



Synergetic Hepatoprotective Effects of Korean Red Ginseng and *Pueraria Radix* on the Liver Damaged-Induced by Carbon Tetrachloride (CCl₄) in Mice

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Abstract – This study was designed to investigate the synergetic hepatoprotective effects from a mixture of Korean Red Ginseng and *Pueraria Radix* on carbon tetrachloride (CCl₄)-induced hepatotoxicity in mice. Liver toxicity was induced by intraperitoneal administration of CCl₄ (0.6 mg/kg) in 12 groups of ICR mice. The negative control group was given CCl₄ without test samples and the normal group was given no treatment. Among treatment groups, the RGAP treatment (Korean Red ginseng acetic acid extract : *Pueraria radix* water extract, w/w, 38.4:57.6) decreased CCl₄-elevated ALT (101.60 IU/L), AST (833.89 IU/L), and LDH (365.02 IU/L) levels in the serum, and increased the SOD (11.03 unit/mg protein) and CAT (0.37 unit/mg protein) levels and the LPO levels (59.09 μM/g tissue) more than that in the mice group with CCl₄-induced control group hepatotoxicity. These results suggest that administration of a mixture of Korean Red ginseng and *Pueraria radix* decreases CCl₄-induced liver damage and enhances antioxidant activity in mice and imply that administration of the mixture in a certain ratio is more effective than single administration of either Korean Red ginseng or *Pueraria radix* alone.

Keywords – Carbon tetrachloride, Red Ginseng, *Pueraria Radix*, Hepatoprotective, Synergetic

Introduction

Although the modern world benefits from rapid development of industries and the convenience of automation, the side effects and consequences of industrialism such as exposure to risks in industrial fields, fatigue from change of life patterns, oxidative stress, nutritional imbalance, improper diet, and lack of exercise, damage the liver function, resulting in liver disorders, liver cirrhosis, chronic hepatitis, and liver cancer.¹ In particular, liver disease has a high prevalence rate in Korea and poses a threat to the health of its citizens. Thus, studies are actively being conducted on natural substances with therapeutic hepatic effects.²

Currently employed treatment methods of liver disease are largely classified into dietary therapy and medicinal therapy, and in most cases, these two methods are combined. In medicinal therapy, various medicines with different working mechanisms are used, based on the cause and type of liver disease. Typical medicines used in

clinical settings are regeneration promoters of hepatocytes, liver function supplements such as silymarin, biphenyl dimethyl dicarboxylate, ursodeoxycholic acid, glutathione, and glycyrrhizin extracted and separated from natural materials, antiviral medicines such as acyclovir, immunosuppressants such as corticosteroids, 6-mercaptopurine, and azathioprine, and vitamin B complex.³

Ginseng (*Panax ginseng*) is a medicinal plant that grows in the far Eastern regions, including Korea, China, and Japan. Red ginseng (RG) is defined as steamed and dried red-colored ginseng that is over 4 years old. RG has a tough tissue and a red outer surface because of a non-enzymatic-browning reaction during the drying process.⁴ The major ingredients of RG are saponin-family ginsenoside, polyacetylene-family panaxytriol, panaxadiol, and acidic polysaccharides, which have been reported to have effects such as promotion and enhancement of liver regeneration⁵, detoxification of toxic materials and alcohol⁶, enhancement of immunity⁷, antioxidant functions⁸, and anti-cancer functions.^{9,10}

Pueraria radix (PR) belongs to the family *Leguminosae* and is the root of Kudzu (*Pueraria lobata* Ohwi) with its periderm eliminated. It has been used as food and medicine in folk remedies for colds, headaches, appease-

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ment of thirst, diabetes, and diarrhea.¹¹ PR contains isoflavonoid-family daidzein, daidzin, genistein, and puerarin, and triterpenoid-family soyasapogenol A and kuzusapogenol B. The presence of substances such as polysaccharides has also been reported. These substances have been reported to have effects such as sedation, hypotensive functions¹², extension of coronary arteries¹³, suppression of lipid peroxidation¹⁴, detoxification of alcohol¹⁵, and liver protective functions.¹⁶

While there have been many studies on the hepatoprotective effect of separate administration of RG and PR, there is a lack of studies on comparison and analysis of the effect of combined or mixed administration of RG and PR. Therefore, in order to develop a medicine to prevent and treat liver disease, this study investigated the effect of the administration of a mixture of RG, PR, *Meretrix lusoria*, *Artemisia asiatica*, shell calcium extract, milk thistle (including 80% silymarin), and vitamin complex in a certain ratio in liver tissues damaged by CCl₄ in ICR mice. Additionally, this study compared treatment groups to investigate the synergistic effect of PR and RG and the reduction of liver damage.

