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Inhibition of Production of Reactive Oxygen Species and Gene Expression Profiles by *Cirsii Japonici Herba* Extract Treatment in HepG2 Cells

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Abstract

Cirsii Japonici Herba (CJH) extract has been used for hundreds of years in Asian countries as a treatment for pollutant, radiation, and alcohol-induced liver damage. The reducing effect of CJH on hydrogen peroxide-induced reactive oxygen species (ROS) production, the main cause of cell damage or death, was evaluated using the HepG2 cell line. Cell survival was determined using MTS assay. The viability of cells treated with CJH was not significantly different from oxidative-stressed HepG2 cells. A dose-dependent inhibitory effect by CJH on ROS production was shown in oxidative-stressed cells using the H_2DCFDA assay. To identify candidate genes responsible for the anti-oxidative effects of CJH on HepG2 cells, an oligonucleotide microarray analysis was performed. The expressions of five genes were decreased, whereas nineteen genes were up-regulated in CJH plus hydrogen peroxide treated cells, compared to only hydrogen peroxide treated cells. Among them, the expression of 5 genes was decreased in hydrogen peroxide treatment when compared to control. These genes are known to regulate cell survival and progression. On the other hand, it was shown that its main compounds were not a sylimarin or its analogs. The list of differentially expressed genes may provide further insight on the action and mechanism behind the anti-oxidative effects of Cirsii Japonici Herba.

Keywords: Cirsii Japonici Herba, Reactive oxygen species, Gene expression profiles

Excess production of reactive oxygen species (ROS) may lead to cellular injury through nonspecific modification and disruption of proteins, phospholipids and nucleic acids[1]. Critical sites of ROS attack are the cell's plasma membrane and the membranes of intracellular organelles. The disruptive effects of ROS involve membrane lipid per-oxidation and membrane protein modification, which may produce alterations in the membrane structure and function including fluidity, permeability, enzyme activity, ion channels and transport, and receptor proteins[2]. The role of ROS's has been implicated in many human degenerative diseases of aging. Various antioxidants have been found to have some preventive and therapeutic effects on these diseases[3]. Therefore, substantial efforts have been made in recent years to identify both natural and synthetic antioxidants.

Cirsii Japonici Herba (CJH) is the herb of the Composite plant, *Cirsium japonicum* DC. In traditional Oriental medicine, CJH has been a remedy for treating malfunctions of the liver, hemorrhage, scabies, and various abdominal and intestinal disorders[4]. The chemical components of CJH are known as ciryneol A, C, H, ciryneone F, cireneol G, p-coumaric acid, syringin, linarin, beta-sitosterol and daucosterol[5].

Hydrogen peroxide, the cause of the main ROS, is known to cause lipid peroxidation and DNA damage in cells[6]. The present study was therefore designed to investigate whether CJH is capable of reducing the hydrogen peroxide-induced ROS generation and cytotoxicity in the human hepatoma cell line HepG2. This study also measured which genes are differentially expressed in relation to the effect of CJH by using an oligonucleotide microarray assay. We searched to determine whether CJH's bioactive compounds are silymarin or its analogs, well known as the main compound of milk tistle.

HPLC Spectrum of CJH

CJH belongs to the thistle family, but it is in a different genus from *Silybium marianum*. Silymarin or its derivates were isolated from fruits of *Silybum mari-*

