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ANTIOXIDANT AND ANTITUMOR EFFECTS OF MANDA

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Abstract

We have investigated the ability of Manda, an yeast fermentation product of various wild-fruits and vegetables to inhibit lipid peroxidation and antitumor activities in rodents. When mice were pretreated with Manda fractions of molecular weight smaller than 500 for two weeks by intraperitoneal injections (0.4 g/kg body weight), the hepatic lipid peroxidation induced by carbon tetrachloride and/or the pulmonary lipid peroxidation by paraquat have been suppressed, evidenced by the reduced levels of thiobarbituric acid reactive substances in those tissues. These results suggest that Manda exhibited antioxidant effect against free radical-mediated tissue damages. Furthermore, an induction of tumor nodules in the lung by inoculation of LL/2 cells to C57BL/6 mice via tail vein has also been reduced by Manda administration: While the control group received only the cancer cells showed 67% of highly vascularized large nodules, the Manda-treated groups before and/or after the LL/2 cell inoculations did not show any large size of nodule, but only yielded much smaller size without neovascularization, suggesting an anticarcinogenic activity of Manda.

Manda is known to be a health food in Japan for a number of years, although its biochemical mechanism or factor(s) associated with its efficacy as a diet supplement is not clearly known. It is a fermentation product consisted of several natural products, such as wild fruits, vegetables, seeds, seaweeds, and cane sugar (1). Needless to mentioned that fruits and vegetables contain vitamins such as ascorbic acid, tocopherol, retinol and β-carotene which are all antioxidants and believed to aid as the protective nutrients against cancer, atherosclerosis, cataract and ageing process (2). Free radicals such as superoxide and hydroxyl radicals are carcinogenic, since these are highly reactive with cellular components including DNA, and thus, antioxidants are likely to counteract against free radicals. A high intake of β-carotene is associated with a decreased risk of lung cancer and also stomach and colon cancers (3). Okuda and Takara (4) have demonstrated in their clinical studies that Manda inhibits the lipolytic activity of toxohormone-L from ascites cells isolated from hepatoma bearing patients, and further shown to prevent liver injury. Recent studies by Hwang *et al.* (5) have also shown that Manda exhibited the anticancer activities *in vivo* and *in vitro*: An oral administration of Manda to the tumor bearing mice prolonged their survival rate about 40% compared to the control group. Furthermore, γ-globulin level of the mice injected with sarcomas-180 cells was elevated when they were given Manda. The activities of natural killer cells against YAC-1 cells and lymphokine-activated killer cells against P815 cells were also significantly increased in the Manda treated group.

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In the present study, as a part of our continuous investigations to understand the health efficacy of natural food products, we have undertaken to investigate effect of Manda for free radial-mediated tissue damage experimentally induced to ICR mice and antitumor effect in C57BL/6 mice. Histological observations comparing the neovascularization of tumor nodules before and after Manda-treatment have been also presented.

Materials and methods

Materials. Paraquat dichloride (PQ), carbon tetrachloride (CCl_4), thiobarbituric acid and corn oil were obtained from Sigma (St. Louis, MO, U.S.A), and 1,1,3,3-tetraethoxypropane was from Aldrich (Milwaukee, WI, U.S.A). All other reagents used were highest grade available. Manda was obtained from MANDA Fermentation Co. Ltd. in Japan. For the oxidative stress experiment, Manda was fractionated; briefly, 50 g of Manda were added to 50 ml of distilled water and mixed vigorously for 1 h at room temperature. The mixture was then centrifuged at 10,000 rpm for 30 min and the supernatant was filtered through YC 05 ultrafiltration membrane. The filtrate, Manda fraction (MF), containing molecular weight smaller than 500 was freeze-dried and stored at 4°C until use. However, for the antitumor effect experiment, the original Manda was used without further fractionation.

Animals. Male ICR mice (8-9 weeks, weighing approximately 20 g) used for the antioxidant experiments were obtained from the Laboratory Animal Center of Seoul National University. The C57BL/6 mice (weighing 20-25 g) used to inoculate tumor cells were kindly provided from the Korea Institute of Science and Technology in Daejeon. All experimental mice were housed in a standard cages under the controlled temperature (25±2℃) and relative humidity of 55±10 %, with an alternating 12 h light-and-dark cycle. Animals were fed standard pellets diet and allowed for free access to water, unless otherwise specified.

