J. Toxicol. Pub. Health
Vol. 21, No. 2, pp. 115-119 (2005)

Official Journal of
Korean Society of Toxicology
Available online at http://www.toxmut.or.kr

Antioxidative Activity and Irritation Response of *Lespedeza bicolor*

Yang-Suk Lee[1], Zhiqiang Chang[2], Seung-Chun Park[2], Nac-Ryong Rim[3] and Nam-Woo Kim[1]

[1]*Department of herbal Biotechnology, Daegu Haany University, Gyeongbuk 712-715*
[2]*Laboratory of Veterinary Pharmacology and Toxicology,*
College of Veterinary Medicine, Kyungpook National University, Daegu 702-701
[3]*Division of Science Education, Chonbuk National University, Chonbuk 561-756, Korea*

Received March 22, 2005; Accepted June 3, 2005

ABSTRACT. In the present study, we evaluated the free radical scavenging and xanthine oxidase inhibitory activities exhibited by extracts obtained from the dried stems (and leaves) of *Lespedeza bicolor*. We also assessed its potential irritation activities with regard to cosmetic use. When the DPPH radical scavenging activities of *L. bicolor* were assessed at six different concentrations (0, 50, 100, 250, 500 and 1000 μg/ml), the concentration of *L. bicolor* required to inhibit DPPH radical formation by 50% was found to be 164.90 μg/ml. The effects of *L. bicolor* on the inhibition of xanthine oxidase were determined at seven different concentrations. The 50% effective concentration was found to be 282.75 μg/ml. In the skin irritation test, all animals survived for the duration of the study, and all exhibited normal gains in body weight. The control sites exhibited no response to the control procedures. No edema, erythema, or eschar formation was observed in any of the tested rabbits. In the ocular irritation study, all of the rabbit eyes remained normal. In summary, *L. bicolor* extracts were considered to be non-irritating to the skin and eye.

Keywords: Antioxidative activity, Dermal irritation study, Primary irritation index, *Lespedeza bicolor*.

INTRODUCTION

Natural products have been found, in the search for new compounds for the treatment of diseases, to be excellent sources of lead compounds (Nguyen *et al.*, 2004). *Lespedeza bicolor* is a large, leguminous, deciduous shrub, which ranges in height from 4 to 10 feet (1.2~3 m). It is indigenous to Japan, but also can be currently seen in North America and Eastern Asia. The *Lespedeza* species is often planted and used as food for the northern bobwhite and other upland game birds, and has also been used for many years in Traditional Chinese Medicine. The *Lespedeza* species are easily hybridized with one another, and feature similar constituents (Miyase *et al.*, 1999). The *Lespedeza* species exhibits a panoply of biological activities, including antioxidative and anti-inflammatory effects, and has proven effective in the treatment of acute and chronic nephritis, azothemia, and diuresis (Maximov *et al.*, 2004). The active compounds of this plant are assumed to be flavonoids, which exert a variety of biological and pharmacological effects (Havsteen, 1983). In this paper, we evaluated the free radical scavenging and xanthine oxidase inhibitory properties of *L. bicolor*, as well as its potential irritation effects to know whether it can be usable for cosmetics.

Correspondence to: Nam-Woo Kim, Department of herbal Biotechnology, Daegu Haany University, Gyeongbuk 712-715, Korea
E-mail: tree@dhu.ac.kr

MATERIALS AND METHODS

Preparation of test samples

The analysed samples of *L. bicolor* were collected from mid-September and mid-October, in 2003 to 2004, at Gyeongsan, Korea. Dried stems of *L. bicolor* (200 g) were cut into small pieces and were extracted using 1,500 ml of 70% methanol-water solution (MeOH-H_2O) at 100°C for 7 hours by water circulation. And the extracted solution was concentrated at 80°C in a rotary evaporator and was freeze dried to yield a MeOH-H_2O extracts.

Assays for DPPH radical scavenging activity

The MeOH-H_2O extracts were initially dissolved in 1 ml of deionized water (DDW), followed by dilution with DDW to construct trial samples of different concentra-

tions. The free radical scavenging activity of *L. bicolor* was measured as follows. The reaction mixture contained 1 ml of 1.5 mM DPPH-ethanol solution, 0.9 ml of 10 mM acetate buffer (pH 5.6) and 0.1 ml of either different concentrations of test samples, or DDW (control). The mixture was allowed to react at room temperature for 30 minutes and absorbance values were measured at 517 nm, then converted into percentages of antioxidant activity, which were then expressed as percentage decreases in absorbance, as compared with the control values. This experiment was performed in triplicate.

Assays for the inhibition of xanthine oxidase

The MeOH-H_2O extracts were initially dissolved in 1 ml of methanol (95%) followed by dilution with 0.1 mM phosphate buffer (pH 7.4) in order to yield different concentrations of trial samples. The inhibitory activity of *L. bicolor* on xanthine oxidase was measured as follows: the reaction mixtures contained 100 μl of xanthine water solution (1.286 mM), 40 μl of xanthine oxidase solution (0.0741 units/ml), 100 μl of sample, and enough phosphate buffer to adjust the final volume to 2 ml. The inhibition of xanthine oxidase activity was evaluated by measuring the formation of uric acid from xanthine with a spectrophotometer at 295 nm for 3 minutes. The reaction mixture without sample was measured as a control. Xanthine oxidase inhibitory activity (%) was calculated as (C-S)/C × 100, where C and S represent the activities of the enzyme, both with and without test material. This experiment was performed in triplicate.

