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Hepatoprotective Activities of *Rubus coreanus* Depends on the Degree of Ripening

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Abstract – *Rubus coreanus*, commonly known as 'red raspberry' is used as a traditional oriental medicine in Korea for the management of diseases such as impotence, spermatorrhea and athsma, and for allergies, in combination with other herbal preparations, in many centuries. We undertook a comparison of the hepatoprotective effect of ethanol extracts of the unripe (UREx) and ripe (RREx) *R. coreanus* extract against acetaminophen (AAP) induced hepatotoxicity in rats. UREx reduced the elevated alanine aminotransferase (ALP), aspartate aminotransferase (AST), total bilirubin (TB), alkaline phosphatase (AP) , lipid peroxide and nitric oxide content which had been increased by AAP administration. UREx also increased the cellular glutathione (GSH) content and induced the glutathione-S-transferase (GST), glutathione peroxidase (GSH-Px) content which had been decreased by AAP. RREx did not exhibit strong hepatoprotective effect or antioxidant activity under the same conditions. The experimental results show that the degree of the ripening of *R. coreanus* affects the hepatoprotective activity in the AAP-intoxicated rats. These findings of a protective mechanism are supportive evidence for the utility of unripened *R. coreanus* in traditional medicine for liver ailments.
Keywords – Unripened *Rubus coreanus*, Hepatoprotective effect, Acetaminophen, Liver, Antioxidant effect

Introduction

Acetaminophen (AAP) is widely used as an analgestic and antipyretic drug, but can induce hepatic injury in both humans and experimental animals when given in high doses (Vermeulen *et al*., 1992). At high doses, AAP induces hepatotoxicity by excessive cytochrome p450-generated production of N-acetyl-p-benzoquinoneimine (NAQI) with accompanying depletion of GSH levels (Vermeulen *et al*., 1992). The depletion of cellular glutathione, a natural antioxidant, leaves the cell vulnerable to oxidative insults following AAP overdose. Reactive oxygen species are known to be an early mediator of hepatocyte injury and also the activation of Kupffer cells involved in hepatocyte inflammation. Many studies have reported that the hepatoprotective activity of *Silybum marianum* (Dvorak *et al*., 2003), *Balanitis aegyptiaca* and *Haplophylum tuberculatum* (Ali *et al*., 2001) in AAP-intoxicated rat depend on their antioxidant effects. Various antioxidants have been shown to protect against AAP-toxicity, such as β-carotene (Barnowitz and Maderson, 1995) and α-tocopherol (Amimoto *et al*.,1995). *R. coreanus* is one of the hundred genera in the family Rosaceae, subfamily Rosoidea, and there are currently 250 species established around the world. *R. coreanus*, commonly known as 'red raspberry' is used as a traditional medicine in Korea for the management of impotence, spermatorrhea, enuresis, asthma and allergic diseases. Moreover, a recent study showed that the ethanol extract of *R. coreanus* enhances osteoblast function by promoting osteoblast differentiation and inhibiting bone reabsorbing mediators (Lee and Choi, 2006). The bioactive components isolated from *R. coreanus* are to date rather limited, including 19-α-hydroxyursolic acid, coreanoside-F1, niga-ichigoside F1, F2, 23-hydroxydiphenol, gallic acid and ellagic acid (Oh *et al*., 2007). Niga-ichigoside F1(N1F1) and 23-hydroxydiphenol isolated from the unripened fruit of *R. coreanus* have antigastropathic and antirheumatic effects (Kim *et al*., 2007). Ellagic acid, a plant polyphenol, is present in high concentrations in *R. coreanus*, and has been shown to possess potent anti-oxidative effects as well as broad chemoprotective properties against a variety of carcinogens (Mandal *et al*., 1990). It has been reported that the quantity of the active biomaterials in many plants depends on the cultivar, harvesting season and the extraction procedure (Bogs *et al*., 2005, Watson *et al*.,

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2002). The objective of the present study was to compare the hepatoprotective effect of UREx and RREx against the oxidative stress-induced hepatotoxicity induced by AAP administration in rats. The results that the hepatoprotective effect of *R. coreanus* extracts differed depending on the stage of the ripening of the fruit.

