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Effects of Methanol Extracts of *Rubus Coreanus* Miquel and *Atractylodes Japonica* Koidzumi on Hepatic Toxicity and Immunomodulating Activity in Mice*

Hye-Young Sung, Young-Sun Choi§, Sung-Hee Cho[1], Hyang-Rim Lee[1] and Sang-Won Choi[1]

Department of Food and Nutrition, Daegu University, Gyeongsan, Gyeongbuk 712-714, Korea

[1]Department of Food Science and Nutrition, Catholic University of Daegu, Gyeongsan, Gyeongbuk 712-702, Korea

This study was aimed at investigating hepatic toxicity and immunomodulating effects of defatted methanol extracts of two kinds of medicinal plants, *Rubus coreanus* Miq. and *Atractylodes japonica* Koidz. in mice. Defatted methanol extracts of fruits of *Rubus coreanus* Miq. and rhizome of *Atractylodes japonica* Koidz. were added at the level of 0.5% or 5%(w/w) to cholesterol-supplemented AIN-76 diet. Each diet was fed to 8 ICR male mice for 30 days. Weight gain and food efficiency ratio of the mice fed 5.0% extract of *Rubus coreanus* Miq. were significantly lower than those of the mice fed 0.5% extract. Relative liver weight and activity of plasma alanine aminotransferase were significantly increased only in the mice fed 5% extract of *Atractylodes japonica* Koidz. compared with the others. Splenocyte proliferation was not significantly different between the groups fed 0.5% or 5.0% extract of *Rubus coreanus* Miq. However, splenocyte proliferation was significantly decreased in the mice fed 5.0% extract of *Atractylodes japonica* Koidz. compared with that in the mice fed 0.5%. Production of interleukin-2 by splenocytes from the mice fed 0.5% extract of *Atractylodes japonica* Miq. was significantly higher than the control value and it became lower with 5.0% dietary level. Secretion of interferon-γ was not significantly different among groups. In conclusion, the defatted methanol extract of *Atractylodes japonica* Koidz. was likely to exert immunomodulating effect at the level of 0.5%, but it may exert adverse effects on immune and liver functions at the level of 5.0%.

Key words: *Rubus coreanus* Miq., *Atractylodes japonica* Koidz., Splenocyte proliferation, Cytokine, Hepatic toxicity

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INTRODUCTION

Rubus coreanus Miq. (Rosaceae, Rubi Fructus) is a type of red raspberry and grows wild in Korea and China. Its unripe fruits, well known as 'Bokbunja' in Korea, have been used as a folk medicine. This drug is used for the management of impotence. The fruits are rich in sugars, organic acids and several vitamins, and also include various antioxidants,[1)] terpenoids,[2,3)] tannins[4)] and phenolic acids.[5)] Extracts showed considerable antioxidant activity in various test systems regardless of ripeness *in vitro*.[6)] Both water and ethanol extracts inhibited the growth of various types of cancer cells and stimulated T and B cell activities.[7)]

The rhizome of *Atractylodes japonica* Koidz. has been used in oriental medicine as a diuretic and stomachic. Atractylon, a major component and its derivatives isolated from rhizome were shown to have antihepatic effects.[8)] Sesquiterpenoids, diacetyl atractylodiol and its derivatives were isolated from the non-polar fraction.[9)] Active polysaccharides from *Atractylodes lancea* DC, which is similar to *Atractylodes japonica* Koidz. showed proliferative effects on bone marrow cells, and active polysaccharide seemed to be a modulating compound of the intestinal immune system.[10)]

Most of these activities of *Rubus coreanus* Miq. and *Atractylodes japonica* Koidz. was tested in the *in vitro* assay system. According to Cho *et al.*,[11)] the extracts of *Rubus coreanus* Miq. and *Atractylodes japonica* Koidz. were hypotriglyceridemic at the level of 0.1 and 0.5% of diets in ovariectomized rats. They reported that feeding 2% extract of *Atractylodes japonica* Koidz. increased serum cholesterol level and alanine aminotransferase (ALT) activity and revealed a hepatotoxic effect.

