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Original Article

Protective effects against alcoholic liver damage: potential of herbal juice (HJ), blend of *Zingiber officinale Roscoe* and *Pueraria lobata Ohwi* extracts

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Protective effects against alcoholic liver damage: potential of herbal juice (HJ), blend of *Zingiber officinale Roscoe* and *Pueraria lobata Ohwi* extracts

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Objectives : Alcohol-induced liver disease advances as to reactive oxygen species (ROS) and cellular lipid peroxidation increase. We examined the hepatoprotective effects of Zingiber officinale Roscoe rhizome extract (ZR), Pueraria lobata Ohwi flower extracts (PF), and a newly developed herbal juice (HJ), which was a combination of ZR and PF extracts, against ethanol-induced hepatotoxicity.

Methods: The study utilized the human hepatoma cell line HepG2 cells to validate the hepatoprotective effect of HJ (50~200 μg/mL) against ethanol (EtOH, 700 mM)-induced liver damage.

Results: HJ effectively reduced the protein expression of sterol regulatory element-binding transcription factor 1, adiponectin, and AMP-activated protein kinase in EtOH-induced HepG2 cells. The levels of ROS, total cholesterol, and triglycerides, which are the result of various synthesis and lipogenesis processes induced by EtOH in the liver, were reduced by HJ. Furthermore, the activities of alcohol dehydrogenase and aldehyde dehydrogenase, enzymes linked to alcohol degradation, were more effectively downregulated by HJ treatment compared to treatment with ZR and PF alone, all without causing cytotoxic effects.

Conclusions: HJ protects the liver by inhibiting EtOH-induced lipogenesis, lowering ROS generation, and improving alcohol degradation, which is more effective than ZR and PF alone. Further, in vivo experiments can offer additional evidence regarding the effectiveness, safety, and underlying mechanism of action of HJ.

Keywords : Zingiber officinale Roscoe; Pueraria lobata Ohwi; alcoholic liver damage

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서 론

The liver is one of the largest organs in human body. It is also a very critical organ responsible for regulation of metabolism, immunity, digestion, detoxification, and vitamin storage[1]. It can be damaged by various factors, such as chronic alcohol abuse, fat accumulation in the liver, certain prescription or over-the-counter medications, or even certain herbal compounds[2, 3]. In particular, unhealthy eating habits and excessive drinking these days can cause accelerated liver damage. Therefore, our study aims to analyze the efficacy of several natural extracts in protecting liver damage caused by excessive alcohol intake.

When we consume EtOH through drinking, it can lead to various effects, typically promoting lipogenesis in liver and lowering antioxidant activity. Alcoholic fatty liver is the result of the early response in the liver, and if this condition becomes chronic, it can develop into liver cancer[4]. We focused on the sterol regulatory element binding protein-1 (SREBP-1) in this onset mechanisms EtOH-regulated SREBP-1 promoter can lead to mature SREBP-1 protein[5]. Mature SREBP-1 induces and accumulates fatty acid and cholesterol synthesis in the liver, thereby resulting in an increase in total cholesterol and triglycerides[6,7]. Therefore, in this study, we confirmed whether EtOH -induced SREBP-1 can be inhibited by potential of herbal juice (HJ), blend of Zingiber officinale Roscoe and Pueraria lobata Ohwi extracts, and regulation of upstream proteins adiponectin and AMPK activity[8].

Interestingly, EtOH can also increase reactive oxygen species (ROS) production by disrupting the ratio of reduced glutathione (GSH) and oxidized glutathione (GSSG)[9]. Thereafter, ROS can trigger the inflammatory reaction in liver, and leading to the mitochondrial disfunction, and finally necrosis as well as apoptotic cell death can result in extensive liver damage[10]. Therefore, we investigated whether HJ can reduce liver damage by restoring antioxidant activity.

First of all, the degradation of EtOH after intake, is carried out by these two main enzymes, ADH and ALDH[11]. EtOH metabolism releases AA, and thus the cells exposed to acetaldehyde that can be carcinogenic, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) are then activated to degrade it[12]. Therefore, we intend to reduce liver damage by enhancing the activity of ADH and ALDH after treatment with HJ.

