Journal of Multidisciplinary Engineering Science and Technology (JMEST)
ISSN: 2458-9403
Vol. 11 Issue 7, July - 2024

# Extraction of chavicol derivatives and benzyl glycosides from *Piper betle* with their nitric oxide inhibitory activity

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***Abstract*—Two chavicol derivatives (1 and 2) and two benzyl glycosides (3 and 4) were isolated from the leaves and stems of *Piper betle* using separation and chromatographic methods. Chemical structures of these compounds were determined by extensive analysis of $^{1}$H-, $^{13}$C-, HSQC, and HMBC spectral data as well as comparison with those previously reported data in the literature. This is the first report on the isolation of neotaiwandimerol A (2) and (*S*)-1-phenylethyl *β*-D-glucopyranoside (4) from the *P. betle* plant. Both of compounds 2 and 4 significantly inhibited NO production in LPS-activated RAW 264.7 cells with $IC_{50}$ values of 47.1 and 12.4 µM, respectively.**

***Keywords*— *Piper betle; chavicol derivative; benzyl glycoside; isolation; nitric oxide inhibitor.***

## I. INTRODUCTION

*Piper betle* L. (Piperaceae family) is a perennial climbing plant and widely cultivated worldwide [1]. It has been used in traditional medicines for thousand of years in Asian countries such as China, India, and Vietnam [2]. The leaves are edible, having a strong aromatic flavor and pungent taste [2]. The leaves are also the most valued medicinal benefits which showed anti-inflammation and anti-microbial activities [1]. Phytochemical studies on *P. betle* received much attention over the past decades. Several types of compounds including alkaloids, phenolics, glycosides were found in this plant [3-8]. Of these, chavicol and its derivatives such as hydroxychavicol, eugenol, chavibitol are revealed to be major bioactive compounds [1]. In contribution to clarify anti-inflammatory constituents from *P. betle*, this paper describes the extraction and isolation of two chavicol derivatives, two benzyl glycosides from the leaves and stems of *P. betle*. The anti-inflammatory activity of the isolated compounds was evaluated by inhibiting nitric oxide production in LPS activated RAW 264.7 cells.

## II. MATERIALS AND METHODS

### A. General experiment procedures

The NMR spectra were measured on a Bruker AvanceNEO 600 MHz or a Bruker AvanceIII 500 MHz. Thin layer chromatography was carried out using precoated plates (Silica gel 60 $F_{254}$ and/or Silica gel 60 RP18 $F_{254S}$). Open column chromatography was performed using silica gel (particle size 40–63 µm) and ODS reversed phase resin (particle size 150 µm). Semi-preparative HPLC was acquired on an Agilent 1100 system including autosampler, quaternary pump, DAD detector, and equipped with YMC J'sphere ODS-H80 HPLC column (20×250 mm, particle size 4µm). Isocratic solvent system was used at a flow rate of 3 mL/min and detector acquisition was set at 210 and 254 nm. Organic solvents were used at technical grade, except for HPLC grade solvents used for semi-preparative HPLC.

### B. Plant materials

The leaves and stems of *Piper betle* were collected at Phu Tho province, Vietnam in January 2024. The fresh samples were air dried and pulverized into fine powder.

### C. Extraction and isolation

The dried and powdered plant samples (2.0 kg) were ultrasonically extracted with methanol three times (each time using 3 L methanol, extraction in 1 hour at room temperature). The methanol solutions were filtered and evaporated in vacuum to give dark solid extract (118 g). This crude extract was then well mixed with 1.5 L of distilled water and then successively separated with n-hexane and ethyl acetate. The ethyl acetate soluble fraction (31 g) was subjected on a silica gel column chromatography and then eluted with gradient solvent system of dichloromethane/methanol (0-100% volume of methanol) to give eight fractions, EA1–EA8. Fraction EA3 was repeatedly chromatographed on a silica gel column, eluting with n-hexane/acetone (4/1, v/v) to give three fractions, EA3A– EA3C. Fraction EA3B was further fractionated on a reversed phase RP18 column chromatography, eluting with acetone/water (2/1, v/v) to give two sub-fractions, EA3B1 and EA3B2. Subfraction EA3B1 was purified by semi-preparative HPLC using methanol/water (64% methanol in volume) to give compound **1** (18 mg). Fraction EA5 was subjected on a silica gel column chromatography and eluted with dichloromethane/methanol/water (7/1/0.1, v/v/v) to give five fractions, EA5A–EA5E. Fraction EA5B was first chromatographed on an RP18 column, eluting with methanol/water (1/2, v/v) and then further purified by semi-preparative HPLC using acetonitrile/water (35% acetonitrile in volume) to give compound **4** (12 mg). Fraction EA5C was separated on a silica gel column,

