JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY



# Antioxidant Activity of Vinegar Produced from Distilled Residues of the Japanese Liquor Shochu

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Rice shochu distilled residue (RSDR) is a byproduct of rice shochu production. RSDR was converted into vinegar by acetate fermentation. In our present study, two major antioxidant compounds, tyrosol and ferulic acid, were identified from the RSDR-derived vinegar. Furthermore, we investigated the antioxidant activity of freeze-dried RSDR-derived vinegar, which was *Acetobactor aceti* fermentation powder (AFP), in vitro and in vivo. AFP at 0.25 mg/mL or higher concentrations showed an inhibitory effect against lipid peroxidation and cellular GSH depletion in HepG2 cells induced by $H_2O_2$ ($P < 0.05$). We thus considered the potential of AFP in protecting cells against damage induced by $H_2O_2$. Its antioxidant activity was evaluated in vivo using carbon tetrachloride ($CCl_4$)-induced acute liver injury mouse models. Five consecutive days of oral preadministration of AFP dissolved in PBS at 200, 400, and 800 mg/kg body weight significantly suppressed lipid peroxidation in the liver induced by $CCl_4$ ($P < 0.01$). Consequently, treatment with AFP at 200 mg/kg body weight or higher doses suppressed the elevation of alanine aminotransferase and aspartate aminotransferase levels in serum ($P < 0.05$). These findings suggest that RSDR-derived vinegar can be developed as a health food with an antioxidant effect for the prevention of oxidative injury and cancer.

**KEYWORDS: Rice shochu distilled residue; vinegar; antioxidant activity; tyrosol; ferulic acid; $H_2O_2$; $CCl_4$**

## INTRODUCTION

Reactive oxygen species (ROS), such as the hydroxyl radical ($^{\bullet}OH$), superoxide anion ($O_2^-$), and hydrogen peroxide ($H_2O_2$), are potentially dangerous byproducts of normal cellular metabolism. ROS causes oxidative damage to DNA, cells, and tissues because of its extraordinary reactivity (*1, 2*). ROS-induced oxidation is reduced by cellular antioxidant systems; however, excessive ROS generated under acute and chronic inflammation are considered to be associated with cancer, arterial sclerosis, and cardiac diseases (*3–6*). Furthermore, ROS generated by inflammation is one of the major causes of various diseases. Antioxidants obtained from plants may be a good candidate for treating such diseases and for cancer prevention owing to their scavenging of free radicals.

Shochu is a Japanese traditional distilled liquor that is produced by fermentation with *Saccharomyces cerevisiae* from raw materials such as barley, sweet potato, and rice. It is predominantly produced in the Kyushu region in southern Japan, and approximately 500 000 kL of shochu is produced each year. At the same time, approximately 750 000 kL of SDR is generated. SDR contains large amounts of organic substances and causes pollution if it contaminates environmental water. On the other hand, it can become a new resource when it is converted into more useful functional products because it contains many useful components such as proteins, polyphenols, amino acids, polysaccharides, and organic acids (particularly citric acid) (*7*). In fact, many studies have suggested that SDR has various beneficial effects. For example, Mahfudz et al. reported that SDR has an important role in decreasing cholesterol levels (*8*), and Mochizuki et al. demonstrated that the administration of barley SDR inhibited the progression of orotic acid-induced fatty liver deposits (*9*).

Accordingly, we established a method of producing vinegar from RSDR using acetate fermentation (*10*), in addition to the recycling process of RSDR (*11, 12*). It was reported that black vinegar (kurozu) produced from brown rice in Japan was shown to be beneficial to human health with its antioxidant activity, and dihydrosinapic acid and dihydroferulic acid were identified from kurozu (*13*). We previously showed that RSDR-derived vinegar shows strong $LOO^{\bullet}$-scavenging activity in vitro (*7*). Further, we also reported that freeze-dried RSDR and freeze-dried RSDR-derived vinegar, namely, *Acetobactor aceti* fermentation powder (AFP), at 0.5% (w/w) in the diet showed antitumor activity in vivo (*14*). Thus, we considered the potential of RSDR-derived vinegar for cancer prevention and treatment and carried out further investigations. In the present study, we hypothesized that RSDR-derived vinegar may exhibit preventive effects against some ROS-related diseases. To clarify our hypothesis, we identified antioxidant compounds in RSDR-derived vinegar and AFP and investigated the potential protective effects of AFP against $H_2O_2$-induced oxidative cytotoxicity

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10.1021/jf073040w CCC: $40.75 
Published on Web 04/24/2008

using HepG2 cells in vitro and $CCl_4$-induced liver damage using ddY mouse in vivo.

## MATERIALS AND METHODS

**Materials.** RSDR was provided by a rice shochu distillery in the Kuma region of Kumamoto, Japan and was kept at −20 °C until use. *A. aceti* NBRC 3283, which is a citric acid-tolerant strain, was purchased from the Institute for Fermentation in Osaka, Japan (*15*).

