Pharmacologically Active Components from a Peruvian Medicinal Plant, Huira-Huira (*Culcitium canescens* H. & B.)

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The methanol extract of Huira-Huira (*Culcitium canescens*) showed analgesic effects in acetic acid-induced writhing and tail pressure tests, and it also produced potent prolongation of hypnosis induced by pentobarbital. The latter activity was used as an isolation-guide to determine the active components which were identified as dehydrocacalohastine, cacalohastine and cacalonol.

Keywords *Culcitium canescens*; dehydrocacalohastine; cacalohastine; cacalonol; hypnosis-prolongation; Peruvian medicinal plant

During our assay of traditional herbal drugs having neurotropic effect,[1] an extract of the South American medicinal plant, Huira-Huira (*Culcitium canescens* H. & B., syn. *Senecio canescens* (H. & B.) CUATR.; Compositae) indicated significant effects in pharmacological tests such as prolongation of pentobarbital-induced hypnosis and acetic acid writhing. This perennial plant having pilose leaves is used for the treatment of fever, cough, catarrh, pain around the breast, *etc.* in Peru and nearby countries.[2]

The methanol extract of Huira-Huira showed an analgesic effect in the acetic acid-induced writhing test at oral doses of 2 and 3 g/kg in mice without a dose-dependent manner (Fig. 1). In the tail pressure test, the pain threshold level was increased dose-dependently at the same dose-levels as shown Fig. 2.

Tendency of an antipyretic effect was observed by administration of 2 and 3 g/kg to mice, although it was not significant (Fig. 3).

The extract exerted potent prolongation of pentobarbital-induced hypnosis in mice when administered at 1 g/kg (Fig. 4). Since period of prolongation over 180 min in each mouse was still calculated as just 180 min, the figure does not indicate any dose-dependency.

In the following experiment, the active components were isolated using the prolongation effect of hypnosis as a guide (Chart 1). The extract was partitioned with ethyl acetate and water, and the active ethyl acetate fraction was separated by silica gel column chromatography. The less potent fraction, fr. 1C gave crystals tentatively called compound-1 which were weakly effective. The most potent fraction, fr. 1B was further chromatographed to get the active fraction, fr. 2A. Since fr. 2A was not clearly fractionated by further flash chromatography, the major fractions from fr. 2A were separated repeatedly by medium pressure liquid chromatography (MPLC) to yield active components, compounds-2 and -3.

The molecular formula, $C_{15}H_{14}O_3$ of compound-1 was obtained by high resolution MS (HR-MS). The structure was estimated to be cacalonol by ^1H- and ^{13}C-NMR (see the experimental part) including differential nuclear Overhauser effect (NOE) and 2D-NMRs such as correlation spectroscopy (COSY) and Hetronuclear multiple bond correlation spectroscopy (HMBC), and was

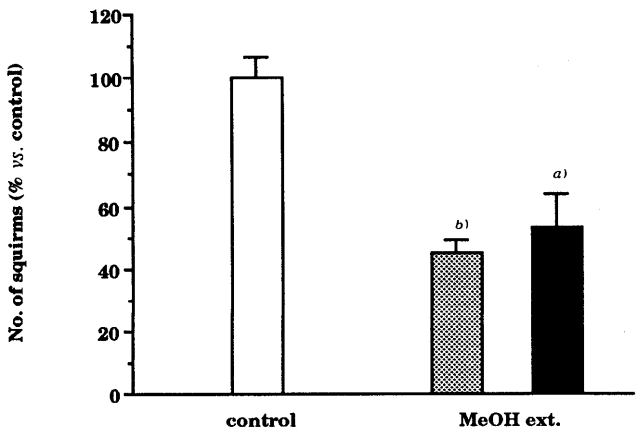

Fig. 1. Analgesic Effect of the Extract on Acetic Acid-Induced Writhing in Mice

Each bar represents the mean ± S.E. The number of squirms in control (35.3 ± 2.3) was taken as 100%. *a*) $p<0.01$, *b*) $p<0.001$. $n=6$. □, control; ▨, 2 g/kg; ■, 3 g/kg.

