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Free radical scavenging and hepatoprotective actions of *Quercus aliena* acorn extract against CCl_4-induced liver

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Abstract

In the present study, we investigated the protective effect of *Quercus aliena* acorn extracts against CCl_4-induced hepatotoxicity in rats, and the mechanism underlying the protective effects. Aqueous extracts of *Quercus aliena* acorn had higher superoxide radical scavenging activity than other types of extracts. The *Quercus aliena* acorn extracts displayed dose-dependent superoxide radical scavenging activity ($IC_{50} = 4.92\ \mu g/ml$), as assayed by the electron spin resonance (ESR) spin-trapping technique. Pretreatment with *Quercus aliena* acorn extracts reduced the increase in serum aspartate aminotransferase (AST) and serum alanine aminotransferase (ALT) levels. The hepatoprotective action was confirmed by histological observation. The aqueous extracts reversed CCl_4-induced liver injury and had an antioxidant action in assays of $FeCl_2$- ascorbic acid induced lipid peroxidation in rats. Expression of cytochrome P450 2E1 (CYP2E1) mRNA, as measured by RT-PCR, was significantly decreased in the livers of *Quercus aliena* acorn-pretreated rats compared with the livers of the control group. These results suggest that the hepatoprotective effects of *Quercus aliena* acorn extract are related to its antioxidative activity and effect on the expression of CYP2E1.

Keywords: *Quercus aliena* acorn, hepatoprotective, antioxidative, CCl_4, CYP2E1, free radicals

Introduction

Reactive oxygen species (ROS) may be essential for cellular functions such as ingestion of bacteria and redox regulation of signal transduction [1]. They are generally harmful causing considerable damage to cellular components such as lipids, proteins, and DNA [2]. Moreover, lipid peroxides promote the formation of additional free radicals in a type of chain reaction [3]. ROS are recognized to the development of various diseases such as atherosclerosis, diabetes, cancer and arthritis [4–5]. Thus a scavenger of ROS is expected to reduce these free radical-mediated diseases. The use of antioxidants, both natural and synthetic, in the prevention and cure of various diseases is expanding. Currently there is considerable interest in the antioxidant activities of dietary antioxidants, such as vitamins E and C, carotenoids, and plant polyphenolics. Liver intoxication has increased as a result of exposure to high levels of environmental toxins, as the liver has an important role in detoxification, and plants contain substances that can protect or treat hepatic injury.

Carbon tetrachloride (CCl_4) is a potent hepatotoxin producing centrilobular hepatic necrosis. It is bio-transformed to the trichloromethyl radical ($\cdot CCl_3$) by the cytochrome P450 system in liver microsomes,

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often leading to liver injury [6–8]. Antioxidative action plays an important role in protection against CCl_4-induced liver injury [9]. Although several isoforms of cytochrome P450 may metabolize CCl_4, attention has focused largely on the cytochrome isoform P450 2E1 (CYP2E1), which is ethanol-inducible [10]. Alterations in the activity of CYP2E1 affect susceptibility to hepatic injury from CCl_4 [11]. Thus, CCl_4 has been used to evaluate the free radical scavenging action of antioxidants *in vivo*.

The *Quercus aliena* acorns are distributed worldwide and are used in foods, such as types of bread and noodles, and cakes. *Quercus aliena* acorn extracts contain a mixture of gallic acid, digallic acid, gallotannin and minor components such as crude fats, proteins, fibers, minerals, and tannins [12]. In the present study, we examined the antioxidant action of *Quercus aliena* acorn extract and its hepatoprotective effect against CCl_4-induced liver toxicity *in vitro* and *in vivo*; we also identified the antioxidant components.

Material and methods

Preparation of crude Quercus aliena *acorn extracts*

Quercus aliena acorns were collected in Korea and washed thoroughly in tap water, shade-dried and powdered. Samples of 100 g each were extracted twice with 2 L water, 75% ethanol, ethanol, methanol and chloroform for 3 day each time, and the extracts were dissolved in normal saline prior to oral administration.