**Chemicals.** Acetonitrile, methanol, luminol, diethylenetriaminepentaacetic acid (DTPA), and $CCl_4$ were obtained from Wako Pure Chemical Industries (Osaka, Japan). L-Cystine, caffeic acid, *p*-hydroxyphenethyl alcohol (tyrosol), 4-hydroxy-3-methoxycinnamic acid (ferulic acid), trichloroacetic acid, *t*-butylhydroxytoluene (BHT), and *t*-butylhydroperoxide (*t*-BuOOH) were obtained from Sigma-Aldrich (St. Louis, MO). All solvents were of HPLC grade. The remaining compounds were purchased from Oriental Yeast (Tokyo, Japan).

**Fermentation of RSDR into Vinegar and Lyophilization.** A loopful of fresh slant culture of *A. aceti* NBRC 3283 was inoculated into 50 mL of sterilized preculture medium (5 g/L polypeptone, 5 g/L yeast extract, and 50 mL/L ethanol) in Erlenmeyer flasks. After incubation at 30 °C at a rotation speed of 130 rpm for 24 h, 1.35 L of RSDR containing 75 mL of ethanol and 75 mL of the precultured broth was poured into a 3 L jar fermentor (Able, Tokyo, Japan). Cultivation was performed at 600 rpm and 1 vol/vol/min (vvm) of aeration at 30 °C for 24 h. During the cultivation, the pH was automatically maintained at 4.0 by adding 3 N NaOH. After cultivation, the culture broth was centrifuged (H-600N centrifuge, Kokusan Inc. Ltd., Tokyo, Japan) at 8000*g*. The supernatant was RSDR-derived vinegar and was used for the study of the identification of antioxidants. RSDR-derived vinegar was lyophilized using a freeze-dryer (Ulvac Inc. Ltd., Kanagawa, Japan), and AFP was obtained. AFP was dissolved in PBS and adjusted at pH 7.4 and then used for the study of antioxidant activities in vitro and in vivo.

**Identification of Antioxidants in RSDR-Derived Vinegar.** RSDR-derived vinegar was extracted with ethylacetate. One hundred milliliters of the vinegar was subjected to extraction with 40 mL of ethylacetate for 20 min. After centrifugation, the supernatant was collected, and the water phase was re-extracted 3 times with ethylacetate. The ethylacetate extract was concentrated by a rotary evaporator at 40 °C under reduced pressure. The chromatographic separation of the extract was carried out as described by Rechner et al. with some modification (*16*). Purification was performed by RP-HPLC using a Gilson HPLC system. The mobile phase for separation consisted of methanol/$H_2O$/5 N HCl (10:89.9:0.1) (A) and acetonitrile/$H_2O$/5 N HCl (50:49.9:0.1) (B). The gradient applied was as follows: 0−5 min, 100% A; 5−40 min, 50% A and 50% B (1:1 mixture); 40−60 min, 100% B; 60−65 min, 100% B; and 65.1 min, 100% A. The run time was 70 min followed by a 10 min delay prior to the next injection. The column was maintained at 30 °C, and the absorbance was measured at 220 nm. Fractionated samples were collected and dried in a rotary evaporator at 40 °C under reduced pressure. The $LOO^{\bullet}$-scavenging activity was measured in each fraction.

The molecular weight analysis of the purified samples was performed using a LC/MS system (JMS-LC mate, JEOL Ltd., Tokyo, Japan) that used methanol as the moving phase. The purified samples were dissolved in methanol-$d_4$ and measured by $^1H$ NMR and $^{13}C$ NMR analyses (magnet, Oxford Magnet Technology, Oxford, U.K. and analysis, Varian) at 400 MHz. FT-IR absorption spectra of purified samples were obtained using a PerkinElmer Instruments Spectrum One FT-IR spectrometer.

**Total Phenolic Content in AFP.** Total phenolic content in AFP was determined by the Folin−Ciocalteu method (*17*). Approximately 3.4 mL of AFP dissolved in PBS was mixed with 0.2 mL of Folin−Ciocalteu reagent and incubated for 3 min. After incubation, the reaction solution was mixed with 0.4 mL of 10% (w/v) sodium carbonate. The mixture was then incubated for 1 h at room temperature before the absorption was measured at 750 nm (Shimadzu UV160A, UV−vis spectrophotometer, Kyoto, Japan). The total phenolic content was expressed as caffeic acid equivalents.

**Determination of Lipid Peroxyradical-Scavenging Activity.** The $LOO^{\bullet}$-scavenging activities of RSDR-derived vinegar and its fractions, upon RP-HPLC, were determined by the method reported by Akaike et al. (*18*) and Kanazawa et al. (*19*). Briefly, the reaction mixture contained 1.7 mM DTPA, 16.7 $\mu$M luminol, 1 mM *t*-BuOOH, graded concentrations of the sample, and 1 mg/mL hemoglobin in PBS (pH 7.4). Distilled deionized water was used as a control instead of the sample. The assay was started by adding hemoglobin to the reaction mixture, and chemiluminescence was measured using a Labsystems Luminoskan (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan). The suppression of radical generation was described as $IPOX_{50}$, which indicates the required concentration of the sample in the assay mixture to quench 50% chemiluminescence.

