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Protective Effects of *Angelica keiskei* Extracts Against D-Galactosamine (GalN)-induced Hepatotoxicity in Rats

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ABSTRACT - Although the vegetable *Angelica keiskei* (AK) has widely been utilized for the purpose of general health improvement among Korean population, its functionalities are not very well defined. In this study, we examined the effects of methanol extract of AK in rats on the biochemical changes induced by two hepatotoxins, D-galactosamine (GalN) and carbon tetrachloride (CCl_4). AK was orally administered once daily for 7 days to male rats at 200 and 500 mg/kg, before hepatotoxins. Effects of AK were assessed 24 hr later. AK pretreatments at 200 and 500 mg/kg significantly blunted GalN-induced elevation in liver lipid peroxidation, plasma aspartate-transaminase (AST) and alanine-transaminase (ALT) activities. AK also prevented, after 500 mg/kg but not after 200 mg/kg, the GalN-induced elevation in triglyceride, total cholesterol and LDL-cholesterol levels. Differently from against GalN-induced toxicity, AK did further elevate the CCl_4-induced rise in AST, ALT and lipid peroxidation. These results suggest that AK, when pre-administered prior to GalN, exerted protective effects against GalN-induced hepatotoxicity, in contrast however, AK exacerbated that induced by CCl_4. To explore possible mechanism for the toxicity-potentiating effects of AK on CCl_4, the activity of hepatic drug metabolism after AK treatment was assessed. It was observed that AK increased the activity of aniline hydroxaylase, a cytochrome P450 isoenzyme responsible for metabolic activation of CCl_4. This finding suggests that hepatoprotective effects of AK are not equally expected depending on hepatotoxins employed.

Key words: *Angelica keiskei*, hepatotoxicity protection, GalN, CCl_4, rats

Introduction

The vegetable food *Angelica keiskei* (AK, Sin-sun-cho in Korean) has widely been utilized among Korean population for several decades with the belief in various beneficial pharmacological actions. The proposed important effects include amelioration of lipid profiles[1,2], protection against hepatotoxicity[3,4], and reduction of smoking-related stresses[5], among others.

Numerous nutritionally important substances such as flavonoids, coumarins, saponins, and vitamins have been identified to be present in AK[6,7,8]. Although little is known about other pharmacologically active principles of AK mediating beneficial activities, several potent antioxidant flavonoids such as quercetin, isoquercitrin, hyperoside and cynaroside have been proposed[1,4,8]. In a previous study by our group[1], it was demonstrated that administration AK-containing diets in normal rats led to amelioration of plasma lipid profiles. In this study[1], quercetin and isoquercitrin were detectable in both serum and liver when rats were fed on AK-containing diets. As antioxidant principles were detected accompanying the lipid profile changes, such beneficial actions could be ascribed to AK-containing flavonoids.

Antioxidant flavonoids are ubiquitously present in most vegetable foods and fruits[9]. Different flavonoids have been believed to exert preventive action against cardiovascular diseases, inflammation and cancer in humans[10]. More than 5,000 different flavonoids have been identified so far, and tea plants, apples and onions in particular are known to contain rich amount of flavonoids[11].

In studies performed with laboratory animals, it has been reported that AK is effective in reducing hepatotoxicity induced by bromobenzene[4] or carbon tetrachloride[3], both known to be metabolized to free radicals beforehand for induction of hepatotoxicity. As the responsible principle for these protection, a few flavonoids have been identified to date, and more are to be identified in future studies. On the other hand, flavonoids can induce the activities of cytochrome P450 enzyme systems[12]. In light of these facts, it would be highly difficult to predict whether we will achieve protective effects or not, or

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toxicity-enhancing effects in worst cases, if a medicinal preparation contains more than one flavonoid. Furthermore, while some flavonoids are antioxidative, however, others are prooxidative as well depending on the constituents of flavonoids involved[13,14]. Herbal preparations like AK is not likely to contain consistent compositions in active principles depending on the preparation methods, harvest seasons and plant parts used. Then it will not be uniform in AK's action as a hepatoprotective substance against hepatotoxins of different modes of action.