CCl_4-induced hepatotoxicity in rats

Male Wistar albino rats were purchased from the animal house and kept for 1 week on a commercial diet under environmentally controlled conditions (temperature 22 ± 3°C, relative humidity 55 ± 5%) with free access to water and food (Purina Rat Chow 5001, Ralston Purina, St. Louis, MO, USA). A controlled 12 h light/dark cycle was maintained. Rats weighing 180–220 g were used for assessing CCl_4-induced hepatotoxicity. Liver damage was induced with a 1:1 (v/v) mixture of CCl_4 and olive oil, administered s.c. at a dose of 2 ml/kg body weight. The animals were divided into six groups of six rats each. Group 1 received normal saline (10 ml/kg, p.o.) as normal control. Group 2 was injected with CCl_4/olive oil alone (2 ml/kg). Groups 3–6 were administered *Quercus aliena* acorn extract (50, 100, 300 mg/kg) and silymarin p.o., once 30 min before the injection of CCl_4/olive oil (2 ml/kg) s.c. and twice thereafter, 24 and 48 h after injection.

Assay of serum AST and ALT activities

Seventy-two hours after CCl_4 intoxication the animals were anaesthetized with ether, and blood was withdrawn from the carotid artery. The blood was centrifuged at 3000 rpm for 10 min at 4°C to obtain the serum aspartate aminotransferase (AST) and serum alanine aminotransferase (ALT) activities were measured with an auto-biochemistry-detector (EKTA Chem; DT60 II, DTSC II module, DTE II module, Johnson & Johnson Orthoclinical Diagnostics).

Histopathological observation

Immediately after the blood was collected, sections were taken from each lobe of the liver. The tissues were fixed in 10% neutral formalin for at least 24 h, dehydrated in graded (50–100%) alcohols, embedded in paraffin, cut into 4–5 μm thick sections and stained with haematoxylin-eosin for microscopic assessment.

$FeCl_2$-ascorbic acid stimulated lipid peroxidation in rat liver homogenates

Young male wistar albino rats weighing 180 g were used for producing rat liver homogenates [13]. The rats were killed by decapitation and their liver tissues quickly removed. Portion of 2 g of liver tissue were sliced and homogenized in ice cold 10 ml 150 mM KCl-Tris-HCl buffer (pH 7.2). Protein content was determined by the Lowry method [14]. Reaction mixtures were composed of 0.35 ml of liver homogenate, 0.1 ml of Tris-HCl buffer (pH 7.2), 0.05 ml of 4 mM $FeCl_2$, 0.05 ml of 0.1 mM AA and 0.05 ml of various crude extracts. The mixtures were incubated at 37°C for 1 h in capped tubes; then 0.5 ml of HCl (0.1N), 0.2 ml of SDS (9.8%), 0.7 ml of distilled water and 2 ml of TBA (thiobarbituric acid) (0.6%) were added with vigorous shaking. The tubes were placed in a boiling water bath (100°C) for 30 min. After cooling, the flocculent precipitate was removed by adding 5 ml of n-BuOH and centrifuging at 3000 rpm for 15 min. Thereafter the absorbance of the supernatant was measured at 532 nm. Lipid peroxidation was assessed by measuring the formation of TBA-reactive MDA (malondialdehyde) [15]. The 1,1,3,3-tetraethoxypropan was used as a standard for calibrating MDA.

Assay of superoxide anion scavenging activity

Using an ESR spectrometer, we analyzed the superoxide anion radical ($\cdot O_2^-$) formed from the spin adduct of $\cdot O_2^-$ (DMPO-OOH) [16]. The superoxide radical $\cdot O_2^-$ was generated in a hypoxanthine HPX-xanthine oxidase reaction system. The mixtures contained 180 μl of 0.1 M potassium dihydrogen phosphate (Kpi) buffer (pH 7.4), 4 μl of 50 mM hypoxanthine, 2 μl of 5 mM enetriaminepentaacetic acid (DETAPAC), 5 μl of various concentrations of crude drug extract, 2 μl of 100 μM catalase and 4 μl of 9 mM DMPO (5,5-dimethyl-1-pyrroline-oxide). Reactions were initiated by adding 3 μl of 25 units XOD to each

sample. After 2 min, the spin adduct DMPO-OOH was measured with an ESR spectrometer (JEOL-JES-TM200, JEOL, Tokyo). ESR spectra were recorded at 37°C with the field set at 336.75 ± 5.0 mT for superoxide radicals, modulation frequency 100 KHz, modulation amplitude 0.79 × 0.1 mT, response time 0.1 s, sweep time 0.5 min, microwave power 2.0 mM (9.419 GHz), receiver mode 1st.

