

IN VITRO ANTIOXIDANT AND ANTICANCER ACTIVITIES OF EXTRACTS FROM A FERMENTED FOOD

DONG CHUNG KIM

Department of Food Science
Suncheon First College
Suncheon 540-744, Korea

WOO-IK HWANG

Department of Biochemistry
Medical College
Korea University
Seoul 136-701, Korea

AND

MAN-JIN IN¹

Department of Human Nutrition and Food Science
Chungwoon University
Hongsung 350-701, Korea

Received for Publication August 16, 2002
Accepted for Publication March 14, 2003

ABSTRACT

Solvent extracts were prepared from Manda Enzyme[®], one of the fermented health foods, and their activities of radical scavenging and cancer cell growth inhibition were evaluated. Manda Enzyme[®] was extracted with 55% ethanol, and then fractionated into n-hexane, chloroform, ethyl acetate, methanol-soluble and methanol-insoluble fractions. The antioxidant activities were in the order chloroform > ethyl acetate > other fractions and of each fraction were positively related to the amount of total phenolics and the intensity of brown color. The cancer cell growth inhibitory activities were in the order n-hexane > chloroform > other fractions. Proliferation of HRT-18, HCT-48 and HepG2 human cancer cells was inhibited by the treatment of the n-hexane fraction of Manda Enzyme[®] at a concentration of 400 µg/mL to the extent of 75, 89 and 90%, respectively. From these results, it is considered that Manda Enzyme[®] has

¹ Corresponding author. Department of Human Nutrition and Food Science, Chungwoon University, San 29, Namjang-ri, Hongsung-eup, Hong Seong-gun, Chungnam 350-701, Korea. TEL: +82-41-630-3278; FAX: +82-41-632-3278; EMAIL: manjin@mailchungwoon.ac.kr

chemically different ingredients showing strong antioxidant and anticancer activity in vitro.

INTRODUCTION

As oxidation processes are involved in many degenerative physiological events such as cancer, aging, diabetes, cardiovascular disease, the protective defense systems are very important for health (Gutteridge 1995). Free radicals, one of the major oxidants, are of great importance in the aging process, and in disease. Antioxidants are substances that prevent or delay the oxidation of cellular oxidizable substrates. As many biological functions of antioxidants, such as antimutagenicity, anticarcinogenicity and antiaging have been known, antioxidants are one of the important defense systems in living cells. There are two basic categories of antioxidants, namely, synthetic and natural. Synthetic antioxidants such as BHA and BHT have been used as antioxidants since the beginning of the twentieth century. Restrictions on the use of these compounds, however, are being imposed because of their carcinogenicity. Therefore, there is increasing interest in natural dietary antioxidants, including vitamins and nonessential dietary antioxidants. The antioxidant activity of several plant materials and food has recently been reported (Velioglu *et al.* 1998; Nagai *et al.* 2001).

Cancer, one of diseases caused by oxidative damage is one of the most difficult problems in modern medicine. Most of the anticancer medicines used recently are chemically synthetic compounds. Repeated use of these compounds has led to the development of resistance to the agents in the tumor cell lines and adverse effects on human health such as alopecia, leucopenia, sterility and secondary malignancies in clinical trials. Therefore, several attempts have been made to develop new anticancer and cancer prophylactic agents from naturally occurring compounds or fermented food, which have fewer side effects. Also, the possibility that fermented food itself might control cancer by oral diet without any adverse side effect on human health was provided (Biffi *et al.* 1997; Choi and Park 1999).

Numerous fermented foods have been claimed to be physiologically functional as anticancer, anticoagulant and antioxidant activity. One of these is Manda Enzyme[®], which is known to be a health food in Korea and Japan for a number of years. It is manufactured by yeast fermentation of mixtures of several vegetables, fruits, grains and seaweeds and is naturally matured at low temperature for over 39 months. The immunopotentiating effect (Hwang *et al.* 1996), *in vivo* antioxidant and antitumor effects of small size molecules originating from Manda Enzyme[®] (Kim *et al.* 1998), the suppression effect against lipid peroxidation in the senescent rat brain (Kawai *et al.* 1998) and

antithrombin activity against blood coagulation (Kim *et al.* 2000) seem to be in support of the claim for Manda Enzyme®.

