



Anti-inflammatory effect and acute oral toxicity of 4-methoxycinnamyl *p*-coumarate isolated from *Etingera pavieana* rhizomes in animal models

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ABSTRACT

4-methoxycinnamyl *p*-coumarate (MCC) is a phenolic constituent derived from *Etingera pavieana* rhizomes. Continuing from the recent study of the inhibitory activity of MCC on lipopolysaccharide-induced inflammatory response in murine macrophages, in the present study, MCC was extracted and its *in vivo* anti-inflammatory properties were evaluated in rats. To assess the safety of MCC, an acute oral toxicity study of MCC in mice was conducted following the Organization for Economic Cooperation and Development 420 guideline. It was observed that topical application of MCC (3 mg/ear) significantly suppressed ethyl phenylpropiolate-induced rat ear edema. In addition, MCC (25, 75, and 150 mg/kg, *p.o.*) significantly and dose-dependently inhibited carrageenan-induced paw edema in rats. In the acute oral toxicity study, neither mortality nor toxic signs were observed in MCC-treated mice even at the highest dose (2,000 mg/kg, *p.o.*). These results indicate that MCC has anti-inflammatory effects and is relatively safe. Therefore, MCC is a promising candidate for treating inflammation-related diseases.

INTRODUCTION

Inflammation is an essential defense mechanism of the body for the removal of noxious stimuli and invading pathogens (Abbas and Lichtman, 2003; Christgen and Kanneganti, 2020). The inflammatory process involves the migration of immune cells as well as the production and secretion of proinflammatory cytokines and mediators such as histamine, serotonin, prostaglandins (PGs), nitric oxide (NO), reactive oxygen species, tumor necrotic factor- α (TNF- α), and interleukin (Christgen and Kanneganti, 2020; Christiansen *et al.*, 2006; Fujiwara and Kobayashi, 2005; Kuebler,

2005). These inflammatory mediators are well known as essential biochemicals, which are involved in protecting body tissues and organs from pathogens or hazardous stimuli and initiating tissue repair (Bednash and Mallampalli, 2016; Christgen and Kanneganti, 2020; Oehmcke-Hecht and Köhler, 2018). However, excess and lasting production of inflammatory mediators and proinflammatory cytokines is associated with a number of inflammatory diseases (Bednash and Mallampalli, 2016; Christgen and Kanneganti, 2020; Tsuchiya and Hara, 2014).

Zingiberaceae, commonly known as the ginger family, include a large number of different plants comprising about 52 genera and 1,587 species (Srisook and Srisook, 2020). For centuries, various plants in this family have been consumed as food and used as herbs in many countries around the world, especially in the Asian zone (Larsen and Larsen, 2006). A number of natural compounds from plants in Zingiberaceae have been reported to

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show various pharmacological activities (Chongmelaxme *et al.*, 2017; Jurenka, 2009; Martins *et al.*, 2019; Mustafa and Srivastava, 1990), including anti-inflammatory effects. *Zingiberaceae* are recognized as a source of natural compounds for treating inflammation-related diseases, for instance, curcumin isolated from the rhizomes of *Curcuma longa* which has been used as an anti-inflammatory agent for rheumatic diseases and some inflammatory disorders (Yang *et al.*, 2019).

Etilingera pavieana (Pierre ex Gagnep) R.M.Sm, known in Thailand as rew-hom, is one of the *Zingiberaceae* species, widely grown in Thailand, Vietnam, and other Southeast Asian countries (Poulsen and Phonsena, 2017). In Thailand, *E. pavieana* rhizomes are commonly consumed as a spice and herb which are sometimes mixed in salads. The rhizomes are also widely used as herbal medicine for diuresis (Poulsen and Phonsena, 2017) and to relieve fever, gastrointestinal disorders, and pharyngitis (Phonsena, 2007; Poulsen and Phonsena, 2017). *Etilingera pavieana* rhizome extracts have been shown to possess biological effects, for example, anti-inflammatory, antioxidant, and cytotoxic properties (Srisook *et al.*, 2017, 2018; Iawsipo *et al.*, 2018). Moreover, *E. pavieana* rhizomes have been demonstrated to consist of many phytochemical compounds, some of which have pharmacological properties (Wang *et al.*, 2009; Srisook *et al.*, 2017; Iawsipo *et al.*, 2018; Naksang *et al.*, 2020; Srisook *et al.*, 2020). For example, the ethyl acetate fraction of *E. pavieana* and some phenolic compounds exhibits an anti-inflammatory effect in LPS-induced macrophages (Srisook *et al.*, 2017). 4-Methoxycinnamyl *p*-coumarate (MCC), a phenylpropanoid derived from *E. pavieana* rhizomes, has been demonstrated to have anti-inflammatory, antitumor, and antibacterial effects (Tachai and Nuntawong, 2016; Srisook *et al.*, 2017; Iawsipo *et al.*, 2018; Mankhong *et al.*, 2019; Srisook *et al.*, 2020). Its anti-inflammatory effects are related to inhibition of Nuclear factor kappa B (NF- κ B), AP-1, and Akt signaling cascades, resulting in the reduction of iNOS, COX-2, and proinflammatory cytokine expression and their subsequent protein biosynthesis (Mankhong *et al.*, 2019). However, the anti-inflammatory effect and the safety profile of MCC in experimental animals have not been reported. This study aimed to examine the *in vivo* anti-inflammatory effect of MCC against acute inflammation in rats and to investigate the acute oral toxicity of MCC in mice.

