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***Research paper***

# Isolation of Dihydrostilbenes from *Macaranga Heynei* and their Bioactivities

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## Abstract

*Macaranga* is known to contain abundant sources of prenylated flavonoids and stilbenoids which possessed broad spectrum of biological activities including anti-inflammatory, antimicrobial, antioxidant and cytotoxicity. These species are widely distributed in New Guinea, Borneo and from West Africa to the south Pacific islands. Although *Macaranga* comprises a large number of species, this genus has not been widely investigated which prompted us to conduct a study on *Macaranga heynei*. This research is conducted to isolate and characterise the compounds from *M. heynei* as well as to access the antibacterial and cytotoxicity of the isolates. The purification from the leaves of *M. heynei* has successfully yielded three dihydrostilbenes which were analysed by means of NMR, UV-Vis, FTIR, MS and comparison with the literature data. These compounds were characterised as laevifolins A **(1)** and B **(2)** as well as macarubiginosin C **(3)**. Laevifolin A **(1)** exhibited good activity against *S.cohnii subsp. urealyticum* and moderate activity against *S.aureus* ATCC 25923 with the $IC_{50}$ values of 11.65 and 27.13 □M respectively. It also displayed pronounced inhibition against HT-29 cells with an $IC_{50}$ value of 21.20 □M. Meanwhile, laevifolin B **(2)** displayed moderate activity on *S.cohnii subsp. urealyticum* but strong inhibition against *S.aureus* ATCC 25923 with the $IC_{50}$ values of 20.71 and 1.64 □M respectively.

***Keywords***: *antibacterial activity; cytotoxicity; dihydrostilbenes; Macaranga heynei*

## 1. Introduction

Tropical rainforests are of great importance to the humans as they are rich in biological diversity which produces many essential items such as timber, non-timber products, wild fruits as well as traditional remedies. Malaysia is blessed to be one of the countries that have one of the world's oldest rainforest with abundance of bio-resources and various plant families. Amongst the families found in the rainforests are Dipterocarpaceae and Euphorbiaceae [1]. *Macaranga* which belongs to the family of Euphorbiaceae are a large genus and comprises of 300 species [2]. This genus also played a role in traditional medicines, for example the stem water decoction of *M. denticulata* Muell. Arg. was used to wash wounds and as tonic for women after giving birth [3]. This genus is well-known as a rich source of prenylated flavonoids and stilbenoids which possessed broad spectrum of biological activities such anti-viral, antiplasmodial, antimicrobial and cytotoxicity [4,5,6,7].

Stilbenoids are phenolic compounds that were produced by plants in response to pathogen and fungi attack or other stresses. Thus, the content of this class of compounds in the plants increases with the stress exposure. Basically, stilbenoid consists of two aromatic rings which are connected by ethenyl bridge with various substituents. The presence of ethenyl bridge could be in the *cis*- and *trans*-form, nevertheless, the *trans*- isomer is more stable and biologically active. [8,9] On the other hand, the double bond could undergo hydrogenation to give dihydrostilbenoids which were also reported to be active in several bioactivities especially the prenylated dihydrostilbenoids. Although dihydrostilbenoids were not as abundance as the oligostilbenoids, these compounds exhibited potential antioxidant, anti-inflammatory and cytotoxic activities [10,11]. Based on several studies, the presence and the number of prenyl group in the compounds affect the bioactivities of stilbenoids [9,10,11].

*Macaranga heynei* or locally known as mahang biru can be abundantly found in Peninsular Malaysia and Thailand as well as Sumatra. This species can be found at open places and exposed subsoils [12]. The trees are up to 10 m tall with slender and blackish twigs. The leaves are finely ridged with the diameter of 1 mm and the petioles are 5 – 8 cm. The staminate flowers are in clusters with 0.2 mm diameter and 2 sepals whilst the fruits are tiny and in group of 3 or 4. *M. heynei* belongs to the *Javanica* group which consists of 13 species such as *M. costulata*, *M. cumingii*, *M. javanica*, *M. kinabaluensis* and *M. loheri* [13]. Amongst the species, only *M. javanica* has been phytochemically studied and was reported to contain prenylated dihydrostilbenes as well as dihydrophenanthrenes [14]. This genus consists a large number of species, however, only 10% have been phytochemically investi-

gated [15]. Hence, *M. heynei* was selected to develop the dihydrostilbene profile in *Macaranga* as well as to increase the diversity of the bioactive compounds. We herein report the isolation, elucidation as well as the bioactivities of the compounds.

