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Effects of Extract and Isorhamnetin Glycoside from *Brassica juncea* on Hepatic Alcohol-Metabolizing Enzyme System in Rats

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Abstract – The effects of methanol extract of the leaves of *Brassica juncea* and its major component, isorhamnetin 3-*O*-β-D-glucopyranoside on hepatic alcohol metabolizing enzymes were investigated. The methanol extract and isorhamnetin 3-*O*-β-D-glucopyranoside supplementations increased the activities of microsomal ethanol oxidizing system and aldehyde dehydrogenase in a dose-dependent manner, and had mild effects on the activities of alcohol dehydrogenase and catalase. Isorhamnetin 3-*O*-β-D-glucopyranoside alleviated the adverse effect of ethanol ingestion by enhancing the activities of alcohol oxidizing emzymes, microsomal ethanol oxidizing system and aldehyde dehydrogenase.
Keywords – Alcohol detoxification, Isorhamnetin 3-*O*-β-D-glucopyranoside, Microsomal ethanol oxidizing system, Aldehyde dehydrogenase, *Brassica juncea*

Introduction

Excessive alcohol ingestion clearly affects the brain and causes difficulty in walking, blurred vision, slurred speech, slowed reaction, and impaired memories. Chronic alcohol consumption has been highly associated with liver diseases (fatty liver, alcoholic hepatitis and cirrhosis), coronary artery disease and stroke (Pearson, 1996; Conway, 2005). And the highest cancer risk associated with alcohol consumption is seen for the upper aerodigestive tract (oral cavity, pharynx, larynx and esophagus), and heavy drinking, especially combined with smoking, increases the risk of developing these cancers (Seitz and Becker, 2007). Alcohol-related liver cancer, hepatocellular carcinoma primarily develops in people with liver cirrhosis resulting from chronic excessive alcohol drinking (Pöschl and Seitz, 2005).

The amount of dietary fat intake and degree of fatty infiltration are important factors in the pathogenesis of alcoholic steatosis and fibrosis (Lieber and DeCarli, 1970a; Tsukamoto *et al.,* 1986). In rats fed ethanol, hepatic triglycerides strikingly accumulated when the fat content of diet increased above 25% of the calories.

The major organ of alcohol metabolism is the liver. In the liver, alcohol can be oxidized by three enzyme systems, the cytoplasmic alcohol dehydrogenase, microsomal ethanol oxidizing system, and caltalase. These three emzymatic pathways convert alcohol into acetaldehyde, which is rapidly oxidized to acetate by aldehyde dehydrogenase. Acetaldehyde, a reactive molecule that can oxidize and covalently bind to a variety of functional groups, is thought to be a major cause of alcoholic liver damage (Niemelö *et al.,* 1994). Reactive oxygen species and other reactive metabolites generated by alcohol ingestion in the hepatic tissue are effectively scavenged by hepatic antioxidative enzymes such as superoxide dismutase, catalase, glutathione reductase and nonenzymatic antioxidants such as vitamins (Lecomte *et al.,* 1994).

The recommended therapies for alcoholic liver diseases are lifestyle change, proper nutrition, drug (conventional and alternative therapies) and liver transplantation. As a result of continuing studies for developing the natural hepatoprotective agents against ethanol, we found that isorhamnetin 3-*O*-β-D-glucopyranoside significantly enhanced hepatic ethanol metabolism. Isorhamnetin 3-*O*-β-D-glucopyranoside has been reported some biological activities such as immunomodulatory, antiinflammatory and antioxidative effects (Akbay *et al.,* 2003; Ahmed *et al.,* 2005; Wijeratne *et al.,* 2006). In this study, we investigated the effects of the methanol extract of the

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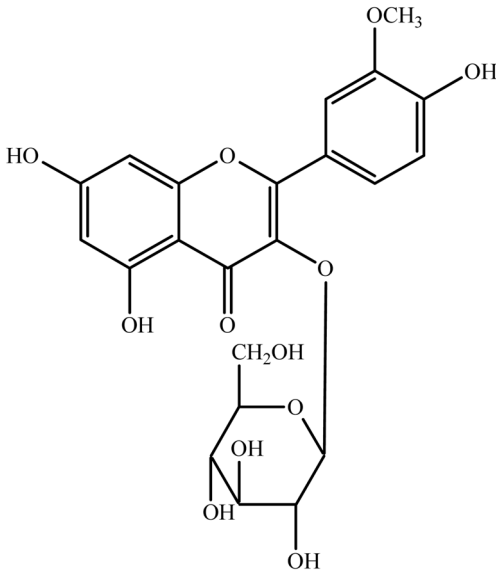

