ORIGINAL ARTICLE

Antinociceptive activity of *Dyera costulata* extract in experimental animals

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Abstract

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The effects of the chloroform extract from the leaves of *Dyera costulata* Hook. f. (*D. costulata*) on nociceptive response using writhing, hot plate and formalin tests in mice and the antipyretic activity in yeast-induced fever in rats, were examined. General behavior was also examined using pentobarbital-induced sleep and locomotor activity in mice. The LD_{50} value of intraperitoneally injected *D. costulata* extract in mice was 0.27 g/kg. Oral administration of *D. costulata* extract (100-400 mg/kg) dose dependently 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 extract (100-400 mg/kg, p.o.) had no significant effect on fever induced by yeast in rats. The *D. costulata* extract (100-400 mg/kg, p.o.) prolonged the duration of pentobarbital-induced sleep but had no significant effect on locomotor activity in mice. These results suggest that the *D. costulata* extract possesses marked analgesic but no antipyretic effect.

Key words : *Dyera costulata*, antinociceptive activity, writhing test, formalin test

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ผลของสารสกัดจากตีนเป็ดแดงต่อการแก้ปวดในหนูทดลอง

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ได้ทำการทดสอบผลทางเภสัชวิทยาของสารสกัดคลอโรฟอร์มจากใบตีนเป็ดแดงในหนูทดลอง โดยสังเกตผลของสารสกัดต่อการระงับปวดซึ่งเกิดจากกรดอะเซติก ความร้อน และฟอร์มาลินในหนูถีบจักร และผลต่อการลดไข้ซึ่งเกิดจากการเหนี่ยวนำโดยยีสต์ในหนูขาว ยังได้สังเกตผลต่อพฤติกรรมทั่วไปโดยใช้การเหนี่ยวนำให้หลับด้วยเพ็นโทบาร์บิตาลและการเคลื่อนที่ในหนูถีบจักร ขนาดของสารสกัดที่ทำให้หนูถีบจักรตาย 50% มีค่า 0.27 มก./กก. เมื่อฉีดเข้าทางหน้าท้องในหนูถีบจักร เมื่อป้อนสารสกัดจากตีนเป็ดแดง (100-400 มก./กก.) เข้าทางปากในหนูทดลอง พบว่าสามารถลดจำนวนของการบิดและยืดของลำตัวเมื่อถูกกระตุ้นโดยกรดอะเซติก และลดการเลียในช่วงเฟสหลังของการทดสอบด้วยฟอร์มาลินในหนูถีบจักร ผลของสารสกัดจะเพิ่มขึ้นตามขนาดของสารสกัดที่ใช้ แต่ไม่มีผลต่อการทดสอบด้วยความร้อน สารสกัด (100-400 มก./กก.) ไม่มีผลในการลดไข้ซึ่งเกิดจากการเหนี่ยวนำโดยยีสต์ในหนูขาว สารสกัดเสริมฤทธิ์การหลับของเพ็นโทบาร์บิตาลให้ยาวนานขึ้น แต่ไม่มีผลต่อการเคลื่อนที่ในหนูถีบจักร จากผลการทดลองนี้เสนอว่า สารสกัดตีนเป็ดแดงมีฤทธิ์แก้ปวดที่เด่นชัด แต่ไม่มีฤทธิ์ลดไข้

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Dyera costulata Hook. f. (*D. costulata*) is a big tree having a yellow heartwood. It occurs in primary evergreen lowland or hill forest in the family of Apocynaceae. It can be found in the South of Thailand, Peninsular Malaysia, Singapore, Sumatra, Borneo and intervening islands. This plant is known in Thai as "Teen-Pet Daeng" (Ridley, 1923; Whitmore, 1973). The bark and leaves of *D. costulata* have been used in folk medicine for treatment of fever by traditional doctors in the South of Thailand. The constituents from the leaves of *D. costulata* have been identified to contain 6 bisindole alkaloids, ochrolifuanines A, E, F and 18-dehydrochrolifuanines A, E, F (Mirand, *et al*., 1983).

