treated with the vehicle olive oil. The animals were sacrificed after 21 days of tumor induction, lungs were excised and metastatic colonies in lung were counted. Percentage of inhibition was calculated by the formula [(Control -Treated)/Control] ×100.

Statistical analysis

Results were provided as mean ± SE for each condition and statistically analyzed by the Student - Newman - Keuls test. $p<0.05$ was considered statistically significant.

Results

The effect of GHE on inhibition of lung cancer was assessed by counting the number of lung tumor colony formed in the respective groups as given in Table 1. The lungs of GHE-treated mice showed less number of tumor colonies (35.2±11.52) compared to those mice. Metastastic tumor bearing mouse treated with GHE showed significant reduction on tumor colony formation in a dose dependent manner (Fig. 1, Fig. 2). Untreated control animals developed a massive number of tumor colonies (97.4±30.2) in the lung. Lung metastasis frequency was significantly reduced by

Table 1. Effect of GHE on lung metastasis induced in mice by B16F10 cells

Treatment	Average no. of lung metastastic foci	Average lung weight (mg)
Vehicle control		160±4
Tumor control	79.40±8.19	410±35
GHE 50 mg/kg	80.20±12.42	456±74
GHE 100 mg/kg	40.60±9.07	356±39
GHE 200 mg/kg	35.2±11.52	338±46

B16F10 melanoma cells (1×10^6 cells/mice) were injected to the lateral tail vein of mice. After GHE treatment for 10 alternative days animals were sacrifice and lung nodule were counted. Values are mean ± standard error, n=10.

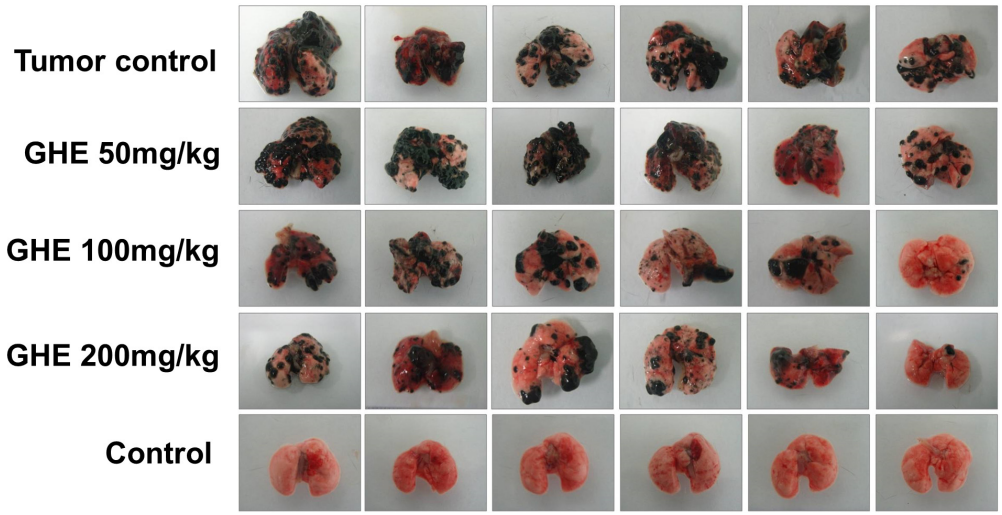

Fig. 1. Effect of GHE on B16F10 lung metastasis. Mice injected with B16F10 cells were orally administrated either with GHE at different concentration and olive oil. After treatment of 10 alternative days lung were excised and tumor colonies were counted.

6.93, 46.80 and 50.53% in mice treated with 50, 100 and 200 mg/kg of GHE, respectively (Fig. 3). In comparison with control, reduction of lung weight also noticed in mice treated with GHE at dose-dependent manner (Table 1).