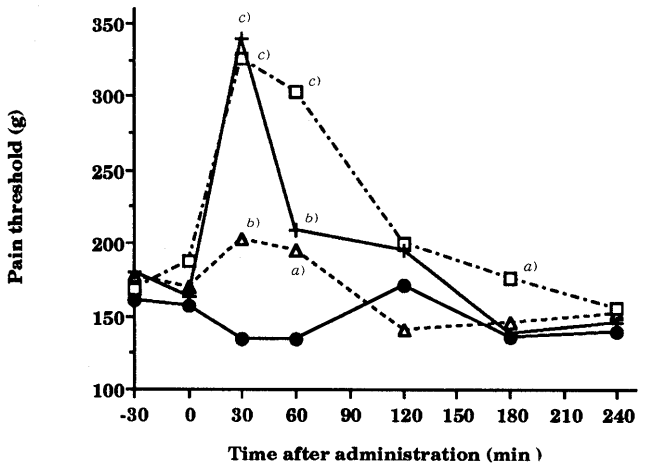

Fig. 2. Analgesic Effect of the Extract on the Pressure Pain Threshold in Mice

a) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$. $n=5$—7. AP, aminopyrine. —●—, control; ---△---, 2 g/kg; –·–□–·–, 3 g/kg; —+—, AP 100 mg/kg.

identified by comparison of the spectral data (UV and ^{1}H- and ^{13}C-NMR) with that already published.[3] Compound-1 has no optical activity and might be an artifact as mentioned in references 3*a* and *b*.

The structures of compounds-2 and -3 were estimated by spectroscopic method and comparison of the spectral data with those of compound-1. Both compounds were

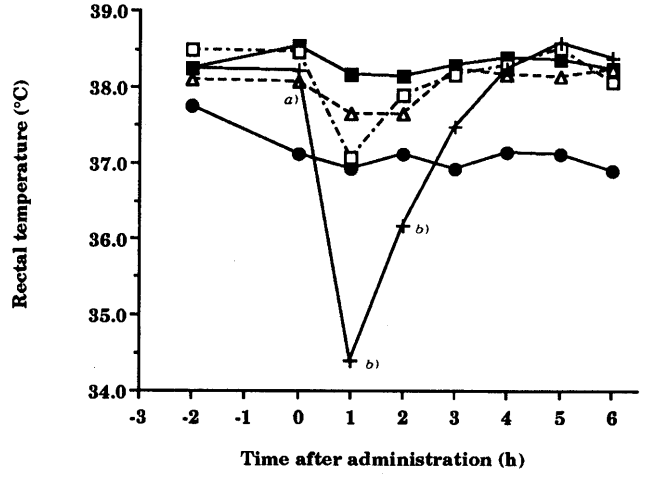

Fig. 3. Antipyretic Effect of the Extract in LPS-Treated Mice

Non-treated mice and LPS-treated mice were used for control and LPS(control), respectively. Significance of each sample expresses the statistical difference from LPS (control). *a*) $p<0.05$, *b*) $p<0.001$. $n=6$—7. AP, aminopyrine. —●—, control; —■—, LPS (control); ---△---, 2 g/kg; -·-□-·-, 3 g/kg; —+—, AP 100 mg/kg.

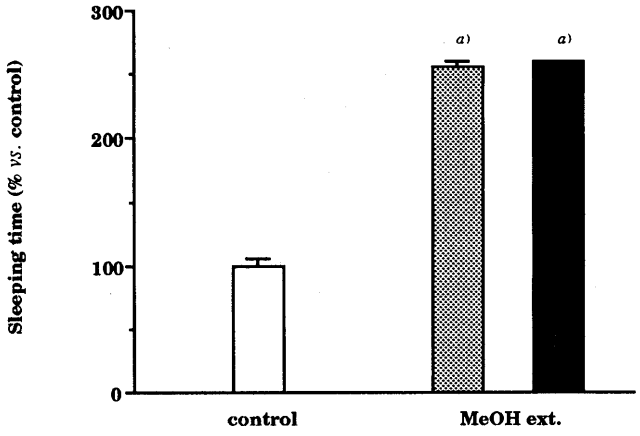

Fig. 4. Effect of the Extract on Prolongation of Hypnosis in Pentobarbital-Treated Mice

Each bar represents the mean ± S.E. The sleeping time of control (69.2 ± 4.1 min) was taken as 100%. Times which exceeded over 180 min in each mouse were still calculated as 180 min. *a*) $p<0.001$. $n=6$. □, control; ▨, 1 g/kg; ■, 2 g/kg.