Although *D. costulat*a has been used for a long time as herbal medicine in the South of Thailand, no pharmacological studies have previously been conducted on analgesic and antipyretic actions of this plant. In the present study, in order to evaluate the potential existence of analgesic and antipyretic activities of the extract obtained from *D. costulata*, we investigated the antinociceptive effects of the extract using the writhing, hot plate and formalin tests in mice, and its antipyretic activity in yeast-induced fever in rats. In addition, we also studied the general behavior using pentobarbital-induced sleep and locomotor activity in mice.

Materials and Methods

Plant material

The leaves of *Dyera costulata* (Apocynaceae) were collected in March, 1999 from Songkhla Province, Thailand. The plant was identified by direct comparison with herbarium specimens in the PSU Herbarium, Department of Biology, Faculty of Sciences, Prince of Songkla University. A voucher specimen of plant material has been deposited in the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, Thailand.

Preparation of the extract from the leaves of *Dyera costulata*

The dried coarsely powdered leaves of *D. costulata* (15.0 kg) were macerated with 30.0 l

of methanol for five days and then filtered and evaporated. The marc was remacerated with methanol (30.0 l) for five days four times and filtered. The combined filtrate was concentrated to a syrupy mass under reduced pressure, mixed with 2% H_2SO_4 (5 × 1000 ml) and filtered. The acidic filtrate was washed with portions of n-hexane (2 × 800 ml), then made basic (pH 8) with 25% NH_4OH and extracted with $CHCl_3$ (6 × 800 ml). The combined $CHCl_3$ extract was washed with water (2 × 1000 ml), dried over anhydrous Na_2SO_4 and evaporated to yield the crude alkaloids 18.058 g. The dried chloroform extract was used as the test extract. One gram of the extract was equivalent to 830.66 g of dried powdered leaves of *D. costulata*. All doses were expressed in terms of dried crude extract (mg/kg body weight).

Animals

All animals used in this study were obtained from the Animal House, Faculty of Sciences, Prince of Songkla University, Hat Yai, Songkhla, Thailand. Male Swiss mice with the weight ranging from 28-38 g were used for all experiments except for yeast-induced fever test, in which male Wistar rats with the weight ranging from 150-220 g were used. The rats were handled for 5-10 min daily for several days before experiments. The 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 *D. costulata* extract was estimated by the up-and-down method in mice (Bruce, 1985). Doses were adjusted by a constant multiplicative factor; viz. 4, for this experiment. The dose for each successive animal was adjusted up or down depending on the previous outcome.

Antinociceptive activity

1. Writhing test

Writhing behaviour was tested, in which 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). The plant extract (100, 200 and 400 mg/kg), a reference analgesic drug aspirin (200 mg/kg) or cosolvent vehicle was orally administered 30 min before acetic acid.

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 °C ± 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 *D. costulata* extract (100, 200 and 400 mg/kg, p.o.), aspirin (200 mg/kg, p.o.) or cosolvent, 20 µl of 2.5% formalin in saline was injected subcutaneously to a hindpaw of the mice. 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

Antipyretic actvity of drug was measured by slightly modifying the method described by Adams *et al*. (1968). Male Wistar rats were fasted overnight with water *ad lib* before the experiments. Pyrexia was induced by subcutaneously injecting 20% (w/v) brewer's yeast suspension (10 ml/kg) into the animals' dorsum region. Nineteen hours after the injection, the rectal temperature of each rat was measured using a digital thermometer (SK-1250MC, Sato Keiryoki Mfg. Co., Ltd., Japan). Only rats that showed an increase in temperature of at least 0.7 °C were used for the

experiments. 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

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

Locomotor activity

Locomotor activity was recorded in an activity cage (Basile, Milan) using a modification of a method previously reported (Capasso *et al*., 1996). The animals were placed in the cage for at least 10 min for acclimatization before oral administration of drugs. Temperature, sound and light conditions were maintained uniform during the course of the experiments. Measurements were performed at 5-min intervals and cumulative counts were recorded for a period of 1 hr. Experiments were carried out from 9 A.M. to 5 P.M.

