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Antinociceptive Effects of *Alpinia katsumadai* via Cyclooxygenase-2 Inhibition

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Abstract − *Alpinia katsumadai* has been widely used in traditional Chinese and Korean medicine to treat a variety of conditions including emesis and gastric disorders such as gastric pain and distended abdomen. To investigate the antinociceptive potential and mechanism of *A. katsumadai*, ethanolic extracts of *A. katsumadai* were assayed on cyclooxygenase-2 and evaluated for analgesic activity based on phenylbenzoquinone (PBQ)-induced writhing and carrageenan-induced hyperalgesia tests. *A. katsumadai* extracts inhibited the cyclooxygenase-2 enzyme activity in a dose-dependent fashion at an IC_{50} value of 0.044 μg/ml. *A. katsumadai* extract (30-300 mg/kg, orally (p.o.) administered) significantly inhibited PBQ-induced writhing. This inhibition was judged not to be a false positive because a Rota-rod test revealed no difference in muscular coordination when compared to the controls. With regard to the carrageenan-induced hyperalgesia, *A. katsumadai* extract (30-300 mg/kg, p.o.) produced a significant, dose-dependent increase in the withdrawal response latencies. Naloxone did not reverse the analgesic effect of *A. katsumadai* extract in the carrageenan-induced hyperalgesia. Taken together, these results suggest that the antinociceptive activity of *A. katsumadai* is not related to the opioid receptor. *A. katsumadai* extract has remarkable, non-opioid-receptor-mediated analgesic effects on PBQ-induced writhing and carrageenan-induced hyperalgesia that occur via cyclooxygenase-2 inhibition.

Keywords: *Alpinia katsumadai*, Analgesic, Anti-inflammatory, Antinociceptive, Phenylbenzoquinone

INTRODUCTION

The plant used in this study, *Alpinia katsumadai*, was selected based on the use of its seeds in traditional Chinese and Korean medicine (Ching, 1978; Kim *et al*., 2000). *A. katsumadai* has been widely used in traditional Chinese and Korean medicine to treat a variety of conditions including emesis and gastric disorders such as gastric pain and distended abdomen. *Alpinia katsumadai* is often used with other herbs such as cinnamon bark and ginger (Dong *et al*., 1992). The extract from *A. katsumadai* seeds is known to have significant antioxidant activity (Lee *et al*., 2003), antiemetic activity (Yang *et al*., 1999) and to inhibit prostaglandin production in LPS-stimulated mouse peritoneal macrophages (Noh *et al*., 1998).

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In addition, we recently reported that *A. katsumadai* has anti-pruritic activities in a thromboxane A_2-involved mouse model (Choi *et al*., 2009). The traditional use of *A. katsumadai* for the treatment of gastric pain and distended abdomen suggest that the antinociceptive and anti-inflammatory activity of *A. katsumadai* may play a role in the use of this plant. However, very little is known about the antinociceptive activities of *A. katsumadai* even though it is already known that some other Alpinia species have antinocicpetive effects. Therefore, in the current investigation, we assessed the analgesic potential of *A. katsumadai* extract in animals as well as the related mechanism of the antinociceptive effects.

MATERIALS AND METHODS

Chemicals and animals

Indomethacin, carrageenan and phenylbenzoquinone

(PBQ) were purchased from Sigma (St. Louis, USA).

All of the mice and rats used in the present study were purchased from Samtako Korea and maintained under SPF conditions until required. The animals were housed under standard conditions (22 ± 2°C, 50 ± 10% humidity and a 12 h light-dark cycle) and maintained with free access to water and a regular diet.

Male Sprague-Dawley rats (6-7 weeks) and male ICR mice (5-6 weeks) were used in the experiments, and all experiments were conducted under the Korea Association for Laboratory Animal Science's guidelines for the care and use of experimental animals.

Preparation of *A. katsumadai* extract

Dried seeds of *A. katsumadai* were purchased at the Kyungdong Oriental Drug Store (Suwon, Korea) in 2007. The materials obtained were authenticated by Professor Sun Yeou Kim of the Department of Medical Science, Graduate School of East West Medical Science, Kyung Hee University, Korea. A voucher specimen (PRI99-121) has been deposited in the Natural Resource Deposit, Amorepacific R&D Center. *A. katsumadai* (200 g) was extracted at 50°C in 95% ethanol for 15 h, after which the extract was filtered and the filtrate was concentrated in a vacuum rotary evaporator under low pressure.

