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Rubus coreanus Extract Attenuates Acetaminophen Induced Hepatotoxicity; Involvement of Cytochrome P450 3A4

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Abstract − Foods of plant origin, especially fruits and vegetables, have attracted attention because of their potential benefits to human health. In this report, *Rubi Fructus* (RF), the dried unripe fruit of *Rubus coreanus* Miq (Rosaceae) and ellagic acid (EA) purified from RF were used to test their potential hepatoprotective effect against acetaminophen (AAP)-induced hepatotoxicity in rats. RF extract (RFext) and EA reduced the elevated levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) in serum and the content of lipid peroxide in liver by AAP administration, while the increment of the cellular glutathione (GSH) content and the induction of glutathione S-transferase (GST) and glutathione peroxidase (GSH-PX) which were decreased by AAP administration. RFext and EA from RFext did not affect the two major form of cytochrome P450s, cytochrome P450 2E1 (CYP2E1) and cytochrome P450 1A2 (CYP1A2), but down-regulated the cytochrome P450 3A4 (CYP3A4) related to the conversion of AAP to N-acetyl-P-benzoquinone imine (NAPQI). These results suggest that RFext and EA from RF exhibit a hepatoprotective effect not only by increasing antioxidant activities but also by down-regulating CYP3A4 in the AAP-intoxicated rat.

Keywords: Hepatoprotective effect, Acetaminophen, *Rubus coreanus*, Ellagic acid, Anti-oxidant effect, Cytochrome P450

INTRODUCTION

Rubus coreanus is one of the hundred genera in the family Rosaceae, subfamily Rosoideae, and there are currently 250 species of *Rubus* established around the world. *Rubus coreanus*, commonly known as "red raspberry", is used as a traditional oriental medicine in Korea. The dried, unripe fruit of *Rubus coreanus* MiqRubi Fructus (RFext) has also been used to improve male reproductive function and to treat several diseases, such as diabetes, many types of infection, colic and burns in combination with other herbal preparations in traditional Korean medicine (Oh *et al*., 2007). The constituents of RF include 19-α-hydroxyursolic acid, sanguine, coreanoside-F1, niga-ichigoside F1, F2, 23-hydroxydiphenol, 2,3-(S)-HHDP-D-glucopyrannose, gallic acid and ellagic acid (Kim *et al*., 2001; Kim *et al*., 2005; Oh *et al*., 2007). Among these, ellagic acid, known as a plant polyphenol, is present in high concentrations in RFext and has been identified to possess strong anti-oxidative effects and also broad chemoprotective properties towards a variety of carcinogens (Mandal and Stoner, 1990). Acetaminophen (AAP) is widely used as an analgestic and antipyretic drug, but it can produce hepatic injury in both human and experimental animals when given in high doses (Vermeulen *et al*., 1992). At therapeutic doses, AAP is biotransformed and eliminated as non-toxic glucuronic acid and sulfate conjugates (Cohen and Khairallah, 1997). Only a small proportion of AAP is converted to n-acetyl-p-benzoguinoneimine (NAPQI), a cytochrome P450 (CYP)-mediated intermediate metabolite, which is normally detoxified by conjugation with GSH. However, at high doses, AAP induces hepatotoxicity by excessive P450-generated production of NAPQI with accompanying depletion of GSH levels. Subsequently, ex-

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cess NAPQI binds covalently to cellular macromolecules (Jollow *et al*., 1973). Therefore, the increased production of the active metabolite NAPQI or depressed stores of GSH enhances toxicity in the liver. Several CYP enzymes are known to play important roles in AAP bioactivation to NADPI. CYP 2E1, CYP 1A2 and CYP 3A4 have been suggested to be primary enzymes for AAP bioactivation in liver microsomes (Raucy *et al*., 1989; Lee *et al*., 1996). A number of antioxidants and antioxidative enzymes, such as coenzyme Q10, α-tocopherol, ascorbic acid and superoxide dismutase have been shown to prevent AAP-induced liver injury *in vivo*.