In this study, it was examined whether administration of AK methanol extract can prevent the hepatotoxicity in rats caused by two hepatotoxins, GalN and CCl_4, of different action modes. While metabolic activation of CCl_4 to a free radical[15] is a prerequisite for its toxicity, GalN acts directly without metabolic transformation[16,17,18].

Materials and Methods

Test substance and reagents

Dryed powdered aerial parts of AK was purchased from a herbal market in Gunsan City and extracted with 10-fold volume of 100% methanol for 24 hr. After filtration with gauze, the extraction solvent was completely removed using a rotary evaporator at 60°C. With this extraction method, semi-solid test substance was obtained at a yield of about 9.2%. AK extract was suspended in 0.5% carboxymethylcellulose (CMC) for suspension and oral administration to rats. Reagents used for analysis of AST and ALT were obtained from Asan Pharmaceutical (Seoul, Korea). Analytical kits for triglyceride, total cholesterol, low and high-density lipoproteins were purchased from Sigma (St. Louis, MO, USA). All other reagents were obtained from Sigma, if not specified otherwise.

Animal treatments

Specific pathogen-free Sprague-Dawley male rats (6 week old) were purchased from Damul Science (Daejon, Korea) and acclimated for one week in the laboratory. The animals were maintained in air-filtered rodent cabinets maintained at a temperature of 23 ± 1°C and relative humidity of 55 ± 5%. Animal cabinets were illuminated with 12 h light/dark cycle. Rats were supplied *ad libitum* with commercial rodent chow (CJ Incorporate, Seoul, Korea) and tap water. AK or vehicle control CMC was force fed with an oral feeding needle for 7 days daily before GalN or CCl_4 administration. Both GalN (1 g/kg dose, dissolved in saline) and CCl_4 (1 ml/kg dose, dissolved in corn oil) were injected into the peritoneal cavity at 1 ml/kg volume on the final day of AK administration. Tissue and plasma samples for analyses were taken 24 hr after GalN or CCl_4. To control animals sterile saline and corn or were injected respectively instead of GalN and CCl_4.

Sample preparations

Rats were fast overnight before sacrifice. They were lightly anesthetized with ether and required volume of blood was taken from the abdominal aortae using disposable syringes treated with heparin. After maximal exsanguination, liver tissues were excised. Plasma was separated by centrifugation for 30 min at 3,600 × g and 3°C, and was used for the level measurements of triglyceride, cholesterol, low-density lipoprotein, high-density lipoprotein, glutamate-oxaloacetate transaminase (AST), and glutamate-pyruvate transaminase (ALT). Obtained plasma and liver samples were kept at −70°C until analysis.

Biochemical analysis

Plasma was used for lipid contents and transaminase activity. Analysis of triglyceride, total cholesterol, low-density lipoprotein-cholesterol LDL-C), high-density lipoprotein chloesterol (HDL-C) was performed with commercial kits from Sigma. Very-low density lipoprotein-cholesterol (VLDL-C) concentrations were calculated by subtracting LDL and HDL levels from that of total cholesterol. Aspartate-transaminase (AST) and alanine-transaminase (ALT) levels were measured according to Reitman and Frankel[19].

Liver tissues were homogenized in 9 volumes of saline for lipid peroxide level. Lipid peroxide (LPO) levels were determined using thiobarbituric acid method of Okhawa et al.[20]. An aliquot of liver tissue was homogenized in 10 volumes of potassium phosphate buffer (100 mM, pH 7.4) for aniline hydroxylase activity. The homogenate was centrifuged at 9,000 × g for 30 min, then the supernatant was obtained and adjusted to contain protein at 10 mg protein/ml with the phosphate buffer. The assay mixture was composed of 1.0 ml potassium phosphate buffer (100 mM, pH 7.4) containing 2 mM aniline, 0.1 mM EDTA, the liver supernatant, and an NADPH generating system (0.33 mM NADP, 8 mM glucose-6-phosphate, 0.1 unit glucose-6-phosphate dehydrogenase, 6 mM $MgCl_2$, preincubated for 5 min at 37°C before addition). Enzyme reaction was started by adding the NADPH generating system to the mixture and continued for 30 min. After the reaction, p-aminophenol formed by the enzyme reaction was extracted according to the ether extraction method[21] and quantified with a spectrophotometer at 610 nm. Protein contents were determined in accordance with Lowry et al.[22] using bovine serum albumin as the standard.