Chemicals

The following drugs were used: morphine sulfate, brewer's yeast (AR grade, Sigma Chem. Co., St. Louis, U.S.A.), aspirin (AR grade, Srichand United Dispensary Co., Ltd., Bangkok, Thailand), sodium chloride (AR grade, Carlo Erba, Germanny), acetic acid (AR grade, J.T, Baker Inc., Phillipsburg, U.S.A.), silica gel (SiO_2 230-400 mesh, ASTM, Merck KGaA, Germany), n-hexane, chloroform, methanol and ethyl acetate (AR grade, Merck KGaA, Germany). *D. costulata* extract and aspirin were dissolved in cosolvent solution (propylene glycol : ethanol : tween 80 : water = 4:1:1:4), and administered orally in a constant volume (10 ml/kg for mice and 5 ml/kg for rats) 30 min before the experiments. Morphine sulfate was 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 ± SEM and analyzed statistically using Student's t-test and paired t-test. A difference was considered significantly at $p<0.05$.

Results

Acute toxicity

In the acute toxicity test, signs of toxicity included lethargy, jerk, convulsion and death. The LD_{50} value of intraperitoneally injected *D. costulata* extract in mice was 0.27 g/kg.

Effects of *D. costulata* on nociceptive responses

Writhing test

Oral administration of the *D. costulata* (100-400 mg/kg) dose dependently attenuated the number of writhings and stretchings induced by intraperitoneal 0.6% acetic acid (Figure 1). The reference drug aspirin (200 mg/kg) also produced significant protective effects towards the acetic acid-induced pain.

Hot plate test

The mean latency of nociceptive responses to thermal stimuli is summarized in Table 1. Neither the *D. costulata* 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 acting analgesic drug, morphine sulfate (10 mg/kg, s.c.) markedly increased pain latency.

Formalin test

Either the *D. costulata* extract or aspirin (200 mg/kg) decreased the licking activity only in the late phase but not in the early phase of formalin-induced pain (Figure 2). In contrast, the reference antinociceptive drug morphine sulfate (10 mg/kg) significantly reduced the licking activity against both phases of formalin-induced nociception.

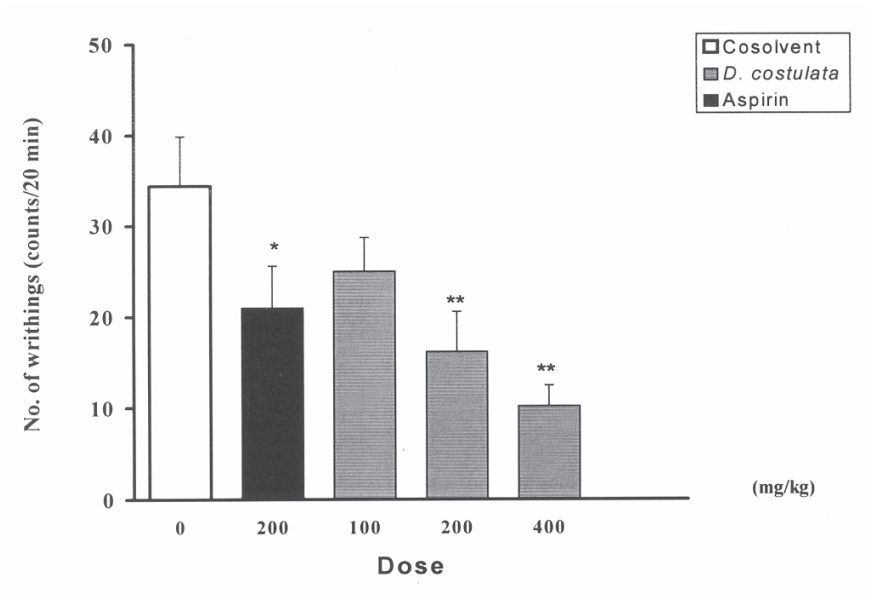

Figure 1. Effect of *D. costulata* extract and aspirin on acetic acid-induced writhing in mice. Each column represents the mean ± S.E.M. (n=10) *p<0.05, **p<0.01 compared to the control group (Student's t-test)

Table 1. Effect of *D. costulata* 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	-	11.7 ± 0.8	9.8 ± 0.7	11.6 ± 1.3	8.2 ± 0.5
D. costulata	100	12.6 ± 1.2	9.8 ± 0.8	10.7 ± 1.2	8.3 ± 0.6
	200	12.0 ± 0.8	10.9 ± 1.0	11.2 ± 1.0	11.0 ± 1.1
	400	12.7 ± 1.0	13.0 ± 0.9	11.9 ± 0.9	10.1 ± 1.3
Aspirin	200	11.1 ± 1.3	12.3 ± 1.2	9.2 ± 1.3	10.0 ± 0.9
Morphine sulfate	10	18.0 ± 2.3*	25.0 ± 2.1**	18.2 ± 2.7	19.8 ± 1.7**

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, ** p<0.01 compared with the control group (Student's t-test).**

Effect of *D. costulata* on yeast-induced fever in rats

The *D. costulata* extract had no significant effect on pyrexia induced by yeast while the reference drug aspirin reversed yeast-induced fever (Table 2).