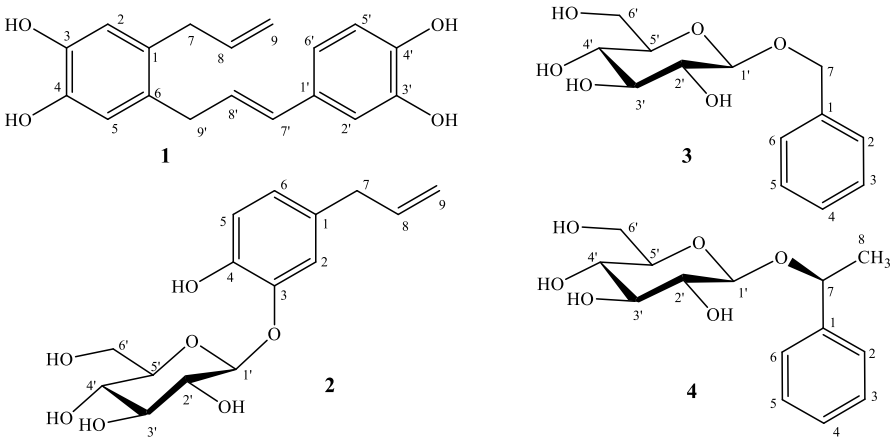

Fig. 1. *Chemical structure of chavicol derivatives and benzyl glycosides isolated from Piper betle*

eluting with dichloromethane/acetone (1/1, v/v) to give two sub-fractions, EA5C1 and EA5C2. Sub-fraction EA5C2 was purified by semi-preparative HPLC, running with acetonitrile/water (32% acetonitrile in volume) to give compound **3** (25 mg). Fraction E5D also load on a silica gel column chromatography and eluted with dichloromethane/acetone (1/1, v/v) to give three sub-fractions, E5D1– E5D3. Finally, sub-fraction E5D3 was purified by semi-preparative HPLC, running with acetonitrile/water (30/70, v/v) to give compound **2** (18 mg).

- Neotaiwandimerol A (**1**): Molecular formula: $C_{18}H_{18}O_4$; Pale yellow amorphous powder; $^1$H-NMR and $^{13}$C-NMR data are given in Table I.
- Hydroxychavicol 3-*O*-*β*-D-glucopyranoside (**2**): Molecular formula: $C_{15}H_{20}O_7$; White amorphous powder; $^1$H-NMR and $^{13}$C-NMR data are given in Table I.
- Benzyl alcohol *O*-*β*-D-glucopyranoside (**3**): Molecular formula: $C_{13}H_{18}O_6$; White amorphous powder; $^1$H-NMR and $^{13}$C-NMR data are given in Table II.
- (*S*)-1-Phenylethyl *β*-D-glucopyranoside (**4**): Molecular formula: $C_{14}H_{20}O_6$; Pale yellow amorphous powder; $^1$H-NMR and $^{13}$C-NMR data are given in Table II.