In the present study, as part of our investigation to examine the physiological functionality of fermented foods, we observed that the organic solvent extracts of Manda Enzyme® have free radical scavenging activities and anticancer abilities as judged by inhibition of cell proliferation.

MATERIALS AND METHODS

Sample and Chemicals

Manda Enzyme® was obtained from Manda Fermentation Co., Ltd. (Hiroshima, Japan). The human rectal cancer cell line (HRT-18), human hepatoma cell line (HepG2) and human colon cancer cell line (HCT-48) were purchased from American Type Culture Collection (Manassa, VA). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS) and antibiotic-antimycotic were from Life Technologies (Grand Island, NY); 1,1-diphenyl-2-picrylhydrazyl (DPPH), *N,N'*-dimethylformamide (DMF) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were products of Sigma Chemical Co. (St. Louis, MO). Other reagents were of analytical grade.

Preparation of Manda Enzyme® (ME) Fractions

The ME (145 g, wet weight) was suspended in 200 mL distilled water. Cold absolute ethanol was added to the suspension to make a final concentration of 55% (w/v). The mixture was stirred at 4°C for overnight and centrifuged at 3000 × g for 15 min. The pellet was discarded and supernatant was concentrated with evaporator under reduced pressure. The concentrate (142 mL) was extracted successively with *n*-hexane, chloroform and ethyl acetate of 360 mL, respectively. The final aqueous layer was concentrated under reduced pressure, mixed slowly with 120 mL of methanol, incubated at 4°C for 15 h, and divided into methanol soluble and insoluble fractions by centrifugation. All extracts were evaporated and freeze-dried, yielding five ME fractions.

Free Radical Scavenging Activity

Free radical-scavenging activities of ME fractions were assayed using a stable free radical, DPPH dissolved in ethanol, according to the method of Blois (1958). In the presence of a radical scavenger, DPPH is converted to 1,1-diphenyl-2-picrylhydrazine with yellow color. The reaction mixture contained 0.4 mL of 0.2 mM DPPH and 0.2 mL of the ME fraction sample (dissolved in ethanol) at different concentration (0 ~ 1.6 mg/mL) except for methanol-

insoluble fraction (dissolved in DW). Finally, the total volume of the reaction mixture was adjusted to 2.0 mL with ethanol. After the reaction mixture was incubated at 37°C for 30 min, the free radical scavenging activity of each fraction was monitored by decrease of absorbance at 517 nm. The radical scavenging activity was calculated as follows;

$$\text{Radical scavenging activity (\%)} = \left(1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of blank}}\right) \times 100$$

Human Cancer Cell Proliferation Inhibition Activity

The tetrazolium dye colorimetric test is used to monitor cell proliferation indirectly as indicated by the conversion of the tetrazolium salt to the colored product, formazan, the concentration of which can be measured spectrophotometrically (Park *et al.* 1987). Briefly, human cancer cell line was cultured in 48-well microplates [1×10^4 cells/well in 1 mL of complete DMEM (containing 5% FBS, 100 units/mL of penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B, pH 7.4)] for 24 h. After 24 h culture, media were changed with fresh complete DMEM containing ME fractions (0 ~ 4 mg/mL). At the end of 40 h incubation, cells were washed with 0.5 mL of PBS. And then 0.1 mL of MTT (0.5 mg/mL) and 0.2 mL of complete DMEM were added to each well and incubation was allowed to continue for a further 2 h. Finally, 0.7 mL of lysis buffer (50% DMF and 20% SDS, pH 4.6) was added to each well and incubated for another 2 h. The absorbance of each well was read using a spectrophotometer (Beckman model DU-64) at 540 nm.