In this study we evaluated the protective effect of EA and RFext on AAP-induced hepatotoxicity and elucidated the mechanisms(s) underlying the protective effects in rat. The parameters analysed included the serum levels of ALT and AST activity, hepatic GSH, lipid peroxide content and the antioxidant enzyme (GSH-PX, GST) activities. The inhibition of CYP by EA and RFext was also investigated to study the mechanisms of its protective action in AAP-intoxicated rats. The experimental results show that EA pre-treatment significantly protected against AAP-induced hepatotoxicities by its strong antioxidant effects. EA and RFext also down-regulate the expression of CYP 3A4 but not CYP 2E1 and CYP 1A2. Since EA and RFext treatment downregulates CYP 3A4, we suggested that the hepatoprotective effect by EA and RFext is exerted not only by the strong antioxidant effect, but also by inhibiting CYP 3A4 in the AAP-intoxicated rat.

MATERIALS AND METHODS

Preparations of plant material and plant extract

The unripened fruit of *Rubus coreanus* Miq (RF) was collected during June from Buan (Chunra North Province) area in Korea. The plant samples were kept in the hebarium of KRIBB. The EA from RFext was prepared as follow. Eighty liters of 50% ethanol was added to 15 kg RF (containing 0.2-0.3% EA) and extracted after 6 hours at 60^{o}C. This procedure was repeated 2 times and yielded 1.5-2.5% ellagic acid containing crude RFext. A Dianion HP-20 column was used with 50-100% ethanol gradient elutions, and yielded 10% ellagic acid containing RFext. For purification of EA, Prep-HPLC (JA1GEL-ODS, S-343-15) was used with the ethanol mobile phase and EA was detected at OD=250 nm. A greater than 95% pure form of EA was obtained.

Animal treatment

Male Sprague-Dawley (SD) rats (150 ± 10 g) were obtained from Koa-Tech animal company (Seoul, Korea). The animals were allowed free access to rodent chow (Purina, Korea) and tap water, maintained in a controlled environment 22^{o}C and 50 ± 10% relative humidity with a 12 hr dark/light cycle and acclimatized for at least 1 week before use. Ellagic acid from RFext (5, 10 and 20 mg/kg b.w.) and RFext (250 and 500 mg/kg b.w., each containing 5 and 10 mg/kg EA, respectively) were administered orally once daily for 6 consecutive days. For microsomal p450 enzyme assay, 100 mg/kg and 250 mg/kg bw RFext (containing 10 mg and 25 mg) were used.

At day 7, rats were treated with AAP (500 mg/kg b.w.). After 24 hr, hepatotoxicity was assessed by quantifying the serum activities of ALT and AST, the hepatic lipid peroxidation, GSH content and antioxidant enzyme activities. Rats were sacrificed and the liver was removed, rinsed with ice-cold 1.15% KCl-buffer and homogenized at 4 degree centigrade in the same buffer. Homogenates were centrifuged at 1,600 × g for 10 min and supernatants were collected for the measurement of lipid peroxide and GSH contents. The remaining precipitates were dissolved in 1.15% KCl buffer and centrifuged at 15,000 × g for 30 min and the supernatant was collected for glutathione peroxidase activity measurement. Supernatants were centrifuged at 20,000 × g for 60 min. The resulting supernatants were collected for glutathione-S-transferase activity. The serum ALT and AST activities were measured with a spectrophotometric diagnostic kit obtained from the Sigma Co. (Sigma, USA).

Determination of hepatic lipid peroxide and glutathione contents

For the determination of lipid peroxide content Ohkawa *et al*.'s method was used (Ohkawa *et al*., 1978). Lipid peroxide contents were expressed as MDA content using a standard curve determined by malondialdehyde tetrabutylammonium salts. For the determination of glutathione content, the same amount of supernatant (collected after 2,000 rpm centrifugation) and 10% TCA (trichloro acetic acid) were mixed and centrifuged at 3,000 rpm for 20 min. To the 0.1 ml supernatant, 0.5 ml of a mixture of 0.01 M $NaNO_2$ and 0.2 N H_2SO_4 (1:9 v/v) was added. After 5 min, 0.2 ml of 0.5% sulfamic acid ammonium solution was added and mixed. To the mixture, 1% $HgCl_2$, 1 ml of 3.4% sulphanilamide, 0.4 N HCl mixture and 1 ml of n-1-naphthyl ethylene diamine (0.4 N HCl) were added and the OD at 540 nm were determined.

Determination of glutathione peroxidase and glutathione S-transferase activity

Glutathione peroxidase activity was determined as described (Paglia and Valentine, 1967). Changes of 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 (Habig *et al*., 1974).

