

Short Communication

Nitric oxide inhibitory substances from *Curcuma mangga* rhizomes

Kanidta Kaewkroek, Chatchai Wattanapiromsakul, Supinya Tewtrakul*

Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences
Prince of Songkla University, Hat Yai, Songkhla, 90112 Thailand.

Received 27 November 2008; Accepted 23 February 2009

Abstract

Curcuma mangga Val. & Zijp. is a member of the Zingiberaceae family commonly grown in Thailand. It is locally known as mango tumeric because of its mango-like smell when the fresh rhizomes are cut. *C. mangga* is a popular vegetable, the tips of the young rhizomes and shoots are consumed raw with rice. Medicinally, the rhizomes are used as a stomachic and for chest pains, fever, and general debility. It is also used in postpartum care. In the present study, we investigated the anti-inflammatory effect of the extract and compounds from *C. mangga* rhizomes against lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW 264.7 cell line. From bioassay-guided fractionation, the chloroform fraction exhibited the most potent inhibitory activity with an IC_{50} value of 2.1 μ g/ml, followed by the hexane fraction (IC_{50} = 3.8 μ g/ml) and the ethyl acetate fraction (IC_{50} = 23.5 μ g/ml), respectively. Demethoxycurcumin (**1**) and 3-buten-2-one, 4-[(1R, 4aR, 8aR)-decahydro-5, 5, 8a-trimethyl-2-methylene-1-naphthalenyl]-, (3E)-rel- (**2**) were isolated from the chloroform- and hexane fractions, respectively. Bisdemethoxycurcumin (**3**) whose structure is similar to that of **1** was also tested for NO inhibitory activity. Of the tested compounds, compound **1** exhibited the highest activity with an IC_{50} value of 12.1 μ M, followed by **3** (IC_{50} = 16.9 μ M) and **2** (IC_{50} = 30.3 μ M). These results suggest that *C. mangga* and its compounds exert NO inhibitory activity and have a potential to be developed as a pharmaceutical preparation for treatment of inflammatory-related diseases. Moreover, this is the first report of compound **2** that was isolated from *C. mangga* rhizomes.

Keywords: RAW264.7 cell, nitric oxide, *Curcuma mangga*, Zingiberaceae

1. Introduction

Nitric oxide (NO) is produced via the oxidation of L-arginine by family members of nitric oxide synthase (NOS). Inducible NOS (iNOS) is induced in response to various pro-inflammatory cytokines, including interferon- γ (INF- γ), tumor necrosis factor- α (TNF- α), and interleukin-6 (IL-6), and mediates several inflammatory responses. NO synthesized by iNOS has also been considered as an important mediator of carcinogenesis. Over-expression of iNOS that has been detected in human cancer and tumor associated with the production of NO by iNOS may promote cancer progression (Jung *et al.*, 2007). Overproduction of NO has been

reported to cause oxidative damage cell death. Inhibition of NO overproduction has been proved to increase cell survival in several models. For example, addition of iNOS antagonist prevented rat amoeboid microglia from lysing oligodendrocytes *in vitro* (Merrill *et al.*, 1993). NO has been correlated with the inflammatory process, in which multiple cytotoxic effects are related to the ability to increase vascular permeability and edema. This involves changes in local blood flow and increases in pro-inflammatory prostaglandins (Lantz *et al.*, 2005).

The macrophages can be activated by lipopolysaccharide (LPS) and interferon-gamma (IFN- γ). LPS is one of the major constituents of the outer membrane of Gram-negative bacteria, and the immune system is constantly exposed to low levels of LPS through infections. LPS recognition and signal transmission are the key events aimed at eliminating an invading pathogen. The LPS-induced activation of macro-

*Corresponding author.

Email address: supinyat@yahoo.com

phages results in the production of bioactive lipids, reactive oxygen species, and in particular, inflammatory cytokines to fight and clear the bacterial infection. However, LPS mediates both the beneficial and deleterious reaction to the host. The excessive and uncontrolled production of inflammatory mediators triggered by LPS is harmful, and can lead to potentially lethal systemic disorders such as septic shock (Cho *et al.*, 2008). The antigen can activate macrophages to release some inflammatory mediators such as NO, TNF- α , PGE₂ and others. Therefore, the inhibition of NO, PGE₂ and TNF- α production is an important therapeutic consideration in the development of anti-inflammatory agents.