**Protective Effect of AFP against $H_2O_2$-Induced Oxidative Cytotoxicity in HepG2 Cells.** The human hepatoma cell line, HepG2, was purchased from the Health Science Research Resources Bank (Tokyo, Japan), which was cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, 100 $\mu$g/mL streptomycin, and 100 U/mL penicillin at 37 °C in 5% $CO_2$/air.

The effect of AFP, tyrosol, and ferulic acid on cytotoxicity was determined using a 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium (WST-8) quantitative colorimetric assay of the dehydrogenase activity retained in the culture cells. Briefly, the cells were plated at $1 \times 10^4$ cells/well in 96-well plates, and each sample was treated appropriately at 37 °C for 24 h in 5% $CO_2$/air. The cells were then treated for 3 h with 10 $\mu$L of WST-8, and the absorbance of the solution was measured at 540 nm.

The protective effect of AFP, tyrosol, and ferulic acid was evaluated with the viability of cells against oxidative stress induced by $H_2O_2$ using a WST-8 quantitative colorimetric assay. A $H_2O_2$-induced oxidative cytotoxicity method was carried out using the method of Puiggròs et al. (*20*) with some modification. HepG2 cells were treated with AFP between 0 and 2.5 mg/mL at 37 °C in the 5% $CO_2$ incubator for 23 h. Then, the cells were treated with 1 mM $H_2O_2$ for 1 h, and the malondialdehyde (MDA) level and GSH content were analyzed. MDA formation was determined using a thiobarbituric acid (TBA, Nacalai Tesque, Inc., Kyoto, Japan) method. The 0.5 mL aliquots of the treated cell ($1 \times 10^6$ cells/mL) or medium were added to 0.5 mL of 30% (w/v) trichloroacetic acid containing 1 mM BHT (*21*). Then, 3 mL of 0.1 M phosphoric acid and 1 mL of 0.04 M TBA aqueous solution were added to 0.5 mL of supernatant of the cell aliquots in a 10 mL centrifuge tube. The mixture was heated for 60 min in boiling water. After cooling, 4 mL of *n*-butanol was used to extract the chromophore, and the absorbance was measured at 535 and 520 nm. The concentration of thiobarbituric acid reactive substances (TBARS) was proportional to the amount of the MDA extinction coefficient (156 $mM^{-1}$ $cm^{-1}$).

The cellular GSH content of the HepG2 cells was determined by the 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) method (*22*). To remove proteins, 0.4 mL of a metaphosphoric acid solution (1.67 g of metaphosphoric acid, 0.20 g of EDTA, and 30.0 g of NaCl in 100 mL of $H_2O$) was added to the liver homogenates and incubated at 25 °C for 40 min. Then, after removal of protein precipitates by microcentrifugation, 0.4 mL of the supernatant was removed, and 0.4 mL of 300 mM $Na_2HPO_4$ was added in 15 mL test tube. Then, 0.1 mL of DTNB (0.02 w/v%; 20 mg of DTNB in 100 mL of 1% sodium citrate) was added to the blank and samples, and the absorbance of the sample was measured against the blank at 412 nm. The cellular GSH content was determined using a calibration curve prepared with an authentic sample of GSH (Kyoto, Japan). An aliquot of the lysate was used for determining the protein content using a bicinchoninic acid (BCA) Protein Assay kit (Pierce, Rockford, IL). The cellular GSH values were expressed as nmol/mg protein.

**Protective Effect of AFP against Carbon Tetrachloride-Induced Hepatic Damage in ddY Mice.** Male ddY mice (5 weeks of age) were purchased from SLC, Inc. (Shizuoka, Japan). Mice were housed in groups of four to five per cage and were maintained at 22 ± 1 °C and 55 ± 5% relative humidity. Lighting was automatic on a 12 h light/dark cycle. Mice were fed a chemically defined AIN-93G diet without antioxidative compounds, such as BHT. All experiments were carried out according to the Laboratory Protocol of Animal Handling, Kumamoto University.

ddY mice were fed with the AIN-93G diet and water ad libitum for 1 week before the experiment. Then, the mice were divided into six

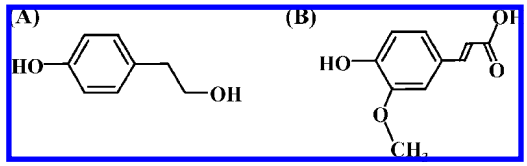


**Figure 1.** Chemical structural formulas of *p*-hydroxyphenethyl alcohol (tyrosol) (**A**) and 4-hydroxy-3-methoxycinnamic acid (ferulic acid) (**B**).

