Vietnam Journal of Chemistry, International Edition, **55**(5): 589-594, 2017
DOI: 10.15625/2525-2321.2017-00513

Isolation and identification of phenolic compounds from the leaf extract of *Cassia alata* L.

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Received 5 September 2017; Accepted for publication 20 October 2017

Abstract

Cassia alata is one of the most important species of the genus *Cassia* which is rich in anthraquinones and polyphenols. This plant is used as a medicinal material of which the leaves are known to have laxative and antibiotic properties. In our study, the methanol leaf extract of *C. alata* showed a significant antibacterial activity against human pathogenic bacteria strains *Staphylococcus aureus* and *Bacillus cereus*. The organic layers such as *n*-hexane, ethyl acetate, and aqueous layers, were prepared by partitioning the methanol extract with *n*-hexane and ethyl acetate successively. We successfully isolated and identified the structures of five compounds from *C. alata* leaves. Their structures were elucidated by MS and NMR spectroscopic methods as well as comparison with literature data. These compounds were determined to be methyl 2,4,6-trihydroxybenzoate (**1**), kaempferol (**2**), **(-)**epiafzelechin **(3)**, kaempferol-3-*O*-glucoside (**4**) and kaempferol-3-*O*-gentiobioside (**5**).

Keywords. *Cassia alata* L., epiafzelechin, kaempferol, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-gentiobioside.

1. INTRODUCTION

C. alata is an important medicinal plant, as well as an ornamental flowering plant in the family *Caesalpinioideae*. The plant is originated from South America, now grows in other tropical countries including Viet Nam. Various parts of this plant are used as a germicide, astringent, purgative, expectorant, and to treat skin diseases, tokelau, herpes circiné [1].

In 2003, the ethanol extract of leaves of *C. alata* was studied against cattle tick *Rhipicephalus annulatus* adult [2]. In 2012, Maikinde et al. researched antifungal and antimicrobial activities of aqueous and methanol extracts of the leaves of *C. alata* [3]. The research of Varghese et al. showed a test for anti-diabetic activity of extracts and components from *C. alata* leaves [4]. The extracts of these leaves were tested for antimicrobial, antimutagenic, antifungal, anti-inflammatory, analgesic and hypoglycaemic activities [5, 6]. In this study, our aim is to identify compounds from the leaves of *C. alata* growing in Vietnam.

2. MATERIALS AND METHODS

2.1. General experimental procedure

Isolation was performed using silica gel column (SiO_2, 60 Å, 15-40 µm and 40-63 µm (Merck, Germany)). Thin layer chromatography (TLC) was executed by silica gel 60 F_{254} 20×20 cm) purchased from Merck, Germany. Nuclear magnetic resonance (NMR) spectra were recorded using a Bruker AMX-500 (500 MHz) spectrometer (Bruker Analytische Messtechnik GmbH, Rheinstetten, Germany) operating at 500 MHz for ^{1}H-NMR and 125 MHz for ^{13}C-NMR, respectively. Results were recorded as follows: chemical shift values were expressed as units acquired in CD_3OD and $(CD_3)_2CO$ with

tetramethylsilane as an internal reference. MS spectra were performed on Mass Spectrometer LTQ Orbitrap XL^{TM} at HUS-VNU.

2.2. Plant materials

The leaves of *C. alata* were collected in Quang Thinh, Lang Giang district, Bac Giang province in October 2015 and in August 2016. Plant material was identified by MSc. Pham Hong Minh from National Institute of Medicinal Materials. The leaves were dried at temperature 50 °C to reach a moisture content of 10 %. The dried materials crushed to the size ranging from 0.3 to 0.5 cm. The plant specimen (Number of 136.16.ĐTKHCN) was stored at R&D Center of Bioactive Compounds, Vietnam Institute of Industrial Chemistry prior to extraction experiments.