Fingerprint of *A. katsumadai* extracts by gas chromatography-mass spectrometry (GC-MS)

An Agilent 6890 gas chromatograph coupled with a JEOL GC-Mate 2 mass spectrometer was used to establish the fingerprint of *A. katsumadai* extracts. The separation was performed on a HP-5MS column (0.25 mm i.d. X 30 m, 0.32 μm coating thickness). The temperature of the column was programmed to increase from 50 to 300°C at 10°C/min during analysis, while the injector temperature and the detector temperature were maintained at 280°C. Helium was applied as the carrier gas at a constant flow rate of 1.0 ml/min. The mass spectrometer was operated at 70 eV and a scan range of 5-500 amu. Some of the compounds were identified from the recorded mass spectra by comparison with the mass spectra from the NIST and Wiley libraries.

Cyclooxygenase-2 enzyme assay

The cyclooxygenase-2 activity was determined by a spectrophotometric assay conducted using recombinant ovine COX-2 enzymes (Cayman Chemicals, Ann Arbor, MI) (Ouellet *et al*., 2001). Briefly, 50 nM COX-2 in 100 mM Tris buffer (pH 8.0) and 0.5 mM heme were added to the appropriate wells. The reaction was then initiated with 100 mM arachidonic acid and 10 mM TMPD (N,N,N',N'-tetrametyl-p-phenylenediamine) after incubation with the extracts of *A. katsumadai* for 5 min under the indicated concentrations. After 5 min of incubation at room temperature absorbance at 590 nm was read. Indomethacin was used as a reference compound.

Phenylbenzoquinone (PBQ)-induced writhing in mice

The writhing test was conducted according to the modified method described by Siegmund *et al*. (1957). Acute pain was induced by intraperitoneal injection of 0.2 ml of 0.02% PBQ 54 min after p.o. administration of the *A. katsumadai* extract or other compounds. Six min after the PBQ injection, the total number of writhes was counted for 6 min. The control animals received an appropriate volume of dosing vehicle (80% saline, 10% ethanol and 10% Tween 80). Indomethacin was used as a positive control.

Carrageenan-induced hyperalgesia test in rats

The carrageenan-induced hyperalgesia test was conducted according to the modified method described by Randall and Selitto (1957) (Siegmund *et al*., 1957). The nociceptive pressure threshold was determined for the left hindpaw (Analgesymeter, Ugo Basile, Milan) using a wedge-shaped mechanical probe to apply graded pressure stimuli to the paw. The cut-off threshold was set at 250 g. The end point was taken as paw withdrawal, vocalization or overt struggling. The nociceptive pressure thresholds were determined in the absence of inflammation. The rats were divided into groups of six according to their nociceptive pressure thresholds, after which carrageenan (0.1 ml, 1%) was injected into the plantar surface of the left hindpaw. The rats received vehicle or compounds 2 h after carrageenan injection and were evaluated for paw hyperalgesia 0, 1, and 2 h after compounds administration. To investigate the involvement of the opioid receptor, the rats were injected with 5 mg/kg of naloxone hydrochloride (s.c.) and *A. katsumadai* extract (p.o.) simultaneously.

Carrageenan-induced hind paw edema test in rats

The carrageenan-induced hind paw edema test was conducted according to the modified method described by Winter *et al*. (1962). Briefly, the rats were divided randomly into groups of five, after which they were injected subcutaneously through the plantar surface of the hind paw with 0.05 ml of 1% carrageenan. Different doses of plant extract were injected p.o. 1 h before the administration of carrageenan. Indomethacin was used as a positive control. Rat paw edema was assessed using a plethysmometer (Ugobasile 7150) before and 3 h after the carra-

geenan injection. The difference in paw volume determined before and after the carrageenan injection was taken to indicate the severity of edema.

Rota-rod test in mice on muscular coordination

The rota-rod (Ugo Basile, model-7600) consisted of a bar with a diameter of 2.5 cm subdivided into five compartments by disks 25 cm in diameter. The bar rotated at a constant speed of 30 revolutions per minute. Mice were tested at 1 h after administration of the extracts. This test requires a high degree of sensory motor coordination and is therefore used to test more subtle neurological deficits than those determined by loss of the righting reflex. All mice utilized were initially tested and shown to be able to pass the rota-rod test prior to drug administration and subsequent evaluation.

