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# Isolation, characterization, and nitric oxide production inhibitory activities of the metabolites from *Psidium guava* L. in Viet Nam

**Ngo Van Hieu[1], Pham Van Cong[1], Ngo Viet Duc[1], Do Thi Thu Hien[1], Duong Thanh Nam[1], Le Van Nhan[1], Hoang Minh Tao[1], Vu Thi Trang[1], Nguyen Quang Trung[1], Nguyen Tien Dat[1], Pham Thi Mai[2], Hoang Le Tuan Anh[1, *]**

[1]*Center for Research and Technology Transfer, Vietnam Academy of Science and Technology (VAST), 18 Hoang Quoc Viet, Cau Giay, Ha Noi, Viet Nam*

[2]*Nha Trang University (NTU), 02 Nguyen Dinh Chieu, Nha Trang, Khanh Hoa, Viet Nam*

[*]Email: *hltanh@ctctt.vast.vn*

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**Abstract.** Thirteen compounds, including catechin (**1**), gallocatechin (**2**), quercetin (**3**), myricetin (**4**), kaempferol 3-*O*-*α*-L-arabinofuranoside (**5**), avicularin (**6**), guaijaverin (**7**), quercetin-3-*O*-*β*-D-galactopyranoside (**8**), quercetin-3-*O*-*β*-D-glucopyranoside (**9**), quercetin-3-*O*-*β*-D-glucuronide (**10**), casuarinin (**11**), ursolic acid (**12**), and 4-methoxy-3,5-dihydroxybenzoic acid (**13**) were isolated from the methanolic extract of the leaves *Psidium guajava* L. by using various chromatographic techniques. Moreover, the structures of these compounds were continuously elucidated by modern spectroscopic methods (MS, $^{1}$H/$^{13}$C-NMR) and compared with those of the reported data. In addition, all the isolated compounds were further evaluated for their inhibitory effects on nitric oxide (NO) production in LPS-induced RAW cells with $N^{G}$-Methyl-L-arginine acetate (L-NMMA) as the positive control. Among of them, compounds **3**, **8**, **10**, and **11** showed inhibitory effects on NO production within $IC_{50}$ values of 19.30 – 42.86 µM, compared to positive control: $IC_{50}$ 7.90 ± 0.63 µM. Interestingly, compound **13** was first isolated from *Psidium guajava* L.

*Keywords: Psidium guajava* L, NO production inhibitory activities, avicularin, guaijaverin, casuarinin, 4-methoxy-3,5-dihydroxybenzoic acid.

*Classification numbers:* 1.1.1, 1.1.6

## 1. INTRODUCTION

*Psidium guajava* L. is a fruit-bearing tree commonly known as guava, which belongs to Myrtaceae family. This plant is distributed in South America, European, Africa, and Asia [1]. According to traditional medicine, its leaves are used to treat diarrhea, dysentery, vomiting, and sore throats, and to regulate menstrual cycles [2]. Recent research indicated its crude extracts and purified compounds showed diverse activities such as anti-inflammatory, antimutagenic, anti-proliferative, antibacterial, and so on [3-6]. Phytochemical studies of *P. guajava* showed the

presence of a variety of chemical constituents such as flavonoids, terpenoids, and tannins [2, 6]. These traits make guava a potential source of antioxidants, which are consumed in functional food and nutraceuticals. Its essential oil was demonstrated to contain plenty of caryophyllene and its derivatives, which make this plant a source of anti-inflammatory agent [6]. In addition, its leaf and bark extract was used as a natural source with a potent anti-diabetic reagent which strongly inhibits α-glucosidase and α-amylase [6]. *P. guajava*, known as Oi, is frequently grown in Viet Nam as fruit trees. However, there are limited studies on this plant [7, 8]. Our study aims to find out active compounds to inhibit NO production. These results will provide scientific evidence on the chemical composition and biological activity of guava in Viet Nam.

Immunity is the human being's defense system to prevent infectious agents. Most malignancies' growth and malignant progression are correlated with inflammation. Targeting inflammation is a promising method for both cancer therapy and cancer prevention because most cell types implicated in cancer-associated inflammation are genetically stable and do not quickly develop drug resistance [9]. A previous study showed the ethanolic extract of *Psidium guajava* significantly inhibited LPS (lipopolysaccharide)-induced production of NO [10]. Traditional medicine also used this plant for the treatment of diseases such as anti-inflammation, diabetes, wounds, ulcers, and so on [10]. This study would like to point out the potential compounds as inflammatory agents. Herein, we displayed the isolation and structural identification of thirteen compounds including ten flavonoids (**1-10**), one tannin (**11**), one triterpenoid (**12**), and one derivative of benzoic acid (**13**) from the methanolic extract. Furthermore, all of them were evaluated the anti-inflammatory activity.

## 2. MATERIALS AND METHODS

### 2.1. General experimental procedures

$^{1}H/^{13}C$-NMR spectra were recorded on a Bruker Advance III 500 MHz NMR spectrometer (Switzerland), and a Bruker Advance Digital 600 MHz NMR spectrometer (Karlsruhe, Germany), with tetramethylsilane (TMS) as an internal standard, using solvents $CDCl_3$ (deuterated chloroform), $CD_3OD$ (deuterated methanol), and DMSO (deuterated dimethyl sulfoxide). High-resolution electrospray ionization mass spectrometry (HR-ESI-MS) data were measured by using the micrOTOF-Q III mass spectrometer (Bruker Daltonics, 255748 Germany). Column chromatography (CC) was utilized on silica-gel (Kieselgel 60, 230-400 mesh, Merck), RP-18 resin (150 µm, Fuji Silysia Chemical Ltd.), Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Thin-layer chromatography (TLC) (silica gel 60 $F_{254}$, 0.25 mm, Merck, Germany) was visualized under UV light (254 and 365 nm) and acid solution ($H_2SO_4$/vanillin), the dried powder was extracted by a multifunctional ultrasonic cleaner.