Statistical analysis

Data were expressed as mean ± S.D. Statistical significance was tested using one-way analysis of variance followed by Duncan's multiple range test. Significance level was set as p-

values < 0.05.

Results and Discussion

It was explored whether AK methanol extract can exert protective effects against hepatotoxicity induced by chemical hepatotoxins that involve oxidative stress as a common mechanism in their toxicity. As a result, contrasting results were obtained: while AK extract protected GalN-induced toxicity the same test substance aggravated that induced by CCl_4.

Biochemical and lipid profile parameters were examined after 1-week AK pre-treatment and acute GalN intoxication. Hepatotoxic action of GalN and the influence of AK extract pre-treatment are illustrated in Fig. 1. Single injection of GalN at 1,000 mg/kg induced significant increase in hepatic lipid peroxide levels, and plasma AST and ALT enzymes. It has been proposed that production of reactive oxygen intermediate are responsible at least in part for GalN-iduced liver injury[23,25,26]. Thus excessive lipid peroxidation of hepatic tissues is well documented after GalN intoxication[26,27]. The affected hepatic cells eventually lose plasma membrane integrity[17,28] leading to the leakage of hepatic transaminase enzymes[29].

The elevation in hepatic lipid peroxide and plasma transaminase activity were equally blunted by pre-treatment of AK extract, with the protection by two tested dose levels were dose-dependent (Fig. 1). AK is known to contain several antioxidant molecules including flavonoids[1,30,31,32]. The herb extract is likely to protect the injury derived from GalN intoxication via preventive actions of such antioxidant. Thus the rats fed AK extract seemed to be less liable to peroxide elevation caused by GalN. These results also suggest that all different antioxidant principle(s) in AK, e.g., flavonoids, vitamin A, vitamin C or β-carotene[33,34] could have collectively played a role to intervent GalN-induced oxidative toxicity.

The liver is the major organ for the synthesis and metabolism of cholesterol, bile acids and phopholipids[35]. Thus the blood lipid profiles can be perturbed by GalN administration and the phenomenon in typically evidenced in the rise of triglyceride, total cholesterol and LDL-cholesterol[36,37]. Such changes are the secondary consequence of excess free radical production that leads to destructive degradation of membrane lipid bio-molecules[38]. Fig. 2 demonstrates the effects of AK on GalN-induced plasma lipid profile changes. AK in a dose-dependent manner, prevented elevations in concentrations of triglyceride, total cholesterol (T-Chol) and low-density lipoprotein-cholesterol (LDL). In high-density lipoprotein-cholesterol (HDL), although statistical significance was not achieved, there was a tendency of reduction by GalN and its prevention by AK.

The effects of AK on the lipid profile change indirectly indicate protective action of anti-oxidants present in the AK