Experimental

Plant materials and preparation of extracts – Ripe and unripe fruit of *R. coreanus* Miguel were collected from Busan (Chunra North province), Korea, on August 10 and June 27, 2007, respectively. The plant samples were kept in the herbarium of KRIBB. The *R. coreanus* extracts (ripe, unripe) were prepared as follows. In brief, the dried unripe (1,000 g) and ripe (1,000 g) fruits were pulverized and extracted two times with 5 L of 50% ethanol generating the following extract yields: UREx 21.2% and RREx 40.2%.

Animal treatment – Male Sprague-Dawley (SD) rats (150 ± 10 g) were obtained from the Koa-Tech animal company (Seoul, Korea). The animals were allowed free access to purina rodent chow and tap water, maintained in a controlled environment (22 degree centigrade) and 50 ± 10% relative humidity with a 12 hr dark/light cycle and acclimated for at least one week before use. Ethanol extracts of UREx or RREx (250 mg/kg body weight, 500 mg/kg body weight) were administered orally once daily for 6 consecutive days. At day 7, rats were treated with acetaminophen (500 mg/kg body weight). After 24 hrs, blood serum was separated by centrifugation at 1600 × g for 10 min at 4 °C. Hepatotoxicity was assessed by quantifying the serum activities of ALT, AST, alkaline phosphatase (ALP) and total bilirubin using an automatic biochemical analyzer in the Clinical Medical Laboratory at the College of Medicine, Chungbuk National University. After collection of blood, the liver was immediately excised, washed with cold saline, blotted and a part of it was minced and homogenized for glutathione peroxidase (GST-PX), glutathione-s-transferase (GST), lipid peroxidation and reduced glutathione determination.

Cytotoxicity of AAP in rat hepatocytes – Primary cultures of hepatocytes from rats were performed using the method of Williams *et al*. (1979). Hepatocytes were pretreated with UREx and RREx at concentrations of 0, 50, 100, 200 and 400 μl/ml for 24 hrs and then incubated with AAP (0.5 mM) for 24 hrs. After the final treatment, cytotoxicity was quantitatively assessed by the measurement of LDH released into the culture medium using an LDH assay kit.

Determination of the hepatic lipid peroxide and glutathione content – For the determination of lipid peroxide, Ohkawa's method was used (Ohkawa *et al*., 1978). Lipid peroxide content was expressed as MDA content using a standard curve determined by malondialdehyde tetrabutylammonium salt. For the determination of the glutathione content in the liver homogenate, Ellman's method was used (Ellman, 1959).

Determination of glutathione peroxidase and glutathione S-transferase activity – Glutathione peroxidase activity was determined as described (Paglia *et al*., 1967). Changes in NADPH absorbance at 340 nm were recorded. One unit of glutathione peroxidase was determined as a change of oxidized NADPH in 1 mg protein. Cytosolic glutathione S-transferase activity was determined using 1-chloro-2,4 dinitrobenzene as a substrate.

Determination of total nitrate/nitrite contents – The serum level of total nitrate/nitrite (nmole/ml) was determined by the acidic Griess reaction after reduction of nitrate to nitrite by vanadium trichloride. The Griess reaction relies on a simple colorimetric reaction between nitrite, sulfonamide and N-(1-naphthyl) ethylenediamine to produce a pink azo-product with a maximum absorbance at 543 nm prior to the Griess reaction. All nitrate is converted to nitrite using vanadium trichloride, as described (Miranda *et al*., 2001). Serum proteins were precipitated by absolute methanol and after centrifugation, supernatants were used for estimation of the nitrate/nitrite level.

Statistical analysis – All experiments were repeated at least three times. Results are reported as the means ± SD. The Microsoft Excel Program was used to evaluate the difference between multiple groups. The Student t-test was used to compare the means of two-specific groups, with $p < 0.05$ considered as significant.

