

Growth inhibitory potential of effective microorganism fermentation extract (EM-X) on cancer cells

CHUNG HIN CHUI^{1,2,3}, GREGORY YIN MING CHENG³, BIN KE⁴, FUNG YI LAU³, RAYMOND SIU MING WONG³, STANTON HON LUNG KOK^{1,2}, SARWAT FATIMA², FILLY CHEUNG², CHOR HING CHENG², ALBERT SUN CHI CHAN^{1,2} and JOHNNY CHEUK ON TANG^{1,2}

¹Central Laboratory, Institute of Molecular Technology for Drug Discovery and Synthesis, ²Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University; ³Department of Medicine and Therapeutics, Prince of Wales Hospital, The Chinese University of Hong Kong, Hong Kong, P.R. China; ⁴EM Research Organization, Okinawa, Japan

Received April 14, 2004; Accepted July 8, 2004

Abstract. The effective microorganism (EM-X) fermentation extract is derived from rice bran and seaweed extract. It has been shown to possess anti-oxidation activity both *in vitro* and *in vivo*. To our knowledge, the possible *in vitro* anti-cancer potential of EM-X has not been demonstrated. Here we showed that the double concentrate of EM-X (EM-X2) at concentrations of 20-30% by volume, had growth inhibitory activity on MDA-MB231 breast cancer cell line and K-562 chronic myelogenous leukaemia cell lines by [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-H-tetrazolium, inner salt] (MTS) assay. No characteristic features of apoptosis could be observed morphologically. Colony formation assay illustrated that both MDA-MB231 breast cancer and K-562 CML cells lost part of their regeneration potential after treatment with EM-X2 at 30% concentration by volume for 24 h. At these concentrations, only slight growth inhibitory effect was observed in 293 human kidney fibroblast cells and in three non-malignant bone marrows. Intracellular nitro blue tetrazolium (NBT) reduction assay showed that both MDA-MB231 breast cancer and K-562 CML cells had about 30% reduction of intracellular NBT after incubation with 30% of EM-X2. Increased activity of superoxide dismutase (SOD) could be detected from both MDA-MB231 and K-562 cell lines after incubating with 30% of EM-X2. Taken together, our data suggested that EM-X could inhibit growth and reduce the regeneration potential of cancer cells, possibly through its antioxidation activity.

Introduction

Cancer is a leading cause of death. Traditional chemotherapy and radiotherapy have many side effects. There is now great interest in the use of natural products in cancer prevention. A well-recognized example is green tea. It has been demonstrated that green tea extracts and green tea catechins such as (-)-epicatechin and (-)-epigallocatechin have anti-cancer activities both *in vitro* and *in vivo* (1). Green tea inhibits vascular endothelial growth factor, fibroblast growth factor and tumour angiogenesis (2,3). In addition, many plant extracts with antioxidation properties are also found to have anti-cancer properties. Examples include the 70% ethanol extract from the seed of *Oenothera biennis* L, one of the species of evening primroses. This ethanol extract of evening primroses (EPE) was shown to induce apoptosis in Ehrlich ascites tumour cells (4).

Effective microorganism fermented extract (EM-X) is a refreshment drink commonly found in East Asia. This is produced by fermentation of papaya, seaweed extract and rice bran using effective microorganisms such as photosynthetic bacteria, lactic acid bacteria and yeast. EM-X is rich in flavonoids, minerals, lycopene, saponin, ubiquinone and α -tocopherol. It was reported that EM-X significantly reduced tumour necrotic factor α and hydrogen peroxide induced release of interleukin-8 from human alveolar epithelial cell. EM-X also inhibited ascorbic acid and ferric chloride-induced phospholipid liposomal non-enzymatic peroxidation in ox brain in a dose dependent manner (5). These data showed that the flavonoid-rich EM-X have antioxidant properties (6) and anti-cancer potentials. We studied the possible growth inhibitory activity of EM-X2, the double concentrate of EM-X, on MDA-MB231 breast cancer cells and the K-562 chronic myelogenous leukaemia cells.

