2.2. Capsaicin-induced pain

The procedure used was similar to that described previously [13]. The animals were placed individually in transparent glass cylinders. Following the adaptation period, 20 µl of capsaicin (1.6 µg/paw) was injected under the skin of the plantar surface of the right hindpaw, using a microsyringe. The animals were observed individually for 5 min following capsaicin injection. The amount of time spent licking the injected paw was timed with a chronometer and was considered as indicative of nociception. Animals were treated with the compounds (10 mg/kg, i.p.) or saline (10 ml/kg, i.p.) 1 h before administration of capsaicin. The control animals received a similar volume of 0.9% NaCl (10 ml/kg, i.p.).

3. Statistical analysis

The results are presented as mean ± s.e.m., and the statistical significance between the groups was analysed by means of an analysis of variance followed by Dunnett's multiple comparison test. *P* values less than 0.05 were considered as indicative of significance. The ID_{50} values (the dose of the compound that reduced responses by 50% in relation to the control values) were estimated by graphical interpolation from individual experiments. ID_{50}'s are presented as mean values and 95% confidence interval. MI is the maximum inhibition at higher dose used (10 mg/kg).

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References

1 Cechinel Filho, V.; Yunes, R. A.: Quím. Nova **2**, 99 (1998)
2 Cechinel Filho, V.; Vaz, Z.; Nunes, R. J.; Calixto, J. B.; Yunes, R. A.: Pharm. Sci. **2**, 199 (1996)
3 Corrêa, R.; Cechinel Filho, V.; Schlemper, V.; Rosa, P. W.; Pereira, C. I.; Nunes, R. J.: Pharm. Sci. **3**, 1 (1997)
4 Cechinel Filho, V.; Corrêa, R.; Vaz, Z.; Calixto, J. B.; Nunes, R. J.; Pinheiro, T.; Andricopulo, A. D.; Yunes, R. A.: Farmaco **53**, 55 (1998)
5 Andricopulo, A. D.; Filho, A. W.; Corrêa, R.; Santos, A. R. S.; Nunes, R. J.; Yunes, R. A.; Cechinel Filho, V.: Pharmazie **53**, 493 (1998)
6 Stiz, D. S.; Souza, M. M.; Golim, V.; Netto, R. A. E.; Corrêa, R.; Nunes, R. J.; Yunes, R. A.; Cechinel Filho, V.: Pharmazie **55**, 12 (2000)
7 Andricopulo, A. D.; Müller, L. A.; Cechinel Filho, V.; Cani, G. S.; Ross, J. F.; Corrêa, R.; Santos, A. R. S.; Nunes, R. J.; Yunes, R. A.: Farmaco **55**, 319 (2000)
8 Campos, F.; Corrêa, R.; Souza, M. M.; Yunes, R. A.; Nunes, R. J.; Cechinel Filho, V.: Arzneim.-Forsch./Drug Res. in press.
9 Calixto, J. B.; Beirith, A.; Ferreira, J.; Santos, A. R. S.; Cechinel Filho, V.; Yunes, R. A.: Phytother. Res. **14**, 401 (2000)
10 Ribeiro, R. A.; Vale, M. L.; Ferreira, S. H.; Cunha, F. Q.: Eur. J. Pharmacol. **391**, 97 (2000)
11 Grabchev, I.; Moneva, I.; Bojinov, V.; Guittonneau, S.: J. Mater. Chem. **10**, 1291 (2000)
12 Collier, R. F.; Dinnen, H. O. J.; Johnson, C. A.; Schneider, C.: Br. J. Pharmacol. **32**, 295 (1968)
13 Sakurada, T.; Katsumata, K.; Yogo, H.; Tan-No, K.; Sakurada, S.; Kisara, K.: Neurosci. Lett. **151**, 142 (1993)

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Bioactive compounds from *Leycesteria formosa*

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As part of our research for natural products with anti-inflammatory potential, we investigated several plant extracts for their ability to inhibit *in vitro* cAMP-phosphosdiesterase type IV (PDE4) activity. The control of inflammation is mediated in part by modulation of cAMP levels and PDE4 is currently considered as an intracellular target for new anti-inflammatory drugs [1, 2].
In a preliminary screening, we selected an ethanolic stem extract of *Leycesteria formosa* Wall. (Caprifoliaceae) for its promising PDE4 inhibition activity. This species, commonly known as "Himalayan honeysuckle", is a cultivated ornamental shrub native of East Asia, especially appreciated in gardens for its hardiness and its white flowers in pendent clusters [3]. Previous phytochemical studies on this species only reported the presence of coumarins [4] and monomeric flavonoids [5].
A bioactivity-directed fractionation led to the isolation of two polyphenolic constituents, identified by means of 1D and 2D NMR (H-H COSY, HMQC, HMBC) experiments and comparison with published data [6–7] as amentoflavone ($3'–8''$ biapigenin) and its $4'''$ methyl derivative, podocarpusflavone A.
Biflavonoids are interesting taxonomic markers in Angiosperms because of their sporadic occurrence. In Caprifoliaceae family for example, only two genera are known for their biflavonoid content: *Viburnum* with amentoflavone exclusively [8] and *Lonicera* with both amentoflavone and ochnaflavone derivatives [9–11]. The occurence of this two biflavonoids in *Leycesteria* and their inhibitory effect on purified PDE4 are reported here for the first time.
Amentoflavone and to some extent, podocarpusflavone A, are good PDE4 inhibitors acting at an under micromolar range (Table). Thus, in view of the recently described correlation between *in vitro* PDE4 inhibition and *in vivo* topical anti-inflammatory properties [12], *Leycesteria formosa* may be considered of potential interest in treating cutaneous inflammation.