Results and Discussion

Acetaminophen induces dramatic depletion of the cellular glutathione levels in the liver. The excess formation of NAPQI (the metabolic product of AAP) causes the depletion of GSH, resulting in the formation of covalent binding of cellular proteins and leading to hepatocellular injury. Recent studies have suggested that the formation of reactive oxygen and nitrogen species are involved in the development of AAP toxicity. Thus, the reported causative factors involved in liver cell injury include the free radicals derived from oxygen and other chemicals, and inflammatory molecules, such as iNOS (Laroux *et al*., 2001). For these reasons the effect of RREx and UREx on the cytotoxicity of AAP was

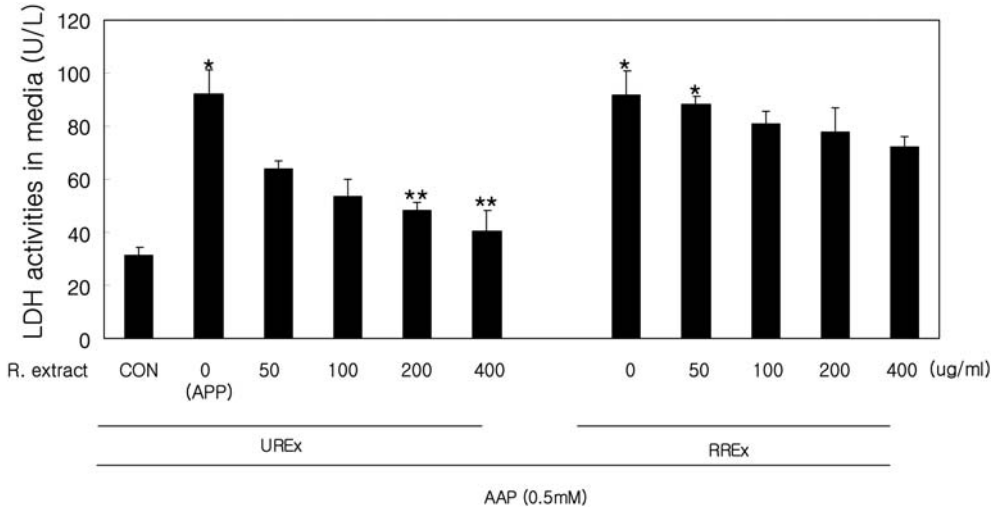

Fig. 1. Effect of *R. coreanus* extracts (UREx, RREx) on the cytotoxicity induced by AAP.
After pretreatment with UREx, or RREx (0, 50, 100, 200, 400 μg/ml) for 24 hrs, rat hepatocytes were treated with AAP (0.5 mM) for 24 hrs. Cytotoxicity was measured by LDH activity in media. Values are expressed as the mean ± SD of six experiments. *Significantly different ($P < 0.05$) from the control group (CON). **Significantly different from AAP (0) at $P < 0.05$.

Table 1. Effects of *R. coreanus* extracts (UREx, RREx) on AAP-induced hepatotoxicity in rats

Treatment		Serum ALT (U/L)	Serum AST (U/L)	Total bilirubin (mg/dL)	Alkaline phosphatase (IU/L)
Normal	–	103.32 ± 3.27	66.15 ± 12.1	0.8 ± 0.05	78.7 ± 11.21
AAP	500 mg/kg b.w	2276.28 ± 101.93	1387.21 ± 116.37	2.3 ± 0.71	121.2 ± 10.37
UREx + AAP	250 mg/kg b.w	1631.31 ± 106.2	1232.23 ± 106.97	1.7 ± 0.32*	102.2 ± 18.67
	500 mg/kg b.w	1078.28 ± 126.49*	676.37 ± 102.37*	1.2 ± 0.47*	89.3 ± 13.32*
RREx + AAP	250 mg/kg b.w	2127.82 ± 78.6	1292.6 ± 127.1	2.1 ± 0.37*	118 ± 12.72
	500 mg/kg b.w	2037.21 ± 102.3	1098.21 ± 98.67	2.0 ± 0.42	120 ± 11.37

