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Indole Alkaloids of a Thai Medicinal Herb, *Mitragyna speciosa*, that has Opioid Agonistic Effect in Guinea-Pig Ileum

Abstract

Recently, we found that mitragynine, a major constituent of *Mitragyna speciosa*, has an opioid agonistic activity, but its weak potency could not explain the opium-like effect of this plant. In the present study, bioassay-guided fractionation of the crude extract of the leaves of *M. speciosa* was carried out to search for potent opioid agonists other than mitragynine. Opioid agonistic activities were evaluated using twitch contraction induced by electrical stimulation in guinea-pig ileum. The crude extract of *M. speciosa* inhibited the twitch contraction in a concentration-dependent manner. The inhibition was reversed by naloxone. The opioid effect was detected only in the crude base fraction, which was followed by the isolation of five indole alkaloids. Among these alkaloids, 7-hydroxymitragynine showed the most potent opioid effect on the electrically-stimulated contraction (pD_2 = 8.38 ± 0.12). The potency, calculated using pD_2 values, was 30- and 17-fold higher than that of mitragynine and morphine, respectively. Antagonism of naloxone on concentration-response curves for 7-hydroxymitragynine confirmed its opioid effect. These results suggest that the opioid effect of *M. speciosa* is mostly based on the activity of 7-hydroxymitragynine.

Key words

Mitragyna speciosa · Rubiaceae · morphine · ileum · opioid receptor · mitragynine · nociceptin · opioid receptor-like 1 receptor

Introduction

Mitragyna speciosa (called kratom in Thailand) has been used in Thailand for its opium-like and coca-like effects [1]. Additionally, it has been used to treat diarrhea and to wean addicts off morphine [1]. These pharmacological effects are supposed to result from an agonistic effect on opioid receptors. This medicinal herb contains many indole alkaloids [2], [3], [4]. Mitragynine (Fig. **1**), a main constituent of the leaves of *M. speciosa* [4], is an indole alkaloid and structurally similar to an *Uncaria* alkaloid that we previously investigated [5], [6]. Macko et al. [7] reported that mitragynine is comparable to codeine as an analgesic in dogs, but they suggest that the analgesic effect of mitragynine is not caused by stimulation of the opioid receptors.

Recently, we found that mitragynine acts on μ-opioid receptors in guinea-pig ileum [8], [9], although *Uncaria* alkaloids do not. In addition, some pharmacological studies have also revealed that mitragynine has an antinociceptive action through the supraspinal μ- and δ-opioid receptors [10], [11], [12], [13], [14]. The analgesic effect of mitragynine, however, is less potent than that of the crude extract of this plant [15]. That is, the opium-like effect of *M. speciosa* cannot be fully explained by that of mitragynine. This finding suggests that minor constituents of *M. speciosa*

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have a very potent analgesic effect. However, this plant has not so far been investigated systematically for isolation of opioid agonistic principles. In the present study, we fractionated the crude extract of *M. speciosa*, and explored active constituents that have opioid agonistic activities using an *in vitro* guinea-pig ileal contraction test.

Materials and Methods

Animals

Male albino Dunkin-Hartley guinea pigs (300–400 g) were purchased from Takasugi Lab. Animals Co. (Kasukabe, Japan). They were housed under controlled environmental conditions with 12 h/12 h light/dark cycles at 23 ± 2 °C and free access to food and water for at least 1 week before the experiments. The experiments were carried out in strict accordance with the "Guiding Principles for the Care and Use of Laboratory Animals" approved by the Japanese Pharmacological Society and the guidelines approved by the Ethical Committee on Animal Care and Animal Experiment of our graduate school.

Isolated ileum preparation

The isolated ileum was prepared and contraction of the ileum was isotonically recorded as described previously [16]. The ileum preparation was set up under 1 g tension in a 5 mL organ bath containing Krebs-Henseleit nutrient solution. The bath was maintained at 37 °C and continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2.

