Journal of Pharmacopuncture

ISSN 2093-6966(Print), **ISSN 2234-6856**(Online)
Journal of Pharmacopuncture 2012;15(1):007-011
DOI: http://dx.doi.org/10.3831/KPI.2012.15.1.007

Original Article

Effect of Egg White Combined with Chalcanthite on Lipopolysaccharide induced Inflammatory Cytokine Expression in RAW 264.7 cells

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Key Words

cytokine; GM-CSF; IL-1β; IL-6; lipopolysaccharide; TNF-α

Abstract

Aim: Historically, mineral compound herbal medicines have long been used in treatments of immune-related diseases in Korea, China and other Asian countries. In this study, we investigated the anti-inflammatory effect of egg white combined with chalcanthite (IS4) on lipopolysaccharide (LPS)-stimulated RAW 264.7 cells.

Methods: RAW 264.7 cells cultured with LPS and various concentrations of IS4 were analyzed to determine the production of pro-inflammatory cytokines and mediators by using enzyme-linked immune sorbent assays (ELISAs).

Results: IS4 concentration inhibited the production of interleukin-1beta (IL-1β), interleukin-6 (IL-6), and granulocyte -macrophage colony-stimulating factor (GM-CSF) induced by LPS. IS4 at high concentrations (25 and 50 μg/ml) inhibited, in concentration-dependent manner, the expression of tumor necrosis factor-alpha (TNF-α) stimulated by LPS.

Conclusion: IS4 has shown an anti-inflammatory effect in RAW 264.7 cells.

Received: Sep 21, 2011 **Accepted:** Feb 24, 2012

1. Introduction

Chalcanthite derived from the old Latin name *Chalcanthum* meaning "flowers of copper" is a richly-colored, blue, water-soluble sulfate mineral commonly found in the late-stage oxidation zones of copper deposits [1]. Copper is the main component of chalcanthite and the third most common mineral in the body, being essential in human growth and development [1-3]. Copper is found throughout the musculo-skeletal system, with largest amount being found in the brain and the liver. It helps to release energy in cells and contributes to the production of melanin; several key enzymes, neurotransmitters and neuroactive compounds; collagen; and red blood cells. It also helps in the functioning of antioxidants, the formation and regulation of melatonin, and the absorption and transport of iron [4]. Copper toxicities caused by inadequate diet or surgical interventions may lead to cardiac hypertrophy, poor neuronal myelination, blood vessel abnormalities, and impaired immune responses [2,3]. Elevated copper levels are associated with morphological and metabolic changes in the liver and in several other tissues and show a wide spectrum of hepatic abnormalities, including hepatitis, cirrhosis, acute liver failure and/or neurological and psychiatric diseases that may progress to death if left untreated [3]. Recent reports indicate that changes in the expression of copper transporters alter the sensitivity of cancer cells to major chemotherapeutic agents, such as cisplatin, although the mechanism behind this phenomenon remains unclear [5]. Regulation of human copper homeostasis has significant therapeutic potential and requires detailed understanding of copper transport mechanisms [6]. Normally, most of the copper entering the blood in ionic form goes directly to the liver and the kidneys before reappearing in blood plasma in newly