Materials and methods

EM-X2 extract. The EM-X2 extract was kindly provided by the EM Research Organization (Okinawa, Japan) in a sterile and liquid form. It was kept at 4°C in the dark and aliquoted until use.

Correspondence to: Professor Gregory Cheng Yin Ming, Department of Medicine and Therapeutics, Prince of Wales Hospital, The Chinese University of Hong Kong, Ngan Shing Street, Sha Tin, Hong Kong, P.R. China, E-mail: gcheng@cuhk.edu.hk
Dr Tang Cheuk On, Johnny, Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Hong Kong, P.R. China
E-mail: bccoang@inet.polyu.edu.hk

Key words: effective microorganism fermentation extract, growth inhibitory potential

Cell lines and cell cultures. MDA-MB231 breast cancer cell line, K-562 chronic myeloid leukemia cell line, and the 293 human kidney fibroblast cell line were routinely maintained in RPMI-1640 culture medium (JRH Biosciences) supplemented with 10% fetal bovine serum (Hyclone) in a 5% carbon dioxide incubator with 37°C (Napco).

Primary bone marrow cultures from non-malignant bone marrow. After informed consent, bone marrow samples were obtained from 3 patients (2 male and 1 female) with non-malignant haematological disorders. Mononuclear cells were enriched and harvested immediately after Ficoll density centrifugation (Pharmacia L.K.B.) and washed with phosphate buffered saline (PBS), cell pellets were resuspended in RPMI-1640 culture medium and cell viability was determined by Trypan blue exclusion assay as described (7).

Growth inhibition assay. Changes in the cellular viability of EM-X2 treated cells were monitored using the MTS activity assay as reported previously (8). Briefly, cells were seeded on day 0 in a 96-well microtiter plate (Yixie from Valency Company). After 24 h of seeding, the culture medium was removed while fresh complete medium with EM-X2 at different concentrations by volume was added. After 48 h of incubation, EM-X2 culture medium was removed and MTS in PBS solution was added. Following 60 min of incubation, optical absorbance at 490 nm was determined (Victor²V 1420 multilabel HTS counter, Perkin Elmer, Wallac) according to the user manual provided (Promega).

Intracellular reduction of nitroblue tetrazolium assay. Changes in the intracellular superoxide anion level were reflected by the intracellular nitro blue tetrazolium (NBT) reduction assay. Briefly, cancer cells were seeded on day 0. On day 1, culture medium was removed and the cells were incubated with phenol red-negative RPMI-1640 complete medium containing NBT (1 mg/ml in phosphate buffered saline) and different concentrations of EM-X2. After 48 h, the culture medium was removed and the cancer cells were washed with phosphate buffered saline. The cells were lysed releasing the reduced NBT into the lysis buffer. Optical absorbance was measured. The degree of NBT reduction reflected the intracellular level of superoxide anion.

Morphological monitoring of EM-X2 treated cells. After EM-X2 treatment, morphological changes and intracellular reduction of NBT from EM-X2 treated cells were monitored by examinations under an inverted microscope (Nikon).

Measurement of superoxide dismutase activity. Briefly, after 48 h of EM-X2 treatment, cells were washed with phosphate buffered saline and then resuspended in lysis buffer. Afterwards, cellular extract was divided into two parts. One part was mixed with xanthine buffered solution containing xanthine and xanthine oxidase. Then NBT solution was added and they were incubated for a further 15 min. The reaction was terminated by adding sodium dodecyl sulfate solution. The optical absorbance was then determined and recorded. The remaining cellular extract was used for protein standardization using the Bradford method (Bio-Rad). Finally, the result of