Experimental

Plant materials – Among the test samples, 4-year-old ginseng was purchased from a large supermarket in the city of Chuncheon and PR was produced by and purchased from Young-cheon in the Gyeongbuk, Korea. Vitamin complex, shell calcium, *M. lusoria*, and *A. asiatica* were supplied by JS F&B Inc (Chuncheon, Korea). Milk thistle (including 80% silymarin) and product “Urusa” of Daewoong Pham. Co., Ltd (Ursodeoxycholic acid content: 100 mg) were generally used as positive controls in Korea. 2 kg of ginseng were put into an RG maker (Korea Maker, Korea), fumigated for 24 h, and dried in a 65 °C dry oven. This yielded 1.98 kg of reddish-black RG. Then, 1 L of distilled water was added to 100 g of RG, and reflux extraction was conducted at 100 °C for 3 h. The mixture was freeze-dried to yield 39 g of RG water extract (RGWE). RG acidic extract (RGAE) was refluxed for extraction using 0.1% acetic acid, and evaporated under reduced pressure to yield a residue (42 g). PR, *M. lusoria*, and *A. asiatica* were extracted using the same method with distilled water, and 40 g, 5 g, and 35 g of extracts were obtained, respectively.

Animals and treatment – Experimental animals used in this study were ICR mice with an average body weight of 37 ± 3 g, purchased from Joong-Ang Experimental Animal Center Inc. They were adapted for 1 week and

separated into 13 groups. The breeding environment for animal experiments was an automatically-controlled animal breeding room with a temperature of 22 ± 2 °C, humidity of 55 ± 5%, odor of less than 20 ppm, illumination of 150-300 lux, and a 12-h light and shade cycle. Solid feed (Samyang Oil and Feed Corp., Korea) and water were freely supplied. The experimentation, breeding, and management of animals were conducted according to the ‘Guide for the Care and Use of Laboratory Animals’, and experiments were performed with the authorization of the Ethics Committee of Hallym University (Hallym-2009-78).

Liver toxicity was induced by intraperitoneal administration of CCl₄ (0.6 mg/kg, CCl₄ : Olive oil, 3:2, v/v; final concentration = 1.0 mg/kg) in 12 groups of mice (all groups except for the normal group, or N group). The negative control group (C group) was given CCl₄ without test samples and the N group was given no treatment. The two positive control groups were administered product “Urusa” of Daewoong Pham. Co., Ltd in Korea (U group, 70 mg/kg) and milk thistle (including 80% silymarin, S group, 50 mg/kg). The RGAP (RGAE : PWE, 38.4:57.6%), RGW (RAW 96.0%), RGA (RGAW 96.0%), and PW (PWE 96.0%) were orally administered 200 mg/kg (b.w) for 10 days (*n* = 7) with vitamin complex (4.0%). In addition, other groups were orally administered 200 mg/kg (b.w) for 10 days (*n* = 7) with various RGAE and PWE ratio (%) and *A. asiatica* (12.5%), milk thistle (12.5%), shell extract (19.2%) and *M. lusoria* (19.2%) including vitamin complex (4.0%). After completion of the experiments, the animals were fasted for 18 h and anesthetized with diethyl ether. The compositions of the oral administration experimental diets are shown in Table 1.

Blood samples were drawn from an orbit vein. Collected blood was separated by centrifugation (350 × *g* at 4 °C for 20 min), and serum was used for blood biomarkers analysis. Right after blood collection, livers were removed and washed in physiological saline solution (0.9% NaCl solution). Water was removed from the livers, weights were measured, and they were stored in -75 °C until the time of analysis.

Measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) activities in serum – The activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was measured with a kit (Asan Pharmaceutical Inc., Korea) manufactured according to Reitman and Frankel method¹⁷, by estimating the absorbance at 505 nm using a spectrophotometer (EL 800 Universal Microplate Reader, BIO-TEK Instrument, Inc., Winooski, VT, USA).