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formed ceruloplasmin. Copper is transported by albumin and transcuprein, a high-affinity copper carrier in the plasma involved in the initial distribution of copper entering blood. Studies have discovered that transcuprein is a macroglobulin regulated by copper and iron availability [3,7]. The main ingredient of chalcanthite is $CuSO_4$, which exists as a series of compounds that differ in their degrees of hydration [1]. The anhydrous form occurs as a rare mineral known as chalcocyanite. The hydrated copper sulfate occurs in nature as chalcanthite ($CuSO_4 \bullet 5H_2O$), and two of more rare ones: bonattite ($CuSO_4 \bullet 3H_2O$) and boothite ($CuSO_4 \bullet 7H_2O$) [1]. Macrophages are known to play an important role in immune reactions, allergies, and inflammation. During inflammation response, macrophages release many different kinds of cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and granulocyte-macrophage colony-stimulating factor (GM-CSF), initiating and maintaining specific immune responses as part of the immune/inflammatory cascade [8]. Lipopolysaccharide (LPS) is a major outer membrane component of gram-negative bacteria and is frequently used as an inflammatory model due to its ability to activate macrophages. LPS stimulates immune responses by interacting with the membrane receptor CD14 to induce cytokine release through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation [7-11]. Therefore, the modulation of macrophage-mediated inflammatory responses is important in order to formulate a new therapeutic approach against these inflammatory diseases. Minerals have been traditionally used as part of oriental herbal medicine treatment, especially for immune-related diseases, in Korea, China, and other Asian countries. According to oriental medicine, chalcanthite has sour, acrid, cold and toxic properties, and is affiliated with the liver meridian. Its main function is to eliminate toxins. Internally, it functions as a purgative and helps the body get rid of excess phlegm and toxins. Chalcanthite can also be applied externally for skin abscesses, inflammation, and the removal of necrotic tissue [12]. The traditionally used dose of chalcanthite is between 0.3 to 0.6 grams of powdered mineral. Larger amounts can be used if chalcanthite is being applied externally [12]. However, chalcanthite is poisonous and should be used with extreme caution. To date, due to safety issues concerning their cytotoxical mechanisms, mineral medicines have rarely been used. Previously, we reported that egg white combined with chalcanthite (IS4) inhibited the growth of NCI-H460 human lung cancer cells by inducing apoptotic cell death via caspase-3 activation. Mixing egg white with mineral drugs is a method to enhance pharmaceutical properties and to reduce toxicity [13]. In this study, the anti-inflammatory effects of IS4 on LPS-stimulated cytokines in mouse RAW 264.7 macrophage-like cells were investigated.

2. Materials and methods

2.1. IS4

Egg white combined with chalcanthite (IS4) was used as the main experimental material. The eggs were from Hamyang, Korea, and the chalcanthite was obtained from Manila, The Philippines. The chalcanthite was heated and dehydrated. During the heating process, chalcanthite uniformly received heat while being carefully watched for color change every 3 to 5 hours until it turned fully grey. The heating duration varied with the amount and the quality of the mineral used and was in the range of 10 to 24 hours. Once the chalcanthite had been dehydrated, it was cooled until the remaining heat was completely released. The moisture content of the chalcanthite ranged from 0% to 5%. After it had been cooled, the chalcanthite was finely pulverized. The pulverized chalcanthite should be stored in a concealed airtight container in a dry place to prevent any moisture from entering. The prepared chalcanthite powder was rapidly mixed with egg whites in a ratio of 30:13. The egg whites had to be homogeneously mixed with a wooden spatula in a ceramic vessel so that the utensils would not interfere with or cause any chemical reactions during the mixing process. After the chalcanthite and egg whites had been sufficiently mixed, the mixture was cooled until the reaction heat was completely released. One-hundred mg of the attained substance was then dissolved in 1 ml of distilled water and was centrifuged at 6,000 rpm for 10 minutes. The upper level was filtered in 0.8μm pore sizes, and the filtered substance diluted in distilled water was used for treatment. The treatment doses of IS4 (12.5, 25, 50 μg/ml) were based on our previous IS4 [13].

2.2. Cell culture

The RAW 264.7 macrophage-like cell lines obtained from ATCC® were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and penicillin-streptomycin at 37 °C under 5% CO_2.

2.3. Reagents

The fetal bovine serum (FBS) was purchased from HyClone (Logan, UT) and high-glucose DMEM, L-glutamine, penicillin, and streptomycin were purchased from GIBCO BRL (Life Technologies, Grand Island, NY). Purified LPS derived from *E. coli* serotype 055:B5, Allo, Tris, sodium dodecyl sulfate (SDS), Triton X-100, Tween 20, phenylmethylsulfonyl fluoride, leupeptin, aprotinin, proteinase K, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2.4. Measurement of cytokines

RAW 264.7 cells were seeded into 24 well plates at 1 x 10^5 cells /well and were cultured in DMEM containing 10% FBS at 37°C for 24 hours. After treatment with LPS (1 μg/ml) for 24 hours, the concentrations of IL-1β and IL-6 in the supernatant were measured by using a commercial enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, San Diego, CA). The concentrations of TNF-α and GM-CSF in the supernatant were measured by using an ELISA kit (Millipore, Billerica, MA). The concentrations were calculated using linear-regression equations obtained from the standard absorbance values.

2.5. Statistical analysis

Numerical data from each experiment were averages from triplicate samples. Experiments were repeated three times to avoid any experimental errors and to ensure the repeatability of the used methods. Data were expressed as means ± SD. Statistical differences were analyzed using the Mann–Whitney U-test in SPSS ver. 10.0 (statistical software package, Chicago, IL). P-values less than 0.05 were considered statistically significant.