The ileum was transmurally stimulated through platinum needle-ring electrodes with monophasic square wave pulses (0.2 Hz) of 0.1 msec duration by a stimulator (SEN-7203, Nihon Kohden, Tokyo, Japan) as reported previously [17]. This transmural stimulation induces a twitch contraction via acetylcholine released from postganglionic cholinergic neurons [8]. Contractions were isotonically recorded by using a displacement transducer (NEC, San-ei Instruments Ltd., Type 45347, Tokyo, Japan), DC strain amplifier (San-ei 6M92, Tokyo, Japan) and a DC recorder (Hitachi, Mod 056, Tokyo, Japan). All concentration-response curves were constructed in a cumulative manner. The height of the twitch response to transmural stimulation was measured before and after drug challenge. The twitch contraction percentage of the response after the addition of each compound was determined as described previously [9]. Agonist potency was expressed as the pD_2 value, which is the negative logarithm of the concentration required to produce 50% of the maximum responses to the drug (EC_{50}). This biological assay for opioid agonists in guinea-pig ileum was introduced by Kosterlitz and co-workers [18]. The solvent (dimethyl sulfoxide 0.5%) did not affect the electrically-stimulated or acetylcholine-induced contraction.

Plant material

The leaves of *M. speciosa* were collected in the campus of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, in December 1991. The plant was identified by Dr. Nijsiri Ruangrungsi, Faculty of Pharmaceutical Sciences, Chulalongkorn University. A voucher sample (#1991Dec-MS) was deposited in the Herbarium of Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Extraction and isolation

Big; young leaves were powdered (165.5 g) and extracted with hot MeOH five times. The solvent was concentrated under reduced pressure to give a crude extract (53.5 g), a part of which was dissolved in 10% aqueous acetic acid. The insoluble material was removed by filtration through Celite to give the AcOH-insoluble fraction solution (AcOH-insoluble fraction, 50.3 g). The aqueous layer was basified with Na_2CO_3 at 0 °C and extracted with $CHCl_3$. The organic layer was washed with water, dried over $MgSO_4$, and then evaporated to give the crude base fraction (2.43 g). The aqueous layer was further extracted with *n*-BuOH, which was concentrated under reduced pressure to yield the *n*-BuOH fraction (4.77 g). A part of the residual aqueous solution (10 mL) was lyophilized to give a hygroscopic solid (ca. 2 g), which was isolated with ethanol using a Soxhlet extractor in order to remove the inorganic materials. The ethanol extract was evaporated to give a residue containing the water-soluble organic materials (water-soluble fraction, 1.08 g). The inhibitory activity of each fraction (at 100 μg/mL) on electrically-induced twitch contraction in guinea-pig ileum was as follows: 37.0 ± 5.2% (crude extract); 7.3 ± 4.5% (AcOH-insoluble fraction); 72.5 ± 8.1% (crude base fraction); −5.7 ± 3.7% (*n*-BuOH fraction); −3.8 ± 4.1% (water-soluble fraction).

The crude base fraction (2.0 g), which exhibited an opioid agonistic effect in the guinea-pig ileum, was purified by SiO_2 column chromatography (6×17 cm) using $CHCl_3$/AcOEt (9:1, 370 mL; fraction A), $CHCl_3$/AcOEt (4:1, 240 mL; fraction B), $CHCl_3$/AcOEt (1:1, 320 mL; fraction C), AcOEt (80 mL; fraction D), MeOH/AcOEt (1:19, 120 mL; fraction E), MeOH/AcOEt (1:4, 160 mL; fraction F), MeOH/AcOEt (1:1, 80 mL; fraction G), and MeOH (150 mL; fraction H). The combined fractions C and D were further purified by SiO_2 column chromatography (3×17 cm) using an *n*-hexane/AcOEt (3:2, 1:1, 1:5, 30 mL each) gradient that afforded 24 fractions. Fractions 2–8 contained mitragynine {1343 mg, 66% based on the crude base, $[\alpha]_D^{24}$: −126° (*c* 1.2, $CHCl_3$)} and fractions 18–22 yielded 7-hydroxymitragynine {40 mg, 2% based on the crude base, $[\alpha]_D^{23}$: +47.9° (*c* 0.55, $CHCl_3$)}. From fraction E, paynantheine {178 mg, 8.9% based on the crude base, $[\alpha]_D^{25}$: +29.4° (*c* 1.2, $CHCl_3$)} was obtained. Fraction F afforded speciogynine {132 mg, 6.6% based on the crude base, $[\alpha]_D^{24}$: +26.8° (*c* 0.85, $CHCl_3$)}. Fraction G was subjected to MPLC (SiO_2, 2.5×10 cm) with MeOH/$CHCl_3$ (1:9, 3 mL/min) to provide speciociliatine {t_R: 18 min, 15 mg, 0.8% based on the crude base, $[\alpha]_D^{24}$: −10.5° (*c* 1.2, $CHCl_3$)}. The isolated compounds were identified by direct comparison with the corresponding authentic samples. The purity (> 99%) of the above compounds was checked by HPLC and ^{1}H-NMR (500 MHz) analyses.