Metastasis remains to be the major cause of death in cancer patients and it has critical importance in cancer therapy. Some drugs used for metastasis therapy have low efficiency, low response rate and it also produce a number of side effects. In the present study we investigate the anti-metastatic effect of garlic hexane extract on lung metastasis induced by B16F10 melanoma cell in experimental mice. GHE extracted from garlic showed that it could significantly abrogate the metastatic formation of nodules in lung by dose-dependent manner (Fig. 1, Fig. 2). More than 100 mg/kg garlic extract exert maximum anti-metastatic effect with 56% (Fig. 3). Though GHE showed inhibition of metastasis, this study opens ups further investigation on mode of action behind this effect.

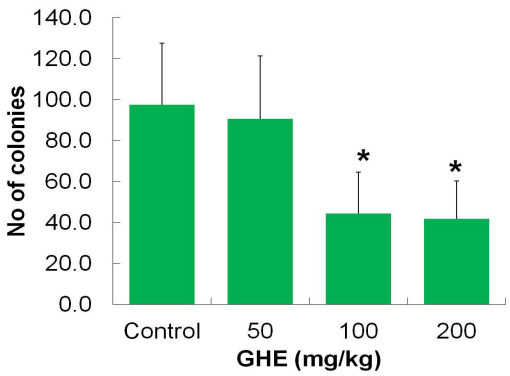

Fig. 2. Effect of GHE on formation of metastatic foci in lung of mice injected with B16F10 cells. B16F10 melanoma cells (1×10^6 cells/mice) were injected through the lateral tail vein of mice. After GHE treatment for 10 alternative days animals were sacrifice and lung nodule were counted. Values are mean ± standard deviation, n=10. Comparisons between control and treatment groups were performed by Student-Newman-Keuls test. *p* values **<0.01, *<0.05.

Discussion

Melanoma is the most aggressive form of skin cancer with high metastatic potential and extraordinary resistance to cytotoxic agents. Despite recent advances, the results of

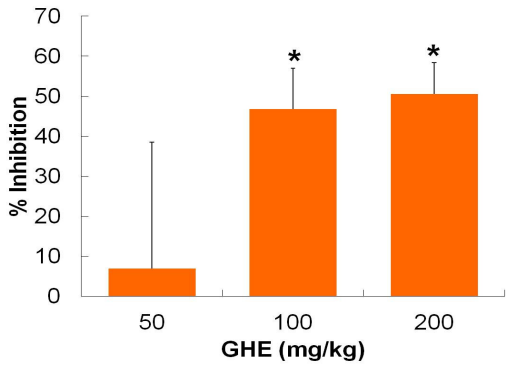

Fig. 3. Inhibitory effect of GHE on lung metastasis induced by B16F10 cells. Values are mean ± standard deviation, n=10. Comparisons between control and treatment groups were performed by Student-Newman-Keuls test. *p* values **<0.01, *<0.05.

chemotherapy for patients with metastatic melanoma remain inadequate because of the relative drug resistance of metastatic cells. Metastasis is a sequential process in which tumor cells detach from the primary growth, invade through the surrounding host tissue into the circulation and subsequently disseminate to distant organs, where they arrest, extravasate and proliferate to form metastatic foci [1]. Metastasis covers 90% of causality of cancer patients' death [4] indicating that anti-metastatic agents are required for efficient cancer therapy. Consequently, identification of novel agents that are nontoxic but can delay onset and/or progression of cancer is highly desirable. Natural products have received increasing attention in recent years for the discovery of novel cancer preventive and therapeutic agents [15].

Many *in vitro* and animal studies have reported a relation between garlic intake supplementation and risk cancer prevention [18, 24]. Recent studies have provided evidence that sarcoma cell migration was inhibited by aged garlic extract [7] and different garlic extracts [9]. However, it is still ambiguous by which agents suppress metastasis of cancer cells from garlic extracts. In the present study, to screen the novel anti-metastasis compound from garlic, we prepared different extracts of garlic such as raw garlic and black garlic. During this process, hexane extract provide novel activity which suppresses the migration of cancer cells [9] and the proliferation of cancer cells [9], and garlic constituent sulfur [16], various solvent extracts were highly effective in suppressing growth of cancer cells in intro [10].