Curcuma mangga Val. & Zijp. is a perennial herb and a member of the Zingiberaceae family, commonly grown in Thailand, Peninsular Malaysia, and Java. It is locally known as "mango tumeric" because of its mango-like smell when the fresh rhizomes are cut (Thai name is Kha-Min-Khao). Due to this characteristic and its palatable taste, *C. mangga* has become a popular vegetable, of which the tips of young rhizomes and shoots are consumed raw with rice. Medicinally, the rhizomes are used as a stomachic and for chest pains, fever, and general debility. It has been reported that compounds from *C. mangga* showed high cytotoxic activity against a panel of human tumor cell lines, such as human leukemia (HL-60), breast cancer (MCF-7) and liver cancer (HepG2) (Abas *et al.*, 2005). Moreover, the ethanol and water extracts from *C. mangga* also possessed anti-allergic activity (Tewtrakul and Subhadhirasakul, 2007). Since the EtOH extract of *C. mangga* rhizomes possessed potent NO inhibitory effect ($IC_{50} = 11.8 \mu\text{g/ml}$), the present study is aimed to isolate the active principles from this plant against NO release using RAW 264.7 macrophage-like cells.

2. Materials and Methods

2.1 Reagents

Lipopolysaccharide (LPS, from *Escherichia coli*), RPMI-1640 medium, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), L-nitroarginine (L-NA), caffeic acid phenethyl ester (CAPE), indomethacin, and phosphate buffer saline (PBS) were purchased from Sigma Aldrich (Sigma Aldrich, Missouri, USA). Fetal calf serum (FCS) was bought from Gibco (Invitrogen, California, USA). Penicillin-streptomycin was purchased from Invitrogen (Invitrogen, California, USA). 96-Well microplates were obtained from Nunc (Nunc, Birkroð, Denmark).

2.2 Plant material and preparation of extract

The fresh rhizomes of *Curcuma mangga* Val. & Zijp. were collected in Songkhla Province, Thailand, in 2006. The voucher specimens are SKP No. 206 03 13 01. The plant material was identified by Dr. S. Subhadhirasakul and the voucher specimens are kept at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University,

Songkhla, Thailand.

2.3 Extraction and isolation of compounds

Twenty kilogram of the fresh rhizomes were cleaned, cut into small pieces, dried in the shade and ground to powder. The powder (1.8 kg) was extracted three times (6 l, each) with EtOH at room temperature. The solvent were removed under reduced pressure to give 309.4 g of crude extract and then partitioned between 90% MeOH and hexane, removed of MeOH, addition of water and partition with chloroform. After that the water layer was partitioned with ethyl acetate (EtOAc). Each partition was evaporated to dryness in vacuo to give residues of hexane (176.5 g), chloroform (39.1 g), EtOAc (2.5 g), and water fractions (91.1 g), respectively. The chloroform fraction (12 g) was then adsorbed on 200 g of silica gel by vacuum liquid chromatography. The column was eluted in ascending polarity manner with hexane, hexane/chloroform, followed by chloroform/methanol mixture to afford ten fractions (A-J). Fraction G (50 mg) was separated by reversed-phase HPLC, using isocratic method with a flow rate of 2 ml/min. The mobile phase consisted of 70% methanol with 30% water; chromatographic separation was accomplished using a C18 column (10 mm, 250 mm). The eluent was monitored at 254 nm, to afford six fractions (A-F). Repeated column chromatography of the fraction F (10 mg) by gel permeation chromatography on Sephadex LH-20 (10% chloroform in methanol) afforded demethoxycurcumin (**1**) (orange powder, 2 mg).

The hexane fraction (80 g) was chromatographed on 1,500 g of silica gel. The column was eluted in ascending polarity with hexane/EtOAc (98:2 to EtOAc 100%; 15 l) to afford eleven fractions (A-K). Fraction D (1.75 g) was subjected to column chromatography on 100 g of silica gel eluted with hexane/EtOAc (98:2 to EtOAc 100%; 2 l) to give ten fractions (A-J). Further column chromatography of the fraction E (160 mg) was on 140 g of silica gel using hexane/dichloromethane (1:1, 800 ml) which afforded seven fractions (A-G). Fraction F (45 mg) was separated by HPLC, using isocratic method with a flow rate of 3 ml/min. The mobile phase consisted of 90% methanol in water; chromatographic separation was accomplished using a C18 column (10 mm, 250 mm). The eluent was monitored at 254 nm, to afford 3-buten-2-one, 4-[(1R, 4aR, 8aR)-decahydro-5, 5, 8a-trimethyl-2-methylene-1-naphthalenyl]-, (3E)-rel- (**2**) (21 mg, white solid).