### *D. Nitric oxide assay*

Evaluation of inhibitory effects of isolated compounds on nitric oxide production in LPS-activated RAW 264.7 cells was performed as previous reports [9]. In brief, the cells were dispensed into a 96-well plate ($2\times10^5$ cells/well) and incubated at 37°C in a humidified atmosphere (5% CO2 and 95% air). After 24 h incubation, the culture medium was replaced with DMEM without FBS and incubated for an additional 3 h. The cells were treated with either compounds or vehicle solution and then stimulated with LPS (1 µg/mL) in the next 2 h. After an additional 24 h incubation, the cell culture medium (100 µL) was mixed with an equal volume of Griess reagent for 10 minutes and the absorbance was read at 540 nm. The amount of nitrite in the medium was calculated from a standard curve, which was constructed by $NaNO_2$ standard serial dilution. Dexamethasone was used as a positive control. Cell viability was determined by adding 10 µL MTT solution (5 mg/mL) and incubating for 4 h. Formazan crystals were dissolved in 50 µL of DMSO. Absorbance was read at 540 nm and compared with the vehicle group. Experiments were performed in triplicate and data are expressed as the Mean ± SD.

## III. Results and Discussion

The powdered leaves and stems of *P. betle* was extracted with methanol and then separated with *n*-hexane and ethyl acetate. The ethyl acetate extract was then fractionated and purified to give four compounds **1**–**4** (Fig. 1).

Compound **1** was isolated as a pale yellow amorphous powder. The $^1$H-NMR spectrum of **1** showed proton signals corresponding for an ABX aromatic proton coupled system [$\delta_H$ 6.82 (1H, d, *J* = 1.8 Hz) 6.69 (1H, d, *J* = 7.8 Hz) 6.66 (1H, dd, *J* = 1.8 and 7.8 Hz)], an AX aromatic proton coupled system [$\delta_H$ 6.61 (1H, s) and 6.56 (1H, s)], five olefinic protons [$\delta_H$ 6.18 (1H, d, *J* = 15.6 Hz), 6.07 (1H, dt, *J* = 15.6, 7.2 Hz), 5.94 (1H, m), 5.01 (1H, d, *J* = 11.2 Hz), 4.97 (1H, d, *J* = 16.4 Hz), ], and two methylene groups [$\delta_H$ 3.34 (2H, d, 7.2 Hz) and 3.28 (2H, d, *J* = 6.6 Hz)]. The $^{13}$C-NMR spectrum of **1** contained signals of 18 carbons including 16 $sp^2$ hybridized carbons ($\delta_C$ 113.6 to 146.2) and two saturated methylene carbons ($\delta_C$ 37.5 and 36.7). Based on the HSQC analysis, the above carbon signals were assigned into seven non-protonated carbons, eight olefinic methines, one olefinic methylene, and two saturated methylene groups. Above NMR data suggested that **1** to be a bis-phenylpropanoid. The HMBC correlations between $H_2$-9 ($\delta_H$ 5.01 and 4.97) and C-8 ($\delta_C$ 139.2)/ C-7 ($\delta_C$ 37.5) indicated the presence of prop-2-en-1-yl group. Then, the HMBC correlations between $H_2$-7 ($\delta_H$ 3.28) and C-1 ($\delta_C$ 130.4)/ C-2 ($\delta_C$ 117.8)/ C-6 ($\delta_C$ 131.0) demonstrated the prop-2-en-1-yl group linked to C-1 of an AX coupled benzene ring. The HMBC correlations between H-2 ($\delta_H$ 6.56) and C-3 ($\delta_C$ 144.4)/ C-4 ($\delta_C$