Several other research studies stated the establishment of the experimental lung metastasis model or pulmonary metastatic model by intravenously injected mouse melanoma B16F10 in C57BL/6 mice [3, 27]. The B16F10 mouse melanoma cell line was chosen on the basis of its high metastatic potential in this study. Specifically, the B16F10 line has been shown to metastasize to the lungs when injected into the lateral tail vein and the majority of cells find themselves in the pulmonary tissue, but some are also localized in other organs [2] and is accepted as useful model for the study of lung metastasis [23]. We showed that the mice received B16F10 cells only implanted, the melanoma cells quite well and the lungs of these hosts were visibly riddled with metastatic tumor nodules within 21 days (Fig. 1). Using C57BL/6 model mouse, we demonstrated that GHE could inhibit the colony forming rate of melanoma cells in dose-dependent manners (Fig. 3). Moreover, GHE significantly inhibited the colony-forming ability of melanoma cells. In conclusion, the results of the present study indicate that oral administration of GHE prevents development of melanoma carcinoma and multiplicity of pulmonary metastatic lesions in C57BL/6 mice without causing weight loss.

Acknowledgement

This work was supported for two years by a Pusan National University Research Grant.

References

1. Bedrosian, I., Faries, M. B., Guerry, D. t., Elenitsas, R., Schuchter, L., Mick, R., Spitz, F. R., Bucky, L. P., Alavi, A., Elder, D. E., Fraker, D. L. and Czerniecki, B. J. 2000. Incidence of sentinel node metastasis in patients with thin primary melanoma (< or = 1 mm) with vertical growth phase. *Ann. Surg. Oncol.* **7**, 262-267.
2. Fidler, I. J. 1973. Selection of successive tumor lines for metastasis. *Nature (London)* 242, 148-149.
3. Gautam, A., Waldrep, J. C. and Densmore, C. L. 2000. Inhibition of experimental lung metastasis by aerosol delivery of PEI – p53 complexes. *Mol. Ther.* **2**, 318-323.
4. Hanahan, D. and Weinberg, R. A. 2000. The hallmarks of cancer. Cell 100, 57-70.
5. Herman-Antosiewicz, A. and Singh, S. V. 2004. Signal transduction pathways leading to cell cycle arrest and apoptosis induction in cancer cells by Allium vegetable-derived organosulfur compounds: a review. *Mutat. Res.* **555**, 121-131.
6. Hsing, A. W., Chokkalingam, A. P., Gao, Y. T., Madigan, M. P., Deng, J., Gridley, G. and Fraumeni, J. F. Jr. 2002. Allium vegetables and risk of prostate cancer: a population based study. *J. Natl. Cancer Inst.* **94**, 1648-1651.
7. Hu, X., Cao, B. N., Hu, G., He, J., Yang, D. Q. and Wan, Y. S. 2002. Attenuation of cell migration and induction of cell death by aged garlic extract in rat sarcoma cells. *Int. J. Mol. Med.* **9**, 641-643.
8. Jeong, J. W., Park, S., Park, C., Chang, Y. C., Moon, D. O., Kim, S. O., Kim, G. Y., Cha, H. J., Kim, H. S., Choi, Y. W., Kim, W. J., Yoo, Y. H. and Choi, Y. H. 2014. N-benzyl-N-methyldecan-1-amine, a phenylamine derivative isolated from garlic cloves, induces G2/M phase arrest and apoptosis in U937 human leukemia cells. *Oncol. Rep.* **32**, 373-381.
9. Kim, E. K., Yun, S. J., Ha, J. M., Jin, I. H., Kim, Y. W., Kim, S. G., Park, D. J., Choi, Y. W., Yoon, S., Kim, C. D. and Bae, S. S. 2011. Inhibition of cancer cell migration by compounds from garlic extracts. *J. Life Sci.* **21**, 767-774.
10. Kim, H. J., Han, M. H., Kim, G. Y., Choi, Y. W. and Choi, Y. H. 2012. Hexane extracts of garlic cloves induce apoptosis through the generation of reactive oxygen species in Hep3B human hepatocarcinoma cells. *Oncol. Rep.* **28**, 1757-1763.