In this study, we investigated the impact of HJ, which is combination of Zingiber officinale Roscoe extract (ZR) and Puerariae Flos extracts (PF) to ameliorate the liver damage. It was observed that by reducing SREBP-1, HJ suppressed lipogenesis, and thereafter attenuated the total cholesterol and triglyceride were reduced. In addition, HJ reduced ROS generation and increased the activities of ADH and ALDH that can degrade acetaldehyde, a product of EtOH. Overall, we confirmed that HJ can prevent and alleviate liver damage through diverse mechanims, and can be used for protection against liver damage.

재료 및 방법

Preparation of HJ

The dried rhizome of Zingiber officinale Roscoe and dried flower of Pueraria lobata Ohwi were purchased from Dong-Yang Herb Inc. (Seoul, Korea). Each herb was extracted in 300 mL of distilled water for 2 hours at 100 ℃. The extraction of each herb was concentrated in a rotary vacuum evaporator and lyophilized. The resulting extracts were named ZR for rhizome of Zingiber officinale Roscoe and PF for flower of Pueraria lobata Ohwi, respectively. HJ, a newly formulated herbal juice, was created by blending ZR and PF in a 2:1 ratio. The yield of ZR and PF powder was 25.99 % and 13.00 %, respectively. The lyophilized powders were stored at -20 ℃ until use.

Reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Tris base, glycine, NaCl, sodium dodecyl sulfate (SDS), and bovine serum albumin (BSA), Alcohol Dehydrogenase Activity kit, and Aldehyde Dehydrogenase Activity kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-SREBP-1, anti-adiponectin, anti-phosphor-AMPK, and anti—AMPK antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-β-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The GSH/GSSG-GloTM Assay kit was purchased from Promega (Madison, WI, USA). Total Cholesterol (AM 202-K) and Triglyceride kit (AM 157S-K) were purchased from ASAN Pharm (Seoul, Korea).

Cell line and cultured condition.

Human hepatocellular carcinoma HepG2 cells were obtained from American Type Culture Collection (Manassas, VA). HepG2 cells were cultured with RPMI-1640 medium containing 10% inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were cultured and incubated at 37℃ in 5% CO2 conditions.

MTT assay for cell viability

To evaluate the cell viability, cells were subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HepG2 cells (5×10^3 cells/well) were seeded on 96 well plate and incubated for overnight in 37℃. Then cells were treated with each extract with indicated concentrations for 24 h. After treatment, 30 μL of MTT solution (5 mg/ml) was added for 2 h and MTT lysis buffer treated for at least 8 h. Lysed MTT formazans were measured by VARIOSKAN LUX (Thermo Fisher Scientific Inc, Waltham, MA) at 570 nm.

Western blot analysis

HepG2 cells (5×10^5 cells/well) were seeded and treated for the various indicated time intervals with HJ (200 μg/ml) and EtOH (700 mM). The cells were harvested and whole cell lysates were prepared to prepare SDS-PAGE gel running. Equal amounts of proteins were loaded and resolved in sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) gel, then electro transferred to nitrocellulose membrane. The membranes were blocked with 5% skimmed milk for 2 h at room temperature and probed with target

primary antibodies for overnight at 4℃. Thereafter, the membranes were incubated with horse radish peroxidase (HRP) on room temperature for 1 h. The membranes were detected by enhanced chemiluminescence (ECL) kit (EZ-Western Lumi Femto, DOZEN).

Glutathione measurement

HepG2 cells (1×10^4 cells/well) were treated with HJ (200 ㎍/ml) and EtOH (700 mM) for 2 h. Then GSH/GSSG was evaluated according to the previously described method[13].

Measurement of ROS production

HepG2 cells (5×10^5 cells/well) were treated with HJ (200 ㎍/ml) and EtOH (700 mM) for 2 h, then 3 mM of N-acetylcysteine (NAC) was treated for 15 min. Intracellular production of ROS was measured using cell-permeable fluorescent 2′,7′-dichlorofluorescin diacetate (H2DCF-DA) and ROS levels were measured as described earlier[14].

