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The Chemopreventive Effects of *Glycrrhiza uralensis Fisch* (Chinese Licorice Root) in Human Breast Cancer Cell

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1. Introduction

Breast cancer is the most common cancer (excluding non-malignant skin melanomas) and the second leading cause of cancer deaths among women and the leading cause of death in women age 4055 years and, it is second only to lung for overall cancer-related deaths (1). However, recently in the USA, a large trial that demonstrated a reduction of approximately 50% in the risk of developing breast cancer led to Food and Drug Administration (FDA) approval of tamoxifen as a preventive agent in women at increased risk (2). The chemoprevention with tamoxifen or raloxifene and extract of herbs or plants has been got more attention for the treatment of breast cancer mortality (3-5). We can find many of these chemicals in oriental herbs or plants. The licorice root has long been employed in Western countries as a flavoring and sweetening agent, as well as a demulcent and expectorant. In oriental medicine, licorice root has been known posses various pharmaceutical functions, including detoxification, antiulcer, anti-inflammation, anti-viral, antiatherogenic, and anticarcinogenic (6). In addition, some components of licorice root demonstrated significant antimicrobial activity *in vitro* (7,8) and antioxidant activity (9,10). Especially, Chinese licorice is called kanzo, north-eastern Chinese licorice and ural liquorice and be dwelled in Northern China Mongolia and Siberia. A perennial glandular herb, Chinese licorice has the stem erect with short whitish hairs and echinate glandular hairs, leaves alternate, fruit in a flat oblong and the root having used in medicine is cylindrical, fibrous, flexible, furrowed and light yellow inside. Previous studies have demonstrated that licorice root extract has multi-potent biological effects. A water extract of licorice root was found to inhibit angiogenesis (11) and Licochalcone-A from licorice root has antitumor activity *in vitro* and *in vivo* in a mouse skin papilloma model (12). In another study, licorice root (*Glycrrhiza glabra*) has biological activity

capable of Bcl-2 phosphorylation and G2/M cell cycle arrest in human breast cancer cell line, MCF-7 (13) and induced apoptosis in MCF-7 and HL-60 cell line, as demonstrated by cleavage of PARP (14). This study is the first time to reveal that the root of licorice, *Glycyrrhiza uralensis Fisch* has anti-proliferation effects in breast cancer cell line.

In recent letters of Journal by Wang H. (15) and Marian V. (16), consumption of whole fruits may provide the antioxidant balance needed to quench reactive oxygen species which have been implicated in tumorigenesis. A EPIC-Norfolk prospective study showed an inverse relation between plasma vitamin C and mortality due to cancer (17). The Diet of unbalanced single-target agents may be less advantageous than dietary of whole extracts and than, it can be permit reverse reaction as cell survival of cancer cell. Therefore, we designed to investigate the anti-tumor effects of $CHCl_3$, EtOAc, C_6H_{14} and CH_3OH -H_2O (70 : 30) extracts of licorice root and elucidates the potential mechanisms using an *in vitro* system. We found that the extracts of licorice root was able to induce apoptosis in MCF-7 cells in dose- and time-dependent manner, which were possibly mediated through cleavage of PARP, up-relicorice rootlation Bax and cleavage of Bcl-2.

2. Material and Methods

1) Materials

The fresh roots of licorice (*Glycrrhiza uralensis Fisch*) were washed, disintegrated, and extracted with $CHCl_3$, EtOAc, C_6H_{14} and CH_3OH -H_2O (70 : 30) for 24h. The crude extracts obtained with subjecting into silica gel chromatography and then, that were evaporated to dryness with a rotary evaporator.

2) Methods

Cell proliferation assay
Apoptosis assay
Flow cytometric analysis of apoptosis
Western blot analysis

3. Results

1) Extracts of licorice root decreased cell proliferation in MCF-7

To analyze the inhibition of Trichloromethane, Ethyl acetate, 70%

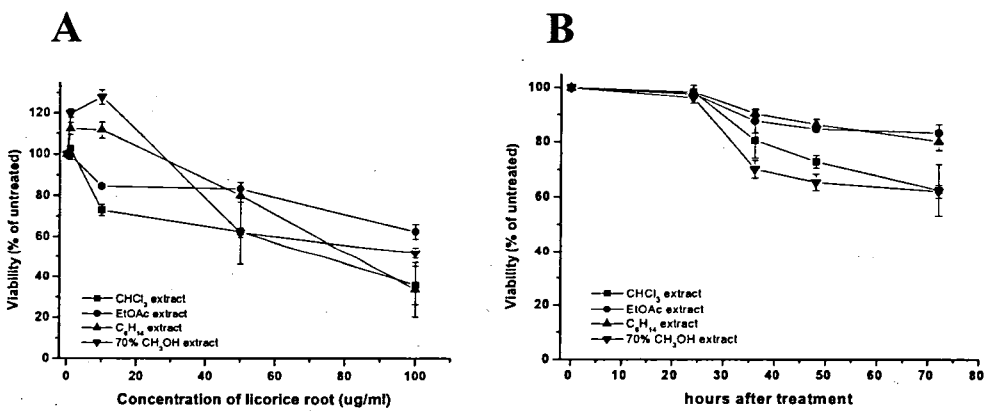

Fig. 1 Inhibition of proliferation by licorice root. Cells were treated with various concentrations of test compound for 72h (A) and various times at 50ug/ml licorice root extracts (B).

