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Protective Effect of *Crataegus pinnatifida* and *Cinnamomum cassia* on Ethanol-induced Cytotoxicity and DNA Damage in HepG2 Cells

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Abstract – Plant extracts produced from branches of *Crataegus pinnatifida* and barks of *Crataegus pinnatifida* inhibited ethanol-induced cytotoxicity and DNA damage in liver cells. Furthermore, these two extracts inhibited the expression and activities of CYP2E1 enzyme. *Cinnamomum cassia* had a better effect on inhibition of DNA damage than *Crataegus pinnatifida*, as well as showed a high tendency to inhibit CYP2E1 expression and catalytic activities. It is considered that extracts produced from *Crataegus pinnatifida* or *Cinnamomum cassia* have an effect to reduce ethanol-induced cytotoxicity and DNA damage in liver cells. Therefore, we suggest to use *Crataegus pinnatifida* and *Cinnamomum cassia* and their ingredients as potential candidate substances to prevent and treat ethanol-induced cytotoxicity and genotoxicity in liver cells.
Keywords – Ethanol, Cytotoxicity, Comet assay, DNA damage, *Crataegus pinnatifida, Cinnamomum cassia,* Plant extract

Introduction

Ethanol induces various kinds of disease including fatty liver, liver cirrhosis, stomach cancer, liver cancer and pancreatic cancer and specific alcoholic diseases including as alcoholism.[1-5] This has triggered a lot of studies for the application of plant-originated natural substances, which has relatively low side effects, to liver diseases.[6,7] At present, about 50% of medicines for liver diseases used for clinical purposes are natural substances or derivatives of natural products.[8-10] There have been a lot of studies on plant extracts or compounds that have protective effect on substances such as CCl_4, galactosamine and paracetamol that induces cytotoxicity in liver cells.[11] Nevertheless, there have been only a handful of reports on natural substances that have cytoprotective and antigenotoxicic effect on ethanol.[12-15] Therefore, based on the result of screening of 120 plant extracts, the authors of this study have found *Crataegus pinnatifida* and *Cinnamomum cassia* as plant extracts that have protective effect on alcohol-induced cytotoxicity in liver cells.[16] This study reports that these two plant extracts reduce ethanol-induced cytotocixity and genotoxicity by inhibiting the expression and activities of cytochrome P450 2E1 (CYP2E1) in liver cells.

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Experimental

Experiment materials – Materials such as ethanol, acetaminophen, Trolox and dimethysufoxide (DMSO) were purchased from Sigma-Aldrich (MO, USA), and the negative control group was tested at the same concentration as DMSO used for the test group (final concentration of DMSO: 1%). Added 70% ethanol 3,000 ml respectively to dried branches of *Crataegus pinnatifida* (CP) 500 g and barks of C*innamomum cassia* (CC) 500 g purchased from Kyungdong Market in Seoul, South Korea, extracted them for 7 days at room temperature, filter them with Whatman paper (No.6) and then conducted vacuum concentration in order to get the extracts of the two materials. Yield (%) of each extract was 8.3% and 10.5% respectively.

Cell culture – The cell line used for *in vitro* study of this experiment was HepG2 cell (HB-8065^{TM}), which is a human liver cancer cell, purchased from ATCC (Rockville, MD, USA) and stored in a liquid nitrogen tank of College of Pharmacy, Kangwon National University. This cell was cultured according to the experimental purpose through frequent subcultures. For cell culture, MEM medium that contains 10% FBS, 1% L-glutamine, 1% penicillin-streptomycin. Most reagents used for cell culture were purchased from GIBCO (Grand Island, NY, USA).

Protective effect test on ethanol-induced cytotoxicity – To evaluate cytoprotective effect, the cytotoxicity of ethanol in HepG2 cells was measured by using a microplate reader.[17] 25,000 cells were put respectively in each well. After the culture in the medium 90 μl in a CO_2 incubator for 24 hours, 13% ethanol test solution 10 μl only or 13% ethanol test solution 10 μl containing plant extract was added to make the cells 100 μl in total. From preliminary screening study[16], the final concentration of the plant extract was 0.4% and the concentration of ethanol was 1.3% for cell viability testing. After the culture for 20 hours, MTT reagent 15 μl was added. After the culture for 4 hours, the medium was removed. After dissolution in DMSO 200 μl, the absorbance was measured at 570 nm. Trolox and acetaminophen were used under the same condition as the substances for comparison of activities.

