

Coumarins and flavonoid from *Murraya paniculata* (L.) Jack: Antibacterial and anti-inflammation activity

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Abstract: The ethyl acetate extract of leaves of *Murraya paniculata* (L.) Jack was described in the previous *in vitro* study on the inhibition effect on the growth of periodontopathic bacteria and the reduction of cytokines from LPS-stimulated macrophages. In this study, four coumarins including murrangatin (1), murrangatin acetate (2), murranganonesenecionate (3), micropubescin (4) and one flavonoid, 3',4',5',7-tetramethoxyflavone (5) were isolated from the leaves of ethyl acetate extract of *M. paniculata*. MTT assay was used to test cytotoxicity on human gingival fibroblast and monocytes. The isolated compounds were evaluated for their antibacterial effect against *Porphyromonas gingivalis* (ATCC33277) and anti-inflammation on lipopolysaccharide-stimulated inflammation using monocyte cells. All isolated compounds exhibited antibacterial activity against *P. gingivalis* (ATCC 33277). Murranganonesenecionate (3) was highly potent anti-inflammation properties. The coumarin constituents from *M. paniculata* leaves might be potential lead molecules for the development of antimicrobial drugs for treating periodontal disease.

Keywords: *Murraya paniculata* (L.) Jack, coumarins, flavonoid, antibacterial, anti-inflammation.

INTRODUCTION

Murraya paniculata (L.) Jack (Rutaceae), which is known in Thai as Kaew, is an 8-12 feet high medium-sized shrub. Its flowers are white and fragrance, which bloom all the year round. Its leaves are green and ovate in shape, with less than 2 inches of blade length, alternately arranged on the branch. Its leaves and roots have traditionally found wide medicinal uses in southeast Asia and China (Imai *et al.*, 1989). Chemical constituents from this plant including flavonoids (Kinoshita and Firman, 1996), flavones (Yang and Du, 1984), indole alkaloids (Kong *et al.*, 1985), coumarins (Kinoshita *et al.*, 1996), monoterpenes (Li *et al.*, 1988), sesquiterpenes (Chowdhury *et al.*, 2008) and cinnamates (Rahman *et al.*, 1997). Coumarins and cinnamates exhibited antioxidant, antimicrobial and anticancer activity (Rahman *et al.*, 1997; Ng *et al.*, 2012). To our knowledge, its ethyl acetate fraction of crude extract from its leaves exerted inhibitory effect on the growth of periodontopathic bacteria (Rodanant *et al.*, 2010) and could reduce the release of cytokines, IL-1 β and TNF- α , from LPS-stimulated macrophages (Rodanant *et al.*, 2012). Previous chemical investigations of plant from Rutaceae family demonstrated the isolation of various phytochemical constituents including coumarins and flavonoids (Jiwajinda *et al.*, 2000; Kinoshita and Firman, 1997). Natural coumarins and flavonoids from plants have been shown to exert antimicrobial and anti-inflammation activities (Creaven *et al.*, 2006; Xu and Lee, 2001; Serafini *et al.*, 2010; Hadjipavlou-Litina *et al.*, 2007). The potency of these secondary metabolites might

benefit the treatment of periodontal disease, a chronic inflammation initiated by bacterial contamination, which continues to breakdown periodontium through the activation of host defence cells (Page, 1991). Although there are extensive uses of antimicrobial and anti-inflammatory drugs, prolonged consumption might exert numerous side effects or developing drug resistance. The need for alternative strategies to lower the incidence of those unwanted phenomenon is warranted. The purpose of this *in vitro* study was to continue our research on evaluation of biologically active compounds of *M. paniculata* leaves for its potential antibacterial and anti-inflammation activities. These could be helpful in providing a rationale for the ethnomedicinal use of the plant. Cytotoxicity of the isolated compounds to human gingival fibroblasts (HGFs) and monocytes (U937) were also investigated.

MATERIALS AND METHODS

Plant materials

M. paniculata leaves were collected in Hua Mark District, Bangkok. Voucher specimen was identified by Associate Professor Nopporn Dumrongsiri and archived in the herbarium of the Faculty of Science, Ramkhamhaeng University.

