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Analgesic activity of the lignans from *Lychnophora ericoides*

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Abstract

Lychnophora ericoides is a Brazilian medicinal plant that is commercially available as an analgesic and anti-inflammatory agent. The extract from roots, which yielded 10 lignans, showed analgesic activity in the mouse writhing test and the lignan, cubebin, was one of the most active. Anti-inflammatory and anti-pyretic activities from cubebin (10 mg/kg) revealed no significant effects. In addition two previously unknown methyl clusin derivatives are reported.

Keywords: *Lychnophora ericoides*; Asteraceae; Lignans; Analgesic activity; Methylclusin; Cubebin

1. Introduction

The genus *Lychnophora* (Asteraceae) is widely distributed in Brazil and some species are popularly known as ''arnica'', ''falsa arnica'' or ''arnica da serra''. The plants are used in folk medicine as analgesic and anti-inflammatory agents (Cerqueira et al., 1987). Phytochemical investigation of *Lychnophora* species has revealed the occurrence of flavonoids and sesquiterpene lactones, some of them showing trypanocidal activity (Chiari et al., 1991, 1996; Bazon et al., 1997; Jordão et al., 1997; Borella et al., 1998; Grael et al., 2000). Among the *Lychnophora* species, *Lychnophora ericoides* (Mart) is the most popular species, whose intact leaves and root powder are commercially used as analgesic and anti-inflammatory agents. A dichloromethane extract of *Lychnophora ericoides* leaves also yielded sesquiterpene lactones and flavonoids (Borella et al., 1998). However, no data are available about the chemical composition or biological activity of the roots of *Lychnophora* species. The folk medicinal usage of this plant and the absence of chemical data about its roots motivated us to study the phytochemical and the analgesic, anti-inflammatory and anti-pyretic activities of the extract and of the major compounds isolated from roots of *Lychnophora ericoides*.

2. Results and discussion

The dichloromethane extract of *L. ericoides* roots showed significant analgesic activity, but the leaf extract had no activity (Fig. 1). The active extract was suspended in $MeOH/H_2O$ and partitioned with hexane and CH_2Cl_2 to yield hexane, CH_2Cl_2 and MeOH fractions. When these fractions were tested in the writhing mouse model only fraction CH_2Cl_2 showed analgesic activity. This active fraction was submitted to CC followed by prep. TLC, prep. HPLC or circular chromatography (Chromatotron) yielding **2** (Koul et al., 1983), **3** (Koul et al., 1984), α-methylclusin **8**, and β-methylclusin **9**, in addition to the major compounds hinoquinin **1** (Lopes et al., 1983), α,β-cubebin **4**, **5** (Batterbee et al., 1969), α,β-methylcubebin **6**, **7** (Rücker et al., 1981) and dihydrocubebin **10** (Tillekeratne et al., 1982) (Fig. 2). Cubebin (**4**,**5**) and methylcubebin (**6**,**7**) were identified as a mixture of β and α anomers as previously described (Rücker et al., 1981; Badheka et al., 1987; Blumenthal et al., 1997) and the other above known compounds were identified by comparison with reported spectroscopic data.

Nearly all relevant spectral details of the **8** + **9** mixture were closely comparable to the corresponding features of the lignan clusin isolated from *Piper clusii* (Koul et al., 1983). All the differences in the ^1H NMR and IR spectra could be attributed to the presence of a methoxyl group at C-9 and suggested the presence of a mixture of an α–β methyl anomers. After HPLC purification, compounds **8** and **9** were confirmed to be isomers ($C_{23}H_{28}O_7$) by

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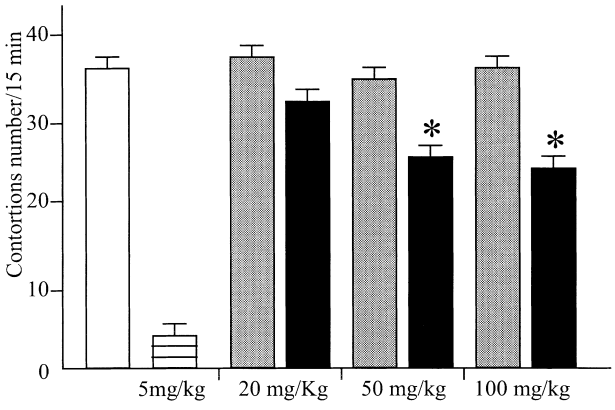
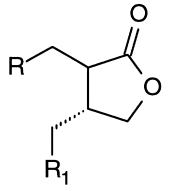