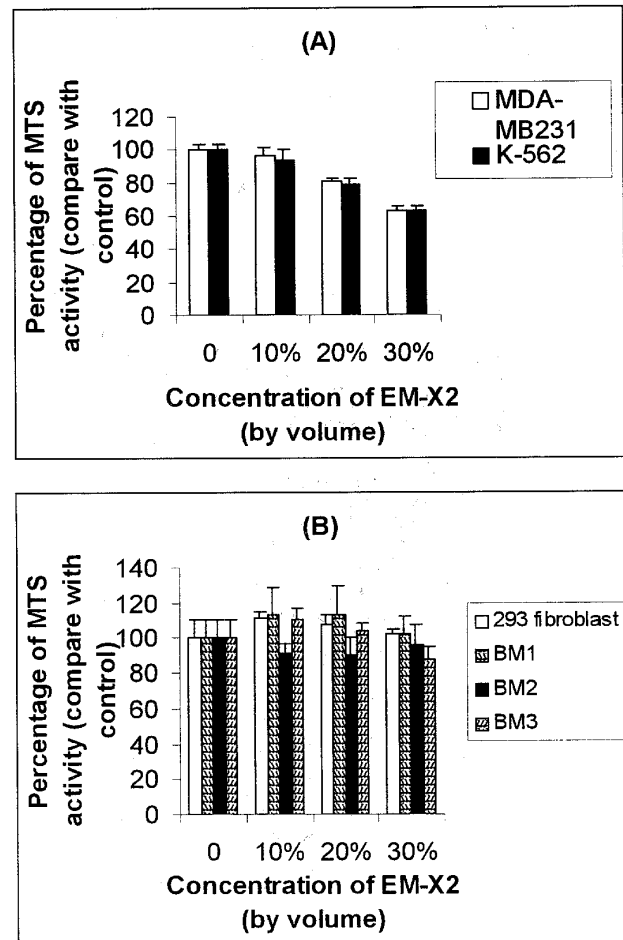


Figure 1. A, Growth inhibitory potential of EM-X2 on MDA-MB231 breast cancer cells and K-562 chronic myelogenous leukaemia cells as assayed by MTS activity after 48 h of EM-X2 incubation. B, Effect of EM-X2 on 293 human kidney fibroblast and bone marrow cells. Results are shown as mean \pm SD of triplicate tests. It is a representative of three independent experiments.

superoxide dismutase activity in terms of arbitrary unit was normalized with cellular protein content and compared with the untreated control.

Colony formation assay. The cancer cell lines MDA-MB231 and K-562 were incubated with various concentrations of EM-X2 for 24 h. The cells were then harvested, washed, stained with Trypan blue and counted. For MDA-MB231 breast cancer cells, they were cultured in completed RPMI-1640 medium while K-562 CML cells were cultured in methylcellulose semi-solid medium (Stem Cell Technology, Canada). Cells were incubated for 7-14 days. For MDA-MB231 cells, they were stained with methylene blue and then counted. For K-562 cells, they were directly counted under an inverted microscope (Nikon).

Results

Growth inhibitory activity of EM-X2 on MDA-MB231 and K-562 cancer cells. After 48 h incubation, we observed that EM-X2 inhibited the growth of both MDA-MB231 breast cancer cell and K-562 CML cell in a dose-dependent manner at 10-30% concentrations by volume (Fig. 1A) using the MTS

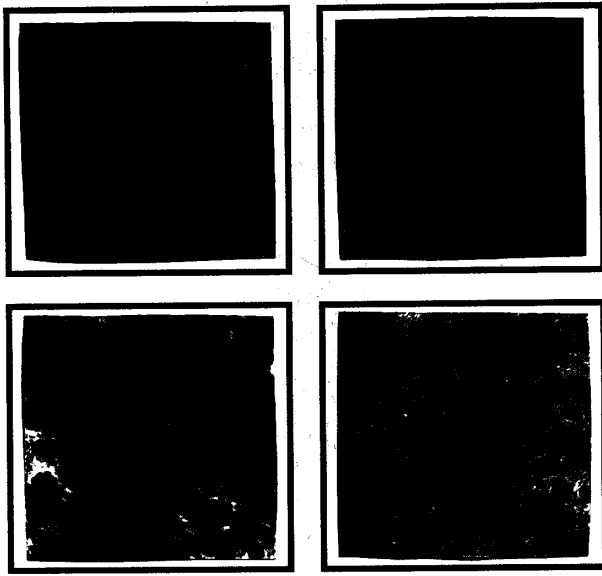
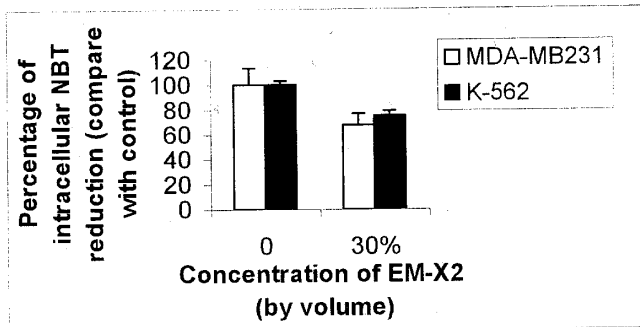


