



Anti-inflammatory and antioxidant activity of Thai mango (*Mangifera indica* Linn.) leaf extracts

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Abstract

Mango (*Mangifera indica* Linn.) has been widely studied as a folk medicines and it has become a common ingredient in cosmeceutical products. However, there was no report on the efficacy of Thai mango leaf extracts against inflammation. Thus, the aim of this study was to ascertain the anti-inflammatory and antioxidant activities of methanol (MeOH) and aqueous (Aq) extracts from the leaves of three mango varieties: Keaw Morakot, Nam Doc Mai, and Mahajanaka. To assay in vitro anti-inflammatory activity, the amount of nitric oxide (NO) released from macrophage cells was indirectly measured. Since free radicals play an important role in the inflammatory process, the antioxidant activity of *M. indica* leaf extracts was measured by ABTS and DPPH radical scavenging, ascorbic acid, and total phenolic assays. Mangiferin was also analyzed by HPLC. The most effective extract was selected and tested for anti-inflammatory activity in an animal model, an acute ear edema test in albino rats. The MeOH extract of Mahajanaka (M-MeOH) reduced nitric oxide production by LPS-stimulated macrophage cell lines RAW 264.7. M-MeOH showed the strongest antioxidant activity and it was, therefore, selected for an in vivo anti-inflammatory assay. An acute ear edema test in albino rats showed that M-MeOH exhibited significant anti-inflammatory activity compared to the control; ethyl phenylpropiolate-induced ear edema ($P < 0.05$). Moreover, mangiferin, a bioactive compound in *M. indica* leaves was detected in MeOH and Aq extracts. We concluded that the MeOH extract of *M. indica* leaves exhibits an anti-inflammatory property, both in vitro and in vivo, due to the presence of efficacious antioxidants.

Keywords *Mangifera indica* Linn. · Anti-inflammation · Antioxidant · Nitric oxide · Ear edema · Mangiferin

Introduction

Inflammation is a necessary part of the body's immune response. It is the body trying to heal itself after an injury and protect itself against foreign invaders (Mohan 2002). When

inflammation happens, various pathological changes occur. Active inflammatory mediators are produced by microbial products or host proteins, such as complements, kinins, and coagulation factors. There are many mediators of inflammation such as histamine, nitric oxide (NO), prostaglandins (PGs), leukotrienes (LTB₄), bradykinin, lipoxins, serotonin, growth factors, and cytokines (Dray, 1995) and the inhibition of these pro-inflammatory mediators is an important goal in the treatment of various inflammatory diseases.

Nitric oxide (NO) is synthesized by nitric oxide synthase (NOS). There are three main NOS isoforms: the first one is neuronal isoforms (nNOS), another one is endothelial isoforms (eNOS), and the last isoform is inducible isoforms (iNOS). The inducible forms of NOS are the pro-inflammatory enzymes, which are responsible for raising the NO levels and for the vasodilation and hypotension observed during inflammatory process. Since the lipopolysaccharides (LPS) stimulated NO secretion, LPS-stimulated RAW 264.7 cells (murine macrophage cell line) have been extensively used to determine inflammatory responses (MacMicking

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et al. 1997). Recently, numerous plant species have been reported for their anti-inflammatory and antioxidant properties. Antioxidation has been proposed as the mechanism underlying these properties.

Many plants have been consumed worldwide for health benefits, and mango (*Mangifera indica* Linn.) has become one of the most popular choices used to treat diseases. The plant is classified in the family Anacardiaceae and is cultivated in many tropical and subtropical regions. *M. indica* offers many important health benefits, for it has anti-cancer (El-Hawary and Rabeh 2014), hypocholesterolemic (Anila and Vijayalakshmi 2003), anti-bacterial (Hannan et al. 2013), and anti-inflammatory properties (Garrido et al. 2004a, b). Among others not only the fruit, but the leaves also have a lot of benefits. While the pharmaceutical information about mango leaves from other countries has been well documented, information about Thai mango leaves is limited. The ability of the fruit extract of mango to exhibit anti-inflammatory activities gained our interest so we tested the properties of the leaf extracts of three varieties of mango grown in Thailand, Keaw Morakot, Nam Doc Mai, and Mahajanaka, which are the commercially important fruits and easy to grow in Thailand. We focused on their antioxidant and anti-inflammatory properties.