groups, namely, normal (no $CCl_4$ treatment), control ($CCl_4$ treated), AFP groups (200, 400, and 800 mg/kg body weight), and tyrosol (13.8 mg/kg (100 $\mu$mol/kg)). Antioxidant components were given daily via a gastric tube for 5 consecutive days, and on day 6, $CCl_4$ in soybean oil (10 v/v %) was injected intraperitoneally to each animal. After 20 h, the mice were sacrificed under anesthesia; blood samples were collected for the analysis of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. Livers were excised from the animals and assayed for the GSH level, GSH-dependent enzyme activity, and MDA formation. Sera were separated from blood, and ALT and AST levels were measured using transaminase CII-test Wako (Osaka, Japan).

The liver sample obtained as stated previously was immediately homogenized in a 10 times the weight of an ice-cold 0.15 M KCl solution. The amount of MDA was determined by using the TBA method as described previously, and 1,1,3,3-tetraethoxypropane was used as an external standard. The GSH level was determined by the DTNB method described previously.

The glutathione reductase (GSH Rd) activity was assayed using a reaction mixture containing 0.99 mL of 0.1 M potassium phosphate buffer (PPB, pH 7.0), 1.1 mM $MgCl_2$, 5 mM oxidized glutathione (GSSG), and 0.1 mM NADPH. The supernatant of the liver homogenate (10 $\mu$L) was added to induce the NADPH conversion reaction, and changes in the absorbance were monitored at 340 nm for 5 min at 25 °C. The specific activity of GSH Rd was expressed as nmol NADPH/min/mg protein. The glutathione peroxidase (GSH Px) activity was expressed as nanomol of oxidized NADP/min/mg protein, using a NADPH $\epsilon_{mol}$ value at 340 nm of 6220 $M^{-1}$ $cm^{-1}$. The reaction was started by the addition of 0.1 mL of 2.5 mM $H_2O_2$, and the conversion of NADPH to NADP was monitored using a continuous-recording spectrophotometer at 340 nm.

**Statistical Analysis.** Student's *t*-test was used for statistical analysis of differences between two groups. The difference was considered significant when $P < 0.05$.

## RESULTS

**Determination of Total Phenolic Content in AFP.** The total phenolic content, which is considered to represent the antioxidant effect, was 28.6 $\mu$g/mg in AFP. Furthermore, AFP exhibited a strong $LOO^{\bullet}$-scavenging activity, and the $IPOX_{50}$ value of the vinegar was 0.005 mg/mL.

**Identification of Antioxidant Compounds in RSDR-Derived Vinegar.** Two major fractions, A and B, showed the strongest $LOO^{\bullet}$-scavenging activity in all fractions at 0.1 and 0.2 $\mu$g/mL, respectively. The spectrometric data of fraction A were as follows: FT-IR 1884, 2880, 2928, 3024, 3130, 3382 $cm^{-1}$; $^1H$ NMR ($CD_3OD$, 400 MHz) $\delta$ 2.63 (2H, s), 3.60 (2H, s), 6.62 (2H, d, $J = 8.00$ Hz), 6.94 (2H, d, $J = 8.00$ Hz); $^{13}C$ NMR ($CD_3OD$, 100 MHz) $\delta$ 38.57, 63.75, 115.45, 130.04, 130.43; MS (EI) *m/z* 122 ($M^+ - OH + H$), 121 ($M^+ - OH$), 108 ($M^+ - 2OH + 4H$), 107 ($M^+ - 2OH + 3H$). These data indicated tyrosol (see **Figure 1A**), and the retention time (RT) of fraction A was the same in RP-HPLC analysis as that of tyrosol using a commercial reagent. The spectrometric data of fraction B were as follows: FT-IR 934, 1665, 1685, 1825, 1966, 2522, 2555, 2844, 3396 $cm^{-1}$; $^1H$ NMR ($CD_3OD$, 400 MHz) $\delta$ 3.94 (3H, s), 6.38 (1H, d, $J = 15.4$ Hz), 6.86 (1H, s), 7.11 (1H, s), 7.23 (1H, s), 7.66 (1H, d, $J = 15.4$ Hz); $^{13}C$ NMR ($CD_3OD$, 100 MHz) $\delta$ 55.63, 110.81, 115.31, 115.31, 115.64, 123.20, 126.96, 146.14, 148.53, 149.66, 170.22; MS (EI) *m/z* 195 ($M^+ + H$), 151 ($M^+ - OH - OCH_3 - 3H$). These data indicated ferulic acid (see **Figure 1B**), and the RT of fraction B was the same as that of the reagent ferulic acid in RP-HPLC analysis. The content of tyrosol and ferulic acid in AFP was 1.87 and 0.29 $\mu$g/mg, respectively.