Table 1. Composition of oral administration experimental diets (%)

Ingredients	Groups								
	RGAP	RGW	RGA	PW	RGWPSM	RGWP-1	RGWP-2	RGWPM-1	RGWPA-2
RGAE ¹⁾	38.4	–	96.0	–	–	–	–	–	–
RGWE ²⁾	–	96.0	–	–	38.4	19.2	38.4	38.4	38.4
PWE ³⁾	57.6	–	–	96.0	19.2	76.8	57.6	45.1	45.1
Vitamin complex	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
<i>A. asiatica</i>	–	–	–	–	–	–	–	–	12.5
Milk thistle	–	–	–	–	–	–	–	12.5	–
Shell extract	–	–	–	–	19.2	–	–	–	–
<i>M. lusoria</i>	–	–	–	–	19.2	–	–	–	–
Total (%)	100	100	100	100	100	100	100	100	100

¹⁾ RGAE : Red ginseng acid extract

²⁾ RGWE : Red ginseng water extract

³⁾ PWE : *Pueraria radix* water extract

According to King method¹⁸, the activity of lactate dehydrogenase (LDH) was measured as follows: substrate that contained 2.31 g of lithium lactate and 2.42 g of *tris*-hydroxy-methylamino-methane per 100 mL and chromogenic reagent that contained 574 mg of NAD and 3.4 mL of 1-methylphenassium metalsulfate were mixed at a ratio of 1:1 (v/v), maintained for 5 min at 37 °C, and reacted for 10 min at 37 °C by adding test sample. The last reaction was terminated by HCl, and activity was measured at a wavelength of 570 nm.

Measurement of activities of superoxide dismutase (SOD), catalase (CAT) and lipid peroxidase (LPO) in liver tissue – After 0.1 M phosphate buffer (pH 7.4) was added to 1 g of removed liver four times for homogenization, centrifugation (600 × g) was performed for 15 min. Using the cytosol fraction acquired from the ultracentrifugation of supernatant for 1 h (105,000 × g), the activity of superoxide dismutase (SOD) was measured according to Croppo *et al.*¹⁹ method and activity of catalase (CAT) was measured according to Lee and Shin method²⁰ at 420 and 240 nm, respectively. According to Ohkawa *et al.* method²¹, activity of lipid peroxidase (LPO) was measured as follows: 20% acetate buffer (pH 3.5) and 8.1% sodium dodecyl sulfate were placed into homogenized triturated liquid, and the color reagent 0.8% thiobarbituric acid was added. Then, the reaction was conducted for 1 h at 95 °C and cooled to room temperature, and *n*-BuOH : Pyridine (15:1, v/v) was added. After 15 min of centrifugation, the red layer was removed and measured at a wavelength of 540 nm.

Histopathological observation of liver tissue – Liver tissue of ICR mice administered CCl₄ was fixed in 10% formalin solution, embedded in paraffin at a thickness of

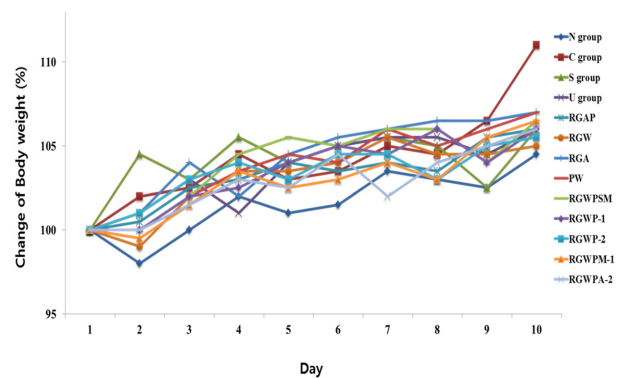


Fig. 1. Body weight change after 10-day oral administration of Red ginseng and *Pueraria radix* extract

¹⁾ Refer to Table 1.

²⁾ Data are expressed as the mean ± SE (*n* = 7)

5 μm, treated with hematoxylin-eosin staining, and observed with an optical microscope.²²

Statistical analysis – The results of this study were expressed as the mean ± standard error (SE) for each experimental group (*n* = 7) by using SAS package, and comparisons among data were carried out using Student's unpaired *t*-tests (*p* < 0.05).