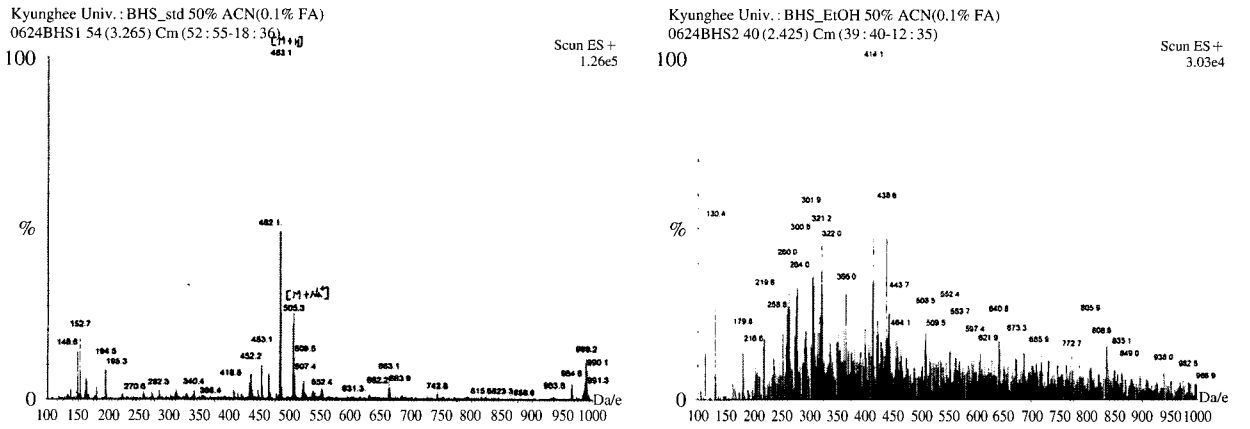

Fig. 1. Mass-spectrometry spectrum of silybinin (A) and the ethanol extract of CJH (B) eluted by HPLC with a similar retention peak time to silybinin

anum by Wagner *et al.*[7]. These compounds showed strong antioxidant effects[8]. Therefore, to determine whether CJH contains silymarin or its derivates, HPLC and LC-MS analyses were performed. The retention time of Silibin, the major class of sylimarin, showed similar retention time of some components in CJH ethanol extracts. Then, the fraction containing the candidate compound was isolated and analyzed by Mass-spectrometry to compare with that of silibin. As shown Fig. 1, silymarin (mainly silybinin) had a main peak at 482.1 (Mw) but the fraction of CJH showed at 414.1 (Mw) and 438.6 (Mw) in Mass-spectrometry analysis. This result suggests that the major active compound in CJH is not sylimarin.

Effect of CJH on ROS Production in HepG2 cells

The intracellular concentration of ROS in HepG2 cells, as assessed by H_2DCFDA oxidation, was increased by hydrogen peroxide treatments. CJH ethanol extract significantly inhibited ROS production in various concentrations (Fig. 2). The CJH ethanol extract was fractionated using buthanol, ethyl acetate and water. Treatment of various concentrations (0.001 -0.1 mg/mL) of a CJH water or CJH ethyl acetate (EtOAc) fraction for 24 hr significantly inhibited the increase of intracellular ROS when cells were stimulated with hydrogen peroxide (Fig. 3 and 4). Especially, it was very strong antioxidative effect in 0.001 mg of CJH EtOAc fraction ($p < 0.001$). But CJH buthanol fraction was not inhibited the increase of ROS (data not shown).

Effect of CHJ on Gene Expression in HepG2 cells

When we examined 8793 genes, 24 genes showed

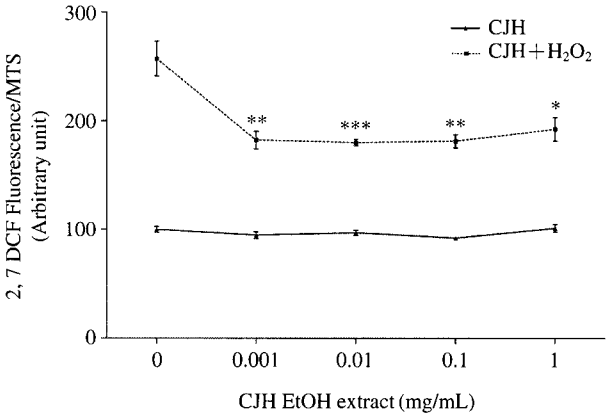

Fig. 2. Inhibition effects on ROS by various concentrations of the CJH ethanol extract in the case of hydrogen peroxide stimulated HepG2 cells. ROS measurement of HepG2 cells in medium containing various concentrations of CJH ethyl acetate extract after 24 hr. ROS of each sample was quantified using DCF fluorescence intensity (excitation 485 nm/ emission 538 nm). Values are the mean ± S.E.M. of three independent experiments duplicate in each run. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to hydrogen peroxide alone.