Determination of Total Phenolics

Total phenolics were determined by reaction with Folin-Ciocalteu reagent (Singleton and Rossi 1965). One milliliter of ME fractions (1 mg/mL) was mixed with 1.0 mL of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) and allowed to stand at 25°C for 5 min; 1.0 mL of sodium bicarbonate (60 g/L) solution was added to the mixture. After incubation at 25°C for 90 min, absorbance was measured at 725 nm. Content of total phenolics were expressed in terms of ferulic acid equivalents.

RESULTS AND DISCUSSION

Preparation of ME Fractions

As the inhibitory effects of crude ME against induced oxidative stress by CCl_4 and growth of solid tumor had been demonstrated (Kim *et al.* 1998), ME was divided into n-hexane-, chloroform-, ethyl acetate-, methanol soluble-, and methanol insoluble-fraction to identify the bioactive compounds showing antioxidant and anticancer activities. The yield and some characteristics regarding antioxidant activity of ME fractions are shown in Table 1. The yields were in the order of methanol soluble-fraction > methanol insoluble-fraction > ethyl acetate-fraction > chloroform-fraction > n-hexane-fraction. And the yields decreased as the hydrophobicity of extraction solvent increased. The amount of total phenolics in the each fractions varied between 0.29 and 8.24%, with the highest amount found in chloroform-fraction and lowest amount in n-hexane-fraction.

TABLE 1.
SOME CHARACTERISTICS OF ME FRACTIONS

Fraction	Yield [%(w/w)]	Color Intensity ^a	Total Phenolics ^b (% as Ferulic Acid)
n-Hexane	0.32	0.074	0.29 ± 0.024
Chloroform	0.39	0.329	8.24 ± 0.17
Ethyl acetate	0.49	0.211	6.83 ± 0.31
Methanol insoluble	2.88	0.077	0.62 ± 0.021
Methanol soluble	67.61	0.068	0.63 ± 0.035

^a Color intensity were expressed as absorbance (at 420 nm) of 0.1% (w/w) solution in ethanol.

^b Data expressed as the mean ± SD (n=3).

Antioxidant Activity of ME Fractions

The DPPH radical was used to measure the antioxidant activity of the ME fractions. The free radical scavenging activities of ME fractions were investigated, and these results are shown in Fig. 1 and Table 2. The order of RC_{50} of ME fractions, i.e. the amount required for 50% decolorization of 4.73 μg DPPH solution, was chloroform-fraction > ethyl acetate-fraction > other fractions. Chloroform- and ethyl acetate-fraction exhibited strong free radical scavenging activity. And also, these activities of chloroform- and ethyl acetate-fraction tended to increase with increasing sample concentration (Fig. 1). The radical scavenging activities of chloroform- and ethyl acetate-fraction were 81.5 and

66.0% when 185 and 118 $\mu\text{g}/\text{mL}$ were added to the system, respectively. On the other hand, other fractions decolorized the DPPH solution only slightly. The radical scavenging action of plant constituents has been found to be linked with phenolic compounds (Madsen *et al.* 1996).

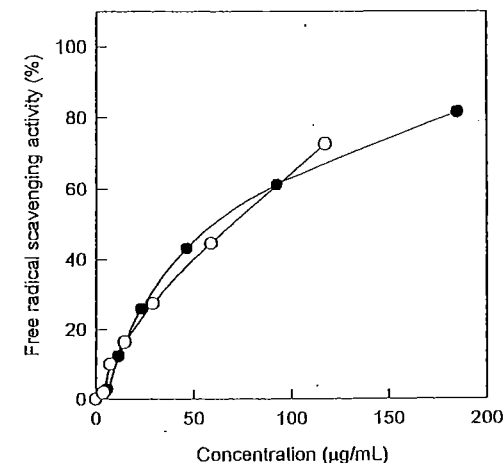


FIG. 1. FREE RADICAL SCAVENGING ACTIVITY OF CHLOROFORM (●) AND ETHYL ACETATE (○) FRACTIONS AS A FUNCTION OF THE CONCENTRATION OF FRACTION IN THE MIXTURE OF THE ME FRACTION AND DPPH

As ME is a fermentation product of plants such as vegetables, fruits and seaweeds, ME also has many phenolic compounds. The antioxidant activities of ME fractions were positively related to the amount of total phenolics and the intensity of brown color. These results implied that radical scavengers of chloroform- and ethyl acetate-fraction might be phenolic compounds.