Chemicals

Chemicals for pharmacological assay were obtained from the following sources: acetylcholine chloride (Dai-ichi Seiyaku Co., Ltd., Tokyo, Japan); morphine hydrochloride (Takeda Chemical Industries, Osaka, Japan); naloxone hydrochloride (Sigma Chemical Co., St. Louis, MO, USA); nociceptin (human) (Peptide Institute, Osaka, Japan). CompB (alias J113397, 1-[(3*R*,4*R*)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl-3-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one]) was a gift from Banyu Pharmaceutical Co. (Tsukuba, Japan).

Statistical analysis

The data are expressed as the mean ± S.E.M. Statistical analyses were performed with two-tailed *t*-test for comparison of two groups, and by a one-way analysis of variance followed by a Bonferroni multiple comparison test for comparison of more than two groups. A P value < 0.05 was considered statistically significant.

Results

Effect of crude extract on electrically-stimulated twitch contraction

First, the opioid agonistic activity of the crude extract of *M. speciosa* was evaluated using the twitch contraction induced by electrical stimulation in guinea-pig ileum. The crude extract (1 – 300 μg/mL) inhibited the twitch contraction in a concentration-dependent manner (Table **1**).

The effect of the opioid receptor antagonist naloxone on the contraction inhibition was examined to verify that the extract acts on opioid receptors. The effect of the crude extract was reversed by naloxone (Table **2**). Naloxone (30 – 300 nM) also inhibited the effect of morphine, but did not affect the effect of verapamil, an L-type Ca^{2+} channel blocker, on the twitch contraction (Table **2**), suggesting that the antagonistic effect of naloxone is specific to the opioid receptors.

This crude extract was successively fractionated into crude base, *n*-BuOH and water fractions. Among them, only the crude base extract was found to exhibit the inhibitory activity on the twitch contraction (Table **1**). The inhibitory effect was concentration-dependent (1 – 100 μg/mL). The AcOH-insoluble, *n*-BuOH- and water-soluble fractions (10 – 300 μg/mL) hardly showed any effect on ileal twitch contraction.

Table **1** Opioid agonistic activities of extracts and constituents of *Mitragyna speciosa* in guinea-pig ileum preparation

Compound	*pD_2 Value*	*Relative Potency*	*Maximum Inhibition*	*Relative Inhibitory Activity*
Morphine (positive control)	7.15 ± 0.05	100%	87.2 ± 1.8%[c]	100%
Crude extract	5.05 ± 0.24	0.8%	42.3 ± 6.0%[b]	49%
Crude base fr	4.32 ± 0.15	0.1%	72.5 ± 8.1%[c]	83%
n-BuOH fr	NE	NE	−11.3 ± 4.0%[a]	−13%
AcOH-insoluble fr	NE	NE	13.6 ± 8.9%	16%
Water-soluble fr	NE	NE	−12.3 ± 7.3%	−14%
7-Hydroxymitragynine	8.38 ± 0.12	1698%	86.3 ± 4.8%[c]	99%
Mitragynine	6.91 ± 0.04	58%	84.0 ± 2.0%[c]	96%
Speciogynine	5.61 ± 0.06	3%	75.1 ± 8.3%[c]	86%
Paynantheine	4.99 ± 0.06	1%	74.9 ± 5.0%[c]	86%
Speciociliatine	5.55 ± 0.15	3%	86.3 ± 2.1%[c]	99%

Effects of samples on electrically-induced twitch contraction were examined in guinea-pig ileum. Potency is expressed as the pD_2 value, which is the negative logarithm (−log g/mL for extracts, −log M for compounds) of the concentration required to produce 50% of the maximum response to each compound (EC_{50}). Relative potency is expressed as a percentage of the pD_2 value of each compound against that of morphine. Maximum inhibition (%), which is elicited by the compound when the response reached a plateau, was calculated by regarding the twitch contraction as 100%. Relative inhibitory activity, which means intrinsic activity on opioid receptors, is expressed as a percentage of the maximum inhibition by each compound against that by morphine. Each value represents mean ± S.E.M. of five animals. In case significant inhibition was not obtained at 30 μM of the compound, the effect was recorded as "not effective (NE)". [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$, significantly different from the values before the addition of each compound (paired t-test). fr: fraction.