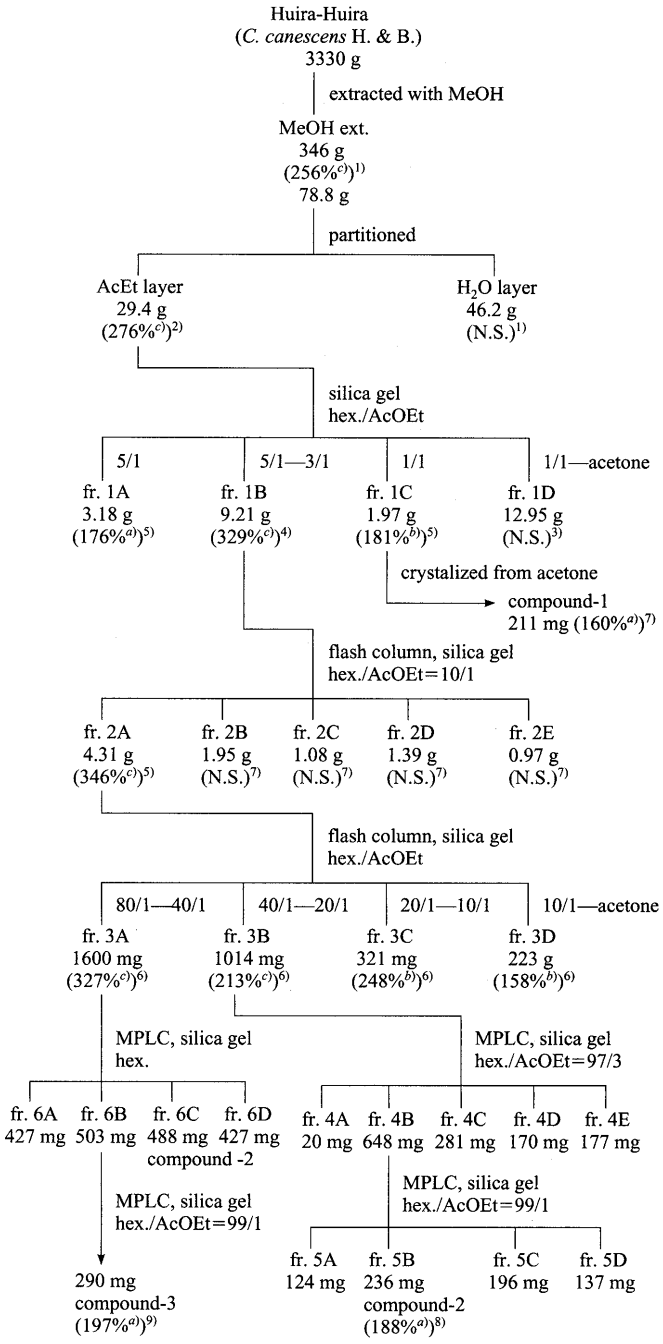

Chart 1

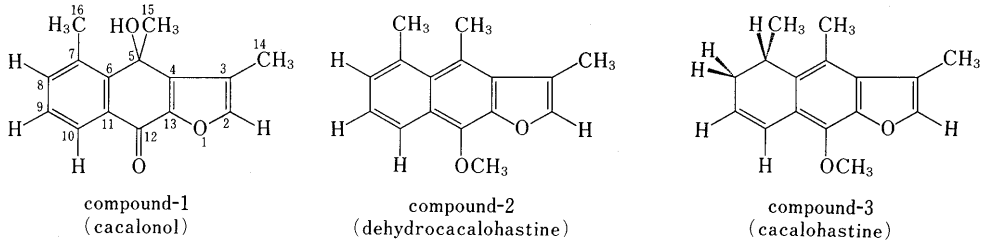

Fig. 5. Structures of Compounds-1—3

Chart 1. Isolation Procedure of the Active Components from Huira-Huira

(Sleeping time, % *vs.* control). 1) 1 g/kg, 2) 500 mg/kg, 3) 230 mg/kg, 4) 170 mg/kg, 5) 100 mg/kg, 6) 60 mg/kg, 7) 50 mg/kg, 8) 30 mg/kg, 9) 10 mg/kg, *p.o.* *a*) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$. $n=5$—6. N.S., no significance.

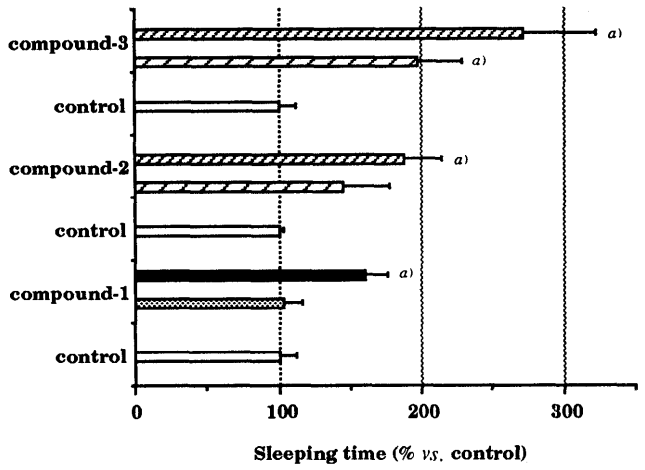

Fig. 6. Effects of Compounds-1—3 on Prolongation of Hypnosis in Pentobarbital-Treated Mice

Each bar represents the mean±S.E. The sleeping time of each control (44.8±5.4, 61.4±1.9 or 42.7±5.0 min) for compounds-1, -2 and -3, respectively, was taken as 100%. *a)* $p<0.05$. $n=6$. □, control; ▨, 10 mg/kg; ▩, 20 mg/kg; ▩, 30 mg/kg; ■, 50 mg/kg.