Fig. 1. Structure of isorhamnetin 3-*O*-*β*-D-glucopyranoside.

leaves of *Brassica juncea* (Cruciferaceae) and its major component, isorhamnetin 3-*O*-*β*-D-glucopyranoside on hepatic enzymes responsible for alcohol metabolism. The seeds of *B. juncea* have been used as a spice and in traditional folk medicine as a treatment agent of several diseases such as arthritis, lumbago, dyspepsia, neuralgia, and rheumatism.

Experimental

Plant material – The leaves of *B. juncea* were purchased from a farm in Dolsan region, Yeosu city, Jeonnam, Korea in April, 2005. A voucher specimen has been deposited at the Herbarium of Sunchon National University.

Extraction and isolation of isorhamnetin 3-*O*-*β*-D-glucopyranoside – The air-dried leaves of *B. juncea* (3.7 kg) was refluxed with methanol 4 times for 3 h and evaporated to obtain extract (996 g). This extract was successively partitioned with dichloromethane, ethyl acetate, *n*-butanol and aqueous fraction using organic solvents of different polarities. The ethyl acetate fraction (18 g) was carried out silica gel column chromatography by elution with CH_2Cl_2-MeOH-H_2O mixtures to gain compounds. Isorhamnetin 3-*O*-*β*-D-glucopyranoside (780 mg) was isolated as one of major component of this plant. Its structure (Fig. 1) was elucidated by the comparison NMR spectral data with reference (Lim *et al.,* 2006). Its NMR data is summarized as follow.

Isorhamnetin 3-*O*-*β*-D-glucopyranoside – ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 7.93 (1H, d, J = 2.04 Hz, H-2'), 7.50(1H, dd, J = 2.04 & 8.4 Hz, H-6'), 6.92 (1H, d, J = 8.4 Hz, H-5'), 6.46 (1H, d, J = 2.04 Hz, H-8), 6.22 (1H, d, J = 2.04 Hz, H-6), 5.56 (1H, d, J = 7.4, anomeric H of glucose), 3.84 (3H, s, -OCH_3); ^{13}C-NMR (DMSO-d_6, 100.5 MHz) δ: 177.4 (C-4), 164.1 (C-7), 161.2 (C-5), 156.4 (C-2), 166.3 (C-9), 149.3 (C-3'), 146.9 (C-4'), 132.9 (C-3), 122.0 (C-6'), 121.0 (C-1'), 115.2 (C-2'), 113.4 (C-5'), 104.0 (C-10), 100.7 (C-1"), 98.7 (C-6), 93.7 (C-8), 77.3 (C-3"), 76.3 (C-5"), 74.3 (C-2"), 69.8 (C-4"), 60.6 (C-6"), 55.6 (-OCH_3).

Animals – Male Sprague-Dawley rats (Daehan BioLink, Eumsung, Korea), weighing 200 ± 10 g, were kept at 20 ± 2 ºC with a 12 hr light cycle. Rats were fed a standard commercial rat diet *ad libitum* and allowed free access to 25% ethanol solution, or an isocaloric sucrose solution for normal group, for 6 weeks. Rats were orally administered daily with the methanol extract at a dose of 250 or 500 mg/kg of body weight or isorhamnetin 3-*O*-*β*-D-glucopyranoside at a dose of 5 or 10 mg/kg of body weight during last two weeks of ethanol treatment. The dosages were chosen within ranges known not to exhibit any special adverse effects on the animals. Metadoxine (20 mg/kg) was used as a reference compound. Animals were starved overnight before sacrifice.

Preparation of enzyme source – Animals were sacrificed by exsanguination under anesthesia with CO_2 gas. Liver was perfused exhaustively with ice-cold saline solution through the portal vein until the organ was uniformly pale and immediately removed and weighed. Liver was homogenized with 4 volumes of ice-cold 0.25 M sucrose solution containing 2 mM mercaptoethanol. The homogenate was centrifuged at 600 × g for 10 min. The supernatant was recentrifuged at 10000 × g for 20 min, and the resulting pellet containing the mitochondrial fraction, was used as the enzyme source of aldehyde dehydrogenase. The supernatant was further centrifuged at 105000 × g for 60 min to obtain the upper fraction as cytoplasm. The pellet was resuspended and centrifuged again at 105000 × g for 60 min to obtain the microsomal fractions at 2 - 4 ºC. The cytoplasmic fraction was used as the enzyme source of alcohol dehydrogenase, and the microsomal fraction was used as the enzyme sources of microsomal ethanol oxidizing system.