11. Kim, H. K., Choi, Y. W., Lee, E. N., Park, J. K., Kim, S. G., Park, D. J., Kim, B. S., Lim, Y. T. and Yoon, S. 2011. 5-Hydroxymethyl furfural from black garlic extract prevents TNF-α-induced monocytic cell adhesion to HUVECs by suppression of vascular cell adhesion molecule-1 expression, reactive oxygen species generation and NF-κB activation. *Phytother. Res.* **25**, 965-974.
12. Kim, K. H., Park, J. K., Choi, Y. W., Kim, Y. H., Lee, E. N., Lee, J. R., Kim, H. S., Baek, S. Y., Kim, B. S., Lee, K. S. and Yoon, S. 2013. Hexane extract of aged black garlic reduces cell proliferation and attenuates the expression of ICAM-1 and VCAM-1 in TNF-α-activated human endometrial stromal cells. *Int. J. Mol. Med.* **32**, 67-78.
13. Lee, S. K., Park, Y. J., Ko, M. J., Wang, Z., Lee, H. Y., Choi, Y. W. and Bae, Y. S. 2015. A novel natural compound from garlic (*Alliums ativum* L.) with therapeutic effects against experimental polymicrobial epsis. *Biochem. Biophys. Res. Commun.* **464**, 774-779.
14. Marsh, C. L., Torrey, R. R., Woolley, J. L., Barker, G. R. and Lau, B. H. 1987. Superiority of intravesical immunotherapy with *Corynebacterium parvum* and *Allium sativum* in control of murine bladder cancer. *J. Urol.* **137**, 359-362.
15. Newman, D. J., Cragg, G. M. and Snader, K. M. 2003. Natural products as sources of new drugs over the period 1981-2002. *J. Nat. Prod.* **66**, 1022-1037.
16. Park, H. S., Kim, G. Y., Choi, I. W., Kim, N. D., Hwang, H. J., Choi, Y. W. and Choi, Y. H. 2011. Inhibition of matrix metalloproteinase activities and tightening of tight junctions by diallyldisulfide in AGS human gastric carcinoma cells. *J. Food Sci.* **76**, T105-T111.
17. Park, S. Y., Seetharaman, R., Ko, M. J., Kim, D. Y., Kim, T. H., Yoon, M. K., Kwak, J. H., Lee, S. J., Bae, Y. S. and Choi, Y. W. 2014. Ethyl linoleate from garlic attenuates lipopolysaccharide-induced pro-inflammatory cytokine production by inducing heme oxygenase-1 in RAW264.7 cells. *Int. Immunopharmacol.* **9**, 253-261.
18. Qingjun, C., Ling, M. T., Feng, H., Cheung, H. W., Tsao, S. W., Wang, X. and Wong, Y. C. 2006. A novel anticancer effect of garlic derivatives: inhibition of cancer cell invasion through restoration of E-cadherin expression. *Carcinogenesis* **27**, 2180-2189.
19. Riggs, D. R., DeHaven, J. I. and Lamm, D. L. 1997. *Allium sativum* (garlic) treatment for murine transitional cell carcinoma. *Cancer* **79**, 1987-1994.
20. Shin, D. Y., Kim, G. Y., Kim, J. I., Yoon, M. K., Kwon, T. K., Lee, S. J., Choi, Y. W., Kang, H. S., Yoo, Y. H. and Choi, Y. H. 2010. Anti-invasive activity of diallyldisulfide through tightening of tight junctions and inhibition of matrix metalloproteinase activities in LNCaP prostate cancer cells. *Toxicol. In Vitro* **24**, 1569-1576.
21. Shin, D. Y., Yoon, M. K., Choi, Y. W., Gweon, O. C., Kim, J. I., Choi T. H. and Choi, Y. H. 2010. Effects of aged black garlic extracts on the tight junction permeability and cell invasion in human gastric cancer cells. *J. Life Sci.* **20**, 528-534.
22. Tannock, I. F., de Wit, R., Berry, W. R., Horti, J., Pluzanska, A., Chi, K. N., Oudard, S., Théodore, C., James, N. D., Turesson, I., Rosenthal, M. A. and Eisenberger, M. A. 2004. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. *N. Engl. J. Med.* **351**, 1502-1512.
23. Teng, M. W., von Scheidt, B., Duret, H., Towne, J. E. and Smyth, M. J. 2011. Anti-IL-23 monoclonal antibody synergizes in combination with targeted therapies or IL-2 to suppress tumor growth and metastases. *Cancer Res.* **71**, 2077-2086.
24. Thomson, M. and Ali, M. 2003. Garlic (*Allium sativum*): a review of its potential use as an anti-cancer agent. *Curr. Cancer Drug Targets* **3**, 67-81.
25. Virginia, L. 2006. The analysis of onion and garlic. *J. Chroma. A* **1112**, 3-22.
26. William, E. D. Jr., Rosenbaum, L. E. and Bosenberg, M. 2011. Decoding melanoma metastasis. *Cancers* **3**, 126-163.
27. Yao, Z., Che, X. C., Lu, R., Zheng, M. N., Zhu, Z. F., Li, J. P., Jian, X., Shi, L. X., Liu, J. Y. and Gao, W. Y. 2007. Inhibition by tyroserleutide (YSL) on the invasion and adhesion of the mouse melanoma cell. *Mol. Med.* **13**, 14-21.
28. Yoon, S. O., Kim, M. M. and Chung, A. S. 2001. Inhibitory effect of selenite on invasion of HT1080 tumor cells. *J. Biol. Chem.* **276**, 20085-20092.
29. Yun, H. M., Ban, J. O., Park, K. R., Lee, C. K., Jeong, H. S., Han, S. B. and Hong, J. T. 2014. Potential therapeutic effects of functionally active compounds isolated from garlic. *Pharmacol. Ther.* **142**, 183-195.