2.3. Extraction and fractionation of constituents from the leaves of *C. alata*

The dried leaves of *C. alata* (11 kg) were extracted three times with 47 L of methanol (MeOH) at room temperature. The plant extract was filtered off using Whatman™ grade 8 filter papers. The solvent was evaporated under reduced pressure using a rotary evaporator at the temperature 50 °C to remove MeOH, giving 2.65 kg of methanol extract. The MeOH extract (2.3 kg) was suspended into 5 L of MeOH/distilled water (8/2) and partitioned three times with 5L of *n*-hexane (HEX). The resulting aqueous layer was evaporated to dry, re-suspended into 1L of distilled water and partitioned three times with 5L of ethyl acetate (EA). Each organic layer was pooled, washed with distilled water, and concentrated to yield 1.0 kg of HEX and 0.391 kg of EA layers.

The EA residue (50 g) was dissolved in 100 ml of MeOH and mixed with 100 g silica gel (40-63 μm). The mixture was fractionated using column chromatography with silica gel (500 g SiO_2, 40-63 μm, 7×30 cm) as a stationary phase and eluted using gradient systems of HEX : EA (95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:45, 60:40, 50:50, 40:60, 20:80, 0:100, 0:100, v/v) and EA:MeOH (98:2, 96:4, 94:6, 92:8, 90:10, 85:15, 80:20, 70:30, 60:40, 50:50, 40:60, 20:80, v/v). All fractions were run for TLC. Based on TLC profile, fractions with similar R_f values were pooled into seventeen fractions EA.1-EA.17.

Fraction EA.5 (0.97 g) was mixed with 2 g of silica gel 60 Å (15-40 μm) and applied on the top of a silica gel column (20 g SiO_2, 15-40 μm, 2 × 10 cm). The column was eluted with gradient solvent systems of HEX: EA (5:5, 4:6, 3:7, 2:8, 1:9, v/v) and finally washed out with MeOH to yield six subfractions EA.5.1-EA.5.6. Subfraction EA.5.2 was fractionated on a Sephadex LH-20 column (2 g SiO_2, 15-40 μm, 2.0×50 cm) which was eluted with MeOH to yield compound **1** (13 mg).

Fraction EA.10 (5.02 g) was dissolved in 50 ml MeOH, then mixed with 10 g SiO_2 (40-63 μm). The mixture was evaporated under reduced pressure using a rotary evaporator at the temperature 50 °C to remove MeOH and subjected on the top of a silica gel column (200 g SiO_2, 60Å, 40-63μm, 5×23 cm) using stepwise gradient elution with HEX : EA (95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:45, 60:40, 50:50, 40:60, 20:80 0:100, v/v) and EA: MeOH (95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:45, 60:40, 50:50, 40:60, 20:80 0:100, v/v) to yield 10 subfractions EA.10.1-EA.10.10. Subfraction EA.10.4 (0.37g) was chromatographed on a silica gel column (12 g SiO_2, 40-63 μm, 1.8×10 cm) and eluted with HEX: EA (80:20, 75:25, 70:30, 65:35, 60:40, 55:45, 50:50, 45:55, 40:60, 35:65, 30:70, v/v) to yield four subfractions EA.10.4.1-EA.10.4.4. Subfraction EA.10.4.2 was recrystallized from MeOH to give compound **2** (56 mg).

Fraction EA.11 (4.26 g) was loaded into a SiO_2 column chromatography (30 g SiO_2, 40-63 μm, 2×18 cm) and was eluted with an increasing gradient of EA up to 100 % (90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100, v/v) to afford six subfractions EA.11.1-EA.11.6. Subfraction EA.11.2 (0.51 g) was chromatographed on a silica gel column (15 g SiO_2, 40-63 μm, 1.8×12 cm), and eluted with HEX : EA (90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100, v/v) to yield two subfractions (EA.11.2.1 - EA.11.2.2). Subfraction EA.11.2.2 was further purified on a Sephadex LH-20 (2×30 cm) with MeOH to yield compound **3** (15 mg).