Materials and methods

Plant materials and extraction

Three varieties of *M. indica* leaves were collected from Ban Hong District, Lamphun Province, Thailand. The plant species and varieties were identified by a botanist of the Queen Sirikit Botanic Garden, Mae Rim, Chiang Mai, Thailand (the plant identification number: Mahajanaka (M) is WP 5059, Nam Doc Mai (N) is WP 5060, and Keaw Morakot (K) is WP5061). The leaves were extracted by two solvents (methanol: MeOH and distilled water: Aq). Ground dry leaves of mango were macerated in both solvents for 24 h at room temperature. Each extract was filtered with Whatman No. 1 and the solvents were removed by vacuum rotary evaporator. Then, the crude extracts were lyophilized in a freeze dryer. They were then weighed to calculate the actual percentage yield (K-MeOH 0.52%, K-Aq 0.25%, N-MeOH 0.91%, N-Aq 0.45%, M-MeOH 0.73%, M-Aq 1.29%).

Animals

Male Wistar rats (*Rattus norvegicus*) age 18–20 days obtained from the National Laboratory Animal Center, Mahidol University, Thailand, were kept in cages and had free access to tap water and a standard pellet diet (SmartHeart® Hamster Food: Complete and Balanced Formula). The laboratory room temperature was maintained at 24–26 °C in a 12-h light/dark

cycle. All procedures encompassing the animals were approved under permission number of Re. 004/13.

Determination of antioxidant activity

DPPH radical scavenging activity

The DPPH radical scavenging assay is to measure a stable free radical with violet-color of DPPH (1,1-diphenyl-2-picrylhydrazyl). A colorless of DPPH is observed after mixing the DPPH solution with an antioxidant. Following this, various concentrations of the extracts were added in methanolic DPPH solution and the absorbance was recorded at 517 nm (Brand-William et al. 1997). Gallic acid was used as a standard radical scavenger. The IC₅₀ (a half maximal inhibitory concentration) of each extract was then calculated and compared to that of gallic acid to obtain gallic acid equivalent antioxidant activity (GAEA).

ABTS radical scavenging activity

ABTS or 2,2'-azinobis (3-ethylbenzo thiazoline-6-sulfonic acid) free radical cation (ABTS⁺) decolorization assay (Re et al. 1999) was used to investigate antioxidant efficacy. A solution of ABTS⁺ was prepared by mixing ABTS with hydrogen peroxide (H₂O₂) for 16–18 h in the dark at a low temperature. The degree of green ABTS⁺ solution was measured at 734 nm. The extracts at various concentrations were added to the ABTS⁺ radical cation solution before measuring the optical density. The IC₅₀ of each extract was then calculated and compared to that of trolox, a vitamin E analog, to establish the difference.

Ascorbic acid assay

Following the method of Schlessier et al. (2002), the content of ascorbic acid (vitamin C) analog was determined by extracting it from the samples using trichloroacetic acid. Dinitrophenylhydrazine reagent was mixed and then the sample was heated to 60 °C. The mixture was then cooled in an ice tank and sulfuric acid (H₂SO₄) was added. After 20 min of incubating in the dark, the amount of vitamin C was recorded at 520 nm before calculating its concentration against (standard) ascorbic acid. The amount of ascorbic acid was expressed as milligram ascorbic acid equivalent per gram of extract (mg AAE/g extract).

Total phenolic assay

To investigate the amount of phenolics in the extracts, the method of Singleton and Rossi (1965) was applied by mixing Folin-ciocalteu reagent in sodium carbonate decahydrate (Na₂CO₃·10H₂O) with tested extracts for 2 h. The reaction

was then spectrophotometrically read at 750 nm. We calculated total phenolic by comparing absorbance with a standard gallic acid monohydrate. The amount of phenolic was expressed as milligram gallic acid equivalent per gram of extract (mg GAE/g extract).