Rats were pretreated with *R. coreanus* extracts (UREx, RREx) orally for 6 days consecutively. Control rats were given saline. Three hours after the final treatment, rats were treated with AAP (500 mg/kg, IP). Hepatotoxicity was determined 24 hrs later by quantifying the serum activity of ALT, AST, total bilirubin and alkaline phosphatase. Each value represents the mean ± SD of six rat.
* Significantly different from control (AAP) at < 0.05 by Student t-test.

examined in rat hepatocytes. As shown in Fig. 1, AAP (0.5 mM) treated rat hepatocytes significantly released LDH (0 ug/ml R. extract), indicating that AAP induced cytotoxity in primary rat hepatocytes. *R. coreanus* extracts (RREx, UREx) treatment without AAP did not exhibit a high level of LDH release in primary rat hepatocytes. LDH release was markedly decreased by pretreatment with UREx (50 - 400 ug/ml) up to the control level at a cytotoxic concentration of AAP (Fig. 1). However, unlike the case with UREx, RREx pretreatment did not protect AAP treated primary hepatocytes efficiently, since higher levels of LDH activity were shown in the 50 - 400 ug/ml RREx pretreated primary hepatocytes. These results suggest that UREx showed more potent protective effect against AAP-induced hepatotixicity than RREx in pretreated rat primary hepatocytes.

We then examined the effects R. coreanus extracts (UREx, RREx) on the hepatotoxicity of AAP in rat. The data for the serological markers of liver function, including AST, ALT, total bilirubin (TB) and alkaline phosphatase (AP) are shown in Table 1. These serum marker results are clear indications of the protective effect of *R. coreanus* against AAP-induced liver damage. All serum markers (ALT, AST, TB, AP) were significantly increased in the AAP-intoxicated rats**.** UREx (250 mg/kg bw, 500 mg/kg bw) treated rats showed significantly reduced ALT and AST levels ($p < 0.05$), while the same amount of RREx treatment did not result in reduced ALT and AST levels. Additionally, significant lower levels of total bilirubin and alkaline phosphatase were observed after treatment with UREx (250, 500 mg/kg bw), while the same dose of RREx treatment did not show any

decrease of the enzyme levels in the AAP-intoxicated rats (Table 1). Elevated serum ALT and AST levels have been reported as a sign of liver cell damage (Reitman and Frankel, 1967). Since UREx treatment reduced the serum ALT and AST levels in UREx pretreated rats, UREx may stabilize the cell membrane and prevent a leakage of intracellular enzymes into the blood. Long-term UREx treatment would be expected to reduce the liver damage caused by oxidative insult and thus improve liver function.

ROS are considered to be an early mediator of hepatocyte injury and the activation of Kupffer cells. It was reported that antioxidants may protect against AAP-induced hepatotoxicity through anti-peroxidation, iNOS inactivation or induction of protective enzyme expression (Parola *et al*., 1992). These endogeneous antioxidant protective factors, such as GST-PX, GST and superoxide dismutase (SOD) are activated in the systematic response to oxidative cell injury by means of these free radical scavengers. These enzymes play an important role in the elimination of the ROS derived from the redox process of xennobiotics in liver tissues (Machlin and Bendich, 1987). Lipid peroxidation is reported to be a mechanism of cellular injury. Therefore, to examine the consequence of AAP-induced oxidative damage to cellular macro-molecules and to determine the possible effects of *R. coreanus* extracts (RREx, UREx), we analyzed the formation of malondialdehyde (MAD) as a marker for membrane lipid peroxidation. Exposure of AAP alone increased the amounts of cell-associated MAD in intoxicated rats (Table 2). Consistent with the serum levels of the functional liver markers (ALT, AST, TB, AP), pretreatment with UREx significantly prevented the MAD production induced by AAP, while RREx did not. These results show that UREx treatment inhibit the membrane lipid peroxidation triggered by the injurious radicals generated by AAP. GSH is known to have a protective effect against AAP-induced toxicity. Therefore, the influence of UREx and RREx on the AAP-induced depletion of GSH in the intoxicated rats were also investigated. As expected, the exposure to AAP (500 mg/kg bw) resulted in a dramatic depletion of GSH (Table 2). However, in the presence of UREx, but not RREx, the loss of intracellular GSH was prevented in a dose-dependent manner (Table 2). The effects of pretreatment of UREx and RREx on the AAP-induced decrease of GST-PX and GST activity are shown in Table 2.

