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Bleeding Time Prolongation Effect of Methanol Extract of *Viscum album* var. *coloratum*

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Abstract – The methanol extract of *Viscum album* var. *coloratum*, Korean mistletoe, showed potent prolongation effects on the bleeding time in rats *in vivo*, and whole blood clotting time and plasma recalcification time in rats *ex vivo*. The prolongation effect on the bleeding time of Korean mistletoe is comparable to that of *Viscum album* L., European mistletoe, 185.6% and 176.5%, respectively. However, the water extracts of the both plants did not show any prolongation effects. Platelet activating factor (PAF) receptor binding assay was carried out to elucidate the action mechanisms of the extracts, and both of the methanol extracts did not show any inhibitory activity. The LD_{50} of the methanol extracts of both mistletoes are more than 2 g/kg. These results suggest that the mehtanol extract of Korean mistletoe might be a potential candidate to develop new drug to improve microcirculation.
Keywords – *Viscum album* var. *coloratum, Viscum album,* bleeding time, platelet activating factor

Introduction

European mistletoe is believed to have hypotensive, vasodilator, sedative, antispasmodic and anticancer activities in Europe (Reynolds, J. E., 1999; Franz, H., 1985). Korean mistletoe also have been used in traditional medicine for the preparation of anti-inflammatory and hypotensive in Korea (Kim, J. K., 1989). In order to know the improvement effect of microcirculation of *Viscum album* var. *coloratum* (Korean mistletoe, Loranthaceae), we carried out the bleeding time test in rats *in vivo*, whole blood clotting time, plasma recalcification time tests *ex vivo,* and the platelet activating factor (PAF) receptor binding assay with the titled plant and *Viscum album* var. L. (European mistletoe, Loranthaceae). Present study is on the prolongation effect on bleeding time of two mistletoes *in vivo*.

Experimental

Plant materials – The crude drug of Korean mistletoe was collected in March 1995 from the mountain of Chiac, Wonju, Kangwon-do, Korea. The dried voucher specimen has been deposited in the herbarium of the Institute for Life Sciences, University of Ulsan (No. AS-95-3). European mistletoe was commercially available, Mistel (Mistelkraut, Herba Vici albi), Caesar & Loretz Gmbh.

Extraction – Dried whole plants of Korean (1 kg) and European (1 kg) mistletoes were extracted with cold water and with methanol at 4°C for 24 hours by 3 times for water and methanol extracts, respectively. The water and methanol extracts of the Korean mistletoe were 210 g and 257 g, whereas those of the European one were 204 g and 242 g, respectively.

Analysis of typical compounds, homoflavoyadorin B and flavoyadorin B – HPLC was performed to analyze the typical compounds of the extracts. The systems are consisted in Waters 600 controller, 717 plus Autosampler, Waters 996 Photodiode Array Detector, Waters C_{18} radial pack column (7.9×100 mm) and Milenium software data module. About 10 mg of extracts were accurately weighed and dissolved in 4.0 ml of acetonitrile. Samples were passed through a streile-filter and every 10 μl was injected to HPLC system via an autosampler. Detector: 254 nm, solvent A: 0.6% citric acid, solvent B: acetonitrile, flow rate: 1.0 ml/min, gradient: 0 (min) 85% A 15% B; 10 (min) 80% A 20% B; 10 (min) 70% A 30% B; 5 (min) 60% A 40% B; 5 (min)

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50% A 50% B; 5 (min) 20% A 80% B; 5 (min) 15% A 85% B. Standard compounds, homoflavoyadorin B and flavoyadorin B were isolated from the methanol extract of Korean mistletoe to use as standard compounds. The structure identification was performed by comparison of their ^{1}H-NMR, ^{13}C-NMR and mass spectra with data reported in the liteature (Fukunaga, T., *et al.* 1989).

Bleeding time test – The bleeding time of sample-treated rats was measured as originally described by Hornstra (Hornstra, G., *et al.* 1981) and the method is briefly discribed as follows; samples were dissolved in water for the water extracts and suspended in 1% CMC solution for methanol extracts given orally once a day for 12 days. At the following day of the last medication, animals were anaesthetized with sodium pentobarbital (25 mg/kg, intraperitoneally). The tail was transsected at 3 mm from the tip, and the tail was immersed 5 cm deep vertically in saline at 37°C. The period between transsection and the moment the third bleeding stopped was taken as the bleeding time. Data are presented as mean±S.E. and were analyzed by ANOVA (one-way analysis of variance). Differences were considered as "significant" when $p<0.05$.

After bleeding time tests, nine volumes of blood were collected from the heart directly into 1 volume of 3.13% sodium citrate solution to assess the prolongation effects of whole blood and plasma clotting time by the samples (Han, Y.N., *et al.* 1987). After the mixture, 1 ml of whole blood and 100 μl of saline or sample solutions, was

Table 1. Bleeding time, whole blood clotting time and plasma clotting time of the rats given 125, 250 and 500 mg/kg p.o. of methanol extracts of Korean and European mistletoes

Group	Samples	Control	Sample doses by p.o. (mg/kg)[g]		
			125	250	500
BT[a]	MKM[d]	144.8±25.5	299.7±73.1* (107.0)[f]	395.7±87.5** (173.3)	413.6±58.8*** (185.6)
	MEM[e]		244.2±28.8** (68.6)	290.4±49.3** (103.0)	399.2±88.5** (175.7)
WBCT[b]	MKM	129.4±7.5	175.9±7.1**** (35.9)	218.8±7.2***** (69.1)	204.7±8.0**** (58.2)
MEM			192.9±11.1**** (49.1)	188.8±5.3***** (45.9)	193.3±5.0***** (48.8)
PCT[c]	MKM	167.9±10.9	283.9±22.2***** (69.1)	326.1±19.2***** (94.2)	319.6±17.9***** (90.4)
	MEM		208.6±14.6**** (24.2)	256.0±11.0***** (52.5)	311.6±16.3***** (85.6)