Alcohol dehydrogenase activity – Alcohol dehydrogenase activity of the cytoplasmic fraction of liver was measured spectrophotometically at 340 nm by the method of Borson *et al.* (1997) with some modification. The reaction mixture consisted of 50 mM glycine buffer, pH 7.4, containing 13.3 mM NAD^+, 10 mM ethanol and 100 μL of cytoplasmic fraction at a final volume of 4.0 mL.

Enzyme activity was defined as nmols of NADH produced/mg protein/min.

Aldehyde dehydrogenase activity – Aldehyde dehydrogenase activity of the liver mitochondrial fraction was measured spectrophotometically by monitoring the rate of NADH formation at 340 nm (Inoue *et al.,* 1980). The enzyme reaction was carried out in a 70 mM sodium pyrophosphate buffer containing 60 mM propionaldehyde as a substrate and 13.3 mM NAD^+ as a cofactor in the presence of 16.7 mM pyrazole as an inhibitor of ADH in a total volume of 4.0 mL. After incubation for 5 min at 37 °C, NADH was measured at 340 nm. Enzyme activity was defined as nmols of NADH produced/mg protein/min.

Microsomal ethanol oxidizing system activity – Activity of microsomal ethanol oxidizing system was measured spectrophotometically at 340 nm by the method of Lieber and DeCarli (1970b) with minor modification. 1 mL of 0.3 μM NADPH, 50 μM ethanol, 5 μM $MgCl_2$, 80 μM phosphate buffer (pH 7.4) were put into outer well of a 50 mL Erlenmeyer flask type reaction apparatus at 37 °C, and 0.6 mL of 0.16 M potassium phosphate buffer, pH 7.0, containing 15 mmole of semicarbazide-HCl was added into center well. The reaction was initiated by putting 1.0 mL of microsome into the outer well. After 30 min of incubation, the reaction was stopped by adding 70% TCA to the outer well, followed by sitting at room temperature for 24 h. The resulting acetaldehyde-semicarbazone complex was measured at 224 nm, and the enzyme activity was calculated from a standard curve. Enzyme activity was expressed as nmols of acetaldehyde produced/mg protein/min.

Catalase activity – Catalase activity was measured by monitoring the enzymatic decomposition rate of H_2O_2 at 240 nm as reported by Abei (1974). The reaction mixture (final volume of 3.0 mL) consisted of 50 mM phosphate buffer, pH 7.0, 30 mM hydrogen peroxide and 100 mL mitochondrial fraction. Enzyme activity was defined as the disappearance of H_2O_2 nmol/mg protein/min.

Protein assay and statistical analysis – Protein content was determined by Folin phenol reagent using bovine serum albumin as a standard (Lowry *et al.,* 1970). Statistical differences between the experimental groups were determined by using ANOVA and Duncan's multiple range test.

Results and Discussion

Alcohol-related disorders are one of the challenging current health problems combined with medical, social and economic consequences in the world. Chronic alcohol abuse potentially results in serious illness, including fatty liver, cirrhosis, cardiovascular disease, and so on. Alcohol is readily absorbed from the gastrointestinal tract, circulated rapidly, and distributed uniformly throughout the body. 80 - 90% of the alcohol absorbed is rapidly metabolized to toxic acetaldehyde in the liver. Acetaldehyde, the main metabolite of alcohol accumulates in the liver and blood as a result of excessive alcohol consumption. And aldehyde dehydrogenase metabolizes the bulk of acetaldehyde into acetic acid in the liver. Acetaldehyde caused depletion of mitochondrial glutathione, impair mitochondrial β-oxidation of fatty acids, and promote formation of reactive oxygen species (Lluis *et al.,* 2003).