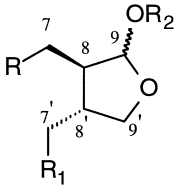
Fig. 1. Effects of root and leaf extracts on acetic acid-induced writhing in mice. Animals were treated orally with vehicle (▭), indomethacin (▤) or extracts from leaves (▩) or roots (■), at the doses indicated in the figure. All treatments were made 30 min before the stimulus. The data represent the mean±SEM for 6 animals per group. $^{*}P<0.05$.

mass spectroscopy ($[M]^{+}$ m/z 416) and elemental analysis. The presence of a methoxyl group at C-9 in each compound was evident from the three proton singlet at 3.30 (β) and 3.31 (α) in their ^{1}H NMR spectra. The orientation of the methoxyl group was established by comparing the signal for the C-9 protons with those of α- and β-methyl or ethyl cubebins (Rücker et al., 1981; Badheka et al., 1987; Blumenthal et al., 1997) and with synthetic enantiomerically pure lignans (Rehnberg and Magnusson, 1990). Compounds **8** and **9** showed the acetal proton (C-9) at 4.53 (*d*, $J=4.5$ Hz) and 4.72 (*s*), respectively, and these chemical shifts were in agreement with the same β-OMe (4.63 ppm, *d*, $J=4.46$ Hz) and α-OMe (4.68, *s*) orientation proposed by Rehnberg and Magnusson (1990). In the HMBC of **8**, the proton signals at $\delta=4.53$ and 6.3 showed correlations with C-9 (51.5); C-7 (33.8) and C-7 (33.8); C-1 (130.7), respectively. In addition, measurement of their optical rotations confirmed that compounds **8** and **9** are β-methylclusin and α-methylclusin. Finally, we present ^{13}C NMR spectral data for lignan 3 whose ^{1}H NMR spectral data were only previously described (Koul et al., 1984).

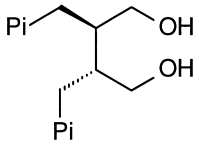
The analgesic activity of the major compounds isolated are shown in Fig. 3, in which it can be observed that cubebin and methylcubebin at doses of 10, 20 and 40 mg/kg exerted a significant analgesic activity in the acetic acid-induced writhing in mice. This figure also shows that the analgesic activity of cubebin was slightly more pronounced than that observed for methylcubebin. Although at the doses used (10, 20, 40 mg/kg) these compounds did not produce a dose-dependent antinociception, the dose of 5 mg was ineffective as were the other lignans.

Compared to indomethacin, cubebin, 10 mg/kg, was ineffective in reducing the febrile response induced by intravenously injected LPS (ΔT°C, 3 h after LPS, controls: 1.12±0.04; indomethacin treated: 0.44±0.13; cubebin: l.25±0.11, $n=10$ for each group). Moreover, cubebin slightly reduced the rat paw edema induced by intraplantar injection of carrageenan [Δ paw volume (μl), 3 h after carrageenan injection, controls: 760±0.6; indomethacin treated: 464±0.9; cubebin: 642±0.13, $n=10$ for each group].





	R	R_1		R	R_1
1. Hinoquinin	Pi	Pi	**4**. α-Cubebin	Pi	Pi
2	Tf	Pi	**5**. β-Cubebin	Pi	Pi
3	Tf	Tf	**6**. α-Methylcubebin	Pi	Pi
			7. β-Methylcubebin	Pi	Pi
			8. α-Methylclusin	Tf	Pi
			9. β-Methylclusin	Tf	Pi



10. Dihydrocubebin

Fig. 2. Lignans isolated from the roots of *Lychnophora ericoides*.

