Bioactive compounds from the leaves of *Murraya paniculata* (L.) Jack

สารออกฤทธิ์ทางชีวภาพจากใบแก้ว

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ABSTRACT

Murraya paniculata (L.) Jack is a tropical evergreen rounded shrub found in south, southeast Asia, China and Australia. Recent phytochemical studies have confirmed a positive correlation between several groups of active constituents and the traditional usage. In this work, twenty-six compounds were isolated from the leaves of *M. paniculata*, including two compounds: 2,6,2□,6□-tetramethoxy-4,4□-*bis*-(1,2-*trans*-2,3-epoxy-1-hydroxypropyl) biphenyl (**25**) and medioresinol (**26**) which were firstly reported as constituents in this plant. Moreover, the antibacterial, antioxidant and anti-inflammatory activities of some isolated compounds were investigated. The results showed that only compound **1** exhibited moderate antioxidant activity with IC_{50} 289 µg/mL.

บทคัดย่อ

แก้วมีชื่อทางพฤกษศาสตร์ว่า *Murraya paniculata* (L.) Jack เป็นไม้พุ่มเขตร้อน ที่มีผิวใบเขียวมันเข้ม แก้วพบมากในแถบเอเชียใต้ เอเชียตะวันออกเฉียงใต้ จีนและออสเตรเลีย การศึกษาทางพฤกษเคมีเมื่อไม่นานมานี้ยืนยันความสัมพันธ์ที่ดีระหว่างหมู่แทนที่ที่หลากหลายของสารที่เป็นองค์ประกอบของแก้วกับการใช้งานแบบดั้งเดิม งานวิจัยนี้ได้แยกสารทั้งหมด 26 ชนิดจากใบแก้ว สาร 2 ชนิด ได้แก่ 2,6,2□,6□-tetramethoxy-4,4□-*bis*-(1,2-*trans*-2,3-epoxy-1-hydroxypropyl)biphenyl (**25**) และ medioresinol (**26**) ได้รายงานเป็นองค์ประกอบทางเคมีในใบแก้วเป็นครั้งแรก นอกจากนี้ได้ทดสอบฤทธิ์ต้านแบคทีเรีย ฤทธิ์ต้านอนุมูลอิสระและฤทธิ์ต้านการอักเสบของสารบางชนิด พบว่าเฉพาะสาร **1** แสดงฤทธิ์ต้านอนุมูลอิสระปานกลางด้วยค่า IC_{50} 289 µg/mL

Keywords: *M. paniculata*, antioxidant, anti-inflammatory
คำสำคัญ: ต้นแก้ว ฤทธิ์ต้านอนุมูลอิสระ ฤทธิ์ต้านการอักเสบ

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Introduction

Murraya paniculata (L.) Jack belongs to the family Rutaceae and is a small, smooth tree, growing from 3 to 8 m in height, with very hard wood. Leaves are 8 to 15 cm long. Stems are hairy. Flowers are few, white, very fragrant, 1.5 to 2 cm long, and borne on short, terminal or axillary cymes. Fruits are fleshy, red when ripe, pointed or oval-shaped, 1 to 1.5 cm long. It is commonly cultivated in garden in many tropical and subtropical countries for its glossy green foliage and large clusters of fragrant flowers. An infusion of the leaves and flowers of *M. paniculata* is tonic and stomachic. This plant has been used in traditional medicine to treat several diseases. In Thai rural areas, leaves and root barks of *M. paniculata* are still widely used for treating tapeworm disease, coughs, giddiness, hysteria, thirst, burning of the skin and rheumatism (Sastri, 1962; Chopra *et al.*, 1956; Ghani, 2003). Moreover, decoction of leaves also used as mouthwash for toothaches (Rahman *et al.*, 1997). Several authors have reported different pharmacological activities for *M. paniculata* such as anti-inflammatory, anticancer activity of mahanine, mahanimbicine, mahanimbine on the human cell lines such as human breast MCF-7, human cervical HeLa and murine leukemia cell lines (Maja *et al.*, 2015), antioxidant activity of carbozole alkaloids (mahanimbine and koenigine) which showed high antioxidant activities, antimicrobial activity of alkaloids (mahanine, mahanimbicine and mahanimbine) on the growth of antibiotic resistant bacteria, and antidiabetic activity of murrayacinine, isomahanimbine, mahanimbicine and mahanimboline (Ganesan *et al.*, 2013).