## 2. Material and methods

### 2.1 Analysis

The elucidation on pure compounds (**1**), (**2**) and (**3**) were done by using infrared (IR), ultraviolet-visible (UV-Vis), one and two dimensional nuclear magnetic resonance (1D and 2D NMR) spectroscopy. The NMR analysis was done in acetone-$d_6$ and the chemical shift, δ was recorded in ppm.

### 2.2 Plant material

The leaves of *Macaranga heynei* were collected from Pulau Pangkor, Perak, Malaysia in December 2015 and identified by Dr. Shamsul Khamis. The voucher specimen SK2875/15 was deposited at Herbarium of Universiti Putra Malaysia.

### 2.3 Extraction and isolation

The dried leaves of *M. heynei* were ground and macerated in methanol at room temperature for 24 hours and repeated for three times. The methanolic crude extract (300 g) was dissolved in 80% methanol and further partitioned by hexane and ethyl acetate subsequently. The ethyl acetate crude extract (150 g) was fractionated by using vacuum liquid chromatography (VLC) with an eluent hexane-EtOAc in increasing polarity to give seven combined fractions (MH 1-7). 1 g of MH 2 was purified twice by radial chromatography (RC) with the solvent system hexane-EtOAc and $CHCl_3$-EtOAc to give **(1)** (48 mg) and **(2)** (60 mg). Compound **(3)** (20.1 mg) was obtained from the separation by VLC (twice), sephadex LH-20 gel column (MeOH), radial (twice) and column chromatography (CC) ($CHCl_3$-EtOAc).

### 2.4 Antibacterial

Minimal inhibitory concentration (MIC) was performed in accordance to Weigand and co-researchers [16]. Sterile Mueller Hinton Broth (MHB) (50µL) was loaded to each 96-well from column 2-10, 100µL in the sterility control wells (column 12) and 50µL in the growth control well (column 11). Compounds **(1-3)** (100µL) were pipetted into respective wells (column 1). Two-fold serial dilution was made and 50µL of sample was transferred from the first well to the next. 50µL of the mixed solution was pipetted out from the last well of each row out (column 10). The overnight grown microorganisms were adjusted to a standardized final $OD_{625nm}$ of approximately 0.08-0.10 to meet the 0.5 McFarland standard of 1 x $10^8$ CFU/ mL and mixed by using a vortex and diluted to 1:100 to give a working concentration 5 x $10^5$ CFU/ mL in each well. Adjusted bacterial inoculum (50µL) was dispensed into each well except the sterility control wells (column 12). The final volume in each well should be 100µL. The same serial dilution for positive and negative control wells of each plate were applied. Appropriate blank was included. The microtiter plates were incubated at 37 °C for 18-24 hours (bacterial strains). Resazurin (10µL of 1.1 mM) was added into the wells to give a final concentration of 0.1mM. The microtiter plates were re-incubated for at least 4 hours and analysed by a microplate reader at an $OD_{570nm}$ and $OD_{600nm}$. Results were recorded as positive (blue colour) or negative (pink colour).

### 2.5 Cytotoxicity

Sulforhodamine B (SRB) assay [17] was used to assess the cytotoxicity of the compounds given. Cell suspension (100 µL) were seeded into a 96-wells plate with a concentration of 1-5x$10^4$ and incubated (37°C, 5% $CO_2$) for 24 hours. Then, 100 µL of six concentrations (0.94-30 µg/mL) of serially diluted compounds were added in their respective wells. Hydrogen peroxide was used as the positive control, enriched media was used as the negative control and an enriched media added with DMSO was used as the vehicle control. The plate was incubated for 48 hours. Then, the cells were fixed with 50 µL of 50% cold (4°C) tricholoroacetic acid (TCA) and it was incubated on 4°C for 1 hour. The plate was washed with running tap water for 5 times and left to dry overnight. SRB stain (100 µL, 0.4%) dissolved in 1% acetic acid was added into each well and the plate was incubated in a room temperature for 30 minutes. The plate was then washed with 1% acetic acid for 4 times to remove excess unbounded stain and it was left to dry overnight. Tris base (pH ~ 10.5) (200 µL of 10 mM) were added into each well and the plate was shaken for 15 minutes. The plate was read with a maximum sensitivity OD of 564 nm. The graph of viability against concentration was plotted and $IC_{50}$ values was determined.