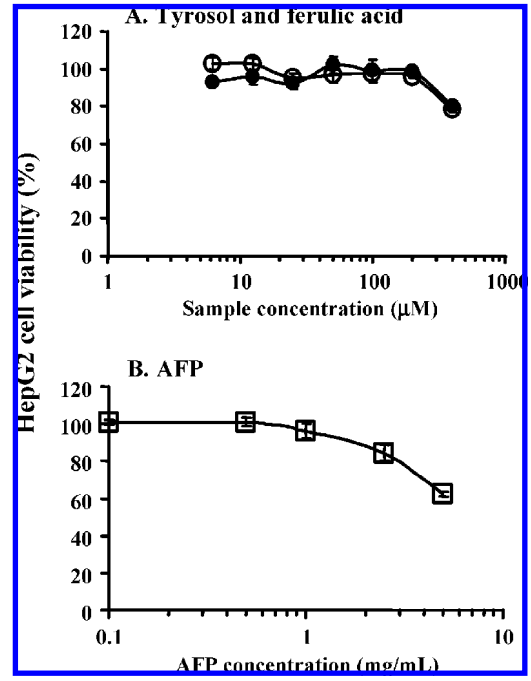


**Figure 2.** Cytotoxic activity of tyrosol, ferulic acid, and AFP against HepG2 cells. (**A**) Cytotoxic activities of tyrosol (●) and ferulic acid (○). (**B**) Cytotoxic activity of AFP (□). Values are mean ± SD ($n = 3$).

**Protective Effect of AFP against $H_2O_2$-Induced Oxidative Cytotoxicity in HepG2 Cells.** The cytotoxicity of AFP against HepG2 cells was determined using the WST-8 method, as described previously. As shown in **Figure 2A**, tyrosol and ferulic acid showed no toxic effects on HepG2 cells up to 200 $\mu$M. AFP showed no toxic effects on HepG2 cells up to 2.5 mg/mL (see **Figure 2B**). Therefore, AFP in the range of 0.5–2.5 mg/mL was used for the cell culture experiments.

The antioxidant activity of AFP against lipid peroxidation and GSH depletion induced by $H_2O_2$ in HepG2 cells was measured. As shown in **Figure 3**, when the cells were exposed to 1 mM $H_2O_2$, their MDA concentration, an index of lipid peroxidation, was significantly increased. Tyrosol and ferulic acid at more than 50 $\mu$M significantly suppressed MDA formation as compared to the $H_2O_2$-treated control group (see **Figure 3A**). The AFP pretreatment group significantly suppressed MDA formation (see **Figure 3B**).

Similarly, $H_2O_2$ treatment induced a significant decrease in the GSH level in the cells. However, pretreatment with AFP at 0.25, 0.5, and 1.25 mg/mL resulted in the recovery of the GSH level in the cells comparable to the level of normal (nontreated) cells (see **Figure 4B**). Pretreatment of cells with tyrosol or ferulic acid at 50, 100, and 200 $\mu$M significantly suppressed the decrease in GSH level upon $H_2O_2$ treatment (see **Figure 4A**).

**Protective Effect of AFP against Carbon Tetrachloride-Induced Liver Damage in ddY Mice.** To elucidate the antioxidant activity of AFP in vivo, we further investigated the protective effect of AFP on $CCl_4$-induced liver damage using a ddY mouse model; we used tyrosol for the control. Treatment with AFP or tyrosol showed no effects on the body weight of

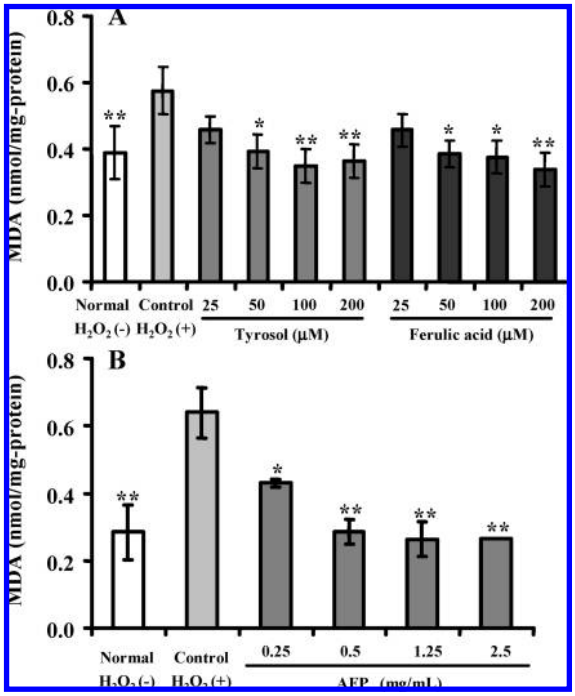


**Figure 3.** Protective effect of tyrosol, ferulic acid (**A**), and AFP (**B**) on $H_2O_2$-induced lipid peroxidation in HepG2 cells. Normal HepG2 cells were not treated with $H_2O_2$, and control cells were treated with $H_2O_2$. Values are mean ± SD ($n = 5$). *, $P < 0.05$ and **, $P < 0.01$ vs the control group.