Therefore, we investigated in this study the effects of methanol extracts of *Rubus coreanus* Miq. and *Atractylodes japonica* Koidz. on hepatic toxicity and immunomodulating

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§ To whom correspondence should be addressed.
(E-mail : yschoi@daegu.ac.kr)

activity in mice to evaluate not only their safety but also effects on immune function. Specifically, a low level (0.5%) and a high level (5.0%) of defatted methanol extracts of *Rubus coreanus* Miq. and *Atractylodes japonica* Koidz., respectively, were fed to mice, and hepatic toxicity and mitogen-induced splenocyte proliferation and production of interleukin (IL)-2 and interferon (IFN)-γ by T cells were examined.

MATERIALS AND METHODS

1. Preparation of Methanol Extracts

Methanol extracts of *Rubus coreanus* Miq. (RC) and *Atractylodes japonica* Koidz. (AJ) were prepared by the procedure as described by Cho *et al.*[11] One hundred grams of ground dry fruits of *Rubus coreanus* Miq. and rhizome of *Atractylodes japonica* Koidz. each were extracted three times with 1 L of 80% aqueous methanol at room temperature. Extracts were filtered and concentrated by evaporating under reduced pressure. The concentrates were redissolved with 100 mL of 80% aq. methanol and washed twice with 200 mL of n-hexane to remove lipids. Yields of the defatted methanol extracts from RC and AJ were 8.9% and 10.2%, respectively. These defatted extracts were added at the level of either 0.5% or 5.0% to experimental diets.

2. Animals and Diets

Male ICR-mice, approximately five-weeks old, were kept on a pellet diet for a week and were assigned to one of 5 groups. The mice were kept individually in wire-mesh cages in a room maintained at 20±2 ℃ and 50±5% relative humidity. Experimental diets as shown in Table 1 included AIN-76 based control diet,[12] 0.5% (0.5RC) and 5.0% (5.0RC) extract of *Rubus coreanus* Miq., and 0.5% (0.5AJ) and 5.0% (5.0AJ) extract of *Atractylodes japonica* Koidz. The mice were fed experimental diets ad-libitum for 30 days. These experiments were performed according to the guidelines of animal experimentation approved by Daegu University.

Table 1. Composition of experimental diets(g/kg)

Ingredients	Control	0.5RC	5.0RC	0.5AJ	5.0AJ
Casein	160	160	160	160	160
Corn starch	440	435	390	435	390
Sucrose	150	150	150	150	150
Lard	70	70	70	70	70
Soybean oil	75	75	75	75	75
AIN mineral mix[1)]	40	40	40	40	40
AIN vitamin mix[2)]	10	10	10	10	10
Cholesterol	5	5	5	5	5
α-Cellulose	50	50	50	50	50
Rubus coreanus Miq.	0	5	50	0	0
Atractylodes japonica Ext.	0	0	0	5	50

Control: basal cholesterol diet (AIN76+0.5% cholesterol)
0.5RC: 0.5% of methanol extract *Rubus coreanus* Miq.+basal cholesterol diet
5.0RC: 5.0% of methanol extract *Rubus coreanus* Miq.+basal cholesterol diet
0.5AJ: 0.5% of methanol extract *Atractylodes japonica* Koidz.+basal cholesterol diet
5.0AJ: 5.0% of methanol extract *Atractylodes japonica* Koidz.+basal cholesterol diet
[1)] AIN-93G mineral mix (Harlan Teklad, USA)
[2)] AIN-93G vitamin mix (Harlan Teklad, USA)

3. Splenocyte Proliferation Assay

Splenocyte proliferation induced by mitogens, lipopolysaccharide (LPS) and concanavalin A (Con A) were carried out using MTT (3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma Co., USA) assay. Splenocytes were diluted to a concentration of 5×10^6 cells/mL and 100 μL of cell suspension was dispensed in 96-well microtiter plate (NUNC, Denmark). Then 100 μL of EMEM containing LPS (10 μg/mL, Sigma Co.) or Con A (10 μg/mL, Sigma Co.) was added to each well and incubated for 72 h at 37 ℃, 5% CO_2 and 95% air. Medium was removed and 20 μL MTT solution (5 mg/mL PBS buffer) was dispensed in each well, and cells were incubated for 4 h. Medium was separated and 100 μL of 0.04 N HCl/isopropanol was added and mixed. Absorbance was measured at 540 nm. Ratio of absorbance with mitogen to absorbance without mitogen was designated stimulation index (SI) of splenocyte proliferation.