Alcohol dehydrogenase (ADH) activity assay

The alcohol dehydrogenase (ADH) activity was measured according to the manufacturer protocol. HepG2 cells were treated with HJ (0, 50, 100, 150, 200 ㎍/ml) and EtOH (700 mM) for 2 h. The cells were incubated with ADH assay buffer and then the absorbance of the solution was measured at 450 nm, until absorbance if the most active sample was greater than the value of the highest standard (10 nmol/well). A positive control included in the kit was used.

Aldehyde dehydrogenase (ALDH) activity assay

The aldehyde dehydrogenase (ALDH) activity was measured according to the manufacturer protocol. HepG2 cells were treated with HJ (0, 50, 100, 150, 200 ㎍/ml) and EtOH (700 mM) for 2 h. The cells were incubated with ADH assay buffer and then the absorbance of the solution was measured at 450 nm, until absorbance of the most active sample was greater than the value of the highest standard (10 nmol/well). A positive control included in the kit was used.

Oil red O staining

HepG2 cells (5×10^5 cells/well) were treated with HJ (200 ㎍/ml) and EtOH (700 mM) for 2 h. Then cells were fixed by 10% formalin for 1 h and washed by 60% isopropanol, then dried on room temperature. After drying, the cells were stained by 60% oil red O solution for 1 h and washed with distilled water. The cells were observed using by Nikon ECLIPSE Ts2 (magnification, 20×). Thereafter the cells were lysed with 60% isopropanol and measured by VARIOSKAN LUX (Thermo Fisher Scientific Inc, Waltham, MA) at 550 nm.

Total cholesterol measurement

HepG2 cells (1×10^6 cells/well) were treated with HJ (200 ㎍/ml) and EtOH (700 mM) for 2 h. Then the cells were dissolved by RIPA buffer (50 mM Tris-Hcl (ph 7.8), 150 mM NaCl, 1% triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 2mM EDTA). Cell lysate was mixed with buffer and incubated for 5 min at 37℃. The absorbance was measured at 500 nm within 60 min. Total cholesterol (mg/dL) level was derived through

the following calculation method.

$$Total\ cholesterol\left(\frac{mg}{dL}\right) = \frac{Sample\ abs}{Standatd\ sol\ abs} \times (Standard\ solution\ concentration = 300\ mg/dL)$$

Triglyceride measurement

HepG2 cells (1×10^6 cells/well) were treated with HJ (200 μg/ml) and EtOH (700 mM) for 2 h. Then cells were dissolved in RIPA buffer (50 mM Tris-Hcl (ph 7.8), 150 mM NaCl, 1% triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 2mM EDTA). The cell lysate was mixed with buffer and incubated for 10 min at 37℃. The absorbance was then measured at 550 nm within 60 min. Total cholesterol (mg/dL) level was derived through the following calculation method.

$$Triglyceride\left(\frac{mg}{dL}\right) = \frac{Sample\ abs}{Standatd\ sol\ abs} \times (Standard\ solution\ concentration = 300\ mg/dL)$$

Statistical analysis

All numerical values are represented as the mean ± SD. Statistical significance of the data compared with the untreated control was determined using student's t-test. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA) and determined using the Image J computerized densitometry system for image quantification. The relative band densities were determined using an Image J computerized densitometry system. Significance was set at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

결 과

HJ and constituent extracts can prevent EtOH-induced liver damage in human liver HepG2 cells.

To evaluate the protective effects of ZR, PF, and HJ (Mixture of ZR and PF), we measured cell viability after EtOH treatment. Although

EtOH caused a decrease in cell viability, but ZR, PF, and HJ treatment could increase it significantly in a dose dependent manner. (Fig1. A-C).