### 2.2. Plant materials

The leaves of *P. guajava* were collected from Dong Du town, Gia Lam district, Han Ni, Viet Nam in January 2022. 8 kg of the material was sun dried till the bulk was unaltered. This plant was identified by Dr. Do Thanh Tuan (Thai Binh University of Medicine and Pharmacy). Its specimen (PG2022) was deposited at Center for Research and Technology Transfer, VAST, Hanoi, Viet Nam.

### 2.3. Extraction and isolation

The dried powder of the leaves of *P. guajava* (1.5 kg) were extracted three times with methanol at a room temperature (2 h × 45 L) by using a multifunctional ultrasonic cleaner. Extracts were combined, filtered, and concentrated under reduced pressure to obtain a crude extract (PG 500 g). This crude extract was suspended in distilled water and successively partitioned with *n*-hexane, $CH_2Cl_2$, and EtOAc to give *n*-hexane (PGH, 250 g), $CH_2Cl_2$ (PGC, 45 g), EtOAc (PGE, 30 g) extracts, respectively, and water layer after removing solvents. Fraction PGE (30 g) was submitted to a silica gel column and eluted with gradient solvent of $CH_2Cl_2$/methanol (100/1-1/1, v/v) to get eleven fractions (PGE1-PGE11). Fraction PGE2 (50 mg) appeared as a pale orange crystal which was washed and crystallized with acetone to obtain compound **1** (8 mg). Fraction PGE4 (120 mg) was purified on a Sephadex LH-20 column and eluted with methanol to get compound **2** (5 mg) and two fractions (PGE4.1-PGE4.2). Fraction PGE4.2 (50 mg) was purified on a silica gel column using a solvent system of $CH_2Cl_2$/methanol (90/1, v/v) to yield compound **3** (6.3 mg). Fraction PGE5 (500 mg) was separated on a silica gel column with a solvent system of $CH_2Cl_2$/methanol (9/1, v/v) to afford five fractions (PGE5.1-PGE5.5). Compound **4** (8.1 mg) was obtained after eluting fraction PGE5.3 (80 mg) on a Sephadex LH-20 column with methanol. Fraction PGE5.4 (80 mg) was purified on a silica gel column with a solvent gradient of EtOAc/acetone/methanol (50/1/1 - 10/1/1, v/v/v) to afford compound **5** (7.3 mg). Fraction PGE8 (1 g) was separated on a silica gel column with an isocratic solvent system of EtOAc/acetone/methanol (70/1/1, v/v/v) to yield eight fractions (PGE8.1-PGE8.8). Fraction PGE8.3 (200 mg) appeared yellow crystals which were washed and recrystallized by acetone to obtain compound **6** (30 mg). Fraction PGE8.4 (63 mg) was further separated by using a Sephadex LH-20 column and eluting with methanol to afford compound **7** (6.3 mg). Fraction PGE10 (160 mg) was submitted to a silica gel column and eluted with a solvent system of EtOAc/acetone/methanol (60/1/1, v/v/v) to obtain compound **8** (7 mg), and three fractions (PGE10.1-PGE10.3). Compound **9** (8.3 mg) was afforded from fraction PGE10.2 (70 mg) by using an RP-18 column and eluting with a gradient solvent system of methanol/water (1/1-10/1, v/v). Fraction PGE11 (250 mg) was purified by a silica gel column with a solvent system of EtOAc/methanol/water/formic acid (50/1/1/1, v/v/v/v) to get compound **10** (15 mg), and four fractions (PGE11.1-PGE11.4). Compound **11** (13 mg) was isolated on a Sephadex LH-20 column and eluted with methanol from fraction PGE11.1 (40 mg). Fraction PGE11.2 (43 mg) was further purified on a silica gel column and eluted with a solvent system of EtOAc/methanol/water/formic acid (10/1/1/1, v/v/v/v) to get compound **12** (8 mg). With the same condition, compound **13** (6.5 mg) was isolated from fraction PGE11.3 (68 mg).

**Catechin (1):** pale orange crystals, *m/z* $[M-H]^-$ 289.0717, $^1$H-NMR (500 MHz, $CD_3OD$) $\delta_H$ (ppm): 4.58 (1H, d, $J$ = 7.5 Hz, H-2), 3.99 (1H, m, H-3), 2.53 (1H, dd, $J$ = 5.0, 16.0 Hz, H-4a), 2.87 (1H, dd, $J$ = 8.0, 16.0 Hz H-4b), 5.95 (1H, d, $J$ = 2.5 Hz, H-6), 5.88 (1H, d, $J$ = 2.5 Hz, H-8), 6.86 (1H, d, $J$ = 1.5 Hz, H-2'), 6.78 (1H, d, $J$ = 8.0 Hz, H-5'), and 6.73 (1H, dd, $J$ = 1.5, 8.0 Hz, H-6'). $^{13}$C-NMR (125 MHz, MeOD) $\delta_C$ (ppm): 82.9 (C-2), 68.8 (C-3), 28.5 (C-4), 157.9 (C-5), 96.4 (C-6), 157.6 (C-7), 95.6 (C-8), 156.9 (C-9), 100.9 (C-10), 132.3 (C-1'), 115.3 (C-2'), 146.3 (C-3'), 146.3 (C-4'), 116.1 (C-5'), and 120.0 (C-6').

**Gallocatechin (2):** pale brown powder, *m/z* $[M-H]^-$ 305.0667, $^1$H-NMR (600 MHz, $CD_3OD$) $\delta_H$ (ppm): 4.55 (1H, d, $J$ = 7.2 Hz, H-2), 3.99 (1H, m, H-3), 2.52 (1H, m, H-4a), 2.84 (1H, m, H-4b), 5.88 (1H, d, $J$ = 2.4 Hz, H-6), 5.94 (1H, d, $J$ = 2.4 Hz, H-8), and 6.42 (2H, s, H-2'/H-6'). $^{13}$C-NMR (150 MHz, MeOD) $\delta_C$ (ppm): 82.9 (C-2), 68.8 (C-3), 28.1 (C-4), 156.8 (C-5), 96.3 (C-6), 157.8 (C-7), 95.5 (C-8), 157.6 (C-9), 100.7 (C-10), 131.6 (C-1'), 107.2 (C-2'/C-6´), 146.9 (C-3'/C-5'), and 134.0 (C-4').