4. Determination of Cytokines by ELISA

The culture supernatants used for the assay of IL-2 and IFN-γ were collected after 72 h incubation of splenocytes with Con A. IL-2 and IFN-γ were determined using murine IL-2 ELISA kit (Amersham Biosciences, UK) and IFN-γ ELISA kit (Pierce, USA) by sandwich method, respectively.

5. Statistical Analysis

The results were expressed as means±standard error of the mean (SEM), and the SPSS release 11.0 software package was used for the statistical analyses. Differences among groups that were significant at $P<0.05$ using one-way ANOVA were tested by Duncan's multiple range tests at $P<0.05$. Difference of means between the groups fed 0.5% or 5.0% of each extract was tested by student's t-test.

RESULTS

1. Weight Gain and Food Efficiency Ratio

Although food intake was not significantly different among the groups, weight gain and food efficiency ratio were lower in 5.0RC group compared with 0.5RC group,

Table 2. Weight gains, food intakes and food efficiency ratios of groups.

Group	Initial weight (g)	Final weight (g)	Weight gain (g/day)	Food intake (g/day)	FER (g/g)
Control	27.47±0.484^{NS}	34.21±1.068^{ab}	0.240±0.046^{ab}	3.933±0.190^{NS}	0.061±0.011^{ab}
0.5RC	27.63±0.547	36.05±1.314^{a}	0.301±0.049^{a}	3.911±0.205	0.075±0.011^{a}
5.0RC	27.73±0.507	31.87±1.617^{b}	0.148±0.061^{b}	3.712±0.334	0.037±0.017^{b}
0.5AJ	28.11±0.405	35.66±0.697^{a}	0.256±0.026^{ab}	4.316±0.202	0.060±0.006^{ab}
5.0AJ	27.69±0.412	32.85±1.192^{ab}	0.184±0.036^{ab}	3.998±0.342	0.043±0.008^{ab}

Control: basal cholesterol diet (AIN76+0.5% cholesterol), 0.5RC: 0.5% of methanol extract *Rubus coreanus* Miq., 5.0RC: 5.0% of methanol extract *Rubus coreanus* Miq., 0.5AJ: 0.5% of methanol extract *Atractylodes japonica* Koidz., 5.0AJ: 5.0% of methanol extract *Atractylodes japonica* Koidz.
Values are mean±SEM (n=8), and those in the same column not sharing common superscript letters are significantly different at $P<0.05$ by Duncan's test.
NS: not significant

whereas those were not significantly different between 5.0AJ group and 0.5AJ group (Table 2).

2. Organ Weights, and Serum ALT and AST Changes

Relative weights of spleen and thymus shown in Table 3 were not significantly different among groups. Spleen and thymus are major organs of the immune system. In mammals, T cells mature in the thymus and B cells mature in the bone marrow. Spleen specializes in filtering blood and trapping blood-borne antigens; thus, it can respond to systemic infections. The initial activation of B and T cells takes place in the T cell-rich periarteriolar lymphoid sheath of spleen.

Relative liver weight of mice fed 5.0% extract of *Atractylodes japonica* Koidz. was significantly higher than those of the other groups. Plasma alanine aminotransferase (ALT) activity shown in Table 4 was significantly elevated in the mice fed 5.0% extract of *Atractylodes japonica* Koidz. compared with the mice fed 0.5% extract of *Atractylodes japonica* Koidz. and the mice fed extract of *Rubus coreanus* Miq. Considering that serum ALT activity is the circulating marker of hepatocyte injury, the 5% dietary level of *Atractylodes japonica* seemed toxic to liver. Increased liver weight of 5.0AJ group shown in Table 3 may have been associated with hepatic injury. ALT activity was not significantly different between the groups fed 0.5% or 5.0% extract of *Rubus coreanus* Miq. Rather plasma ALT activity in mice fed 5% extract of *Rubus coreanus* Miq. tended to be lower compared with the control mice. Plasma aspartate aminotransferase (AST) activity was not significantly different among the groups.

