J. Pharm. Pharmacol. 1999, 51: 1441–1446
Received May 14, 1999
Accepted July 6, 1999

Isolation of Opioid-active Compounds from *Tabernaemontana pachysiphon* leaves

K. INGKANINAN, A. P. IJZERMAN*, T. TAESOTIKUL† AND R. VERPOORTE

*Division of Pharmacognosy, Leiden/Amsterdam Centre for Drug Research, Gorlaeus Laboratories, Leiden University, P. O. Box 9502, 2300 RA, Leiden, The Netherlands, *Division of Medicinal Chemistry, Leiden/Amsterdam Centre for Drug Research, Gorlaeus Laboratories, Leiden University, P. O. Box 9502, 2300 RA, Leiden, The Netherlands, and †Department of Pharmacology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand*

Abstract

A procedure for prefractionation of crude plant extracts by centrifugal partition chromatography (CPC) has been developed to enable rapid identification of known-positive compounds or false-positive compounds and to increase the chance of identifying minor unknown-active compounds. The study explored the use of CPC as a tool in the prefractionation step before investigation of bioactivity.

Fractions obtained by CPC from an ethanolic extract of *Tabernaemontana pachysiphon* Stapf (Apocynaceae) were screened by means of an opiate-receptor-binding assay and an adenosine A_1-receptor-binding assay. Fractions containing fatty acids, which had false-positive effects on the assay, were identified, as were unknown-positive fractions from which two opioid-active compounds, tubotaiwine and apparicine, were subsequently isolated. The affinities (K_i) of tubotaiwine and apparicine at the opiate receptor were $1{\cdot}65 \pm 0{\cdot}81$ and $2{\cdot}65 \pm 1{\cdot}56$ μmol, respectively. Both alkaloids had analgesic activity in the abdominal constriction test in mice.

CPC prefractionation led to the rapid isolation of two opioid-active compounds, tubotaiwine and apparicine, from the unknown-positive fraction; false-positive fractions were rapidly identified. Both tubotaiwine and apparicine had affinity for adenosine receptors in the micromolar range and also had in-vivo analgesic activity in mice.

Because high-throughput screening is available in the pharmaceutical industry, the number of samples to be tested is no longer a limiting step, and so the use of centrifugal partition chromatography (CPC) has been studied as a prefractionation tool before bioactivity screening of crude plant extracts (Ingkaninan et al 1999a). In the previous study we compared a series of CPC systems for the general prefractionation of crude plant extracts. With the system chosen, fatty acid fractions, which interfere with some of the bioassays, could be identified (Ingkaninan et al 1999b).

CPC is a counter current liquid–liquid partitioning chromatographic method in which the stationary phase is immobilized by centrifugal force and the mobile phase is pumped through at high flow rates. CPC has distinct advantages in the isolation of natural products. Because it does not involve solid adsorbents such as silica gel, the denaturation of sensitive compounds is minimized and there is no irreversible retention. It has high capacity and can be used for analytical- and preparative-scale separations. The theoretical aspects and the application of CPC have been extensively discussed by Foucault (1995).

In this study, the application of CPC prefractionation is presented. An ethanolic extract of leaves from *Tabernaemontana pachysiphon* Stapf (Apocynaceae) was used as the model for the study. We used receptor ligand-binding assays to screen fractions of this extract obtained by CPC prefractionation. The active fractions containing fatty acids were identified, as were newly found positive fractions on which investigation is continuing.

Correspondence: R. Verpoorte, Division of Pharmacognosy, Leiden/Amsterdam Centre for Drug Research, Gorlaeus Laboratories, Leiden University, P. O. Box 9502, 2500 RA, Leiden, The Netherlands.

Materials and Methods

Plant materials
The plants listed in Table 1 were cultivated at the Division of Pharmacognosy, Leiden University, Leiden, The Netherlands. The herbarium specimens are kept at Rijksherbarium, Leiden, The Netherlands.

Extraction
The fresh leaves were cut into small pieces and macerated with ethanol (5 mL g^{-1}). After one week the mixtures were filtered and the ethanol filtrates were evaporated to dryness under reduced pressure.

Chemicals
All solvents used were of analytical grade or were distilled before use. All chemicals were purchased from commercial sources except for some alkaloids, i.e. catharanthine, ibogaine, ibogamine, tabernamine, vindoline and vindolinine, which were isolated from plants or plant cell cultures in our laboratory.