Protective effect test on liver cell DNA damage – To evaluate the degree of DNA damage, comet assay was conducted for the cultured HepG2 cells.[18,19] Protective effect on DNA damage was tested by comparing the ethanol test solution containing the extracts of *Crataegus pinnatifida* and *Crataegus pinnatifida* with pure ethanol of the same concentration. For the experiment, 1.5×10^6 of the cells were put in a 6-well culture dish, and after 24 hours, 13% ethanol test solution only or 13% ethanol test solution containing the plant extract of the prescribed concentration was added respectively. The final concentrations of the extract and ethanol were 0.5~2% and 1.3% respectively. After 45 minutes, the medium was completely removed and replaced with a new medium 3 ml, and the new medium was cultured in a 37 ºC CO_2 incubator for an hour. Harvested the cells by adding trypsin EDTA 1 ml in each culture dish. Collected the test tube by putting the medium 2 ml. After centrifugation at 1,100 rpm for 3 minutes, the supernatant was removed and suspended by adding 0.5% LMPA (low melting point agarose) 300 μl respectively.

After dropping in this solution 50 μl on the slide that was processed with 0.65% NMPA (normal melting point agarose) 130 μl, the cover slide was closed. After hardening in a refrigerator for about 30 minutes, the cover slide was removed. After dropping 0.5% LMPA 130 μl again on the slide, the cover slide was closed and hardened in the refrigerator for 30 minutes. After removing the cover slide, lysis was conducted for 60 minutes by soaking it in lysis buffer (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10, 10% DMSO, 1% Triton X-100). After soaking it in electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13) for 20 minutes, the slide was arranged to the both poles of the electrophoresis device and electrophoresis was conducted at 25 V and 300 mA for 15 minutes. After taking out the slide, it was soaked and neutralized in 0.4 mM tris (pH 7.5) for 30 minutes. After drying the slide and dropping ethidium bromide (2 μg/ μl) 20 μl on the slide, the cover slide was closed. After observing it with a fluorescence microscope by using a 515~560 nm excitation filter and a 590 nm barrier filter, 25 cells per slide were analyzed by using KOMET 5.5 (Kinetic image, England), which is an image analyzer. The degree of DNA damage was analyzed by using tail length (TL) as the measurement parameter.

CYP2E1 RT-PCR – After culturing the cells in a 6-well plate (5×10^6 cells/well) for 24 hours, the ethanol test solution only or the ethanol test solution containing plant extracts of the prescribed concentration was added in the plate to adjust the final concentrations of the plant extract in the medium to 50~200 μg/ml and the concentration of ethanol to 1.3%. After culturing the cells for 5 hours and harvesting them by using trypsin EDTA, centrifugation was conducted at 1,000 rpm for 3 minutes. After removing supernatant, RNAs were extracted by using Total RNA Extraction Kit (Bio-Rad) according to the method recommended by the manufacturer. After measuring the absorbance at both 260 nm and 280 nm and calculating the concentration of RNAs, the extracts were stored at −70 ºC. cDNAs were RT reacted and synthesized for 50 minutes at 42 ºC and 5 minutes at 99 ºC by using Gene Cycler Thermal Cycler (Biorad, Gerculis, CA). The primer sequences (β-actin bp 396, 5' CTACAATGAGCTGCTG CGTGTGG 3', 5' TAGCTCTTCTCCAGGGAGGA 3', CYP2E1 bp 356, 5'TGCCATCAAGGATAGGCAAG 3', 5' AATGCTGCAAAATGGCACAC 3') were used for this experiment were as follows.[20]

As for genes, β-actin was amplified for 20~30 cycles under the condition of 1 cycle for 3 minutes at 94 ºC, for 20 seconds at 94 ºC, for 20 seconds at 52 ºC and for 40 seconds at 72 ºC, and CYP2E1 was amplified for 37 cycles under the condition of 1 cycle for 4 minutes at 95 ºC, for 1 minute at 94 ºC, for 20 seconds at 60 ºC and for 2 minutes at 72 ºC. After amplification, electrophoresis was conducted for the reaction mixture 8 - 10 μl by using 1.5% agarose gel and the band was confirmed by dying it with ethidium bromide for 20 minutes.