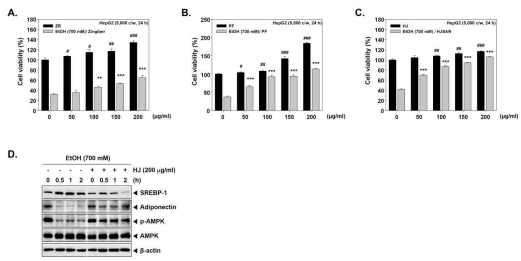

Figure 1. Protective effects of HJ constitutive extracts on human HepG2 liver cells. Cell viability of HepG2 cells under (A) ZR, (B) PF, and (C) HJ were evaluated by MTT assay. (D) Whole cell extracts were analyzed by Western blot analysis for SREBP-1, Adiponectin, p-AMPK and AMPK. ZR: *Zingiber officinale Roscoe* extracts; PF: *Pueraria lobata Ohwi* extracts; HJ, blend of *Zingiber officinale Roscoe* extracts and *Pueraria lobata Ohwi* extracts.

HJ inhibited EtOH-induced lipid synthesis related proteins in HepG2 cells.

Then we confirmed whether HJ could inhibit EtOH-induced lipid synthesis in HepG2 cells. The cells were treated with both HJ and EtOH with 0, 0.5, 1, 2 h. We found that SREBP-1 involved in lipid synthesis was

increased upon EtOH treatment and showed the highest activity at 2 h. However, it was confirmed that the activity was suppressed by HJ. Adiponectin and p-AMPK can decrease lipogenesis, although EtOH-induced inhibition can be recovered by HJ (Fig. 1D).

HJ suppressed ROS production through induction of GSH level.

ROS, another product of EtOH, can lead to reduced antioxidant activity. Therefore, we confirmed the ROS inhibitory effect of HJ in HepG2 cells. First, we measured GSH, GSSG level and GSSG/GSH ratio. EtOH reduced GSH level, whereas increased GSSG level. Conversely, it was confirmed that HJ increased GSH and decreased GSSG despite EtOH (Fig 2A-C). Then also, ROS production was increased 1.3% to 15.6% by EtOH, however, HJ can reduce it to 5.4%. And it was compared to the positive control, N-acetylcysteine (NAC) which has been known as antioxidant (Fig. 2D).

HJ induced the alcohol degradation by stimulating alcohol dehydrogenase and aldehyde dehydrogenase activity.

We measured alcohol dehydrogenase and aldehyde dehydrogenase activities to evaluate the action of HJ. Both enzymes are known to cause important contributions in the process of alcohol degradation, and interestingly dose-dependent HJ treatment increased the activity of both enzymes (Fig. 3A and B).

HJ inhibited lipogenesis after exposure to EtOH in HepG2 cells.

To evaluate the inhibitory effects of HJ on

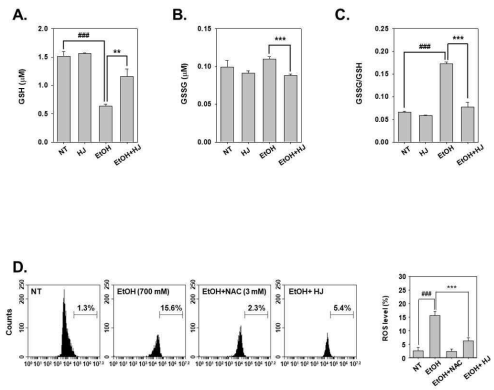

Figure 2. Inhibition of EtOH-induced ROS by HJ. (A) GSH, (B) GSSG, and (C) GSSG/GSH levels were measured under HJ treatment. (D) ROS level was evaluated by flow cytometer. GSH: glutathione; GSSG: oxidized glutathione; ROS: reactive oxygen species; HJ, blend of *Zingiber officinale Roscoe* extracts and *Pueraria lobata Ohwi* extracts

lipogenesis, we measured lipid levels using oil red O staining. The cells were treated with both HJ and EtOH for 2 h, staining and lysis. Oil Red O, which entered the fat, was then dissolved and the absorbance was measured. It was confirmed that EtOH increased staining of the lipids, however, it was inhibited by HJ treatment (Fig. 3C). Next, total cholesterol and triglyceride levels were evaluated. HepG2 cells were treated with HJ and EtOH for 2 h, then dissolved in RIPA buffer. We found that EtOH increased both total cholesterol and triglyceride levels in HepG2 cells, however, exposure to HJ reduced their levels (Fig. 3 D and E).

HJ, a combination extracts, exhibited higher potential for inhibiting liver damage than ZR and PF alone.