Objective of the study

The aim of this study was to isolate chemical constituents from the leaves of *M. paniculata* and investigate antibacterial, antioxidant and anti-inflammatory activities of certain isolated constituents.

Methodology

Plant material

Leaves of *M. paniculata* were collected in May 2015 from Nakhonnayok, Thailand. The fresh leaves were air dried for 24 h. The dried leaves were roughly ground and kept in a dry and dark container until use.

Preparation of crude extracts

5 kg of *M. paniculata* leaves were extracted in 3 L of hexane at room temperature for 7 d. After that, the extract was filtered through filter paper (No.1, Whatman) and then the residue was extracted with 3 L of CH_2Cl_2, EtOAc and MeOH for 7 d, respectively. The extract was filtered through filter paper. The organic solvent was removed in a rotatory evaporator at 45 °C to give the hexane, CH_2Cl_2, EtOAc and MeOH extracts 196, 197, 137 and 351 g, respectively.

145 g of the methanol extract was suspended in water (1 L) and then hexane (3 L) was added to extract for 3 times. After that, EtOAc was added to extract for 5 times. The organic solvent was removed in a rotatory evaporator at 45 °C to give hexane and EtOAc crude extracts, respectively.

Chromatographic Separation

Hexane (100 g), CH_2Cl_2 (190 g) and EtOAc (112 g) crude extracts were subjected to silica gel quick column using gradient solvent starting from hexane and increased polarity by mixing with EtOAc and MeOH. Each fraction was examined and combined by TLC.

Each fraction of hexane, CH_2Cl_2 and EtOAc crude extract was subjected to normal phase column using a gradient elution of hexane with increasing amounts of EtOAc up to 100 % EtOAc followed by increasing amount of MeOH. Fractions with similar TLC spots were combined and were repeatedly chromatographed on silica gel column to obtain compounds **10**, **11** and **15**.

The EtOAc crude from the MeOH crude extract was subjected to normal phase column using a gradient elution of $CHCl_3$ with increasing amount of MeOH up to 50% MeOH. After that, some fractions were subjected to reverse phase column using a gradient elution of MeOH with decreasing amount of water down to 100% MeOH. Furthermore, some fractions were purified by semi-preparative HPLC using a mobile phase of MeOH-H_2O or MeCN-H_2O to afford compounds **1**-**9**, **12**-**14** and **16**-26.

Spectroscopic Technique

^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded at room temperature in $CDCl_3$ using a Bruker AVANCE 400 spectrometer and chemical shifts are given in δ (ppm) relative to TMS as an internal standard.

Antioxidant activity

Free radical-scavenging activities of 11 compounds (**1**, **2**, **5**, **7**-**12**, **15** and **18**) were evaluated in comparison with ascorbic acid using DPPH assay. In brief, 0.05 mg/mL solution of DPPH in MeOH was prepared. This solution (100 µL) was added to 50 µL of different compounds in MeOH at different concentrations (62.5, 125, 250, 500, 1000 µg/mL). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min then, absorbance was measured at 517 nm by using microplate reader (BioTek PowerWave XS2). A reference standard compound being used was ascorbic acid and the experiment was done in triplicate. The IC_{50} value of the sample, which is the concentration of sample required to inhibit 50% of the DPPH free radical, was calculated using log dose inhibition curve. The percent DPPH scavenging effect was calculated by using the following equation:

$$\text{Percent inhibition} = (A_0 - A_1 / A_0) \times 100$$

Where A_0 = the absorbance of control reaction

A_1 = the absorbance in the presence of test or standard sample

Antibacterial activity

Compounds **10**, **11** and **15** were tested against bacteria pathogens: *Propionibacterium acnes* (KCCM41747), *Staphylococcus aureus* (ATCC25923), *Streptococcus sobrinus* (KCCM11898), *Streptococcus mutans* (ATCC25175), and *Salmonella typhi* (ATCC442). The antibacterial activity was evaluated by agar diffusion method (Chung *et al.*, 1990). Muller Hinton agar medium was prepared and poured into the petri dishes. Then it was incubated with a swap of bacterial culture and spread throughout the medium uniformly with a sterile cotton swap. Using a sterile cork borer (10 mm diameter) wells were made in agar medium. The test compounds were introduced into the wells and all the

