ORIGINAL ARTICLE

Antinociceptive activity of the alkaloid extract from *Kopsia macrophylla* leaves in mice

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Abstract

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The effects of the alkaloid extract from the leaves of *Kopsia macrophylla* Hk. f. K. (*K. macrophylla*) on nociceptive response using writhing, hot plate and formalin test and the antipyretic activity in yeast-induced fever in mice, were examined. General behavior was also examined using pentobarbital-induced sleep in mice. The LD_{50} value of intraperitoneally injected *K. macrophylla* extract in mice was 318.46 mg/kg. Oral administration of *K. macrophylla* extract at the dose of 400 mg/kg significantly decreased the number of contortions and stretchings induced by acetic acid and licking activity of the late phase in the formalin test but not in the heat-induced pain in mice. The *K. macrophylla* extract (100-400 mg/kg, p.o.) had no effect on fever induced by yeast in mice. The alkaloid extract of *K. macrophylla* prolonged the duration of pentobarbital-induced sleep in mice. These results suggest that the alkaloid extract of *K. macrophylla* possesses analgesic action via peripheral pathway but no antipyretic activity.

Key words : *Kopsia macrophylla*, alkaloid extract, antinociceptive, analgesic

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ฤทธิ์แก้ปวดของสารสกัดแอลคาลอยด์จากใบเข็มดงในหนูถีบจักร

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ทดสอบผลของสารสกัดแอลคาลอยด์จากใบเข็มดงในหนูถีบจักรต่อการระงับปวด ซึ่งเกิดจากกรดอะเซติก ความร้อน และฟอร์มาลิน และผลต่อการลดไข้ซึ่งเกิดจากการเหนี่ยวนำโดยยีสต์ นอกจากนี้ยังได้ทดสอบผลต่อพฤติกรรมทั่วไปในหนูถีบจักร โดยการเหนี่ยวนำให้หลับด้วยเพ็นโทบาร์บิตาล ขนาดของสารสกัดที่ทำให้หนูถีบจักรตาย 50% เมื่อฉีดเข้าทางหน้าท้อง มีค่าเท่ากับ 318.46 มก./กก. เมื่อป้อนสารสกัดจากใบเข็มดงขนาด 400 มก./กก. เข้าทางปากในหนูถีบจักร พบว่าสามารถลดจำนวนของการบิดและยืดของลำตัวเมื่อถูกกระตุ้นโดยกรดอะเซติกและลดการเลียในช่วงเฟสหลังของการทดสอบด้วยฟอร์มาลินในหนูถีบจักรอย่างมีนัยสำคัญ แต่ไม่มีผลต่อการทดสอบด้วยความร้อน สารสกัดจากใบเข็มดงขนาด 100-400 มก./กก. ให้ทางปาก ไม่มีผลในการลดไข้ซึ่งเกิดจากการเหนี่ยวนำโดยยีสต์ในหนูถีบจักร สารสกัดแอลคาลอยด์จากใบเข็มดงทำให้ระยะเวลาในการหลับของเพ็นโทบาร์บิตาลยาวนานขึ้น จากการทดลองนี้เสนอว่าสารสกัดแอลคาลอยด์จากใบเข็มดงมีฤทธิ์แก้ปวดผ่านทางส่วนปลาย แต่ไม่มีฤทธิ์ลดไข้