Treatment of Animal for antioxidant experiments. The animals were experimentally induced for oxidative stress by administration of CCl_4 or PQ by intraperitoneal injections (i.p.). The CCl_4 solution was prepared by mixing an appropriate amount of CCl_4 in corn oil, and a single injection of 0.1 ml (containing 0.1 ml of the original CCl_4/kg body weight) was given. PQ was dissolved in 0.9% NaCl and 0.1 ml of the solution (calculated on the basis of 40 mg/kg body weight) was administered to each mouse. To find out the optimum period to induce maximum level of oxidative stress, the animals were sacrificed at 6, 15, 24 and 48 h after the single injection of CCl_4 (or PQ). It was found that in both cases, 15 h period of induction yielded the highest level of oxidative stress (Figs. 1 and 2). MF solution was prepared by dissolving the freeze-dried MF in distilled water at a concentration of 2 g/ml, and the amount of MF containing equivalent to 0.4 g/kg was administered to mice once a day for 2 weeks.

Animals were divided into the following four groups:

1. The control group who received 0.1 ml of either corn oil or saline;
2. The CCl_4 (or PQ)-treated group who received 0.1 ml containing the specified amount of either compounds;
3. The MF-treated group who received daily injections of MF (0.4 g/kg) for 2 weeks; and finally,
4. The MF plus CCl_4 (or PQ)-treated group who received daily injections of MF (0.4 g/kg) for two weeks followed by an injection of CCl_4 (or PQ) 4 h after the last administration of MF.

All animals were then sacrificed 15 h after CCl_4 (or PQ) injection, and the liver from the CCl_4-treated and the lung from the PQ-treated were taken immediately to quantify the lipid peroxidation activities.

Lipid peroxidation assay. The tissues (liver or lung) were homogenized in a glass-teflon homogenizer with precooled 1% KCl. to make 10%-homogenate for the liver and 20%-homogenate for the lung. The extent of lipid peroxidation was determined by the method of Uchiyama and Mihara (6) using the thiobarbituric acid reaction. The results were expressed as nmol of thiobarbituric acid reactive substances (TBARS) per g tissue, using 1,1,3,3-tetraethoxypropane as the standard.

Statistical analysis. The values in the figures and tables were expressed as means $\pm$S.D. One way ANOVA and Scheffe F-test were performed to determine the significant differences among individual groups. The student's t-test was also used whenever it is appropriate.

Tumor cell inoculations and Manda treatments. The LL/2 tumor cells were cultured in 5% DMEM medium in a CO_2 incubator, and adjusted to 5×10^5 cells/0.1 ml with 0.9% NaCl just before the use. To produce tumor metastasis in C57BL/6 mouse lung, two different methods had been employed initially by injecting 0.1 ml of the cell suspension to mice; the first method is to inject the cells through tail vein, and the other, to inject subcutaneously on the right shoulder of the mice. Since the former method was found to yield tumor metastasis in their lungs much more extensively than the latter, all the subsequent experiments were carried out by inoculating cells via tail veins. The mice were divided into three different groups, 6 mice for each: The 1st group, the control who was inoculated the tumor cells and was received 0.2 ml saline daily for 21-days by gavage; the 2nd group, the Manda-treated for 7-days before the cell inoculation and continuously for another 21-days subsequently; and the third group, the Manda-treated for 21-days only after the cell inoculation. All these animals were finally sacrificed at 21-days after the inoculation of 5×10^5 cells via tail veins, and lung tissues were excised for histological examinations.

Tissue preparation. The excised lungs were rinsed with physiological saline solution, and fixed in 4% paraformaldehyde or 10% bufferd neutral formalin solutions. Subsequently, the tissue pieces were dehydrated by treating them with solutions containing increasing concentrations of ethanol, and were embedded into paraffin by the use of xylene. The paraffin blocks were sectioned with microtome for about 5 μm thickness and they were carefully placed on microscopic slides. The slides were then immersed into xylene and treated with the decreasing concentrations of ethanol. Finally, the specimens were double stained with hematoxylin and eosin, and finally mounted in Euparal. The slides were examined by a Nikon compound microscope (Japan) and photographed.