In terms of GST-PX and GST activity, both were reduced in AAP-intoxicated rats compared to the control group. However, increasing doses of UREx administration resulted in a significant increase in GST-PX and GST activity, while RREx did not (Table 2). In acetaminophen-treated mice, there was reportedly a direct correlation between nitric oxide (NO) synthesis and liver cell injury, as measuared by the serum levels of nitrate and nitrite (Michael *et al*., 2001). It was also reported that toxic doses of AAP led to the induction of nitric oxide synthase (NOS), and that pretreatment of mice with a ntric oxide synthase inhibitor, aminoguanidine, decreased this toxicity (Gardner *et al*., 1998). NO is produced in Kupffer and endothelial cells during endotoxaemia and inflammation. NO plays an important role in various kinds of tissue injury, either directly, or by interacting with reactive oxygen intermediates to form a more toxic species. Excessive NO can inactivate antioxidants and antioxidases by reacting with active groups of the molecules. Our experimental findings show that pretreatment of rats with UREx resulted in significantly less nitric oxide (nitrate + nitrite) than with RREx in AAP-intoxicated rats (Fig. 2). These findings indicate that the degree of fruit ripening affects the production of the inflammatory mediator nitric oxide in AAP-intoxicated rats.Based on our experimental

Table 2. Effect of *R. coreanus* extracts (UREx, RREx) on AAP-induced oxidative stress in rats

Treatment		Lipid Peroxidation (MDA nmole/g)	GSH-Px (U/mg protein)	GST (U/mg protein)	GSH (μmole/g protein)
Normal	–	2.42 ± 0.32	10.32 ± 0.93	6.70 ± 0.32	3.32 ± 0.98
AAP	500 mg/kg b.w	6.31 ± 0.21	3.28 ± 0.83	2.11 ± 0.28	1.82 ± 0.21
UREx + AAP	250 mg/kg b.w	5.03 ± 0.72	5.94 ± 0.44	3.06 ± 0.21*	2.32 ± 0.37
	500 mg/kg b.w	3.36 ± 0.18*	8.19 ± 0.14*	4.51 ± 0.26*	2.96 ± 0.29*
RREx + AAP	250 mg/kg b.w	6.37 ± 0.38	4.98 ± 0.76	2.38 ± 0.32	1.92 ± 0.42
	500 mg/kg b.w	5.16 ± 0.27	5.72 ± 0.75	3.47 ± 0.63	2.18 ± 0.26

Rats were pretreated with *R. coreanus* extracts (UREx, RREx) orally for 6 days consecutively. Control rats were given saline. Three hours after the final treatment, rats were treated with AAP (500 mg/kg, IP). Lipid peroxide, glutathione content and GSH-Px, GST activities were determined as described in materials and methods. Each value represents the mean ± SD of six rats.
*Significantly different from control (AAP) at < 0.05 by Student t-test.