CYP2E1 catalytic assay – After putting 2.5 pM cytochrome P450 2E1 (human recombinant isozyme, Biocatalytics) in the reaction mixture (0.2 mM-nitrophenol, 7.5 mM glucose, 2 units/ml of glucose-6-phosphate dehydrogenase, 0.4 mM $NADP^+$, 5 mM $MgCl_2$, 100 mM photassium phospate buffer (pH 6.8), the ethanol test solution only or the ethanol test solution containing plant extracts

of the prescribed concentration was added to adjust the final concentrations of the plant extract in the medium to 50~200 μg/ml and the concentration of ethanol to 1.3%. After culturing it at 37 ºC for 30 minutes, the reaction was stopped by using TCA, and the absorbance was measured at 526 nm.[21]

Statistical analysis – Most experiments were conducted 3 times, and a significance test was conducted for acquired data by using Student's t-test

Results

Protective effect on ethanol-induced cytotoxicity – Table 1 shows the protective effect of the extracts of *Crataegus pinnatifida* and *Cinnamomum cassia* on 1.3% ethanol-induced cycotoxicity. It has been confirmed that if cell viability gets higher than 100%, alcohol-induced cytotoxicity is reduced. Compared to the 1.3% ethanol only, the 1.3% ethanol containing 0.4% *Crataegus pinnatifida* or *Cinnamomum cassia* had a better protective effect; that is, the protective effects of *Crataegus pinnatifida* and *Cinnamomum cassia* were 63.6% ($p < 0.05$) and 42.6% ($p < 0.01$) respectively. Although the activation of *Cinnamomum cassia* was slightly lower than *Crataegus pinnatifida,* the statistical significance was very high. The protective effects of Trolox, which is an antioxidant used as the substance for comparison of activities, and acetaminophen, which is a hepatotoxic substance, were 23.6% ($p < 0.05$) and –24.7% ($p < 0.01$).

Protective effect on ethanol-induced DNA damage – Table 2 shows the protective effect of the extracts of *Crataegus pinnatifida* and *Cinnamomum cassia* on 1.3% ethanol-induced liver cell DNA damage. Compared to the 1.3% ethanol only, the 1.3% ethanol containing 0.5~2.0% *Crataegus pinnatifida* or *Cinnamomum cassia* had a better protective effect; that is, the protective effect of *Crataegus pinnatifida* was 19.3~21.6%, without concentration-dependent tendency, and statistically significant in 1% concentration ($p < 0.05$), and the protective effect of *Cinnamomum cassia* was 14.5~30.4%, concentration-dependent tendency, and statistically significant in 2% concentration ($p < 0.05$).

Table 1. Protection of *Crataegus pinnatifida* and *Cinnamomum cassia* extract on ethanol-induced cytotoxicity in HepG2 cells

	OD 570 nm					
Treatment[1]		Ind. Value		Mean ± SD	Cell viability (%)	Protection (%)
Negative control	1.092	1.022	1.046	1.053 ± 0.036	100	–
1.3% Ethanol	0.824	0.898	0.909	0.877 ± 0.046	83.3	–
CP	1.189	1.499	1.617	1.435 ± 0.221*	136.3	63.6
CC	1.136	1.350	1.266	1.251 ± 0.108**	118.8	42.6
Trolox	1.072	1.027	1.154	1.084 ± 0.064*	103.0	23.6
Acetaminophen	0.640	0.699	0.641	0.660 ± 0.034**	62.7	–24.7

[1]0.4% of extract in final concentration of 1.3% ethanol, *$p < 0.05$, **$p < 0.01$ (Student's t-test), CP: *Crataegus pinnatifida,* CC: *Cinnamomum cassia*

Table 2. Inhibition of *Crataegus pinnatifida* and *Cinnamomum cassia* extract on ethanol-induced DNA damage in HepG2 cells