dynamic changes in expression resulting from CJH addition compared to hydrogen peroxide only treatment. The expressions of 5 genes were decreased, whereas 19 genes were increased upon CJH treatment. The expressions of 5 genes (Cysteine-rich, angiogenic inducer 61; CYR61, TIA1 Cytotoxic granule-associated RNA binding protein; TIA1, Myeloid/lymphoid or mixed-lineage leukemia translocated to 2; MLLT2, Nuclear factor of activated T-cells 5 tonicity-responsive; NFAT5, Inositol 1, 4, 5-triphosphate receptor type 2; IP3R2) were decreased over 2-fold in hydrogen peroxide treatment compared to control,

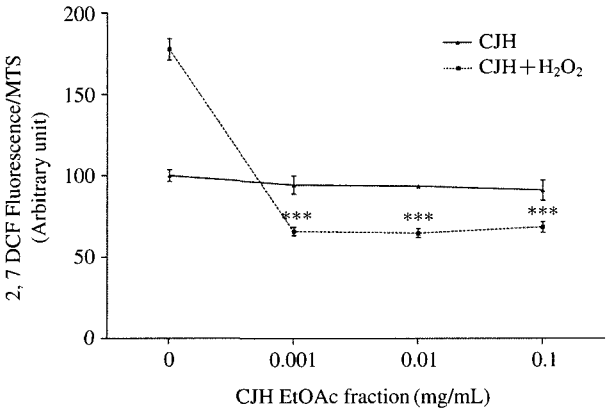

Fig. 3. Inhibition effects on ROS by various concentrations of the CJH ethyl acetate fraction in the case of hydrogen peroxide stimulated HepG2 cells. ROS measurement of HepG2 cells in medium containing various concentrations of CJH ethyl acetate fraction after 24 hr. ROS of each sample was quantified using DCF fluorescence intensity (excitation 485 nm/emission 538 nm). Values are the mean ± S.E.M. of three independent experiments duplicate in each run. ***$P<$ 0.001 compared to hydrogen peroxide alone.

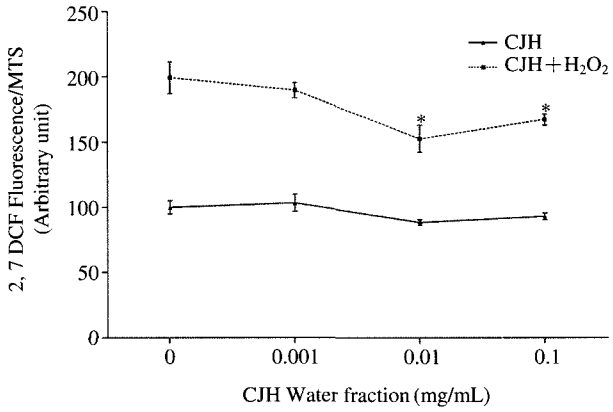

Fig. 4. Inhibition effects on ROS by various concentrations of the CJH water fraction in the case of hydrogen peroxide stimulated HepG2 cells. ROS measurement of HepG2 cells in medium containing various concentration of CJH water fraction after 24 hr. ROS of each sample was quantified using DCF fluorescence intensity (excitation 485 nm/emission 538 nm). Values are the mean ± S.E.M. of three independent experiments duplicate in each run. *$P<0.001$ compared to hydrogen peroxide alone.