Methanol and Hexane extracts of licorice root on the growth of MCF-7 cells, we measured DNA synthesis in presence of licorice root. After 72 h of treatment, all extracts of licorice root inhibited the proliferation of MCF-7 cells in a dose- and time-dependent manner in Figure 1. These results were used in all further experiments.

2) Extracts of licorice root induced apoptosis in MCF-7 cells

To analyze the contribution of apoptosis to this process, Hoechst 33258 staining were performed. When MCF-7 cells were treated with 50ug/ml licorice root extracts for 48hr, cell exhibited typical morphological changes of apoptosis. As shown in Figure 2, the test compound induced chromatin condensation and nuclear fragmentation (arrows). The cells shrank, turned round, and had a relatively smaller volume than control cells. And, considering that licorice root decreased cell proliferation and induced cell death, cell cycle analyses were performed with flow cytometry. MCF7 cells accumulated in the sub-G1 phase gradually from 24 to 72hr after treatment with the test compound, whereas the number of cells in G1 phase decreased in same manner. All of licorice root extracts were found to be effective on the apoptosis of MCF-7 cell.

3) Effects on PARP expression and cleavage in MCF-7 cells

To assess the role of PARP in this apoptotic process, the expression of PARP was examined by western blot analysis. The 116Kd PARP was cleaved to its active 85 Kd in MCF-7 cells treated Trichloromethane, Ethyl acetate as well as Hexane and 70% Methanol extracts of licorice

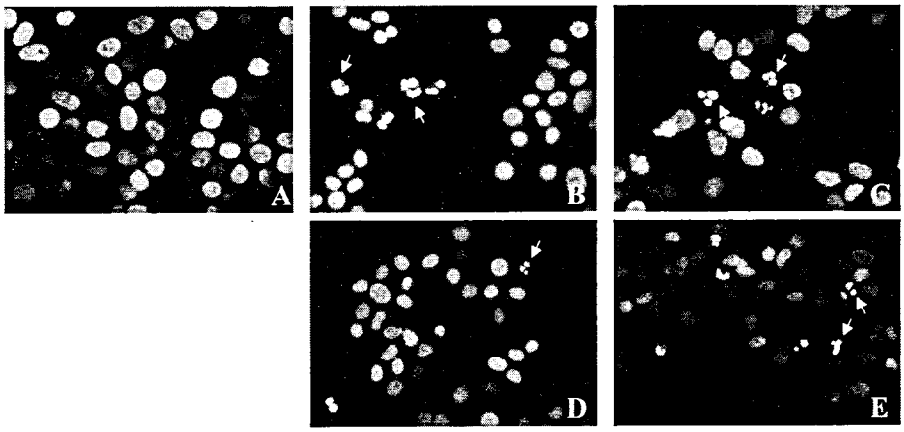

Fig. 2 Detection of apoptotic morphological changes in MCF-7 cells treated with licorice root. Normal MCF-7 cells (A); treated with $CHCl_3$ (B), EtOAc (C), C_6H_{14} (D) and CH_3OH -H_2O (70 : 30) (E) extracts of licorice root for 48h.

root induced PARP degradation fragment in MCF7 cells. This result showed that licorice root induced apoptosis, since treatment with licorice root resulted in cleavage of PARP, the substrate of several ICE-like proteases.

4) Effects on Bcl-2/Bax expression

To determine the effect of licorice root on apoptotic pathways, we evaluated transcription factor Bcl-2 and Bax expression by western blot. Licorice root caused Bcl-2 cleavage in MCF-7 cells and the Bax protein level was increased 72 h after treatment. The 26Kd Bcl-2 was cleaved to its active 22Kd in MCF-7 cells treated licorice root. The results suggested that Trichloromethane, Ethyl acetate, Hexane and 70% Methanol extracts of licorice root induced apoptosis in MCF-7 cells might be mediated through cleavage of Bcl-2 and up-relicorice rootlation of Bax pathway.

4. Discussion

Apoptosis or programmed cell death is an essential physiological process that plays a critical role in development and tissue homeostasis (20). The goals of cancer chemoprevention are to inhibit the induction or suppress the progression of preneoplastic lesions to invasive cancer by using specific natural or synthetic chemicals. Further understanding of the effects of potential chemopreventive agents on specific components of the pathways that lead to apoptosis may provide a rational approach to use such agents alone or in combination with other agents to

enhance apoptosis as a strategy for effective chemoprevention of cancer (21). There are two main pathways leading to apoptosis. The first of these depends upon the participation of mitochondria and the second involved in the interaction of a death receptor with its ligand. Pro- and anti-apoptotic members of the Bcl-2 family relicorice rootlate the mitochondrial pathway (22).