	Tail length (μm)				
Treatment[1]		Ind. Value		Mean ± SD	Inhibition (%)
Negative control	15.1	16.1	16.8	16.0 ± 0.9	–
1.3% ethanol	101.0	96.8	89.7	95.8 ± 5.7	–
CP					
0.5%	70.4	74.2	90.8	78.5 ± 10.9	21.6
1%	86.8	74.3	74.0	78.4 ± 7.3*	21.6
2%	89.0	85.0	67.2	80.4 ± 11.6	19.3
CC					
0.5%	81.6	90.1	81.1	84.3 ± 5.1	14.5
1%	77.9	78.8	88.2	81.6 ± 5.7	17.8
2%	70.7	80.0	64.2	71.6 ± 7.9*	30.4

[1]0.5~2% of extract in final concentration of 1.3% ethanol, *$p < 0.05$, **$p < 0.01$ (Student's t-test)

Table 3. The effect of *Crataegus pinnatifida* and *Cinnamomum cassia* on catalytic activity of CYP2E1

	OD526 nm				
Treatment[1]	Ind. Value			Mean ± SD	Inhibition (%)
1.3 % ethanol	0.124	0.115	0.112	0.117 ± 0.006	–
CP (μg/ml)					
50	0.022	0.039	0.037	0.033 ± 0.009**	72.1
100	0.011	0.032	0.002	0.015 ± 0.015**	87.2
200	0.019	0.013	0.014	0.015 ± 0.003**	87.2
CC (μg/ml)					
50	0.017	0.032	0.039	0.029 ± 0.011**	74.9
100	0.023	0.024	0.022	0.023 ± 0.001**	80.3
200	0.004	0.004	0.000	0.003 ± 0.002**	97.7

[1]50~200 μg/ml of extract in final concentration of 1.3 % ethanol, *$p < 0.05$, **$p < 0.01$ (Student's t-test), CP: *Crataegus pinnatifida,* CC:*Cinnamomum cassia*

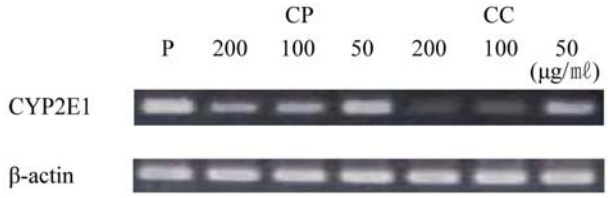

Fig. 1. RT-PCR analysis of CYP2E1 expression in HepG2 cells after being induced by 1.3% ethanol without or with 50~200 μg/ml of extract. P: 1.3% ethanol treatment, *Crataegus pinnatifida* (CP) and *Cinnamomum cassia* (CC).

Inhibition of CYP2E1 enzyme expression – As shown in Fig. 1, CYP2E1 expression enhanced by 1.3% alcohol was inhibited concentration-dependently by treating 50~200 μg/ml concentration of *Crataegus pinnatifida,* or *Cinnamomum cassia* or the both substances. *Cinnamomum cassia* had evidently a better inhibitory effect than *Crataegus pinnatifida*.

Inhibition of CYP2E1 enzyme activation – The calibration curve of CYP2E1 enzyme reaction with nitrophenol as the substrate shows linear relationship ($r^2 = 0.960$) with concentration of substrate (Data were not shown). As a result of enzyme catalytic assay in Table 2, the effect of the extracts of *Crataegus pinnatifida* and *Cinnamomum cassia* on the activation of CYP2E1 enzyme was quantified by absorbance. By treating the extracts (50~200 μg/ml) of *Crataegus pinnatifida* and *Cinnamomum cassia,* the activation of CYP2E1 enzyme was inhibited significantly by 72.1~87.2% ($p < 0.01$) and 74.9~97.7% ($p < 0.01$) respectively. The inhibitory effect was relatively high in the both extracts. Like the result of RT-PCR analysis on the inhibition of CYP2E1 expression, *Cinnamomum cassia* had a slightly better inhibitory effect on CYP2E1 activation than *Crataegus pinnatifida*.