The isolation of compound **3** is as follow; the ethanol extract of *Curcuma longa* rhizomes (500 mg) was chromatographed on silica gel (60 g). The column was eluted in ascending polarity with chloroform, followed by chloroform/methanol to afford bisdemethoxycurcumin (**3**) (6 mg, yellow powder). The structures of these three compounds (**1-3**) were elucidated by spectroscopic techniques and compared with the reported spectral data (Weyerstahl *et al.*, 1995; Paramasivam *et al.*, 2009).

2.4 Assay for NO inhibitory effect and cytotoxicity test from RAW264.7 cells

Inhibitory effect on NO production by murine macrophage-like RAW264.7 cells was evaluated using a modified method from that previously reported (Tewtrakul and Subhadhirasakul, 2008). Briefly, the RAW264.7 cell line (purchased from Cell Lines Services) was cultured in RPMI medium supplemented with 0.1% sodium bicarbonate and 2mM glutamine, penicillin G (100 units/ml), streptomycin (100 µg/ml) and 10% FCS. The cells were harvested with trypsin-EDTA and diluted to a suspension in a fresh medium. The cells were seeded in 96-well plates with 1×10^5 cells/well and allowed to adhere for 1 h at 37°C in a humidified atmosphere containing 5% CO₂. After that the medium was replaced with a fresh medium containing 100 µg/ml of LPS together with the test samples at various concentrations (3-100 µg/ml for crude extracts and 3-100 µM for pure compounds) and was then incubated for 48 h. NO production was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent and detected the colour at a wavelength of 570 nm.

Cytotoxicity was determined using the MTT colorimetric method. Briefly, after 48 h incubation with the test samples, MTT solution (10 µl, 5 mg/ml in PBS) was added to the wells. After 4 h incubation, the medium was removed, and isopropanol containing 0.04 M HCl was then added to dissolve the formazan production in the cells. The optical density of the formazan solution was measured with a microplate reader at 570 nm. The test compounds were considered to be cytotoxic when the optical density of the sample-treated group was less than 80% of that in the control (vehicle-treated) group. L-NA (NO synthase inhibitor), CAPE (NF-κB inhibitor) and indomethacin (non-steroidal anti-inflammatory drug, NSAID) were used as positive controls. The stock solution of each test sample was dissolved in DMSO, and the solution was added to the medium RPMI (final DMSO is 1%). Inhibition (%) was calculated using the following equation and IC₅₀ values were determined graphically ($n = 4$):

$$\text{Inhibition (\%)} = \frac{A - B}{A - C} \times 100$$

A-C: NO₂ concentration (µM) [A: LPS (+), sample (-); B: LPS (+), sample (+); C: LPS (-), sample (-)].

3. Results and Discussion

Ethanol extract, hexane-, chloroform-, ethyl acetate- and water fractions from *C. mangga* rhizomes were investigated for their inhibitory activities against LPS induced NO production in RAW 264.7 cell lines. Among these species, the chloroform fraction exhibited the most potent inhibitory activity with an IC₅₀ value of 2.1 µg/ml, followed by the hexane fraction (IC₅₀ = 3.8 µg/ml), EtOAc fraction (IC₅₀ = 23.5 µg/ml) and water fraction (IC₅₀ > 100 µg/ml), respectively (Table 1).