Radioligand receptor-binding assays

Opiate-receptor-binding assay. The opiate receptor-binding assay was performed on membranes from rat cortical brains. The membranes were prepared according to the method of Lohse et al (1984) except that they were incubated with 2 Int. units mL^{-1} adenosine deaminase at 37°C before storage, as described by Pirovano et al (1989). Protein concentrations were measured by the bicinchonic acid method (Smith et al 1985).

The assays were performed as described by Cox (1997) using 1·5 nM [^{3}H]naloxone as the radio-ligand (K_d 2·1 nM). Briefly, the incubation mixture consisted of [^{3}H]naloxone (100 μL), sample or 10^{-5} M morphine (100 μL), Tris–HCl buffer (pH 7·4, 50 mM; 100 μL) and rat brain homogenate (100 μL) containing the brain tissue (100 μg). After incubation at 25°C for 30 min the mixture was cooled on ice and then filtered through glass-fibre filters (GF/B Whatman) under reduced pressure. The filters were washed six times with ice-cold Tris–HCl buffer (pH 7·4, 50 mM; 2 mL). After addition of scintillation fluid (3·5 mL) the activity of the washed filters was counted for 4 min in a Hewlett–Packard Tri-Carb 1500 liquid scintillation counter. Non-specific binding was determined in the presence of 10^{-5} M morphine. Radioligand binding data were analysed with the software package Prism (Graph Pad, San Diego, CA).

Table 1. The plant material used in the experiments.

Species	Herbarium specimen
Tabernaemontana dichotoma Roxb. ex Wall.	Fcog 940129
T. divaricata (L.) R.Br. ex Roem. & Schult.	Fcog 880004
T. elegans Stapf	Fcog 810338
T. pachysiphon Stapf	Fcog 930095
T. pandacaqui Poir.	Fcog 870630

Adenosine A_1-receptor-binding assay. The adenosine A_1-binding assay was performed on membranes of rat cortical brains prepared as described above. The assays were performed with [^{3}H]1,3-dipropyl-8-cyclopentylxanthine (DPCPX; 0·4 nM) as the radioligand (K_d 0·39 nM) as originally described by Lohse et al (1987). Non-specific binding was determined in the presence of 10^{-5} M N^6-cyclopentyladenosine (CPA).

Centrifugal partition chromatography (CPC)
A modular Sanki (Kyoto, Japan) centrifugal partition chromatograph (type LLN) was used. It consists of a power supply (model SPL), a centrifuge (model NMF), and a triple-head constant flow pump (model LBP-V). A Panasonic pen-recorder (model VP 67222A) was connected to a UVIS 200 detector (Linear Instruments, Reno, NV). Fractions were collected by means of a LKB 2211 Superrac fraction collector. In all experiments, six partition cartridges (total internal volume 125 mL) were used. The pressure was limited to 60 bar. The flow rate in all experiments was 2 mL min^{-1}. A fraction was collected every 4 min. Samples were dissolved in not more than 4 mL of each of the two phases before injection. The void volume was rejected.

Isolation of the opioid-active compounds from an ethanolic extract of T. pachysiphon
The first separation step was conducted by CPC with the solvent system heptane–ethyl acetate–methanol–water 6 : 1 : 6 : 1 (v/v) (Table 2). After dissolution in 4 mL of each of the two phases, 800 mg of the dried ethanolic extract of *T. pandacaqui* was injected into CPC. The first 120 mL, excluding the void volume were eluted in ascending mode. The mode of elution was then reversed to descending mode and another 120 mL were eluted.

The eluate was grouped into fractions 1–9 according to the results of TLC analysis. Fraction 5

Table 2. Volumes, weights and the results from the radioligand-binding assays of the fractions obtained from prefractionation of the ethanolic extract of *T. pachysiphon* (800 mg) by CPC (solvent system heptane–methanol–ethyl acetate–water, 6 : 1 : 6 : 1, v/v).