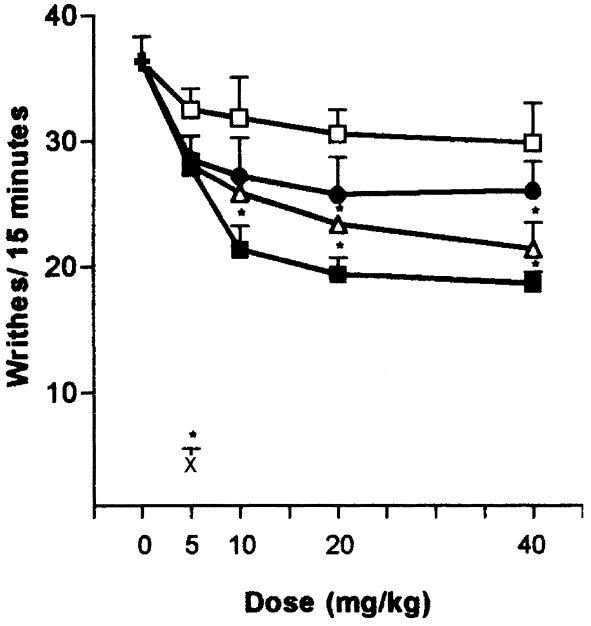


Fig. 3. Effects of the major isolated compounds on acetic acid-induced writhing in mice. Animals were treated orally with vehicle (+), indomethacin (**X**), dihydrocubebin (□), hinoquinin (●), methylcubebin (△) or cubebin (■), at the doses indicated in the figure. All treatments were made 30 min before the stimulus. The data represent the mean±SEM for 6 animals per group. $^{*}p < 0.05$.

Although lignans occur in Vernoniae (Asteraceae) (Bohlmann and Jakupovic, 1990), the present study reports for the first time the presence of these compounds in the Brazilian species of the Asteraceae. The analgesic effects induced by the root extracts, containing cubebin and methylcubebin, support the popular use of the "arnica". Comparison to similar major lignans revealed that the cyclic hemiacetal is important for the analgesic activity. These results show that, like indomethacin, cubebin has antinociceptive activity. This property of indomethacin is related to its capacity to inhibit cyclooxygenase-1 and 2 (COX-1 and COX-2) (Vane, 1971; Vane and Botting, 1996), the enzymes that convert arachidonic acid to prostaglandins (Xie et al., 1992) and are involved in modulating fever (Milton and Wendlandt, 1970) and inflammatory pain (Ferreira, 1972, 1990). However, since cubebin at this dose (10 mg/kg) did not show any anti-pyretic and anti-edematogenic activities, it is not plausible that it acts by this way, and further studies are necessary to clarify the mechanism of the analgesic effect of cubebin.

3. Experimental

3.1. General

Prep. TLC was carried out on Si gel PF-254 (Merck) and CC on Si gel 60H (0.005–0.045 mm). HPLC analyses were performed on a Shimadzu LC-6A apparatus with a UV detector SPD-6AV. Optical rotations were measured on a Polamat Jasco DIP-370. The ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a Brüker DPX-300 in $CDCl_3$ with TMS as int. standard. FT-IR was obtained on a Nicolet-protégé-460. EI-MS (70 eV) and elemental analysis were recorded on Hewlett–Packard 5988-A and Perkin–Elmer CNH-2400 instruments, respectively. Edema was measured with a plethysmometer (Ugo Basile).

3.2. Plant material

Roots and leaves from *Lychnophora ericoides* were collected in Delfinópolis, Minas Gerais, Brazil and were identified by Prof. Dr João Semir, Campinas University, where the voucher specimen is deposited (NPL-123).

3.3. Isolation of constituents

Air-dried and powdered roots (170 g) were extracted with CH_2Cl_2 at room temperature. The extract (9 g) was suspended in 200 ml of MeOH–H_2O (8:2) and extracted with hexane (3×50 ml) followed by CH_2Cl_2 (3×75 ml) and yielded 3 g of a hexane fraction, 4.2 g of a CH_2Cl_2 fraction and 0.5 g of a MeOH fraction. The CH_2Cl_2 fraction was submitted to flash CC (252 g), eluted with a hexane–EtOAc gradient of increasing polarity and this gave 5 pooled frs. Fr. A (120 mg) was purified by TLC ($CHCl_3$–MeOH, 97:3) and furnished **4**+**5** (40 mg). Fr. B (2.0 g), Fr. D (350 mg) and Fr. E (120 mg) were individually submitted to circular chromatography and yielded the indicated compounds: Fr. B, cubebin (250 mg), methylcubebin (100 mg) and hinoquinin (20 mg); Fr. D, **2** (20 mg) and **8**+**9** (40 mg) and fr. E, **3** (18 mg). Fr. C (500 mg) was purified by prep. HPLC (ODS-Shimadzu, 5.0×250 mm column, MeOH–H_2O 60:40, $\lambda = 280$ nm, flow 8 ml/min) afforded hinoquinin (40 mg) and dihydrocubebin (90 mg). Finally the mixture of α–β-methylclusin was submitted to prep. HPLC (Silica, S/L (H) Shimadzu 5.0×250 nm column, hexane–ETOAc 7:3, $\lambda = 280$ nm flow 8 ml/min) and yielded β-methylclusin (15 mg) and α-methylclusin (7 mg).