Fraction EA.16 (12.1 g) was subjected to silica gel column chromatography (240 g SiO_2, 40-63 μm, 5 × 32 cm), eluting with a gradient solvent system of 80-100 % HEX:EA (80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100, v/v) to obtain twenty seven subfractions EA.16.1-EA.16.27. Subfraction EA.16.4 (0.36 g) was purified by a silica gel column chromatography (7.2 g SiO_2, 60 Å (40-63 μm), 2 × 14 cm) eluting with HEX: EA 9:1 and then fractionated on a Sephadex LH-20 column (2×30 cm) eluting with MeOH to yield compound **4**. Subfraction EA.16.15 (1.23 g) was chromatographed on a silica gel column (33 g SiO_2, 60 Å, 40-63 μm, 2×21 cm) eluting by a gradient of HEX: EA (90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100, v/v) and EA : MeOH

(90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100, v/v) to yield eight subfractions (EA.16.15.1-EA.16.15.8). Subfraction EA.16.15.5 was passed through on Sephadex LH-20 (2×30 cm) with an elution of MeOH to yield compound **5**.

2.4 Structural characterization of compounds isolated from the leaf of *C. alata*

Compound **1** (methyl 2,4,6-trihydroxybenzoate)

MS: m/z 184.14 $[M^+]$ ($C_8H_8O_5$).

^{1}H-NMR (500 MHz, $(CD_3)_2CO$), δ (ppm)): 9.99 (2H, s, 2-OH, 6-OH), 9.93 (4-OH), 5.93 (2H, s, H-3, H-6), 4.04 (3H, brs, -CH_3).

^{13}C-NMR (125 MHz, $(CD_3)_2CO$), δ(ppm): 170.22 (-COO-), 162.90 (C-4), 164.75 (C-2, C-6), 95.42 (C-3, C-5), 93.23 (C-1), 52.05 (-CH_3).

Compound **2** (kaempferol)

ESI-MS: *m/z* 285.05 $[M-H]^-$ ($C_{15}H_9O_6$).

^{1}H-NMR (500 MHz, CD_3OD), δ (ppm): 8.08 (2H, d, *J* = 7.0 Hz, H-2', H-6',), 6.91 (2H, d, *J* = 7.0 Hz, H-3', H-5'), 6.40 (1H, d, *J* = 2.5 Hz, H-8), 6.18 (1H, d, *J* = 2.0 Hz, H-6).

^{13}C-NMR (125 MHz, CD_3OD), δ (ppm): 177.39 (C-4), 165.58 (C-7), 162.53 (C-5), 160.57 (C-4'), 158.28 (C-8a), 148.06 (C-2), 137.14 (C-3), 130.68 (C-2', C-6'), 123.74 (C-1'), 116.31 (C-3', C-5'), 104.56 (C-4a), 99.26 (C-6), 94.46 (C-8).

Compound **3** ((-) epiafzelechin)

ESI-MS: *m/z* 292.96 $[M+NH_4]^+$, *m/z* 273.23 $[M-H]^-$ ($C_{15}H_{13}O_5$).

^{1}H-NMR (500 MHz, $(CD_3)_2CO$), *δ* (ppm): 8.28 (1H, brs, 4'-OH), 8.16 (1H, brs, 5-OH), 7.99 (1H, brs, 7-OH), 7.38 (2H, d, *J* = 8.5 Hz, H-2', H-6'), 6.83 (2H, d, *J* = 8.5 Hz, H-3', H-5'), 6.04 (1H, d, *J* = 2.5 Hz, H-6), 5.94 (1H, d, *J* = 2.5 Hz, H-8), 4.95 (1H, s, H-2), 4.24 (1H, m, H-3), 3.65 (1H, d, *J* = 5.5 Hz, 3-OH), 2.89 (1H, dd, *J* = 16.5, 4.5 Hz, H-4β), 2.76 (1H, dd, *J* = 16.5, 3.0 Hz, H-4α).