TABLE I. $^{1}$H- AND $^{13}$C-NMR SPECTROSCOPIC DATA OF COMPOUNDS **1** AND **2** IN $CD_3OD$

| No. | **1** | | **2** | |
|---|---|---|---|---|
| | [a]$\delta_C$ | [b]$\delta_H$ (mult., *J* in Hz) | [c]$\delta_C$ | [d]$\delta_H$ (mult., *J* in Hz) |
| 1 | 130.4 | - | 133.2 | - |
| 2 | 117.8 | 6.56 (s) | 119.3 | 7.05 (d, 2.0) |
| 3 | 144.4 | - | 146.6 | - |
| 4 | 144.4 | - | 146.6 | - |
| 5 | 117.9 | 6.61 (s) | 117.0 | 6.78 (d, 8.0) |
| 6 | 131.0 | - | 124.8 | 6.75 (dd, 8.0, 2.0) |
| 7 | 37.5 | 3.28 (d, 6.6) | 40.5 | 3.29 (d, 7.0) |
| 8 | 139.2 | 5.94 (m) | 139.2 | 5.96 (ddd, 16.0, 10.0, 6.5) |
| 9 | 115.3 | 4.97 (d, 16.4)<br>5.01 (d, 11.2) | 115.6 | 5.06 (dd, 16.0, 2.0)<br>5.02 (dd, 10.0, 2.0) |
| 1′ | 131.5 | - | 104.5 | 4.75 (d, 7.5) |
| 2′ | 113.6 | 6.82 (d, 1.8) | 74.9 | 3.44-3.51 (m) |
| 3′ | 146.2 | - | 78.3 | 3.44-3.51 (m) |
| 4′ | 145.7 | - | 71.3 | 3.44-3.51 (m) |
| 5′ | 116.3 | 6.69 (d, 7.8) | 77.7 | 3.44-3.51 (m) |
| 6′ | 119.3 | 6.66 (dd, 1.8, 7.8) | 62.4 | 3.90 (dd, 12.0, 2.0)<br>3.74 (dd, 12.0, 6.0) |
| 7′ | 131.5 | 6.18 (d, 15.6) | | |
| 8′ | 127.6 | 6.07 (dt, 15.6, 7.2) | | |
| 9′ | 36.7 | 3.34 (d, 7.2) | | |

Measured at [a]150 MHz, [b]600 MHz, [c]125 MHz, [d]500 MHz.

144.4) and deshielded-carbon signals of C-3 ($\delta_C$ 144.4) and C-4 ($\delta_C$ 144.4) indicated the presence of hydroxy groups at C-3 and C-4. Next, in an ABX-coupled benzene ring, the HMBC correlations between H-2′ ($\delta_H$ 6.82)/ H-6′ ($\delta_H$ 6.66) and C-4′ ($\delta_C$ 145.7), H-5′ ($\delta_H$ 6.69) and C-1′ ($\delta_C$ 131.5)/ C-3′ ($\delta_C$ 146.2), together with deshielded-carbon signals of C-3′ ($\delta_C$ 146.2) and C-4′ ($\delta_C$ 145.7) also indicated the presence of other hydroxy groups at C-3′ and C-4′. The HMBC correlations between H-2′ ($\delta_H$ 6.82)/ H-6′ ($\delta_H$ 6.66) and C-7′ indicated a double bond at C-7′/C-8′ and linkage between C-7′ and C-1′. Additionally, a coupling constant value between H-7′ and H-8′ (*J* = 15.6 Hz) confirmed the *E*-geometric configuration of double bond C-7′/C-8′. Finally, the HMBC correlations between $H_2$-9′ ($\delta_H$ 3.34) and C-7′ ($\delta_C$ 131.5)/ C-8′ ($\delta_C$ 127.6)/ C-1 ($\delta_C$ 130.4)/ C-5 ($\delta_C$ 117.9)/ C-6 ($\delta_C$ 131.0) established linkage between two phenylpropanoid moieties by C-9′/C-6. Therefore, the chemical structure of **1** was determined to be neotaiwandimerol A. This is a bis-phenylpropanoid and previously isolated from *Piper* species such as *P. taiwanense* and *P. hymenophyllum* [10, 11]. However, this is the first report on the isolation of this compound from *P. betle*.