초록 : Melanoma B16F10 cell에 의해서 유도된 mouse모델에서 마늘 헥산 추출물의 암전이에 억제 효과

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암전이는 현재까지 적당한 치료제가 거의 없었기 때문에 암에 의한 사망의 주요한 원인 중의 하나로 인식되고 있다. 최근 본 연구팀은 마늘 추출물과 순수분리한 성분에 대한 암전이 억제 시험 결과 마늘의 추출물 또는 성분이 암전이를 억제시켰으며, 역학조사에서도 마늘을 많이 섭취한 사람은 암의 발생을 억제시키는 것으로 보고되어 있다, 본 연구의 암전이 실험에서는 C57BL/6 mouse의 꼬리 정맥에 melanoma B16F10세포를 주사하여 폐에 전이를 유도하였다. 암세포 주사 1일 후에 마늘의 헥산 추출물 50, 100 및 200 mg/kg body weight를 2일 간격으로 21일 동안 구강투여 한 다음 암전이 억제효과를 조사하였다. GHE를 처리하지 않은 대조구에서는 폐에서 암 colony가 97.4±30.2으로 대량 생성되었다. GHE를 50, 100 및 200 mg/kg의 농도로 경구투여시에 암전이 빈도는 각각 6.93, 46.80 및 50.53% 억제하였다. 또한 100 mg/kg body weight 경구투여 시에는 폐로 암전이 억제율이 약 53% 이상으로 매우 높았다. 폐에서 melanoma cell colony의 발생율과 면적은 마늘 헥산 추출물의 농도가 높을수록 감소하였다. 결론적으로 C57BL/6 mice의 암전이 모델에서 마늘 헥산추출물의 구강투여는 폐에 암전이를 억제시켰으나, 향후 그 기작에 대한 연구가 수행되어야 할 것으로 생각된다.