Antioxidative and anti-inflammatory activities of *Phaseolus aureus*

***In Taik Hong*[1], Beom Jun Kim[1], Duck Cho Yu[1] , Jung Haeng Kim[1], Jung Han Kim[2], Moon Young Heo[2], Song Jin Lee[2] and Hyun Pyo Kim[2]**

[1]Research Center, Koreana Cosmetics Co., Cheon An City, 333-830, [2]College of Pharmacy, Kangweon National University, Chuncheon, 200-701, Korea.

Keyword : Phaseolus aureus, flavonoid, vitexin, isovitexin, antioxidative activity, anti-inflammatory acitvity

Abstract

Phaseolus aureus (mung bean), Leguminosae, has been used as an antidote from the ancient time. Especially, it has been widely used for cleaning face and skin in oriental countries. Although several constituents such as fatty acids, phytoallexin and phaseol derivatives were reported in *P.aureus* and related species including seedlings, there has been a few report to describe its biological activity. Therefore, in this investigation, the ethanol extract from *P. aureus* was obtained and its biological activities including the antioxidative and anti-inflammatory activities were studied. The 70% ethanol extract from *P. aureus* showed dose-dependent antioxidative activity (52.3% inhibition at 4 mg/ml) against lipid peroxidation assay, while the extract did not show the inhibitory activity of superoxide radical formation. The extract also showed the topical anti-inflammatory activity against croton-oil and arachidonic acid induced mouse ear edema test (18-19% inhibition at 7.5 mg/ear) as well as mild inhibitory activity against picryl chloride induced delayed hypersensitivity in mouse. For investigating active principles, vitexin and isovitexin (apigenin C-glycoside) as flavonoids, and adenosine were isolated from the extract using silica gel chromatography. The actual contents of vitexin and isovitexin were found to be 3.7 and 2.4 mg/g extract, respectively. Vitexin and isovitexin showed the antioxidative activity. They showed the topical anti-inflammatory activity, although the activities were not potent compared to the reference compounds. These results suggested that vitexin and isovitexin may be, at least in part, the compounds contributing the antioxidative activity in vitro and the topical anti-inflammatory activity of *P.aureus in vivo*. All results of present study might be one of the scientific rationale in using mung bean for skin care from the ancient time.

Introduction

Phaseolus aureus (mung bean), Leguminosae, has been used as an antidote from the ancient time. Especially, it has been widely used for cleaning face and skin in oriental countries. In an ancient literature(1), *P. aureus* is a major ingredient of the cosmetic preparation called Ock-Yong-Seo-Si-San. However, there has been a few report to describe its biological activity. Several constituents have been reported such as fatty acids, several phytoalexins, phaseol and phaseoline derivatives in *P.aureus* and related species including seedlings.(2-5) Recently, Shin *at el.*(6) isolated triacontanol, stigmasterol, β-sitosterol and vitexin from mung been. In this investigation, the ethanol extract from *P.aureus* was obtained and its biological activities including the antioxiative and anti-inflammatory activities were studied. In addition, the major constituents such as vitexin and isovitexin were isolated and their biological activities were also studied.

Materials and Methods

Melting points were determined on a Fisher-Johns melting point apparatus and uncorrected. UV/visible spectra were obtained using a Hitachi U-2000 spectrometer. ^{1}H- and ^{13}C-NMR spectra were obtained on a Varian 200 MHz NMR using TMS as an internal standard. Mass spectrum was obtained using HP 5988A MS model (thermospray, positive ion mode). The purity of the isolated compounds was checked using TLC (Kiesel gel 60, F_{254} glass plate, Merck) and HPLC. Silica gel for column chromatography was 70-230 mesh (Merck). HPLC was equipped with Shimatzu LC-9A pumps including gradient controller and ODS reverse phase column. Croton-oil, arachidonic acid (AA), prednisolone and indomethacin were products of Sigma Chem. Co. (USA). Picryl chloride was from Nacalai tesque (Japan). The other reagents used were the highest purity chemicals available. Male ICR mice were acclimated in an animal chamber under the conditions of 21-23°C, 12 hr/12 hr (L/D) cycle at least for 7 days before use. Animals were feeding with pellet lab, chaw and tap water *ad libitum*.