Table **2** Effects of naloxone on twitch contraction inhibited by crude extract and constituents of *Mitragyna speciosa* in guinea-pig ileum preparation

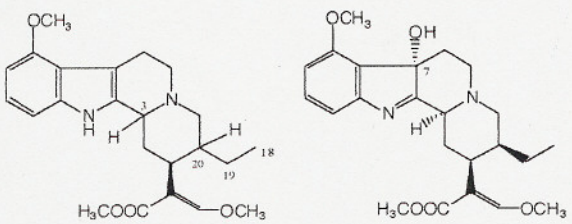
Compound	*(Concentration)*	*Contraction (%) Inhibited by Compounds*	*Contraction (%) Reversed by Naloxone*	
			30 nM	*300 nM*
Crude extract	(300 μg/mL)	56.5 ± 11.2	65.7 ± 8.4	83.2 ± 5.2[b]
7-Hydroxymitragynine	(100 nM)	24.4 ± 4.4	56.3 ± 8.2[b]	129.5 ± 8.1[c]
Mitragynine	(3 μM)	18.9 ± 2.3	29.9 ± 2.5	117.4 ± 5.7[c]
Speciogynine	(30 μM)	22.6 ± 9.1	25.9 ± 9.0	42.3 ± 12.0
Paynantheine	(30 μM)	43.0 ± 5.8	42.4 ± 5.8	41.6 ± 6.5
Speciociliatine	(30 μM)	25.2 ± 6.4	19.1 ± 5.4	25.2 ± 5.2
Morphine	(1 μM)	15.3 ± 2.0	68.0 ± 5.3[c]	121.6 ± 6.1[c]
Verapamil	(3 μM)	7.9 ± 2.6	7.9 ± 2.6	6.1 ± 1.6

Each value represents mean ± S.E.M. of five animals. [b] $P < 0.01$, [c] $P < 0.001$, significantly different from the values before the addition of naloxone (Bonferroni multiple comparison test).

Effect of 7-hydroxymitragynine on electrically-stimulated twitch contraction

Silica gel column chromatography of the crude base fraction isolated five alkaloids: 7-hydroxymitragynine, mitragynine, speciogynine, speciociliatine and paynantheine (Fig. **1**). Each alkaloid inhibited the electrically-induced twitch contraction in a concentration-dependent manner (Fig. **2**).

Among them, 7-hydroxymitragynine showed the most potent effect on the ileal contraction (Figs. **2** and **3**, Table **1**). The potency was 30- and 17-fold higher than that of mitragynine and morphine, respectively. Naloxone (30–300 nM) restored the twitch contraction inhibited by 7-hydroxymitragynine (Table **2**). Pretreatment with naloxone (1–10 nM) shifted the concentration-response curves for 7-hydroxymitragynine to the right (Fig. **4**). The slope factors for 7-hydroxymitragynine and morphine in a Schild analysis were 0.91 ± 0.20 and 1.08 ± 0.27, respectively. These values are not significantly different from unity, suggesting the competitive inhibition. The pA_2 value was 8.95 ± 0.30 for 7-hydroxymitragynine, and 9.38 ± 0.36 for morphine, the difference being not statistically significant. 7-Hydroxymitragynine (100 nM) did not affect the acetylcholine-induced contraction in the ileum (Fig. **5**).



C3-H	C20-H	
α	α	Mitragynine
α	β	Speciogynine
β	α	Speciociliatine
α	β	Paynantheine ($\Delta^{18\text{-}19}$)

Fig. **1** Chemical structures of constituents of *Mitragyna speciosa*.

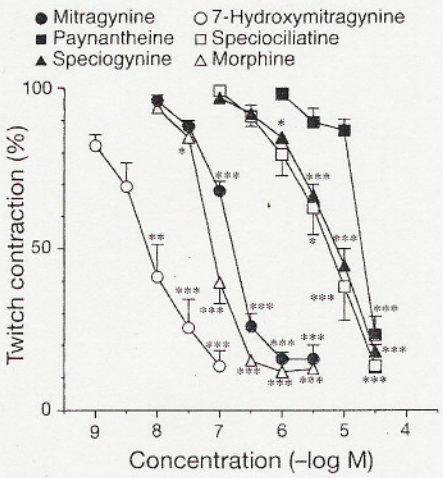


Fig. **2** Effects of constituents of *Mitragyna speciosa* on electrically-induced twitch contraction in guinea-pig ileum. All concentration-response curves were constructed in a cumulative manner. Data are expressed as contraction percentage of the twitch contraction before the addition of samples. Each value represents mean ± S.E.M. of five animals. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significantly different from the values before the addition of each sample (Bonferroni multiple comparison test).