Hepatic microsome isolation and P450s enzyme activity assays

Sprague Dowley (SD) rats were orally administrated (with) 100 mg/kg b.w or 250 mg/kg b.w RFext (containing 10 mg and 25 mg EA) for 6 consecutive days for enzymatic assay. For the enzyme activity assay, rat microsome fractions were isolated. Hepatic microsomes were prepared as described previously (Ahn *et al*., 1996). The induction or inhibitory effect of ellagic acid on CYP1A2, CYP3A4 and CYP2E1 enzyme activities were measured by assaying ethoxyresorifin-O-deethylase (EROD) (Burk *et al*., 1990), testosterone 6β- hydroxylase (Wood *et al*., 1983) and PNP hydroxylase (Koop *et al*., 1989), respectively. Cytochrome P450 reductase activity was determined as described by Phillips and Langdon (Phillips *et al*.).

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. Student's t-test was used to compare the means of two-specific groups, with $p < 0.05$ considered as significant.

RESULTS

Effects of RF extract and EA on AAP-induced hepatotoxicity in rats

The effects of pretreatment with RFext and EA on the AAP induced elevation of ALT and AST activities are shown in Table I. A single dose of AAP (500 mg/kg) caused hepatotoxicity in the rats, as indicated by 20 folds increase in their ALT and AST serum levels after AAP administration (Table I). EA pretreatment prevented the AAP-induced elevation of the ALT and AST serum level in a dose dependent manner. Low doses of EA (5 mg/kg) or RFext (250 mg/kg) partially prevented the elevation of the ALT and AST serum levels without statistical significance. However, high doses of EA (20 mg/kg) or 500 mg/kg RFext showed more than 50% prevention of hepatotoxicity.

Effects of EA on lipid peroxidation and depletion of GSH in AAP-intoxicated rats

Lipid peroxidation is known to be a potential mechanism of cellular injury. Therefore, to examine the consequence of AAP-induced oxidative damage to cellular macromolecules and to determine the possible effects of EA and RFext, we analyzed the formation of malondialdehyde (MDA) as a marker for membrane lipid peroxidation. Exposure of AAP alone for 6 hr increased the amount of cell-associated MDA in intoxicated rats (Table I). Consistent with the serum levels of ALT and AST, the presence of EA significantly prevented the MDA production induced by AAP and this inhibition was strongly dependent upon the doses of EA and RFext. These results indicate that EA and RFext were able to 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 EA and RFext on the AAP-induced depletion of GSH in the intoxicated rats was also investigated. As expected, ex-

Table I. Effect of EA and RF extract on AAP-induced heptotoxicity in rats

Treatment	Serum ALT (unit/ml)	Serum AST (unit/ml)	Liver lipid peroxidation (MDA nmole/g)
Normal	103.32 ± 3.27	66.15 ± 12.21	2.42 ± 0.36
EA (20 mg/kg)	98.28 ± 1.79	72.36 ± 28.7	3.01 ± 0.27
AAP	2,276.28 ± 101.93	1,387.21 ± 116.37	6.31 ± 0.21
AAP + 5 mg/kg EA	1,878.21 ± 201.36	1,078.36 ± 176.23	5.26 ± 0.39
AAP + 10 mg/kg EA	1,036.73 ± 127.37[a]	537.31 ± 69.36[a]	3.25 ± 0.02[a]
AAP + 20 mg/kg EA	426.76 ± 78.28[a]	276.18 ± 48.44[a]	3.30 ± 0.21[a]
AAP + 250 mg/kg RFex.	1,632.31 ± 106.2	1,232.23 ± 106.97	5.03 ± 0.72[a]
AAP + 500 mg/kg RFex.	1,078.28 ± 126.49[a]	676.37 ± 102.37[a]	3.36 ± 0.18[a]

Rats were pretreated with EA or RF extract (containing 2% EA) orally for 6 days consecutively. Control rats were given with saline. Three hours after the final treatment, rats were treated with acetaminophen (AAP; 500 mg kg^{-1}, i.p.). Hepatotoxicity was determined 24 hr later by quantifying the serum activity of ALT and AST and the level of hepatic lipid peroxidation. Each value represents the mean ± SD of five rats.
[a]Significantly different from control (AAP) at $p < 0.05$ by student t-test.

posure of AAP (500 mg/kg b.w.) resulted in a dramatic depletion (>80%) of GSH. However, in the presence of EA and RFext, the loss of intracellular GSH was largely prevented in a dose-dependent manner (data not shown). These results suggest that EA reduced AAP-induced oxidative stress in the cells.