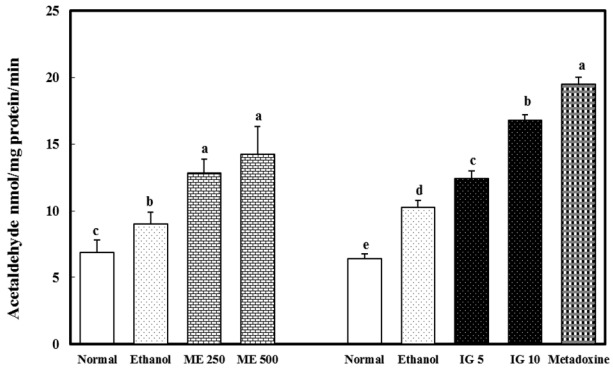

Fig. 2. Effects of methanol extract and isorhamnetin 3-*O*-β-D-glucopyranoside of *Brassica juncea* on hepatic alcohol dehydrogenase activity in rats treated with ethanol. Rats were fed a standard commercial rat diet *ad libitum* and allowed free access to 25% ethanol solution, or an isocaloric sucrose solution for normal group, for 6 weeks. Rats were orally administered daily with the methanol extract (ME) at a dose of 250 or 500 mg/kg of body weight or isorhamnetin 3-*O*-β-D-glucopyranoside (IG) at a dose of 5 or 10 mg/kg of body weight during last two weeks of ethanol treatment. The values are mean ± S.D. of 6 replications. Means sharing the same superscript letter are not significantly different ($p > 0.05$).

The hepatic alcohol dehydrogenase activity was little altered by the alcohol treatment, but was mildly affected by methanol extract and isorhamnetin 3-*O*-β-D-glucopyranoside, while metadoxine used as a positive control showed good effect (Fig. 2). The microsomal ethanol oxidizing system accounts for only a small part of total metabolism because of a low catalytic efficiency relative to alcohol dehydrogenase. However, long-term alcohol ingestion increased the enzyme activity up to 10-fold, which is largely responsible for the metabolic tolerance to ethanol (Lieber, 1987). Therefore, microsomal ethanol oxidizing system is greatly involved in alcohol decomposition in heavy drinking or chronic alcohol ingestion condition, although most of the ingested alcohol is normally metabolized by alcohol dehydrogenase. The methanol extract and isorhamnetin 3-*O*-β-D-glucopyrano-

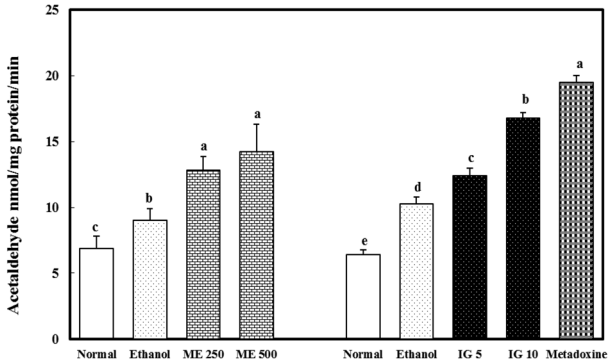

Fig. 3. Effects of methanol extract and isorhamnetin 3-*O*-*β*-D-glucopyranoside of *Brassica juncea* on hepatic microsomal ethanol oxidizing system activity in rats treated with ethanol. Treatments and abbreviations are in Fig. 2. The values are mean ± S.D. of 6 replications. Means sharing the same superscript letter are not significantly different ($p > 0.05$).

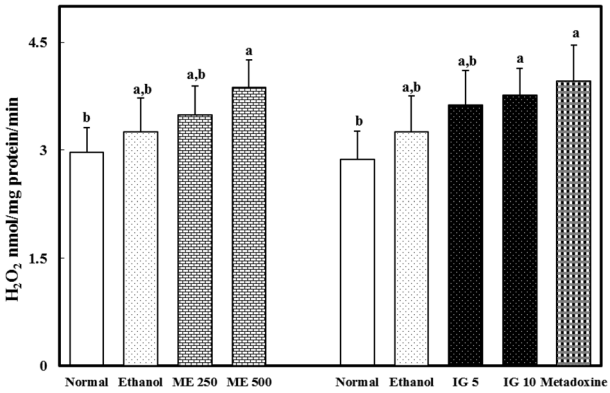

Fig. 4. Effects of methanol extract and isorhamnetin 3-*O*-*β*-D-glucopyranoside of *Brassica juncea* on hepatic catalase activity in rats treated with ethanol. Treatments and abbreviations are in Fig. 2. The values are mean ± S.D. of 6 replications. Means sharing the same superscript letter are not significantly different ($p > 0.05$).

side greatly increased the microsomal ethanol oxidizing system activity dose-dependently compared with alcohol alone group, even though its effects were lower than metadoxine used as a positive control (Fig. 3).