**Quercetin (3):** pale orange powder, *m/z* [M-H]$^-$ 301.0354, $^1$H-NMR (500 MHz, DMSO) $\delta_H$ (ppm): 6.20 (1H, s, H-6), 6.42 (1H, s, H-8), 7.69 (1H, s, H-2'), 6.87 (1H, d, *J* = 8.5 Hz, H-5'), and 7.55 (1H, d, *J* = 8.5 Hz, H-6'). $^{13}$C-NMR (125 MHz, DMSO) $\delta_C$ (ppm): 146.8 (C-2), 135.8 (C-3), 175.9 (C-4), 160.8 (C-5), 98.2 (C-6), 163.9 (C-7), 93.4 (C-8), 156.2 (C-9), 103.1 (C-10), 122.0 (C-1'), 115.1 (C-2'), 145.1 (C-3'), 147.6 (C-4'), 115.6 (C-5'), and 120.1 (C-6').

**Myricetin (4):** brown crystals, *m/z* [M-H]$^-$ 317.0303, $^1$H-NMR (600 MHz, $CD_3OD$) $\delta_H$ (ppm): 6.20 (1H, d, *J* =1.8 Hz, H-6), 6.40 (1H, d, *J* =1.8 Hz, H-8), and 7.36 (2H, s, H-2'/H-6'). $^{13}$C-NMR (150 MHz, MeOD) $\delta_C$ (ppm): 148.0 (C-2), 137.4 (C-3), 177.3 (C-4), 162.5 (C-5), 99.2 (C-6), 165.5 (C-7), 94.4 (C-8), 158.2 (C-9), 104.5 (C-10), 123.1 (C-1'), 108.5 (C-2'/C-6'), 146.7 (C-3'/C-5'), and 137.4 (C-4').

**Kaempferol-3-*O*-*α*-L-arabinofuranoside (5):** brown amorphous powder, *m/z* [M-H]$^-$ 417.0827, $^1$H-NMR (600 MHz, $CD_3OD$) $\delta_H$ (ppm): 6.23 (1H, d, *J* = 2.4 Hz, H-6), 6.43 (1H, d, *J* = 2.4 Hz, H-8), 7.98 (2H, d, *J* = 7.2 Hz, H-2', H-6'), 6.95 (2H, d, *J* = 7.2 Hz, H-3', H-5'), 5.49 (1H, s, H-1''), 4.34 (1H, t, *J* = 3.6 Hz, H-2''), 3.93 (1H, m, H-3''), 3.84 (1H, m, H-4''), 3.51 (1H, m, H-5''a), and 3.52 (1H, m, H-5''b). $^{13}$C-NMR (150 MHz, MeOD) $\delta_C$ (ppm): 158.6 (C-2), 135.0 (C-3), 179.4 (C-4), 163.1 (C-5), 100.0 (C-6), 166.3 (C-7), 94.9 (C-8), 159.4 (C-9), 109.8 (C-10), 122.7 (C-1'), 132.0 (C-2'/C-6'), 116.6 (C-3'/C-5'), 161.6 (C-4'), 109.7 (C-1''), 83.4 (C-2''), 73.3 (C-3''), 88.2 (C-4''), and 62.6 (C-5'').

**Avicularin** (**6):** yellow crystals, *m/z* [M-H]$^-$ 433.0779, $^1$H-NMR (500 MHz, DMSO) $\delta_H$ (ppm): 6.20 (1H, d, *J* = 2.0 Hz, H-6), 6.40 (1H, d, *J* = 2.0 Hz, H-8), 7.48 (1H, d, *J* = 2.0 Hz, H-2'), 6.84 (1H, d, *J* = 8.0 Hz, H-5'), 7.56 (1H, dd, *J* = 2.0, 8.0 Hz, H-6'), 5.58 (1H, s, H-1''), 4.15 (1H, m, H-2''), 3.72 (1H, m, H-3''), 3.56 (1H, m, H-4''), 3.26 (1H, m, H-5''a), and 3.28 (1H, m, H-5''b). $^{13}$C-NMR (125 MHz, DMSO) $\delta_C$ (ppm): 156.9 (C-2), 133.3 (C-3), 177.7 (C-4), 161.2 (C-5), 98.6 (C-6), 164.1 (C-7), 93.5 (C-8), 156.3 (C-9), 103.9 (C-10), 120.9 (C-1'), 115.5 (C-2'), 145.0 (C-3'), 148.4 (C-4'), 115.5 (C-5'), 121.6 (C-6'), 107.8 (C-1''), 82.1 (C-2''), 76.9 (C-3''), 85.8 (C-4''), and 60.6 (C-5'').

**Guaijaverin (7):** pale brown crystals, *m/z* [M-H]$^-$ 433.0776, $^1$H-NMR (600 MHz, $CD_3OD$) $\delta_H$ (ppm): 6.21 (1H, d; *J* = 2.4 Hz, H-6), 6.40 (1H, d; *J* = 2.4 Hz, H-8), 7.76 (1H, d; *J* = 2.4 Hz, H-2'), 6.88 (1H, d, *J* = 8.4 Hz, H-5'), 7.59 (1H, dd, *J* = 2.4, 8.4 Hz, H-6'), 5.17 (1H, d, *J* = 6.6 Hz, H-1''), 3.92 (1H, m, H-2''), 3.66 (1H, m, H-3''), 3.83 (1H, m, H-4''), 3.46 (1H, m, H-5a''), and 3.83 (1H, m, H-5b''). $^{13}$C-NMR (150 MHz, MeOD) $\delta_C$ (ppm): 158.7 (C-2), 135.7 (C-3), 179.4 (C-4), 163.0 (C-5), 99.9 (C-6), 165.9 (C-7), 94.7 (C-8), 158.4 (C-9), 105.6 (C-10), 123.0 (C-1'), 117.5 (C-2'), 145.9 (C-3'), 149.9 (C-4'), 116.2 (C-5'), 122.9 (C-6'), 104.7 (C-1''), 72.9 (C-2''), 74.1 (C-3''), 69.1 (C-4''), and 67.0 (C-5'').