Primary skin irritation study in rabbit

Four adult rabbits (male) of the New Zealand strain, weighing between 1.75 and 2.25 kg, were selected for this study. Prior to dosing, application sites were prepared by clipping the hair from the saddle area of the rabbits. Two abraded areas located diagonally across this area on each rabbit, were prepared by making minor epidermal incisions with a hypodermic needle. MeOH-H_2O extracts were dissolved in DDW to yield 50 mg/ml of sample solution. 0.5 ml of the sample solution was then applied under a 2-square-centimeter surgical gauze patch on an intact skin area and an abraded skin area on each rabbit, after which and 0.5 ml of DDW was also applied under the gauze patch on the remaining skin test areas to serve as a control. After applying of the patches, the trunks of each rabbit were wrapped with bandages, and the animals were restrained for 24 hours. At the end of the exposure period, the patches were removed, and the reactions were scored at 24 and 72 hours after the application.

Ocular irritation study in rabbit

Four adult rabbits (male) of the New Zealand strain, weighing between 1.75 and 2.25 kg were selected for this study. MeOH-H_2O extracts were dissolved in DDW to yield 50 mg/ml of sample solution. 0.1 ml of sample solution was applied to the conjunctival sac of the left eye of each test rabbits and 0.1 ml of DDW was applied into the right eye, as a control. The upper and lower eyelids were gently held together for few seconds, then released. Examinations for gross signs of eye irritation were conducted at 1, 2, 3, 4, and 7 days after the initial application.

RESULTS

Assays for DPPH radical scavenging activity

The DPPH radical scavenging activities of *L. bicolor* were evaluated at six different concentrations (0, 50, 100, 250, 500 and 1000 μg/ml), as shown in Table 1. All *L. bicolor* samples exhibited radical scavenging properties to different extents, and DPPH reduced gradually with increasing concentrations of *L. bicolor*. The concentration of *L. bicolor* required to inhibit DPPH radical formation EC_{50} was found to be 164.90 μg/ml.

Table 1. Free radical scavenging activity of *Lespedeza bicolor*

Concentration (μg/ml)	Free radical scavenging activity (%)
0	0
50	15.31 ± 9.26
100	35.30 ± 10.48
250	76.50 ± 7.64
500	83.67 ± 2.65
1000	84.90 ± 43.49
EC_{50} (μg/ml)	164.90 ± 59.74

Values were expressed as the Mean ± S.D. of 3 independent experiments; EC_{50} values were determined by linear regression analysis.

Table 2. Inhibition of xanthine oxidase activity of *Lespedeza bicolor*

Concentration (μg/ml)	Inhibition of xanthine oxidase (%)
0	0
25	5.38 ± 5.92
50	15.14 ± 5.16
125	35.02 ± 12.82
250	45.64 ± 6.49
500	62.94 ± 3.73
750	72.32 ± 8.11
EC_{50} (μg/ml)	282.75 ± 68.76

Values were expressed as the Mean ± S.D. of 3 independent experiments; EC_{50} values were determined by linear regression analysis.

Table 3. Effects of *Lespedeza bicolor* on skin irritation in rabbit

Site	Control site								Test site							
Change	Erythema & eschar				Edema				Erythema & eschar				Edema			
Phase (hrs)	Intact		Abraded		Intact		Abraded		Intact		Abraded		Intact		Abraded	
	24	72	24	72	24	72	24	72	24	72	24	72	24	72	24	72
No.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
No.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
No.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
No.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ΣMean score	0				0				0				0			
Total	0								0							
P.I.I.	0								0							

P.I.I.: primary irritation index = total/4.

Assays for the inhibition of xanthine oxidase

The effects of *L. bicolor* on the inhibition of xanthine oxidase were assessed at seven different concentrations as shown in Table 2. All concentrations of *L. bicolor* samples exhibited inhibitory effects on xanthine oxidase to different extents, and the formation of uric acid from xanthine reduced gradually with increases of the concentration of *L. bicolor*. *L. bicolor* inhibited xanthine oxidase activity by EC_{50} at a concentration of 282.75 μg/ml.

Skin irritation study in rabbit

All animals survived for the duration of the study and exhibited gains in body weight. No overt signs of toxicity were detected in any of the animals during the course of the study. After the application of *L. bicolor* to rabbits, we noted a light brown staining at the treated skin sites, which had no effect on our evaluation of skin responses. The control sites exhibited no responses to the control procedures. No edema, erythema, or eschar formation was detected in any of the rabbits (Table 3). Therefore, *L. bicolor* was considered to be non-irritating to the skin.