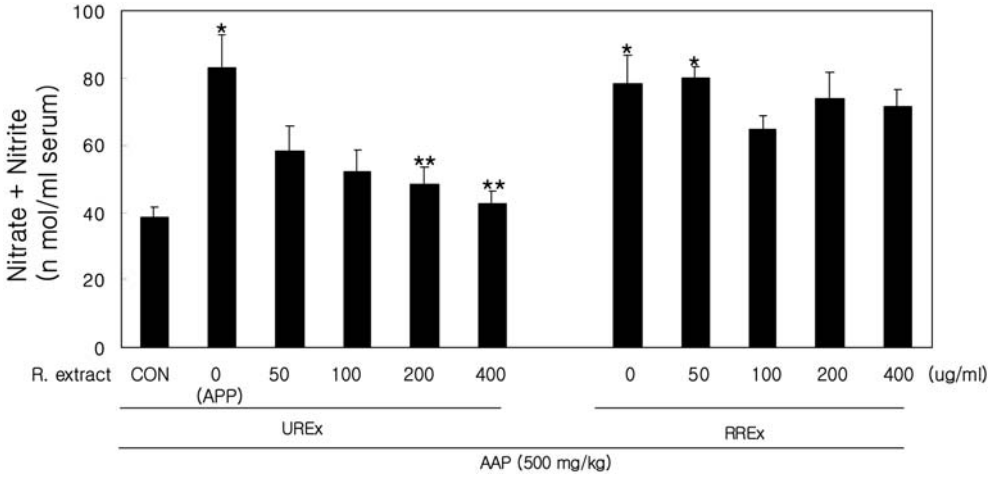

Fig. 2. Effect of *R. coreanus* extracts (UREx, RREx) on the AAP induced nitrate/nitrite production in rats.
Rats were pretreated with *R. coreanus* extracts orally for 6 days consecutively. Control rats were given saline. Three hours after the final treatment, rats were treated with AAP (500 mg/kg, IP). Serum nitrate plus nitrite was measured 24 hrs after AAP treatment. Results are expressed as the mean ± SD of six rat. *Significantly different ($P < 0.05$) from the control group (CON). **Significantly different from control (AAP) at < 0.05.

results, UREx exerts its hepatoprotective activity by scavenging free radicals and inhibiting NO formation. These effects of dietary UREx may be able to prevent the hepatic injury caused by AAP. Therefore, UREx from *R. coreanus* has potential as a hepatoprotective agent against acute or chronic chemical- induced hepatotoxicity. The physiological properties of *R. coreanus* were shown to be altered by fruit ripening process. Cha *et al*. (2001) reported that the production of moisture, crude protein, crude fat and fiber were lower in unripe than ripe fruit. The concentrations of glucose and fructose increased in ripe fruit, while citric acid showed the highest concentration in the unripe fruit. Furthermore, the major amino acid contents (glutamic acid, aspartic acid, arginine, leucine) were higher in unriped fruits. Ellagic acid, a phenolic compound, is found in a wide variety of fruits and nuts, including raspberries, strawberries and blackberries (Priyadarsini *et al*., 2002). Ellagic acid is of particular interest in that it has been reported to have antioxidant (Priyadarsini *et al*., 2002), anti-inflammatory (Lino *et al*., 2002), anit-fibrotic and anticancer properties (Thresiamma and Kuttan, 1996). Ellagic acid has also been reported to inhibit the elevation of NO production induced by endrin (Akubue and Stoh, 1992), independent of its antioxidant properties. Our experimental results obtained from the HPLC analysis of the ellagic acid showed that the ellagic acid amount in UREx was 10 times higher compared to RREx (3.82 mg/g ext. vs 0.28 mg/g ext.),very similar to Yang's results (Yang *et al*., 2008), suggesting that ellagic acid might be a bioactive component possessing hepatoprotective properties. Recent report on hepatoprotective activity of ellagic acid and sylimarin showed that both exerted similar effects on paracetamol induced liver toxicity in mice (Girish *et al*., 2009). These results suggested that ellagic acid is main component in UREx which exert hepatoprotective effect in experimental animals. Furthermore, our recent results demonstrated that ellagic acid and ellagic acid containing UREx showed hepatoprotective activity in AAP intoxicated rat by inhibiting cytochrome p450 3A4 (CYP 450 3A4) during the conversion of AAP to NAPQI (Biomedicine and Pharmacotherapy, accepted). These results suggested that ellagic acid or ellagic acid containing UREx can be developed as drugs for the treatment of liver diseases as is other phytochemicals like sylimarin.

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