Results

Effects of CCl_4 and PQ treatment for the level of lipid peroxidation in mice. Fig. 1 shows time-dependent effect of CCl_4 treatment on lipid peroxidation in mice liver. The amount of TBARS, an index for lipid peroxidation, started to increase soon after the CCl_4 administration reaching to its maximum activity at 15 h and it decreased rather rapidly reaching to the control level at around 24 h after the CCl_4. There was no changes in the level of TBARS in the liver of the control animal received only 0.1 ml of corn oil (data not shown). In the case of PQ administration, the profile for the time-dependent increase of TBARS activity in the mouse lung was very much similar to that of the CCl_4-treated, peaking at around 14-15 h after PQ administration (Fig. 2). These results indicated that exogenous administration of both CCl_4 and PQ induced the oxidative stress rather transiently, and thus the subsequent studies were all carried out at 15 h post induction.

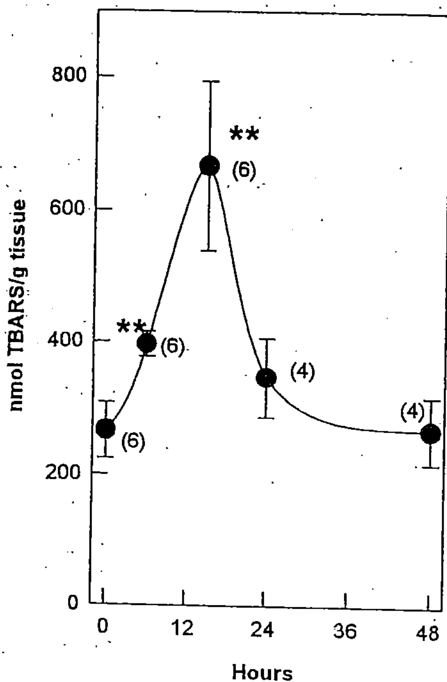

Figure 1

Effect of CCl_4 treatment on the lipid peroxidation in mice livers. Mice were treated with CCl_4 (0.1 ml/kg,i.p.), and sacrificed at 6, 15 24 and 48 h after the treatment. The level of lipid peroxidation was determined by measuring TBARS as described in the text.

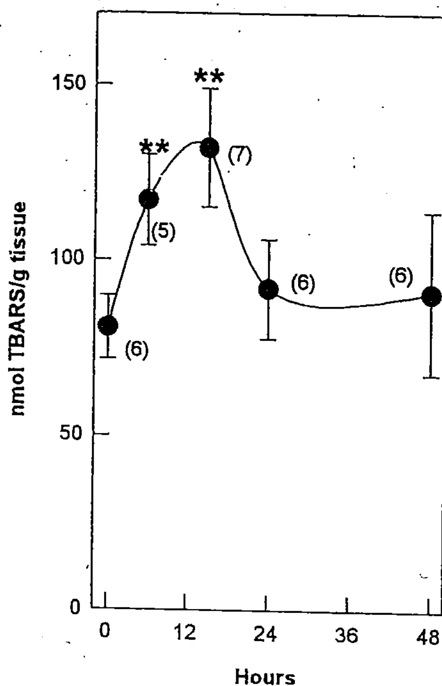

Figure 2

Effect of paraquat treatment on the lipid peroxidation in mice lungs. Mice were treated with paraquat (40 mg/kg, i.p.), and sacrificed at 6, 15, 24 and 48 h after the treatment. The level of lipid peroxidation was determined as described in Fig. 1.

Values in parentheses represent the number of mice. Symbals, **, indicate $p<0.01$ versus control group (by Student's t-test).