In this study, using MCF-7 human breast cancer cell lines, we have shown the effect of licorice root, *Glycrrhiza uralensis Fisch* on cell proliferation, and on the induction of apoptosis in a cell-specific manner. In these results, we have found that $CHCl_3$, EtOAc, C_6H_{14} and CH_3OH -H_2O (70 : 30) extracts of licorice root inhibited cell viability in MCF-7 in a concentration- and time-dependent manner. We demonstrated that $CHCl_3$, EtOAc, C_6H_{14} and CH_3OH -H_2O (70 : 30) extracts of licorice root induced apoptosis through the cleavage of Bcl-2 protein and the increase of Bax protein expression. Cellular stress induced pro-apoptotic Bcl-2 family members to translocate from the cytosol to the mitochondria, where they induce the release of cytochrome c, while the anti-apoptotic Bcl-2 proteins work to prevent cytochrome c release from mitochondria, and thereby preserve cell survival (21). Especially, unlike Bcl-2, the cleaved Bcl-2 fragment was no longer functional for dimerization with either Bcl-2 or Bax (23).

Recent study showed that alcohol extracts of Licorice root (*Glycyrrhiza glabra*) can inhibit cell growth, and induce cell cycle arrest at transition G2/M phase in tumor cell lines (22). However, we couldnt observe that the extracts of licorice root were capable of inducing cell cycle arrest in MCF-7 cells. In order to confirm this result, flow cytometric analysis was performed. All extracts of licorice root did not induce cell cycle arrest in any phase. But, sub-G1 population, number of apoptotic cell is increased significantly in MCF-7 cells treated with licorice root. Accumulating with sub-G1 DNA content has a tendency to the result of proliferation assay because non-sub-G1 cell is decreased in the way of viability treated with licorice. In addition, a nuclear enzyme involved in DNA repair and maintenance of genome integrity and post-translational ribosylation of proteins, whereby apoptosis occurs, PARP cleaves several substrates. This occurred with cleavage of 116 Kd PARP to 85 Kd proteolytic fragments in MCF-7 cells treated with $CHCl_3$ and EtOAc extracts of licorice root. And also, C_6H_{14} and CH_3OH -H_2O (70 : 30) extracts of licorice root just induced PARP degradation fragment in MCF-7 cells. It appeared that the pattern of PARP cleavage differed from that observed in MCF-7 cells treated with $CHCl_3$ and EtOAc extracts of licorice root and did not lead to a persistent fragment. It could be hypothesized that PARP degradation continues after its first cleavage.

In summary, $CHCl_3$, EtOAc, C_6H_{14} and CH_3OH -H_2O (70 : 30) extracts of licorice root induces apoptosis through overexpression of Bax and cleavage of Bcl-2 in human breast cancer cells. It is concluded that the root of licorice, *Glycyrrhiza uralensis Fisch* might be a good

chemopreventive natural product for the human breast cancer.

5. Acknowledgment

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6. References

(1) SL Parker, et al. Cancer Statistics. CA Cancer J. Clin. 1998, 48, 2629
(2) A Decensi. European Journal of Cancer 2000, 36, 694-709
(3) Konstantin Christov, et al. Breast Cancer Research and Treatment 2003, 77, 253-264
(4) Hu H, et al. Int J Cancer 102 : 250-3, 2002
(5) Cho JH, et al. Carcinogenesis 23 : 1163-9, 2002
(6) Wang ZY, et al. Nutr Cancer 39 : 1-11, 2001
(7) Demizu S, et al. Chem Pharm Bull 36 : 3474-3479, 1998
(8) Friis-Møller Alice, et al. Planta Medica 68 : 416-419, 2002
(9) Belinky PA, et al. Atherosclerosis 137 : 49-61, 1998
(10) Vaya J, et al. Free Radical Biol Med 23 : 302-313, 1997
(11) Kobayashi S, et al. Biol Pharm Bull 18 : 1382-6, 1995
(12) Shibata S, et al. Planta Medica 57 : 221-4, 1991
(13) Mohamed M Rafi, et al. J Agric Food Chem 50 : 677-684, 2002
(14) Rafi MM, et al. Anticancer Res : 20 : 653-8, 2000
(15) Wang H, et al. J Agric Food Chem 44 : 701-705, 1998
(16) Marian V, et al. Nature 405 : 903 904, 2000
(17) Khaw KT, et al. Lancet 357 : 657-63, 2001
(18) Soto AM, et al. Environ Health Perspect 102 : 380-383, 1994
(19) Kang KS, et al. Mol Carcinog 21 : 225-33, 1998
(20) Zimmermann KC, et al. Pharmacol Ther 92(1) : 57-70, 2001
(21) Sun SY. Drug News Perspect 14(2) : 75-80, 2001
(22) Rafi MM, et al. J Agric Food Chem 50 : 677-84, 2002
(23) Lin H, et al. Exp Cell Res 261 : 180-6, 2000