As shown in Fig. 4, the catalase activity which plays only a minor role under normal physiological conditions (DeMaster *et al.,* 1981) was mildly increased by methanol extract, isorhamnetin 3-*O*-*β*-D-glucopyranoside, and metadoxine in the experimental conditions. The hepatic aldehyde dehydrogenase enzyme rapidly converts acetaldehyde to acetate and use NAD^+ as a cofactor. This enzyme activity significantly decreased in ethanol-treated rats, which was greatly attenuated by methanol extract and isorhamnetin 3-*O*-*β*-D-glucopyranoside, dose-dependently (Fig. 5). Overproduction of reactive oxygen species produced by alcohol metabolism causes to deplete

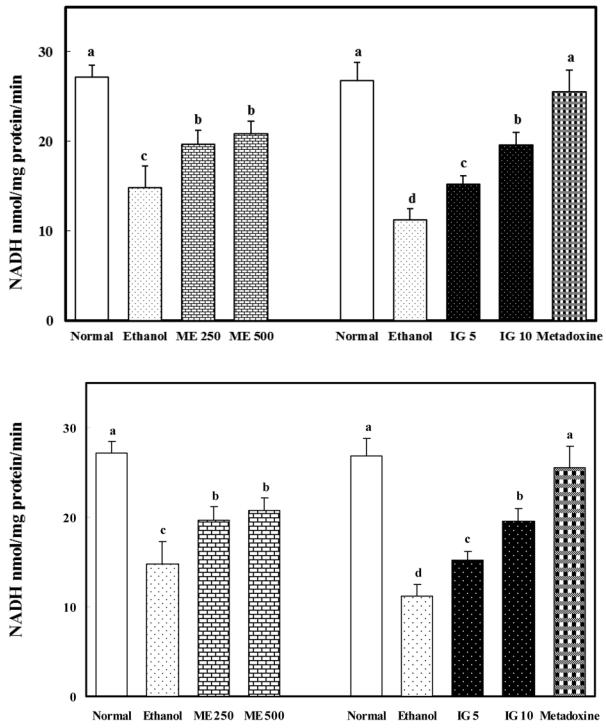

Fig. 5. Effects of methanol extract and isorhamnetin 3-*O*-*β*-D-glucopyranoside of *Brassica juncea* on hepatic aldehyde dehydrogenase in rats treated with ethanol. Treatments and abbreviations are in Fig. 2. The values are mean ± S.D. of 6 replications. Means sharing the same superscript letter are not significantly different ($p > 0.05$).

the antioxidant defense system, resulting in lipid peroxidation, protein oxidation, DNA damage and adduct formation (Niemelö *et al.,* 1994). In previous study, isorhamnetin 3-*O*-*β*-D-glucopyranoside provided mild protection against lipid peroxide level increased by bromobenzene, hepatotoxic chemical (Hur *et al.,* 2007).

Among the medicinal plants used in traditional Chinese medicine, the roots of *Pueraria lobata* have been used to treat alcohol abuse safely and effectively and its extract suppressed the free-choice ethanol intake of ethanol-preferring golden Syrian hamsters (Rezvani *et al.,* 2003). Two isoflavones, daidzin and daidzein accounted for antidipsotropic effect of the root of this plant. And also St. John's wort which has been used to treat mild to moderate depression, showed potential antidipsotropic effect (Rezvani *et al.,* 1999). This medicinal plant has been shown to be effective in reducing alcohol intake. In previous studies on hepatic alcohol-metabolizing enzymes, hispidulin 7-*O*-neohesperidoside isolated from *Cirsium japonicum* var. *ussuriense* and gallic acid from *Orostachys japonicus* showed the good detoxification effect by enhancing the activities of alcohol-oxidizing enzymes (Park *et al*., 2004; Hur and Park, 2006). So, medicinal plants are considered

as a useful candidate for development of antidipsotropic agents and treatment agents for diseases associated with alcohol.

In conclusion, the methanol extract of the leaves of *B. juncea* and isorhamnetin 3-*O*-β-D-glucopyranoside, one of the major components of this plant, increased the hepatic microsomal ethanol oxidizing system and aldehyde dehydrogenase activities associated with ethanol oxidation.

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