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Kopsia macrophylla Hk. f. K. (*K. macrophylla*) is a small tree and known in Thai as Khem-Dong, Khem-Pa, Phut-Pa or Phut-Dong in the family Apocynaceae. It has been known as cool medicine preparations in folk medicine. For example, the root and the stem have been used for relief of fever and toxicemia by Thai traditional doctors (Pongbunrod, 1979; Wuthammawech, 1997). *Kopsia fructicosa (K. fructicosa)* is one of other species of genus *Kopsia*, found as a native plant in Myanmar. Its root has been used to poultice ulcerated nose in tertiary syphilis (Perry, 1980). Furthermore, two other species, *K. larutensis* and *K. singapurensis* also showed similar properties (Perry, 1980). Some chemical constituents from *Kopsia* spp. have been reported. Most of them are indole alkaloids e.g., kopsine, kopsingine, kopsaporine, kopsingarine (Perry, 1980), kopsinginine, kopsinine (Kam *et al*., 1993), harmane, kopsilongine, kopsamine, leuconolam, pleiocarpine, buchtienine and eburnamonine (Kam *et al*., 1998). The pharmacological activities of the compounds from *Kopsia* spp. have been studied. Intravenous injection of kopsingine (0.2-10.0 mg/kg) from *K. teoi*, produced dose-related decreases in the the mean arterial blood pressure and heart rate in anesthetized spontaneously hypertensive rats (Mok *et al*.,1998). Kopsiflorine, isolated from *K. dasyrachis*, enhanced cytotoxicity of vincristine in drug-resistant KB cells (Rho *et al*., 1999). However, no pharmacological studies of *K. macrophylla* have previously been conducted on antipyretic and analgesic actions of this plant. In the present study, we evaluated the potential existence of antipyretic and analgesic activities of the alkaloid extract obtained from *K. macrophylla* by investigating the antinociceptive effects using the writhing, hot plate and formalin tests and the antipyretic activity in yeast-induced fever in mice. Furthermore, the general behavior using pentobarbital-induced sleep in mice was also observed.

Materials and Methods

Plant material

The leaves of *Kopsia macrophylla* Hk. f. K. (*K. macrophylla*) (Apocynaceae) were collected in Krabi Province, Thailand. The plant was identified

by comparing its habit with that described in Tree Flora of Malaya (Whitmore, 1973). Voucher specimens of crude drugs have been deposited in the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand.

Preparation of the alkaloid extract from the leaves of *Kopsia macrophylla*

The dried coarsely powdered leaves of *K. macrophylla* (5.5 kg) were macerated with 10.0 l of methanol for three days and then filtered and evaporated to give a syrupy mass. The marc was remacerated with methanol (10.0 l) three times, filtered and evaporated. The combined filtrates were concentrated to a syrupy mass under reduced pressure and then combined with 2% sulfuric acid solution. After shaking and filtering, the acidic filtrate was made basic (pH 10) with 25 % ammonia solution, and then extracted with portions of chloroform. The combined chloroform extracts were washed with water, dried over anhydrous sodium sulfate and evaporated to yield a coarse green powder of crude alkaloids (138.69 g or 2.52%). All doses were expressed in terms of dried crude alkaloid extract (mg/kg body weight) except when otherwise specified.

Animals

Male Swiss Albino mice with the weight ranging from 30-40 g were obtained from Southern Laboratory Animal Facility, Prince of Songkla University, Hat Yai, Songkhla, Thailand. All animals were housed for at least one week in the laboratory animal room prior to testing. Food and water were given *ad libitum* unless otherwise specified.

Acute toxicity

The 50% lethal dose of the alkaloid extract of *K. macrophylla* was estimated by the up-and-down method in mice (Bruce, 1985). Doses were adjusted by a constant multiplicative factor; viz. 1.5, for this experiment. The dose for each successive animal was adjusted up or down depending on the previous outcome.

Antinociceptive Activity

1. Writhing test

The alkaloid extract of *K. macrophylla* (100, 200 and 400 mg/kg), a reference analgesic drug, (aspirin, 200 mg/kg), or cosolvent vehicle was orally administered 30 min before writhing behaviour was tested. Briefly, 0.6% acetic acid solution (10 ml/kg body weight) was injected intraperitoneally and the number of writhings and stretchings was counted over a 20-min period as previously reported (Koster *et al*., 1959; Hendershot & Forsaith, 1959).

2. Hot plate test

The hot plate test was carried out according to the method described by Woolfe & MacDonald (1944). Mice were placed on a hot plate maintained at 55±1°C. Latency of nociceptive response such as licking, flicking of a hind limb or jumping was measured. Starting thirty minutes after p.o. administration of the test agents except morphine (15 min after administration), the nociceptive response was measured every 15 min over a 60 min period. Morphine sulfate was injected subcutaneously. The cut-off time was 45 sec. Only the mice that showed nociceptive responses within 15 sec were used for the experiments.