RT-PCR analysis of CYP2E1

Total RNA was isolated from rat liver homogenates with a Trizol RNA isolation kit (Invitrogen). cDNA was synthesized from the total RNA with an AMV RNA PCR kit (Takara, Japan). Reverse transcription (RT) reactions were carried out in 2.5 mM $MgCl_2$, 10^{-}x PCR buffer, RNase free dH_2O, 10 mM dNTP, 0.25 unit AMV reverse transcriptase, 2.5 pmol/μl oligo dT in a final volume of 20 μl. (and carried out) 10 min at 30°C, 30 min at 50°C for annealing, 2 min at 95°C, 5 min at 5°C PCR cocktail contained 2 μl of RT reaction mixture, 2.5 mM $MgCl_2$, 10 × PCR buffer, 2.5 mM dNTP, 0.05 μM each of the mixed oligonucleotide primer and 2.5 unit *Taq* DNA polymerase (Takara, Japan) in a final volume of 50 μl. The primers used for the rat CYP2E1 gene [17] were sense: 5′-ACCACCAGCACAACTCTGAGATA-TGG-3′ and antisense: 5′-CAATTCCATGCG GGC-CAGGCCTTCTCC-3′ (436 bp), and for the rat β-actin gene, sense: 5′-CATCCC CCAAAGTTCTAC-3′ and antisense: 5′-CCAAAGCCTTCATACATC-3′ (347 bp). PCR conditions were: Denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min before a final extension at 72°C for 7 min. The PCR products were analyzed by electrophoresis on a 1% agarose gel, followed by staining with ethidium bromide.

Statistical analysis

All experimental data are expressed as means ± S.E. Duncan's multiple range test was applied for assessing the significance of differences between groups. $P < 0.05$ was regarded as significant.

Results

Superoxide radical scavenging activity

The superoxide radical scavenging activities of different extracts are shown in Table I and Figure 1. The order of superoxide radical scavenging activity was: Water > 75% ethanol > methanol > ethanol > chloroform. As shown in Table I, the 50% scavenging concentration (ic50) of the aqueous extract was 4.92 μg/ml. Measurement of ESR signals showed that *Quercus aliena* acorn extracts reduced the DMPO-OOH signal in a dose-dependent manner (Figure 2). Comparison of the ESR signals generated using different concentrations of aqueous *Quercus aliena* acorn extract with the manganese oxide (Mn^{2+}) signal as internal standard revealed that the aqueous extracts were strongly inhibited at 5 μg/ml.

Table I. Superoxide radical scavenger activity assay.

Sample	Concentration (μg/ml)	Inhibition (%)	IC_{50} (μg/ml)
Water ex.	5	50.3	4.92
	10	69.3	
	100	94.9	
75% EtOH ex.	5	44.9	6.53
	10	61.6	
	100	95.7	
EtOH ex.	5	20.7	48.62
	10	31.9	
	100	79.5	
MeOH ex.	5	39.5	8.67
	10	53.8	
	100	88.7	
CH_2Cl_2 ex.	10	6.5	>>200
	100	1.0	
	200	2.8	

CCl_4-induced hepatotoxicity

We examined the protective properties of water extract of *Quercus aliena* acorn in rats treated with CCl_4. In these studies the *Quercus aliena* acorn extracts were administered once, 30 min prior to CCl_4 administration, and twice 24 and 48 h after its administration. The activities of AST and ALT obtained in CCl_4-treated hepatitis in rats are summarized in Table II. AST and ALT levels were greatly elevated by injection of CCl_4/olive oil, and treatment with 50, 100, and 300 mg/kg *Quercus aliena* acorn extract or silymarin protected against this effect. The protective effects of *Quercus aliena* acorn extract were confirmed by histological observation (Figure 3). Injection of CCl_4:olive oil induced ballooning degeneration, centrilobular necrosis, bridging necrosis, and apoptosis of the hepatocytes (Figure 3A and B), and

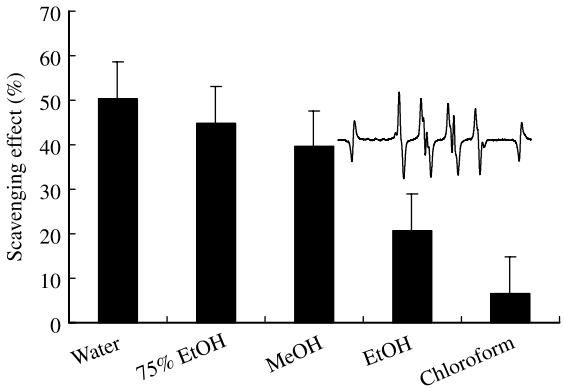

Figure 1. Inhibition of superoxide radical formation by various extracts of acorn. Inset shows the ESR spectrum of the superoxide radical.