	Fraction								
	1	2	3	4	5	6	7	8	9
Volume (mL)	16	32	32	40	16	16	24	24	40
Weight (mg)	40	20	10	10	450	160	15	10	5
Opiate-receptor-binding assay[a]	60 ± 15	51 ± 17	44 ± 20	42 ± 14	17 ± 2	48 ± 8	68 ± 16	44 ± 2	21 ± 8
Adenosine A_1-receptor-binding assay[b]	78 ± 15	20 ± 6	17 ± 10	16 ± 10	65 ± 17	69 ± 3	93 ± 12	42 ± 11	49 ± 11

[a]Percentage of specific binding of [^{3}H]naloxone remaining on opiate receptors after incubation with the fractions (0·1 mg mL^{-1}; 100 μL). Data are means ± s.d. of results from three separate experiments performed in duplicate.[b]Percentage of specific binding of [^{3}H]DPCPX remaining on adenosine A_1 receptors after incubation with the fractions (0·1 mg μL^{-1}; 100 mL). Data are means ± s.d. of results from three separate experiments performed in duplicate.

(450 mg) was subjected to a second CPC separation with the solvent system chloroform–methanol–water 1 : 1 : 1 (v/v) (Table 3). The first 192 mL, excluding the void volume were eluted in descending mode. The mode of elution was then reversed to ascending mode and 88 mL were eluted. The eluate was grouped into fractions 5/1–5/8 according to the results of TLC analysis.

Fractions 5/2 and 5/3 were combined and evaporated to dryness under reduced pressure. The substrate was then purified by preparative TLC (20 cm × 20 cm × 2 mm silica gel F_{254} plates; Merck, Darmstadt, Germany) with toluene–ethanol–diethylamine, 8 : 1 : 1 (v/v) as mobile phase. The adsorbent containing the main compound was scraped from the plate and extracted with methanol.

The main compound from fraction 5/7 was purified by preparative TLC with ethyl acetate–isopropanol–ammonium hydroxide 8 : 2 : 0·2 (v/v) as mobile phase by the procedure described above.

TLC analysis

The fractions were spotted on to 20 cm × 20 cm silica gel F_{254} plates (Merck) and developed in saturated normal chambers. Three mobile phases were used—chloroform–methanol 9 : 1 (v/v), cyclohexane–chloroform–diethylamine 6 : 3 : 1 (v/v) and dichloromethane–ethanol–water 4 : 4 : 0·5 (v/v). Initial visual detection was conducted under UV illumination at 254 and 366 nm. The plates were then sprayed with one of three detection reagents: modified anisaldehyde–sulphuric acid spray reagent; Dragendorff spray reagent; or ferric chloride–perchloric acid spray reagent. (3·25% $FeCl_3$ in 35% $HClO_4$). After spraying, the plates were heated with a hot air blower. Colour changes during heating were recorded.

Table 3. Volumes, weights and activity in an opiate-receptor-binding assay of fractions obtained from the separation of fraction 5 of the ethanolic extract of *T. pachysiphon* (solvent system chloroform–methanol–water 1 : 1 : 1, v/v).

	Fraction							
	5/1	5/2	5/3	5/4	5/5	5/6	5/7	5/8
Volume (mL)	16	128	48	8	8	8	24	40
Weight (mg)	90	20	3	185	90	12	22	13
Activity		+[a]	+				+	

[a]+ indicates an active fraction in the opiate-receptor-binding assay (percentage of remaining specific binding of [^{3}H]naloxone less than 20%)

Structural identification of the active compounds

UV spectra (in methanol) were acquired with a Cary 1Bio UV–Visible spectrophotometer. GC–MS was performed with a Finnigan ITD 700 (Finnigan, San Jose, CA) in EI mode coupled with a Packard 438A gas chromatograph. ^{1}H and ^{13}C NMR analysis was performed at 300 and 75 MHz, respectively, on a Bruker DPX-300 spectrometer in deuterated chloroform. TMS was used as an internal chemical shift reference for ^{1}H NMR spectra.

Tubotaiwine was obtained as a white amorphous powder. MS m/z (rel. int.) 326 (6), 142 (10), 124 (100); UV (methanol): 228, 292, 328 nm; ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 0·72 (3H, t, J = 7·1 Hz, H-18), 0·90 (2H, qd J = 7·1, 7·0 Hz, H-19), 1·90 (1H, m, H-14b), 2·05 (1H, m, H-6b), 2·09 (1H, m, H-14a), 2·22 (1H, td, J = 7, 3·2 Hz, H-20), 2·72 (1H, m, H-3b), 2·78 (1H, m, H-6a), 3·10, (1H, td, J = 11·9, 8·0 Hz, H-5b), 3·19 (1H, bs, H-15), 3·50 (1H, m, H-3a), 3·57 (1H, m, H-5a), 3·79 (3H, s, CO_2Me), 4·41 (1H, bs, H-21), 6·84 (1H, d, J = 7·7 Hz, H-12), 6·93 (1H, t, J = 7·7 Hz, H-10), 7·17 (1H, t, J = 7·7 Hz, H-11), 7·28 (1H, d, J = 7·7 Hz, H-9), 8·83 (1H, bs, H-1); ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 11·15 (C-18), 22·98