Extraction and isolation procedure : *P.aureus* grown in middle part of Korea was collected and powdered after removing outershells. The powdered *P. aureus* (600 g) were soaked in 70% ethanolic aqueous solution (5,000 ml) at 20°C for 3 days. After filtration, the ethanolic filtrate was evaporated to dryness under vaccum.(75 g) This extract was used for further biological study. The ethanol extract (50 · g) was dissolved in small amount of methanol and poured on silica gel column. Fractions were collected using ethyl acetate : methanol : water (100 : 13.5 : 10) as a mobile phase and evaporated to give compound A, B and C. Recrystallization from each solvent gave pure compound A (38 mg), B (29 mg) and C (12 mg).

Compound A (vitexin,5,7,4'-trihydroxyflavone-8-C-β-D-glucopyranoside) : Yellow needles (recrystallized from methanol), m.p.= 268-269°C (rep.(7), 269-270°C), ^{1}H-NMR (DMSO-d_6), δ 4.70 (1H,d,j = 10.0 Hz, C_1"-H), 6.27 (1H,s,C_6-H), 6.78 (1H,s,C_3-H), 6.89

(2H,d,J = 8.4 Hz,C_3'-H and C_5'-H), 8.02 (2H,d,J = 8.4 Hz,C_2'-H and C_6'-H), 10.36, 10.85 (2H,2brs,C_7-OH and C_4'-OH), 13.17 (1H,s,C_5-OH). ^{13}C-NMR δ 182.5 (C-4), 164.3 (C-2), 162.9 (C-7), 161.5 (C-4'), 160.7 (C-5), 156.3 (C-9), 129.3 (C-2' and C-6'), 121.9 (C-1'), 116.1 (C-3' and C-5'), 104.9 (C-8)[a], 104.3 (C-10)[a], 102.7 (C-3), 98.4 (C-6), 82.1 (C-5''), 78.9 (C-3''), 73.6 (C-1''), 71.1 (C-2''), 70.8 (C-4''), 61.5 (C-6''). [a]Interchangeable values, Mass m/z, 433 $[M + H]^+$, 313., No reaction upon 2N HCl hydrolysis.(8)

Compound B (isovitexin, 5,7,4'-trihydroxyflavone-6-C-β-D-glucopyranoside) : Yellow needles (recrystallized from acetone), m.p.= 236-237°C (rep.(7), 239°C), ^{1}H-NMR(DMSO-d_6), δ 4.87 (1H,d,J = 10.0 Hz, C_1''-H), 6.51 (1H,s,C_8-H), 6.79 (1H,s,C_3-H), 6.92 (2H,d,J = 8.8 Hz,C_3'-H and C_5'-H), 7.93 (2H,d,J = 8.8 Hz,C_2'-H and C_6'-H), 10.37, 10.59 (2H,2brs,C_7-OH and C_4'-H), 13.56 (1H,s,C_5-OH). ^{13}C-NMR δ 182.4 (C-4), 163.9 (C-2), 163.6 (C-7), 161.5 (C-5), 161.0 (C-4'), 156.6 (C-9), 128.8 (C-2' and C-6'), 121.4 (C-1'), 116.3 (C-3' and C-5'), 109.2 (C-6), 103.7 (C-10), 103.1 (C-3), 93.9 (C-8), 81.8 (C-5''), 79.2 (C-3''), 73.3 (C-1''), 70.8 (C-2''), 70.4 (C-4''), 61.7 (C-6''). Mass m/z 433 $[M+H]^+$, 313., No reaction upon 2N-HCl hydrolysis.(8)

Compound C (adenosine) : Colorless cubics (recrystallized from methanol), m.p.=236-237°C (rep.(7), 234-236°C), directly compared with authentic sample in NMR, TLC and HPLC. ^{1}H-NMR (DMSO-d_6) δ 8.37(s), 8.16(s), 7.37(brs), 5.89 (d,J = 6.3 Hz), 5.45(m), 5.20(d), 4.63(m), 4.16(m), 3.98(m), 3.65(m). On D_2O exchange, peaks of d 7.37, 5.45 and 5.20 were disappeared. ^{13}C-NMR (DMSO-d_6) δ 156.1, 152.3, 149.0, 139.9, 119.3, 87.8, 85.8, 73.3, 70.5, 61.5.