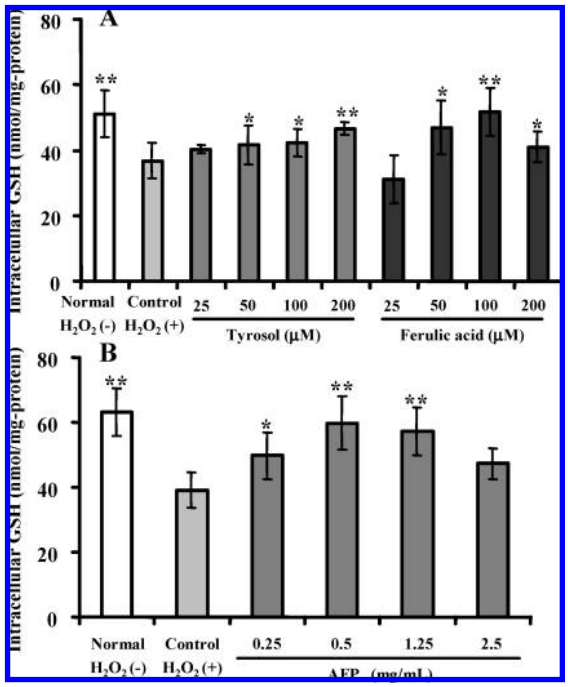


**Figure 4.** Protective effect of tyrosol, ferulic acid (**A**), and AFP (**B**) on $H_2O_2$-induced cellular GSH depletion in HepG2 cells. Normal HepG2 cells were not treated with $H_2O_2$, and control cells were treated with $H_2O_2$. Values are mean ± SD ($n = 5$). *, $P < 0.05$ and **, $P < 0.01$ vs the control group.

$CCl_4$-treated mice (data not shown). As shown in **Figure 5**, 13.8 mg/kg tyrosol given orally significantly decreased the $CCl_4$-induced MDA formation. AFP pretreated mice showed a significantly decreased MDA production in the liver induced by $CCl_4$ (see **Figure 5**).

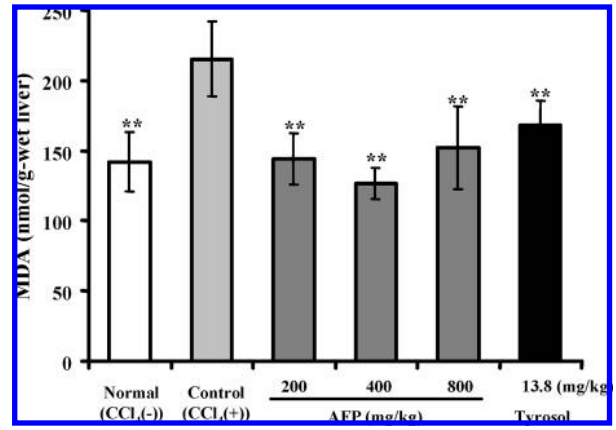


**Figure 5.** Suppressive effect of tyrosol and AFP on $CCl_4$-induced MDA production in mouse liver. ddY mice were orally administered tyrosol, AFP, or PBS (control) for 5 days prior to the ip administration of 10 v/v% $CCl_4$. Normal mice were not treated with $CCl_4$ administration, and control mice were treated with $CCl_4$. Values are mean ± SD ($n = 5$). **, $P < 0.01$ vs the control group.

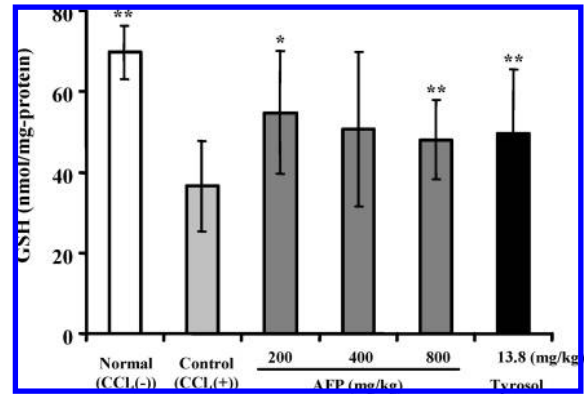


**Figure 6.** Suppressive effect of tyrosol and AFP on decrease of GSH content induced by $CCl_4$ in mouse liver. ddY mice were orally administered tyrosol, AFP, or PBS (control) for 5 days prior to the ip administration of 10 v/v% $CCl_4$. Normal mice were not treated with $CCl_4$ administration, and control mice were treated with $CCl_4$. Values are mean ± SD ($n = 5$). *, $P < 0.05$ and **, $P < 0.01$ vs the control group.

Likewise, pretreatment with both tyrosol (13.8 mg/kg) and AFP (400 and 800 mg/kg) significantly suppressed the decrease in the GSH level (see **Figure 6**).