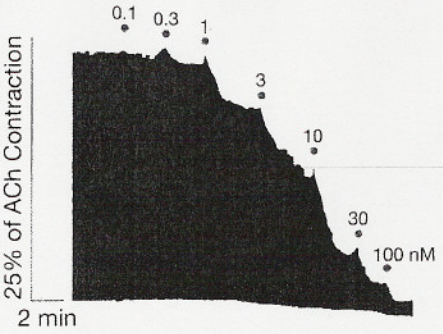


Fig. **3** Typical recording showing the effect of 7-hydroxymitragynine on electrically-induced twitch contraction in guinea-pig ileum. Ordinate scale represents 25% of the maximum response to acetylcholine (3 μM). Abscissa scale represents 2 min.

To verify the involvement of opioid receptor-like 1 (ORL1) receptors in the effect of 7-hydroxymitragynine on ileal contraction, the effect of CompB, a non-peptidic ORL1 receptor antagonist, on the twitch contraction inhibited by 7-hydroxymitragynine was examined. CompB did not affect the inhibition by 7-hydroxymitragynine (Table **3**).

Effects of isolated alkaloids on electrically-stimulated twitch contraction

The other alkaloids mitragynine, speciogynine, paynantheine and speciociliatine exhibited the inhibitory effect on electrically-induced twitch contraction in guinea-pig ileum (Fig. **2**, Table **1**). Naloxone restored the inhibition elicited by mitragynine as reported previously [8], but did not restore that by speciogynine, paynantheine and speciociliatine (Table **2**). Speciogynine and paynantheine inhibited acetylcholine-induced contraction in guinea-pig ileum, but speciociliatine did not affect acetylcholine-induced contraction.

Discussion

Opioid effect of extract

M. speciosa has been traditionally used as a substitute for opium in tropical areas [1]. We found that mitragynine, a major constituent of this plant, elicits an opioid agonistic effect in guinea-pig ileum [8], [9]. In the present study, we attempted to find opioid agonistic principles other than mitragynine. The opioid agonistic activities of the crude and the fraction extracts were evaluated using the twitch contraction induced by electrical stimulation in guinea pig ileum. The crude extract inhibited the twitch contraction, which was reversed by naloxone. This result demonstrates that it has an opioid agonistic effect.

Opioid effect of alkaloids

Based on the results of activities of various fractions, the active components were extracted from the crude base fraction. This fraction extract was chromatographed to yield five compounds. They were identified as 7-hydroxymitragynine, mitragynine, speciogynine, paynantheine and speciociliatine by direct comparison with the corresponding authentic samples. Each alkaloid inhibited the electrically-induced twitch contraction in a concentration-dependent manner. The opioid agonistic effect of mitragynine was also obtained as reported previously [8], [9].

7-Hydroxymitragynine is an oxidized derivative of mitragynine and minor constituent in the leaves of *M. speciosa* [19]. The inhi-

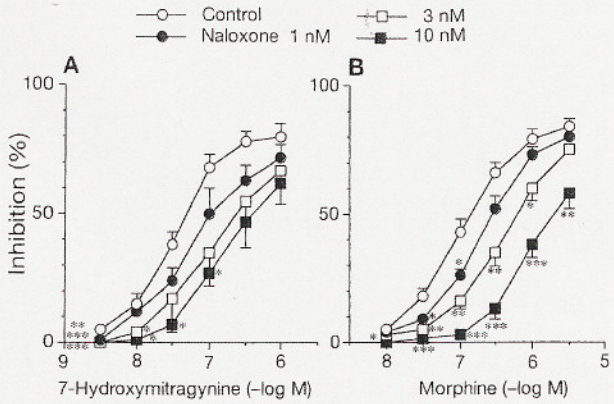


Fig. **4** Effect of naloxone (1–10 nM) on concentration-response curves for 7-hydroxymitragynine (**A**) and morphine (**B**) in guinea-pig ileum. All concentration-response curves were constructed in a cumulative manner. Data are expressed as inhibition percentage of the twitch contraction before the addition of samples. Each value represents mean ± S.E.M. of five animals. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significantly different from the corresponding control group (Bonferroni multiple comparison test).