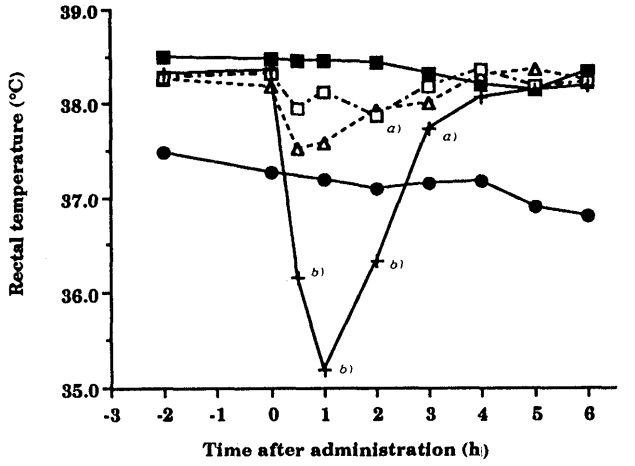

Fig. 7. Antipyretic Effect of Compound-3 in LPS-Treated Mice

Non-treated mice and LPS-treated mice were used for control and LPS(control), respectively. Significance of each sample expresses the statistical difference from LPS (control). *a)* $p<0.05$, *b)* $p<0.001$. $n=7$—8. AP, aminopyrine. —●—, control; —■—, LPS (control); ---△---, 20 mg/kg; -·-□-·-, 50 mg/kg; —+—, AP 100 mg/kg.

identified with dehydrocacalohastine and cacalohastine, respectively, by comparing with the ^{1}H- and ^{13}C-NMR data.[4] This was the first report of isolation of these compounds from *C. canescens*.