Results

Measurement of increase in body weight and liver weight – Increases in the body weight of the experimental animals are presented in Fig. 1. Significant differences in weight increases of N group, comparison group, and experimental groups were not observed. Weight increase was somewhat greater in the C group administered CCl₄ than in the N group, while the liver damage in S, U, and experimental groups restricted their body weight increase.

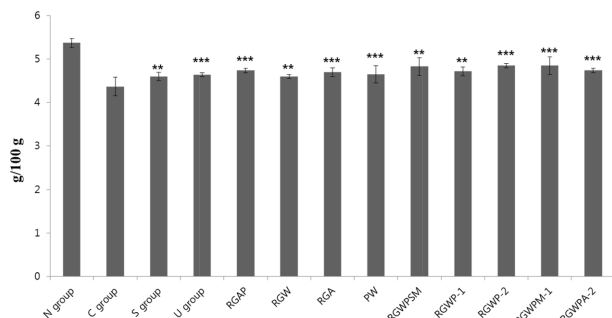


Fig. 2. Liver weight per 100 g of body weight, to examine the effect of CCl_4 and administration of extract of Red ginseng and *Pueraria radix* on the weight of the organ

1) Refer to Table 1.

2) Data are expressed as the mean \pm SE ($n = 7$)

Statistical significance of differences was calculated between C group and experiment groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

In addition, liver weight per body weight of 100 g was measured to examine the effect of various ways of ingestion of RG and PR against CCl_4 and CCl_4 administration on the organ weight (Fig. 2). Significant differences in liver weight increase of N group and experimental groups were not observed.

Change in the activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) in serum –

The results of the examination of the marker enzymes of liver function alanine aminotransferase (ALT) and aspartate aminotransferase (AST) after inducing liver toxicity with CCl_4 are presented in Fig. 3. The activities of ALT and AST were 41.04 and 300.23 IU/L in the N group and 455.33 and 6887.74 IU/L in the C group, respectively. This demonstrates the induction of liver toxicity by CCl_4 , and that the experimental group administered CCl_4 also had increased activity, verifying that liver damage was induced by CCl_4 . ALT activity decreased in both the S group (22.80 IU/L) and in the U group (154.78 IU/L). A significant decrease in ALT activity was also seen in experimental groups, and among them, RGWPSM, RGWP1, 2, RGWPM-1, and RGWPA-2 (130.67-148.00 IU/L) had 1.04-1.18-fold decreased activity compared to the U group ($p < 0.05$). Among experimental groups, the RGAP group, in which the treatment was a mixture of acetic acid extract of RG and PR (101.60 IU/L), showed a 1.52-fold decrease in ALT activity compared to the U group, exhibiting the greatest decrease in activity. However, no experimental group decreased to the level of the N or S group in terms of ALT activity. In the case of AST, the S and U groups exhibited a decrease in activity of 788.78 and 1006.22 IU/L, respectively. As seen in Fig. 3, the RGAP group (833.89 IU/L) experienced a decrease in

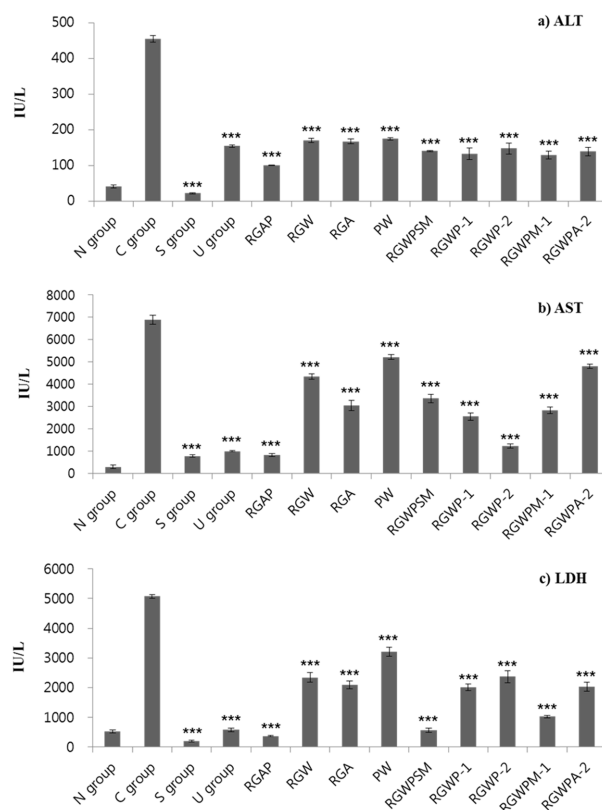


Fig. 3. Effect of Red ginseng and *Pueraria radix* on the activity of serum alanine transaminase (a; ALT), aspartate transaminase (b; AST), and lactate dehydrogenase (c; LDH) in CCl_4 -treated ICR mice