Then, we compared inhibitory potential of ZR, PF, and HJ individually in HepG2 cells. Similar to

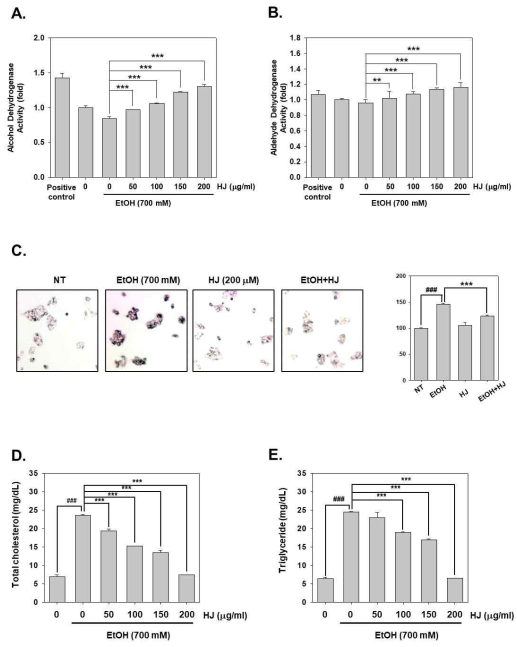

Figure 3. Induction of alcohol degradation activity by HJ in HepG2 cells. (A) Alcohol dehydrogenase activity and (B) aldehyde dehydrogenase activity was evaluated with dose-dependent HJ treatment. (C) Suppression of lipid by HJ were evaluated using Oil red O staining. (D) Total cholesterol and (E) triglyceride were evaluated with dose-dependent HJ treatment. HJ, blend of *Zingiber officinale Roscoe* extracts and *Pueraria lobata Ohwi* extracts.

before, HepG2 cells were treated with ZR, PF, and HJ (200 μg/ml) with EtOH for 2 h. The cells were analyzed for alcohol dehydrogenase and aldehyde dehydrogenase activity. Both alcohol degradation activity increased even when ZR and PF were treated alone, however, HJ, a mixture of both extracts, showed the highest activity (Fig. 4A and B). Then, inhibitory effects on lipogenesis were also compared and evaluated. The cell lysate was analyzed for total cholesterol and triglyceride. Both total cholesterol and triglyceride levels in HepG2 cells were increased after EtOH exposure but, all three extracts can reduce both of them, and HJ was found to be particularly effective (Fig. 4C and D).

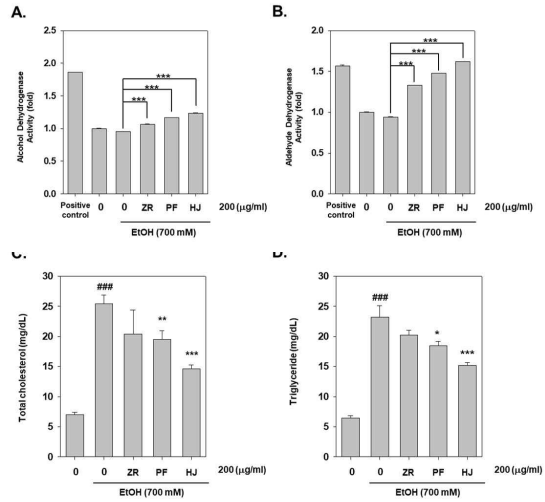

Figure 4. Comparison of ZR, PF, and the HJ on alcohol dehydrogenase, aldehyde dehydrogenase activity and lipogenesis in HepG2 cells. ZR and PF, and a mixture of the two, HJ, were compared, respectively. Alcohol degradation activity was measured as (A) alcohol dehydrogenase activity and (B) aldehyde dehydrogenase activity. Then lipid production was evaluated by measuring analyses of (C) total cholesterol and (D) triglyceride levels. ZR: *Zingiber officinale Roscoe* extracts; PF: *Pueraria lobata Ohwi* extracts; HJ, blend of *Zingiber officinale Roscoe* extracts and *Pueraria lobata Ohwi* extracts

결 론

Today, due to various causes, one of our major organs, the liver, is constantly prone to damage[2),3)]. The problem is, that if the damage becomes chronic, it can develop into cirrhosis and even liver cancer in severe cases[4)]. Therefore, in our study, we confirmed

the protective effects of HJ in preventing and alleviating ethanol-induced liver damage, and the findings suggest that it can contribute to liver improvement.