MATERIALS AND METHODS

Preparation of MCC

Etilingera pavieana was collected from Chanthaburi Province, Thailand, and authenticated by Dr. B. Chewprecha, Botanist, at the Department of Biology, Faculty of Science, Burapha University. The voucher specimen (KS-SCBUU-0012-2) was kept at the Faculty of Science, Burapha University. MCC was separated from the ethyl acetate fraction of the ethanolic rhizome extract from *E. pavieana* as previously described by Srisook *et al.* (2017).

Experimental animals

Sprague Dawley rats and Institute of Cancer Research mice were purchased from Nomura Siam International Co., Ltd., Thailand. The female mice were used in the acute oral toxicity study, while the male rats were used in the anti-inflammatory activity study. The rats and mice were housed separately in open-mesh cages in a

temperature-controlled room maintained at 21°C + 1°C with a 12:12 light-dark cycle. Standard diet and drinking water were available *ad libitum*. All animals were kept for at least 1 week to acclimatize prior to starting the experiments. Approval from the Animal Ethics Committee, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand (no. 44/2561), and by the Institutional Animal Care and Use Committee, Burapha University (no. IACUC 005/2562), was obtained for all experimental procedures.

In vivo anti-inflammatory activity study of MCC

Ethyl phenylpropionate(EPP) -induced rat ear edema

The EPP-induced ear edema was conducted as described by Brattsand *et al.* (1982). Nine male rats (40–60 g) were divided into three groups (three rats each): vehicle control, reference drug, and MCC-treated groups. Topical administration of acetone (20 μ l/ear), phenylbutazone (1 mg/ear), or MCC (3 mg/ear) to the ears of each of the rats was performed prior to ear edema induction. Immediately after that treatment, inflammation was induced by topical application of EPP (1 mg/ear) to both ears of each rat. Ear thickness was measured using a digital Vernier caliper just prior to and at 15, 30, 60, and 120 minutes after EPP application. The level of ear edema formation was assessed by ear thickness.

Carrageenan-induced rat paw edema

The carrageenan-induced hind paw edema experiment was performed according to the method described by Winter *et al.* (1962). Thirty male rats (weight 100–120 g) were divided into five groups ($n = 6$ /group) including control, aspirin, and three different doses of MCC-treated groups. The vehicle (5% Tween 80), aspirin (300 mg/kg), or MCC (25, 75, and 150 mg/kg) was administered orally to each rat 1 hour before injection of 1% carrageenan prepared in a normal saline solution into the subplantar side of the right hind paw of each rat.

Paw volume of the injected feet was assessed with a plethysmometer (Ugo Basile, Italy) after injection of carrageenan at 0, 1, 3, and 5 hours. After paw volume measurement, all rats were immediately sacrificed. The right hind paws of all rats were collected for histopathological examination.

Acute toxicity study in mice

The acute toxicity test was carried out according to the OECD guideline 420: acute oral toxicity-fixed dose procedure [OECD (The Organisation of Economic Co-operation and Development), 2001]. After acclimatization for 7 days, the female mice were divided into two groups of five each. The test group was treated orally with MCC (2,000 mg/kg). The control group was given an equal volume of 5% Tween 80. Mortality and signs of toxicity were monitored at 1, 2, 4, and 6 hours after oral administration and then once daily for 14 days after dosing. The body weights of the mice were recorded prior to and at 7 and 14 days after treatment. On day 15, all remaining mice were anesthetized and sacrificed. Necropsy and a gross pathological examination of internal organs, including thymus, bronchi, lungs, spleen, liver, heart, kidneys, stomach, intestine, sex organs, and adrenal glands, were performed.

Statistical analysis

Data are reported as mean \pm standard error of the mean (SEM). Statistically significant differences between groups were

analyzed by one-way analysis of variance followed by the *post hoc* least significant difference test. Differences were considered statistically significant when p values < 0.05 .