In vitro anti-inflammatory activity

Cell culture

Murine macrophage cells (RAW 264.7) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) containing streptomycin and penicillin (100 µg/ml and 100 U/ml, respectively) at 37 °C in a 5% CO₂ incubator.

Cell viability

A modified MTT assay was used to assess the viability of RAW 264.7 cells (Mosmann 1983). A portion of MLE at different concentrations (1.25, 2.5, 5, 10, 20, and 40 µg/ml) was added to 96-well plate containing 5×10^5 cells each. After mixing 20 µl of MTT solution in phosphate-buffered saline, the wells were further incubated for 4 h at 37 °C. To solubilize any deposited formazan, a volume of 200 µl of dimethyl sulfoxide (DMSO) was added to each well and the optical density (OD) was recorded at 550 nm by a microplate reader.

Determination of nitric oxide production

We indirectly measured the amount of nitric oxide (NO) released from macrophage cells by pre-incubating RAW 264.7 cells (5×10^5 cells/well) with lipopolysaccharide (LPS) (2 µg/ml) for 24 h. Following this, a volume of 100 µl of cell culture medium was collected and mixed with 100 µl of Griess reagent. After incubating the mixture at 25 °C for 10 min, the level of NO was measured at 550 nm using a microplate reader. All experiments were performed in triplicate (Griess reagent: 1% sulfanilamide and 0.1% naphthyl ethylenediamine dihydrochloride in 2.5% phosphoric acid) (Yoon et al. 2009; Lee et al. 2013; Joo et al. 2014).

In vivo anti-inflammatory activity

The most effective extract from in vitro study was selected and tested for anti-inflammatory activity in an animal model. The EPP-induced ear edema method was used to test for acute inflammation in male Wistar rats (age 18–20 days) using the adapted method of Keardrit et al. (2010). Firstly, the rats weighing 35 to 40 g were divided into four groups (6 rats each). Both ears of each rat in the first three groups were topically applied with the tested extracts or

phenylbutazone (PHBZ) at a dose of 1 mg/ear and served as two treatment groups and one positive control group. The negative control group was applied with the vehicle. After pre-treatment, induction of ear edema was carried out by topically applying ethyl phenylpropionate (EPP) dissolved in acetone (50 mg/ml) to the inner and outer surfaces of both ears (20 µl/ear). Ear thickness was measured using a digital vernier caliper at 15, 30, 60, and 120 min after the edema induction.

Mangiferin analysis

Mangiferin was analyzed by HPLC following Gholkar and Laddha (2015) with some modifications. An Agilent system (quaternary pump, VWD detector at 257 nm, degasser) coupled with the Zorbax eclipse XDB-C18 column (4.6 × 150 mm) was used. The mobile phase consisted of acetonitrile: water containing 0.1% v/v of o-phosphoric acid (15:85). The flow rate was 1.0 ml/min; the injection volume was 20 µl. The retention time of a standard mangiferin was used to identify the peak of mangiferin being presented in the tested extracts. The amounts of mangiferin were determined by comparing the peak area of the tested samples with those peak areas from the standard (0.01–0.10 mg/mL).

Data analysis

All data are expressed as mean ± standard error of mean (S.E.M). One-way ANOVA followed by Tukey's post hoc test for multiple comparisons in SPSS version 17 was used, while a statistical significance was considered when $P < 0.05$. In vitro assays were performed in triplicate. The IC₅₀ (concentration that produced 50% inhibition) values were calculated using linear regression.

Results

DPPH and ABTS radical scavenging activity

The IC₅₀ of DPPH and ABTS scavenging activities in the leaf extracts of the three varieties of mango grown in Thailand are shown in Table 1. M-MeOH had the lowest IC₅₀ of DPPH and ABTS (0.129 ± 0.015 and 0.275 ± 0.008 mg/ml, respectively). The lowest IC₅₀ correlates with the highest antioxidant capacity. The IC₅₀ values of DPPH of M-MeOH was significantly lower than the K-Aq and M-Aq ($P < 0.05$), while, an IC₅₀ value of ABTS of M-MeOH was significantly lower than other extracts, except for K-Aq. From our result, it was found that the IC₅₀ of trolox, a standard antioxidant, was 0.211 mg/ml and it had the representative regression coefficient (R^2) at 0.9951, while the linear regression equation was