Table 3. Relative weights of spleen, thymus and liver of experimental groups. (g/100 g B.W.)

Group	Spleen	Thymus	Liver
Control	0.206±0.019^{NS}	0.138±0.019^{NS}	5.036±0.208^{b}
0.5RC	0.226±0.014	0.157±0.016	4.876±0.186^{b}
5.0RC	0.187±0.014	0.177±0.023	4.779±0.586^{b}
0.5AJ	0.215±0.013	0.157±0.011	5.014±0.212^{b}
5.0AJ	0.219±0.015	0.144±0.015	5.993±0.381^{a}

Control: basal cholesterol diet (AIN76+0.5% cholesterol), 0.5RC: 0.5% of methanol extract *Rubus coreanus* Miq., 5.0RC: 5.0% of methanol extract *Rubus coreanus* Miq., 0.5AJ: 0.5% of methanol extract *Atractylodes japonica* Koidz., 5.0AJ: 5.0% of methanol extract *Atractylodes japonica* Koidz.
Values are mean±SEM (n=8), and those in the same column not sharing common superscript letters are significantly different at $P<0.05$ by Duncan's test.
^{NS}not significant

Table 4. Plasma ALT and AST activities in mice. (Karman)

Group	ALT	AST
Control	125.66± 8.63^{ab}	252.20±37.86^{NS}
0.5RC	119.42±16.04^{b}	290.56±27.88
5.0RC	96.36± 7.18^{b}	269.10±22.92
0.5AJ	119.74±11.05^{b}	314.37±40.67
5.0AJ	170.71±31.40^{a}	334.43±33.33

Control: basal cholesterol diet (AIN76+0.5% cholesterol), 0.5RC: 0.5% of methanol extract *Rubus coreanus* Miq., 5.0RC: 5.0% of methanol extract *Rubus coreanus* Miq., 0.5AJ: 0.5% of methanol extract *Atractylodes japonica* Koidz., 5.0AJ: 5.0% of methanol extract *Atractylodes japonica* Koidz.
ALT: alanine aminotransferase, AST: aspartate aminotransferase
Values are mean±SEM (n=8), and those in the same column not sharing common superscript letters are significantly different at $P<0.05$ by Duncan's test.
NS: not significant

3. Effect on Splenocyte Proliferation

Con A- or LPS -induced splenocyte proliferation in the mice fed 5.0% of *Rubus coreanus* Miq. and 0.5% *Atractylodes japonica* Koidz. tended to be increased compared with the control mice (Fig. 1). Splenocyte proliferation induced by Con A was significantly lower in the mice fed 5.0% extract

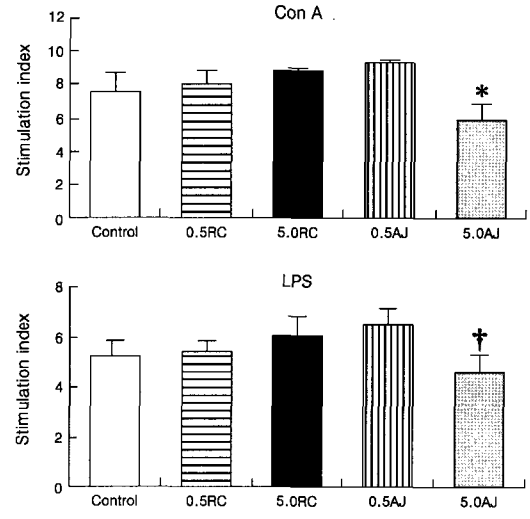

Fig. 1. Effects of experimental diets on LPS- or Con A- induced stimulation index of splenocytes

Control: basal cholesterol diet (AIN76+0.5% cholesterol), 0.5RC: 0.5% of methanol extract *Rubus coreanus* Miq., 5.0RC: 5.0% of methanol extract *Rubus coreanus* Miq., 0.5AJ: 0.5% of methanol extract *Atractylodes japonica* Koidz., 5.0AJ: 5.0% of methanol extract *Atractylodes japonica* Koidz.
Con A: concanavalin A, LPS: lipopolysaccharide
Values are means with SEM bar (n=8).
* $P<0.05$ compared to 0.5AJ group by t-test,
† $P<0.10$ compared to 0.5AJ group by t-test.