1) Refer to Table 1

2) Data are expressed as the mean \pm SE ($n = 7$)

Statistical significance of differences was calculated between C group and experiment groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

activity that was 1.21 times greater than that in the U group. Other experimental groups did not show a distinct decrease in activity of AST, and AST activity was not decreased to the level of the N or S groups.

Changes in the activity of lactate dehydrogenase (LDH) in serum –

Lactate dehydrogenase (LDH) in serum is an enzyme of the glycolytic pathway, which is distributed in the heart, muscles and liver.²³ Activity of LDH markedly increased in C group with CCl_4 -induced liver toxicity and no administration of test samples (C group, 5072.56 IU/L), while its activity significantly decreased in the S group (192.22 IU/L), U group (577.89 IU/L), and the experimental groups (364.67-3206.44 IU/L) ($p < 0.05$). In particular, among experimental groups, the RGWPSM and RGAP groups showed decreases 1.02 and 1.58 times greater, respectively, than the U group. However, these groups showed less of a decrease than the S group did.

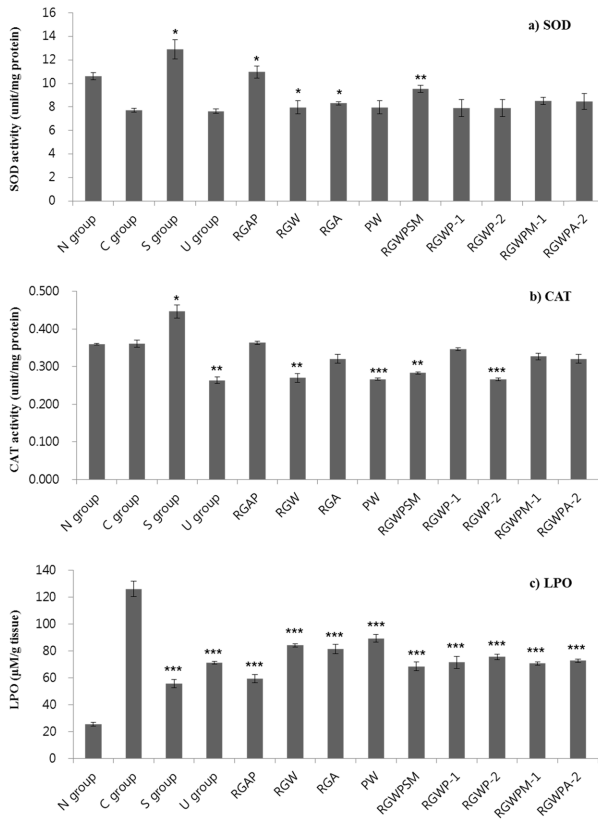


Fig. 4. Effect of Red ginseng and *Pueraria radix* on the activity of superoxide dismutase (a; SOD), catalase (b; CAT), and lipid peroxidase (c; LPO) in CCl₄-treated ICR mice