Table 1 DPPH and ABTS radical scavenging activity of *M. indica* leaf extracts (MLE)

<i>M. indica</i> leaves	Antioxidant activity (IC ₅₀ mg/ml)	
	DPPH	ABTS
K-MeOH	0.245 ± 0.006 ^{ab}	0.447 ± 0.002 ^b
N-MeOH	0.146 ± 0.007 ^{ab}	0.395 ± 0.008 ^b
M-MeOH	0.129 ± 0.015 ^a	0.275 ± 0.008 ^a
K-Aq	0.258 ± 0.014 ^b	0.317 ± 0.024 ^a
N-Aq	0.229 ± 0.059 ^{ab}	0.518 ± 0.002 ^c
M-Aq	0.270 ± 0.012 ^b	0.670 ± 0.011 ^d

Values are mean ± S.E.M. of triplicate. Different superscripts (a–d) in the same column are significantly different ($P < 0.05$)

$y = 2.7725x + 44.148$. Furthermore, an IC₅₀ value of gallic acid was 0.05 mg/ml ($y = 0.1111x + 0.0445$, $R^2 = 0.9995$).

Ascorbic acid and total phenolic contents

The results for ascorbic acid contents showed higher values in the methanolic extracts and a significant increase when compared with the distilled water extracts. M-MeOH had the highest value (71.524 ± 2.341 mg AAE/g extract). The total phenol content was also higher in the methanolic extracts. The highest value occurred in the M-MeOH (0.295 ± 0.022 mg GAE/g extract) (Table 2).

Effect of MLE on macrophage RAW 264.7 cell viability

Figure 1 illustrates the viability of RAW 264.7 cells in the presence of MLE. With this result, concentrations of the samples which produced 95% of cell viability were 1.25 and 2.5 µg/ml. On the contrary, less number of cell viability was significantly observed in the presence of the extracts at concentrations of 5–40 µg/ml when compared to 1.25 and 2.5 µg/ml ($P < 0.05$). Thus, the extracts at concentrations up to 2.5 µg/ml were selected for subsequent NO inhibition experiment.

Inhibitory efficacy against nitric oxide production

The inhibitory efficacy of MLE was evaluated against NO production in LPS-stimulated RAW 264.7 cells. Accumulation of nitrite in the cells increased due to the stimulation by LPS. Our results indicate that the percentage of inhibition of NO production induced by LPS increased in a concentration-dependent manner when treated with MLE, with 0.075, 0.15, 0.3125, 0.625, 1.25, and 2.5 µg/ml of MLE. Interestingly, the M-MeOH effectively inhibited NO production, at as high as just over 80%, when compared with other five extracts (Fig. 2).

Table 2 Ascorbic acid and total phenolic contents of MLE

<i>M. indica</i> leaves	Ascorbic acid (mg AAE/g extract)	Total phenolics (mg GAE/g extract)
K-MeOH	28.000 ± 0.719 ^c	0.268 ± 0.006 ^{ab}
N-MeOH	55.238 ± 1.098 ^b	0.290 ± 0.028 ^b
M-MeOH	71.524 ± 2.341 ^a	0.295 ± 0.022 ^b
K-Aq	1.810 ± 0.476 ^d	0.221 ± 0.007 ^{ab}
N-Aq	2.000 ± 0.436 ^d	0.225 ± 0.005 ^{ab}
M-Aq	6.571 ± 0.660 ^d	0.188 ± 0.001 ^a

Values are mean ± S.E.M. of triplicate. Different superscripts (a–d) in the same column are significantly different ($P < 0.05$)

EPP-induced ear edema in rats: acute inflammation

Inhibition of acute inflammation by MLE was clearly seen in Table 3. Edema thickness of the phenylbutazone group and the extract at both doses used (1 and 2 mg/ear) was significantly reduced when compared to a negative control group. The percentage inhibition of ear edema in rats receiving a low dose of the extract was lower than that of rats treated with phenylbutazone (1 mg/ear) but the extract at a high dose (2 mg/ear) showed higher percentage inhibition (87.88%) at 120 min after topical application when compared with phenylbutazone (79.80%).