Figure 2. Upper panel, Intracellular NBT reduction assay on both MDA-MB231 breast cancer cells and K-562 chronic myelogenous leukaemia cells after 24 h of EM-X2 incubation. Lower panel, Photomicrograph showing untreated MDA-MB231 breast cancer cells and untreated K-562 chronic myelogenous leukaemia cells (left) when compared with 30% of EM-X2 treatment (right). Results are shown as mean \pm SD of triplicate tests. Figures are representative of three independent experiments.

activity assay. However, characteristic feature of apoptosis such as cell shrinkage could not be detected in either cancer cell line (Fig. 2). At these EM-X2 concentrations, only slight growth inhibitory activity was detected on 293 human kidney fibroblasts and non-malignant bone marrow cells (Fig. 1B). We have tested whether there was any direct redox reaction between the EM-X2 and MTS. The results were negative, showing that the changes in MTS from yellowish green to brown was due to the enzymatic activity which reflected the viability of cells. There was no significant change in the physical property (e.g. pH value) of culture medium after diluting with EM-X2.

Intracellular reduction of nitroblue tetrazolium assay. Both MDA-MB231 breast cancer cells and K-562 CML cells showed significant decrement in intracellular NBT reduction after 48 h of incubation with EM-X2 at 30% concentration by volume. We noticed that the decrement of intracellular NBT reduction was almost even among cell population when compared with untreated control. Thus the result was not due to the difference in cell number. Representative results are shown in Fig. 2. No direct

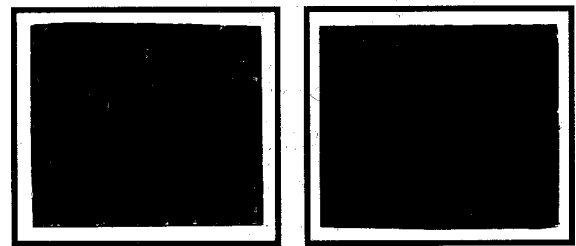
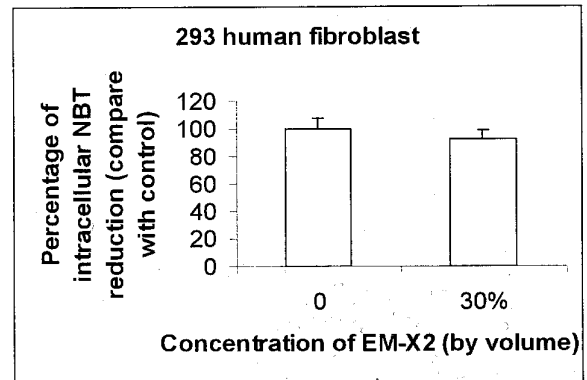


Figure 3. Upper panel, Intracellular NBT reduction assay on 293 human kidney fibroblast after 24 h of EM-X2 incubation. Lower panel, Photomicrograph showing untreated 293 human kidney fibroblast (left) when compared with 30% of EM-X2 treatment (right). Results are shown as mean \pm SD of triplicate tests. Figures are representative of three independent experiments.

chemical reaction was found between EM-X2 and NBT. Interestingly, the NBT reduction activity in the human kidney fibroblast 293 was not significantly affected by EM-X2 treatment (Fig. 3).