Extraction and isolation

The air-dried plant leaves (1.64kg) were pulverized and extracted thoroughly with *n*-hexane, ethyl acetate (EtOAc) and methanol (MeOH). The EtOAc extract (103.76g) was subjected to repeat silica column chromatography using dichloromethane/MeOH as eluent

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to give four coumarins (1-4) and one flavone (5). By comparison of the spectroscopic data with those previously reported, the chemical structures of isolated compounds were identified as murrangatin (1, 23.5 mg) (Ito and Furukawa 1987), murrangatin acetate (2, 18.3 mg) (Ito and Furukawa 1987), murranganonesenecioate (3, 8.3mg) (Ito and Furukawa 1987), micropubescin (4, 10.7mg) (Bhan *et al.*, 1973), and 3',4',5',7-tetramethoxyflavone (5, 9.6 mg) (Cushman *et al.*, 1991) (fig. 1). Each of the isolated compounds was dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, USA) to obtain the solutions of 1mg/ml (w/v) concentrations. The stock solutions were kept at -20°C.

Microorganisms

The antibacterial activities of all isolated compounds were determined against *Porphyromonas gingivalis* ATCC 33277 (*Pg* ATCC 33277). The medium used for the activation of the microorganisms was Brain heart infusion broth (BHI). Brucella (Oxoid®, UK) was used as cultivation media. The microorganisms were inoculated into BHI and incubated at 37°C in 5% CO₂ atmosphere (Anaerobic system; Forma Scientific, Inc.) for 7 days. The bacterial suspension was then diluted with BHI broth medium to obtain approximately 1×10⁸ cfu/ml of bacteria. This working concentration was obtained transmittance comparable to 0.5 McFarland turbidity standard using spectrophotometer at 540 nm.

Antibacterial activity

The growth inhibition tests were performed using agar diffusion technique. One hundred microliter (μl) of BHI-suspended microorganism was distributed on the agar medium (25ml/plate) using small-size glass beads. Once the agar surface was dried, papers discs 6mm diameter (Whatman International, UK), soaked with 10μl of the isolated compound solution on each side of the disc, were placed on the agar surface. Chlorhexidine 2% and DMSO were used as positive and negative control respectively. Each plate was incubated in anaerobic chamber at 37°C in 5% CO₂ atmosphere for 7 days. All tests were performed in triplicate and the antibacterial activity was expressed as the mean diameters (mm) of inhibition zone produced by the isolated compound.

Cytotoxicity assay

The stock samples were diluted with Dulbecco's Modified Eagle's Medium (DMEM) or RPMI 1640 (for assay human gingival fibroblasts (HGFs) and monocytes (U937), respectively) to desired concentrations (10, 20, 50, 100 μg/ml). The final concentration of DMSO in each sample did not exceed 1% v/v (Prayong *et al.*, 2008). The cytotoxic activity of the extracts were tested in HGFs and U937 by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Mosmann, 1983) with minor modification.

Briefly, the HGFs or Phorbol 12-myristate 13-acetate (PMA)-stimulated U937 were seeded in 96 well-plates (200μl/well at a density of 1×10⁵ cells/ml) incubated at 37°C in 5% CO₂ atmosphere for 24 hours (for HGFs) and 48 hours (for U937). At 24 hours, cells were washed with sterile phosphate buffer (PBS) 100μl and treated with 100 μl of isolated compound solution at various concentrations (five wells were included for each treatment) then incubated at 37°C in 5% CO₂ atmosphere for another 24 hours. Discarded the supernatant and washed plate with sterile PBS 1X then one hundred microlitre of MTT solution (0.5mg/ml) was added to each well and incubated at 37°C for another 2 hours. Then the medium was aspirated. In each well, the formed formazan crystals were solubilized with 100μl of DMSO, leave for 30 minute at room temperature. An absorbance of formazan was reported as optical density, which was read at 540 nm by ELISA reader (Model series UV 900 Hdi, USA).

Anti-inflammation assay

The HGFs or PMA-stimulated monocytes were seeded in 24 well-plate (1000μl/well at a density of 1×10⁵ cells/ml) incubated at 37°C in 5% CO₂ atmosphere for 24 hours (for HGFs) and 48 hours (for U937). Cells were pretreated with 50 μg/ml of each isolated compounds for 2 hours at 37°C in 5% CO₂ atmosphere, followed by 0.5μg/ml LPS treatment for 24 hours at 37°C in 5% CO₂ atmosphere then the cell supernatants were collected. Two control groups were also performed: (1) positive control consisting of 0.5 μg/ml dexamethasone (DM) and 0.5 μg/ml LPS, (2) negative control-comprising cells incubated in 1% DMSO in DMEM (for HGFs) or RPMI1640 (for U937). For assaying purposes, the cell supernatants were used for measuring the levels of IL-1β and TNF-α proteins using ELISA kit according to the manufacturer's instructions. ELISA results were recorded using microplate reader (Model series UV 900 Hdi, USA) at wavelengths of 450 nm and 550 nm. The IL-1β or TNF-α concentration (pg/ml) were derived from the standard curve. Each sample was measured in duplicate in two separate experiments.