The chemical structures of compound A (vitexin) and B (isovitexin) were shown in Fig. 1. In addition, the ethanolic extract (10 g) was dissolved in distilled water and partitioned with chloroform and evaporated to dryness (380 mg). The dried residue was dissolved in small amount of chloroform and poured on silica gel column. As a mobile phase, chloroform : methanol (95 : 5) was used. Fractions were collected and evaporated to dryness. Recrystallization from chloroform gave colorless needles (56 mg), which was subjected to HPLC using the conditions described below. Fractions were collected and evaporated to give pure β-sitosterol (8 mg), which was identified by direct comparison with an authentic sample.

Component analysis : The each component of the extract was quantitatively analyzed using HPLC according to the following conditions. Vitexin and isovitexin were analyzed using MeOH : H_2O : ethyl acetate (34.6 : 60 : 5.4) as amobile phase at UV 254 nm (flow rate = 1 ml/min). Adenosine was quantitated using 10% sod. citrate buffer, pH 3.8 at UV 254 nm (flow rate = 1ml/min). b-Sitosterol was analyzed using acetonitrile : MeOH (70 : 30) at UV 210 nm (flow rate = 2 ml/min). Retention times for vitexin, isovitexin, adenosin and β-sitosterol were 8, 10, 16 and 22 min, respectively. Protein concentration was measured by Folin-Lowrey assay(9) and amount of carbohydrate was determined by the phenol-sulfuric acid method.(10) Total lipid was determined by ether extraction method, and ash content was determined by heating in electric muffle fur-

nace at 600°C for 6 hrs. For evaluating the anti-inflammatory activity of the extract and flavonoids isolated, the ethanol extract of *P. aureus* and flavonoids, vitexin and isovitexin, were dispersed in a standard vehicle containing parraffin oil, sodium dodecyl sulfate and water. The final concentration was 5% w/w of the ethanol extract and flavonoids, respectively.

Antioxidative activity : A lipid peroxidation system was induced by Fenton's reagent. Each test sample (0.1ml) and ethyl linoleate (10 μl) were added to incubation medium (4.89 ml) containing 2% sod. dodecyl sulfate, 1 μM ferrous chloride and 0.5 μM hydrogen peroxide. The known synthetic antioxidant, butylated hydroxytoluene (BHT) was used as a reference compound. The incubation medium was kept at 55°C for 16 hrs. Each reaction mixture (0.2 ml) was transferred into a test tube, followed by addition of 4% BHT (50 μl) to prevent further oxidation. Antioxidative activity of the sample was measured using thiobarbituric acid (TBA) assay according to the method of Ohkawa *et al.*(11) Inhibitory activity aganist superoxide radical generation was measured following the procedure of Fugita *et al.*(12) The sample solution (2 ml) was added to 2 ml of 60 μM 1,1-diphenyl-2-picryl hydrazyl (DPPH) ethanol solution and kept at room temperature for 30 min. The absorbance was measured at 520 nm.

Anti-inflammatory activity : For measuring the topical anti-inflammatory activity, mouse ear edema assay was employed. According to the modified method of Kim *et al.*(13), based on the original procedure of Tonneli *et al.*(14), preparations of the ethanol extract and flavonoids were topically applied to right ears of mice (18-22 g) three times at 3 hrs interval. Thirty minutes after the final treatment of the test compounds, 2.5% croton-oil or 2% arachidonic acid dissolved in acetone (25 μl/ear) was applied topically to ears of mice. And the ear thickness was measured 5 hrs after croton-oil treatment or 1 hr after AA treatment. Percent inhibition of ear edema was calculated compared to the control group having vehicle and inflammagen only. Inhibitory activity against delayed hypersensitivity was measured according to the method of Tarayre *et al.*(15) Briefly, 3% picryl chloride (acetone) was applied to abdomen of mice (18-22 g). One week later, 3% picryl chloride was applied to ears of mice and ear thickness was measured 24 hrs after the treatment of picryl chloride solution. Preparations of the test compounds were applied to ears of mice daily for 7 days starting from 0 day. The differences between ear thickness of the extract-treated group and the control group treated with picryl chloride and vehicle only were regarded as an inhibitory activity.