plates were incubated at 37 ºC for 24 h. The experiment was performed three times under strict aseptic conditions. Sensitivity of the organism was determined by measuring the diameter of the zone of inhibition. Each assay was repeated for three times and the mean value was taken for analyses. The control experiment was carried out with chloramphenicol antibiotic.

Anti-inflammatory

Cell culture: RAW 264.7 murine macrophages cells were maintained in DMEM medium supplemented with 10% FBS, penicillin (100 units/mL), streptomycin (100 µg/mL), and were incubated at 37 ºC in a humidified atmosphere containing 5% CO_2. The medium was routinely changed every two days. RAW 264.7 cells were passaged by trypsinization until they attained confluence.

Cell viability assay: Cell viability was determined by MTT assay. RAW 264.7 macrophages were treated with different concentrations of compounds **1**-**12**, **15**, **17**, **18** and **21**-**23** (0.5-100 µM) in the presence of 1 µg/mL LPS for 24 h. The untreated group received an equal amount of DMSO, which resulted in a final concentration of 0.2% DMSO in the culture medium. After 24 h incubation, 0.5 mg/mL MTT was added to each well, and the cells were incubated for another 4 h at 37 ºC with 5% CO_2. The supernatant was discarded and 100 mL of DMSO was added to the cells to dissolve the formazan. The absorbance of each group was measured by using a microplate reader at 570 nm. The optical density of MTT-formazan formed in untreated cells was taken as 100% of viability.

Griess assay: Nitrite accumulation, an indicator of NO synthesis, was measured in culture media based on a diazotization reaction using the Griess reagent. Cells were seeded into a 96-well plate at the density of $5x10^4$ cells/mL. After incubation, RAW 264.7 cells were pretreated with various concentrations of compounds **1**-**12**, **15**, **17**, **18** and **21**-**23** (5-100 µM) with or without 1 µg/mL of LPS for 24 h. An aliquot (100 µL) of the supernatant was mixed with an equal volume of Griess reagent, and incubated at room temperature for 10 min, and then the absorbance at 540 nm was measured in a microplate reader. Fresh culture media were used as blanks in all experiments. Nitrite concentration was determined by a sodium nitrite standard curve.

Results and Discussion

Compounds isolated from *M. paniculata*

The crude extracts of the leaves from *M. paniculata* were separated using chromatographic techniques furnishing 26 compounds, including two compounds which were firstly reported in this plant: 2,6,2□,6□-tetramethoxy-4,4□-*bis*(1,2-*trans*-2,3-epoxy-1-hydroxypropyl)biphenyl (**25**) (Yan *et al*., 2016) and medioresinol (**26**) (Zhang *et al*., 2012). In addition, fifteen coumarins (**1**-**15**), seven flavones (**18**-**24**), and two cinnamates (**16**, **17**) were isolated. The structures of all compounds are displayed in Figure 1.

Antioxidant activity

Free radical-scavenging activities of 11 compounds (**1**, **2**, **5**, **7**-**12**, **15** and **18**) were evaluated in comparison with ascorbic acid using a DPPH assay. Based on this protocol, DPPH radicals react with proton donated substances leading to the change of purple of DPPH radicals to yellow. The change of the strong absorption band of DPPH

detected at 517 nm would directly relate to antioxidant activity. The results showed only compound **1** exhibited moderate antioxidant activities with IC_{50} 289 µg/ mL. The percentage of DPPH radical scavenging activity of separated compounds is presented in Table 1.

Antibacterial activity

Three major compounds (**10**, **11** and **15**) could be tested due to the availability of sample. The antibacterial activity of these three compounds was tested *in vitro* against *P. acnes*, *S. aureus*, *S. sobrinus*, *S. mutans*, and *Sa. typhi* and chloramphenicol was used as a positive control. The results are shown in Table 2. All three compounds exhibited weak activity against all bacteria with the inhibition zone about 7.00-9.00 mm.