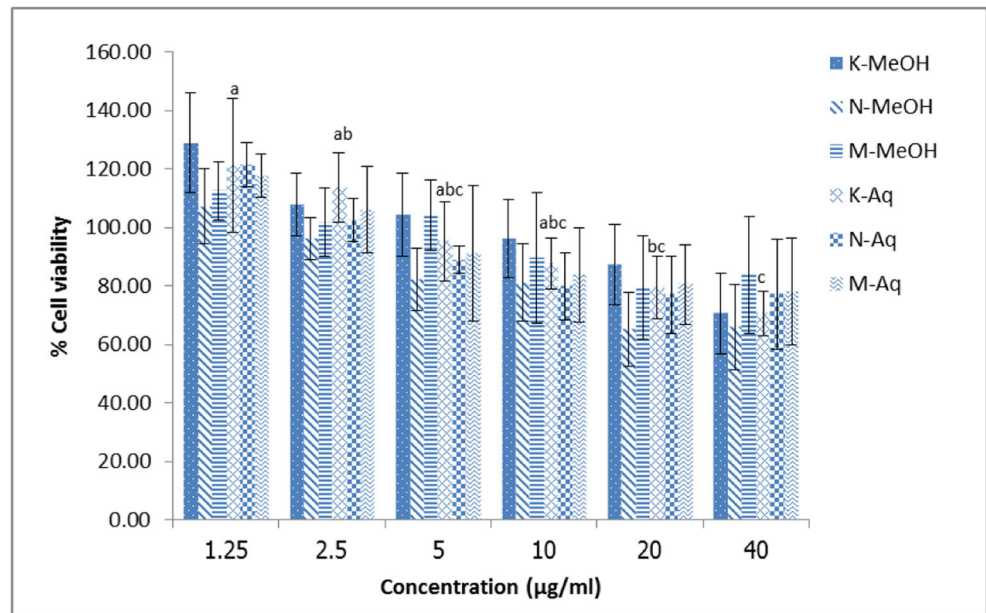
HPLC

The HPLC chromatograms of a standard mangiferin and tested extracts are highlighted in Fig. 3. The regression equation of a standard mangiferin was $Y = 56,680.2882X + 193.99831$, with a correlation coefficient (r^2) of 0.99561. Mangiferin was found in extracts whereas high mangiferin contents were found in the MeOH extracts, M-MeOH had the highest mangiferin contents (31.423% W/W) (Fig. 4). Therefore, mango leaves extracted with MeOH are a good source of mangiferin.

Discussion

Macrophages have a major effect on inflammatory responses via the generation of various pro-inflammatory molecules, especially NO. If NO over-produced, it causes various inflammatory diseases. Since LPS isolated from the cell walls of gram-negative bacteria is extensively used to activate macrophage in a variety of anti-inflammatory experiments (Nicholas et al. 2007), this study used the LPS to stimulate nitric oxide production from RAW 264.7 cells. Therefore, we used LPS-treated macrophage cells to screen various anti-inflammatory plants. MLE effectively reduced

Fig. 1 Effect of MLE on the viability of LPS-stimulated RAW 264.7 cells. Data represent the means \pm S.E.M. of three independent experiments. Superscript letters (a, b, and c) indicate significant difference ($P < 0.05$)



NO, which shows that it might be useful for suppressing the inflammatory process. The anti-inflammatory property of these plants might involve in their underlying antioxidant mechanisms. Chain-breaking and removal properties have been proposed to be key mechanisms against inflammation (Rice-Evans and Diplock 1993). The former is the primary antioxidant, which donates an electron to the free radical presented in the system. The latter acts as secondary antioxidant which involves in removing initial ROS/reactive nitrogen species and quenching the chain-initiating catalyst. Moreover, antioxidants also prevent any damage being occurred in human body through

different ways, including electron donation, co-antioxidants, metal ion chelation, and gene expression regulation (Krinsky 1992). Phenolic compounds, which are found in all plants, are an essential part of the human diet. Thus, they act as antioxidant properties, which inhibit the production of NO and peroxynitrite (Conforti and Menichini, 2011).