Changes of intracellular superoxide dismutase activity. After EM-X2 treatment, any change in the intracellular superoxide dismutase activity was determined in the form of its lysed cellular extract. Under normal physiological condition, superoxide dismutase catalyzes the conversion of superoxide anion to hydrogen peroxide and molecular oxygen. The relative activity of intracellular superoxide dismutase was increased about 10-15% when compared with untreated control after 48 h of 30% of EM-X2 incubation (Fig. 4, upper).

EM-X2 reduced the regeneration potentials of cancer cells. After incubating with 30% of EM-X2, both MDA-MB231 breast cancer and K562 leukaemia cells showed a reduction in colony formations by anchorage dependent assay and semi-solid medium colony formation assay respectively (Fig. 4, B).

Discussion

EM-X is a refreshment drink in Japan and it can be mixed with coffee or tea during consumption. Recently, the antioxidation property of EM-X has been demonstrated *in vitro* (5) and *in vivo* (9). Aruoma *et al* showed that oral administration of EM-X to rats for seven days inhibited ferric-nitrosyltriacetic acid (Fe-NAT)-dependent oxidation of fatty acids while docosapentanoic, docosahexanoic, oleic, arachidonic,

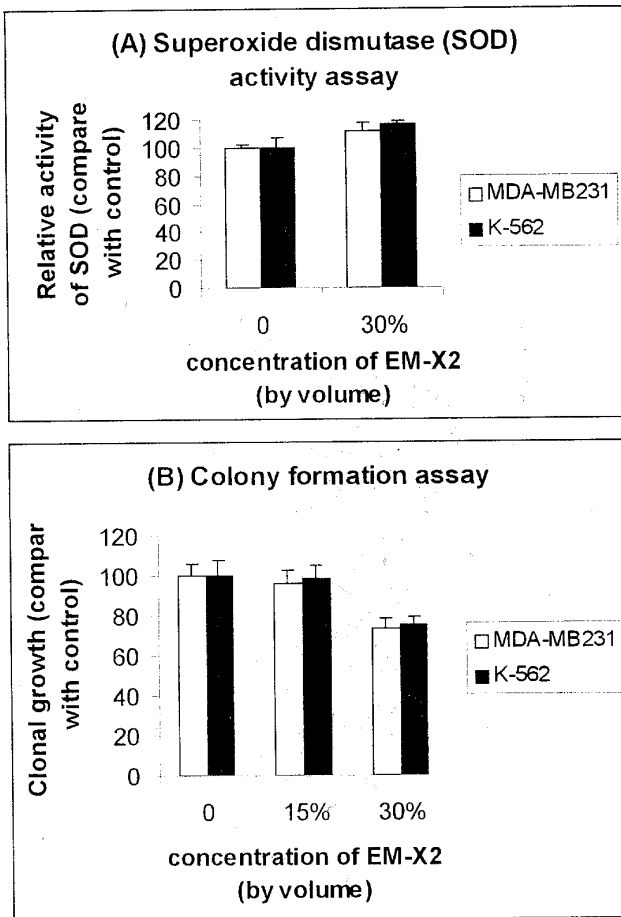


Figure 4. A, Measurement of superoxide dismutase activity from cellular extract. Cells were either untreated or treated with EM-X2 and their superoxide dismutase activity was estimated using xanthine-oxidase system. Results were compared with untreated control. B, Effect of EM-X2 on clonal growth of MDA-MB231 breast cancer and K-562 chronic myelogenous leukaemia cell lines. Colony formation assay for both MDA-MB231 breast cancer cells and K-562 chronic myelogenous leukaemia cells were performed after incubated with either 15% or 30% of EM-X2 for 24 h. Results are expressed as mean \pm SD of three independent studies. See Materials and methods for details.

eicosadienoic and linoleic acids were protected in the kidneys and liver. As α -tocopherol and glutathione levels were mainly unaffected, the EM-X effect was mainly due to the *in vivo* inhibition of lipid peroxidation. This is likely mediated by the bioactive flavonoids in EM-X (9). In addition, EM-X was shown to protect the retinal neurons in rats from the excitotoxicity of the glutamate agonist, N-methyl-D-aspartate (10). These antioxidant activities of EM-X might have anti-cancer potentials.