(C-19), 26·56 (C-14), 28·92 (C-15), 39·18 (C-20), 41·45 (C-6), 44·76 (C-3), 51·43 (OCH_3), 52·17 (C-5), 53·22 (C-7), 64·43 (C-21), 95·80 (C-16), 110·22 (C-12), 120·01 (C-9), 121·76 (C-10), 128·27 (C-11), 134·29 (C-8), 143·23 (C-13), 168·04 (CO), 168·23 (C-2).

Apparicine was obtained as a white amorphous powder, MS m/z (rel. int.) 264 (100), 222 (40), 208 (60); ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 1·45 (3H, dd, J = 6·8, 2·2 Hz, H-18), 1·89 (1H, ddt, J = 13·6, 7·1, 2·5 Hz, H-14b), 2·16 (1H, dddd, J = 13·6, 11·2, 7·9, 5·4 Hz, H-14a), 3·06 (1H, dddd, J = 13·1, 11·2, 7·1, 1·3 Hz, H-3b), 3·19 (1H, bd, J = 15·9 Hz, H-21b), 3·40 (1H, ddd, J = 13·1, 7·9, 2·1, H-3a), 3·80 (1H, ddt, J = 15·9, 2·0, 2·0 Hz, H-21a), 3·91 (1H, brs, H-15), 4·26 (1H, d, J = 17·8 Hz, H-6b), 4·50 (1H, d, J = 17·8 Hz, H-6a), 5·24 (1H, dd, J = 6·8, 2 Hz, H-19), 5·26 (1H, s, H-17b), 5·39 (1H, s, H-17a), 7·06 (1H, ddd, J = 8·1, 7·0, 1·0 Hz, H-10), 7·18 (1H, ddd, J = 8·1, 7·0, 1·1 Hz, H-11), 7·28 (1H, bd, J = 8·1 Hz, H-12), 7·42 (1H, bd, J = 7·9 Hz, H-9), 7·86 (1H, bs, H-1).

Animals

Experiments were performed on Swiss albino mice purchased from The National Laboratory Animal Centre (NLAC), Salaya, Mahidol University, Nakorn Pathom, Thailand. All animals were acclimatized for at least one week before the experiments.

Analgesic testing

The analgesic properties of the compounds isolated were tested by use of the abdominal constriction model in mice as described by Collier et al (1968). Abdominal constriction was induced in Swiss albino mice of either sex, 20–30 g, by intraperitoneal injection of 0·75% acetic acid (0·1 mL/10 g). Test substances and control vehicle were injected intraperitoneally into the mice 30 min before acetic acid, and the number of constrictions was noted for 15 min beginning 5 min after injection of the acid. Student's *t*-test was used to determine the significance of differences between the control group and the experimental groups.

Results and Discussion

Selection of the model used for the study

The first stage of this study was selection of a suitable model for prefractionation and the screening process. Because *Catharanthus roseus* (L.) G. Don and a few of *Tabernaemontana spp*. were available in our laboratories and *Tabernaemontana spp*. were reported to have analgesic activity (Taesotikul et al 1989; Henriques et al 1995), some alkaloids common to both genera were screened by the opiate-receptor-binding assay and compared with morphine (Table 4). Interestingly, the iboga-classed alkaloids (Kisakurek & Hesse 1980) from genus *Tabernaemontana*, i.e. ibogaine, ibogamine and tabernamine, were highly inhibitive in this radioligand-binding assay, and catharanthine, the iboga alkaloid from *C. roseus*, despite the different stereochemistry, was still active. It has been reported that ibogaine has therapeutic potential in the treatment of opiate addiction (US patent 4499090) and it has been reported to bind with high affinity to the opioid agonist site (Codd 1995). Because of the high chance of obtaining opioid-active compounds from *Tabernaemontana spp*, we screened the ethanolic extracts from five species of this genus by means of opiate-receptor-binding assay (Table 5). All showed the displacement effects in the assay. These extracts also had a positive effect on the adenosine A_1 receptors. *T. pachysiphon*, which was the most readily available extract, was chosen for further investigation.