The chloroform- and hexane fractions were chromatographed further to obtain demethoxycurcumin (**1**) and 3-buten-2-one, 4-[(1R, 4aR, 8aR)-decahydro-5, 5, 8a-trimethyl-2-methylene-1-naphthalenyl]-, (3E)-rel- (**2**), respectively (Figure 1). These two compounds together with compound **3** (bisdemethoxycurcumin) were tested for their NO inhibitory effects (Table 2). The result indicated that compound **1** (demethoxycurcumin) exhibited the highest activity against NO release with an IC₅₀ value of 12.1 µM, followed by **2** (IC₅₀ = 30.3 µM). Bisdemethoxycurcumin (**3**), derivative of **1**, which was isolated from the rhizomes of *C. longa* was also tested for anti-inflammatory effect against NO release. It was found that **3** also possessed marked activity with an IC₅₀ value of 16.9 µM. Compounds **1** (IC₅₀ = 12.1 µM) and **3** (IC₅₀ = 16.9 µM) exhibited higher effect than indomethacin (IC₅₀ = 25.0 µM) and L-NA, a positive control (NO synthase inhibitor, IC₅₀ = 61.8 µM), whereas compound **2** (IC₅₀ = 30.3 µM) possessed comparable activity to that of indomethacin (IC₅₀ = 25.0 µM) and higher effect than that of L-NA (IC₅₀ = 61.8 µM) but lower than that of CAPE, NF-κB inhibitor (IC₅₀ = 5.6 µM). The previous study reported that demethoxycurcumin (**1**) and bisdemethoxycurcumin (**3**) inhibited LPS-stimulated NO production in a dose-dependent

Table 1. NO inhibitory effects of fractions^a from *Curcuma mangga* rhizomes.

Sample	% inhibition at various concentrations (µg/ml)					IC ₅₀ (µg/ml)
	0	3	10	30	100	
EtOH extract	0.0±3.5	-12.0±3.7	40.3±5.7	93.6±3.7**	-	11.8
Hexane fraction	0.0±3.6	26.1±2.4	65.1±2.8**	89.5±1.6**	-	3.8
Chloroform fraction	0.0±3.6	34.2±2.4**	84.0±1.6**	100.7±2.3**	-	2.1
EtOAc fraction	0.0±3.0	17.4±3.6	27.1±2.8	60.6±1.7**	-	23.5
Water fraction	0.0±3.0	-	-		40.0±1.5**	>100

Statistical significance, * $p < 0.05$, ** $p < 0.01$

^aEach value represents mean ± S.E.M. of four determinations.

^bCytotoxic effect was observed.

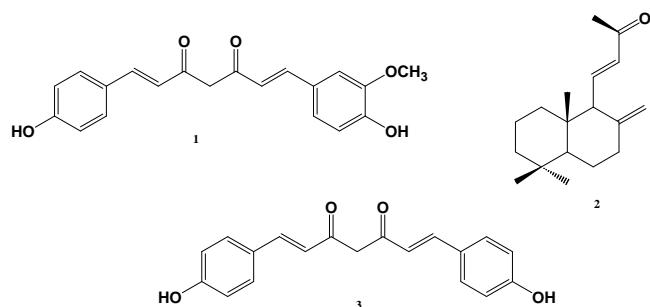


Figure 1. Structures of compounds **1** and **2** isolated from *C. mangga* rhizomes and its derivative (**3**)

manner, the IC_{50} values were 6.9 and 8.4 μ M, respectively (Guo *et al.*, 2008). This result is concurrent with the present study that demethoxycurcumin (**1**, $IC_{50} = 12.1 \mu$ M) possessed higher effect than bisdemethoxycurcumin (**3**, $IC_{50} = 16.9 \mu$ M).

The effect of compounds **1-3** on RAW264.7 cell viability were also determined using the MTT assay. The result showed that at the highest dose (100 μ M), the cell viability were less than 80% when treated with compounds **1** and **3**; whereas that of compound **2** was non toxic (Table 2). Compound **2** was first isolated from the rhizomes of *Hedychium acuminatum*, which is the plant in Zingiberaceae family (Weyerstahl *et al.*, 1995). This compound has never been reported for its biological activity. Therefore, this is the first report on biological study of compound **2** against NO release.

In conclusion, the present study supports the traditional use of *C. mangga* rhizomes for treatment of inflammatory-related diseases. The compounds that are responsible for this activity are 3-buten-2-one, 4-[(1*R*, 4*aR*, 8*aR*)-decahydro-5, 5, 8*a*-trimethyl-2-methylene-1-naphthalenyl]-, (3*E*)-rel- (**2**) and demethoxycurcumin (**1**). Moreover, this is the first report of compound **2** that was isolated from *C. mangga* rhizomes.

Table 2. NO inhibitory effects of compounds^a from *Curcuma mangga* rhizomes.