First, we evaluated the toxicity of HJ and its constituents, Zingiber officinale Roscoe extract (ZR) and Pueraria lobata Ohwi extracts (PF) by measuring the cell viability. HJ, as well as ZR and PF alone, showed no toxicity towards the cells, but rather restored viability after EtOH treatment. Through these results, we were able to confirm the potential recovery ability of the damaged hepatocytes HepG2 of each extract and HJ.

Next, we investigated the activity of SREBP-1, which occurs in the early stage of ethanol-induced liver damage mechanism. SREBP-1 synthesizes fatty acids and cholesterol in the liver, and is regulated by the activity of adiponectin and AMPK, which are upstream proteins[8]. In EtOH-stimulated hepatocyte HepG2, SREBP-1 level was increased with increasing time intervals, but on the contrary, adiponectin and p-AMPK activities were decreased at the same condition. However, despite EtOH stimulation, HJ inhibited fatty acid and cholesterol synthesis in hepatocytes by decreasing SREBP-1 and increasing the activities of adiponectin and p-AMPK. Through these results, we were able to identify one of the mechanisms by which HJ can potentially inhibit liver damage. Thereafter, we measured total cholesterol and triglyceride levels in hepatocyte HepG2. Because of EtOH exposure, lipogenesis was promoted and both total cholesterol and triglyceride levels were significantly increased[6),7)]. However, HJ could significantly decrease both productions of lipogenesis I in a concentration dependent manner. Moreover, the superiority of HJ was observed in comparison to ZR and PF. ZR and PF also reduced total cholesterol and triglyceride, however, HJ showed the greatest reduction. These results suggested that HJ, a mixture of the two extracts ZR and PF, has more superior liver protective efficacy.

EtOH can also interfere with the balance of reduced glutathione (GSH) and oxidized glutathione (GSSG), increasing reactive oxygen species (ROS) in hepatocytes[9]. Excessively increased levels of ROS can damage hepatocytes, and it can lead to necrosis and cell death[10]. Therefore, we investigated whether HJ can recover the ratio of GSH-GSSG and ROS production in hepatocytes HepG2. In EtOH-stimulated hepatocytes, ratio of GSH was significantly decreased, and GSSG ratio was increased at the same time. This imbalance resulted in an increase ROS production from 1.3% to 15.6%. However, HJ increased GSH ratio with reducing GSSG under EtOH stimulation. As a result, HJ reduced EtOH-induced ROS production 15.6% to 5.4%. Thus, these results confirmed that HJ can reduce liver damage by increasing the antioxidant activity.

Next, we investigated whether alcohol degradation can be promoted by HJ. We measured the activities of alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH), enzymes that can degrade alcohol, in EtOH and HJ treated hepatocytes HepG2[11),12)]. It was found that compared to EtOH treatment, the activities of ADH and ALDH increased upon HJ treatment,

and thus these results suggested that EtOH degradation in hepatocytes was promoted by HJ. In addition, compared to ZR and PF, HJ showed a higher activity of ADH and ALHD, and these findings indicated that HJ has a higher alcohol degradation ability.

결 론

In our study, we confirmed the hepatocyte protective efficacy of HJ, a mixture of two naturally derived extracts, Zingiber officinale Roscoe extract (ZR) and Pueraria lobata Ohwi extracts (PF). The results showed that HJ inhibited lipogenesis and liver damage through diverse molecular mechanisms, and caused the inhibition of EtOH-induced production of fatty acid and ROS. Moreover, HJ showed significant hepatocyte protective effects by increasing alcohol degradation activity. Based on these data, the outstanding hepatoprotective effect of HJ seems to be further enhanced by ZR, accompanied by the elevated alcohol degradation and the reduction in lipid metabolism-related factors of PF extract.

감사의 말씀

N/A

이해관계

The authors declare no conflict of interest

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