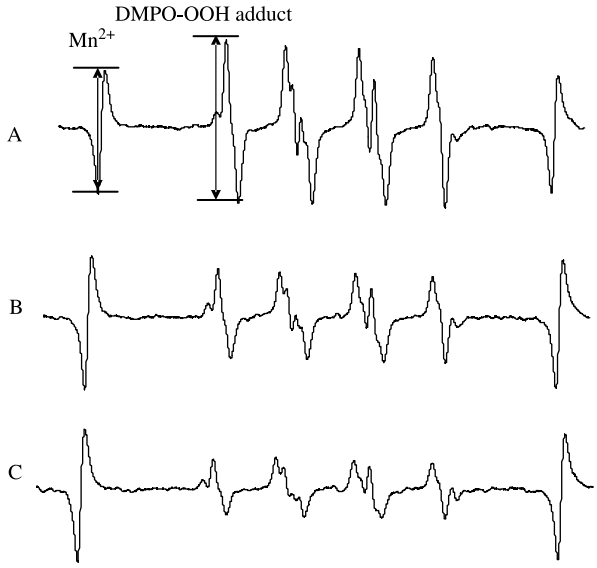

Figure 2. ESR signals of the superoxide radical (DMPO-OOH) measured by ESR spectrometry. Mn^{2+}: Manganese oxide signal as standard. The effect of different concentrations of aqueous extract of acorn. A, 5 μg/ml; B, 10 μg/ml; C, 100 μg/ml.

100 mg/kg of *Quercus aliena* acorn extracts had a significant protective effect against this hepatotoxicity (Figure 3C). The ballooned hepatocytes were of variable size but much larger than normal hepatocytes, and they occasionally appeared as confluent areas (Figure 3). Treatment of the rats with *Quercus ilex* acorn extract on day 3 or day 5 reduced the CCl_4-induced increases in lipid peroxides, but the effect did not quite reach statistical significance (data not shown).

$FeCl_2$-ascorbic acid induced lipid peroxidation in rat liver homogenates

A rat liver homogenate was incubated with ascorbic acid/Fe^{2+}($FeCl_2$-AA) to cause non-enzymatic lipid peroxidation and the effects of crude extracts on this system were determined. For the purpose of investigating the effects of *Quercus aliena* acorn on the *in vitro* lipid peroxidation, the extracts were incubated with a rat liver homogenate in the presence of $FeCl_2$-AA. The lipid peroxide concentration was determined from the absorbance due to the MDA-TBA adduct (a complex of malondialdehyde with thiobarbituric acid) at 532 nm [18,19].

On incubation for 1 h at 37°C, most of the water extracts inhibited the formation of TBA-RS at concentrations of 100–600 μg/ml. There was a significant increase in the level of MDA relative to the control without $FeCl_2$-AA. 100, 300 and 600 μg/ml of *Quercus aliena* acorn aqueous extract had significant anti-lipid peroxidation activity with inhibition rates of 43.8, 52.1 and 66.9%, respectively (Table III and Figure 4). As shown in Figure 4, 600 μg/ml *Quercus aliena* acorn extracts had inhibitory activity equivalent to 100 μg/ml vitamin E. The $FeCl_2$-ascorbic acid mixture is known to stimulate lipid peroxidation by rat livers, as well as by rat liver microsomes and mitochondria *in vitro* [20]. Thus *Quercus aliena* acorn extracts were effective in decreasing MDA production in the livers of rats treated with $FeCl_2$-ascorbic acid mixture.