STATISTICAL ANALYSIS

The data was expressed as mean ± S.D. The multiple comparisons was performed by One-Way ANOVA and subsequently followed by Dunnett's test. The criterion for statistical significance was set at *p*<0.05.

RESULTS

Antibacterial activity

Compounds 1-5, at the concentration of 100 μg/ml, have been assayed for antibacterial activity against *P. gingivalis*. The inhibitory effects of compounds 1-5 against the organism, expressed by zone of inhibition, are given in table 1. The screening results indicated that all isolated pure compounds from EtOAc extract of *M. paniculata* were active against *P. gingivalis*. Compound 1

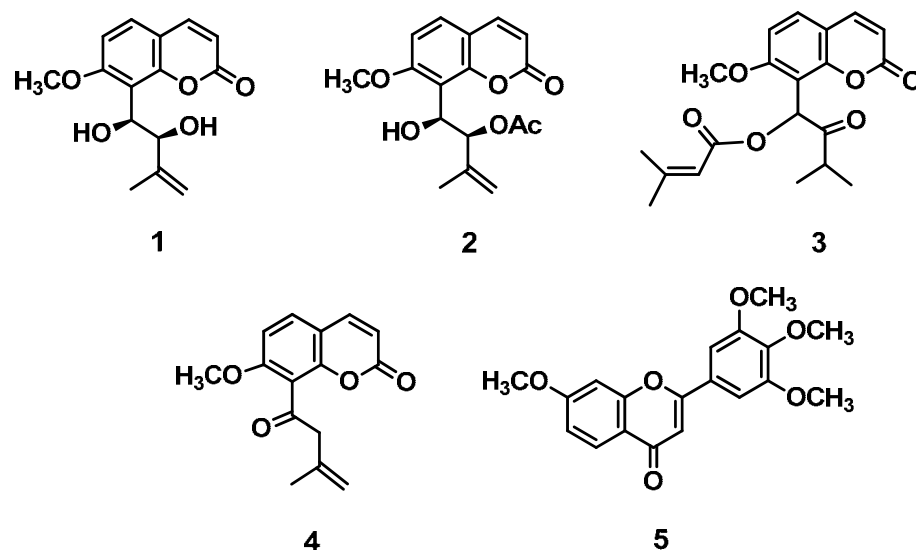


Fig. 1: Chemical structures of isolated compounds from the leaves of EtOAc extract of *M. paniculata*; murrangatin (1), murrangatin acetate (2), murranganonesenecioate (3), micropubescin (4) and 3',4',5',7-tetramethoxyflavone (5).

gave the widest inhibition zone (22 ± 0 mm) followed by compound 2 (20 ± 0.7 mm), nevertheless the zone was significantly narrower than that of standard drug (CHX 2%) ($p=0.014$).

In vitro Cytotoxicity of pure compounds

The cytotoxicity results of pure compounds on HGFs and U937 were summarized in tables 2 and 3. Pretreatment of unstimulated cells (HGFs and U937) with each fraction prepared from *M. paniculata* for 24 hours revealed the IC_{50} of isolated compounds to HGFs and U937 ranging from 67.21–78.12 μ g/ml and 35.72–64.4 μ g/ml, respectively. All compounds were more toxic to U937 than HGFs.

Table 1: Antibacterial activity of isolated compounds in paper disc method against *P. gingivalis*

Compound	Diameters of inhibition zone (mm)
1	$22 \pm 0^*$ NS
2	$20 \pm 0.7^*$
3	NS $16 \pm 0.22^*$
4	$13 \pm 0.08^*$ NS
5	$13 \pm 0.14^*$ NS
CHX 2% (positive control)	29 ± 0

CHX 2% = Chlorhexidinedigluconate 2%

Values are expressed as Mean \pm S.D. (n=3)

* = statistical significance ($p < 0.05$) (compare to CHX 2%)

NS = not statistical significance

Anti-inflammation assay

The concentration of 50 μ g/ml and 20 μ g/ml of the studied compounds (the concentration giving not less than 80% viability of HGFs and U937, respectively) were used for quantitative evaluation of IL-1 β and TNF- α production.