Compound	% inhibition at various concentrations (μ M)					IC_{50} (μ M)
	0	3	10	30	100	
Compound 1	0.0 \pm 4.9	9.2 \pm 5.7	31.7 \pm 4.4**	93.3 \pm 4.9**	110.0 \pm 3.6**	12.1
Compound 2	0.0 \pm 4.9	16.7 \pm 3.9	39.2 \pm 5.5**	49.2 \pm 3.7**	65.0 \pm 1.0**	30.3
Compound 3	0.0 \pm 4.9	1.7 \pm 3.4	20.0 \pm 2.4**	73.3 \pm 1.4**	107.5 \pm 3.5**	16.9
Indomethacin	0.0 \pm 3.6	14.5 \pm 2.7	30.2 \pm 1.6**	47.6 \pm 2.3**	80.3 \pm 1.5**	25.0
L-Nitroarginine (L-NA)	0.0 \pm 9.9	11.7 \pm 4.6	20.2 \pm 5.9	34.7 \pm 1.8*	71.6 \pm 2.6**	61.8
Caffeic acid phenethyl- ester (CAPE)	0.0 \pm 9.9	30.7 \pm 3.2	68.6 \pm 3.4**	98.7 \pm 1.2**	98.9 \pm 2.1**	5.6

Statistical significance, * p <0.05, ** p <0.01

^aEach value represents mean \pm S.E.M. of four determinations.

^bCytotoxic effect was observed.

Acknowledgements

The authors are grateful to the Thailand Research Fund (TRF), the Commission on Higher Education (CHE) and the Prince of Songkla University (PSU) for financial support and further the Faculty of Pharmaceutical Sciences, PSU, for providing laboratory facilities.

References

Abas, F., Lajis, N.H., Shaari, K., Israf, D.A., Stanslas, J., Yusuf, U.K. and Raoft, S.M. 2005. A labdane diterpene glucoside from the rhizomes of *Curcuma mangga*. *Journal of Natural Products* 68, 1090-1093.

Cho, Y-C., Kim, H.J., Kim, Y-J., Lee, K.Y., Choi, H.J., Lee, I-S. and Kang, B.Y. 2008. Differential anti-inflammatory pathway by xanthohumol in IFN- γ and LPS-activated macrophages. *International Immunopharmacology* 8, 567-573.

Guo, L.Y., Cai, X.F., Lee, J.J., Kang, S.S., Shin, E.M., Zhou, H.Y., Jung, J.W. and Kim, Y.S. 2008. Comparison of suppressive effects of demethoxycurcumin and bisdemethoxycurcumin on expressions of inflammatory mediators *in vitro* and *in vivo*. *Archives of Pharmacal Research* 31, 490-496.

Jung, K.H., Ha, E., Kim, M.J., Won, H.J., Zheng, L.T., Kim, H.K.g., Hong, H.J., Chung, J.H. and Yim S.V. 2007. Suppressive effects of nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) expression by *Citrus reticulata* extract in RAW 264.7 macrophage cells. *Food and Chemical Toxicology* 45, 1545-1550.

Lantz, R.C., Chen, G.J., Solyom, A.M., Jolad, S.D. and Timmermann, B.N. 2005. The effect of turmeric extracts on inflammatory mediator production. *Phytomedicine* 12, 445-452.

Merrill, J.E., Ignarro, L.J., Sherman, M.P., Melinek, J., Lane, T.E. 1993. Microglial cell cytotoxicity of oligodendrocytes is mediated through nitric oxide. *Journal of Immunology* 151, 2132-2141.

Paramasivam, M., Poi, R., Banerjee, H. and Bandyopadhyay, A. 2009. High-performance thin layer chromatographic method for quantitative determination of curcuminoids in *Curcuma longa* germplasm. Food Chemistry 113, 640-644.

Tewtrakul, S. and Subhadhirasakul, S. 2007. Anti-allergic activity of some selected plants in the Zingiberaceae family. Journal of Ethnopharmacology 109, 535-538.

Tewtrakul, S. and Subhadhirasakul, S. 2008. Effects of compounds from *Kaempferia parviflora* on nitric oxide, prostaglandin E₂ and tumor necrosis factor-alpha productions in RAW264.7 macrophage cells. Journal of Ethnopharmacology 120, 81-84.

Weyerstahl, P., Schwieger, R., Schwope, I. and Hashem, M.A. 1995. Synthesis of some naturally occurring drimane and dinorlabdane derivatives. Liebigs Annalen 7, 1389-1392.