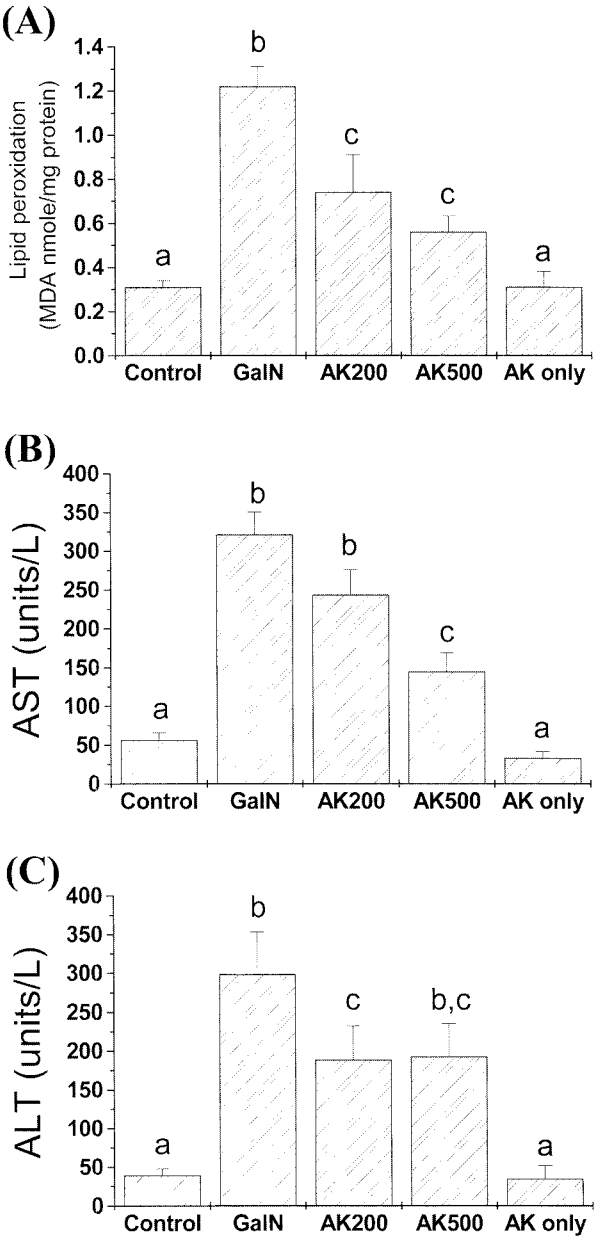

Fig. 1. Effects of *Angelica keiskei* (AK) on GalN-induced hepatotoxicity, (A) lipid peroxidation of liver tissues (B) plasma AST level (C) plasma ALT level, N = 10, Different alphabets denote statistically different values among groups ($p < 0.05$). GalN, D-galactosamine 1 g/kg ip alone; AK 200, AK 200 mg/kg/day + D-galactosamine 1 g/kg ip; AK 500, AK 500 mg/kg/day + D-galactosamine 1 g/kg ip.

extract. It was demonstrated that AK feeding to normal rats per se lowered blood levels of triglyceride, T-Chol and LDL-cholesterol, while increasing HDL-cholesterol[1,2]. Thus the ameliorating effects of AK on these parameters in GalN-intoxicated rats can be a sum of two distinct actions: lipid level changes by AK itself and protective effects against GalN-induced toxic changes.