General behavior

As shown in Table 3, oral administration of *D. costulata* extract dose-dependently (100-400 mg/kg) prolonged the duration of pentobarbital-induced sleep but caused no significant change in spontaneous motor activity in mice.

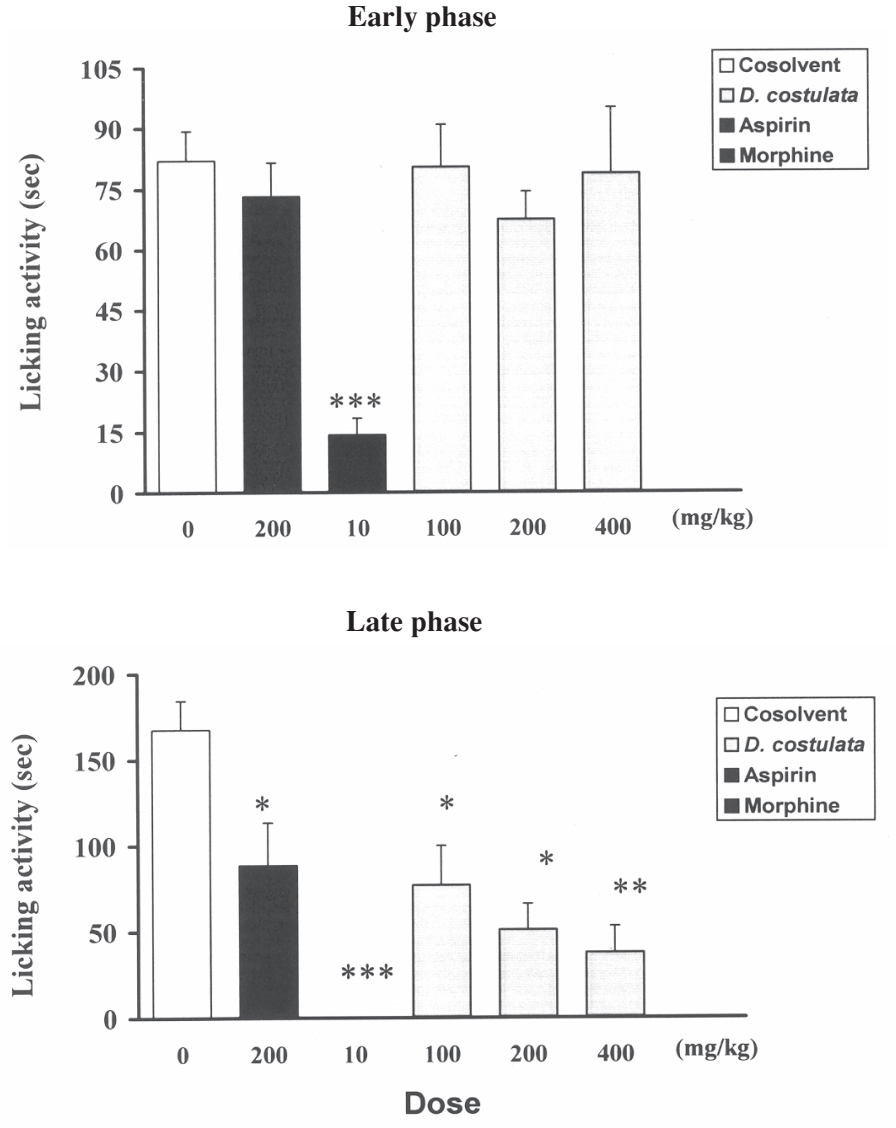

Figure 2. Effect of *D. costulata* extract, aspirin and morphine on hindpaw licking in the formulin test in mice. Each column represents the mean±S.E.M. (n=10) *p<0.01 **p<0.001 and **p<0.0001, compared to the control group (Student's t-test)

Discussion

The results demonstrate that the chloroform extract obtained from the leaves of *D. costulata* attenuated nociceptive responses to chemical stimuli in the acetic acid-induced writhing and in the formalin test in mice but had no effect on yeast-induced hyperthermia in rats.