3. Results

3.1. IL-1β expression in RAW 264.7 cells

To test the effect of IS4 on LPS-induced IL-1β production, we cultured RAW 264.7 cells with LPS (1 μg/ml) and various concentrations of IS4 (12.5, 25, 50 μg/ml) for 24 hours. The IL-1β concentration in the supernatants was determined by using an ELISA. As shown in Fig. 1, stimulation with LPS significantly increased the expression of IL-1β in RAW 264.7 cells when compared to the untreated control ($P<0.001$). Interestingly, the cells treated with IS4 inhibited expression of IL-1β in a concentration-

dependent manner, which showed 25% inhibition with 12.5 μg /ml, 46% inhibition with 25 μg/ml (P<0.05), and 55% inhibition with 50 μg/ml (P<0.05).

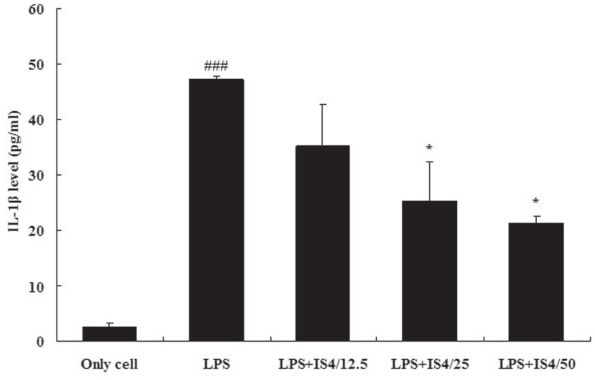

Figure 1 Inhibitory effect of IS4 on IL-1β production in RAW 264.7 cells. Cells were incubated with LPS (1 μg/ml) for 24 hr. Data are expressed as percentages of the control values. Values shown are means ± SD of three independent experiments, each run in triplicate. ### Statistically significant difference from the only cell at P<0.001. * Statistically significant difference from the LPS-treated control at P<0.05.

3.2. IL-6 expression in RAW 264.7 cells

To test the effect of IS4 on LPS-induced IL-6 production, we cultured RAW 264.7 cells with LPS (1 μg/ml) and various concentrations of IS4 (12.5, 25, 50 μg/ml) for 24 hours. The IL-6 concentration in the supernatants was determined by using an ELISA. As shown in Fig. 2, stimulation with LPS showed 99.9% increase in the expression of IL-6 in RAW 264.7 cells when compared to the untreated control (P<0.001). Moreover, the cells treated with IS4 inhibited IL-6 expression in a concentration-dependent manner, which showed 26% inhibition with 12.5 μg/ml (P<0.05), 48% inhibition with 25 μg/ml (P<0.01), and 70% inhibition with 50 μg/ml (P<0.001).

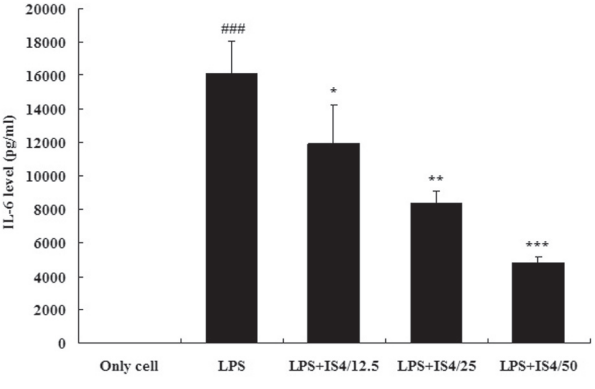

Figure 2 Inhibitory effect of IS4 on IL-6 production in RAW 264.7 cells. Cells were incubated with LPS (1 μg/ml) for 24 hr. Data are expressed as percentages of the control values. Values shown are means ± SD of three independent experiments, each run in triplicate. ### Statistically significant difference from the only cell at P<0.001. *,**,*** Statistically significant difference from the LPS-treated control at P<0.05, P<0.01, and P<0.001, respectively. Means ± SD were calculated from five individual results.