The M-MeOH had a higher level of total phenolics when compared with other extracts in this experiment. It was highly potent for antioxidation and confirmed the result of DPPH and ABTS radical scavenging activity by having the lowest IC₅₀. Both of these radicals are commonly used to evaluate

Fig. 2 Effect of MLE on NO production by LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were stimulated with 2 µg/ml of LPS. Values are mean of three identical experiments ($n = 3$) \pm S.E.M.

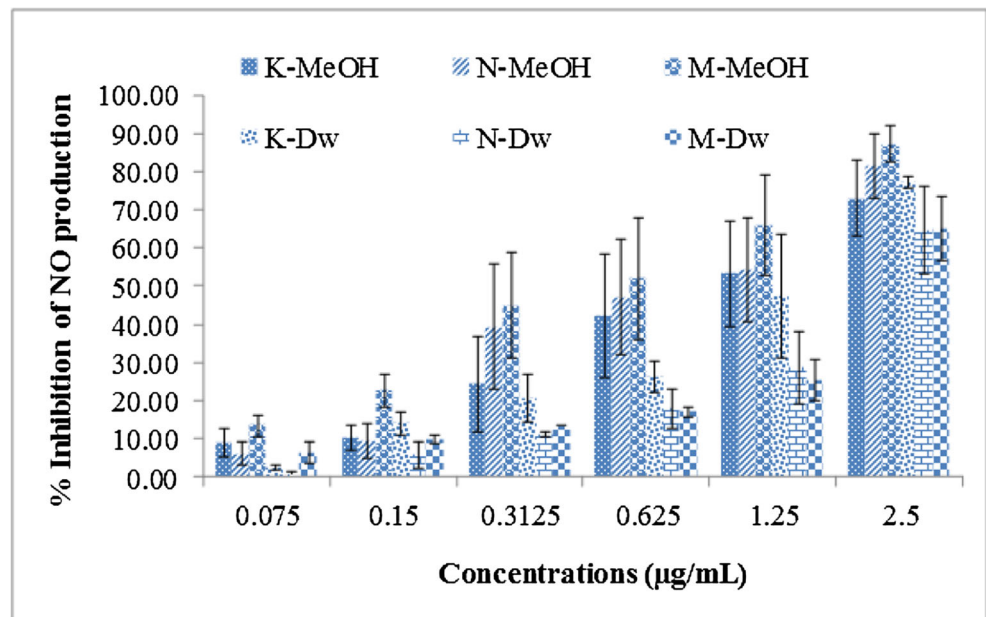


Table 3 Effect of MLE on EPP-induced ear edema in rats

Groups	Edema thickness (μm)				% inhibition			
	15 min	30 min	60 min	120 min	15 min	30 min	60 min	120 min
Negative control	378 \pm 16.0 ^a	393 \pm 17.6 ^a	243 \pm 16.1 ^a	165 \pm 13.8 ^a	—	—	—	—
Phenylbutazone 1 mg/ear	126 \pm 6.7 ^b	140 \pm 7.3 ^{bc}	70 \pm 15.8 ^b	33 \pm 9.9 ^b	66.52	64.41	71.23	79.80
M-MeOH 1 mg/ear	156 \pm 7.1 ^b	173 \pm 8.4 ^b	93 \pm 23.9 ^b	50 \pm 15.1 ^b	58.59	55.93	61.64	69.70
M-MeOH 2 mg/ear	135 \pm 15.7 ^b	111 \pm 10.8 ^c	86 \pm 12.3 ^b	20 \pm 3.7 ^b	64.32	71.61	64.38	87.88

Values are mean \pm S.E.M. of triplicate. Different superscripts (a–c) in the same column are significantly different ($P < 0.05$).