3. Formalin test

Thirty minutes after administration of the alkaloid extract of *K. macrophylla* (100, 200 and 400 mg/kg, p.o.), aspirin (200 mg/kg, p.o.) or cosolvent except morphine (15 min after administration), 20 µl of 2.5% formalin in saline was injected subcutaneously to a hindpaw of the mice. Morphine sulfate was injected subcutaneously. The time spent licking the injected paw was recorded and the data were expressed as total licking time in the early phase (0-5 min) and the late phase (15-30 min) after formalin injection (Hunskaar *et al*., 1985).

Antipyretic activity

The effect of drug on yeast-induced fever was measured by modifying the method described by Adams *et al*. (1968). Male Swiss Albino mice were fasted overnight with water *ad lib* before the experiments. Pyrexia was induced by sub-

cutaneously injecting 20% (w/v) brewer's yeast suspension (10 ml/kg) into the animals' dorsum region. Twenty-four hours after the injection, the rectal temperature of each mouse was measured using a digital thermometer (SK-1250 MC, Sato Keiryoki Mfg. Co., Ltd., Japan). The probe was attached to a digital display and was inserted 2 cm into the rectum. Test agent or cosolvent vehicle was administered orally and the temperature was measured at 1, 2, 3, 4 and 5 hr after drug administration.

Pentobarbital-induced sleep

Mice was injected with pentobarbital (50 mg/kg) intraperitoneally to induce sleep. The duration of sleep was measured as the period between the loss and the recovery of the righting reflex. The alkaloid extract of *K. macrophylla* (100, 200 and 400 mg/kg), and cosolvent vehicle were administered orally 30 min before pentobarbital (Ferrini *et al*., 1974).

Chemicals

The following drugs were used: morphine sulfate, brewer's yeast, pentobarbital sodium (AR grade, Sigma Chem. Co., St. Louis, U.S.A.), aspirin (AR grade, Srichand United Dispensary Co., Ltd., Bangkok, Thailand), formalin, sodium chloride (AR grade, Carlo Erba, Germanny), acetic acid (AR grade, J.T, Baker Inc., Phillipsburg, U.S.A.), methanol, chloroform (AR grade, Merck KGaA, Germany). *K. macrophylla* extract and aspirin were suspended in cosolvent solution (propylene glycol : ethanol : tween 80 : water = 4:1:1:4), and administered orally in a constant volume (10 ml/kg) 30 min before the experiments. Pentobarbital sodium was dissolved in 0.9% sodium chloride solution and administered intraperitoneally. Morphine sulfate was also dissolved in 0.9% sodium chloride solution and administered subcutaneously. All drug solutions were prepared immediately before starting the experiments.

Statistical Analysis

Data are expressed as means ± S.E.M. and were analyzed statistically by one-way ANOVA, followed by Dunnett's test. A difference was considered significant at $p<0.05$.

Results

Acute toxicity

In acute toxicity test, the signs of toxicity included irritability, muscle spasm, convulsion and death. The LD_{50} value of intraperitoneally injected of the alkaloid extract of *K. macrophylla* in mice was 318.46 mg/kg.

Effects of *K. macrophylla* extract on nociceptive responses

Writhing test

Oral administration of the alkaloid extract of *K. macrophylla* (100-400 mg/kg) significantly attenuated the number of writhings and stretchings induced by intraperitoneal 0.6% acetic acid at the dose of 400 mg/kg. The reference drug aspirin (200 mg/kg) also produced significant protective effects towards the acetic acid-induced pain (Table 1).

Hot plate test

Neither the *K. macrophylla* extract (100, 200 and 400 mg/kg, p.o.) nor aspirin (200 mg/kg, p.o.) significantly exerted protective effects on heat-induced pain in mice. By contrast, a centrally

Table 1. Effect of the *K. macrophylla* extract and aspirin on acetic acid-induced writhing in mice.

Drug	Dose (mg/kg, p.o.)	No. of writhings (counts/20 min)
Cosolvent	-	52.4±3.7
Aspirin	200	21.6±4.5*
K. macrophylla	100	55.5±4.9
	200	52.7±3.5
	400	30.7±5.6*

The *K. macrophylla* extract was orally administered. After 30 min, 0.6% acetic acid solution (10 ml/kg) was intraperitoneally injected in mice. Immediately after injection, the number of writhings was counted over a 20-min period. Each datum represents the mean±S.E.M. from 10 mice. *p<0.05, compared with the control group (Dunnett's test).

acting analgesic drug, morphine sulfate (10 mg/kg, s.c.) markedly increased pain latency (Table 2).