3.3. TNF-α expression in RAW 264.7 cells

To test the effect of IS4 on LPS-induced TNF-α production, we cultured RAW 264.7 cells with LPS (1 μg/ml) and various concentrations of IS4 (12.5, 25, 50 μg/ml) for 24 hours. The TNF-α concentration in the supernatants was determined by using an ELISA. As shown in Fig. 3, stimulation with LPS significantly increased the expression of TNF-α in RAW 264.7 cells when compared to the untreated control (P<0.001). Moreover, the cells treated with high doses of IS4 (25 and 50 μg/ml) inhibited TNF-α expression in a concentration-dependent manner, which showed 38% inhibition with 12.5 μg/ml (P<0.05), 32% inhibition with 25 μg/ml (P<0.05), and 52% inhibition with 50 μg/ml (P <0.01).

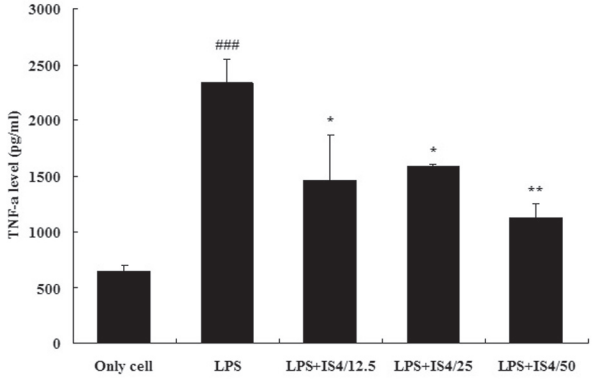

Figure 3 Inhibitory effect of IS4 on TNF-α production in RAW 264.7 cells. Cells were incubated with LPS (1 μg/ml) for 24 hr. Data are expressed as percentages of the control values. Values shown are means ± SD of three independent experiments, each run in triplicate. ### Statistically significant difference from the only cell at P<0.001. *,** Statistically significant difference from the LPS-treated control at P<0.05 and P<0.01, respectively.

3.4. GM-CSF expression in RAW 264.7 cells

To test the effect of IS4 on LPS-induced GM-CSF production, we cultured RAW 264.7 cells with LPS and various concentrations of IS4 (12.5, 25, 50 μg/ml) for 24 hours. The GM-CSF concentration in the supernatants was determined by using

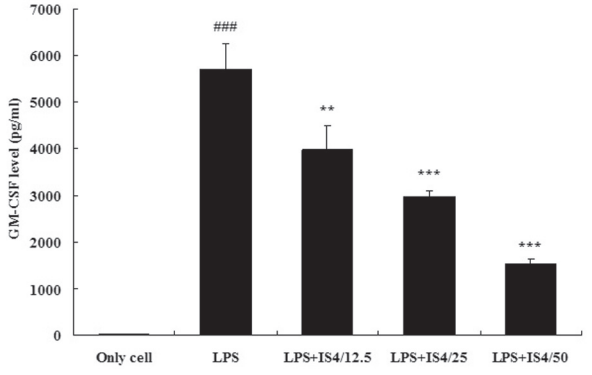

Figure 4 Inhibitory effect of IS4 on GM-CSF production in RAW 264.7 cells. Cells we-re incubated with LPS (1 μg/ml) for 24hr. Data are expressed as percentag-es of the control values. Values shown are means ± SD of three independe-nt experiments, each run in triplicate. ### Statistically significant differen-ce from the only cell at P<0.001. **,*** Statistically significant difference from the LPS-treated control at P<0.01 and P<0.001, respectively.

an ELISA. As shown in Fig. 4, stimulation with LPS (1 μg/ml) remarkably increased the expression of GM-CSF in RAW 264.7 cells when compared to the untreated control ($P<0.001$). Moreover, the cells treated with IS4 inhibited of GM-CSF expression in a concentration-dependent manner, which showed 30% inhibition with 12.5 μg/ml ($P<0.01$), 48% inhibition with 25 μg/ml ($P<0.001$), and 73% inhibition with 50 μg/ml ($P<0.001$).

The results were analyzed using Korea Basic Science Institute (KBSI)'s EAS (Expression Analysis System).