Fig. 3 HPLC chromatograms of standard mangiferin at 0.04 mg/ml (A) and mangiferin in MLE (0.1 mg/ml): K-MeOH, N-MeOH, M-MeOH, K-Aq, N-Aq, and M-Aq

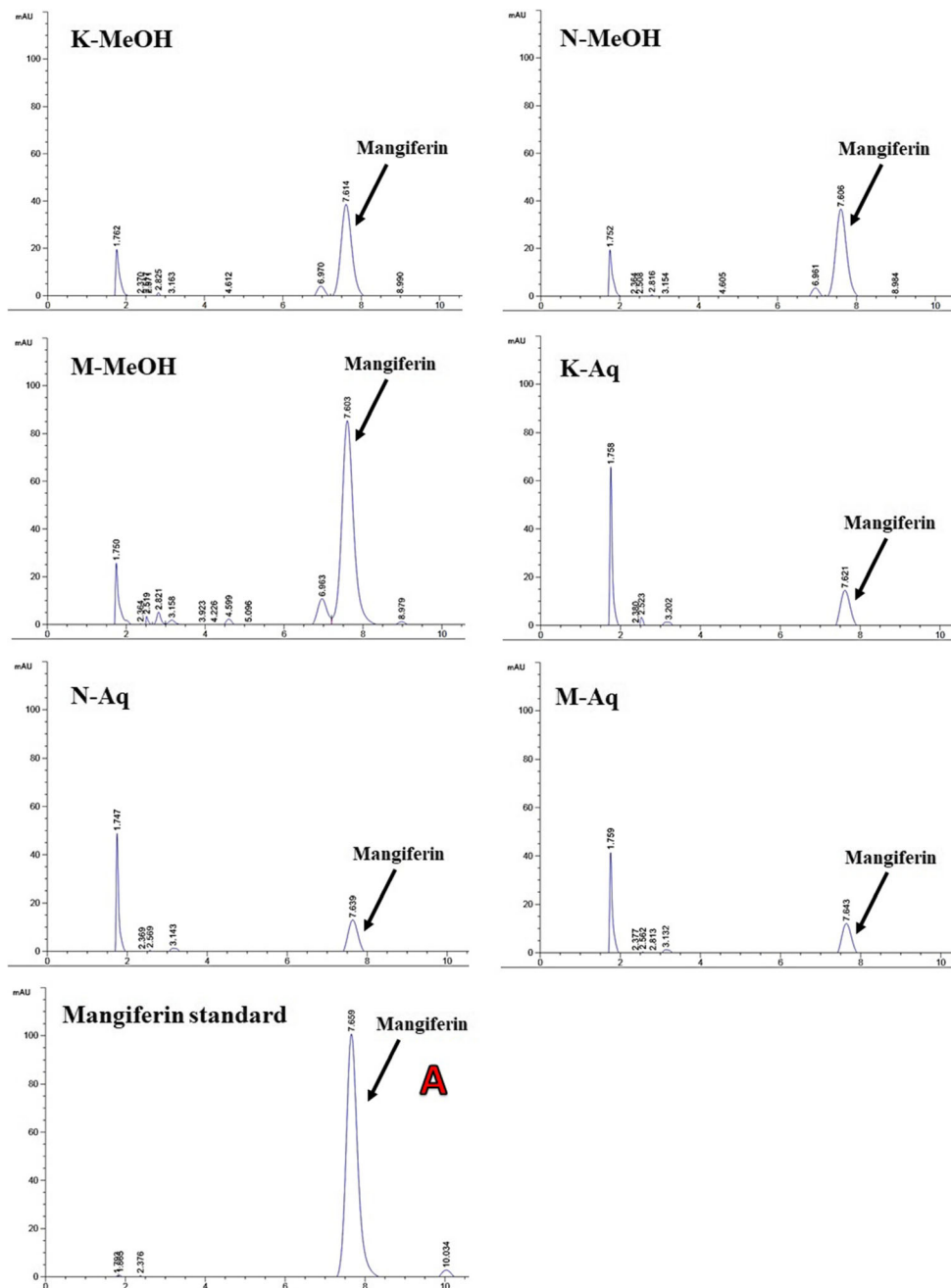
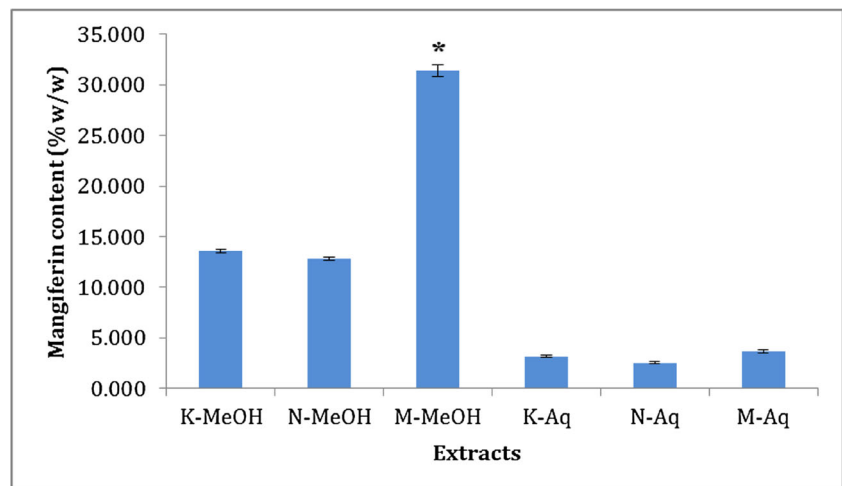