Statistical data analysis

All data are presented as means ± S.E. Statistical significance ($p < 0.05$) was determined by one-way analysis of variance (ANOVA) followed by Dunnett's test. $p < 0.05$ relative to the control group was considered to be significant.

RESULTS

The fingerprint of *A. katsumadai* extracts established by gas chromatography-mass spectrometry (GC-MS)

The fingerprint of *A. katsumadai* extracts was obtained by GC-MS (Fig. 1). Based on the chromatogram of the extracts, four main component peaks were detected: eucalyptol (retention time: 7.00 min), hexadecanoic acid (retention time: 19.14 min), oleic acid (retention time: 20.57 min) and 4,6-heptadien-3-one, 1,7-diphenyl (retention time: 24.09 min).

Effects of *A. katsumadai* on cyclooxygenase-2 enzyme activity

We reported that the extracts of *A. katsumadai* suppressed the production of PGE_2 in LPS-treated macrophages (Noh *et al*., 1998). Recently, we showed that the suppression of PGE_2 production was due to reduced expression of COX-2 (Choi *et al*., 2009). Therefore, this study was conducted to examine the direct effects of *A. katsumadai* extracts on COX-2 enzyme. The results revealed that *A. katsumadai* extracts significantly suppressed COX-2 enzyme activity (Table I) and that the IC_{50} of the extract against COX-2 was 0.044 μg/ml.

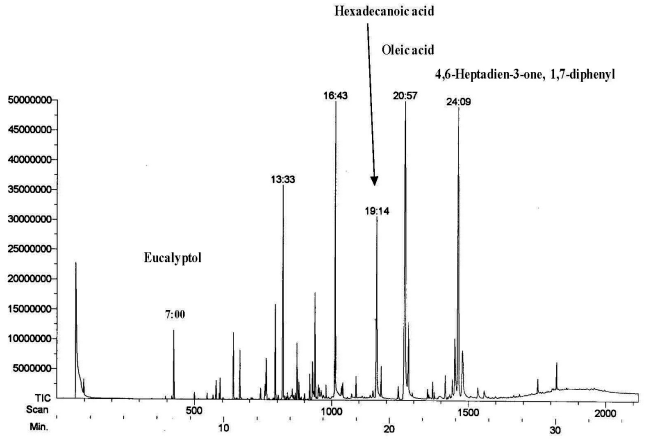

Fig. 1. The fingerprint of the *A. katsumadai* extracts obtained by GC-MS showing several compounds and four main compounds were identified: Eucalyptol (retention time ¼ 7.00 min), Hexadecanoic acid (retention time ¼ 19.14 min), Oleic acid (retention time ¼ 20.57 min) and 4,6-Heptadien-3-one, 1,7-diphenyl (retention time ¼ 24.09 min).

Table I. COX-2 inhibitory effect of *A. katsumadai*

A. katsumadai (μg/ml)	% inhibition (± S.D.)
0.003	−5.26 ± 1.55
0.006	0.533 ± 2.62
0.013	9.30 ± 6.36
0.025	29.9 ± 1.82
0.05	50.1 ± 2.89
0.1	60.0 ± 3.02
Indomethacin[a]	68.4 ± 5.45

[a]Indomethacin was used as a reference compound (10 μM).

Effects of *A. katsumadai* on phenylbenzoquinone (PBQ)-induced writhing test in mice

To assess the in vivo analgesic activity of *A. katsumadai* extracts, they were first evaluated in mice using the phenylbenzoquinone (PBQ)-induced writhing test. Intraperitoneal administration of PBQ induced 22 ± 2 (n=6) writhes in control rats during the 6 min observation period. However, the animals that received *A. katsumadai* at doses of 30, 100 and 300 mg/kg body wt. writhed 16 ± 1 (n=6), 15 ± 2 (n=6), and 11 ± 1 (n=6) times, respectively (Fig. 2A). For comparison, a group of animals administered 1 mg/kg body wt. indomethacin 30 min prior to noxious stimulation was evaluated and the number of writhes was found to be 13 ± 3 (n=6). Only treatment with *A. katsumadai* at 300 mg/kg body wt. induced a significant reduction in the writhing response ($p < 0.05$, ANOVA , Dunnett's test).