of *Atractylodes japonica* Koidz. than in the mice fed 0.5% extract of *Atractylodes japonica* Koidz. (P=0.044). Splenocyte proliferation induced by LPS was also lower in the group fed 5.0% extract of *Atractylodes japonica* Koidz. than in the group fed 0.5% of *Atractylodes japonica* Koidz. (P=0.051). This result implies that dietary level of 5.0% extract of *Atractylodes japonica* Koidz. could be toxic to lymphocytes. On the other hand, this dose-dependent effect by *Atractylodes japonica* Koidz. was not observed with *Rubus coreanus* Miq.

4. Cytokine Production in Splenocytes

As shown in Fig. 2, Con A-induced IL-2 secretion into culture medium was increased 86.2% with addition of 0.5% extract of *Atractylodes japonica* Koidz. in diet as compared with the control, but tended to be decreased with 5.0% extract. Feeding 0.5% extract of *Rubus coreanus* Miq. did not affect IL-2 production by Con A-induced splenocytes. Secretion of interferon-γ was not significantly different among the groups. The result indicates that feeding 0.5% extract of *Atractylodes japonica* Koidz. in diet stimulated cell-mediated immune response such as secretion of IL-2 as well as splenocyte proliferation.

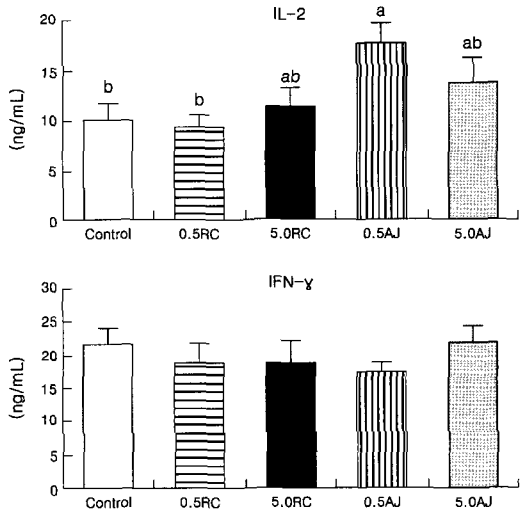

Fig. 2. Production on IL-2 and IFN-γ of splenocytes induced by Con A

Control: basal cholesterol diet (AIN76+0.5% cholesterol), 0.5RC: 0.5% of methanol extract *Rubus coreanus* Miq., 5.0RC: 5.0% of methanol extract *Rubus coreanus* Miq., 0.5AJ: 0.5% of methanol extract *Atractylodes japonica* Koidz., 5.0AJ: 5.0% of methanol extract *Atractylodes japonica* Koidz.
Values are means with SEM bar (n=8), and those not sharing common superscript letters are significantly different at $P<0.05$.

DISCUSSION

The 5.0% extract of *Rubus coreanus* Miq. in diet lowered food efficiency ratio compared with 0.5% (0.037 vs 0.075 g/100 g B.W.). The 5.0% extract of *Atractylodes japonica* Koidz. in diet tended to lower food efficiency ratio compared with 0.5% (0.043 vs 0.060 g/100 g B.W). Cho *et al.*[11] observed decreased weight gain and food efficiency in ovariectomized rats fed 0.1%, 0.5% or 2.0% of extract of *Rubus coreanus* Miq. or 2.0% of *Atractylodes japonica* Koidz. in diets compared with ovariectomized control rats. They speculated that lowered weight gain could have been resulted from estrogenicity of methanol extracts of *Rubus coreanus* Miq. and *Atractylodes japonica* Koidz., because methanol extracts of *Rubus coreanus* Miq. or *Atractylodes japonica* Koidz. exhibited estrogenicity in MCF-7 estrogen-dependent human breast cancer.[13] Since the similar result was observed in male mice fed 5.0% extracts of *Rubus coreanus* Miq., but not in those fed 0.5%, it can be speculated that 5.0% methanol extract corresponding to 6.6 g/kg body weight could decrease bioavailability of other nutrients.