Anti-inflammatory activity

Constituents of *M. paniculata* leaves (compounds **1-12**, **15**, **17**, **18** and **21-23**) were screened for cell viability by MTT assay and all compounds were further tested on NO production at concentration ranging of 0.5–100 µM. % Cell viability of the systems incubated with all compounds was about 60-120. With the range of 0.5-100 µM, all compounds revealed no cytotoxicity with RAW 264.7 cells (Figure 2). All compounds were thus selected for next study on the inhibition of NO production. Figure 3 indicated that all compounds did not inhibit NO production with 80-140 % nitric oxide.

Table 1. Antioxidant activity of some isolated compounds from leaves of *M. paniculata*

compound	% antioxidant at concentrations					IC_{50} (µg/mL)
	62.5 µg/mL	125 µg/mL	250 µg/mL	500 µg/mL	1000 µg/mL	
1	26.69 ± 1.48	37.45 ± 2.05	45.72 ± 1.02	69.63 ± 1.53	74.39 ± 0.71	289
2	12.98 ± 1.68	13.20 ± 2.46	9.58 ± 1.29	33.60 ± 2.73	14.22 ± 0.52	-
5	12.86 ± 3.97	13.31 ± 1.42	10.37 ± 2.90	35.75 ± 1.74	16.15 ± 1.92	-
7	13.99 ± 1.09	16.03 ± 2.08	14.33 ± 1.53	38.02 ± 0.86	27.14 ± 1.29	-
8	10.93 ± 1.71	11.16 ± 1.09	10.71 ± 1.56	11.27 ± 1.99	11.50 ± 1.74	-
9	13.88 ± 1.53	9.69 ± 0.34	11.61 ± 1.87	17.51 ± 0.59	17.17 ± 0.34	-
10	9.46 ± 5.73	12.07 ± 0.59	10.48 ± 2.39	13.43 ± 0.68	13.99 ± 0.71	-
11	14.22 ± 2.26	12.52 ± 2.05	13.99 ± 1.93	16.37 ± 1.37	17.96 ± 3.41	-
12	13.09 ± 2.90	10.59 ± 2.05	11.05 ± 1.89	12.75 ± 2.23	12.24 ± 4.57	-
15	12.18 ± 4.49	13.99 ± 0.39	17.39 ± 1.04	20.34 ± 0.98	24.42 ± 2.46	-
18	14.22 ± 1.09	11.95 ± 1.71	12.86 ± 1.04	13.54 ± 5.79	14.67 ± 0.52	-
Ascorbic acid	97	97	97	97	97	45