The effects of the compounds on hypnosis induced by pentobarbital are shown in Fig. 6. Compound-3 (cacalohastine) had the most potent effect, and prolonged the hypnosis by 197% ($p<0.05$) over control at an oral dose of 10 mg/kg. Similar potency was observed by administration of 30 mg/kg of compound-2 (dehydrocacalohastine). Compound-1 required 50 mg/kg for 160% ($p<0.05$) prolongation.

This medicinal plant has been used for the treatment of fever, however, the antipyretic effect of compound-3 was not clear in mice at doses of 20 and 50 mg/kg (Fig. 7), which was a similar observation to that found with the extract. Because of limitation in the amount of available sample, analgesic effect which was exerted in the extract was not tested for these compounds.

To determine whether the prolongation of the hypnosis was due to inhibition of drug metabolism in liver, compounds-1, -2 and -3 were investigated for their effect on aminopyrine N-demethylase activity in liver microsomes of mice. They did not inhibit the enzyme, and therefore were suggested to have some neuronal effect.

Experimental

Melting points were determined by a Yanagimoto micro melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO J-20 polarimeter. IR spectra were recorded on a Hitachi 260-10 spectrophotometer, UV spectra on a Hitachi U-3400 spectrophotometer, and MS spectra on a JEOL HX-110A spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded on JEOL-JNM-GSX 400, GSX 500 and A500 spectrometers. The following abbreviations are used: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; m, multiplet; br, broad; sh, shoulder. Column chromatographies were performed on Wakogel C-200 and Silica gel 60. Pre-packed column (Kusano CPS-HS-221-5) was used for MPLC.

Isolation Huira-Huira (*Culcitium canescens* H. & B.) was provided by Mr. T. Shiota of Peru, in July, 1991, and was identified by himself and Satake, one of the authors. The dried aerial part (3.33 kg) was extracted with methanol at room temperature to obtain the methanol extract (346 g). The extract (78.8 g) was then partitioned with ethyl acetate and water, and the active ethyl acetate fraction (29.4 g) was applied to silica gel chromatography. Hexane–ethyl acetate eluants (fr. 1A—C) showed the activity. Crystals obtained from fr. 1C (1.97 g) were purified by crystallizaion with acetone to yield compound-1 (211 mg). The most potent fraction, fr. 1B eluted with hexane–ethyl acetate 5/1—3/1, was flash chromatographed on silica gel. The active fractions from fr. 2A (4.31 g) were further purified repeatedly by MPLC (silica gel, hexane, hexane–ethyl acetate 97/3 or 99/1). Compounds-2 and 3 were obtained in the amounts of 724 and 290 mg, respectively.

Compound-1: mp 207—208.5 °C (light yellow needles from acetone; lit.[3a] mp 201—203 °C). HR-FAB-MS *m/z* (%): 281.0584 $(M+K)^+$ (err. +0.4 mmu) for $C_{15}H_{14}O_3K$, 243.1030 $(M+1)^+$ (err. +0.9 mmu) for $C_{15}H_{15}O_3$. Optical rotatory dispersion (ORD) (400—650 nm) in methanol: 0.0° ($c=1.23$ mg/ml). IR ν_{max}^{KBr} cm^{-1}: 3390, 1655, 1585, 1535, 1415, 1225, 1085, 795, 775. UV λ_{max}^{MeOH} nm (log ε): 210 (4.16), 236 (3.89), 256 (3.84), 310 (4.14). ^{1}H-NMR (DMSO-d_6) δ: 1.73 (3H, d, $J=1.0$ Hz, H_3-15), 2.25 (3H, d, $J=1.0$, H_3-14), 2.76 (3H, s, H_3-16), 5.98 (1H, d, $J=1.0$, 5-OH), 7.39 (1H, dd, $J=7.8$, 7.5 Hz, H-9), 7.48 (1H, ddd, 7.5, 1.5, 0.7, H-8), 7.88 (1H, q, $J=1.0$ Hz H-2), 8.00 (1H, ddd, $J=7.8$, 1.5, 0.5 Hz, H-10). ^{13}C-NMR (DMSO-d_6) δ: 8.75 (C-14), 21.31 (C-16), 27.31 (C-15), 70.12 (C-5), 120.88 (C-3), 124.26 (C-10), 127.32 (C-9), 131.18 (C-11), 137.06 (C-8), 137.57 (C-7), 143.46 (C-13), 144.60 (C-4), 146.71 (C-2), 146.76 (C-6), 172.02 (C-12); assignment of C-4 and -11 and C-6 and -7 are reversed in reference 3*c*.

