Effects of compounds isolated from *Curcuma mangga* on nitric oxide production in RAW264.7 macrophage cells

ผลของสารสำคัญจากเหง้าขมิ้นขาวต่อการสร้าง nitric oxide ใน RAW264.7 macrophage cells

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ABSTRACT

In the present study, we investigated the anti-inflammatory effect of compounds from *C. mangga* rhizomes against lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW 264.7 cell line. Demethoxycurcumin (**1**) and 3-buten-2-one, 4-[(1*R*, 4a*R*, 8a*R*)-decahydro-5, 5, 8a-trimethyl-2-methylene-1-naphthalenyl]-, (3*E*)-rel- (**2**) were tested for NO inhibitory activity. Of the tested compounds, compound **1** exhibited the highest activity with an IC_{50} value of 12.1 µM, followed by **2** (IC_{50} = 30.3 µM). These results suggest that *C. mangga* and its compounds exert NO inhibitory activity and has 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.

บทคัดย่อ

งานวิจัยนี้ได้ศึกษาฤทธิ์ต้านการอักเสบของสารบริสุทธิ์จากเหง้าขมิ้นขาว โดยใช้ lipopolysaccharide (LPS) เป็นตัวเหนี่ยวนำการสร้างสารไนตริกออกไซด์ (NO) ใน RAW 264.7 cell line เมื่อนำสารบริสุทธิ์ demethoxycurcumin (**1**) และ 3-buten-2-one, 4-[(1*R*, 4a*R*, 8a*R*)-decahydro-5, 5, 8a-trimethyl-2-methylene-1-naphthalenyl]-, (3*E*)-rel- (**2**) มาทดสอบฤทธิ์ยับยั้งการสร้าง NO จากผลการทดสอบพบว่าสาร **1** มีฤทธิ์ดีที่สุด โดยมีค่า IC_{50} เท่ากับ 12.1 µM ตามด้วยสาร **2** ซึ่งมีค่า IC_{50} เท่ากับ 30.3 µM ตามลำดับ จากผลการศึกษาดังกล่าวสรุปได้ว่าขมิ้นขาวและสารบริสุทธิ์ที่แยกได้มีฤทธิ์ยับยั้งการหลั่ง NO และมีฤทธิ์ดีพอที่จะสามารถนำมาพัฒนาเป็นยาเพื่อใช้รักษาโรคที่เกี่ยวกับการอักเสบได้เป็นอย่างดี และงานวิจัยชิ้นนี้เป็นงานวิจัยแรกที่ได้รายงานเกี่ยวกับสาร **2** ซึ่งแยกได้เป็นครั้งแรกในเหง้าขมิ้นขาว

Key Words: RAW264.7 cells, Nitric oxide, Prostaglandin E_2, *Curcuma mangga*, Zingiberaceae

คำสำคัญ: RAW264.7 cell ไนตริกออกไซด์ พรอสตาแกลนดินอี 2 *Curcuma mangga* Zingiberaceae

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Introduction

Nitric oxide (NO) is produced via the oxidation of L-arginine by family of nitric oxide synthase (NOS). iNOS is induced in response to various pro-inflammatory factor-α (TNF-α) and interleukin-6 (IL-6), and mediates cytokines including interferon-γ (INF-γ), tumor necrosis 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 (Hee 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 was able to prevent rat amoeboid microglia from lysing oligodendrocytes *in vitro* (Jantaratnotai et al., 2005). 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). Cyclooxygenase activation resulting from high levels of prostaglandin E_2 can react with superoxide anions to form peroxynitrite (Cruz-Vega et al., 2002). Increased production of prostaglandins during an inflammatory response is achieved by induction of cyclooxygenase 2 (COX-2). COX-2 expression is mediated by NF-κB activation (Lantz et al., 2005). PGE_2 is derived from the catalyzation of arachidonic acid by COX-2. The cells that produce large amount of PGE_2 are monocytes and macrophages. The macrophages can be activated by lipopolysaccharide (LPS) and interferon-γ (IFN-γ) (Tewtrakul and Subhadhirasakul, 2008). 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 macrophages 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). This antigen can activate macrophages to release some inflammatory mediators such as NO, TNF-α, PGE_2 and so on. Therefore, the inhibition of NO, PGE_2 and TNF-α production is an important therapeutic consideration in development of anti-inflammatory agents.

Curcuma mangga Val.&Zijp. is a perennial herb in 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* is then 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 *C. mangga* rhizomes have long been used for treatment of inflammation and possessed marked anti-NO activity, we thus investigated the inhibitory activity of compounds isolated from this plant against NO releases using RAW264.7 macrophage cells.

Materials and methods

2.1. Reagents

Lipopolysaccharide (LPS, from *Escherichia coli*), RPMI-1640 medium, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), L-nitroarginine (L-NA), caffeic acid phenethylester (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, Birkrød, Denmark). Other chemicals were from Sigma Aldrich (Sigma–Aldrich, Missouri, USA).