Statistics : Student t-test was used for evaluating the statistical significance.

Results and discussion

In this study, the 70% ethanol extract of *P. aureus* was prepared and the several constituents including flavonoids were isolated from this extract. And biological activities were investigated for the extract and flavonoids. From the extract, compound A, B, C

and β-sitosterol were isolated. Compound A and B have the same molecular weight (432, $C_{21}H_{20}O_{10}$) and were revealed to be apigenin-glycosides by interpreting NMR spectra. These glycosides could not be hydrolyzed by acid treatment, which supported that these compounds were C-glycosides of apigenin. Compounds A and B were finally identified as vitexin and isovitexin, respectively, which were identical with the previous results of Osterdahl (16), Oh *et al.* (17) and Shin *et al.* (6) This is the first report of isolation of isovitexin from *P. aureus* and related species as far as we have searched literatures and patents. Compound C was identified as adenosine by direct comparison with an authentic sample. Adenosine is frequently isolated from various plant species. Table 1 demonstrated the analysis results of the extract by HPLC and other assay methods. As already noted, the ethanol extract of *P. aureus* was shown to contain relatively high amounts of vitexin (0.37%, w/w) and isovitexin (0.24%, w/w). Table 2 represented the antioxidative activity of the extract and flavonoids. As expected, the extract showed dose-dependent antioxidative activity. Especially, at 4 mg/ml, the extract showed the potent activity (52.3% inhibition). Vitexin and isovitexin showed mild antioxidative activity, although they did not show the concentration dependent inhibition. This result may be explained by the fact that some flavonoid derivatives show the biphasic inhibition of lipid peroxidation because flavonoids possess antioxidative activity as well as prooxidative activity.(18) The antioxidative activity of isovitexin seemed to be higher than that of vitexin. However, the extract, vitexin and isovitexin did not show the inhibition of superoxide radical formation (Data not shown). Table 3 and 4 represented the topical anti-inflammatory activity of the extract. Although the activity was found to be weak compared to the potent activity of the reference compounds, prednisolone and indomethacin, the extract showed anti-edematic activity as well as anti-hypersensitivity. Mouse ear edema assay is a frequently used animal model for topical application of the various compounds.(19) In croton-oil induced ear edema, 12-O-tertradecanoylphorbol-13-acetate (TPA) is suggested to produce edema and leukotriene B4 is found in ear area. In AA induced ear edema, eicosanoids such as prostaglandins and leukotriens are involved in the inflammation. The ethanol extract of *P. aureus* did show the anti-inflammatory activity against both inflammagen, which suggested that the extract might reduce the inflammation in the skin induced by various inflammagens. Because the extract also showed the inhibitory activity against delayed hypersensitivity, the extract may be a useful agent to treat various skin troubles. In order to find the active principles of the extract, vitexin and isovitexin were also tested for the anti-inflammatory activity. Isovitexin showed the anti-inflammatory activity (Table 5) against croton-oil induced edema as well as arachidonic acid induced edema, while vitexin only showed the antiedematic activity against AA-induced edema. Previously, several groups including us demonstrated that flavonoids showed the anti-inflammatory activity and the anti-allergic activity (13, 20-22). The most active ones were flavones/flavonols. These previous findings were well correlated with our results in this investigation. And these results suggested that vitexin and isovitexin may be, at least in part, the compounds contributing the antioxidative activity and *in vitro* and the

topical anti-inflammatory activity *in vivo*. All of these results of our study might be one of the scientific rationale of in using P. aureus (mung bean) for skin care from the ancient time. In conclusion, the ethanol extract from *P.aureus* was prepared and several constituents including flavonoids, vitexin and isovitexin, were isolated. They showed the antioxidative activity *in vitro* as well as topical anti-inflammatory activity *in vivo*.