**Quercetin-3-*O*-*β*-D-galactopyranoside (8):** pale brown oil, *m/z* [M-H]$^-$ 463.0882, $^1$H-NMR (500 MHz, DMSO) $\delta_H$ (ppm): 6.19 (1H, d, *J* = 1.8 Hz, H-6), 6.40 (1H, d, *J* = 1.8 Hz, H-8), 7.53 (1H, d, *J* = 2.4 Hz, H-2'), 6.81 (1H, d, *J* = 7.5 Hz, H-5'), 7.66 (1H, dd, *J* = 2.0, 7.5 Hz, H-6'), 5.36 (1H, d, *J* = 7.5 Hz, H-1''), 3.57 (1H, m, H-2''), 3.36 (1H, m, H-3''), 3.65 (1H, m, H-4''), 3.35 (1H, m, H-5b''), and 3.37 (1H, m, H-5b''). $^{13}$C-NMR (125 MHz, DMSO) $\delta_C$ (ppm): 156.3 (C-2), 133.4 (C-3), 177.4 (C-4), 161.2 (C-5), 98.7 (C-6), 164.3 (C-7), 93.5 (C-8), 156.2 (C-9), 103.8 (C-10), 121.0 (C-1'), 115.9 (C-2'), 144.8 (C-3'), 148.5 (C-4'), 115.2 (C-5'), 121.9 (C-6'), 101.8 (C-1''), 71.2 (C-2''), 73.2 (C-3''), 67.9 (C-4''), 75.8 (C-5''), and 60.1 (C-6'').

**Quercetin-3-*O*-*β*-D-glucopyranoside (9):** orange crystals, *m/z* [M-H]$^-$ 463.0881, $^1$H-NMR (600 MHz, DMSO) $\delta_H$ (ppm): 6.18 (1H, s, H-6), 6.40 (1H, s, H-8), 7.60 (1H, s, H-2'), 6.83 (1H, d, *J* = 8.4 Hz, H-5'), 7.54 (1H, d, *J* = 8.4 Hz, H-6'), 5.44 (1H, d, *J* = 7.8 Hz, H-1''), 3.34 (1H, m, H-2''), 3.26 (1H, m, H-3''), 3.56 (1H, m, H-4''), 3.22 (1H, m, H-5''), 3.08 (1H, m, H-6''a), and

3.12 (1H, m, H-6"b). $^{13}$C-NMR (150 MHz, DMSO) $\delta_C$ (ppm): 156.4 (C-2), 133.2 (C-3), 177.3 (C-4), 161.1 (C-5), 99.1 (C-6), 165.9 (C-7), 93.7 (C-8), 155.9 (C-9), 103.3 (C-10), 120.9 (C-1'), 115.4 (C-2'), 145.2 (C-3'), 149.0 (C-4'), 116.3 (C-5'), 121.3 (C-6'), 101.0 (C-1"), 74.1 (C-2"), 76.5 (C-3"), 69.9 (C-4"), 77.5 (C-5"), and 60.9 (C-6").

**Quercetin-3-*O*-*β*-D-glucuronide (10):** yellow crystals, *m/z* [M-H]$^-$ 477.0674, $^1$H-NMR (600 MHz, DMSO) $\delta_H$ (ppm): 6.18 (1H, d, *J* = 1.8 Hz, H-6), 6.38 (1H, d, *J* = 1.8 Hz, H-8), 8.24 (1H, d, *J* = 2.4 Hz, H-2'), 6.82 (1H, d, *J* = 8.4 Hz, H-5'), 7.36 (1H, dd, *J* = 2.4, 8.4 Hz, H-6'), 5.24 (1H, s, H-1"), 3.23 (1H, m, H-2"), 3.23 (1H, m, H-3"), 3.23 (1H, m, H-4"), and 3.35 (1H, m, H-5"). $^{13}$C-NMR (150 MHz, DMSO) $\delta_C$ (ppm): 157.5 (C-2), 133.9 (C-3), 177.5 (C-4), 160.9 (C-5), 98.9 (C-6), 164.7 (C-7), 93.7 (C-8), 156.5 (C-9), 103.6 (C-10), 120.7 (C-1'), 117.8 (C-2'), 148.4 (C-3'), 144.8 (C-4'), 115.4 (C-5'), 120.5 (C-6'), 102.8 (C-1"), 74.1 (C-2"), 74.2 (C-3"), 76.6 (C-4"), 74.1 (C-5"), and 172.0 (C-6").