CCl_4 has long been utilized as one of the standard chemical

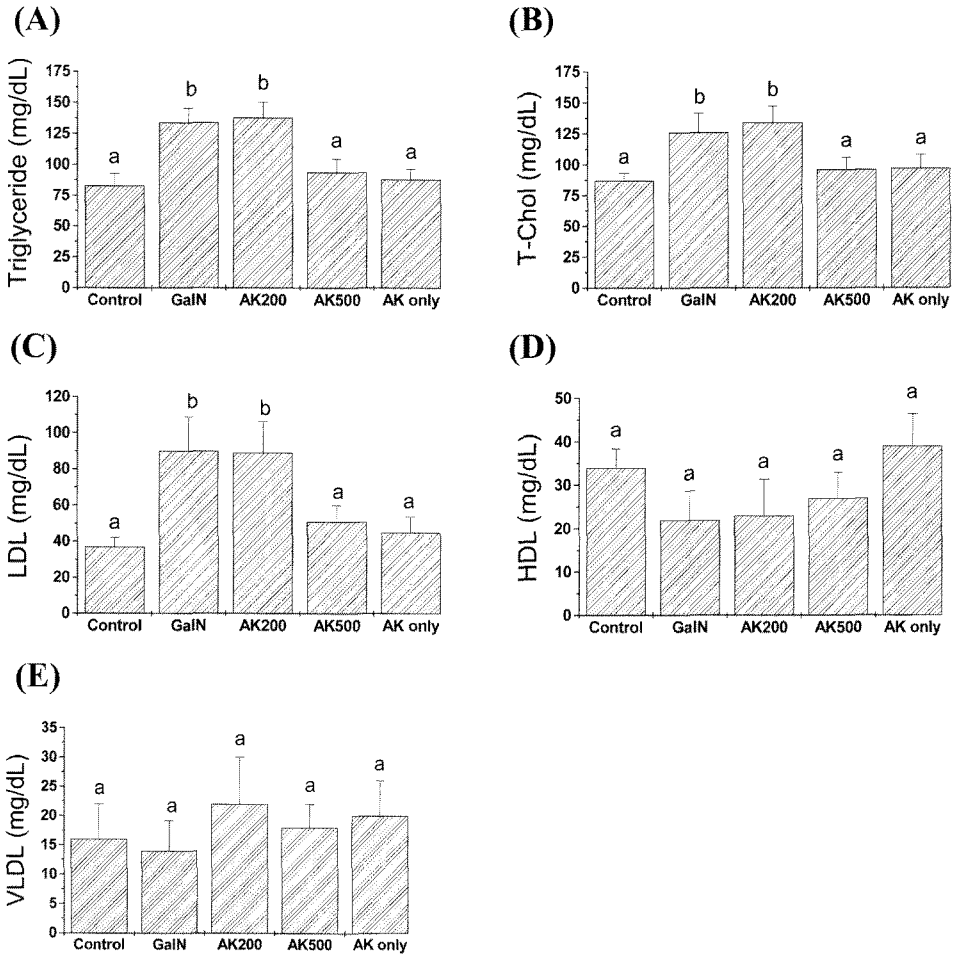

Fig. 2. Effects of *Angelica keiskei* (AK) on GalN-induced plasma lipid profile changes, (A) triglyceride (B) total cholesterol (C) LDL (D) HDL (E) VLDL, Different alphabets denote statistically different values among groups ($p < 0.05$). N = 10, GalN, D-galactosamine 1 g/kg ip alone; AK 200, AK 200 mg/kg/day po + D-galactosamine 1 g/kg ip; AK 500, AK 500 mg/kg/day po + D-galactosamine 1 g/kg ip.

hepatotoxins. The metabolism of CCl_4 to thrichloromethyl ($\cdot CCl_3$) and peroxy trichloromethyl ($\cdot OOCCl_3$) free radicals is known to cause an acute liver damage involving cirrhosis, steasis and necrosis[39]. Free radicals formed strongly bind with highly unsaturated fatty acids forming alkoxy (R·) and peroxy radicals (ROO·) that can generate lipid peroxides. The free radicals then, in the presence of oxygen, lead to peroxidation of lipids and causes functional and morphological damage to the cell membrane[40]. Prominent elevation in serum transaminase levels and hepatic lipid peroxidations are observed after CCl_4[41,42,43].

Fig. 3 shows effects of AK on CCl_4-induced hepatotoxicity. As expected CCl_4 administration at 1 ml/kg induced marked increase in levels of hepatic lipid peroxide (Fig. 3A) and two plasma transaminses (Figs. 3B and 3C). In contrast to the expectation made on the basis of protection against GalN toxicity, AK failed to prevent these elevations. Surprisingly enough, AK further raised CCl_4-induced elevation in lipid peroxidation and transaminase levels, indicating potentiation of toxicity rather than prevention. AK itself did not influence these parameters.

It is fairly well documented that the formation of CCl_4-derived reactive free radicals are catalyzed by cytochrome P450 enzymes[44,45]. The reaction is carried out mainly specifically by CYP2E1, a subclass of cytochrome P450[46]. One can expect that anti-oxdant-containing AK will prevent hepatoxic events of CCl_4 if the toxic mechanism should involve free radical production. However because pretreament with AK simply aggravated CCl_4-induced toxicity, additional pharmacological mechanisms must be considered ascribable to AK extract.