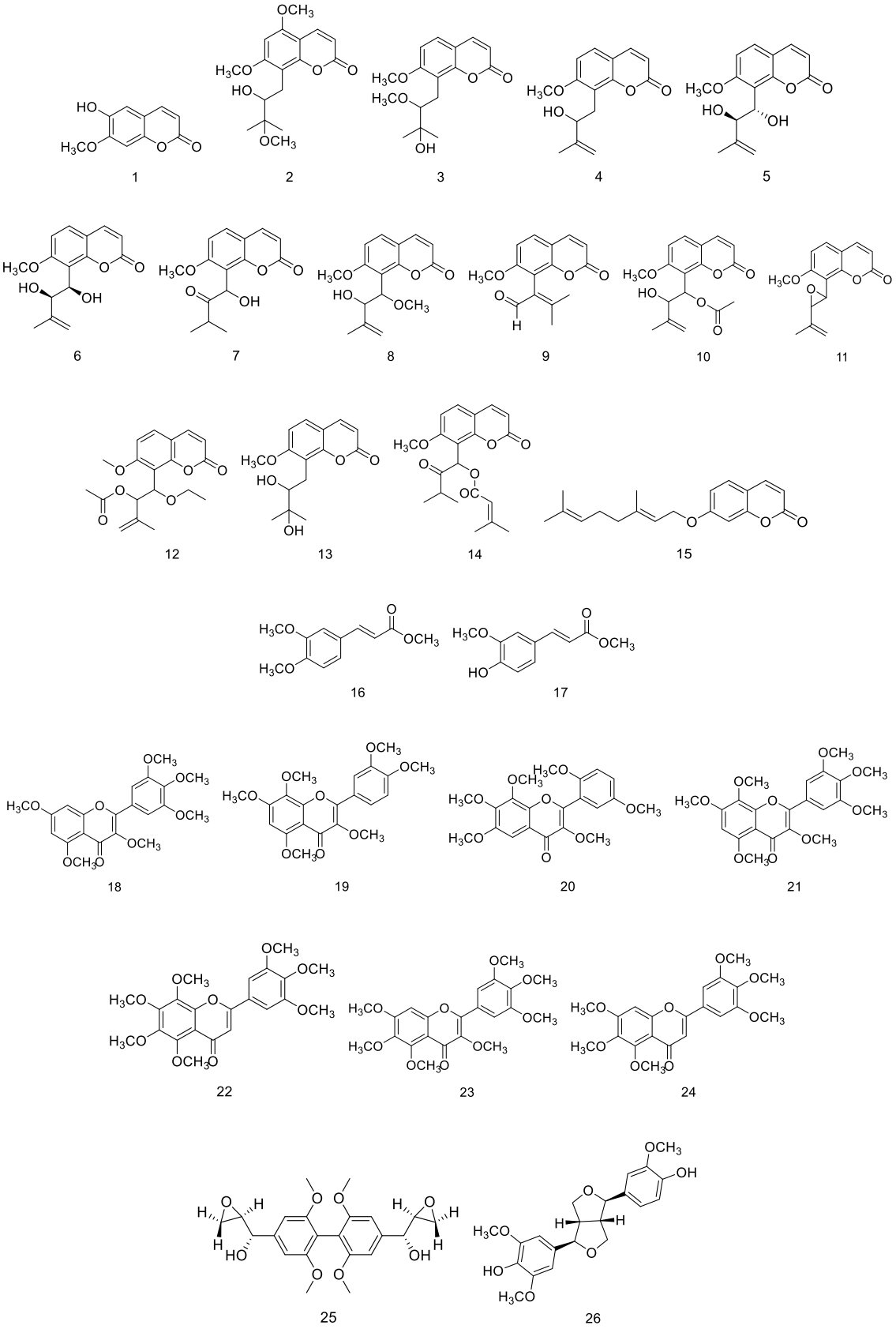

Figure 1. Chemical structures of compounds **1-26**

Table 2. Antibacterial activity of major compounds from the leaves of *M. paniculata*

compound	Inhibition zone average (mm) ± SD				
	P. acnes	*S. aureus*	*S. sobrinus*	*S. mutans*	*S. typhi*
10	7.00±0.00	7.33±0.47	9.00±0.00	7.67±0.47	7.67±0.47
11	7.33±0.47	8.67±0.00	8.00±0.00	7.67±0.47	7.67±0.47
15	7.00±0.00	7.33±0.47	7.00±0.00	7.67±0.47	8.00±0.82
chloramphenicol	23.00±0.00	21.00±0.00	18.00±0.00	19.00±.00	20.00±0.00
acetone	-	-	-	-	-