The release of both pro-inflammatory cytokines from HGFs and U937 were induced by LPS (1 μ g/ml) treatment. All studied compounds did not have any inhibitory effects on both IL-1 β and TNF- α production from HGFs (data not shown). All isolated compounds were not capable of negatively regulating the secretion of TNF- α from U937. Among all fractions, compound 3 did show the best IL-1 β suppression capacity, nevertheless it was significantly less effective than dexamethasone (positive control) ($P=0.00$). Table 4 showed the examination of inhibitory effects of isolated compounds on cytokines production from LPS-stimulated U937.

Table 2: *In vitro* cytotoxicity of coumarins and flavonoid from *M. paniculata* against human gingival fibroblasts

Compound	Cytotoxic to HGF (μ g/ml)	
	IC_{50}	IC_{80}
1	69.66	73.54
2	67.21	72.52
3	77.85	62.04
4	81.03	58.46
5	78.12	57.64

Table 3: *In vitro* cytotoxicity of coumarins and flavonoid from *M. paniculata* against monocytes (U937)

Compound	Cytotoxic to monocyte (U937) (μ g/ml)	
	IC_{50}	IC_{80}
1	46.91	21.44
2	35.72	27.01
3	37.61	24.45
4	40.5	25.6
5	64.4	30.12

Table 4: Effects of coumarins and flavonoid from *M. paniculata* on cytokine release

Compound	Cytokine release (pg/ml)			
	TNF- α		IL-1 β	
1	2050.99 \pm 10.06*		220.12 \pm 5.24*	
2	2172.1 \pm 30.99*		235.55 \pm 15.50*	
3	NS	1682.58 \pm 49.75*	150.21 \pm 6.21*	NS
4	2186.96 \pm 53.40*		NS	214.28 \pm 5.82*
5	2082.82 \pm 71.87*		NS	244.23 \pm 6.28*
LPS	1610.52 \pm 10.64*		275.09 \pm 4.95*	
DEXA	8.13 \pm 0.46		32.09 \pm 1.72	

LPS = Lipopolysaccharide, DEXA = Dexamethasone

Values are expressed as Mean \pm S.D. (n=4)

* = statistical significance ($p < 0.05$) (compare to DEXA)

NS = not statistical significance

DISCUSSION

Natural secondary metabolites coumarins and flavonoids have been reported to possess antimicrobial activity (Debeljak *et al.*, 2007; Xu and Lee, 2001). In this study, the EtOAc extract of the leaves of *M. paniculata*, which showed antibacterial activity against various species of periodontopathic bacteria (Rodanant *et al.*, 2010), was further purified by silica gel column chromatography to afford five compounds including 4 coumarins and 1 flavanone. Two coumarins (compounds 1 and 2) exhibited better antibacterial potency than crude extract (Rodanant *et al.*, 2010). This might reflect the potency of coumarin as a major constituent exhibiting antibacterial effect from this plant or this might explain that the presence of another compound in crude extract might prevent direct contact between this phenolic compound (coumarin) and bacteria. This result is consistent with other study, which showed that phenolic compounds did exert strong antimicrobial effect (Kinza Aslam *et al.*, 2010).

The efficiency to diffuse of test substances is one of a key factor to mention when using agar disc diffusion assay since the diameter of the inhibition zone was used to determine antimicrobial potency. This might explain the narrow inhibition zone of compound 5 since flavanones solution might not diffuse well on the prepared nutrient agar (Zheng *et al.*, 1996). This study also examined the inhibitory effect of coumarins and flavonoid on IL-1 β production from LPS-stimulated macrophages, the result was not in agreement with the previous reviews which showed that these two phytochemicals possess strong anti-inflammation activity (Serafini *et al.*, 2010; Fylaktakidou *et al.* 2004). Although positive result did reveal on the reduction of IL-1 β release from LPS-stimulated macrophages which compound 3 showed the most potent activity, the potency is very weak compare to the reference compound (dexamethasone). This weak potency might be due to the pattern of the substitution on C-3, C-4 and C-7 position of the chemical structure of coumarin (Keating and O'Kennedy, 1997). This might be

interesting to further examination on antimicrobial mode of action of *M. paniculata* leaf-derived materials or their constituents to establish antimicrobial agents in form of mouthwash or local drug delivery as an adjunct to treat periodontal disease.

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