4. Discussion

Previously, we had tested the efficacy of IS3 (heat treated chalcanthite), IS4 (egg white combined with chalcanthite), and IS5 (raw chalcanthite) on NCI-H460 human lung cancer cells. Among them, the egg-white treated IS4 most successfully inhibited the growth of cells by inducing apoptotic cell death via caspase-3 activation [13]. Other than being a quality protein source, egg whites contain many essential minerals, including potassium, magnesium, calcium, phosphorus, copper, zinc, and iron. They are also a good source of riboflavin and selenium, along with essential vitamins such as folate, B12, niacin betaine, and choline [14]. Historically, in medieval times in the West, egg whites were used for medicinal purposes, such as treating wounds, skin disorders, and mending broken bones [15]. Today, egg whites are considered to be one of the most nutrition-dense foods and are widely recommended for children, the elderly, athletes, and patients. Copper has been used in France since the 18th century to stabilize egg foams. Reactive sulfur items, such as egg whites beaten in a copper bowl, create tighter bonds. The bonds created are so tight that the sulfur atoms from the egg whites are prevented from reacting with any other materials [15]. Avidin is a tetrameric biotin-binding glycoprotein found in raw egg white. In chicken egg white, avidin makes up approximately 0.05% of the total protein (approximately 1.8 mg per egg) [16]. The tetrameric protein contains four identical subunits (homotetramers), each of which can bind to biotin (vitamin B_7) with a high degree of affinity and specificity [16]. Research in the 1970s helped establish the avidin-biotin system as a powerful tool in biological sciences [17]. Avidin's affinity for biotin is applied in wide-ranging biochemical assays, including western blot, ELISA, ELISPOT and pull-down assays. Avidin immobilized onto solid supports is also used as decontamination medium to capture biotin-labeled proteins or nucleic acid molecules [17]. For example, lectins, proteins that bind specific sugar molecules on glycoproteins and glycolipids, are expressed at various levels on the surfaces of tumor cells. Conjugation of cytotoxic agents to glycoproteins recognized by lectins could be useful in the treatment of tumors. Avidin, a highly glyco-positively charged protein, contains terminal N-acetylglucosamine and mannose residues that bind to some lectins [18]. In a 1998 study, the ability of avidin targeting different tumor models was tested through radioactively-labeled biotin conjugation. Avidin pre-targeting, followed by injection of radioactive biotin led to avidin-biotin conjugation *in-vivo*. As a result, radio-labeled avidin were highly and rapidly localized in the tumors, and the uptakes of radioactivity in normal tissues and other organs were low, yielding high tumor-to-non tumor activity ratios [18]. Previously, functional avidin was thought to be found only in raw eggs as the biotin avidity of the protein is destroyed by cooking. A 1966 study claimed that the structure of avidin remained stable at temperatures below 70°C. At temperatures above 70°C, avidin's structure was rapidly disrupted, and by 85°C, extensive loss of structure and ability to bind biotin was found [19]. However, a 1992 study disagreed, concluding that protein denaturation was not equivalent to loss of biotin-binding activity [20]. Based on these findings, we examined the effect of IS4 on LPS-stimulated inflammatory cytokines in mouse RAW 264.7 macrophages. Concentrations of pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α, and GM-CSF, were determined by using an ELISA. As shown in the results, stimulation with LPS significantly increased the expressions of cytokines in RAW 264.7 cells when compared to the untreated control. Moreover, cells treated with IS4 (12.5, 25 and 50 μg/ml) inhibited expressions of IL-1β, IL-6 and GM-CSF in concentration-dependent manner. In addition, high doses of IS4 (25 and 50 μg/ml) inhibited TNF-α expression, also in a concentration-dependent manner.

5. Conclusion

Egg white combined with chalcanthite may be considered as a possible future candidate for an anti-inflammatory agent. Further studies are needed to evaluate the inhibitory mechanisms toward pro-inflammatory cytokines and should focus on *in-vivo* biological activity assessment of egg white combined with chalcanthite. It would also be interesting to biotinylate the apoptotic, anti-inflammatory cytokine-stimulating agent chalcanthite and have avidin from the egg whites work as a pre-targeting vehicle using avidin-biotin conjugation mechanisms.

Acknowledgment

This work was supported by Regional Innovation Center (RIC) grant from the Traditional and BioMedical Research Center, Daejeon University (RRC04730, 2005), by Ministry of Knowledge Economy (MKE), Republic of Korea.

Appendix A. Supplementary data

An online supplementary data associated with this article can be found at doi:10.1016/j.cyto.2008.03.010.

Appendix B. Toxicity data

Single and repeated oral dose toxicity tests of IS4 were performed and confirmed its safety. The results are currently under review and will be published in the near future.