^{13}C-NMR (125 MHz, $(CD_3)_2CO$), δ (ppm): 157.63 (C-4'), 157.62 (C-7), 157.57 (C-5), 157.19 (C-8a), 131.49 (C-1'), 129.13 (C-2', C-6'), 115.46 (C-3', C-5'), 99.74 (C-4a), 96.19 (C-6), 95.72 (C-8), 79.47 (C-2), 66.85 (C-3), 29.13 (C-4).

Compound **4** (kaempferol-3-*O*-glucoside)

ESI-MS: *m/z* 449 $[M+H]^+$ ($C_{21}H_{21}O_{11}$).

^{1}H-NMR (500 MHz, CD_3OD), δ (ppm): 8.07 (2H, d, *J* = 8.0 Hz, H-2', H-6'), 6.90 (2H, d, *J* = 8.0 Hz, H-3' and H-5'), 6.42 (1H, d, *J* = 2.0 Hz, H-8), 6.26 (1H, d, *J* = 2.0 Hz, H-6), 5.27 (1H, d, *J* = 7.5 Hz, H-1''), 3.70 (1H, dd, *J* = 12.0, 2.0, H-6"$_a$), 3.52 (1H, dd, *J* = 12.0, 2.0, H-6"$_b$), 3.44 (1H, m, H-2"), 3.41 (1H, m, H-3"), 3.20 (1H, m, H-5").

^{13}C-NMR (125 MHz, CD_3OD), δ (ppm): 179.53 (C-4), 166.26 (C-7), 163.11 (C-5), 161.59 (C-4'), 159.07 (C-8a), 158.56 (C-2), 135.47 (C-3), 132.28 (C-2', C-6'), 122.83 (C-1'), 116.32 (C-3', C-5'), 105.70 (C-4a), 104.11 (C-1"), 99.97 (C-6), 94.80 (C-8), 78.44 (C-5"), 78.07 (C-3"), 75.75 (C-2"), 71.38 (C-4"), 62.65 (C-6").

Compound **5** (kaempferol-3-*O*- gentiobioside)

ESI-MS: *m/z* 611.16 $[M+H]^+$ ($C_{27}H_{31}O_{16}$).

^{1}H-NMR (500 MHz, CD_3OD), δ (ppm): 8.11 (2H, d, *J* = 8.5 Hz, H2', H-6'), 6.90 (1H, d, *J* = 8.5 Hz, H-3', H-5'), 6.42 (1H, d, *J* = 2.0 Hz, H-8), 6.21 (1H, d, *J* = 2.0 Hz, H-6), 5.25 (1H, d, *J* = 7.4, H-1"), 4.16 (1H, d, *J* = 7.5 Hz, H-1"'), 3.96 (1H, dd, *J* = 12, 1.5 Hz, H-6"$_b$), 3.65 (dd *J* = 12.0, 7.0, H-6"$_a$) 3.74 (1H, dd *J* = 12.0, 2.0, H-6"'$_b$), 3.58 (dd *J* =, 12.0, 5.5, H-6"'$_a$), 3.49-3.37 (4H, m, H-2", H-3", H-4", H-5"), 3.25 (1H, dd, *J* = 9.0, 9.0 Hz, H-4"'), 3.20 (1H, t, *J* = 9.0 Hz, H-3"'), 3.09 (1H, dd, *J* = 8.5, 8.0 Hz, H-2"'), 3.04 (1H , ddd, *J* = 9.0, 5.5, 2.5, H-5"').