RESULTS

Effects of MCC on EPP-induced rat ear edema

After topical administration of EPP, the vehicle group showed the greatest extent of ear edema. Figure 1 shows the effects of the test compound MCC and phenylbutazone, the reference anti-inflammatory drug, on EPP-induced rat ear swelling. Both MCC (3 mg/ear) and phenylbutazone (1 mg/ear) significantly reduced the ear edema when compared with the vehicle control group at all assessment times ($p < 0.001$).

Anti-inflammatory effects of MCC on carrageenan-induced rat paw edema

The results of the effects of MCC and aspirin (reference drug) on paw edema induced by carrageenan are shown in Figure 2. Various doses of MCC (25–150 mg/kg) produced dose-dependent inhibition of paw edema at every time point after carrageenan injection ($p < 0.05$ – $p < 0.001$). After 5 hours of injection, MCC at the highest dose (150 mg/kg) and aspirin showed significant levels of edema inhibition, 44.89% and 67.82%, respectively.

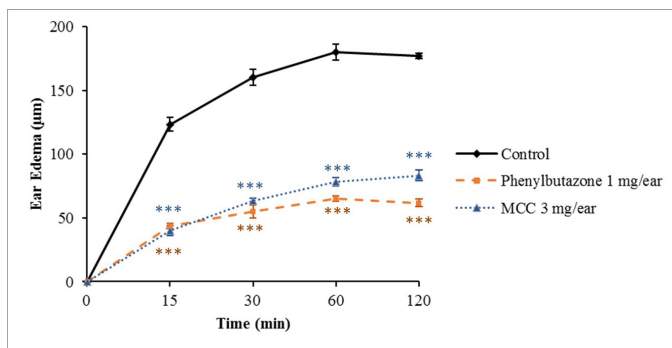


Figure 1. Effects of MCC and phenylbutazone on rat edema induced by EPP. Results are shown as mean \pm SEM ($n = 6$). ***Significantly different from control, $p < 0.001$.

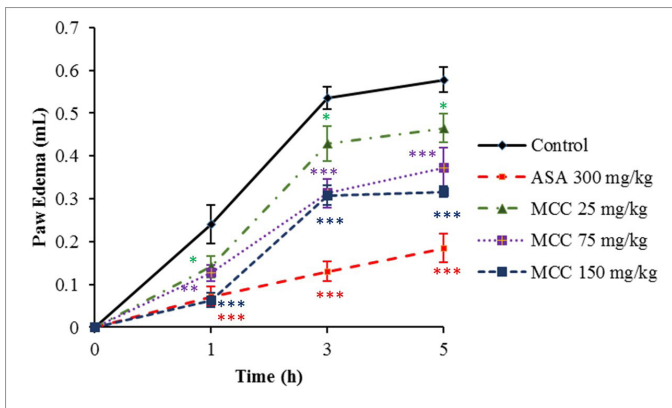


Figure 2. Effects of MCC and aspirin (ASA) on rat paw edema induced by carrageenan injection. Results are shown as mean \pm SEM ($n = 6$). $p < 0.05$, $p < 0.01$, and *** $p < 0.001$ significantly different from control.

Figure 3 shows the histopathological images of rat paw tissues at 5 hours after carrageenan injection. Marked edema with infiltration of inflammatory cells was observed in the tissues from carrageenan-injected paws of the control group. The MCC- (150 mg/kg) treated group as well as the reference drug group (aspirin 300 mg/kg) exhibited less interstitial edema than the control rats.

Acute toxicity study of MCC in mice

In the acute oral toxicity test, a single treatment of MCC (2,000 mg/kg) did not show mortality in any of the tested mice during the period of observation. No signs of toxicity or general behavioral changes were observed. Body weights on days 7 and 14 after treatment were not significantly different between the MCC-treated mice and the control mice. The organ weights of female mice in the MCC-treated group had no significant difference from the control group (Table 1).