Investigation of active compounds in fractions from the extract of T. pachysiphon

Prefractionation of the crude ethanolic extract from *T. pachysiphon* by CPC with the solvent system heptane–ethyl acetate–methanol–water, 6 : 1 : 6 : 1 (v/v), resulted in nine fractions (Table 2). These fractions were tested in both the opiate- and adenosine A_1-receptor-binding assays. The radioligand remaining in each sample was less than in the control. Fractions causing more than 80% displacement of specific radioligand binding were

Table 4. Percentage of specific binding of [^{3}H]naloxone remaining on opiate receptor after incubation with alkaloids (10 or 100 μM; 100 μL) from *Catharanthus roseus* and *Tabernamontana spp*.

	10 μM	100 μM
Ajmalicine	97 ± 5	94 ± 11
Catharanthine	85 ± 8	8 ± 4
Ibogaine	79 ± 18	8 ± 6
Ibogamine	91 ± 12	26 ± 8
Morphine	17 ± 11	4 ± 3
Tabernamine	93 ± 10	24 ± 5
Vincristine	100 ± 0	98 ± 3
Vindoline	96 ± 6	89 ± 18
Vindolinine	80 ± 18	45 ± 11

Data are means ± s.d. of results from three separate experiments performed in duplicate.

Table 5. Percentage of specific binding of [^{3}H]-ligand remaining on receptors after incubation with ethanolic extracts (0·1 or 1 mg mL^{-1}; 100 mL) from *Tabernaemontana* leaves in an opiate-receptor-binding and adenosine A_1-receptor-binding assays.

	Opiate-receptor-binding assay		Adenosine A_1-receptor-binding assay
	0·1 mg mL^{-1}	1 mg mL^{-1}	0·1 mg mL^{-1}
T. dichotoma	40 ± 4	2 ± 3	62 ± 9
T. divaricata	56 ± 13	18 ± 3	79 ± 9
T. elegans	80 ± 8	13 ± 7	62 ± 18
T. pachysiphon	38 ± 5	11 ± 9	59 ± 24
T. pandacaqui	58 ± 8	8 ± 7	64 ± 10

Data are means ± s.d. of results from three separate experiments performed in duplicate.

regarded as active. Opioid activity was found in fraction 5. Fractions 2, 3 and 4 were active in the adenosine A_1-receptor-binding assay (Table 2). However, fractions 2, 3 and 4 from CPC were known to contain linoleic acid, a non-competitive compound in the adenosine A_1-receptor-binding assay (Ingkaninan et al 1999b). Because TLC analysis confirmed the presence of linoleic acid, adenosine A_1-receptor-binding activity was not investigated further.

Two opioid-active compounds from fraction 5 were isolated by successive CPC and preparative TLC guided by the opiate-receptor-binding assay (Table 3). The UV, ^{1}H NMR, 2D-COSY and ^{13}C NMR spectra of the first compound obtained from fractions 5/2 and 5/3 were identical with those of tubotaiwine (Figure 1), the indole alkaloid investigated stereochemically by Schripsema et al (1987).

The compound obtained from fraction 5/7 was identified as apparicine, an indole alkaloid (Figure 1); again the spectral data (UV, MS and ^{1}H NMR) were identical with those reported elsewhere (Joule et al 1965; Heatley et al 1980). Surprisingly, both opioid-active alkaloids isolated from *T. pachysiphon*, tubotaiwine and apparicine, were of the aspidospermatan and not the iboga type, as expected.

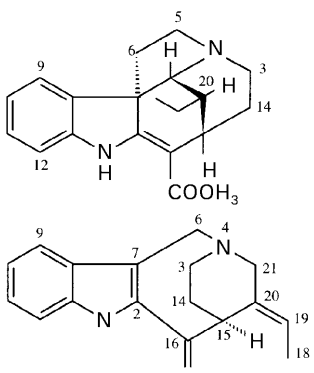

Figure 1. The structures of tubotaiwine (upper) and apparicine.

Analgesic activity of tubotaiwine and apparicine

The affinity (K_i) of tubotaiwine and apparicine for the opiate receptor were 1·65 ± 0·81 and 2·65 ± 1·56 μmol, respectively (Table 6 and Figure 2). The sodium shift (Pert & Snyder 1974) indicated agonist properties for tubotaiwine; these were not apparent for apparicine (Table 6). It should be noted that we observed a much lower K_i ratio for morphine than that reported in the literature (Pert & Snyder 1974; Arens et al 1982).