**Casuarinin (11):** brown oil, *m/z* [M-H]$^-$ 935.0796, $^1$H-NMR (600 MHz, DMSO) $\delta_H$ (ppm): 6.95 (2H, s, H-2a/ H-6a), 6.57 (1H, s, H-6c), 6.29 (1H, s, H-6d), 6.23 (1H, s, H-6e), 5.38 (1H, d, *J* = 4.8 Hz, H-1), 4.54 (1H, m, H-2), 5.26 (1H, m, H-3), 5.28 (1H, d, *J* = 2.4 Hz, H-4), 5.21 (1H, m, H-5), 3.98 (1H, d, *J* = 13.0 Hz, H-6), and 4.58 (1H, d, *J* = 13.0 Hz, H-6). $^{13}$C-NMR (150 MHz, DMSO) $\delta_C$ (ppm): 69.6 (C-1), 75.1 (C-2), 68.5 (C-3), 72.8 (C-4), 65.7 (C-5), 63.5 (C-6), 103.4 (C-3c), 105.0 (C-3d), 106.5 (C-3e), 108.8 (C-2a/C-6a), 114.5 (C-1b), 115.4 (C-1c), 115.5 (C-1d/C-1e), 115.8 (C-3b), 116.5 (C-1a), 118.8 (C-2b), 122.9 (C-2c), 125.0 (C-2d), 125.8 (C-2e), 133.9 (C-5b), 134.7 (C-5c), 136.1 (C-5d), 137.7 (C-5e), 138.8 (C-4a), 142.6 (C-4b), 143.9(C-4c), 144.2 (C-4d), 144.68 (C-4e), 144.73 (C-6b), 144.8 (C-6c), 145.4 (C-6d/C-6e), 145.8 (C-3a/C-5a), 163.0 (C-7a), 164.4 (C-7b), 167.4 (C-7c), 167.9 (C-7d), and 168.4 (C-7e).

**Ursolic acid (12):** white amorphous powder, *m/z* [M-H]$^-$ 455.3531, $^1$H-NMR (500 MHz, $CD_3OD$+$CDCl_3$) $\delta_H$ (ppm): 1.62 (1H, m, H-1a), 1.41 (1H, m, H-1b), 1.60 (1H, m, H-2a), 1.80 (1H, m, H-2b), 3.16 (1H, m, H-3), 0.73 (1H, m, H-5), 1.59 (1H, m, H-6a), 1.68 (1H, m, H-6b), 1.36 (1H, m, H-7a), 1.60 (1H, m, H-7b), 1.51 (1H, m, H-9), 1.93 (1H, m, H-11a), 1.65 (1H, m, H-11b), 5.25 (1H, dd, *J* = 5.5, 12.5 Hz, H-12), 1.30 (1H, m, H-15a), 1.70 (1H, m, H-15b), 1.35 (1H, m, H-16), 2.01 (1H, m, H-18), 1.92 (1H, m, H-19), 1.03 (1H, m, H-20), 1.48 (1H, m, H-21a), 1.99 (1H, m, H-21b), 1.48 (1H, m, H-22a), 1.99 (1H, m, H-22b), 0.98 (3H, s, H-23), 0.96 (3H, s, H-24), 0.78 (3H, s, H-25), 0.65 (3H, s, H-26), 1.11 (3H, s, H-27), 0.87 (3H, s, H-29), and 0.94 (3H, s, H-30). $^{13}$C-NMR (125 MHz, $CD_3OD$+$CDCl_3$) $\delta_C$ (ppm): 39.8 (C-1), 27.7 (C-2), 79.5 (C-3), 39.6 (C-4), 56.5 (C-5), 19.3 (C-6), 34.1 (C-7), 40.4 (C-8), 48.6 (C-9), 37.9 (C-10), 24.2 (C-11), 126.6 (C-12), 139.3 (C-13), 43.1 (C-14), 29.0 (C-15), 25.1 (C-16), 48.6 (C-17), 54.1 (C-18), 40.1 (C-19), 40.2 (C-20), 31.6 (C-21), 37.9 (C-22), 28.7 (C-23), 16.3 (C-24), 16.0 (C-25), 17.6 (C-26), 24.1 (C-27), 180.4 (C-28), 17.7 (C-29), and 21.5 (C-30).

**4-Methoxy-3,5-dihydroxybenzoic acid (13):** orange oil, *m/z* [M-H]$^-$ 183.0299, $^1$H-NMR (600 MHz, $CD_3OD$) $\delta_H$ (ppm): 7.06 (2H, s, H-2, H-6), and 3.834 (3H, s, methoxy).

### 2.4. Nitric oxide (NO) production inhibition assay

The inhibition of NO production on the murine macrophage RAW264.7 cell was determined as described previously [11]. Briefly, RAW 264.7 cells ($1\times10^5$ cells/well) were seeded in a 96-well plate and stimulated with or without LPS (lipopolysaccharide) (1 μg/ml) (*Escherichia coli* 055:B5) in the presence or absence of samples at different concentrations for 24 h. Then, supernatants were reacted with Griess reagent (100 μL). The absorption at 540 nm was measured by a microplate reader. For this study, L-NMMA ($N^G$-Methyl-L-arginine acetate) was used as a positive control [11].

## 3. RESULTS AND DISCUSSION

Compound **1** was obtained as pale orange crystals, its molecular formula was determined as $C_{15}H_{14}O_6$ by the HR-ESI-MS spectrum at *m/z* 289.0717 $[M-H]^-$ (calcd for $C_{15}H_{13}O_6^-$, 289.0712). The 1D-NMR data predicted compound **1** as a flavan-3-ol type. The $^1$H-NMR indicated *meta*-coupling signals of A ring system at $\delta_H$ 5.95 (1H, d, *J* = 2.5 Hz, H-6), and 5.88 (1H, d, *J* = 2.5 Hz, H-8). Together with an ABX aromatic system in B ring including: 6.86 (1H, d, *J* = 1.5 Hz, H-2'), 6.78 (1H, d, *J* = 8.0 Hz, H-5'), and 6.73 (1H, dd, *J* = 1.5, 8.0 Hz, H-6'). Its DEPT-135 data indicated the presence of a characteristic methylene group at $\delta_C$ 28.5 which was assigned to carbon C-4 of C ring. From the above evidence, compared with those published catechin data [12], compound **1** was determined as catechin.

Compound **2** was possessed as a pale brown powder, its molecular formula was examined as $C_{15}H_{14}O_7$ by the HR-ESI-MS data at *m/z* 305.0667 $[M-H]^-$ (calcd for $C_{15}H_{13}O_7^-$, 305.0661). The NMR data is very similar to those of compound **1** except for the replacement of an ABX system in B ring of **1** by an AX system B ring in **2**. This was demonstrated by one couple protonic singlet type at $\delta_H$ 6.42 (2H, H-2'/H-6'). Additionally, the observation of an ion peak at *m/z* 305.0667 $[catechin + OH]^-$ which was fully reasonable to the presence of an added hydroxy group in **2**. This NMR data was compared to those reported spectrum of gallocatechin [13], and compound **2** was confirmed as gallocatechin.