As the possible reason for the unexpected failure to prevent CCl_4 toxicity, one would naturally suspect possible induction of dug-metabolizing enzymes by AK following repeated administration. It was observed that aniline hydroxylase activity, a specific indicator of CPY2E1 activity, was elevated in a dose-dependent manner (Fig. 4). Thus the potentiation of CCl_4-hepatotoxicity by AK is likely to be a result of cytochrome

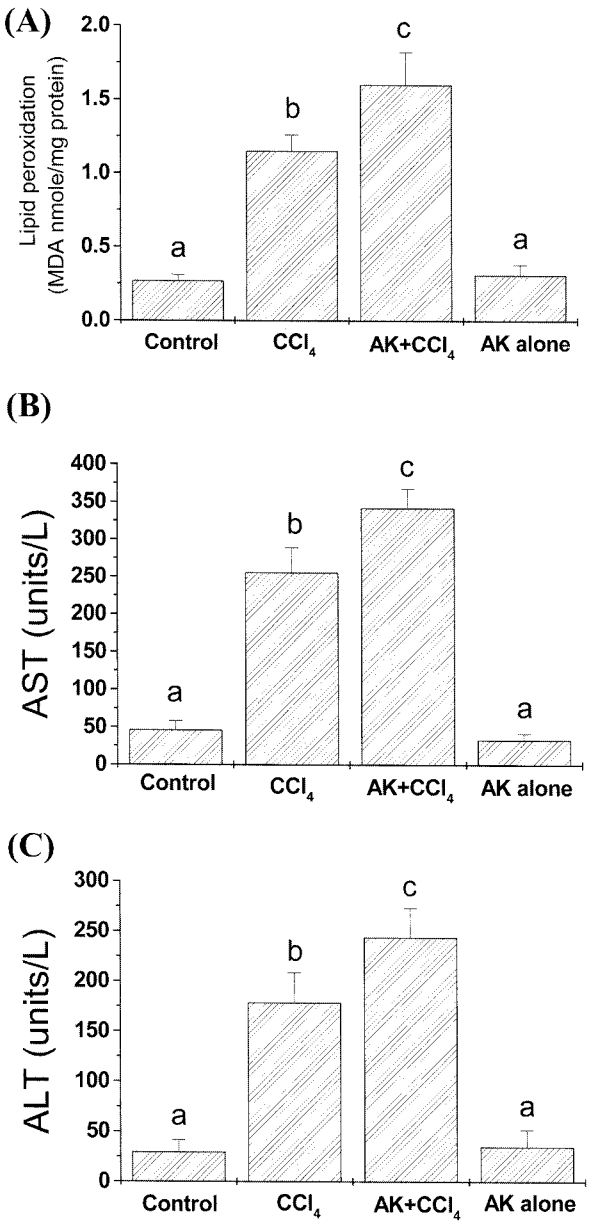

Fig. 3. Effects of *Angelica keiskei* (AK) on CCl_4-induced hepatotoxicity, (A) lipid peroxidation of liver tissues (B) plasma AST level (C) plasma, ALT level, Different alphabets denote statistically different values among groups ($p < 0.05$). N = 10, CCl_4, CCl_4 1 ml/kg ip alone; AK + CCl_4, AK 500 mg/kg/day po + CCl_4 1 ml/kg ip; AK alone, AK 500 mg/kg/day po.

P450 induction by AK pretreament. Although it is not clear yet whether enzyme induction is the most important single mechanism, it may explain at least in part the AK potentiation of CCl_4-induced hepatotoxicity. Some of the major active principles of AK reported up to now include flavonoids, and flavonoids can induce cytochrome P450[12]. There are several studies that report increases in CYP2E1 activities in particular after flavonoid administration in rats[47,48]. Carbon tetrachloride (CCl_4) induces liver injury initially via lipid peroxidation

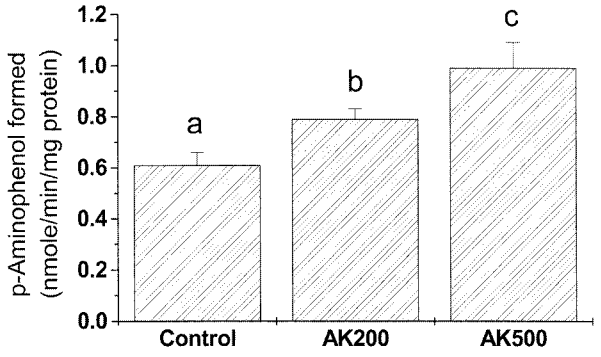

Fig. 4. Effects of *Angelica keiskei* (AK) on liver aniline hydroxylase activity, Different alphabets denote statistically different values among groups ($p < 0.05$). N = 10, AK 200, AK 200 mg/kg/day po p; AK 500, AK 500 mg/kg/day po.