References

1. Huh, Z., in Dong-Eu-Bo-Gam (1613), Namsandang, Seoul, (1966)
2. Woodward, M. D., New isoflavonoids related to kievitone from *Phaseolus vulgaris*. Phytochem. 18, 2007 (1979)
3. O'Neill, M. J. Adesanya, S. A. and Roberts, M.F., Isosojagol, a coumestan from *Phaseolus coccineus*. Phytochem. 23, 2704 (1984)
4. Altshul, M. A. and Alahiya, M. D., b-Sitosterol and flavonoids from *Phaseolus vulgaris* pods., Khim. Prir. Soedin. 236 (1983)
5. Muraru, R., Seshadri, T. R. and Vydeeswaran, S., Components of the seed coats of *Phaseolus mungo* and *Phaseolus radiatus*., Curr. Sci. 42, 605(1973)
6. Shin, S-H., Kang, S. S. and Kwon, K.S., Studies on the components of the seeds of *Phaseolus radiatus*., Yakhak Hoiji 34, 282 (1990)
7. Dictionary of natural products, Chapman & Hall, London. (1994)
8. The systemic identification of flavonids, Marbry, T. J. *et al.* (eds.), Chapt. III, Springer-Verlag, Berlin (1970), pp 23-25
9. Lowrey, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. L., J. Biol. Chem. 193, 265 (1951)
10. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. Anal. Chem. 28, 350 (1956)
11. Ohkawa, H., Ohishi, N. and Yagi, K. Anal. Biochem. 95, 351 (1979)
12. Fugita, Y., Uehara, I., Morimoto, Y., Nakajima, M., Hatano, C., Okuda, T. Yakugaku Zasshi 108, 129-135 (1988)
13. Kim, H. K., Namgoong, S. Y. and Kim, H. P. Arch. Pharm. Res. 16, 18 (1993)
14. Tonneli, G., Thiabalt, L. and Ringler, I. Endocrinol. 77, 625 (1965)
15. Tarayre, J. P., Barbara, M., Aliaga, M. and Tisne-Versailles, J. Arzneim.-Forsch./Drug Res. 40(II), 10, 1125 (1990)
16. Osterdahl, B. G., Chemical studies on Bryophytes. 19. Application of 13C NMR in structural elucidation of flavonoid C-glycosides from Hedwigia ciliate., Acta Chem. Scand. B32, 93 (1978)
17. Oh I. S., Whang W. K. and Kim, I.H. Constituents of Crataegus pinnatifida va. psilosa leaves (II), Flavonoids from BuOH fraction., Arch. Pharm. Res. 17, 314-317 (1994)
18. Shibamoto, T., Hagiwara, Y., Hagiwara, H. and Osawa, T. in Food phytochemicals for cancer prevention II., Ho *et al.* (eds.), American Chem. Soc., pp 154-163 (1994)
19. Bouclier, M., Cavey, D., Kail, N. and Hensby, C. Pharmacol. Rev. 42, 127 (1990)
20. Kim, C. J. and Chung, J. M. Yakhak Hoiji 34, 348 (1990)
21. Lee, S. J., Son, K. H., Chang, H. W., Do, J. C., Jung, K. Y., Kang, S. S., and Kim, H. P. Arch. Parm. Res. 16, 25 (1993)
22. Lee, S. J., Baek, H. J., Lee, C. H., and Kim, H. P. Arch. Pharm. Res. 17, 31 (1994)