We first showed that EM-X2 had growth inhibition activity on MDA-MB231 and K-562 cell lines. The degree of growth inhibition was dose dependent in the tested EM-X2 concentrations of 10% to 30% by volume. We also observed that only slight growth inhibition could be detected in 293 human kidney fibroblast and bone marrow cells patients with non-malignant haematological disorders. The dose of EM-X2 which could inhibit the growth of both MDA-MB231 and K-562 cancer cells, had little toxicity on normal fibroblast and bone marrow cells. Since no characteristic

features of apoptosis were observed, it was possible that the cancer cells were arrested at certain phase(s) of cell cycle rather than going to the sub-G1/G0 apoptotic phase. Colony formation potency of both cancer cell lines was reduced with EM-X2, which also supported the above postulate.

A hypothesis has been put forward that the inhibition of tumour cell growth can be attributed to the increase in the steady state levels of hydrogen peroxide as a result of the increased dismuting activity of manganese superoxide dismutase (11). The EM-X fermentation extract contains components derived from plants such as saponin, flavonoid, γ -olizanol and it is rich in various types of vitamins. The above ingredients could directly eliminate free radicals, or increases the activity of superoxide dismutase by electrons transfer (12). As anticipated, EM-X2 could decrease intracellular NBT reduction and simultaneously increase superoxide dismutase activity. We speculated that the antioxidation property of EM-X2 could play an important role on the growth inhibition of both MDA-MB231 and K-562 cancer cells. Whether other possible mechanisms are involved would require further investigation.

In conclusion, our results suggested that the EM-X fermentation extract might have anti-cancer potential. Recently, we have shown that the fruit extract of *Gleditsia sinensis*, a traditional Chinese medicine, also has anti-angiogenic activity (13). We also discovered that both basic fibroblast growth factor and nitric oxide might be involved in the *Gleditsia sinensis* fruit extract induced growth inhibition (14). Currently, we are studying the possible antiangiogenic potential of EM-X on human umbilical cord blood endothelial cell model (15). Nude mice xenograft experiments should be carried out to investigate the possible anticancer activity of EM-X in an animal model. Since EM-X is a common refreshment drink, whether it could exert chemoprevention effects is of particular interest.

Acknowledgements

We acknowledge the support by the Area of Strategic Development (ASD) programme of the Hong Kong Polytechnic University (work programme A012). The work described in this paper was supported by the Areas of Excellence Scheme established under the University Grants Committee of the Hong Kong Special Administrative Region, China (Project no. AoE/P-10/01). Special appreciation to Professor G.Y.M. Cheng who sponsors Dr C.H. Chui for the purchase of the biological safety cabinet (Nuair, serial no.: 15371092700), the inverted microscope (Nikon, serial no.: 310844) and those related laboratory accessories. Special appreciation is also given to Professor A.S.C. Chan and Dr J.C.O. Tang who sponsor Dr C.H. Chui for the purchase of the Napco carbon dioxide cell culture incubator, micro-centrifuge and the Victor²V 1420 multilabel HTS counter (Wallac, Perkin Elmer). Dr C.H. Chui is supported by the post of 'Research Associate' from the project of 'Catalytic asymmetric synthesis of bioactive derivatives and the investigation of their anti-cancer activities' (1.12.37.8656) kindly offered by Professor A.S.C. Chan. Special thanks to the EM Research Organization (Okinawa, Japan) which provided the fermentation extract of EM-X2 for our study.