Table II. Effect of EA and RF extract on the activities of GST and GSH-PX in AAP-intoxicated rats

Treatment	GSH-PX (units/mg protein)	GST (units/mg protein)
Normal	10.32 ± 0.93 (100)	6.70 ± 0.32 (100)
Control (AAP)	3.28 ± 0.83 (0)[a]	2.11 ± 0.28 (0)[a]
AAP + 5 mg/kg EA	6.64 ± 0.34 (47)	2.62 ± 0.13 (11)
AAP + 10 mg/kg EA	9.16 ± 0.38 (83)[a]	5.20 ± 0.47 (67)[a]
AAP + 20 mg/kg EA	9.83 ± 0.31 (93)[a]	5.61 ± 0.33 (76)[a]
AAP + 250 mg/kg RFex.	5.94 ± 0.44 (37)[a]	3.06 ± 0.21 (20)[a]
AAP + 500 mg/kg RFex.	8.19 ± 0.18 (69)[a]	4.51 ± 0.26 (52)[a]

Rats were pretreated with ellagic acid or RF extract (containing 2% EA) orally for 6 days consecutively. Control rats were given with saline. Three hours after the final treatment, rats were treated with acetaminophen (AAP; 500 mg kg^{-1}, i.p.). Antioxidant enzyme activities of glutathione S-transferase and glutathione peroxidase were determined as described in materials and methods. Each value represents the mean ± SD of five rats. [a]Significantly different from control (AAP) at $p < 0.05$ by student t - test.

Effects of EA and RF extract on AAP-induced depletion of GSH-PX and GST in intoxicated rats

The effects of pretreatment with EA and RF extract on the AAP-induced decrease of GSH-PX and GST activity are shown in Table II. The GSH-PX activity in liver tissues was reduced in AAP-intoxicated rat compared to the control group (10.32 ± 0.93 vs 3.28 ± 0.83 units/mg protein). However, increasing doses of EA (5 mg, 10 mg and 20 mg/kg b.w.) administration resulted in a significant increase in GST-PX activity, almost to the normal level (6.64 ± 0.34, 9.16 ± 0.38 and 9.83 ± 0.31 units/mg protein), respectively ($p < 0.05$). GST enzyme activities were reduced in the AAP-treated rats (6.70 ± 0.32 u/mg vs 2.11 ± 0.28 u/mg) while the pretreatment with the increasing extracts resulted in a significant increase in each of the enzyme ac-

Table III. Effect of dietary RF extract on the catalytic activities of microsomal total cytochrome p450 and NADPH-cytochrome P450 reductase

Treatment	Total cytochrome P450		NADPH cytochrome-P450 reductase activity	
	pmol/mg protein	% of control	pmol cyt c reduced/min/mg protein	% of control
Control	338.2 ± 7.829	100.0 ± 2.31	471.8 ± 24.72	100 ± 5.24
100 mg/kg RF ext.	337.1 ± 19.14	99.7 ± 5.66	470.8 ± 31.23	99.8 ± 6.62
250 mg/kg RF ext.	321.5 ± 19.45	95.1 ± 5.75	499.0 ± 33.21	105.8 ± 7.04

Rats were fed with RF extract (containing 10% EA) in the diet and microsomes were prepared as described in materials and methods. P450 levels and NADPH-cytochrome P450 reductase activity were determined as described in materials and methods. Data are expressed as mean ± SD (n=5 for all assays).

Table IV. Effect of EA and RF extract on hepatic microsomal ethoxyresorufin-O-desthylase, testosterone 6β-hydroxylase and PNP hydroxylase activities

Treatment	Ethoxyresorufin-O-desthylase (EROD)	Testosterone 6β-hydroxylase (TβH)	PNP hydroxylase (PNP)
	(pmol/mg/protein/min) CYP1A	(pmol/mg/protein/min) CYP3A4	(pmol/mg/protein/min) CYP2E1
In vivo (Induction)	119.0 ± 18.47	10.61 ± 0.543	17.27 ± 2.590
Control	(100 ± 15.5)	(100 ± 5.12)	
RF extract 100 mg/kg	130.5 ± 28.36	6.71 ± 0.363	15.04 ± 1.949
(EA 10 mg/kg)	(109.7 ± 23.8)	(63.3 ± 3.42)	
RF extract 250 mg/kg	112.6 ± 18.01	5.65 ± 2.388	20.22 ± 1.949
(EA 25 mg/kg)	(94.7 ± 15.1)	(53.3 ± 22.5)	
In vitro (Inhibition)	115.7 ± 15.25	9.542 ± 1.008	12.52 ± 2.689
Control	(100 ± 13.2)	(100 ± 10.6)	(100 ± 21.5)
500 μg/ml RF extract	40.9 ± 11.06	2.23 ± 0.602	29.54 ± 1.362
	(35.3 ± 9.56)	(22.3 ± 6.31)	(236 ± 10.9)
EA 200 μg/ml	90.5 ± 27.37	7.65 ± 0.370	16.44 ± 3.723
	(78.3 ± 23.7)	(80.2 ± 3.88)	(131 ± 29.7)