Table 1. Significantly differentially expressed genes in Control vs. hydrogen peroxide treated HepG2 cells

Gene name	Fold*	ID#
Nuclear factor of activated T-cells 5, tonicity-responsive (NFAT5)	−5.4	NM_006599.1
Heterogeneous nuclear ribonucleoprotein A1 (HNRPA1)	−2.9	AW450929
Protein kinase, lysine deficient 1 (PRKWNK1)	−2.8	NM_018979.1
Krit1	−2.8	U90268
Coagulation factor VII	−2.8	NM_000131.2
Inositol 1, 4, 5-triphosphate receptor, type 2 (ITPR2)	−2.7	AA834576
TIA1 Cytotoxic granule-associated RNA binding protein (TIA1)	−2.6	NM_022037.1
Cysteine-rich, angiogenic inducer, 61 (CYR61)	−2.3	NM_001554.1
Translocated promoter region (to activated MET oncogene) (TPR)	−2.3	BF110993
Coagulation factor V (proaccelerin, labile factor) (F5)	−2.3	NM_000130.2
Myeloid/lymphoid or mixed-lineage leukemia translocated to, 2 (MLLT2)	−2.2	NM_005935.1
fetal Alzheimer antigen (FALZ)	−2.2	NM_004459.2
Malignant fibrous histiocytoma amplified sequence 1 (MFHAS1)	−2.2	BF739959
Erbb2 interacting protein (ERBB2IP)	−2.1	NM_018695.1
Serine/arginine repetitive matrix 2 (SRRM2)	−2	AI655799
Zinc finger protein 91 (HPF7, HTF10) (ZNF91)	−4	NM_003430.1
cDNADKFZp564D113	−6.2	AL049250.1
6.2 kd protein (LOC54543)	2	NM_019059.1
Calmodulin 3 (phosphorylase kinase, delta) (CALM3)	2.3	NM_005184.1
Cysteine-rich protein 1 (intestinal) (CRIP1)	2.9	NM_001311.1
Vesicle-associated membrane protein 5 (myobrevin) (VAMP5)	2.2	NM_006634.1
BCL2-antagonist/killer 1 (BAK1)	3.2	NM_001188.1

*Fold means ratio of hybridization intensity. The genes with negative value are abundant in non-treated HepG2 cells, while those with positive values are abundant in hydrogen peroxide treated HepG2 cells.

whereas the expression these genes were recovered over 2 fold upon CJH treatment (Table 1 and 2). Therefore these genes were closely associated with anti-oxidative effects by CJH treatments.

Discussion

In this study, we showed that the main active compound of CJH is not silymarin or silybinin through

Table 2. Significantly differentially expressed genes in the CJH ethanol extract plus hydrogen peroxide vs. hydrogen peroxide only treated HepG2 cells

Gene name	Fold*	ID
Polypyrimidine tract binding protein 1 (PTBP1)	−3.3	AC006273
Solute carrier family 29 (nucleoside transporters), member 1 (SLC29A1)	−3.1	AF079117.1
Claudin 6 (CLDN6)	−2.1	NM_021195.1
Cleft lip and palate associated transmembrane protein 1 (CLPTM1)	−2	BC004865.1
Kallikrein 10 (KLK10)	−2.4	BC002710.1
Cysteine-rich, angiogenic inducer, 61 (CYR61)	2	NM_001554.1
NCK-associated protein 1 (NCKAP1)	2	NM_013436.1
Homer, neuronal immediate early gene, 1B (SYN47)	2	BE550452
DCAAUD05	2	AV712064
RAN binding protein 2 (RANBP2)	2.2	D42063.1
Serologically defined colon cancer antigen 1 (SDCCAG1)	2.2	NM_004713.1
Myeloid/lymphoid or mixed-lineage leukemia translocated to, 2 (MLLT2)	2.3	NM_005935.1
ATPase, Ca^{++} transporting, type 2C, member 1 (ATP2C1)	2.3	AB037768.1
Cyclin-dependent kinase inhibitor 1B (p27, Kip1) (CDKN1B)	2.3	BC001971.1
Iaminin, beta 1	2.4	NM_002291.1
Nuclear factor of activated T-cells 5, tonicity-responsive (NFAT5)	2.4	NM_006599.1
Protein kinase, lysine deficient 1 (PRKWNK1)	2.5	NM_018979.1
TRIAD3 protein (TRIAD3)	2.5	NM_019011.1
Nucleosome assembly protein 1-like 1 (NAP1L1)	2.6	AL162068.1
Inositol 1, 4, 5-triphosphate receptor, type 2 (ITPR2)	3.1	AA834576
Zinc finger protein 267 (ZNF267)	2.4	AU150728
TIA1 Cytotoxic granule-associated RNA binding protein (TIA1)	2.2	NM_022037.1
Nuclear receptor subfamily 3, group C, member 1 (NR3C1)	2.2	X03348.1
Coagulation factor V (proaccelerin, labile factor) (F5)	2.5	NM_000130.2