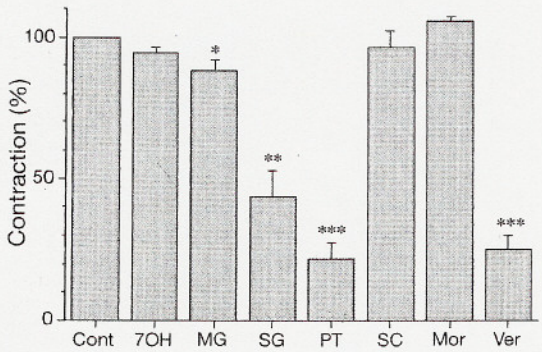


Fig. **5** Effects of the constituents of *Mitragyna speciosa* on acetylcholine-induced twitch contraction in guinea-pig ileum. Data are expressed as contraction percentage of the response to acetylcholine (3 μM) in the absence of samples. Each value represents mean ± S.E.M. of five animals. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significantly different from the control group (Bonferroni multiple comparison test). Cont: control; 7OH: 7-hydroxymitragynine 100 nM; MG: mitragynine 3 μM; SG: speciogynine 30 μM; PT: paynantheine 30 μM; SC: speciociliatine 30 μM; Mor: morphine 1 μM; Ver: verapamil 3 μM.

bitory effect of 7-hydroxymitragynine was abolished by naloxone, suggesting the involvement of opioid receptors. This was confirmed by the parallel shift of the concentration-response curve for 7-hydroxymitragynine by naloxone. No significant difference between the pA_2 values of naloxone on concentration-response curves against 7-hydroxymitragynine and morphine was observed. The results demonstrate that 7-hydroxymitragynine acts on the same receptor as morphine does. In Fig. **4**, the maximum response to 7-hydroxymitragynine seems to be slightly suppressed with the higher concentration of naloxone. But the slope factor is not significantly different from unity, suggesting that naloxone antagonizes the effect of 7-hydroxymitragynine competitively.

Among the components isolated in this study, 7-hydroxymitragynine exhibited the most potent activity. The potency, calculated using pD_2 values, is about 30- and 17-fold higher than that of mitragynine and morphine, respectively. Taken together with this potency, it is suggested that the opioid effect of *M. speciosa* is based on the activity of 7-hydroxymitragynine.

Speciogynine and paynantheine inhibited the twitch contraction in a naloxone-insensitive manner, and also inhibited the contraction induced by direct stimulation of muscarinic receptors on ileal smooth muscle. Therefore, both alkaloids directly act on ileal smooth muscle, leading to the electrically-induced inhibition in guinea-pig ileum.

Speciociliatine, a minor constituent of this plant, is the C3 stereoisomer of mitragynine. This compound inhibited the twitch contraction in a naloxone-insensitive manner, and did not inhibit the acetylcholine-induced contraction. Therefore, speciociliatine inhibits acetylcholine release from the presynaptic nerve through a mechanism other than stimulation of opioid receptors.

Involvement of ORL1 receptor

ORL1 receptors share much sequence homology with classical opioid receptors [20]. The ORL1 receptors in the central nervous system have been reported to play an important role in the regulation of physiological functions such as pain perception [20]. Nociceptin, an endogenous agonist for ORL1 receptors, inhibits electrically-induced twitch contraction in guinea-pig ileum. So we verified the involvement of ORL1 receptors in the inhibitory effects of *Mitragyna* alkaloids on the twitch contraction by using CompB, a non-peptidic ORL1 receptor antagonist [21]. The inhibitory effects of 7-hydroxymitragynine and speciociliatine were not affected by CompB, therefore suggesting that these *Mitragyna* alkaloids do not act on ORL1 receptors.

Table **3** Effects of CompB on twitch contraction inhibited by 7-hydroxymitragynine and speciociliatine in guinea-pig ileum preparation

Compound	*(Concentration)*	*Contraction (%) Inhibited by Compounds*	*Contraction (%) Reversed by CompB*	
			30 nM	*300 nM*
7-Hydroxymitragynine	(100 nM)	24.9 ± 4.2	26.6 ± 4.1	27.5 ± 2.8
Speciociliatine	(30 μM)	15.6 ± 1.6	16.1 ± 3.2	15.5 ± 5.7
Morphine	(1 μM)	45.4 ± 12.2	45.2 ± 9.0	43.7 ± 7.7
Nociceptin	(300 nM)	30.8 ± 5.0	50.9 ± 6.3	79.7 ± 4.7[c]

Each value represents mean ± S.E.M. of five animals. [c] $P < 0.001$, significantly different from the values before the addition of CompB (Bonferroni multiple comparison test).

In conclusion, 7-hydroxymitragynine was isolated as an active principle by activity-guided fractionation of the methanol extract of the leaves of *M. speciosa*. In addition, this alkaloid is a non-peptidic opioid agonist that is structurally different from other opioid agonists.

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