Effects of Manda fraction in mice induced oxidative stress by CCl_4 and PQ. In this study, animals were pretreated with MF (Mr <500) for 2 weeks before CCl_4 (0.1ml/kg) injection and sacrificed 15 h after CCl_4. As shown in Fig. 3, while the CCl_4-treatment itself increased TBARS approximately 3.8-fold that of the control treated with corn oil (~703 vs. ~223 nmol, $p<0.05$), the treatment of MF prior to CCl_4 administration resulted significant reduction in the level of the liver TBARS (~703 vs. ~414 nmol/g, $p<0.01$). On the other hand, the level of TBARS in mice treated with MF alone were about the same as the control group. The pretreatment of MF to the mice before inducing oxidative stress by PQ (40 mg/kg) also reduced TBARS in their lungs (~125 vs. ~84 nmol, $p<0.05$) (Fig. 4). However, it is noted that the level of TBARS reduction in the lung by MF pretreatment was more effective than that on the liver reaching to the control level, while about 60% of the reduction was seen in the liver (compare Figs. 3 and 4).

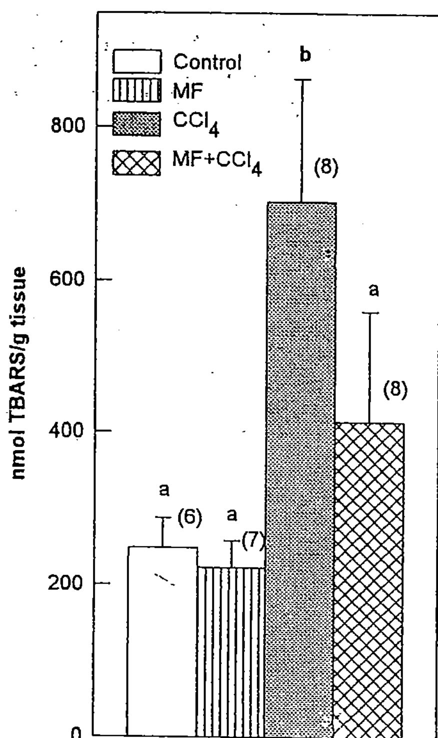

Figure 3

Effect of MF on the CCl_4-induced lipid peroxidation in mice livers. Control mice received 0.1 ml of corn oil, and Manda group received MF (0.4 g/kg, i.p.) once a day for 2 weeks. The CCl_4 group received a single dose of CCl_4. For the $MF+CCl_4$ group, CCl_4 was given 4 h after the last administration of MF. The animals were killed 15 h afer the CCl_4 treatment. Details of experimental conditions are described in the text.

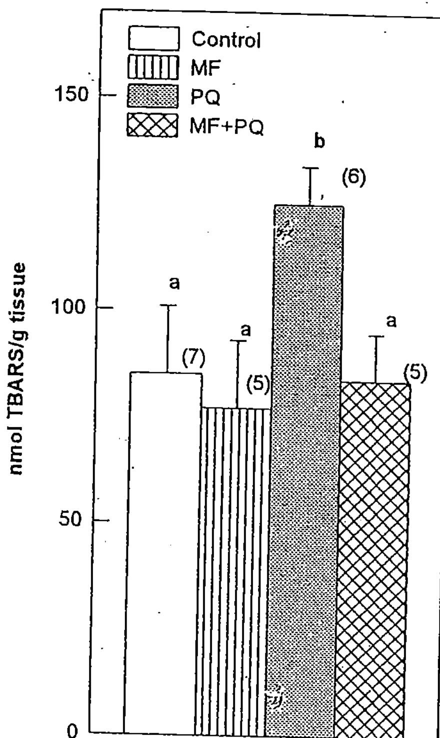

Figure 4

Effect of MF treatment on the PQ-induced lipid peroxidation in the mice lungs. Experimental conditions are samed as Fig. 3, except that PQ is used instead of CCl_4, and the control group received 0.1 ml of saline.

Values in parentheses represent the number of mice.

[a,b] Means with the differenent superscripts represent significant difference treated by the one-way ANOVA followed by Scheffe F-test ($p<0.05$).