Anticancer Activity of ME Fractions

To determine the inhibitory effects of ME fractions on the growth of human cancer cells, ME fractions were added to the culture medium of HT-18 cells and then the viability of cancer cells was measured by MTT assay. Among the ME fractions, n-hexane- and chloroform-fraction showed inhibitory effect on the growth of HT-18 cells, i.e., 50.0% and 24.2% of inhibition were observed at 200 $\mu\text{g}/\text{mL}$ of n-hexane- and chloroform-fraction, respectively, (Fig. 2). The inhibition rate of n-hexane-fraction were observed 20, 50, 75% with the addition of 100, 200, 400 $\mu\text{g}/\text{mL}$, while those of chloroform-fraction were 10, 24, 50%, respectively (Fig. 3A). Both n-hexane- and chloroform-fraction also had inhibitory effects on the proliferation of HCT-48 cells and HepG2 cells (Fig. 3B

and C). The growth inhibitory activities of n-hexane- and chloroform-fraction were increased with increasing the concentration of each fraction and these trends of inhibitory effect on cancer cell growth were similar to HT-18, HCT-48 and HepG2 cells.

TABLE 2. SCAVENGING ACTIVITIES OF ME FRACTIONS ON FREE RADICALS

Fraction	RC ₅₀ on DPPH Radical (µg) ^a
n-Hexane	> 1200
Chloroform	137.2 ± 1.9
Ethyl acetate	161.7 ± 8.5
Methanol insoluble	> 2800
Methanol soluble	> 1600

^a The amount of sample that required decolorization of DPPH(4.73 µg) solution by 50%(RC₅₀) was determined by linear regression. Results were presented as the mean ±SD of three replicates.

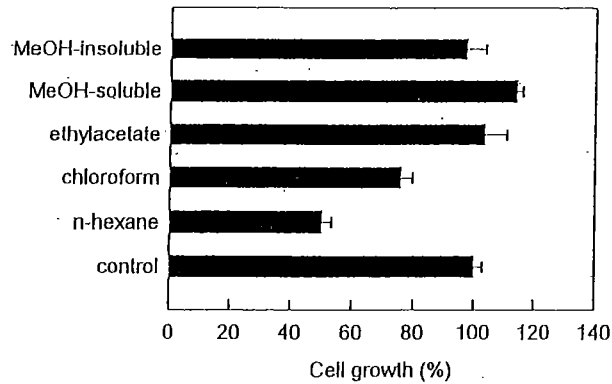


FIG. 2. EFFECT OF ME FRACTIONS ON HUMAN RECTAL CANCER CELL LINE (HT-18) GROWTH

For the MTT assay, cancer cells were preincubated in 48-well microplates (1 × 10⁴ cells/well) for 24 h and then incubated with ME fractions (200 µg/mL) for 40 h. Data represent the mean ± SD (n=3).