Supplementation of 5% extract of *Atractylodes japonica* Koidz. increased relative liver weight and increased plasma alanine aminotransferase significantly compared to 0.5%. Kiso *et al.*[8] reported that extract of *Atractylodes* rhizomes exhibited antihepatic activity by *in vitro* assay method using primary cultured rat hepatocytes. In a study on toxicological evaluation of fucoidan in Wistar rats, when the dose was increased to 900 and 2500 mg/kg body weight per day, clotting time was significantly prolonged.[14] Therefore toxic effects could be dependent on the dose. In our previous study carried out in ovariectomized rats,[11] 2% extract of *Atractylodes japonica* Koidz. caused significantly increased ALT and AST activities compared with ovariectomized control rats. In this study intake level of 5.0% extract of *Atractylodes japonica* Koidz. was equal to 6604 mg/kg body weight per day. Intake level of 0.5% equal to 687 mg/kg body weight per day showed no adverse effect.

ALT activity was not significantly different between the groups fed 0.5% or 5.0% extract of *Rubus coreanus* Miq. Rather plasma ALT activity in mice fed 5% extract of *Rubus coreanus* Miq. tended to be lower compared with the control mice. Methanol extract of *Rubus coreanus* Miq. included a relatively high amount of polyphenolic compound (4.70~5.02 g/100 g dry weight) and exerted electron donating ability and nitrite scavenging activity and SOD-like activity.[6] This kind of physiological activities of *Rubus coreanus* Miq. may protect liver. Yau *et al.*[15] reported that pre-treatment of hepatocytes with an aqueous extract of Fructus Rubi for 24 h significantly reversed *tert*-butyl hydroperoxide-induced cell damage through its antioxidative activity

The rhizome extract of *Atractylodes japonica* Koidz. exhibited a particular inhibition on the proliferation of cultured human tumor cell lines *in vitro*.[16] It has been reported that families of *Atractylodes japonica* Koidz. prevent stomach damage through an anti-ulcer effect and an inhibitory action on gastric secretion. Atractylon, a major active constituent of rhizome extract of *Atractylodes japonica* was reported to inhibit Na^+,K^+-ATPase activity.[17] Aqueous extract of *Atractylodes japonica* was shown to suppress PGE_2 production by inhibition of the LPS-stimulated enhancement of cyclooxygenase-2 enzyme activity and inducible nitric oxide synthase expression in macrophages.[18] Extracts of *Rubus coreanus* Miq. fruits inhibited the growth of human hepatocarcinoma and human gastric cancer cells and stimulated T and B cell activities.[7,19] These results indicate that extracts of *Rubus coreanus* fruits and *Atractylodes japonica* rhizome have immunomodulating activity.

Lectin such as Con A has been widely used as a mitogen for many years to activate T cells. LPS can stimulate the proliferation of B cells irrespective of their antigenic specificity at high concentrations. In this study, proliferation of splenocyte, induced by either Con A or LPS, from mice fed 5.0% extract of *Atractylodes japonica* Koidz. was significantly decreased compared with that of 0.5% extract of *Atractylodes japonica* Koidz. The result may indicate an immunotoxicity of *Atractylodes japonica* Koidz. at the level as high as 5% of diet.

Interleukin-2 (IL-2) is a potent polyclonal autocrine and paracrine T-lymphocyte growth factor. IL-2 is secreted by Type 1 helper (T_H1) cells stimulated by antigen or mitogen. Interferon-γ (IFN-γ), activated CD4 and CD8 T lymphokine is produced by T_H1, TC, and NK cells.[20] Murine helper T cells can be classified as a function of the cytokines they secrete. Cytokines represent those responsible for regulating T_H1 (cell-mediated) and T_H2 (antibody mediated) immune responses. T_H1 cells have been shown to elaborate IL-2, IFN-γ and transforming growth factor(TGF)-β and to affect cell-mediated immune responses.[21] Interference with IL-2 production or IL-2 receptor-mediated signal transduction would be expected to have a major suppressive effect on T-cell proliferation.

In conclusion, defatted methanol extract of *Atractylodes japonica* Koidz. may exert adverse effects on immune and liver functions at the level of 5.0%. On the other hand, defatted methanol extract of *Rubus coreanus* Miq. did not affect hepatic toxicity and immunomodulating activity in mice at either 0.5% or 5.0% of diets.

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