Formalin test

The alkaloid extract of *K. macrophylla* reduced the licking activity only in the late phase but not in the early phase as shown in Table 3. Aspirin (200 mg/kg) also produced similar effects on formalin-induced pain. In contrast, the reference antinociceptive drug morphine sulfate (10 mg/kg, s.c.) significantly reduced the licking activity against both phases of formalin-induced nociception.

Effect of *K. macrophylla* extract on yeast-induced fever in mice

The alkaloid extract of *K. macrophylla* had no significant effect on pyrexia induced by yeast in mice while a reference drug aspirin reversed yeast-induced fever (Table 4).

Table 2. Effect of the *K. macrophylla* extract, aspirin and morphine on nociceptive response induced by heat in mice.

Drug	Dose (mg/kg, p.o.)	Latency of nociceptive response (sec)			
		15	30	45	60 min
Cosolvent	-	8.9±0.7	10.1±1.3	11.0±1.2	12.6±1.2
K. macrophylla	100	11.0±0.8	12.1±1.6	12.4±1.9	13.2±2.0
	200	12.6±1.3	9.2±0.8	12.3±1.1	12.4±1.2
	400	12.5±0.9	11.0±1.7	10.9±1.4	12.6±1.3
Aspirin	200	11.6±1.3	9.9±0.7	10.4±1.5	13.2±1.2
Morphine sulfate	10	18.5±1.6*	26.4±3.3*	31.6±3.4*	19.8±2.0*

Beginning thirty min after oral administration of test agents (or 15 min after morphine injection, s.c), the nociceptive response was measured every 15 min over a 60-min period. Each datum represents the mean latency of nociceptive responses (sec) ± S.E.M. (n = 10)
*** p<0.05 compared with the control group (Dunnett's test).**

Table 3. Effect of the *K. macrophylla* extract, aspirin and morphine on hindpaw licking in the formalin test in mice.

Drug	Dose (mg/kg, p.o.)	Early Phase (sec)	Late Phase (sec)
Cosolvent	-	77.8±7.6	116.5±14.8
Aspirin	200	67.8±3.3	66.9±6.9*
Morphine	10	2.8±1.1**	0.0±0.0**
K. macrophylla	100	82.7±4.3	123.4±18.4
	200	89.5±5.7	101.5±14.0
	400	59.8±6.7	63.3±8.5*

Thirty min after test drug administration (p.o.), (or 15 min after morphine injection, s.c.) 2.5% formalin was subcutaneously injected to a hindpaw in a volume of 20 µl. Each datum represents the mean licking time ± S.E.M. from 10 mice in the early phase (0-5 min) and the late phase (15-30 min) after formalin injection. *p<0.05, **p< 0.01 compared with the control group (Dunnett's test).

Table 4 . Effect of the *K. macrophylla* extract and aspirin on brewer's yeast-induced fever in mice.

Drug	Dose (mg/kg, p.o.)	Average rectal temperature (°C)					
		0	1hr	2hr	3hr	4hr	5hr
Cosolvent	-	36.5±0.1	36.0±0.2	36.1±0.2	35.7±0.2	35.7±0.1	35.7±0.1
K. macrophylla	100	36.5±0.3	34.9±0.6	34.6±0.5	34.9±0.5	35.0±0.3	34.9±0.3
	200	36.1±0.5	35.0±0.6	34.4±0.6	34.8±0.4	34.5±0.5	34.5±0.5
	400	36.2±0.4	35.7±0.4	34.8±0.5	35.0±0.4	34.9±0.2	34.8±0.4
Aspirin	200	36.7±0.1	34.7±0.3	34.3±0.3	33.9±0.2*	33.8±0.2*	34.4±0.2

Twenty percent of yeast suspension was subcutaneously injected into the dorsum region of mice. Twenty-four hours after injection, rectal temperature was measured (time 0) and then drugs were orally administered. The temperature was again measured at 1, 2, 3, 4 and 5 hr after drug administration. Each datum represents the mean rectal temperature (°C) ± S.E.M. (n = 10) *p<0.05, compared with the control group (Dunnett's test).