3.4. Compound *3*

^{13}C NMR (75 MHz, $CDCl_3$), δ 35.0 (C-7), 38.9 (C-7′), 41.1 (C-8′), 46.5 (C-8), 56.1 (-OMe), 60.8 (-OMe), 71.1 (C-9′), 105.8 (C-2, C-6), 106.3 (C-2′, C-6′), 133.6 (C-1), 133.9 (C-1′), 136.9 (C-4), 137.0 (C-4′), 153.3 (C-3, C-5), 153.4 (C-3′, C-5′), 178.5 (C-9).

3.5. β-Methylclusin (8 R, 8′R, 9S)-9 β-methoxy-3,4,5-trimethoxy-3′,4′-methylenedioxy-8.8′.9.O.9′-lignan-Δ: 1,3,5,1′,3′,5′ (*8*)

Oil. (Found: C, 66.45; H 6.81. $C_{23}H_{28}O_7$ requires: C, 66.34; H, 6.73). $[\alpha]_D^{23} = -3.5^{\circ}$ ($CHCl_3$, **c** 0.2). $\nu_{max}^{CHCl_3}$

cm^{-1}. 2951, 2921, 2850, 1589, 1504, 1489, 1463, 1421, 1244, 1127, 941. ^1H NMR (300 MHz, $CDCl_3$): δ 2.05-1.90 (1 H, *m*, H-8), 2.75-2.30 (3H, *m*, H-7, H-7′, H-8′), 3.30 (3H, *s*, C9-OMe), 3.85 (9H, *s*, Ar-OMe), 3.55 (1H, *dd, J*=8.5 Hz, 6.6 Hz H-9), 3.97 (1H, *t, J*=8.2 Hz, H-9′), 4.53 (1H, *d, J*=4.5 Hz, H-9), 5.90 (2H, *s*, OCH_2O), 6.3 (2H, *s*, H-2, H-6), 6.6 (3H, *m*, H-2′, H-5′, H-6′). ^{13}C NMR (75 MHz, $CDCl_3$) δ 33.8 (C-7), 38.7 (C-7′), 43.2 (C-8′), 51.5 (C-8), 54.6 (C9-OMe), 56.3 (C3-OMe, C5-OMe), 60.8 (C4-OMe), 73.0 (C-9′), 101.1 (OCH_2O), 105.1 (C-9), 105.9 (C-2, C-6), 108.1 (C-2′), 108.8 (C-5′), 121.1 (C-6′), 130.7 (C-1), 133.9 (C-1′), 139.8 (C-4), 145.5 (C-4′), 148.3 (C-3′), 153.1 (C-3, C-5). EIMS 70 eV, *m/z* 416 $[M]^+$ (6), 279 (7), 236 (4), 182 (100), 181 (33), 136 (13), 135 (40).

3.6. α-Methylclusin (8R, 8′R, 9S) -9α-methoxy-3,4,5-tri-methoxy-3′,4′-methylenedioxy-8.8′.9.O.9′ lignan-Δ: 1,3,5, 1′,3′,5′ (**9**)

Oil (Found: C 66.20; H 6.79. $C_{23}H_{28}O_7$ requires C 66.34; H 6.73). $[\alpha]_D^{23} = -19.3°$ ($CHCl_3$, **c** 0.1). ^1H NMR (300 MHz, $CDCl_3$): δ 2.16–2.04 (1H, *m*, H-8), 2.72–2.34 (3H, *m*, H-7, H-7′, H-8′), 3.31 (3H, *s*, C9-OMe), 3.64 (9H, *s*, Ar-OMe), 3.64 (1H, *t, J*=8.1 Hz, H-9′), 4.02 (1H, *t, J*=8.0 Hz, H-9′), 4.72 (1H, *s*, H-9), 5.91 (2H, *sl*, OCH_2O), 6.32 (2H, *s*, H-2, H-6), 6.65–6.57 (3H, *m*, H-2′, H-5′, H-6′). ^{13}C NMR (75 MHz, $CDCl_3$) δ 38.5 (C-7), 39.3 (C-7′), 45.7 (C-8′), 52.4 (C-8), 53.9 (C9-OMe), 56.8 (C3, C5-OMe), 61.2 (C4-OMe), 71.9 (C-9′), 100.9 (OCH_2O), 105.8 (C-2, C-6), 107.9 (C-9,), 108.4 (C-2′), 109.1 (C-5′), 121.7 (C-6′), 131.0 (C-1), 133.5 (C-1′), 139.1 (C-4), 145.7 (C-4′), 147.8 (C-3′), 154.0 (C-3, C-5). EIMS 70 eV, *m/z* 416 $[M]^+$ (5), 236 (4),182 (100), 181 (33), 136 (19), 135 (26).