The GSH-dependent enzymes are also an important cellular antioxidant. The GSH-dependent enzymes, GSH Px and GSH Rd, in the mouse liver after $CCl_4$ treatment elicited 70 and 50% reductions in enzyme activity, respectively. Pretreatment with 13.8 mg/kg tyrosol and 800 mg/kg AFP significantly protected the $CCl_4$-induced suppression in GSH Px activity in the mouse liver (see **Figure 7**). This acute liver damage was suppressed in the mice pretreated with 13.8 mg/kg tyrosol or 200, 400, and 800 mg/kg AFP (see **Figure 8**).

## DISCUSSION

Shochu is a traditional Japanese distilled liquor. At the same time, much larger quantities of SDR are produced as a waste product, and this waste poses social and industrial problems. However, SDR contains potentially useful components for further fermentation, such as a vinegar. The aim of the present study was focused on identifying antioxidant compounds in the RSDR-derived vinegar and clarifying the antioxidant activity of AFP both in vitro and in vivo.

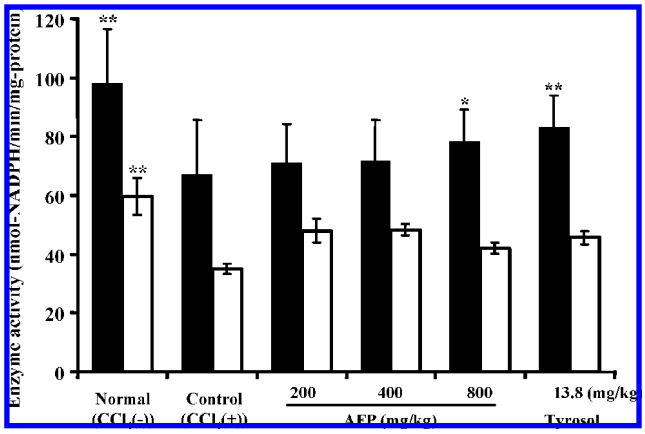


**Figure 7.** Protective effect of tyrosol and AFP on suppression of GSH-dependent enzyme activities induced by $CCl_4$ in mouse liver. ddY mice were orally administered tyrosol, AFP, or PBS (control) for 5 days prior to the ip administration of 10 v/v% $CCl_4$. Normal mice were not treated with $CCl_4$ administration, and control mice were treated with $CCl_4$. GSH Px activity in liver of mice (■) and GSH Rd activity in liver of mice (□). Values are mean ± SD ($n = 5$). *, $P < 0.05$ and **, $P < 0.01$ vs the control group.

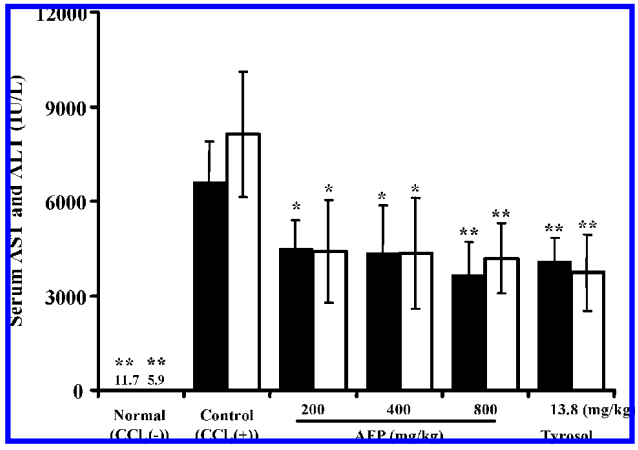


**Figure 8.** Protective effect of tyrosol and AFP on increase of serum ALT and AST levels induced by $CCl_4$. ddY mice were orally administered tyrosol, AFP, or PBS (control) for 5 days prior to the ip administration of 10 v/v% $CCl_4$. Normal mice were not treated with $CCl_4$ administration, and control mice were treated with $CCl_4$. ALT (■) and AST (□). Values are mean ± SD ($n = 5$). *, $P < 0.05$ and **, $P < 0.01$ vs the control group.

Two major antioxidant compounds, tyrosol and ferulic acid, were identified in RSDR-derived vinegar. Their contents in AFP were 1.87 and 0.29 μg/mg, respectively. Tyrosol is one of the major phenolic compounds in olive oil and olive oil mills, and ferulic acid is known to exist in rice. However, these compounds were first identified in RSDR-derived vinegar. Tyrosol and ferulic acid also were found in AFP at 5.98 and 0.14 μg/mg, respectively. It was reported that tyrosol was formed from L-tyrosine by *S. cerevisiae* during ethanol fermentation (*23*). Thus, tyrosol was found in high amounts in AFP and freeze-dried RSDR.