Table 1 shows the weight of organs after various combinations of treatments. First of all, there was no significant changes in their weight between the control and the MF-treated liver or lung. However, the liver weight of the CCl_4-treated mice increased 34% of the control (2.04 vs. 1.52 g). On the other hand, the weight of the PQ-treated mice lung decreased (0.178 vs. 0.22 g), indicating weights of the organs are unlikely to be related with the status of oxidative stress. It is interesting to note that the changes of organ weights by CCl_4 or PQ administration can be restored to the normal level by MF treatment in both cases, although its mechanism is not clear at this time.

Table 1

Effect of organ weight following tretment of Manda, CCl_4 and paraquat to mice.

treatment	liver	lung
	(g)	(g)
control	1.52 ± 0.352[a] (4)	0.22 ± 0.024[a] (4)
MF	1.35 ± 0.309[a] (4)	0.20 ± 0.038[a] (4)
CCl_4	2.04 ± 0.393[a] (5)	n.d.
PQ	n.d.	0.178 ± 0.017[a] (5)
MF plus CCl_4	1.55 ± 0.1444[a] (4)	n.d.
MF plus PQ	n.d	0.23 ± 0.011[a] (4)

Experimental conditions are same as Figs. 1 and 2.
The results are expressed in means ± S.D.
Values in parentheses indicate the number of mice used.
"n.d." indicates "not determined".
[a]These values represent means with no significant difference at 5% level (one-way ANOVA followed by Scheffe F-test).

Formation of metastasis in mice lung by inoculation of LL/2 cells. To find out an efficient methods to yield tumor metastasis in mouse lung, two methods were employed to inoculate LL/2 cells, namely, the tail vein injection and the subcutaneous inoculation on the shoulder. As shown in Figs. 5A and B, the tail vein injection yielded a lot of tumor nodules and active angiogenesis on the surface of the lung, and 4 out of 6 animals in the group showed extremely large tumor nodules. Consistently, histological examination revealed highly neovascularized metastasis and engourgement of red blood cells (Figs. 5C and D). In contrast to it, only 50% of the subcutaneously injected group showed increased cell mass with irregular shaped or even normal shaped lung (data not shown). These experiments thus showed the tail vein injection method to be more effective in yielding lung metastasis, and thus, all the followeing experiments were carried out by the tail vein injection method.

Antitumor effect of Manda in mouse lung subsequent to the inoculation of LL/2 cells. Fig. 6A and B show the lung tissues which were pretreated with Manda for 7-days before the LL/2 cell inoculation and continuous treatment for the next 21-days following the inoculation. In this group, 5 out of 6 showed almost normal appearance of the lung, but one animal exhibited some invasive metastases. Histological examinations also clearly indicated that four out of five mice had a typical normal lung structure (Figs. 6C and D) and the other one with minor degree of micrometastases.

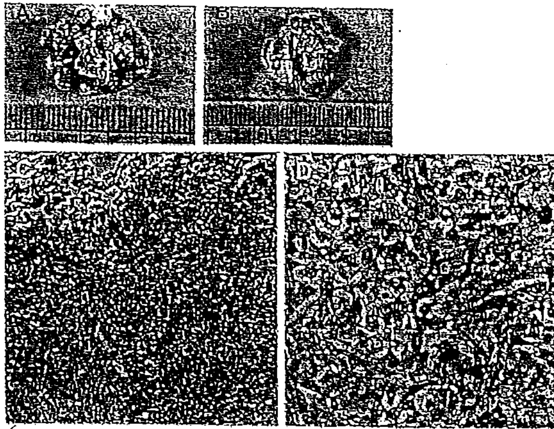

Figure 5

Photographs of lung noduls from the control mice received LL/2 cells. Mice were injected with LL/2 cells through the tail vein as described in the text. Irregular shaped lung and lots of tumor nodules are seen (A and B). Histological micrographs with magnifications of X 410 (D) and X 1,640 (D) show extensive areas of neovascularization.