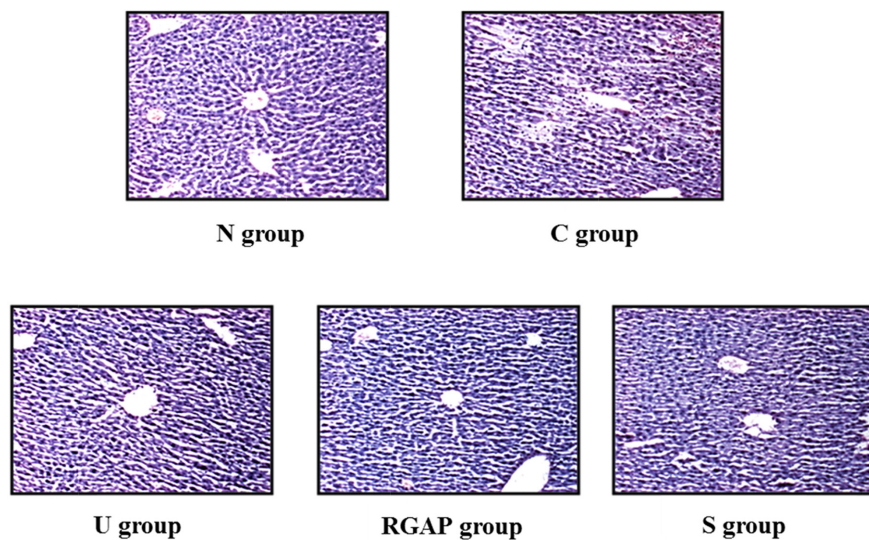
¹⁾ Refer to Table 1

²⁾ Data are expressed as the mean \pm SE ($n = 7$)

Statistical significance of differences was calculated between C group and experiment groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Change in activities of superoxide dismutase (SOD) and catalase (CAT) in liver tissue – Change in the activity of antioxidant enzyme superoxide dismutase (SOD) and catalase (CAT), which eliminate superoxide and hydrogen peroxide after induction of liver toxicity with CCl₄, is presented in Fig. 4. Activity of SOD decreased in C group (7.74 U/mg) with CCl₄-induced liver toxicity and no administration of test samples, while its activity increased in S and experimental groups. Judging from the fact that administration of S group (13.01 U/mg) significantly increases CCl₄-induced SOD, the effect of silymarin was also recognized. No activation of SOD was confirmed in U group (7.88 U/mg), which showed lower activity than experimental groups. On the contrary, it was confirmed that the RGAP (11.32 U/mg), RGA (8.32 U/mg), and RGWPSM groups (9.81 U/mg) had significantly improved activity, with a level similar to that of the S group ($p < 0.05$), even though it still falls short of the level of the S group. In most experimental groups, CAT activity recovered to the level of the N group (0.36 U/mg), which is equal to or higher than that of the U group (0.26 U/mg), though not significant.

Change in activity of lipid peroxidase (LPO) in liver tissue – Changes in lipid peroxidase (LPO) levels generated by toxic substances that attack cell membranes are presented in Fig. 4. Induction of toxicity was judged to be performed well as the activity of LPO increased in CCl₄-administered C group. In this study, however, LPO decreased more significantly in the S (56.01 μ M/mg), U



X 200

Fig. 5. Histopathological observation of liver tissues (H&E, $\times 200$)

(71.16 $\mu\text{M}/\text{mg}$), and experimental groups than in the C group (126.43 $\mu\text{M}/\text{mg}$), though falling short of N group (25.57 $\mu\text{M}/\text{mg}$) ($p < 0.05$). Yet, among the experimental groups, RGAP group (59.37 $\mu\text{M}/\text{mg}$), recovered to the level similar to that of S group, though still falling short of the N group.

Optical microscope observations – Histological assessment was used to complete the study of the hepatoprotective effects of RGAE and PWE on CCl_4 -induced acute liver damage (Fig. 5). From observations under an optical microscope, the histology of the liver sections of N group showed normal hepatic cells. The liver sections of CCl_4 -intoxicated mice revealed extensive liver injuries, characterized by severe hepatocellular degeneration and necrosis, fatty changes, ballooning degeneration, and the loss of cellular boundaries. While, no liver damage was observed in the S and U groups. In addition, the histopathological hepatic lesions were markedly ameliorated and verifying the hepatoprotective effect by pretreatment with RGAE and PWE. This was in good agreement with the results of serum ALT and AST with hepatic oxidative stress levels (Fig. 3 and 4). Thus, RGAP was deemed to prevent liver damage as well as than the existing hepatoprotective agent of the hepatoprotective effects of AP on CCl_4 -induced acute liver damage.

Discussion

Lee *et al.*¹ reported that administration of RG in ICR mice with CCl_4 -induced liver toxicity markedly decreased ALT and AST activity, which increased by the supply of CCl_4 . Kim *et al.* reported that administration of hot water extract of PR in ICR mice with oral administration of alcohol decreased ALT and AST activity.²⁴ Lee *et al.* reported that after inducing liver damage with ethanol administration, treatment with hot water extract of PR reduced ALT and AST activity.²⁵ Thus, RG and PR are known to prevent or treatment liver damage. Given that the RGAP group administered a mixture of RGAE and PWE experienced remarkable reduction in serum ALT and AST activity, it is concluded that there is synergy between the two test samples, and that the acid extract of RG has a better synergy with PR.