Compound **3** was existed as a pale orange powder, its molecular formula was defined as $C_{15}H_{10}O_7$ by an ion peak at *m/z* 301.0354 $[M-H]^-$ (calcd for $C_{15}H_9O_7^-$, 301.0348). The NMR spectrum data suggested compound **3** as a flavonol. Its $^1$H-NMR indicated protonic signals including: *meta*-located protons at $\delta_H$ 6.20 (1H, s, H-6), and 6.42 (1H, s, H-8) has suggested 5,7-disubstituted at A ring. Along with the presence of an ABX aromatic system 7.69 (1H, s, H-2'), 6.87 (1H, d, *J* = 8.5 Hz, H-5'), and 7.55 (1H, d, *J* = 8.5 Hz, H-6') suggesting 3,4-disubstituted in B ring. Furthermore, the $^{13}$C-NMR spectrum demonstrated the presence of fifteen carbon signals at $\delta_C$ ppm including five methine carbons 98.2 (C-6), 93.4 (C-8), 115.1 (C-2'), 115.6 (C-5'), and 120.1 (C-6'), seven oxygenated quaternary carbon signals 146.8 (C-2), 135.8 (C-3), 160.8 (C-5), 163.9 (C-7), 156.2 (C-9), 145.1 (C-3'), and 147.6 (C-4'), two quaternary carbons 103.1 (C-10), and 122.0 (C-1'), one carbon ketone 175.9 (C-4). There was the similarity between the NMR data of compound **3** and those reported spectrum of quercetin [14]. Compound **3** was identified as quercetin.

Compound **4** was obtained as brown crystals, and its molecular formula was determined as $C_{15}H_{10}O_8$ by the existence an ion peak at *m/z* 317.0303 $[M-H]^-$ (calcd for 317.0297, $C_{15}H_9O_8^-$). Its NMR spectra were very similar to the NMR data of compound **3** except for the main difference in B ring. The observation of a couple protonic signal singlet type at $\delta_H$ 7.36 (2H, H-2'/H-6') suggests that 3,4,5-trisubstituted in B of **4**. Moreover, the HR-ESI-MS indicated an ion peak at *m/z* 317.0303 which was totally reasonable to chemical formula $C_{15}H_{20}O_8$, thus can identify the appearance of an added hydroxy group in **4**. The NMR data of compound **4** was completely similar to myricetin [15] by comparing with those published data, compound **4** was assigned as myricetin.

Compound **5** was possessed as brown amorphous powder, and its chemical formula was determined as $C_{20}H_{18}O_{10}$ based on an ion peak at *m/z* 417.0827 $[M-H]^-$ (calcd for $C_{20}H_{17}O_{10}^-$, 417.0822). The $^1$H-NMR revealed the AA'BB' aromatic system in B ring at $\delta_H$ including: *ortho*-coupling signals 7.98 (2H, d, *J* = 7.2 Hz, H-2', H-6'), 6.95 (2H, d, *J* = 7.2 Hz, H-3', H-5'), *meta*-coupling proton signals 6.23 (1H, d, *J* = 2.4 Hz, H-6), 6.43 (1H, d, *J* = 2.4 Hz, H-8) has suggested 5,7-disubstituted in A ring. The presence of an anomeric proton signal at 5.49 (1H, d,

*J* = 3.6 Hz, H-1") to assign a *C*-sugar unit which was determined as an arabinofuranoside unit by comparing with those published data. The $^{13}$C-NMR indicated twenty carbon signals, of which fifteen carbon signals were assigned to an aglycone and five carbon signals to a sugar unit. The aglycone unit was established by six methine carbons at $\delta_C$ ppm 100.0 (C-6), 94.9 (C-8), 132.0 (C-2'/C-6'), and 116.6 (C-3'/C-5'), six oxygenated quaternary carbons at 158.7 (C-2), 135.0 (C-3), 163.1 (C-5), 166.3 (C-7), 158.4 (C-9), and 161.6 (C-4'), two non-protonated carbon signals at 105.6 (C-10), and 123.0 (C-1'), along with one carbon ketone signal at 179.4 (C-4). Additionally, the typical carbon signals of a sugar unit (109.7, 83.4, 73.3, 88.2, and 62.6) were determined as an arabinofuranosyl moiety by comparing with those published data [16]. The NMR data of compound **5** was totally similar to kaempferol-3-*O*-*α*-L-arabinofuranoside, compound **5** was defined as kaempferol-3-*O*- *α*-L-arabinofuranoside.

Compound **6** was obtained as yellow crystals, and its molecular formula of $C_{20}H_{18}O_{11}$ was determined by HR-ESI-MS with an ion peak at *m/z* 433.0779 $[M-H]^-$ (calcd for $C_{20}H_{17}O_{11}^-$, 433.0771). Its NMR data were very similar to compound **3** except for the existence of an anomeric proton signal at $\delta_H$ 5.58 (1H, s, H-1") to assigned to a *C*-sugar unit. Moreover, the $^{13}$C-NMR showed carbon signals of a sugar moiety ($\delta_C$ 107.8, 82.1, 76.9, 85.8, 60.6) were typical to *C*-arabinofuranosyl moiety, together with the existence of fifteen carbon signals of an aglycone unit, of which five methine carbons 98.6 (C-6), 93.5 (C-8), 115.5 (C-2'), 115.5 (C-5'), and 121.6 (C-6'), seven oxygenated non-protonated carbons 156.9 (C-2), 133.3 (C-3), 161.2 (C-5), 164.1 (C-7), 156.3 (C-9), 145.0 (C-3'), and 148.4 (C-4'), one ketone carbon 177.7 (C-4), two quaternary carbons 103.9 (C-10), and 120.9 (C-1'). Based on the above evidence and comparison with those published data [17], compound **6** was determined as avicularin.