Compound **2** was isolated as a white amorphous powder. The $^{1}$H-NMR spectrum of **2** showed signals including an ABX aromatic coupled protons [$\delta_H$ 7.05 (1H, d, *J* = 2.0 Hz), 6.78 (1H, d, *J* = 8.0 Hz), 6.75 (1H, dd, *J* = 8.0 and 2.0 Hz)], three olefinic protons [$\delta_H$ 5.96 (1H, ddd, *J* = 16.0, 10.0, and 6.5 Hz), 5.06 (1H, dd, *J* = 16.0 and 2.0 Hz), 5.02 (1H, dd, *J* = 10.0 and 2.0 Hz)], a methylene group [$\delta_H$ 3.29 (2H, d, *J* = 7.0 Hz)], an anomeric proton [$\delta_H$ 4.75 (1H, d, *J* = 7.5 Hz)], and other carbinol protons [$\delta_H$ 3.44–3.51 (4H, m), 3.90 (1H, dd, *J* = 12.0 and 2.0 Hz), 3.74 (1H, dd, *J* = 12.0 and 6.0 Hz)]. The $^{13}$C-NMR spectrum of **2** observed signals of 15 carbons. Of these, an anomeric carbon ($\delta_C$ 104.5) and five carbinol carbons ($\delta_C$ 78.3, 77.7, 74.9, 71.3, 62.4) suggested the presence of glucopyranosyl group. Additionally, a coupling constant value of anomeric proton *J* = 7.5 Hz indicated for *β*-glucopyranosyl linkage. Other nine carbons including eight $sp^2$ hybridized carbons and a saturated methylene group suggested for a phenylpropanoid backbone. The signals of three olefinic protons ($\delta_H$ 5.96, 5.06, and 5.02) were assigned for a vinyl group, indicating a double bond at C-8/C-9. Moreover, signals of ABX benzene ring and two deshielded carbons ($\delta_C$ 146.6 and 146.6) expected that aglycone of **2** to be hydroxychavicol. The sugar moiety, therefore, should be linked to aglycone by *O*-glycosidic linkage either at C-3 or C-4. Later, comparison of the $^{13}$C-NMR data of **2** with those reported in the literature confirmed the structure of **2** to be hydroxychavicol 3-*O*-*β*-D-glucopyranoside [12]. This compound was previously isolated from the leaves of *P. betle* [4].

The $^{1}$H-NMR spectrum of compound **3** revealed the presence of phenyl group by appearance of five aromatic proton signals [$\delta_H$ 7.44 (2H, d, *J* = 8.0 Hz), 7.34 (2H, t, *J* = 8.0 Hz), 7.27 (1H, t, *J* = 8.0 Hz)]. The $^{13}$C-NMR spectrum of **3** showed signal of 13 carbons including six carbons of glucopyranosyl group ($\delta_C$ 103.3, 78.1, 78.0, 75.1, 62.8), six carbons of a phenyl group ($\delta_C$ 139.1, 129.3×2C, 129.2×2C, and 128.7) and an oxygenated methylene ($\delta_C$ 71.7). Thus, the structure of **3** was established to be benzyl alcohol *O*-*β*-D-glucopyranoside. The NMR data of **3** consisted with those reported in the literature [13].

The $^{1}$H and $^{13}$C-NMR spectra of **4** showed closely similar with those of **3** by signals of a glucopyranosyl group and a phenyl group. The NMR spectral data of **4** exhibited signals of methyl group [$\delta_C$ 24.6 and $\delta_H$ 1.48