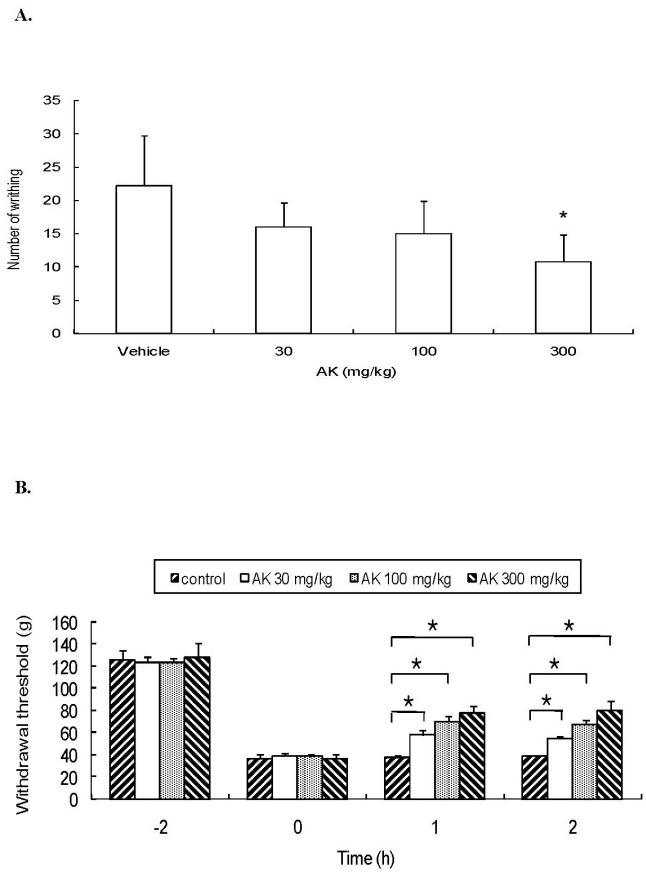

Fig. 2. (A) Effect of *A. katsumadai* (AK) plant extract and indomethacin on PBQ induced writhing in mice (n=6; mean ± SE). As compared with control: *$p < 0.05$, ANOVA (p=0.011, F=4.87), Dunnett's test. (B) Effect of different doses of the extract of *A. katsumadai* on the hind paw withdrawal threshold of rats (n=5; carrageenan-induced hyperalgesia test, mean ± SE). As compared with control of each indicated time point: *$p < 0.05$, ANOVA (1 h: p=0.000, F=16.21, 2 h: p=0.000, F=15.67), Dunnett's test.

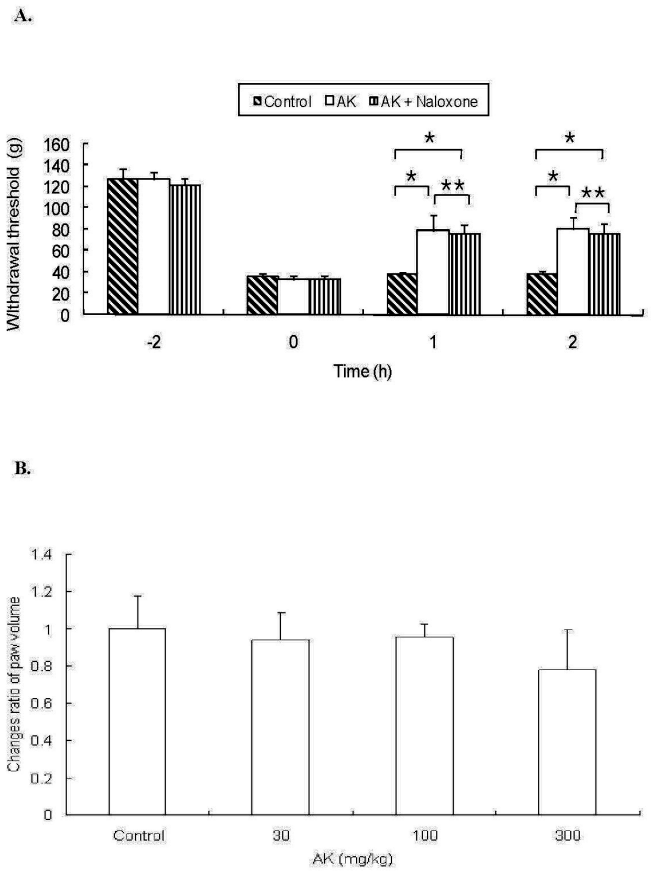

Fig. 3. (A) Effect of naloxone on the reaction time of rats induced by 300 mg/kg *A. katsumadai* (AK) extract (n=5-6; carrageenan-induced hyperalgesia test, mean ± SE). As compared with control of each indicated time point: *$p < 0.05$, ANOVA (1 h: p=0.007, F=7.48, 2 h: p=0.003, F=9.16), Dunnett's test, **No differences. (B) Effect of *A. katsumadai* plant extract on carrageenan induced paw edema in rats (n=5, mean ± SE). ANOVA (p=0.195, F=1.76).