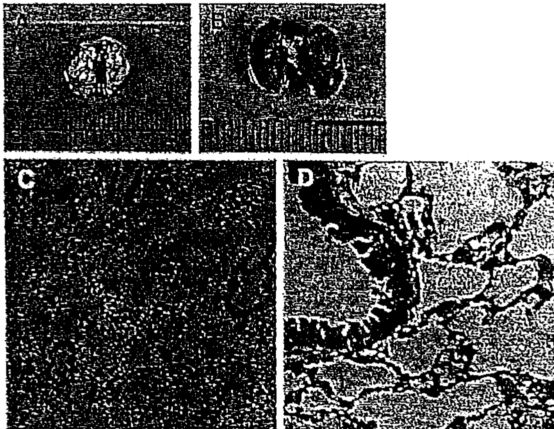

Figure 6

Photographs of lung from mice received Manda for 7-days before and 21-days after the LL/2 cell injection. Lung sections without any tumor nodule are seen (A and B). Histological micrographs of the lung show with magnifications: X 410 (C) and X 1,640 (D). The arrow indicates a tumor nodule with a lot of blood vessels.

The mice received Manda for 21 days only after the cell inoculation without prior manda treatment, showed apparently normal shaped lung macroscopically (Figs. 7A and B), but upon microscopical examinations (magnifications of X~400 and X~1,640), 5 out of 6 revealed perivascular cuffling of metastases without neovascularization (Figs. 7C and D). These results clearly indicate that the 7 days Manda pretreatment and subsequent LL/2 cell inoculation is much more effective to reduce metastasis in mouse lung.

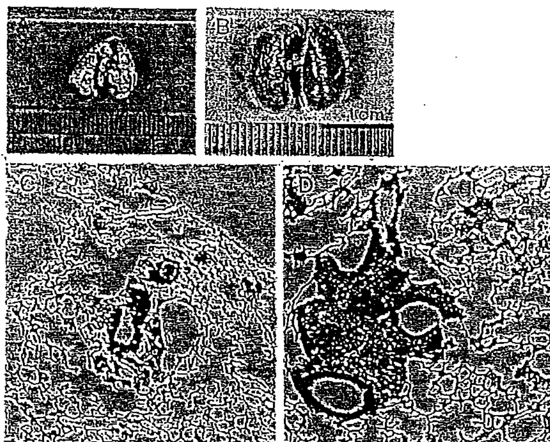

Figure 7

Photographs of lung nodule from mice treated with Manda for 21 days after the LL/2 cell injection. Normal shaped lungs are seen (A and B). Histological examinations indicate a small nodule formed around bronchioles and pulmonary blood vessels with magnifications for both X 410 (C and D).

Discussion

In the present study, we have demonstrated that both an oxidative stress and the transplantable lung tumor nodules in experimental animals can be alleviated by administrations of Manda and its fraction. We have employed CCl_4 and PQ to yield an oxidative stress. CCl_4 is proposed to produce free radicals interacting with membranous lipid during its metabolism, thereby inducing lipid peroxidation (7). This process is being considered as a major mechanism for CCl_4 hepatotoxicity. Therefore, the compound has often been used as a free radical-generating agent to estimate *in vivo* antioxidant activity of experimental drugs (8,9). It is also generally accepted that lung injury caused by PQ is due to the generation of free radicals (10). Several investigators have demonstrated that biological or non-biological antioxidants ameliorate the PQ-induced toxic effects including lipid peroxidation (11,12).

Our results show that CCl_4 or PQ treatment resulted in a significant elevation of lipid peroxidation measured by TBARS in mice livers and lungs (Figs. 1-4). On the contrary, treatment of mice with MF for 2 weeks before CCl_4 or PQ administration resulted in a reduction of the elevated levels of lipid peroxidation in both cases. Another line of study carried out with C57BL/6 black mice bearing lung tumor nodules also showed that oral administration of unfractionated Manda to the animals before and after tumor cell inoculation inhibited the formation of the nodules in their lungs (Figs. 6A,B, and 7A,B). This observation have been further confirmed by microscopical examinations of tumor histology. Although there are some variations in size of the tumor nodules, nevertheless, comparing the two Manda treated groups, for 28-days and for 21-days, there are clear differences in their histology (Figs. 6C,D and 7C,D). Almost complete remission of tumor nodules with little or no neovascularization are seen in the former group who received 7 extra days of Manda treatment before the cell inoculation, suggesting Manda has an ability to prevent proliferation of cancer cells as well.