TABLE II. $^{1}$H- AND $^{13}$C-NMR SPECTROSCOPIC DATA OF COMPOUNDS **3** AND **4** IN $CD_3OD$

| No. | **3** | | **4** | |
|---|---|---|---|---|
| | [a]$\delta_C$ | [b]$\delta_H$ (mult., *J* in Hz) | [a]$\delta_C$ | [b]$\delta_H$ (mult., *J* in Hz) |
| 1 | 139.1 | - | 144.0 | - |
| 2 | 129.2 | 7.44 (d, 8.0) | 127.9 | 7.44 (d, 8.0) |
| 3 | 129.3 | 7.34 (t, 8.0) | 129.4 | 7.34 (t, 8.0) |
| 4 | 128.7 | 7.27 (t, 8.0) | 128.6 | 7.25 (t, 8.0) |
| 5 | 129.3 | 7.34 (t, 8.0) | 129.4 | 7.34 (t, 8.0) |
| 6 | 129.2 | 7.44 (d, 8.0) | 127.9 | 7.44 (d, 8.0) |
| 7 | 71.7 | 4.69 (d, 12.0)<br>4.95 (d, 12.0) | 76.0 | 5.07 (q, 7.5) |
| 8 | | | 24.6 | 1.48 (d, 7.5) |
| 1′ | 103.3 | 4.38 (d, 7.5) | 101.1 | 4.09 (d, 7.5) |
| 2′ | 75.1 | 3.36 (dd, 9.0, 7.5) | 75.2 | 3.25 (dd, 9.0, 7.5) |
| 3′ | 78.1 | 3.33 (t, 9.0) | 77.9 | 3.27 (t, 9.0) |
| 4′ | 71.7 | 3.35 (t, 9.0) | 71.8 | 3.26 (t, 9.0) |
| 5′ | 78.0 | 3.34 (m) | 78.0 | 3.11 (m) |
| 6′ | 62.8 | 3.71 (dd, 5.0, 11.5)<br>3.91 (dd, 2.0, 11.5) | 62.8 | 3.68 (dd, 5.5, 12.0)<br>3.89 (dd, 2.0, 12.0) |

Measured at [a]125 MHz and [b]500 MHz.

(3H, d, *J* = 7.5 Hz)] and oxygenated methine group [$\delta_C$ 76.0 and $\delta_H$ 5.07 (1H, q, *J* = 7.5 Hz)] instead of an oxygenated methylene group as in compound **3**. This evidence suggested aglycone of **4** to be 1-phenylethanol. The carbon chemical shift values of C-7 ($\delta_C$ 76.0), C-8 ($\delta_C$ 24.6), and C-1′ ($\delta_C$ 101.1) indicated for 7*S*-configuration which were similar with those previously reported data recorded in the same deuterated solvent $CD_3OD$ [reported for *S*-isomer: $\delta_{C-7}$ 76.0, $\delta_{C-8}$ 24.6, and $\delta_{C-1'}$ 101.1; and for *R*-isomer: $\delta_{C-7}$ 77.7, $\delta_{C-8}$ 22.3, and $\delta_{C-1'}$ 102.5] [14]. Consequently, compound **4** was determined to be (*S*)-1-phenylethyl *β*-D-glucopyranoside. Both abovementioned *S* and *R* form of **4** have been isolated from natural sources [14]. But this is also the first report on the isolation of this compound from *Piper* genus.

Compounds **1**–**4** were evaluated for their anti-inflammatory activity by inhibition of nitric oxide production in the LPS-activated RAW 264.7 cells. Firstly, the effects of these compounds on the RAW 264.7 cells were examined at concentration as high as 100 µM. Compounds **1**-**4** did not significantly show cytotoxic effects (percentage of cell viability over 95%). The inhibitory activity of the compounds on the NO production was then investigated at diluted concentrations in range of 0.2-100 µM. Compounds **1** and **2** significantly inhibited NO production with IC50 values of 47.1±1.6 and 12.4±0.7 µM, respectively. Compounds **3** and **4** were inactivity ($IC_{50}$ values over 100 µM)

In summary, two hydroxychavicol derivatives [ neotaiwandimerol A (**1**) and hydroxychavicol 3-*O*-*β*-D-glucopyranoside (**2**)] and two benzyl glycosides [ benzyl alcohol *O*-*β*-D-glucopyranoside (**3**) and (*S*)-1-phenylethyl *β*-D-glucopyranoside (**4**)] were isolated from the leaves and stems of *P. betle*. This is the first report on the isolation of compounds **1** and **4** from the species *P. betle*. Compounds **1** and **2** inhibited NO production in LPS activated RAW 264.7 cells with $IC_{50}$ values of 47.1 and 12.4 µM, respectively. The results consisted with previous reports that hydroxychavicol derivatives could be active ingredients of *P. betle* in the NO inhibitory activity.

## ACKNOWLEDGMENT

This research was supported by Hanoi University of Natural Resources and Environment under Grant number 13.01.24.H.01.

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