Effects of *A. katsumadai* on carrageenan-induced hyperalgesia in rats

To determine if *A. katsumadai* had an analgesic effect, the rats were evaluated by means of a carrageenan-induced mechanical hyperalgesia study. The results revealed significant prolongation of the paw withdrawal threshold in rats treated with *A. katsumadai* extract at 1 and 2 h post-treatment (Fig. 2B). To determine if *A. katsumadai* produced an analgesic activity independent of the endogenous opiate system, the interaction between *A. katsumadai* and naloxone was evaluated. Furthermore, the results revealed that subcutaneous administration of naloxone did not significantly reduce the paw withdrawal threshold increased by the 300 mg/kg *A. katsumadai* extract (Fig. 3A).

Effects of *A. katsumadai* on carrageenan-induced hind paw edema test in rats

Measurement of footpad edema is a convenient method of assessing inflammatory response. *A. katsumadai* was assessed for acute anti-inflammatory action by examining its ability to reduce carrageenan-induced paw swelling. Animals injected with carrageenan and treated with vehicle displayed significant edema in the ipsilateral paw 3 h post-carrageenan treatment ($p < 0.05$ vs pre-carrageenan values; data not shown). Furthermore, p.o. administration of *A. katsumadai* extract (30-100 mg/kg) did not cause significant inhibition of the edema induced by sub-plantar carrageenan injection. A high dose (300 mg/kg) of *A. katsumadai* induced a slight suppression of the edema, but this effect was not statistically significant ($p > 0.05$ vs control, ANOVA, Dunnett test; Fig. 3B).

Table II. Treatment with chlorpromazine with the extract of *A. katsumadai* (p.o.) in rota-rod tests in mice

Treatments	Dose (mg kg^{-1})	Number of fallen mice[a]
Vehicle[b]	0	0/5
Alpinia katsumadai	300	0/5
Chlopromazine	10	5/5

[a]Within 2 min, [b]Vehicle: ethanol-tween 80-saline (1:1:8).

Effect of *A. katsumadai* on rota-rod test in mice

To exclude the possible non-specific action of the extract, animals were subjected to the rota-rod test to measure motor performance according to the method described by Duham and Miya, with minor modification (Dunham and Miya, 1957).

High-dose oral administration of *A. katsumadai* extracts (300 mg/kg) did not cause the latency to decrease significantly in the rota-rod test ($p > 0.05$). However, the reference drug, chlorpromazine (10 mg/kg), reduced the latency (Table II). Taken together, these findings indicate that the effects of *A. katsumadai* extracts on PBQ-induced writhing were not caused by impaired motor functions.

DISCUSSION

Analgesic therapeutic alternatives have been found to be only partially effective, and their application is commonly associated with severe side-effects that hinder their continuous use (Mendell and Sahenk, 2003). The interest in identifying new alternatives to treat inflammatory pain has increased greatly in recent years (Calixto *et al*., 2004), and naturally occurring plants seem to represent good choices for this purpose.

As shown in Fig. 1, the fingerprint of *A. katsumadai* showed the existence of eucalyptol (retention time: 7.00 min), hexadecanoic acid (retention time: 19.14 min), oleic acid (retention time: 20.57 min) and 4,6-heptadien-3-one, 1,7-diphenyl (retention time: 24.09 min), which is consistent with the results of a previous study conducted to evaluate *A. katsumadai* (Kuroyanagi, 1983; Yang *et al*., 1999).

As shown in Table I, *A. katsumadai* extracts showed strong inhibitory effects against COX-2 enzyme activity, as indicated by an IC_{50} of 0.044 μg/ml. These findings were somewhat surprising, and it is not clear which components of *A. katsumadai* are responsible for this highly potent inhibition. Diarylheptanoids from other *Alpinia* species have showed inhibitory effects against COX-2 enzyme and it has previously been reported that *A. katsumadai* contained diarylheptanoids, which was also confirmed by our fingerprint experiment (Yang *et al*., 1999) (Fig. 1). Taken together, these findings indicate that the diarylheptanoids from *A. katsumadai* may be potential COX-2 inhibitors.