*Fold means ratio of hybridization intensity. The genes with negative value are abundant in hydrogen peroxide treated cells, while those with positive value are abundant in CJH ethanol extract plus hydrogen peroxide treated cells.

HPLC and LC-MS analysis of CJH. In addition, we also demonstrated that CJH water or ethyl acetate fractions significantly inhibited the increase of intracellular ROS when cells were stimulated with hydrogen peroxide. Subsequently, the oligonucleotide microarray assay was performed to understand the molecular mechanism of CJH's effect on ROS inhibition. Results of the oligonucleotide microarray showed that the expressions of 5 genes (CYR61, TIA1, MLLT2, NFAT5, IP3R2) were decreased in hydrogen peroxide treatment alone but increased following CJH treatment in the presence of hydrogen peroxide. Previous studies have shown that CYR61 supports cell adhesion, induces cell migration, enhances growth factor-induced mitogenesis, and promotes cell survival under apoptotic conditions[9,10]. The cytotoxic granular proteins TIA1 were known to be positive in most tumor cells and CD8-positive cells. NFAT5 is transcription factor which regulates the transcription of osmo-compensatory genes[11]. The IP3R2 gene encodes calcium channels located in the endoplasmic reticulum (ER) membrane (releasing calcium from the ER) and that is involved in cell transformation and progression[12,13]. All of these genes had no reports related to regulation of ROS production but had been known to regulate cell survival, progression and other various physiological functions.

At present, the precise regulatory mechanism of ROS inhibition by CJH is unknown. However, an important role for the inhibition of ROS in protection of liver damage has been recognized[14]. In all types of liver damage there is consistent evidence of enhanced production of free radicals and/or significant decrease of antioxidant defense. As a consequence, a large number of studies have focused on the pathogenic significance of oxidative stress in liver injury as well as on therapeutic intervention with antioxidant and metabolic scavengers[15-18]. In hepatocytes, ROS may play a role in a large cascade of reactions, such as Ca^{++} accumulation, circulatory status and transport functions, nitric oxide (NO) synthesis and metabolism, cytokine gene expression, caspase activity, growth factor synthesis and activity, DNA fragmentation, Na^{+} influx, etc. Hence, a significant and sufficiently steady increase of ROS production leads to perturbation of the normal redox state and of cell metabolism, with subsequent impairment of various cell functions and activities, possibly causing irreversible damage.

In conclusion, our results demonstrated that CJH acted as an antioxidant of HepG2 cells and that its bioactive components were not a silymarin or sily-

marin analogs which are well known as the components of *Silybum marianum*. In addition, the precise mechanism of CJH action to inhibit ROS production and the major bioactive component of CJH remains to be identified.