after production of CCl_4-derived free radicals[16]. For this, CCl_4 should be first metabolically activated by liver microsomal NADPH-cytochrome systems to trichloromethy free radical (CCl_3). D-galactosamine (GalN) is a unique hepatotoxin that induces hepatitis-like liver injury[16]. Involvement of oxidative stress is one of the mechanisms for GalN hepatotoxicity[23], although This chemical reduces the pool of uracil nucleotides in hepatocytes thus leading to diminished RNA and protein synthesis[17,23].

In contrast to the present observation with CCl_4, Jung et al.[3] found protective effects of AK against CCl_4 hepatotoxicity in rats. Test substance used in their study was AK aqueous juice, while methanol extract was utilized in this study. The discrepancy in AK's protective efficacy may derive from the fact that methanol extraction could have concentrated certain principles that are active in P-450 enzyme induction, eg., variuos flavonoids. In this case the flavonoids could or could not be identical to those presumably exert hepato-protection against GalN.

Hepato-preventive effects of AK methanol extract were examined in rats against the toxic injuries induced by GalN and CCl_4 that possess free radical producing activity as a common hepatotoxic mechanism. It was expected that AK could prevent hepatic injuries caused by both hepatotoxins because it has well been demonstrated that AK contains antioxidant substances. Interestingly, discrete results were obtained: while AK prevented GalN-induced toxicity, the same test material failed to ameliorate that of CCl_4-induced one. These results imply that AK contains hepato-protective substances(s), but its beneficial role can not always be guaranteed depending upon the toxicity-causing agents.

요 약

아직 분명히 규명되지 않은 부분이 많지만, 신선초가 다양한 측면에서 건강증진 효과가 있다고 믿어져 왔고 따라

서 일반인들이 지금까지 사용해 오고 있다. 이 연구에서는 신선초의 메타놀 추출물을 투여한 후, 전형적인 간독성 유발물질인 갈락토사민과 사염화탄소를 투여한 랫드에서 일반적 간독성지표와 지질대사능 지표를 측정함으로써 간장보호작용이 있는지 시험하였다. 신선초를 독성유발전 7일간 매일 1회씩 200 및 500 mg/kg의 용량으로 경구투여 하고, 간독성물질을 복강내로 투여 한 후 독성유발 24시간에 혈장과 간장조직에 대한 분석을 수행하였다. 시험한 두 용량(200 및 500 mg/kg)은 갈락토사민에 의해 유발된 지질과산화물 증가, 혈장 AST 및 ALT 활성의 증가를 감소시켰다. 또한 신선초 500 mg/kg은 갈락토사민이 유발한 혈장 중성지질, 총 콜레스테롤 및 저밀도지단백 콜레스테롤의 농도 증가현상을 감소시켰다. 갈락토사민에 의해 유발된 독성에 대한 효과와는 달리, 사염화탄소로 유발된 AST, ALT 및 과산화지질의 증가현상이 신선초 전투여에 의해 더욱 증가하였다. 이 결과는 갈락토사민에 의한 독성을 신선초 전투여가 감소시킬 수 있지만, 반대로 사염화탄소 유발 간독성은 더 악화시킴을 의미한다. 신선초의 사염화탄소 유발 간독성 증강효과에 대한 기전을 이해하기 위해 신선초를 투여한 랫드의 간장내 aninline hydroxylase의 활성을 측정하였다. 그 결과 사염화탄소가 간독성을 발휘하는 데에 필요한 조건인 대사체로의 변환을 매개하는 이 효소의 간장내 활성이 증가함을 관찰하였다. 종합적으로 신선초가 간장보호효과를 발휘하지만 간독성이 어떤 독소에 의해 유발되었느냐에 따라 항상 보효효과가 있지는 않음을 의미한다.

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