Discussion

As a result of screening the effect of 120 Korean plant extracts on ethanol-induced cytotoxicity in liver cells, it has been found that *Crataegus pinnatifida* and *Cinnamomum cassia* have a high protective effect on cytotoxicity.[16] Therefore, this study conducted a comparative assay on the cytoprotective effect of pure ethanol and ethanol test solution containing a certain concentration of these two plant extracts. Ethanol is a chemical substance that induces fatty liver, liver cirrhosis or liver cancer in human bodies. It is required to find a proper pharmaceutically active material that reduces ethanol-induced cytotoxicity in liver cells. The cytotoxicity of ethanol is associated with acetaldehyde or ROS generated by catalytic activities of alcohol dehydrogenase and microsomal cytochrome P450 that is involved in the metabolism of ethanol.[22] It is reported that CYP2E1 induction by ethanol plays a pivotal role for the pathogenesis of alcoholic liver diseases.[23]

As a strong CYP2E1 inducing agent, ethanol generates superoxide radical and hydrogen peroxide, and is oxidized into acetaldehyde, generating free radicals. These radicals cause lipid peroxidation and single strand breaks of DNAs along with various types of genetic toxicity including cytotoxicity and adduct formation.[24,25] Therefore, the inhibition of ethanol-induced CYP2E1 can be the mechanism of reducing the cytotoxicity of ethanol. There is a report that Dilinoleoyl phosphatidyl choline (DLPC) reduces alcohol-induced cytotoxicity in HepG2 cells through CYP2E1 inhibition[26], and there is another report that diallyl disulfide, which is a main ingredient of garlic is effective in the treatment of ethanol-induced liver damage preventing

hepatotoxicity incurred by the metabolism of CYP2E1.[27]

Crataegus pinnatifida, which is a tree so-called Chinese hawthorn, has a lot of thorns. Its fruit contains polyphenols, procyanidines, chlorogenic acid and flavonoids[28,29], but nothing special has been reported on its stem. Among the components of its fruit, flavonoid like quercetin is known for protective effect on ethanol-induced cytotoxicity in liver cells as CYP2E1 inhibitor.[30-33] Meanwhile, *Cinnamomum cassia*, so-called cinnamon, contains cinnamaldehyde and eugenol.[34] There is a report that among the components of *Cinnamomum cassia*, O-methoxycinnamaldehyde, which is cinnamic aldehyde inducing agent, is effective for CYP2E1 inhibition.[35] Therefore, putting the results of RT-PCR analysis and enzyme catalytic assay on cytoprotective effect and CYP2E1 inhibition together, it is considered that not only the antioxidative activity of *Crataegus pinnatifida*[28,36,37] and *Cinnamomum cassia*[38,39] but also the inhibitory effect of *Crataegus pinnatifida* and *Cinnamomum cassia* on on the generation of active oxygen incurred by the metabolism of ethanol through CYP2E1 expression and activation result in the inhibition of oxidative DNA damage.

As mentioned in the result of the comet assay for the detection of DNA single strand breaks, ethanol reduces the genomic stability by causing damage, such as the generation of ROS, to DNAs in cells.[40] Such genotoxicity is deeply associated with the development of cancer in the organs chronically exposed to alcohol such as stomach and liver. Therefore, it is considered that CYP2E1 metabolism inhibitory substances of *Crataegus pinnatifida* and *Cinnamomum cassia*on can contribute to the improvement of the genomic stability of human bodies by reducing ethanol-induced DNA damage.[41] Except for the results of cytoprotective effect, *Cinnamomum cassia* had a better effect on inhibition of DNA damage than *Crataegus pinnatifida*, as well as showed a high tendency to inhibit CYP2E1 expression and catalytic activities. Therefore, it is also required that studies on the mechanism of ROS generation, antioxidant enzyme activity change and pathophysiologic change induced by *Crataegus pinnatifida* and *Cinnamomum cassia* in liver cells of animal models.

In summary, this study compared and confirmed the protective effect of the extracts produced from branches of *Crataegus pinnatifida* and barks of C*innamomum cassia* on the ethanol-induced cytotoxicity and DNA damage in liver cells. The protective effect of these two extracts on ethanol-induced cytotoxicity and genotoxicity may result from the inhibition of CYP2E1 expression and activation. Therefore, we need to have great interest in *Crataegus pinnatifida, Cinnamomum cassia* and their new possible bioactive components as potential candidate substances for the treatment of ethanol-induced cytotoxicity and genotoxicity in liver cells.

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