DISCUSSION

From our previous study, MCC, a phenolic compound derived from *E. pavieana* rhizomes, has demonstrated its *in vitro* anti-inflammatory effect in LPS-stimulated macrophages (Mankhong et al., 2019) and in TNF- α -induced human endothelial cells (Srisook et al., 2020). This study is the first to demonstrate the anti-inflammatory effect of MCC in *in vivo* rat models. Although

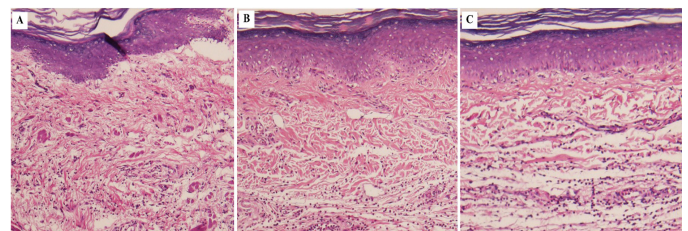


Figure 3. Histopathological feature of rat paw tissues at 5 hours after carrageenan injection. Tissue sections from the rat hind paw were stained with H&E for histopathological examination at 5 hours after carrageenan injection (100 \times magnifications). (A) Control group. (B) Aspirin- (ASA-) treated group. (C) MCC- (150 mg/kg) treated group.

Table 1. Organ weights of female mice in the acute toxicity study of MCC.

Organ	Organ weight (mg)	
	Control	MCC (2,000 mg/kg)
Brain	607.92 \pm 16.81	578.54 \pm 20.08
Lung	333.94 \pm 18.16	321.84 \pm 6.52
Heart	175.00 \pm 3.83	183.58 \pm 4.89
Liver	1,724.60 \pm 65.91	1,707.54 \pm 57.00
Spleen	156.44 \pm 16.22	177.06 \pm 13.50
Adrenal gland	6.86 \pm 0.30	6.96 \pm 0.83
Kidney	235.89 \pm 5.68	242.76 \pm 10.78
Ovary	20.61 \pm 1.42	21.70 \pm 2.04
Uterus	199.68 \pm 32.18	205.08 \pm 21.70

Each value represents the mean \pm SEM ($n = 5$).

No significant difference between the MCC-treated group and the control group.

further toxicity studies are needed, MCC safety data from the acute toxicity study shows the potential of that compound for drug development.

In this study, the *in vivo* anti-inflammatory effect of MCC using two experimental models of acute inflammation was examined. EPP-induced ear edema in rodents is generally used as a screening and evaluation model for testing the anti-inflammatory effect of compounds (Brattsand *et al.*, 1982). EPP causes ear swelling by stimulating the production and secretion of several inflammatory mediators, for example, histamine, bradykinin, PGs, and serotonin (Carlson *et al.*, 1985), leading to vasodilatation and an increase in vascular permeability. The data obtained from the present study demonstrate the anti-inflammatory effect of MCC as this phenolic compound significantly inhibited EPP-stimulated rat ear edema formation. The second model of the present study is hind paw edema induced by carrageenan, an acute inflammation model which is a conventional method for investigating the anti-inflammatory effect of test compounds. Carrageenan injection into a rat's paw causes a biphasic inflammatory mediator release response. The first phase (0–2.5 h after carrageenan injection) results from the release of histamine, bradykinin, and serotonin. The late phase at 2.5–6 hours after injection is attributed to the formation and secretion of inflammatory mediators, especially PGs and NO (Liao *et al.*, 2012; Mansouri *et al.*, 2015). In the present study, MCC reduced paw edema caused by carrageenan injection, thereby demonstrating the anti-inflammatory effect of MCC in this model. In our previous *in vitro* study, we showed that MCC induces inactivation of the NF- κ B and Akt signaling cascades, resulting in the inhibition of the expression of inflammatory mediators, NO and PGE₂, in murine RAW 264.7 macrophage cells (Mankhong *et al.*, 2019). Recently, MCC also exhibited the downregulation of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1, endothelial adhesion molecules which mediate leukocyte emigration into inflamed sites (Srisook *et al.*, 2020). These activities might be the mechanisms of the anti-inflammatory action of MCC observed in the EPP-induced ear edema and carrageenan-induced paw edema in rat models.

In the acute oral toxicity study, MCC (2,000 mg/kg) did not show mortality or produce any signs of acute toxicity in female mice during the 14 days of the study which would indicate that the LD₅₀ value with oral administration of MCC is higher than 2,000 mg/kg for mice. However, further toxicity studies should be performed to confirm its safety in long-term use.

CONCLUSION

In conclusion, MCC has an anti-inflammatory effect in rats. MCC's activity against acute inflammation might be due to suppression of the production and release of inflammatory mediators. MCC does not exhibit acute oral toxicity in mice, indicating its relative safety. Taken together, these findings strongly support the conclusion that there is significant potential for developing MCC as a new therapeutic agent for the treatment of inflammation-related disorders.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

CONFLICT OF INTEREST

Authors declared that they do not have any conflicts of interest.

ETHICAL APPROVALS

Approval from the Animal Ethics Committee, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand (no. 44/2561), and by the Institutional Animal Care and Use Committee, Burapha University (no. IACUC 005/2562), was obtained for all experimental procedures.

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