## 3. Results and discussion

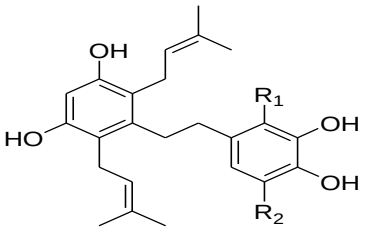
The isolation from the leaves of *M. heynei* has yielded three known dihydrostilbenes characterised as laevifolins A **(1)** and B **(2)** (Fig. 1) as well as macarubiginosin C **(3)** (Fig. 2). The earlier reports by our group have discussed on the elucidation of laevifolins A **(1)** and B **(2)** which both have similar skeletal structure [18,19]. We, therefore, will discuss the elucidation of macarubiginosin C **(3)** only.

The $^1$H NMR spectrum of compound **(3)** exhibited a pair of *meta*-coupled doublet signal at $\delta_H$ 6.63 and 6.53 as well as a singlet signal at $\delta_H$ 6.21 indicating the occurrence of two aromatic rings A and B. These rings were connected by two sets of methylene protons at $\delta_H$ 2.77 and 2.59 resembling the basic skeleton of dihydrostilbene. In addition, the presence of two prenyl groups were detected by the triplet signals at $\delta_H$ 5.35 and 5.15 representing methine protons, doublet signals of methylene protons at $\delta_H$ 3.36 (2H) and 3.33 (2H) as well as broad singlet at $\delta_H$ 1.77 (3H), 1.67 (3H) and 1.73 (6H) indicating methyl protons. This structure was close to that of laevifolin B **(2)**, however this compound was lacking of one prenyl group. There are 28 signals representing 29 carbons were observed in $^{13}$C-APT NMR spectrum which consists of four oxyaril carbons at $\delta_C$ 141 – 154 ppm indicating the presence of four hydroxyl groups in the structure. Seven quaternary carbons including aromatic and olefinic carbons were disclosed at the range of $\delta_C$ 110.4 – 139.6 ppm supporting the presence of two prenyl groups. Nonetheless, the signal at $\delta_C$ 72.5 showed the appearance of quaternary aliphatic carbon which suggesting this compound might consist of aliphatic cyclic in the structure. It was supported by the additional signal in $^1$H NMR spectrum in which two mutually coupled triplet signals at $\delta_H$ 2.67 and 1.79 representing two sets of methylene proton as well as one broad singlet signal of methyl protons at $\delta_H$ 1.27 (6H). The presence of an aliphatic ring was confirmed from the $^1$H-$^{13}$C HMBC (Fig. 3) correlation whereby the prenyl group in ring A has gone through cyclisation to form a dihydrodimethylpyran ring. Meanwhile, the rest of the signals at $\delta_C$ 16.9 – 35.5 ppm are the methylene carbons which connect both aromatic rings and dihydrodimethylpyran ring as well as methyl carbons from the prenyl groups. The $^1$H and $^{13}$C-APT NMR spectra were in good agreement with the literature data and was characterised as macarubiginosin C [20].

As for the biological activities, laevifolins A **(1)** and B **(2)** were reported for their antibacterial activity (*Bacillus subtilis*, *Enterobacter aerogenes*, *Escherichia coli*, *Pseudomonas*

*aeruginosa, Salmonella typhi, Shigella dysenteriae, Staphylococcus aureus*, and *Vibrio cholera*) and cytotoxicity of murine leukemia (P-388 cells). Meanwhile, macarubiginosin C **(3)** was only tested on P-388 cells [14,20].