Effect of Quercus aliena *acorn extract on CYP2E1 expression*

CCl_4 is metabolized to the trichloromethyl free radical ($\cdot CCl_3$) by CYP2E1. To assess the expression of CYP2E1 mRNA, we performed RT-PCR using total RNA isolated from liver homogenates. The RT-PCR showed that the level of CYP2E1 mRNA was markedly increased in liver homogenates of the CCl_4-treated group (Figure 5) and this effect was reduced by pretreatment with *Quercus aliena* acorn extract or silymarin (Figure 5, lane 3–6). CYP2E1 mRNA was scarcely detectible in the liver homogenates of the rats pretreated with 100 mg/kg *Quercus aliena* acorn extract (Figure 5 lane 4).

The hepatotoxicity of CCl_4 is the result of reductive dehalogenation, catalyzed by cytochrome P450, forming the highly reactive trichloromethyl free radical ($\cdot CCl_3$) [21,22]. This free radical, in the presence of oxygen, leads to auto-oxidation of fatty acids and functional and morphological changes in the cell membrane [23]. It is thought that metabolic activation of CCl_4 is mainly mediated by CYP2E1

Table II. Effect of the water extracts from *Acorn* on CCl_4-induced hepatitis in rats.

Groups	Concentration (mg/kg)	3 days later		5 days later	
		ALT(U/L)	AST(U/L)	ALT(U/L)	AST(U/L)
Normal	–	52.0 ± 3.5	131.3 ± 16.1	51.5 ± 6.4	106.5 ± 16.3
CCl_4	–	99.8 ± 30.9	243.3 ± 42.5	59.5 ± 3.5	154.5 ± 6.4
CCl_4 ± Acorn	50	57.8 ± 2.2	129.0 ± 19.7⋆	65.0 ± 5.6	96.0 ± 7.8
CCl_4 ± Acorn	100	61.7 ± 21.9	146.7 ± 40.1	60.0 ± 4.2	99.5 ± 6.4
CCl_4 ± Acorn	300	56.0 ± 5.4	133.3 ± 18.8	57.0 ± 5.6	93.0 ± 7.8
CCl_4 ± Silymarin	50	49.3 ± 9.1	96.3 ± 7.1⋆	52.5 ± 2.1	102.5 ± 4.9

⋆Values represent the mean ± S.D. ($n = 6$). Among the group, values with different letters ate significantly different at $P < 0.05$ by Duncan's multiple range test.

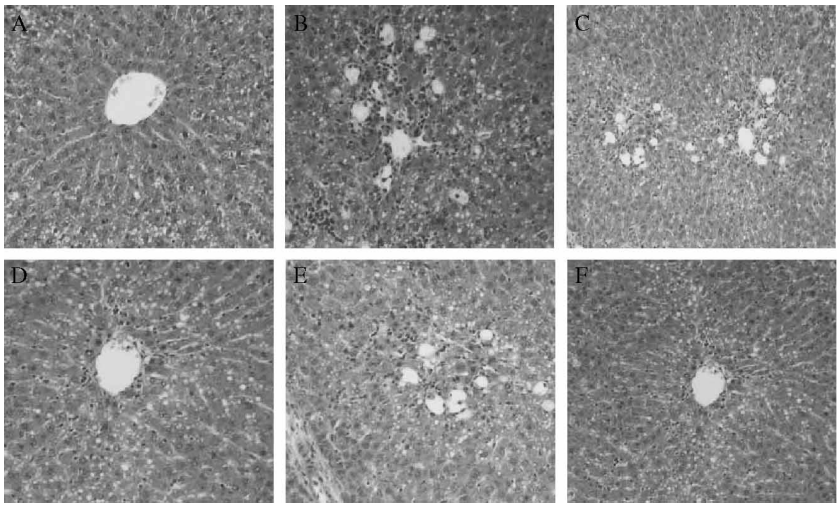

Figure 3. Sections of the livers of CCl_4-treated rats showing the central vein (C.V.) and hepatic cells (hematoxylin-eosin stain, original magnification, 100x). A, control group; B, CCl_4/olive oil (1:1, 2 ml/kg); C, CCl_4 + acorn extract (50 mg/kg); D, CCl_4 + acorn extract (100 mg/kg); E, CCl_4 + acorn extract (300 mg/kg); F, CCl_4 + silymarin (50 mg/kg). Each photograph is representative of the results from at least 3 rats.

[11,24,25]. Inhibition of CYP2E1 gene expression reduced the formation of reactive metabolites, and decreased tissue injury [26]. Our results showed a good correlation between decreased expression of CYP2E1 and protection against CCl_4-induced hepatotoxicity in the liver homogenates of *Quercus aliena* acorn-pretreated rats (Figure 5).