D. costulata extract exerted protective action in the writhing test relatively similar to the reference peripheral analgesic compound, aspirin. The extract showed more marked inhibitory effect than aspirin. This test is generally used for screening of antinociceptive effect (Koster *et al.*, 1959; Hendershot & Forsaith, 1959). Thus the extract may possess analgesic action.

Table 2. Effect of *D. costulata* extract and aspirin on brewer's yeast-induced fever in rats.

Drug	Dose (mg/kg, p.o.)	Average rectal temperature (°C)					
		0	1hr	2hr	3hr	4hr	5hr
Cosolvent	-	37.3 ± 0. 2	37.4 ± 0.2	37.2 ± 0.4	37.0 ± 0.1	36.8 ± 0.2	37.1 ± 0.3
D. costulata	100	37.8 ± 0.3	37.8 ± 0.5	38.0 ± 0.6	37.9 ± 0.5	37.8 ± 0.5	37.7 ± 0.4
	200	37.6 ± 0.4	37.4 ± 0.5	37.6 ± 0.5	37.6 ± 0.4	37.3 ± 0.4	37.2 ± 0.3
	400	37.6 ± 0.3	37.6 ± 0.3	37.7 ± 0.4	37.6 ± 0.4	37.6 ± 0.3	37.3 ± 0.3
Aspirin	200	37.5 ± 0.2	36.7 ± 0.3*	36.4 ± 0.3**	36.5 ± 0.4**	36.4 ± 0.4*	36.0 ± 0.2**

Twenty percent of yeast suspension was subcutaneously injected into the dorsum region of rats. Nineteen 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 = 4-5) *p<0.05, **p<0.01 compared with the before test agent administration (paired t-test).

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 *D. costulata* extract failed to affect the response. These findings, therefore, suggest that the apparent antinociceptive action of the *D. costulata* extract 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). The effects of drugs on the licking responses in the early and late phases reportedly represent antinociceptive action on sensory receptor stimulation and anti-inflammatory action, respectively (Dubuisson and Dennis, 1977; Hunskaar and Hole, 1987). The *D. costulata* extract produced a dose-related reduction of licking activity only in the late phase but not affecting the responses in the early phase, which more pronounced than those of aspirin, suggesting the anti-inflammatory action of this extract. It is interested that in both the writhing and formalin test model, the *D. costulata* extract showed more marked antinociceptive responses than those of the reference drug, aspirin. The active compounds contained in the extract need to be determined.

Unfortunately, the *D. costulata* extract had no significant effect on yeast-induced fever in rats, which does not support the indication for use of *D. costulata* to decrease fever in folk medicine.

It seems that a sedative effect of *D. costulata* extract could apparently account for the antinociceptive responses in the tests used in this study; although the extract showed no significant effect on locomotor activity these were tendency for locomotor activity to be reduced. Thus, the sedative effect of *D. costulata* extract on analgesic responses cannot be excluded.

In conclusion, these results suggest that the *D. costulata* extract possesses analgesic but no antipyretic effect. However, it is interesting to determine the active constituents contained in the extract which possess analgesic activity.

Acknowledgments

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Table 3. Effect of *D. costulata* extract on pentobarbital-induced sleep and locomotor activity in mice.

Drug	Dose (mg/kg, p.o.)	(A) Duration of pentobarbital-induced sleep (min)	(B) Locomotor activity (counts/30 min)
Cosolvent	-	70.9 ± 4.2	792.5 ± 251.5
D. costulata	100	86.0 ± 7.8	646.0 ± 298.4
	200	97.9 ± 4.9**	237.1 ± 75.6
	400	103.1 ± 11.7*	220.9 ± 59.0

(A) *D. costulata* 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. (n = 10).
(B) Thirty min after test agents administration (p.o.), changes in spontaneous motor activity were measured over a 30-min period. Each datum represents the mean ± S.E.M. from 10 mice. *p<0.05, **p<0.01 compared with the control group (Student's t-test).

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