Indeed, it has been previously reported that Manda is a scavenger for free radicals such as hydroxyl, superoxide and diphenyl-p-picrylhydrazyl radicals *in vitro* (13), and inhibits formation of TBARS in iron-induced epileptic foci in rats (14). In

addition, long-term administration of Manda to rats significantly suppressed an age-related increase of lipid peroxidation (15). All these findings together with the present observations point out that Manda has an antioxidant activity against free radical-induced tissue damage.

Free radicals have long been thought to contribute to cause carcinogenesis (16). It has been also postulated that free radical-induced damage to the vascular endothelium facilitates tumor cell metastasis. This event may be secondary to up-regulation of endothelial cell adhesion molecules for cancer cells (17). Injured endothelial cells could release also proteases that can degrade the basement membrane and facilitate cancer invasion (18). Free radicals which attack endothelial cells can be produced by external agents, host cells, and cancer cells (19). Based on our experimental results, it is concluded that the Manda by scavenging free radicals could contribute, in part, to prevent oxidative stress and of tumor nodule metastasis.

Acknowledgement

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References

1. Y. MATSUURA, M. TAKAGAKI and S. MATSUURA, S. Preparation of ME from various vegetables. Satellite symposium of the 2nd international congress of pathophysiology. 32, 1994.
2 G. BLOCK and L. LANGSETH. Food Technology, 48: 80-84, 1994.
3. R.J.B. KING(1996). Cancer biology. Longman. pp. 42-48.
4. H. OKUDA and M. TAKARA. Basic and clinical studies on ME. Satellite symposium of the 2nd International congress of pathophysiology. 35, 1994.
5. W.I. HWANG, Y.K. HWANG, J.Y. LEE, J.Y. LEE and H. OKUDA. Natural Product Science 2: 29-36, 1996.
6. M. UCHIYAMA and M. MIHARA. Anal. Biochem. 86: 271-278, 1978.
7. M.L. BERGER, H. BHATT, B. COMBES and R.W. ESTABROOK. Hepatology. 6: 36-45, 1986.
8. S.P. IP and K.M. KO. Biochem. Pharmacol. 52: 1687-1693, 1996.
9 G.H. WOLFGANG, R.A. JOLLY, W.J. DONARSKI, R. OCHOA and T.W. PETRY. J. Biochem. Toxicol. 5: 167-174, 1990.
10. J.S. BUS, S.D. AUST and J.E. GIBSON. Environ. Health Perspect. 16: 139-146, 1976.
11. Z.E. SUNTRES and P.N. SHEK. Biomed. Environ. Sci. 8: 289-300, 1995.
12. N.A. VAN DER WALS, L.L. SMITH, J.F. VAN OIRSCHOT and B.S. VAN ASBECK. Am. Rev. Respir. Dis. 145: 180-186, 1992.
13. M. KAWAI, S. MATSUURA and A. MORI. Free radical scavenging action of Manda. Clin. Report. 28: 393-397, 1993.
14. M. KAWAI, S. MATSUURA(1997). Manda scavenges free radicals and inhibits lipid peroxidation in iron-induced epileptic focus in rats, in HIRAMATSU, M., YOSHIKAWA, T., and INOUE, M. (eds.), Food and Free Radicals, Plenum Press, New York, pp. 471-480.
15. M. KAWAI, S. MATSUURA, M. ASANUMA and N. OGAWA. Neurochem. Res. 23: 459-465, 1998.
16. M.A. TRUSH and T.W. KENSLER(1991). Role of free radicals in carcinogen activation, in Sies, H.(ed), Oxidative stress: oxidants and antioxidants, Academic Press, London, pp. 279-315.
17. F.W. ORR and D.J.A. WARNER. Inv. Metastasis. 7: 183-196, 1987.
18. R. LAFRENIE, S. G. SHAUGHNESSY and W. ORR. Cancer Metastasis Rev. 11: 377-388, 1992.
19. T.P. SZATROVSKI and C.F. NATHAN. Cancer Res. 51: 794-798, 1991.

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