Kim reported that polysaccharide fractions of ginseng suppressed the activity of LDH in liver cells of ICR mice with CCl_4 -induced toxicity²⁴, and Lee *et al.* reported that administration of RG in ICR mice with CCl_4 -induced toxicity remarkably reduced the activity of LDH, although falling short of the level of the comparison group.¹ Another study verified that administration of PR in ICR

mice with artificial toxicity induced by benzo-(α)-pyrene significantly reduced the activity of LDH.²⁶ This study confirmed that mixed extract of RGAE and PWE decreased the activity of LDH, and especially, RGAP with RGAW and water extract of PR in the ratio of around 1:1.5 ratio (w/w) experienced a greater effect than N group.

Lee and Shin reported that administration of PR MeOH extract in ICR mice supplied with lipid peroxide increased activity of both SOD and CAT.²⁰ Sung *et al.* reported that when white ginseng and RG extracts were administered to ICR mice with acute oxidative liver damage induced by excessive acetaminophen (APAP), which is widely used as a painkiller and an antipyretic, the activity of SOD and CAT increased. Thus, RG and PR not only reduce serum ALT and AST activity increased by damage in liver tissue, but also possess an anti-oxidant effect that eliminates oxygen free radicals.²⁷ Mixed administration of RGAE and PWE has higher efficiency in eliminating superoxide and hydrogen peroxide.

After a 2-week administration of RG extract to ICR mice with CCl_4 -induced toxicity, Lee *et al.* reported a marked suppression of increased LPO content, showing the same result as this study.¹ Sung *et al.* verified that reduction of LPO content is a result of administration of RGWE to ICR mice with a high-cholesterol diet, showing that RGWE prevents oxidative damage.²⁸ Lee and Shin reported that administration of methanol extract of PR to ICR mice supplied with lipid peroxides decreased LPO content. RG and PR are also known to have an antioxidant effect, and considering that RGAP administered with a mixture of RGAE and PWE resulted in reduction of LPO content, RGAP is deemed to protect the body and liver from damage.²⁰

In previous reports, acetic and citric acid-treated RG was known to increase PPD series to ginsenoside Rg_3 , Rh_2 , Rd and suggested that this pre-treatment could increase specific saponins in RG, resulting in the manufacture of ginsengs presenting various physiological activities. On the other hand, PPD series did not change to ginsenoside Rg_3 under neutral conditions.^{29,30} As well as, Bae *et al.* reported that RG water extract was incubated at 60 °C in acidic conditions, its protopanaxadiol ginsenosides were transformed to ginsenoside Rg_3 and $\Delta 20$ -ginsenoside Rg_3 and acid-treated ginseng extract showed potent cytotoxicity against tumor cell lines and inhibited the growth of *Helicobacter pylori*.³¹ Acid extraction of RG had an effect both on the content increase of ginsenoside and on the structural transformation of the internal components. In addition, Kim *et al.* reported that non-organic acid-treated steamed ginseng was not different

from the steamed ginseng treated by several organic acids based on their appearances and RG produced by this pre-treatment showed improved flavor compared to currently available RG through a sensory test.^{32,33} We suggested that acid extraction is the most effective method for the extraction of RG for specific components in RG.

In order to investigate the physiological effects of various mixtures of RG and PR in ICR mice with CCl₄-induced liver damage, this study measured the activity of ALT, AST, and LDH in serum, and SOD, CAT, and LPO in tissues. Administration of CCl₄ induced liver toxicity, increasing the levels of ALT, AST, and LDH, which were lowered by acid extract of RG and water extract of PR. From examining the activity of antioxidant enzymes SOD and CAT, which eliminate peroxide and LPO generated by CCl₄ in tissue, it was observed that RGAP increased antioxidant activity in the body by eliminating superoxide generated by CCl₄ and peroxide, and enhanced the protective function of the body by suppressing necrosis of liver cells. These results suggested that administration of a mixture of RGAE and PWE decreases CCl₄-induced liver damage and enhances antioxidant activity in the body, and imply that administration of a mixture of RGAE and PWE in a certain ratio is more effective than single administration of either RGAE and PWE alone.

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