Discussion

Reactive oxygen species and free radicals play a substantial role in the etiology and pathogenesis of a variety of diseases such as hypertension and cardiovascular disease [27–29]. Any compound, natural or synthetic, with antioxidant properties that might contribute towards the partial or total alleviation of this damage, may have a significant role in maintaining health when continuously taken as a component of dietary foods, spices and drugs. Therefore, removing the superoxide radical is probably one of the most effective defences of a living organism against disease. Table I shows the superoxide radical scavenging activity of different *Quercus aliena* acorn extracts. The order of superoxide radical scavenging activity was: Water > 75% ethanol > methanol > ethanol > chloroform. As shown in Table I, the 50% scavenging concentration (IC_{50}) of the aqueous extract of acorn was 4.92 μg/ml. The scavenging activity of the extract of Acorn was demonstrated by the fact it reduced the intensity of the DMPO-OOH signal generated in the HPX-XOD system. This suggests that *Quercus aliena* acorns contain a free radical scavenging activity that can exert a beneficial action against pathological alterations caused by the presence of CCl_4, which appears to be metabolized to a free radical during intoxication [23]. *Quercus aliena* acorn extracts had no hydroxyl radical scavenging activity (data not shown).

Studies on the hepatoprotective experimental model indicated that CCl_4 is first metabolized by cytochrome P450 in the liver endoplasmic reticulum to the highly reactive trichloromethyl free radical ($\cdot CCl_3$) [30]. The free radical, in the presence of oxygen, leads to auto-oxidation of the fatty acids present in the cytoplasmic membrane phospholipids

Table III. The inhibitory effects of aqueous extracts of *Acorn* on $FeCl_2$-Ascorbic acid induced lipid peroxidation in a rat liver homogenate *in vitro*⋆.

Groups	Concentration (μg/ml)	MDA (nmol/mg protein)	Inhibition rate (%)
Normal	–	1.52 ± 0.64	–
$FeCl_2$-AA	–	3.88 ± 1.07	–
$FeCl_2$-AA + A	100	2.17 ± 0.80**	43.8
$FeCl_2$-AA + A	300	1.96 ± 1.26**	52.1
$FeCl_2$-AA + A	600	1.27 ± 1.04**	66.9
$FeCl_2$-AA + Vit E	100	1.31 ± 0.69**	66.0

⋆Values represent the mean ± S.D. ($n = 5$). Among the group, values with different letters ate significantly different at $P < 0.05$ by Duncan's multiple range test.

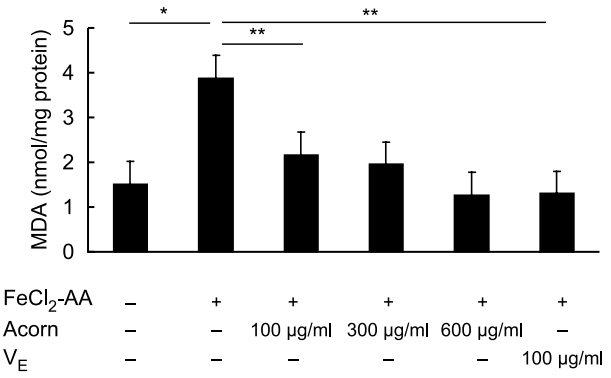

Figure 4. The inhibitory effects of aqueous extracts of acorn on $FeCl_2$-ascorbic acid induced lipid peroxidation in a rat liver homogenate. Values with different letters are significantly different at $\star\ P < 0.05$, $\star\star\ P < 0.01$ by Duncan's multiple range test. $n = 5$.

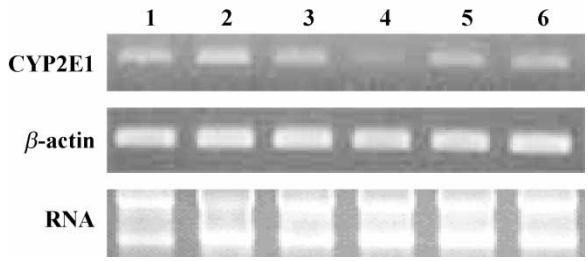

Figure 5. Inhibitory effect of acorn extracts on the level of cytochrome P450 2E1 mRNA. CYP2E1 mRNA was detected by RT-PCR using total RNA from the livers of rats that had been exposed to control (1), CCl_4 (2 ml/kg) (2), CCl_4 after administration of acorn extract (50, 100, 300 mg/kg) (3–5), CCl_4 after administration of silymarin (50 mg/kg) (6), The data are representative of the results from at least 3 rats.