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 Assoc.Prof.Dr. Sanan 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 from Curcuma mangga extract

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 (17.18% w/w) 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, 57.04% w/w), chloroform (39.1 g, 12.63% w/w), EtOAc (2.5 g, 0.80% w/w) and water fractions (91.1g, 29.44% w/w), 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**) (2 mg, 0.02% w/w, orange powder).

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-[(1*R*, 4a*R*, 8a*R*)-decahydro-5, 5, 8a-trimethyl-2-methylene-1-naphthalenyl]-, (3*E*)-rel- (**2**) (21 mg, 0.03% w/w, white solid).

The ethanol extract of *Curcuma longa* (500 mg) was chromatographed on 60 g of silica gel. The column was eluted in ascending polarity with chloroform, followed by chloroform/methanol mixture to afford bisdemethoxycurcumin (**3**) (6 mg, 1.2% w/w, yellow powder). The structures of these compounds (**1**-**3**) were elucidated by using the spectroscopic technique and compared with the reported spectral data (Paramasivam et al., 2009; Weyerstahl et al., 1995).

2.4. Assay for NO inhibitory effect 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% CO_2. After that the medium was replaced with a fresh medium containing 100 µg/ml of LPS together with the test samples at various concentrations (3–100µg/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. 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 IC_{50} values were determined graphically ($n = 4$):

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

A-C: NO_2^- concentration (μM) [*A*: LPS (+), sample (-); *B*: LPS (+), sample (+); *C*: LPS (-), sample (-)].

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

Sample	% inhibition at various concentrations (μg/ml)					IC_{50} (μg/ml)
	0	1	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
L-Nitroarginine (L-NA)	0.0 ± 9.9	11.7 ± 4.6	20.2 ± 5.9	34.7 ± 1.8**	71.6 ± 2.6**	61.8 (13.5)[c]
Caffeic acid phenethylester (CAPE)	0.0 ± 9.9	30.7 ± 3.2*	68.6 ± 3.4**	98.7 ± 1.2[b]**	98.9 ± 2.1[b]**	5.6 (1.4)[c]

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

[a]Each value represents mean ± S.E.M. of four determinations.

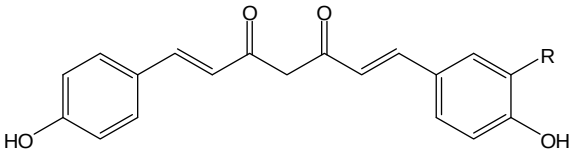
[b]Cytotoxic effect was observed.

[c]Values in parenthesis are IC_{50} (μg/ml).

Results and discussion

The effect of compounds on RAW264.7 cell viability were determined by MTT assay. The survival of cells was not significantly affected by treatment with compounds **1**, **2** and **3** at concentrations ranging from 3 to 30 μM. However, higher doses of the compounds **1** and **3** (100 μM) decreased cell survival (< 80%), whereas compound **2** at 100 μM did not affect.

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 IC_{50} value of 2.1 µg/ml, followed by the hexane- (IC_{50} = 3.8 µg/ml), EtOAc- (IC_{50} = 23.5 µg/ml) and water fractions (IC_{50} > 100 µg/ml), respectively (Table1).



1: R = OCH_3

3: R = H

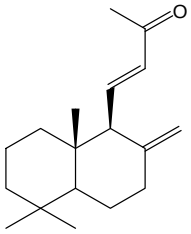


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Fig. 1. Structures of compounds **1** and **2** isolated from *C. mangga* rhizomes and its derivative (**3**)

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

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

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

[a]Each value represents mene ± S.E.M. of four determinations.

[b]Cytotoxic effect was observed.

The chloroform- and hexane fractions were chromatographed further to obtain demethoxycurcumin (**1**) and 3-buten-2-one, 4-[(1*R*, 4a*R*, 8a*R*)-decahydro-5, 5, 8a-trimethyl-2-methylene-1-naphthalenyl]-, (3*E*)-rel- (**2**), respectively (Fig. 1). These two compounds 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 IC_{50} value of 12.1 µM, followed by **2** (IC_{50} = 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 IC_{50} value of 16.9 µM. Compounds **1** (IC_{50} = 12.1 µM) and **3** (IC_{50} = 16.9 µM) exhibited higher effect than indomethacin (IC_{50} = 25.0 µM) and L-NA, a positive control (NO synthase inhibitor, IC_{50} = 61.8 µM whereas compound **2** (IC_{50} = 30.3 µM) possessed comparable activity to that of indomethacin (IC_{50} = 25.0 µM) and higher effect than that of L-NA (IC_{50} = 61.8 µM). 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.

Conclusions

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*, 4a*R*, 8a*R*)-decahydro-5, 5, 8a-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.

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