In this study, the compounds were assayed on different bacteria and cancer cells. Laevifolin A **(1)** exhibited good activity against *S.cohnii subsp. urealyticum* with an $IC_{50}$ value of 11.65 □M and moderate activity against *S.aureus* ATCC 25923 with an $IC_{50}$ value of 27.13 □M. Conversely, laevifolin B **(2)** displayed moderate activity on *S.cohnii subsp. urealyticum* with an $IC_{50}$ value of 20.71 □M but showed strong inhibition against *S.aureus* ATCC 25923 with an $IC_{50}$ value of 1.64 □M. Meanwhile, macarubiginosin C **(3)** did not show any $IC_{50}$ on both strains. Although the structure of both laevifolins A **(1)** and B **(2)** were close to each other, opposite results were observed on different strains. It might be due to the 3D structure of those compounds whereby two prenyl groups at C-2 at ring A and C-2' at ring B of laevifolin A **(1)** were close to each other which caused steric hindrance in the middle of the skeleton (Fig. 4). Meanwhile, laevifolin B **(2)** was less hindered (Fig. 5). The antibacterial assay on laevifolins A **(1)** and B **(2)** on *S.aureus* (clinical isolate) have been reported before by Ilmiawati and co-workers [14] with MIC values 35 □M. As for cytotoxicity, laevifolin A **(1)** displayed pronounced inhibition on HT-29 cells with an $IC_{50}$ value of 21.20 □M but no $IC_{50}$ observed in laevifolin B **(2)**. The steric hindrance enhanced the activity due to the accessibility of laevifolin A **(1)** to exert its effect on the cancer cells [21]. Contrarily, laevifolin B **(2)** which can be considered as weak due to its loose structure, could not penetrate the cells. This is further supported by Tanjung and co-workers on P-388 cells in which laevifolin A **(1)** exhibited significant result with an $IC_{50}$ value of 4.3 ± 0.6 □M [20].




**(1)** $R_1$ = prenyl, $R_2$ = H
**(2)** $R_1$ = H, $R_2$ = prenyl

**Fig. 1**: Structures of laevifolins A **(1)** and B **(2)**

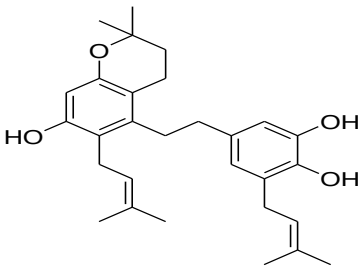



**Fig. 2:** Structure of macarubiginosin C **(3)**

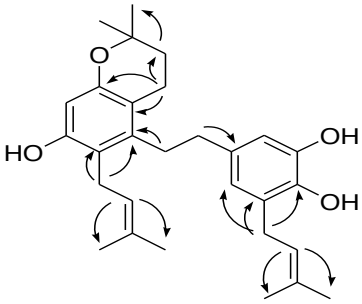



**Fig. 3:** Selected HMBC correlation ( ) of macarubiginosin C **(3)**

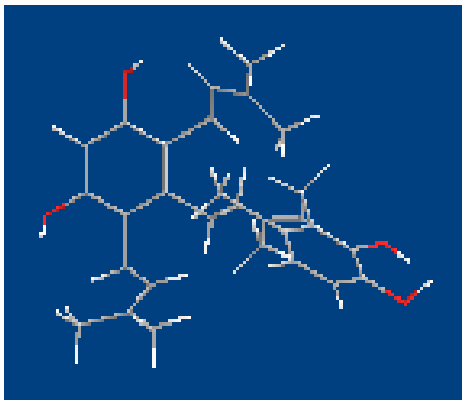


**Fig. 4**: 3D model of laevifolin A **(1)**

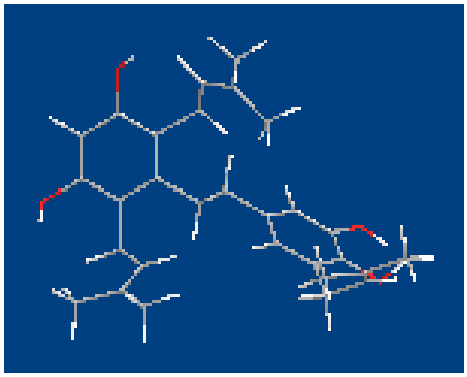


**Fig. 5**: 3D model of laevifolin B **(2)**

## 4. Conclusion

Phytochemical study of *M. heynei* led to the isolation and characterisation of three dihydrostilbenes, laevifolins A **(1)** and B **(2)** as well as macarubiginosin C **(3)**. Laevifolins A **(1)** and B **(2)** exhibited pronounced inhibition on *S. aureus* ($IC_{50}$ = 27.13 and 1.64 □M) and S. *cohnii subsp. urealyticum* ($IC_{50}$ = 11.65 and 20.71 □M) respectively. However, only laevifolin A **(1)** displayed inhibition against human colorectal adenocarcinoma (HT-29) cells with an $IC_{50}$ value of 21.2 □M. The current study is beneficial to seek the potential usage of this plant in antibacterial and cancer chemoprevention research.

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