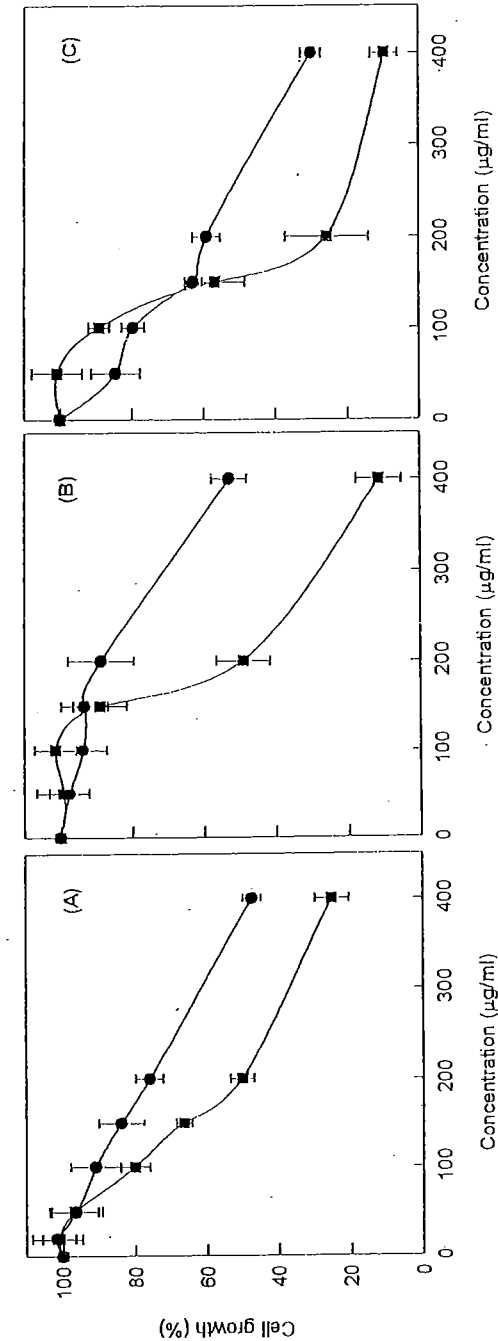


FIG. 3. EFFECT OF n-HEXANE (●) AND CHLOROFORM (■) FRACTIONS ON HUMAN RECTAL (PANEL A), HUMAN COLON (PANEL B) AND HUMAN HEPATOMA (PANEL C) CANCER CELL GROWTH AS A FUNCTION OF THE CONCENTRATION OF FRACTION IN THE DMEM MEDIUM

Cell growth was measured by MTT assay. The percentage of cell growth in the control group was 100%. Data represent the mean ± SD (n=3).

To observe the effects of n-hexane- and chloroform-fraction against cancer cell growth according to culture time, HT-18 cells containing 100, 200 and 400 $\mu\text{g}/\text{mL}$ of two fractions were respectively cultured for 24, 48 and 72 h, and cell growth is illustrated in Fig. 4. The growth rate of HT-18 cancer cells was inhibited in proportion to the incubation time. These results show that organic extract of ME potently inhibited the proliferation of some human cancer cell lines *in vitro*. The types of cytotoxic effect by anticancer reagents include: (1) only dose-dependent, (2) only time-dependent, and (3) simultaneously dose- and time-dependent (Ohoshi and Sugeno 1975). From the above results, it is considered that the effects of organic extract of ME are simultaneously dose- and time-dependent. Therefore, if dose and time are well controlled *in vitro*, it is expected that organic extract of ME is successful medicine to inhibit growth or to kill off human cancer cells.

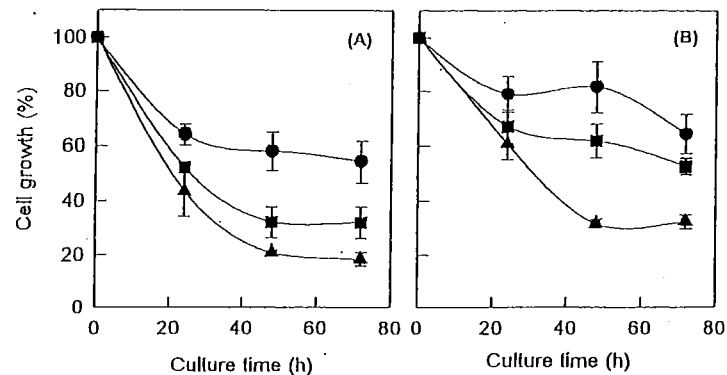


FIG. 4. ANTIPROLIFERATION EFFECT OF n-HEXANE (PANEL A) AND CHLOROFORM (PANEL B) FRACTIONS ON HUMAN RECTAL CANCER CELL LINE AS A FUNCTION OF CULTURE TIME

Cancer cells were preincubated in 48-well microplates (1×10^4 cells/well) for 24 h and then incubated with n-n-hexane and chloroform fractions (100, 200 and 400 $\mu\text{g}/\text{mL}$), respectively. Data represent the mean \pm SD (n=3).