Table 4. Effects of *Lespedeza bicolor* on eye irritation in rabbit

Animal No.	Tissue	Time after application					
		Day 1	Day 2	Day 3	Day 4	Day 7	A.O.I
1	Cornea	0	0	0	0	0	0
	Iris	0	0	0	0	0	0
	Conjunctiva	0	0	0	0	0	0
2	Cornea	0	0	0	0	0	0
	Iris	0	0	0	0	0	0
	Conjunctiva	0	0	0	0	0	0
3	Cornea	0	0	0	0	0	0
	Iris	0	0	0	0	0	0
	Conjunctiva	0	0	0	0	0	0
4	Cornea	0	0	0	0	0	0
	Iris	0	0	0	0	0	0
	Conjunctiva	0	0	0	0	0	0
M.O.I.		0	0	0	0	0	0
Day-7 I.O.I.		0					

M.O.I.: Mean Ocular irritation Index; I.O.I.: Individual Ocular irritation Index; A.O.I.: Acute Ocular irritation Index.

Ocular irritation study in rabbit

After the application of the sample to the rabbits' eyes (ocular membranes), all of the rabbit eyes appeared normal. No abnormal changes, such as lacrimation, reddening, swelling, or pus formation were observed for up to 7 days after exposure (Table 4). Therefore, *L. bicolor* was considered to be non-irritating to the eye.

DISCUSSION

Free radicals, powerful oxidants, are species that contain unpaired electrons. Free radical are generated in a variety bioorganic redox processes, and may induce oxidative damage in various body components (e.g., lipids, proteins, nucleic acids and saccharides) and may also be involved in processes which result in mutations (Yen, 1995). Furthermore, radical reactions play a significant role in the development of chronic diseases including cancer, hypertension, cardiac infarction, arteriosclerosis, rheumatism, cataracts, and others (De Souza *et al.*, 2004). Free radicals may also constitute a contributory factor in the progressive decline of immune system function (Pike and Chandra, 1995). Cooperative defense systems that protect the body from free radical damage include antioxidant nutrients and enzymes (Halliwell, 1996). One effective way of protecting the

body against oxidative stress is to increase antioxidant levels (De Souza *et al.*, 2004). Therefore, the evaluation of the antioxidative properties of candidate materials for the prevention of oxidative damage remains a highly active research area. DPPH is a stable free radical which is often used to in the evaluation of the antioxidant activity in several natural compounds (Yokozawa *et al.*, 1998). Antioxidants, on interaction with DPPH, transfer electrons or hydrogen atoms to DPPH, thus neutralizing its free radical character. Xanthine oxidase (XO) is the enzyme which is responsible for the formation of uric acid from the purines hypoxanthine and xanthine, and is also responsible for the medical condition known as gout. Gout is caused by the deposition of uric acid in the joints which results in painful inflammation. Conversely, the inhibition of XO leads to a remission in gout (Chiang *et al.*, 1994). XO also function as an important biological source of oxygen-derived free radicals, which contribute to oxidative damage to living tissues. This variety of oxidative damage is involved in many pathological processes, including inflammation, atherosclerosis, cancer and aging (Cos *et al.*, 1998). XO inhibitors may potentially prove useful in the treatment of gout or other XO-induced diseases (Goodman Gilman *et al.*, 1990).

The free radical scavenging and antioxidant exhibited by *L. bicolor* may be associated with the presence of flavonoids constituents. Numerous positive effects of flavonoids have been described previously. These include antioxidant (Middleton, 1998; Nijveldt *et al.*, 2001; Rice, 1995), antiviral (Middleton, 1998; Selway, 1986), anticancer (Middleton, 1998; Plaumann *et al.*, 1996), anti-inflammatory (Hayek *et al.*, 1997), antiallergic (Gabor, 1986; Middleton, 1998), anti-atherogenic (Hayek *et al.*, 1997), antithrombotic, and other effects (Berger *et al.*, 1992). Flavonoids are well-known antioxidants, and have drawn a tremendous amount of attention among researchers, as candidate agents for the treatment of free radical-mediated diseases.

Flavonoids are also effective inhibitors of several enzymes including XO, cyclooxygenase, and lipooxigenase (Nguyen *et al.*, 2004; Hoorn *et al.*, 2002). Miyase *et al.* (1999a, b) isolated several phenolic compounds, including isoflavonoids and stilbenoids, which exhibited showed strong antioxidative and antiallergic properties, from *L. homoloba*.

In the present study, the extract of *L. bicolor* was also found to exhibit potent antioxidant effects against the DPPH radical, as well as xanthine oxidase. These results show the extract of *L. bicolor* can be used for cosmetics and herbal bath. Therefore, we are carried out to evaluate the irritation test in rabbits. According to the results of our primary skin irritation and ocular irritation tests, the extract of *L. bicolor* can be considered to be a non-irritating natural material for medicine, cosmetics and herbal bath.

ACKNOWLEDGMENTS

This work was supported by grant number [R12-2003-002-05001-0] from the Basic Research Program and grant number [M02-2004-0000-20335-0] from Research Intern Program of the Korean Science and Engineering Foundation.

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