Compound-2: mp 78—79 °C (colorless needles from acetone; lit.[3a] 76—78 °C). IR ν_{max}^{KBr} cm^{-1}: 1630, 1445, 1390, 1360, 1105, 755. UV λ_{max}^{EtOH} nm (log ε): 220 (4.22), 247 (4.74), 252 (4.81), 323 (3.89), 335 (3.89), 351 (3.89). ^{1}H-NMR ($CDCl_3$) δ: 2.50 (3H, *d*, $J=1.3$ Hz, H_3-14), 2.97 (3H, s, H_3-16), 3.14 (3H, s, H_3-15), 4.21 (3H, s, H_3-17), 7.21 (1H dt, $J=6.8$, 1.3 Hz, H-8), 7.28 (1H, dd, $J=8.6$, 6.8 Hz, H-9), 7.41 (1H, q, $J=1.3$, H-2), 8.20 (1H, ddd, $J=8.6$, 1.3, 0.5 Hz, H-10). ^{13}C-NMR ($CDCl_3$) δ: 12.42 (C-14), 19.42 (C-15), 26.81 (C-16), 61.14 (C-17), 116.57 (C-3), 120.53 (C-10), 122.93 (C-5), 123.67 (C-9), 126.74 (C-11), 128.16 (C-8), 130.46 (C-4), 130.86 (C-6), 135.20 (C-7), 136.74 (C-12), 143.21 (C-13), 143.59 (C-2).

Compound-3: mp 87—88 °C (colorless needles from acetone; lit.[4b] mp 84.0—85.5 °C). $[\alpha]_D^{20}$ +93.8° (calcd by ORD in methanol, $c=1.41$ mg/ml; lit.[4b] +90.5°). IR ν_{max}^{KBr} cm^{-1}: 2950, 1600, 1470, 1330, 1225, 1110, 1000. UV λ_{max}^{EtOH} nm (log ε): 224 (4.25), 241 (4.13), 249 (4.08), 2.74 (sh, 4.21), 284 (4.35), 294 (sh, 4.27). ^{1}H-NMR ($CDCl_3$) δ: 1.07 (1H, d, $J=7.1$ Hz, H_3-16), 2.23 (1H, ddd, $J=17.1$, 6.4, 1.5 Hz, H_a-8), 2.38 (3H, d, $J=1.2$ Hz, H_3-14), 2.52 (3H, s, H_3-15), 2.51—2.57 (1H, m, H_b-8), 3.25 (1H, qdt, $J=7.1$, 6.9, 1.2 Hz, H-7), 4.05 (3H, s, H_3-17), 5.91 (1H, dddd, $J=9.7$, 6.4, 2.4, 1.0 Hz, H-9), 6.91 (1H, dd, $J=9.7$, 3.1 Hz H-10), 7.29 (1H, q, $J=1.2$ Hz H-2). ^{13}C-NMR ($CDCl_3$-d_6) δ: 11.31 (C-14), 13.65 (C-15), 19.27 (C-16), 27.79 (C-7), 30.71 (C-8), 61.05 (C-17), 116.85 (C-3), 121.00 (C-11), 121.32 (C-10), 122.08 (C-5), 124.63 (C-9), 128.35 (C-4), 134.09

(C-6), 138.88 (C-12), 141.57 (C-2), 145.81 (C-13).

Pharmacological Assay Male ddy mice (5 weeks) weighing 22—35 g bred at Japan SLC, Inc. (Hamamatsu, Japan) were allowed free access to food and water, and were housed under a 12 h light/dark cycle at 22—25 °C. Food was withheld 2 h before the experiments. Test samples were suspended in saline with 5% gum arabic and/or 10% Tween 80.