Experimental

1. Plant material

A sample of aerial parts was collected at the Botanical Garden of Metz (France) in January 1998. A voucher specimen was deposited at the corresponding Herbarium.

2. Extraction and isolation

The air-dried and powdered stems (52 g) were exhaustively extracted with boiling 95% EtOH. The alcoholic filtrated solution was concentrated *in*

Table: PDE4 inhibition of an ethanolic stem extract, a polyphenolic fraction and two biflavonoids isolated from *Leycesteria formosa*

Samples	CI_{50} (µg/ml)	CI_{50} (µM)
Ethanolic stem extract	62.10	–
Polyphenolic fraction	23.70	–
Podocarpusflavone A	2.26	4.10
Amentoflavone	0.145	0.27

vacuo, the crude extract (4.2 g) dissolved in MeOH, and directly subjected to preparative HPLC (Gilson, France) using a reversed phase column (Microsorb® RP_{18}, 21.4×50 mm, 3 µm). Elution was performed using an aqueous methanol gradient (from 25% to 75% MeOH in 30 min, 12 ml/min). The fraction eluted between 10–15 min corresponded to the polyphenolic fraction. Two major constituents were isolated from the latter and purified using a Sephadex LH-20 column eluted with MeOH, affording amentoflavone (20 mg, Rt: 10.7 min) and podocarpusflavone A (30 mg, Rt: 12.2 min).

3. Biological assays

Cytosolic PDE4 was purified by anion exchange chromatography from the media layer of bovine aorta by a modification [13] of a previouly described method [14]. Its activity was measured by radioenzymatic assay [15] at a substrate concentration of 1µM cAMP in the presence of 15000 cpm of [^{3}H]-cAMP (Amersham) as a tracer. The buffer solution contained the following components: 50 µM Tris-HCl pH 7.5, 2 mM magnesium acetate, 50 µg/ml bovine serum albumin and 1mM EGTA. To prevent the interaction of contaminating PDE3 in the assay of isolated PDE4, studies with the use of [^{3}H]-cAMP as a substrate were always carried out in the presence of 100 µmol/l cGMP. The extract, the purified fractions and the isolated compounds were dissolved in DMSO, with a final concentration (1%) which did not affect PDE activity. The inhibition study on PDE activity included 6 concentrations of the drug. The results were expressed as percentage of inhibition of substrate hydrolysis. The CI_{50} value was calculated by non linear regression (Prism software) and represented the mean value of 3 determinations.

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References

1 Sekut, L.; Yarnall, D.; Stimpson, S. A.: Clin. Exp. Immunol. **100**, 126 (1995)
2 Hanifin, J. M.; Chan, S. C.; Cheng, J. B.; Tofte, S. J.; Henderson, W. R.; Kirby, D. S.; Weiner, E. S.: J. Invest. Dermatol. **107**, 51 (1996)
3 Brosse, J.: Atlas des arbustes, arbrisseaux et lianes de France et d'Europe Occidentale, Bordas, Barcelone 1983
4 Plouvier, V.: C. R. Acad. Sci. Ser D , **270**, 1526 (1970)
5 Glennie, C. W.; Harborne, J. B.: Phytochemistry **106** , 1325 (1971)
6 Agrawal, P. K.: Carbon-13 NMR of Flavonoids. Elsevier Science Publishers B. V., Amsterdam 1989
7 Harborne, J. B.: The Flavonoids : Advances in Research since 1986, Chapman & Hall, London 1994
8 Lobstein, A.: PHD, University Louis Pasteur, Strasbourg 1995
9 Sultana, S.; Kamil, M.; Ilyas, M.: J. Indian Chem. Soc. **61**, 730 (1984)
10 Son, K. H.; Park, J. O.; Chung, K. C.; Chang, H. W.; Kim, H. P.; Kim, J. S.; Kang, S. S.: Arch. Pharm. Res. **15**, 365 (1992)
11 Flamini G.; Braca, A.; Cioni, P. L.; Morelli, I.; Tomè, F.: J. Nat. Prod. **60**, 449 (1997)
12 Goyarts E.; Mammone, T.; Muizzuddin, N.; Marenus, K.; Maes, D.: Skin Pharmacol. Appl. Skin Physiol. **13**, 86 (2000)
13 Komas, N.; Lugnier, C.; Stoclet, J. C.: Br. J. Pharmacol. **104**, 495 (1991)
14 Lugnier, C.; Schoeffter, P.; Le Bec, A.; Strouthou, E.; Stoclet, J. C.: Biochem. Pharmacol. **35**, 1743 (1986)
15 Keravis, T. M.; Wells, J. N.; Hardman, J. G.: Biochim. Biophys. Acta **613**, 116 (1980)

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