Table 5. Effect of the *K. macrophylla* extract on pentobarbital-induced sleep in mice.

Drug	Dose (mg/kg, p.o.)	Duration of pentobarbital-induced sleep (min)
Cosolvent	-	64.0±4.5
K. macrophylla	100	65.2±4.3
	200	81.0±4.9*
	400	93.2±5.8*

***K. macrophylla* extract was orally administered. After 30 min, pentobarbital (50 mg/kg, i.p.) was injected, and sleeping time was measured. Each datum represents the mean ± S.E.M. from 10 mice. *p<0.05, compared with the control group (Dunnett's test).**

Effect of *K. macrophylla* extract on pentobarbital-induced sleep in mice

As shown in Table 5, the alkaloid extract of *K. macrophylla* dose-dependently (100-400 mg/kg, p.o.) prolonged the duration of pentobarbital-induced sleep in mice.

Discussion

The results demonstrate that the alkaloid extract obtained from the leaves of *K. macrophylla* attenuated nociceptive responses to chemical stimuli in the acetic acid-induced writhing and in the formalin test in mice.

The alkaloid extract of *K. macrophylla* exerted protective action in the writhing test similar to the reference peripheral analgesic compound, aspirin. This test is generally used for screening of antinociceptive effect (Koster *et al*., 1959; Hendershot & Forsaith, 1959). Thus the active compound(s) in the alkaloid extract of *K. macrophylla* leaves may possess analgesic action.

Thermic painful stimuli are known to be selective to centrally, but not peripherally, acting analgesic drugs (Chau, 1989). In the present study, morphine, a centrally acting analgesic drug, produced an inhibitory effect on the nociceptive response in this test, while the alkaloid extract of *K. macrophylla* and aspirin, peripherally acting analgesic drug, failed to affect the response. These findings, therefore, suggest that the apparent antinociceptive action of the active compound(s)

in the alkaloid extract of *K. macrophylla* may be mediated through peripheral but not central mechanism(s).

The formalin test is another pain model, which assesses the way an animal responds to moderate, continuous pain generated by injured tissue (Tjolsen, 1992). Centrally acting drugs such as morphine inhibited both of the early and late phases equally while peripherally acting drugs such as aspirin only inhibited the second phase. (Dubuisson and Dennis, 1977; Hunskaar and Hole, 1987; Shibata *et al*., 1989). The alkaloid extract of *K. macrophylla* produced significantly reduction of licking activity at the dose of 400 mg/kg in the late phase only but did not affect the responses in the early phase, suggested that its antinociceptive action may be mediated by peripheral mechanism.

Most of the nonsteroidal anti-inflammatory drugs such as aspirin possess the analgesic, antipyretic and anti-inflammatory actions (Robert and Morrow, 2001). But in our study, the alkaloid extract of *K. macrophylla* exhibited the antinociceptive effect in writhing and formalin models but it had no significant reduction in yeast-induced fever. In addition, it did not significantly reduce the carrageenin-induced paw edema in rats (data not shown). So it is possible that its analgesic action may be involved in some different mechanism(s) from those of the nonsteroidal anti-inflammatory drugs. Although the roots and stems of *K. macrophylla* have been used as cool medicine preparations for relief of fever in folk medicine (Pongbunrod, 1979; Wuthammawech, 1997), in our study it was found that the alkaloid extract from the leaves of *K. macrophylla* did not suppress the fever induced by yeast in mice. It is possible that some active components in the roots and stems may be different from those of the leaves.

It seems that a sedative effect of the alkaloid extract of *K. macrophylla* could apparently account for the antinociceptive responses in the tests used in this study. Thus, the sedative effect of the alkaloid extract of *K. macrophylla* on analgesic responses cannot be excluded. As the alkaloid extract apparently depressed the central nervous system, it is expected some potential anticonvulsive activities of the *K. macrophylla* extract but unfortunately no significant suppressive effect on convulsion-induced by pentylenetetrazole in mice was observed (data not shown).

In conclusion, these results suggest that the alkaloid extract of *K. macrophylla* possesses analgesic action via peripheral nervous pathway but has no antipyretic activity.

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