It has been reported that n-hexane fraction of *Doenjang* (a natural fermented Korean soy paste) had the highest inhibitory effect on the growth of HT-29 human colon carcinoma cells (Park *et al.* 2000). In this work, n-hexane-extract of ME also possessed the highest anticancer activity *in vitro*.

With these results, the *in vitro* antioxidant and anticancer effects of ME were confirmed and it was shown that different fractions of ME have strong antioxidant and anticancer activity *in vitro*. The isolation and further identification of effective compounds are in progress.

ACKNOWLEDGMENTS

Financial support from Manda Fermentation Co., Ltd. (Hiroshima, Japan) is deeply appreciated.

REFERENCES

- BIFFI, A., CORADINI, D., LARSEN, R., RIVA, L. and DI FRONZO, G. 1997. Antiproliferative effect of fermented milk on the growth of a human breast cancer cell line. *Nutr. Cancer* 28, 93-99.
- BLOIS, M.S. 1958. Antioxidant determinations by the use of a stable free radical. *Nature* 181, 1199-1200.
- CHOI, W.-Y. and PARK, K.-Y. 1999. Anticancer effects of organic chinese cabbage *Kimchi*. *J. Food Sci. Nutr.* 4, 113-116.
- GUTTERIDGE, J.M.C. 1995. Lipid peroxidation and antioxidants as biomarkers of tissue damage. *Clin. Chem.* 41, 1819-1828.
- HWANG, W.I., HWANG Y.-K., LEE, J.-Y., LEE, J.-Y. and OKUDA, H. 1996. Antitumor and immunopotentiating effects of Manda Enzyme. *Natural Product Sci.* 2, 29-36.
- KAWAI, M., MATSUURA, S., ASANUMA, M. and OGAWA, N. 1998. Manda[®], a fermented natural food, suppresses lipid peroxidation in the senescent rat brain. *Neurochem. Res.* 23, 455-461.
- KIM, D.C., OKUDA, H., HWANG, W.I. and JUNG, J. 2000. Isolation and partial characterization of a polysaccharide with antithrombin activity against blood coagulation in Manda[®], a fermented natural food. *Agric. Chem. Biotechnol.* 44, 235-239.
- KIM, J.-H., PARK, M.-K., LEE, J.-Y., OKUDA, H., KIM, S. and HWANG, W.I. 1998. Antioxidant and antitumor effects of Manda. *Biochem. Arch.* 14, 211-219.
- MADSEN, H.L., NIELSEN, B.R., BERTELSEN, G. and SKIBSTED, L.H. 1996. Screening of antioxidative activity of spices. A comparison between assays based on ESR spin trapping and electrochemical measurement of oxygen consumption. *Food Chem.* 57, 331-337.

- NAGAI, T., SAKAI, M., INOUE, R., INOUE H. and SUZUKI, N. 2001. Antioxidative activities of some commercial honeys, royal jelly, and propolis. *Food Chem.* 75, 237–240.
- OHOSHI, A. and SUGENO, H.G. 1975. *Culture of Human Cancer Cells*, Asagurashoten, Tokyo, Japan.
- PARK, J.G., KRAMER, B.S., STEINBER, C.J., COLLINS, J.M., MINNA, J.D. and GAZDAR, A.F. 1987. Chemosensitivity testing of human colorectal carcinoma cell lines using a tetrazolium-based colorimetric assay. *Cancer Res.* 47, 5875–5879.
- PARK, K.-Y., LEE, J.-M., MOON, S.-H. and JUNG, K.-O. 2000. Inhibitory effect of *Doenjang* (fermented Korean soy paste) extracts and linoleic acid on the growth of human cancer cell lines. *J. Food Sci. Nutr.* 5, 114–118.
- SINGLETON, V.L. and ROSSI, J.A. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* 16, 144–158.
- VELIOGLU, Y.S., MAZZA, G., GAO, L. and OOMAH, B.D. 1998. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. *J. Agric. Food Sci.* 46, 4113–4117.