Compound **7** was acquired as pale brown crystals, and its chemical formula was determined as $C_{20}H_{18}O_{11}$ based on an ion peak at *m/z* 433.0776 $[M-H]^-$ (calcd. for $C_{20}H_{17}O_{11}^-$, 433.0771). The 1D-NMR of compound **7** was very similar to the nuclear magnetic resonance data of compound **3** except for the main difference is the chemical shift values of a sugar moiety at $\delta_H$ 5.17 (1H, d, *J* = 6.6 Hz, H-1"), along with carbon signals at $\delta_C$ 104.7, 72.9, 74.1, 69.1, and 67.0 to assign an arabinopyranosyl moiety by comparing to those published data [17]. From the above evidence, compound **7** was determined as guaijaverin.

Compound **8** was obtained as pale brown oil, and its molecular formula was defined as $C_{21}H_{20}O_{12}$ based on an ion peak *m/z* 463.0882 $[M-H]^-$ (calcd. for $C_{21}H_{19}O_{12}^-$, 463.0877). The $^1$H-, and $^{13}$C-NMR of compound **8** were very similar to the NMR data of compound **3** except for the chemical shift values of the sugar moiety for the bellow following evidence: an anomeric proton at $\delta_H$ 5.36 (1H, d, *J* = 7.5 Hz, H-1"), along with the appearance of the typical carbon signals ($\delta_C$ 101.8, 71.2, 73.2, 67.9, 75.8, 60.1) was very similar to a galactopyranosyl moiety, comparison with those published data [18]. Compound **8** was confirmed as quercetin-3-*O*-*β*-D-galactopyranoside.

Compound **9** was obtained as orange crystals, and its chemical formula was determined as $C_{21}H_{20}O_{12}$ based on an ion peak at *m/z* 463.0881 $[M-H]^-$ (calcd. for $C_{21}H_{19}O_{12}^-$, 463.0887). Its 1D-NMR was very similar to the nuclear magnetic resonance spectrum of compound **3** except for the main difference in chemical shift values of the sugar moiety. The $^1$H-NMR data indicated a coupling constant $J_{H\text{-}1",\ H\text{-}2"}$ = 7.8 Hz at $\delta_H$ 5.44 (1H, d, H-1"). Furthermore, the $^{13}$C-NMR showed characteristic carbon signals ($\delta_C$ 101.0, 74.1, 76.5, 69.9, 77.5, 60.9) which are totally reasonable to a glucopyranosyl unit by comparing with those published data [19]. From the above evidence, compound **9** was identified as quercetin-3-*O*-*β*-D-glucopyranoside.

Compound **10** was archived as yellow crystals, and its molecular formula was defined as $C_{21}H_{18}O_{13}$ based on an ion peak at *m/z* 477.0674 $[M-H]^-$ (calcd for $C_{21}H_{17}O_{13}^-$,477.0669). Its NMR spectrum data were very similar to compound **3** except for the main difference in the chemical shift values of the sugar region. The $^1$H-NMR revealed an anomeric protonic signal at $\delta_H$ 5.24 (1H, m, H-1") to assign a *C*-sugar, along with carbon signals ($\delta_C$ 102.8, 74.1, 74.2, 76.6, 74.1, 172.0) were confirmed as a glucuronosyl unit by comparing with those published data [19]. Compound **10** was identified as quercetin-3-*O*-*β*-D-glucuronine.

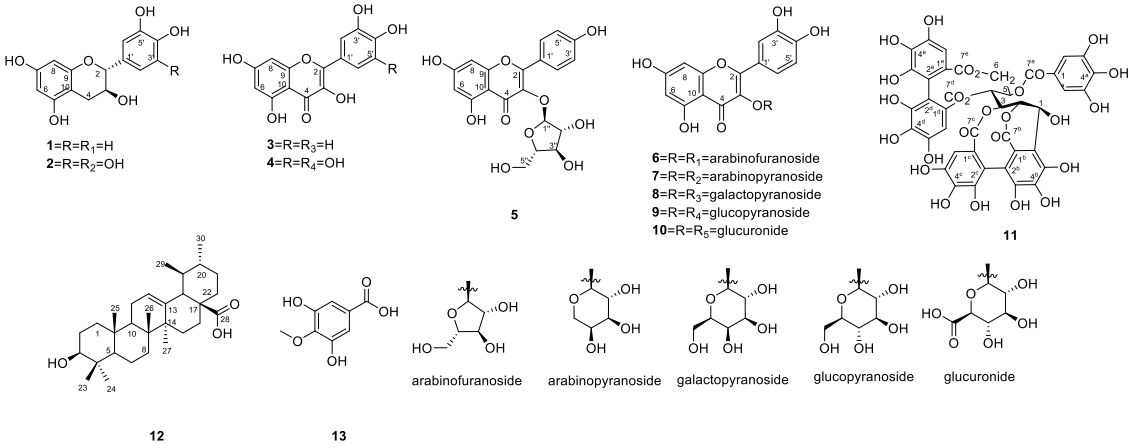


*Figure 1.* Chemical structures of compounds **1-13** isolated from *P. guajava.*

Compound **11** was archived as an amorphous, and its chemical formula was determined as $C_{41}H_{28}O_{26}$ based on an ion peak at *m/z* 935.0796 $[M-H]^-$ (calcd for $C_{41}H_{27}O_{26}^-$, 935.0791). Its $^1$H-NMR showed three singlet characteristic signals of aromatic ring at $\delta_H$ 6.57 (1H, H-6c), 6.29 (1H, H-6d), 6.23 (1H, s, H-6e), along with a couple doublet proton 6.95 (2H, s, H-2a/H-6a) suggesting a 3,4,5-trisubstituted form to assign to an AA' coupling system. Together with the carbohydrate residue at 5.38 (1H, d, $J$ = 4.8 Hz, H-1), 4.54 (1H, m, H-2), 5.26 (1H, m, H-3), 5.28 (1H, d, $J$ = 2.4 Hz, H-4), and 5.21 (1H, m, H-5). Furthermore, the $^{13}$C-NMR indicated carbon signals ($\delta_C$ 75.1, 68.5, 72.8, 65.7, 63.5) were very similar to a glucosyl moiety in **11**. These results and the existence of five ester groups illustrated by the $^{13}$C-NMR data indicate that the glucosyl moiety in **11** is an open-chain form. The *β*-configuration at C-1 was deduced by the coupling constant $J_{H\text{-}1,\ H\text{-}2}$ = 4.8 Hz in the $^1$H-NMR spectrum of compound **11**. The NMR spectrum data of compound **11** was totally similar to casuarinin by comparing with those published data [20], compound **11** was determined as casuarinin.