Methods

Preparation of Herbal Extract

CJH was purchased from Pyung-Hwa Herbal Medicines Company (Seoul, South Korea) and confirmed by the Department of Herbology at the College of Oriental Medicine, Kyung-Hee University, South Korea. Dried CJH 125.5 g was pulverized to powder and extracted by 70, 85% and 100% ethanol for 10 min using a sonicator (Elma Inc., Model D-7700 Singen/Htw.; power 230 W). The ethanol extracts were then mixed together and concentrated by an evaporator (Rotary Vacuum Evaporator N-N Series, EYELA Co. Japan) and then lyophilized by a lyophilizer (Freezone plus 6, Labconco, Kansas, MO, USA). The dried CJH extraction was 27.32 g (yield 21.8%). Fifteen grams of the dried extraction was successively dissolved in 150 mL of ethyl acetate, water, and buthanol. Each fraction was then concentrated and lyophilized. The dried extraction was 0.1 g (yield 0.22%) in ethyl acetate fraction, 0.3 g (yield 0.65%) in buthanol fraction and 2.3 g (yield 5%) in water fraction. Each dried extract was dissolved in distilled water or DMSO and centrifuged at 10,000 g for 10 min. The supernatant was passed through a 0.22 μm filter (Corning, Costar, NY, USA) reaching a final concentration of 100 mg/mL. The solution was aliquoted and stored at 4°C for future use.

The High-Performance Liquid Chromatography (HPLC) Analysis of CJH

The dried ethanol extract of about 240 mg of CJH was accurately weighed, put in a test tube, then dissolved in 4 mL of 50% methanol (HPLC reagent, J.T. Baker Co. Ltd, U.S.A.) then filtered using a 0.45 μm syringe filter (PVDF, Waters, U.S.A.). The standard materials weights were 10 mg, and were dissolved according to the analysis condition of standard materials. The dissolved standard solution was diluted as 0.1, 0.5, 1.0, 1.5, 2.0 mg/mL respectively, and then a standard HPLC chromatogram was obtained. The relationship between the concentration and the peak-area was measured by the minimum square method (R^2 value). The HPLC apparatus was a Waters Breeze System (717+ Autosampler, 2487 dual λ absorbance detector, 1525 binary HPLC Pump, Waters Co., Milford, USA), and the associated Waters Breeze System (Ver. 3.20, Waters Co., Milford, U.S.A.) was used for data acquisition and integration.

Cell Culture

HepG2, human hepatoma cells (KCLB #58065, South Korea) were cultured in RPMI 1640 media (Invitrogen Life Technologies, Rochville, MD, USA) with 10% fetal bovine serum (FBS), 1% penicillin and 1% streptomycin. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

MTS Assay

Cell growth was measured by MTS assay using the Cell Titer 96® Aqueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI, USA). Briefly, 100 μL of the supernatant was added to each 96-well plate. 20 μL of MTS solution was added to each of the 96-well plates and incubated at 37°C in a humidified 5% CO_2 atmosphere for 1 hr. The absorbance was read at 490 nm using a microplate reader (Molecular Device, Sunnyvale, CA, USA). Each sample was repeated.

RNA Extraction

Total RNA was extracted from cultured cells using the RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The extract was assayed for RNA quality and the concentration was determined with a spectrophotometer (DU500, Beckman Instruments Inc. Fullerton, TX, USA). The RNA was stored at −80°C until analysis.

Affymetrix GeneChip® Arrays Processing

cRNA labeling, hybridization and washing were performed using standard procedures as previously described[19].

Microarray Data Analysis

Microarrays were scanned at 570 nm, 3 μm resolution with a gene chip scanner (Affymetrix) and analyzed as previously described[20]. For quantification of relative transcript abundance, the average difference value (Avg Diff) was used. The signal intensity for each gene was calculated as the average intensity difference represented by ((PM-MM)/(number of probe pairs)). All chips were normalized by the mean of the total sums of Avg Diff values across all chips used in the experiment, thus allowing for a comparison between the conditions. An Avg Diff level lower than 200 fluorescence units was considered below the limit of accurate quantification based on extensive quality control experiments performed in-house. Above 200 fluorescence units, based on our in-house validation dataset, any gene having an absolute ratio or change

factor >2, was considered significantly differentially expressed.

Statistical Analysis

All data except microarray results were expressed as the mean $\pm$ S.E.M. of independent experiments. Statistical significance was compared between the CMJ treatment group and the control by the Student's *t*-test. Results with $p<0.05$ were considered statistically significant.

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