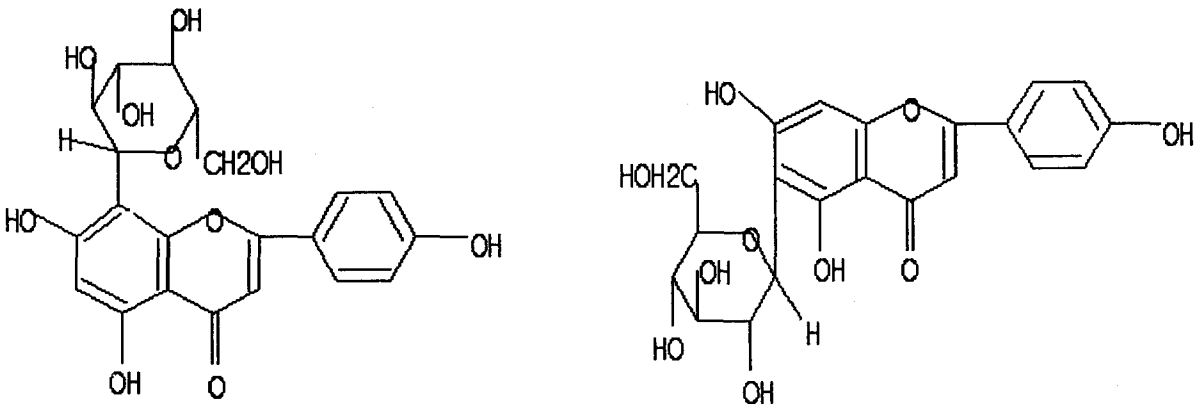

Fig 1. Chemical structure of vitexin and isovitexin

Table 1. Composition of the ethanol extract of *P. aureus*

Components	Contents (㎎/g)[a]
Vitexin	3.7
Isovitexin	2.4
Adenosine	1.3
β-Sitosterol	0.5
Lipid	125.0
Protein	89.0
Carbohydrate	433.0
Ash	92.0

[a]Typical result of one of the several extractions

Table 2. Inhibition of lipid peroxidation of the extract and flavonoids

Groups	Concentration	Absorbance	% Inhibition
Control	-	0.170 ± 0.004	-
BHT	0.2 μM	0.184 ± 0.010	-
	2.0 μM	0.172 ± 0.022	-
	20.0 μM	0.037 ± 0.001**	78.2
P. aureus	0.04 ㎎/㎖	0.152 ± 0.010	10.5
	0.40 ㎎/㎖	0.122 ± 0.010**	28.2
	4.0 ㎎/㎖	0.081 ± 0.005**	52.3
Vitexin	0.2 μM	0.137 ± 0.025	19.4
	2.0 μM	0.153 ± 0.035	10.0
	20.0 μM	0.147 ± 0.028	13.5
Isovitexin	0.2 μM	0.124 ± 0.007**	27.0
	2.0 μM	0.146 ± 0.006*	14.1
	20.0 μM	0.135 ± 0.028	20.5

* : $P < 0.05$, ** : $P < 0.01$, significantly diffenent from control (n = 3)

Table 3. Mouse ear edema inhibition of the EtOH extract of *P. aureus*

Groups	Dose (㎎/ear)	Thickness increased[a] mm, (% inhibition)	Thickness increased[b] mm, (% inhibition)
Control	-	0.25 ± 0.02 (-)	0.16 ± 0.03 (-)
Prednisolone	0.5	0.16 ± 0.03* (36)	NT
Indomethacin	0.5	NT	0.08 ± 0.02** (50)
5% extract	3 × 50	0.20 ± 0.02* (18)	0.13 ± 0.01* (19)

[a]Croton-oil induced ear edema, [b]arachidonic acid induced ear edema,
NT : not tested
* : $P < 0.05$, ** : $P < 0.01$, significantly different from control (n = 6).

Table 4. Inhibition of delayed hypersensitivity by the EtOH extract of *P. aureus*

Groups	Dose (㎎/ear)	Thickness increased mm, (% inhibition)
Control	-	0.22 ± 0.04
Prednisolone	7 × 0.1	0.05 ± 0.04** (77)
5% extract	7 × 50	0.16 ± 0.03* (27)

* : $P < 0.05$, ** : $P < 0.001$, significantly different from control (n = 10).

Table 5. Mouse ear edema inhibition of vitexin and isovitexin

Groups	Dose (㎎/ear)	Thickness increased[a] mm, (% inhibition)	Thickness increased[b] mm, (% inhibition)
Control	-	0.23 ± 0.03 (-)	0.16 ± 0.03 (-)
Prednisolone	0.5	0.16 ± 0.02**(30)	NT
Indomethacin	0.5	NT	0.06 ± 0.03***(63)
5% Vitexin	3 × 50	0.23 ± 0.01 (-)	0.10 ± 0.02* (38)
5% Isovitexin	3 × 50	0.19 ± 0.02* (19)	0.11 ± 0.01* (29)

[a]Croton-oil induced ear edema, [b]arachidonic acid induced ear edema,

NT : not tested

* : P<0.05, ** : P<0.01, *** : P<0.001 significantly different from control (n=8).