[23] and causes functional and morphological changes in the cell membrane. It is thought that metabolic activation of CCl_4 is mainly mediated by CYP2E1 [10]. CYP2E1 has been shown to be largely responsible for the conversion of CCl_4 to its toxic metabolites [31], and pretreatment of rats with CYP2E1 inhibitors can protect against CCl_4-induced hepatotoxicity [32]. Our results also showed that there is a good correlation between the decreased expression of CYP2E1 and the level of protection against CCl_4-induced hepatotoxicity in the liver homogenates of 100 mg/kg pretreated group (Figure 5).

Furthermore, influx of extracellular Ca^{2+}-ions into the cell is claimed to be an important step leading to cell death. Therefore, examination of the preventive action against liver damage caused by CCl_4, may give an indication of the liver-protective action of drugs in general. Silymarin, used as positive control, is a flavonoid extracted from the milk thistle, *Silybum marianum*, that has demonstrated protective effects against oxidative peroxidation in several experimental models and in human hepatic damage [33,34]. This compound has shown a protective effect against the hepatotoxicity of CCl_4 [35,36]. The results of this study show that water extracts of *Quercus aliena* acorn (100 mg/kg) have a protective action against CCl_4-induced hepatotoxicity similar to that of silymarin. This was also confirmed by histological observation. It has been hypothesized that one of the principal causes of CCl_4-induced liver injury is lipid peroxidation by free radical derivatives of CCl_4. Thus, antioxidant activity or inhibition of the generation of free radicals is important for protection against CCl_4-induced liver lesions [37].

Green acorns are known to contain high concentrations of pyrogallol [38]. There is a report of anaphylactic reactions to ingestion of *Quercus ilex* acorn nut [39]. We used *Quercus aliena* acorn extract at dosage levels of 50, 100 and 300 mg/kg body weight and these did not have any side-effect. Perhaps this is because we only used young rats, not old rats, as experimental material, and administered *Quercus aliena* acorn extract in a moderate manner, with 300 mg/kg body weight as the maximum dosage.

The *in vitro* lipid peroxidation in a liver homogenate proceeds in an enzymatic and a non-enzymatic manner. The former process is NADPH-dependent, but the latter process is induced by ascorbate in the presence of Fe^{2+}/Fe^{3+}, even with the boiled liver homogenate. It was reported that Fe^{2+} and ascorbic acid stimulated lipid peroxidation in rat liver microsomes and mitochondria. In order to clarify the mechanism of action of these drugs, *in vitro* experiments were undertaken. According to the results, all of the drugs inhibited $FeCl_2$-ascorbic acid-stimulated lipid peroxidation in rat liver homogenates. The water extract of 100, 300, 600 μg/ml exhibited 43.8, 52.1, 66.9% inhibitory action against the lipid peroxidation, respectively. So, we also investigated the anti-lipid peroxidation in rat liver homogenates and the active oxygen scavenging activity of crude drugs, which at least in part, can explain the mechanism of the hepatoprotective effect of *Quercus aliena* acorn extract. Treatment of rats with *Quercus aliena* extract at 3 days or 5 days prevented CCl_4-induced increases in lipid peroxides. These results suggest that lipid peroxidation may continue at later time points in response to oxidative stress induced by CCl_4. Lipid peroxidation at these later times may not be due to the CCl_4 radical or ion, but instead may involve activation of Kupffer cells and infiltration of neutrophils [40]. Activated Kupffer cells produce reactive oxygen species and blocking Kupffer cell function has been shown to be protective against CCl_4-induced hepatotoxicity [41].

In conclusion, extracts of *Quercus aliena* acorn exhibited free radical scavenging activities and a liver protective effect against CCl_4-induced hepatotoxicity, and possessed anti-lipid peroxidative. The identities of the compounds responsible for the hepatoprotective

effect, anti-lipid peroxidation, and superoxide radical scavenging activity of *Quercus aliena* acorn extracts merit further study.

Acknowledgements

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