[a]BT: Bleeding time (Mean±S.E., second).
[b]WBCT: Whole blood clotting time (Mean±S.E., second).
[c]PCT: Plasma clotting time (Mean±S.E., second).
[d]MKM: methanol extract of Korean mistletoe.
[e]MEM: methanol extract of European mistletoe

[f]Prolongation (%) = $\frac{\text{Clotting time of Samples-Clotting time of control}}{\text{Clotting time of control}} \times 100$

[g]No. of animals tested: Control, 24, the methanol extracts of Korean and European mistletoes, 15 and 6 animals/group.
*P<0.1, **P<0.05, ***P< 0.005, ****P< 0.0005, *****P< 0.0001.

Table 2. The change of body weight on day 12 after the treatment of methanol extracts of Korean and European mistletoes to the mice

Samples (mg/kg, p.o.)	Body weight on day 12 after samples treated (g)[d]			
	control	125	500	1000
MKM[a]	195.3±10.2 (20.9)[c]	201.1±4.4 (20.6)	203.2±7.4 (21.7)	197.3±8.3 (18.9)
MEM[b]		202.2±7.0 (21.0)	192.2±7.5 (17.4)	201.8±8.1 (22.2)

[a]MKM: Methanol extract of Korean mistletoe (Mean±S.E., second).
[b]MEM: Methanol extract of European mistletoe (Mean±S.E., second).

[c]( ): body weight gain (%) = $\frac{\text{B.W. gain on day 12 - B.W. gain on day 1}}{\text{B.W. gain on day 1.}} \times 100$

[d] No. of animals tested: Control, 24, the methanol extracts of Korean and European mistletoes, 15 and 6 animals/group.

preincubated at 37°C for 3 min., 200 μl of 1.7% $CaCl_2 \cdot H_2O$ was added, and was incubated at 37°C until the reaction mixture is clotted. The same procedure was applied for plasma clotting time except 100 μl of plsama, 50 μl of saline or sample solutions, and 50 μl of 25 mM $CaCl_2$ H_2O. The clotting time was defined as the period from when the inducer is added to when the whole blood or plasma is clotted. The prolongation effect was obtained by the following equation.

$$\text{Prolngation}(\%) = \frac{\text{Clotting time of Samples} - \text{Clotting time of control}}{\text{Clotting time of control}} \times 100$$

Determination of LD_{50} – The mistletoe extracts (in 1% CMC) at 7 different doses ranging from 0.06-4 g/kg were given to each group of 10 ICR male mice, weighing between 17-25 g. The dose volume was 10 ml/kg and animals were observed for 14 days. LD_{50} values were graphically estimated by probit analysis.

The [^{3}H]PAF binding to rabbit platelets was carried out according to Valone's method (Valone, F.H., *et al.* 1982) with some modifications (Yang, H.O., *et al.* 1995).

Results and Discussion

The HPLC analysis revealed that the methanol extract of the Korean mistletoe contains flavoyadorinin B (0.12± 0.003%) and homoflavoyadorinin B (0.958±0.016%), whereas these two compounds were not detected in that of European mistletoe under the same conditions. This fact is also supported by the literature study (Fukunaga, T., *et al.* 1989).

Besides the bleeding time of the methanol extract of *Viscum album* var. *coloratum* (Korean mistletoe) treated groups were increased by 185.6% (413.6±58.8(s), n=15, $p<0.005$) compared to that of the control group (144.8± 25.5(s), n=24), the methanol extract of European mistletoe treatment prolonged bleeding time by 175.7% (399.2±88.5, n=6, $p<0.05$) at the same dose of 500 mg/kg (Table 1). However, the water extracts of Korean and European mistletoes did not show any prolongation effects. (Data are not shown) There were not any significant differneces in the change of body weight on day 12 after the treatment of the MeOH extracts to rats compared to those of the control group (Table 2).

The MeOH extract of Korean mistletoe showed the prolongation effects of whole blood clotting time and plasma recalcification time by 58.2% (204.7±8.0(s), n=15, $p<0.0005$) and 90.4% (319.6±17.9(s), n=15, $p<0.0001$) compared to those of the control groups, 129.4±7.5(s), n=24 and 167.9±10.9(s), n=24, respectively. These prolongation effects are comparable to those of European mistletoe at the same dose of 500 mg/kg (Table 1). The water extracts of Korean and European mistletoes did not show any prolongation effects in these tests.

When given orally, the LD_{50} values of the MeOH extracts of Korean and European mistletoes were more than 2 g/kg. There were not any significant differneces in the change of body weight on day 14 after the treatment of the methanol extracts to mice compared to those of the control group. To define the mode of action, platelet activating factor (PAF) receptor binding assay was carried out. The methanol extracts of Korean and European mistletoes have no inhibitory activity on the PAF receptor binding to rabbit platelet using [^{3}H]PAF as a ligand (Data are not shown). These results suggest that the prolongation effects of bleeding time of mistletoes are not contributed by inhibitory activity of platelet activating factor receptor binding. In conclusion, the methanol extract of Korean mistletoe might be a potential candidate to develop new drug to improve blood circulation.

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