For *in vivo* experiment, SD rat were pretreated with RF extract (containing 10% EA) (100 mg/kg, 250 mg/kg) for 6 days and killed after final treatment. For *in vitro* experiment, RF extract (containing 10% EA) and 99.5% EA from RF extract were used.

tivities, almost to the normal level (at $p < 0.05$), as shown in Table II. Increasing concentrations of RFext (250 mg, 500 mg/kg b.w. each containing 5 mg, 10 mg EA respectively) administration also resulted in a significant increase in GST-PX and GST activities (Table II).

Effect of EA on AAP bioactivation-related cytochrome P450s enzymes activities *in vitro*

In this experiment, we tested the effect of EA and EA containing RFext on CYP activities in liver microsomes. After treating with the RFext, the total amounts of CYP and NADPH- CYP reductase activity and the inhibition or the activation of CYP 1A2, 2E1 and 3A were investigated. For CYP subtype substrate, ethoxyresorufin-O-desthylation (EROD), p-nitrophenol (PNP) hydroxylase and testosterone 6 β–hydroxylase (TβH) activities were tested for CYP1 family, CYP3A4 and CYP2E1, respectively. The total amounts of CYP and NADPH- CYP reductase activities were not changed after treatment with a low or high concentration of RFext, (100 mg/kg b.w. or 250 mg/kg b.w. ; each containing 10% EA) (Table III). For the *in vivo* induction test of EA, EA-containing RFext doses (100 mg/kg b.w. and 250 mg/kg b.w.; each containing 10% EA) were orally administered and the induction of EROD, TβH and PNP hydorylase activities were observed (Table IV). For the *in vitro* inhibition test of EA, 500 μg/ml RFext and 200 μg/ml EA were reacted with liver microsomes from rats for the EROD, TβH and PNP activity assays (Table IV). In the induction experiment, there were no significant differences on EROD activities after treatment with either a low or high dose (100 mg/kg b.w. and 250 mg/kg b.w.) of RFext. However, a high concentration (500 μg/ml) of RFext exerted a potent inhibition of EROD activities, suggesting that high concentrations of EA-containing RFext itself inhibited the activity of EROD in the liver and also the drug toxicity induced by CYP1 family. The activity of PNP hydroxylase was not significantly induced by the treatment with high concentrations of RFext. However, RFext (500 μg/ml) and EA (200 μg/ml) were significantly stimulated the activity of PNP hydroxylase. These results suggest that EA exerts part of its effect on AAP-induced hepatotoxicity as a ROS scavenger, not by relating with the activity of CYP2E1. The result of TβH activity by CYP3A4 in the induction experiment showed that the enzyme activity was decreased significantly at both concentrations of the RFext (100, 250 mg/kg). Theses results imply that EA down-regulated the expression of CYP3A4 in liver in rats intoxicated by AAP. In the inhibition experiment, the finding that 500 μg/ml RFext significantly inhibited the activity of TβH compared to 200 μg/ml EA implied that other constituents in the RFext other than EA also inhibit CYP3A4.

DISCUSSION

Our results provide strong evidence that the pretreatments of EA and EA-containing RFext at dosages from 5-20 mg/kg b.w. inhibited the acute liver injury induced by high doses of AAP in rats, as shown by the reduction of serum enzyme activities (ALT, AST) and hepatic lipid peroxidation. EA and RFext pretreatment did enhance the hepatic GSH levels and induce the activities of cytosolic glutathione-S- tansferase and GSH-PX. A pretreatment of EA and RFext affected on CYP isozymes that only the CYP3A, not CYP1A or 2E1, is down-regulated by EA and the EA-containing RFext. The protective effects of EA and EA-containing RFext against the high dose AAP induced liver injury in rats due to enhancing the antioxidant activities and the down-regulation of CYP3A4 activity.

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