References

1. Nordstrom DK, Alpers CN. Negative pH, efflorescent mineralogy, and consequences for environmental restoration at the Iron Mountain Superfund site, California. *Proc Natl Acad Sci USA*. 1999;96(7):3455-62.
2. Gupta A, Chattopadhyay I, Mukherjee S, Sengupta M, Das SK, Ray K. A novel COMMD1 mutation Thr174Met associated with elevated urinary copper and signs of enhanced apoptotic cell death in a Wilson Disease patient. *Behavioral and Brain Functions*. 2010;15(6):33.
3. Gupta A, Lutsenko S. Human copper transporters: mechanism, role in human diseases and therapeutic potential. *Future Medicinal Chemistry*. 2009;1(6):1125-42.
4. United Kingdom Copper Development Association, Technical

Note 34, Copper and Human Health - A Review. 1984; Retrieved from http://www.copperinfo.co.uk/health/downloads/pub-34-copper-and-human-health.pdf on 10 Mar. 2011.
5. Blair BG, Larson CA, Safaei R, Howell SB. Copper transporter 2 regulates the cellular accumulation and cytotoxicity of cisplatin and carboplatin. *Clin Cancer Res.* 2009;15(13): 4312-21.
6. Ishida S, Lee JK, Thiele DJ, Herskowitz I. Uptake of the anticancer drug cisplatin mediated by the copper transporter Ctr1 in yeast and mammals. *Proc Natl Acad Sci USA.* 2002; 99:14298-302.
7. Hong YH, Chao WW, Chen ML, Lin BF. Ethyl acetate extracts of alfalfa (Medicago sativa L.) sprouts inhibit lipopolysaccharideinduced inflammation in vitro and in vivo. *Journal of Biomedical Science.* 2009;146(16):64.
8. Tsai JT, Liu HC, Chen YH. Suppression of inflammatory mediators by cruciferous vegetable-derived indole-3-carbinol and phenylethyl isothiocyanate in lipopolysaccharide-activated macrophages. *Mediators of Inflammation.* 2010;2936-42. doi:10.1155/2010/293642.
9. Shin SL, Lee SW, Kwon JH, Moon SH, Lee SJ, Lee CK, et al. Cordyce-pin Suppresses Expression of Diabetes Regulating Genes by Inhibition of Lipopolysaccharide-induced Inflammation in Macrophages. *Immune Network.* 2009;9(3):98-105.
10. Kimura A, Naka T, Nakahama T, Chinen I, Masuda K, Nohara K, et al. Aryl hydrocarbon receptor in combination with Stat1 regulates LPS-induced inflammatory responses. *J Exp Med.* 2009;206(9):2027-35.
11. Liu N, Lo LS, Askary SH, Jones L, Kidane TZ, Trang T, et al. Transcuprein is a macroglobulin regulated by copper and iron availability. *The Journal of Nutritional Biochemistry.* 2007;18(9):597-608.
12. Acupuncture Today. Herbs & Botanicals, Chalcanthite (dan fan). 2011; Retrieved from http://acupuncturetoday.com/herbcentral/chalcanthite.php.
13. Choi EA, Kim KH, Yoo BC, Yoo HS. Induction of Apoptotic Cell Death by Egg white combined-Chalcanthite on NCI-H460 Human Lung Cancer Cells. *Journal of Pharmacopuncture.* 2009;12(3):49-59.
14. Lunsford, P. The Health Benefits and Nutritional Value of Egg Whites. Associated content. 2008; Retrieved from http://www.associatedcontent.com/article/949095/the_health_benefits_and_nutritional.html?cat=5 on 10 Mar. 2011.
15. McGee, Harold. On Food and Cooking: The Science and Lore of the Kitchen. New York: Scribner, edited by Vinay, 2004: 102-3.
16. Dictionary 3.0. Encyclopedia. Wikipedia Meaning and Definition on Avidin. 2010; Retrieved from http://www.dictionary30.com/encyclopedia.php?subject=Avidin on 10 Mar. 2011.
17. Eakin RE, McKinley WA, Williams RJ. Egg-white injury in chicks and its relationship to a deficiency of vitamin H (biotin). *Science.* 1940;92(2384):224-5.
18. Yao Z, Zhang M, Sakahara H, Saga T, Arano Y, Konishi J. Avidin Targeting of Intraperitoneal Tumor Xenografts. *J Natl Cancer Inst.* 1998;90(1):25-9.
19. Pritchard AB, McCormick DB, Wright LD. Optical rotatory dispersion studies of the heat denaturation of avidin and the avidin-biotin complex. *Biochem Biophys Res Commun.* 1966; 25(5):524-8.
20. Durance TD, Wong NS. Kinetics of thermal inactivation of avidin. *Food Research International.* 1992;25 (2):89-92.