The results of the present study also demonstrated that extracts of the seeds of *A. katsumadai* have antinociceptive activity as indicated by the PBQ-induced writhing and carrageenan-induced hyperalgesia test (Fig. 2). The PBQ-induced writhing test, which is generally used to assess the antinociceptive activity in mice, is known to be devoid of limitations due to interference drugs that inhibit writhing other than analgesics, such as antihistaminics, sympathomimetics, and parasympathomimetic-like central nervous system stimulants and adrenergic blockers (Bjorkman *et al*., 1994). Accordingly, this writhing test points out analgesic activity of tested material acting either through a peripheral or a central mechanism of action (Hendershot and Forsaith, 1959). Therefore, some sedatives possess antinociceptive activity (Rang *et al*., 1978). However, the antinociceptive activity of the *A. katsumadai* extract was found to be genuine in the present study. Specifically, the rota-rod test revealed no difference in muscular coordination when compared with the control, but the reference drug, chlorpromazine (10 mg/kg), reduced the latency (Table II).

There is compelling evidence linking bradykinin, prostaglandin E_2, leukotriene B_4, PAF, interleukin 1, 5-hydroxytryptamine and histamine to the pathophysiological processes that accompany tissue damage and inflammation, especially the production of pain and hyperalgesia (Steen *et al*. 1996; Smith *et al*., 1998). Carrageenan-induced hyperalgesia might be provoked via a central or peripheral action of mechanism. The fact that naloxone, which is a classical morphine receptor antagonist (Gracioso *et al*., 1998), did not block the antinociception induced by the *A. katsumadai* extract suggests that the antinociception was not mediated through opioid receptors or the release of endogenous opioid substances (Fig. 3). Thus *A. katsumadai* extract might not produce side effects of opioid tolerance such as physical dependence and withdrawal phenomena.

It has already been reported that two species of *Alpinia*, leaves of *A. zerumbet* (de Araujo *et al*., 2005) and rhizomes of *A. calcarata* (Arambewela *et al*., 2004), exhibit analgesic activities via the opioid receptor. It is quite interesting that the opioid receptor was not involved in the antinociceptive activity of *A. katsumadai* (Fig. 3B). This mechanistic difference is due to the varying compositions of compounds found in the different parts of *A. katsumadai*. For example, the composition of the compounds of the seeds of *A. katsumadai* is different from that of other parts such as the leaves of *A. zerumbet* and rhizomes of *A.*

calcarata. The mechanism underlying this analgesic effect of the seeds of *A. katsumadai* remains unknown, but appears to be related to peripheral nociceptive signaling since the classical morphine receptor antagonist naloxone did not block the antinociception induced by the *A. katsumadai* extract.

A. katsumadai has long been used to treat gastric pain and distended abdomen, but it has not been determined what component and biological activity of *A. katsumadai* are responsible for its analgesic effects. It is possible that the antinociceptive effect of *A. katsumadai* is due to its antioxidant (Lee *et al*., 2003) and anti-inflammatory activities (Noh, 1998; Choi *et al*., 2009). The seeds contain eucalyptol, a-humulene, *trans,trans*-farnesol(I), linalool, camphor, terpinen-4-ol, carvotanacetone, bornyl acetate, geranyl acetate, methyl cinnamate, nerolidol, alpinetin, cardamomin and diarylheptanoids (Yushiro *et al*., 1968; Saiki *et al*., 1978; Kuroyanagi *et al*., 1983; Brown and Rice-Evans, 1998) and it has been reported that eucalyptol and terpinen-4-ol have antinociceptive activity (Santos and Rao, 2000; Moreira *et al*., 2001).

It has been reported that flavonoids and chalcone possess antinociceptive activity (Kekesi *et al*., 2003; de Campos-Buzzi *et al*., 2006); therefore, these compounds in *A. katsumadai* may contribute to its antinociceptive activity. In addition, cardamonin, one of the major active constituents of *A. katsumadai*, was found to inhibit COX-2 and iNOS expression via inhibition of the NF-κB pathway in RAW 264.7 macrophage cells (Lee *et al*., 2006); therefore, this compound may be involved in the antinociceptive activity of *A. katsumadai*.

In conclusion, the results of this study demonstrated that the mechanism of the antinociceptive activity of *A. katsumadai* is not mediated by opioid receptors, but instead via suppression of COX-2, which differs from *A. zerumbet* and *A. calcarata*. Further studies are ongoing to elucidate the components of *A. katsumadai* responsible for its antinociceptive activity.

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