The analgesic activity of apparicine and tubotaiwine were further evaluated in the abdominal constriction test in mice (Table 7). Acetic acid used as an inducer of abdominal constriction causes algesia by liberation of endogenous substances which then excite the pain-nerve endings. The alkaloids (5 and 10 mg kg^{-1}) were found to exert significant inhibitory activity on the abdominal constriction response. The results obtained suggest that the alkaloids have potent analgesic activity. Despite the more than 100-fold difference in

Table 6. K_i values of specific [^{3}H]naloxone binding.

Displacer	K_i of specific [^{3}H]naloxone binding (M)		K_i ratio
	No NaCl	120 mM NaCl	
Morphine	1·87 ± 0·49 × 10^{-8}	5·16 ± 0·12 × 10^{-8}	2·76
Apparicine	2·65 ± 1·56 × 10^{-6}	3·08 ± 0·61 × 10^{-6}	1·16
Tubotaiwine	1·65 ± 0·81 × 10^{-6}	5·79 ± 0·13 × 10^{-6}	3·52

The K_i values were determined by log-probit analysis using 6–8 different concentrations of the displacer. Data are means ± s.d. of results from three individual determinations performed in duplicate.

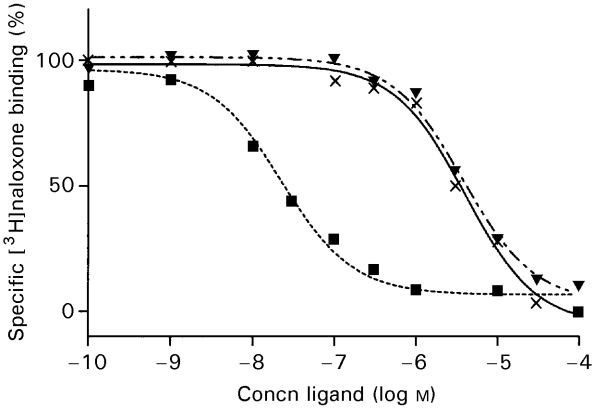

Figure 2. Competition of specific [^{3}H]naloxone binding in rat brain homogenate of morphine (■), tubotaiwine (×) and apparicine (▼). Specific [^{3}H]naloxone binding was assayed by the standard procedure described in the text. The points are averages from one typical experiment performed in duplicate.

Table 7. Effect of acetyl salicylate, morphine, apparicine and tubotaiwine on abdominal constriction response in mice.

Experiment	n	Number of constrictions	Inhibition (%)
Control	10	26·3 ± 1·5	–
Acetyl salicylate 150 mg kg^{-1}	5	8·4 ± 1·5*	68·1
Morphine			
1 mg kg^{-1}	6	17·3 ± 2·2	34·2
5 mg kg^{-1}	6	0	100
10 mg kg^{-1}	6	0	100
Apparicine			
5 mg kg^{-1}	5	17·4 ± 3·3*	33·8
10 mg kg^{-1}	5	4·6 ± 0·5*	82·5
Tubotaiwine			
5 mg kg^{-1}	6	19·5 ± 2·5*	25·8
10 mg kg^{-1}	6	5·1 ± 1·01*	80·6

Values are means ± s.e.m. Drug and test compounds were given intraperitoneally 30 min before injection of 0·75% acetic acid. *$P < 0·05$ compared with control.

affinity in the binding studies, the two alkaloids are only slightly less active than morphine in-vivo.

Both apparicine and tubotaiwine have been found in many *Tabernaemontanae* species (van Beek et al 1984). Arens et al (1982) reported that the opiate-receptor-binding activity of apparicine isolated from *Picralima nitida* cell suspension culture was in the micromolar range. The activity of tubotaiwine in the opiate-receptor-binding assay, and the in-vivo analgesic activity of the two alkaloids, have not previously been reported.

Conclusions

Prefractionation by CPC led to the rapid isolation of two opioid-active compounds, tubotaiwine and apparicine, from the unknown-positive fraction whereas the false-positive fractions were identified at an early stage. Both tubotaiwine and apparicine had micromolar affinity for adenosine receptors and also had in-vivo analgesic activity in mice.

Acknowledgements

We would like to thank the Royal Thai Government for the PhD scholarship awarded to K. Ingkaninan. We are also grateful to Wim Snoeijer for providing the plant materials.

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