Compound **12** was obtained as a white amorphous powder, and its chemical formula was determined as $C_{30}H_{48}O_3$ based on an ion peak at *m/z* 455.3531 $[M-H]^-$ (calcd for $C_{30}H_{47}O_3^-$, 455.3525). The $^1$H-NMR displayed seven methyl groups at $\delta_H$ 0.98 (3H, s, H-23), 0.96 (3H, s, H-24), 0.78 (3H, s, H-25), 0.65 (3H, s, H-26), 1.11 (3H, s, H-27), 0.87 (3H, s, H-29), and 0.94 (3H, s, H-30), together with one olefinic proton signal 5.25 (1H, dd, $J$ = 5.5, 12.5 Hz, H-12). The $^{13}$C-NMR presented thirty carbon signals suggesting that **12** is a terpenoid type. The NMR data was compared to the reported spectral data of ursolic acid [21]. Thus, compound **12** was determined as ursolic acid.

Compound **13** was acquired as white needles, and its chemical formula was determined as $C_8H_8O_5$ based on an ion peak at *m/z* 183.0299 $[M-H]^-$ (calcd for $C_8H_7O_5^-$, 183.0293). The $^1$H-

NMR revealed proton signals of an aromatic ring at $\delta_H$ 7.06 (2H, s, H-2, H-6) together with a methoxy signal at $\delta_H$ 3.83 (3H, s, $OCH_3$). Comparison with the literature NMR spectral data of 4-methoxy-3,5-dihydroxybenzoic acid [22]. Compound **13** was identified as 4-methoxy-3,5-dihydroxybenzoic acid.

The inhibitory activities of NO production of compounds **1-13** were evaluated with $N^G$-Methyl-L-arginine acetate (L-NMMA) as the positive control. All isolated compounds were tested for the inhibition of NO production in LPS-induced RAW 264.7 cells. Compounds **3**, **8**, **10**, and **11** showed the inhibitory effects on NO production within $IC_{50}$ values of 19.30 – 42.86 µM (positive control $IC_{50}$ 7.90 ± 0.63 µM). The above evidence and previous studies [23-26] indicated that quercetin, its derivatives, and tannin are considered to take an important role in the anti-inflammatory activity of *P. guajava*. In general, it was discovered that flavones had more potent anti-inflammatory effects than flavanols, which suggests that the double bond at the C-2 and C-3 positions and the ketone group may be crucial for the inhibitory effects on NO production. Especially, we detected that the OH group at C-5' position in B ring of compound **4** dramatically affects to the anti-inflammatory activity, result to this compound did not show the $IC_{50}$ value compared with the structure of compound **3**. Moreover, we also acknowledged that the presence of sugar moieties influenced their NO production inhibitory activities. For instance, with three compounds **6**, **7**, **9**, have the substituted sugar at C-3 position as arabinofuranosyl, arabinopyranosyl, glucopyranosyl, respectively leading to these compounds having no anti-inflammatory activities.

*Table 1*. NO production inhibitory activities of compounds **3**, **8**, **10**, **11**.

| Compound | **3** | **8** | **10** | **11** | L -NMMA (µM) |
|---|---|---|---|---|---|
| $IC_{50}$ (µM) | 22.73 ± 2.38 | 42.86 ± 2.08 | 19.30 ± 1.87 | 38.63 ± 2.19 | 7.90 ± 0.63 |

## 4. CONCLUSION

In this report, we presented the isolation and structural elucidation of thirteen compounds from *Psidium guajava*, namely, catechin (**1**), gallocatechin (**2**), quercetin (**3**), myricetin (**4**), kaempferol 3-*O*-*α*-L-arabinofuranoside (**5**), avicularin (**6**), guaijaverin (**7**), quercetin-3-*O*-*β*-D-galactopyranoside (**8**), quercetin-3-*O*-*β*-D-glucopyranoside (**9**), quercetin-3-*O*-*β*-D-glucuronide (**10**), casuarinin (**11**), ursolic acid (**12**), and 4-methoxy-3,5-dihydroxybenzoic acid (**13**). Among the isolated compounds, compound **13** was first isolated from *P. guajava*. The results revealed the difference between the chemical composition of guava leaves in Viet Nam and other countries [27, 28]. For instance, in a study of Chinese author found seven compounds from the EtOAc extract including quercetin, avicularin, apigenin, guaijaverin, kaempferol, hyperin, and myricetin [27]. Whereas, our study pointed out more diverse chemical compositions from ethyl acetate extract such as triterpenoid, tanin. All of the compounds were examined for NO production inhibitory activity; compounds **3**, **8**, **10**, and **11** exhibited the inhibition effect on NO production within $IC_{50}$ values ranging from 19.30 - 42.86 µM (positive control: $IC_{50}$ 7.90 ± 0.63 µM) and less affect to normal cells (the percentage of viable cells from 97.39 – 100 % at concentration 20 µM). This is the first time the inhibition of nitric oxide production of the metabolites from *P. guajava* was investigated in Viet Nam.

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***Declaration of competing interest.*** All of the authors declare that this research has no competing financial interests.

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