3.7. Animals

Nociceptive tests were conducted using male Swiss mice (25–30 g), from our own colony, which were housed in a room with controlled temperature (22±2°C).

Fever and edema were measured on male Wistar rats weighing 180–200 g. Animals were housed at 24±1°C under a 12:12 h light–dark cycle (lights on at 06.00 am), and with free access to food and tapwater. All experiments were conducted between 10:00 and 17:00 h in non-fasted animals and are in accordance with the ethical guidelines of the International Association for the Study of Pain (Zimmermann, 1983).

3.8. Mouse writhing test

This test was based on the method of Koster et al. (1959). Contortions were induced by intraperitoneal administration of 0.2 ml of 0.6% acetic acid (v/v) (60 mg/kg) 30 min after oral treatment with indomethacin, 5 mg/kg (diluted in tris[hydroxymethyl]aminomethane. HCl, pH 8.2), cubebin, methyl-cubebin, hinoquinin, dihydrocubebin at doses of 5, 10, 20 and 40 mg/kg (all diluted in saline plus Cremophor RH40 (15% v/v-BASF) or vehicles. The contortions were observed during a 15-min period immediately after the intraperitoneal (i.p.) injection of acetic acid. The data represent the mean ± SEM total number of contortions and were analyzed for statistical significance by one-way analysis of variance followed by Bonferroni's test. The minimum level of significance considered was $P < 0.05$.

3.9. Fever induction and temperature measurements

Fever was induced by intravenous injection of 5 μg/kg of *E. coli* LPS (0111:B4, Sigma Chemical Co.), 30 min after oral treatment either with indomethacin (5 mg/kg), cubebin (5 and 10 mg/kg) or vehicle (as described above).

Body temperature was measured by inserting a thermistor probe (Yellow Springs Instruments no. 402) 3 cm into the rectum, without removing the animals from their home cages, for 1 min every 30 min for up to 6 h. The animals were picked up gently and held manually during the temperature measurements. This procedure was performed at least twice on the day before the experiment to minimize changes secondary to handling. On the day of the experiment, the basal temperature of each animal was determined by four measurements at 30 min intervals, before any injections. Only animals displaying a mean basal rectal temperature between 36.8 and 37.4°C were selected for the study. The experiments were conducted in a temperature-controlled room (28±1°C). All values are reported as means ± SEM and were analyzed for statistical significance by one-way analysis of variance followed by Bonferroni's test. The minimum level of significance considered was $P < 0.05$.

3.10. Induction and measurement of rat paw edema

Rat paw edema was induced by intraplantar injection of 300 μg of carrageenan in a volume of 100 μl (Marine Colloids) diluted in sterile saline in the right hind paws of animals treated with indomethacin, cubebin or vehicle (as described above). The contralateral paw received the same volume of saline and was used as a control. Edema was measured at 1 h intervals up to 4 h following carrageenan injection. Values are reported as mean ± SEM and were analyzed for statistical significance by one-way analysis of variance followed by Bonferroni's test. The minimum level of significance considered was $P < 0.05$.

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References

Badheka, L.P., Prabhu, B.R., Mulchandani, N.B., 1987. Lignans of *Piper cubeba*. Phytochemistry 26, 2033–2036.

Batterbee, J.E., Burden, R.S., Crombie, L., Whiting, D.A., 1969. Chemistry and synthesis of the lignan (−)-cubebin. J. Chem. Soc. (C), 2470–2477.

Bazon, J.N., Lopes, J.L.C., Vichnewski, W., Lopes, J.N.C., Cunha, W.R., 1997. Constituents of *Lychnophora brunioides*. Fitoterapia LXVIII (1), 92–93.

Blumenthal, E.E.A., Silva, M.S., Yoshida, M., 1997. Lignoids, flavonoids and polyketides of *Virola surinamensis*. Phytochemistry 46, 745–749.

Bohlmann, F., Jakupovic, J., 1990. Progress in the chemistry of the Vernonieae (Compositae). Plant Systematics and Evolution 4 (Suppl.), 3–43.