References

1. Sartippour MR, Heber D, Ma J, Lu Q, Go VL and Nguyen M: Green tea and its catechins inhibit breast cancer xenografts. *Nutr Cancer* 40: 149-156, 2001.
2. Sartippour MR, Shao ZM, Heber D, Beatty P, Zhang L, Liu C, Ellis L, Liu W, Go VL and Brooks MN: Green tea inhibits vascular endothelial growth factor (VEGF) induction in human breast cancer cells. *J Nutr* 132: 2307-2311, 2002.
3. Sartippour MR, Heber D, Zhang L, Beatty P, Elashoff D, Elashoff R, Go VL and Brooks MN: Inhibition of fibroblast growth factors by green tea. *Int J Oncol* 21: 487-491, 2002.
4. Arimura T, Kojima-Yuasa A, Watanabe S, Suzuki M, Kennedy DO and Matsui-Yuasa I: Role of intracellular reactive oxygen species and mitochondrial dysfunction in evening primrose extract-induced apoptosis in Ehrlich ascites tumour cells. *Chemico-Biol Inter* 145: 337-347, 2003.
5. Deiana M, Assunta Dessi M, Ke B, Liang YF, Higa T, Gilmour PS, Jen LS, Rahman I and Aruoma OI: The antioxidant cocktail effective microorganism X (EM-X) inhibits oxidant-induced interleukin-8 release and the peroxidation of phospholipids *in vitro*. *Biochem Biophys Res Commun* 296: 1148-1151, 2002.
6. Viana M, Barbes C, Bonet B, Bonet MV, Castro C, Fraile MV and Herrera E: *In vitro* effects of flavonoid-rich extract on LDL oxidation. *Atherosclerosis* 123: 83-91, 1996.
7. Chow LMC, Chui CH, Tang JCO, Teo I, Lau FY, Cheng GYM, Wong RSM, Leung TWT, Lai KB, Yau M, Gou D and Chan ASC: *Gleditsia sinensis* fruit extract is a potential chemotherapeutic agent in chronic and acute myelogenous leukemia. *Oncol Rep* 10: 1601-1607, 2003.
8. Teo ITN, Tang JCO, Chui CH, Cheng GYM, Yau MYC, Lau FY, Wong RSM, Leung TWT, Cheung F, Ho KP, Cheng CH and Chan ASC: Superoxide anion is involved in the early apoptosis mediated by *Gleditsia sinensis* extract. *Int J Mol Med* 13: 909-913, 2004.
9. Aruoma OI, Deiana M, Rosa A, Casu V, Piga R, Peccagnini S, Dessi MA, Ke B, Liang YF and Higa T: Assessment of the ability of the antioxidant cocktail derived from fermentation of plants with effective microorganisms (EM-X) to modulate oxidative damage in the kidney and liver of rats *in vivo*: studies upon the profile of poly- and mono-unsaturated fatty acids. *Toxicol Lett* 135: 209-217, 2002.
10. Aruoma OI, Moncaster JA, Walsh DT, Gentleman SM, Ke B, Liang YF, Higa T and Jen LS: The antioxidant cocktail, effective microorganism X (EM-X), protects retinal neurons in rats against N-methyl-D-aspartate excitotoxicity *in vivo*. *Free Radic Res* 37: 91-97, 2003.
11. Kim KH, Rodriguez AM, Carrico PM and Melendez JA: Potential mechanisms for the inhibition of tumor cell growth by manganese superoxide dismutase. *Antioxid Redox Signal* 3: 361-373, 2001.
12. Nobuyuki S and Teruo H: Research on the antioxidant components of EM-X and the mechanisms of action. Clinical and basic medical research on EM-X: The 1st International EM Medical Conference. International EM Medical Conference Executive Committee Secretariat, pp27-39, 2001.
13. Chow LMC, Chui CH, Tang JCO, Lau FY, Yau MYC, Cheng GYM, Wong RSM, Lai PBS, Leung TWT, Teo ITN, Cheung F, Guo D and Chan ASC: Anti-angiogenic potential of *Gleditsia sinensis* fruit extract. *Int J Mol Med* 12: 269-273, 2003.
14. Chui CH, Tang JCO, Lau FY, Teo ITN, Yau MYC, Wong RSM, Cheng GYM, Ho SKW, Leung TWT, Hui KS, Wong MM, Fatima S, Cheng CH, Cheung F, Tan WQ, Chow LMC, Guo D and Chan ASC: *Gleditsia sinensis* fruit extract induced growth inhibition involved basic fibroblast growth factor and nitric oxide. *Int J Mol Med* 13: 169-173, 2004.
15. Sartippour MR, de Leon E, Rubio R and Brooks MN: The effects of commonly used drugs on angiogenesis. *Anticancer Res* 23: 231-234, 2003.