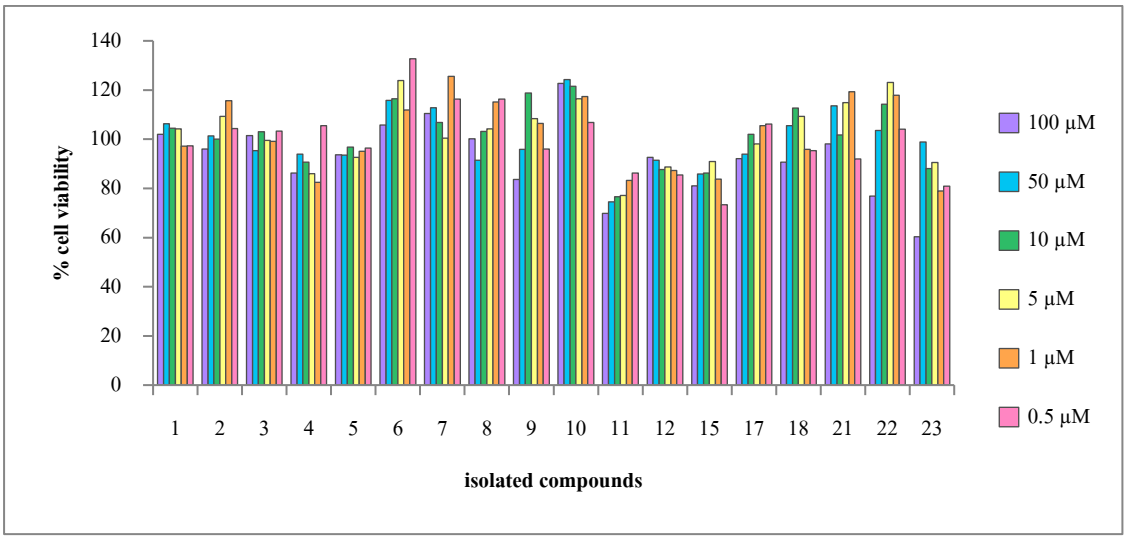

Figure 2. Effect of compounds **1-12**, **15**, **17**, **18** and **21-23** on the viability of RAW 264.7 cells by the MTT assay

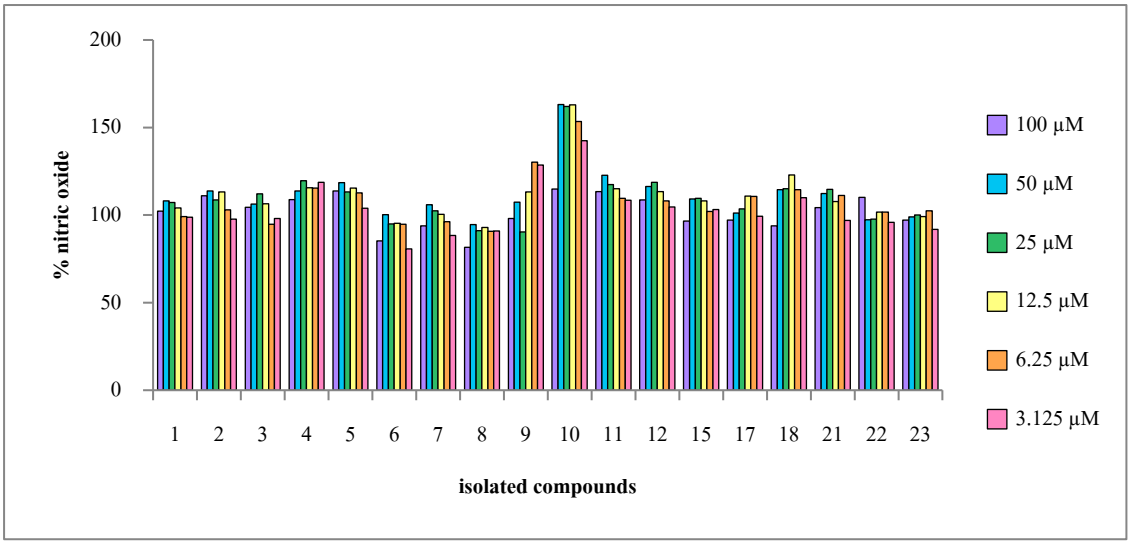

Figure 3. The inhibitory activity on NO production when treated with compounds **1-12**, **15**, **17**, **18** and **21-23**

Conclusions

Phebalosin (**11**), auraptene (**15**) and murrangatin acetate (**10**) are three major components from the leaves of *M. paniculata*. The separation of the MeOH extract by semi-prep HPLC yielded twenty-three known compounds with two firstly reported compounds in this plant. Some constituents were determined for antioxidant, antibacterial and anti-inflammatory activities. All three major compounds presented low activity against all bacteria. For anti-inflammatory, compounds **1**-**12**, **15**, **17**, **18** and **21**-**23** did not reveal cytotoxicity on Raw 264.7 cells and did not show inhibitory activity on NO production. In addition, compound **1** could scavenge DPPH radicals with IC_{50} 289 µg/mL.

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