Borella, J.C., Lopes, J.L.C., Vichnewski, W., Cunha, W.R., Herz, W., 1998. Sesquiterpene lactones, triterpenes and flavones from *Lychnophora ericoides* and *Lychnophora pseudovillosissima*. Biochemical Systematics and Ecology 26, 671–676.

Cerqueira, M.B.S., Souza, J.T., Júnior, R.A., Peixoto, A.B.F., 1987. Ação analgésica do extrato bruto aquoso liofilizado do caule e folhas da *Lychnophora ericoides* Mart. Ciência e Cultura 39 (5/6), 551–553.

Chiari, E., Duarte, D.S., Raslan, D.S., Saúde, D.A., Perry, K.S.P., Boaventura, M.A.D., Grandi, T.S.M., Stehmann, J.R., Anjos, A.M.G., Oliveira, A.B., 1996. In vitro screening of Asteraceae plant species against *Trypanosoma cruzi*. Phytotherapy Research 10 (7), 636–638.

Chiari, E., Oliveira, A.B., Raslan, D.S., Mesquita, A.L., Tavares, K., 1991. Screening in vitro of natural products against blood forms of *Trypanosoma cruzi*. Trans. R. Soc. Trop. Med. Hyg. 85, 372–374.

Ferreira, S.H., 1972. Prostaglandins, aspirin-like drugs and analgesia. Nature New Biol. 240, 200–203.

Ferreira, S.H., 1990. A classification of peripheral analgesics based upon their mode of action. In: Sandler, M., Collins, G. (Eds.), Migraine a Spectrum of Ideas. Oxford Medical Publications, New York.

Grael, C.F.F., Vichnewski, W., Souza, G.E.P., Lopes, J.L.C., Albuquerque, S., Cunha, R.W., 2000. A study of the trypanocidal and analgesic properties from *Lychnophora granmongolense* (Duarte) Semir & Leitão Filho. Phytotherapy Research, 14, 203–206.

Jordão, C.O., Lopes, J.L.C., Albuquerque, S., Vichnewski, W., 1997. Biological activity of the crude extracts and isolated substances from *Lychnophora salicifolia* Mart. Boll. Chim. Farm. 136 (2), 56.

Koster, R., Anderson, M., Beer, E.J., 1959. Acetic acid for analgesic screening. Fed. Proc. 18, 412.

Koul, S.K., Taneja, S.C., Dhar, K.L., Atal, C.K., 1983. Lignans of *Piper clusii*. Phytochemistry 22, 999–1000.

Koul, S.K., Taneja, S.C., Dhar, K.L., Atal, C.K., 1984. Minor lignans of *Piper clusii*. Phytochemistry 23, 2099–2101.

Lopes, L.M.X., Yoshida, M., Gottlieb, O.R., 1983. Dibenzylbutyrolactone lignans from *Virola sebifera*. Phytochemistry 22, 1516–1518.

Milton, A.S., Wendlandt, S.A., 1970. A possible role for prostaglandin E_1 as a modulator for temperature regulation in the central nervous system of the cat. J. Physiol. 207, 76P–77P.

Rehnberg, N., Magnusson, G., 1990. General conjugate-addition method for the synthesis of enantiomerically pure lignans. Total synthesis of (−)- and (+)-burseran, (−)-dihydroxycubebin, (−)-trichostin, (−)-cubebin, (−)-5″-methoxyhinoki and (−)-hinokinin. J. Org. Chem. 55, 4340–4349.

Rücker, G., Langmann, B., Siqueira, N.S., 1981. Inhaltsstoffevon *Aristolochia triangularis*. Planta Med. 41, 143–149.

Tillekeratne, L.M.V., Jayamanne, D.T., Weerasuria, K.D.V., Gunatilaka, A.A.L., 1982. Lignans of *Horsfieldia iryaghedhi*. Phytochemistry 21, 476–478.

Vane, J.R., 1971. Inhibition of prostaglandin biosynthesis as a mechanism of action for aspirin-like drugs. Nature New Biol. 231, 232–235.

Vane, J.R., Botting, R.M., 1996. Mechanism of action of anti-inflammatory drugs. Scand. J. Rheumatol. 102, 9–21.

Xie, W., Robertson, D.L., Simmons, D.L., 1992. Mitogen-inducible prostaglandin G/H synthase — a new target for nonsteroidal anti-inflammatory drugs. Drug Devl. Res. 25, 249–265.

Zimmermann, M., 1983. Ethical guidelines for investigation of experimental pain in conscious animals. Pain 16, 109–110.