Fig. 4 Mangiferin contents of MLE from six extracts of *M. indica*. Values are mean \pm S.E.M. of triplicate



antioxidant activity in vitro. In addition, ascorbic acid or vitamin C content was measured to confirm those properties. In general, vitamin C is essentially responsible for maintaining the normal functions of cells in various organ systems. Therefore, it is known as a powerful antioxidant. The M-MeOH extract in this experiment showed the highest ascorbic acid content. Therefore, we selected this extract to determine its anti-inflammatory property in vivo. EPP-induced ear edema in murine is a good model and usually used for rapid in vivo screening of anti-inflammatory activity. This assay is simple and provides a reliable result with several anti-inflammatory drugs, including phenylbutazone. EPP produces local swelling by causing vasodilatation, vascular permeability and fluid accumulation, and several inflammatory mediators such as histamine, kinins, serotonin (5-HT), and prostaglandins (PGs) are responsible for signs of inflammation. Disruption or prohibition of the synthesis and release of the key mediators of acute inflammation is the mechanism underlying the anti-edematous effect of phenylbutazone. The results obtained from this study suggested a similar mechanism for MLE at a dose of 2 mg/ear (Brattsand et al. 1982; Sireeratawong et al. 2012).

From our preliminary studies, the MLE showed antioxidant and anti-inflammatory properties, due to the mangiferin in MLE. Mangiferin is a xanthone, and this substance is known as the most potent antioxidants. Mangiferin has been reported to possess anti-inflammation property by inhibiting the transcription factor NF- κ B and different pro-inflammatory cytokines. These efficacies provide protective effects against a wide range of pathophysiological conditions and organ dysfunctions (Benard and Chi 2015). Inhibitions of IL-1 (interleukin 1) receptor-associated kinase 1 (IRAK1), NF- κ B, and MAPK (mitogen-activated protein kinase) phosphorylation are the results of mangiferin in ameliorating peptidoglycan or LPS-stimulated peritoneal macrophage. Moreover, the study of Garrido et al. (2004a, b) shows that mangiferin-rich extract from *M. indica* (VIMANG[®]) acted as an anti-

inflammatory agent by reducing ear edema in mice. In addition, mangiferin may reduce inflammation via various mechanisms such as decreases in overpowering cytokines and pro-inflammatory mediators, inhibition of inflammatory cellular activations, regulations of inflammatory gene expressions, and enhancements of the cellular resistance against inflammatory injuries (Sánchez et al. 2000; Garrido et al. 2004a, b; Carvalho et al. 2009).

Conclusions

From the results of this study, we can conclude that the Thai mango leaf extracts, Keaw Morakot, Nam Doc Mai, and Mahajanaka, exhibited anti-inflammatory properties both in vitro and in vivo, and antioxidation proved to be the mechanism underlying those properties. The most effective one was methanolic extract of Mahajanaka.

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Compliance with ethical standard

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures encompassing the animals were conducted with strict adherence to guidelines and procedures reviewed and approved by the Institutional Animal Care and Use Committee of the Biology Department, Faculty of Science, Chiang Mai University, permission number Re. 004/013.

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