With regard to biological activity, tyrosol has a cytoprotective effect against the toxicity of peroxynitrite ($ONOO^-$) (*24, 25*) and inhibits the cell-mediated oxidation of LDL (*26–28*). Furthermore, tyrosol significantly suppresses ROS and $PGE_2$ production and iNOS and COX-2 expression, which are inflammation mediators, from activated macrophages (*29*). On the other hand, ferulic acid suppresses azoxymethane-induced colon carcinogenesis (*30*). Thus, we consider that tyrosol and ferulic acid play an important role in the antioxidant activity of AFP. Therefore, we investigated the antioxidant activity of AFP using $H_2O_2$-induced cellular oxidation in HepG2 cells using tyrosol and ferulic acid as positive controls. Both tyrosol and ferulic acid at 50 μM or higher concentrations significantly suppressed lipid peroxidation and GSH depletion induced by 1 mM $H_2O_2$. AFP at 0.25 mg/mL and higher concentrations showed significant inhibitory effects against lipid peroxidation as well as GSH depletion in the HepG2 cells induced by $H_2O_2$. However, concentrations of tyrosol and ferulic acid in AFP at 0.25 mg/mL (minimum dose in vitro) were far less than 50 μM (i.e., ∼10 and 1%, respectively). Despite very low concentrations of these two compounds in AFP, the antioxidant activity of AFP was comparable to those of pure tyrosol and ferulic acid at 100–200 μM. We found that RSDR-derived vinegar contained other compounds that had a $LOO^•$-scavenging activity; however, their activities were lower than those of tyrosol and ferulic acid (data not shown). Therefore, we considered that the antioxidant activity resulted from the synergistic actions of multiple compounds contained in RSDR-derived vinegar, such as tyrosol and ferulic acid, which showed a $LOO^•$-scavenging activity.

To investigate the antioxidant activity of AFP in vivo, we used $CCl_4$-induced acute liver injury mouse models using tyrosol as a positive control. Five consecutive days of preadministration with AFP at 200, 400, and 800 mg/kg significantly suppressed lipid peroxidation, GSH depletion, and increased the AST and ALT levels in the mice induced by $CCl_4$. Similar results were observed with tyrosol pretreatment at 13.8 mg/kg (100 μmol/kg). However, the tyrosol content of AFP at 800 mg/kg (maximum dose in vivo) was less than about 13.8 mg/kg of tyrosol. Liu et al. reported that the synergistic effects of phytochemicals, such as vitamin C, tyrosol, and ferulic acid, in fruits and vegetables are responsible for their potent antioxidant and anticancer activities and that the best way to acquire these antioxidants is to consume food as a whole (*31, 32*). Taken together, we suggest that the antioxidant activity of AFP in vivo results from the synergistic effects of phytochemicals such as tyrosol, ferulic acid, and unidentified compounds in the vinegar just the same as the in vitro results as described previously. Consequently, these findings suggest that AFP has protective effects toward liver cells by scavenging radicals and enhancing the GSH antioxidant systems.

Presently, SDR disposal is a serious problem in Japan. It is difficult to dispose of SDR because of its high biochemical oxygen demand concentration (i.e., 35 000–85 000 mg/L). SDR has been utilized for fodder and treated by incineration or methane fermentation. However, several serious problems exist, such as the incurrence of high costs and a large environmental load. Food manufacturing byproducts, such as SDR, should be recycled under the Food Recycling Law in Japan. We previously produced RSDR-derived vinegar from RSDR. Also, in our present study, we demonstrated AFP shows a strong antioxidant activity, which contributes to human health in vitro and in vivo. Furthermore, we found that RSDR shows the same effects as AFP (data not shown). Sasaki et al. reported that potato SDR suppresses 7,12-dimethylbenz[*a*]anthracene-induced mammary carcinogenesis (*33*). We suggest that RSDR is a possible resource for new nutraceutical food and that RSDR-derived vinegar is a functional food in this context because of its antioxidant activity in vitro and in vivo.

We identified the antioxidant compounds in RSDR-derived vinegar and demonstrated the various antioxidant activities of RSDR-derived vinegar using AFP. Thus, RSDR and RSDR-derived vinegar may be good candidates for the development of food supplements for the prevention of oxidative injury and cancer.

## ABBREVIATIONS USED

RSDR, rice shochu distilled residues; RSDR-derived vinegar, vinegar produced from rice shochu distilled residues; AFP, freeze-dried RSDR-derived vinegar; ROS, reactive oxygen species; GSH, glutathione; GSH Rd, glutathione reductase; GSH Px, glutathione peroxidase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PBS, 0.01 M phosphate-buffered 0.15 M saline; FBS, fetal bovine serum; RP-HPLC, reversed phase high-performance liquid chromatography.

## ACKNOWLEDGMENT

We thank the rice shochu distillery in the Kuma region of Kumamoto for supplying RSDR.

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**Received for review October 16, 2007. Revised manuscript received March 3, 2008. Accepted March 13, 2008.**

JF073040W