Analgesic Effect by the Acetic Acid-Induced Writhing Test: A slightly modified Whittle's method[5] was used. Samples were given 40 min prior to an intraperitoneal injection of 0.7% acetic acid (0.1 ml/10 g mouse). The number of squirms was counted in each mouse for 15 min beginning 5 min after the injection.

Analgesic Effect by the Tail Pressure Test[6]: The gradient pressure was given at the base of the tail using an analgesy-meter (Ugo Basile, Italy). Prior to the experiment, mice were tested twice, and those having a pressure range of 100—250 g for pain-reaction were used. The reaction time of animals was measured and noted at 30, 60, 120, 180 and 240 min following sample administration.

Effect on Pentobarbital-Induced Hypnosis: Samples were administered 40 min before intraperitoneal injection of 50 mg/kg of sodium pentobarbital (Tanabe Pharmaceutical Co., Ltd.). The time required to regain the righting reflex was measured.

Antipyretic Effect: Mice having a higher body temperature than the mean temperature of the control (saline) were used 12 h after the subcutaneous injection of LPS (lipopolysaccharide). After 14 h of LPS-treatment, samples were administered, and the rectal temperatures were measured with a thermister (Takara Instruments Co., Ltd., Japan).

Effect on Drug Metabolism in Liver: Liver microsomes of the mice were prepared as described previously.[7] The reaction mixture consisted of 0.1 M potassium phosphate buffer (pH 7.4), 0.1 mM EDTA, an NADPH-generating system (0.33 mM NADP, 8 mM glucose 6-phosphate, 0.1 unit of glucose 6-phosphate dehydrogenase and 6 mM $MgCl_2$), liver microsomes (0.3—1.0 mg of protein) and aminopyrine (5 mM) in a final volume of 1.0 ml. The activity of aminopyrine N-demethylase was estimated by determination of formaldehyde-content by the method of Nash.[8] Each sample and the positive control (SKF-525A) were measured using several concentrations up to 0.50 mM.

Statistics: Statistical significance was evaluated by the Student's *t* test.

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References

1) Some earlier publications are: E. Okuyama, K. Umeyama, Y. Saito, M. Yamazaki, M. Satake, *Chem. Pharm. Bull.*, **41**, 1309 (1993); E. Okuyama, T. Nakamura, M. Yamazaki, *ibid.*, **41**, 1670 (1993); E. Okuyama, S. Nishimura, S. Ohmori, Y. Ozaki, M. Satake, M. Yamazaki, *ibid.*, **41**, 926 (1993).
2) J. Soukup, "Vocabulario de los Nombres Vulgares de la Flora Peruana y Catalogo de los Generos," Editorial Salesiana, Lima, Peru, p. 371; N. A. Chávez Velásquez, "La Materia Medica en el Incanato," Editorial Mejía Baca, Lima, Peru, 1977, p. 329.
3) *a*) T. Takemoto, G. Kusano, K. Aota, M. Kaneshima, N. A. El. Emary, *Yakugaku Zasshi*, **94**, 1593 (1974); *b*) K. Naya, Y. Miyoshi, H. Mori, K. Takai, M. Nakanishi, *Chem. Lett.*, **1976**, 73; *c*) S. Abdo, M. de Bernardi, G. Marinoni, G. Mellerio, S. Samaniego, G. Vidari, P. V. Finzi, *Phytochemistry*, **31**, 3937 (1992).
4) *a*) F. Bohlmann, C. Zdero, *Chem. Ber.*, **111**, 3140 (1978); *b*) K. Hayashi, H. Nakamura, H. Mitsuhashi, *Phytochemistry*, **12**, 2931 (1973).
5) B. A. Whittle, *Br. J. Pharmacol.*, **22**, 246 (1964).
6) K. Takagi, M. Harada, *Yakugaku Zasshi*, **89**, 879 (1969).
7) M. Kitada, T. Igarashi, T. Kamataki, H. Kitagawa, *Jpn. J. Pharmacol.*, **27**, 481 (1977).
8) T. Nash, *Biochem. J.*, **52**, 416 (1953).