^{13}C-NMR (125 MHz, CD_3OD), δ (ppm): 179.41 (C-4), 166.204 (C-7), 161.56 (C-5), 161.56 (C-4'), 158.55 (C-8a), 158.99 (C-2),135.522 (C-3), 132.37 (C-2', C-6'), 122.70 (C-1'), 116.23 (C-3', C-5'), 105.75 (C-4a), 104.56 (C-1"'), 104.08 (C-1"), 100.05 (C-6), 94.93 (C-8), 77.93 (C-5"), 77.81 (C-3"'), 77.78 (C-3"), 77.64 (C-5"'), 75.74 (C-2"'), 75.08 (C-2"), 71.25 (C-4"), 71.35 (C-4"'), 69.53 (C-6"), 62.54 (C-6"').

3. RESULTS AND DISCUSSION

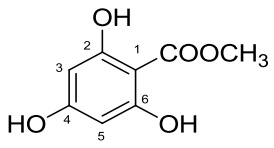
^{1}H-NMR spectral data of the compound **1** showed one methyl group (4.04, s, 3H), two independent aromatic rings at 5.93 ppm (2H, s, H-3 and H-6), and three hydroxyl groups connected to the aromatic ring at C-2, C-4, C-6 positions and appeared at δ 9.99 (2H, s, 2-OH, 6-OH), δ 9.93 (1H, s, 4-OH). The ^{13}C-NMR spectrum of compound **1** displayed that it has eight carbons including six carbons of the phenolic group in a range of δ 93.23-164.75 ppm, one ester group (170.22 ppm), one methyl group connected with ester group at 52.05 ppm. From all these data, compound **1** was identified as methyl 2,4,6-trihydroxybenzoate.

Compound **2** was obtained as yellow crystalline solid. The ^{1}H-NMR and ^{13}C-NMR spectra of compound **2** showed two aromatic rings. Two signals at δ 6.40 ppm (1H, d, *J* = 2.5 Hz, H-8) and δ 6.18 ppm (1H, d, *J* = 2.0 Hz, H-6) showed two doublet protons. There was also the presence of a carbonyl group, which was shown by ^{13}C-NMR chemical shift at δ 177.39 ppm. Four signals at δ 158.28 (C-8a), 148.06 (C-2), 123.74 (C-1'), and

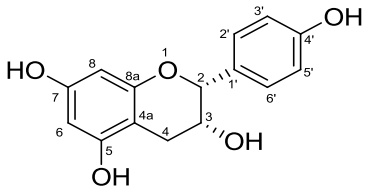
104.56 (C-4a) belong to four aromatic quaternary carbons. The signals at 165.58 (C-7), 162.53 (C-5), 160.57 (C-4'), 137.14 (C-3) are specific to four aromatic carbons connected to hydroxyl groups. By comparing NMR data with those previously reported, **2** was identical to kaempferol [7, 8].

The molecular formula of compound **3** was assumed to be $C_{15}H_{14}O_5$ based on ^{1}H-NMR and ^{13}C-NMR spectra and ESI-MS data. The *m/z* value of the isolated compound, according to the MS data in ESI negative ion mode was found at *m/z* 273.23. The ^{1}H-NMR spectra of compound **3** showed four signals of six aromatic carbons at δ 7.38 (2H, d, *J* = 8.5 Hz, H-2', H-6'), 6.83 (2H, d, *J* = 8.5 Hz, H-3', H-5'), 6.04 (1H, d, *J* = 2.5 Hz, H-6), 5.94 (1H, d, *J* = 2.5 Hz, H-8). The coupling constant between H-2 and H-3 was negligible, so the peak of H-2 at δ 4.95 ppm appears as a singlet. This confirmed that H-2 and H-3 are in a *cis* relationship. In the ^{13}C-NMR spectrum of compound **3,** revealed the presence of fifteen different carbon atoms. Three signals at δ 157.63 (C-4'), 157.62 (C-7), and 57.57 (C-5) are specific to three aromatic carbons connecting to hydroxyl groups. According to these data interpretation and compared with those previously reported, compound **3** was known as (-) epiafzelechin [9, 10].

The ^{1}H-NMR and ^{13}C-NMR spectra characterized compound **4** as a flavonoid. The ^{1}H-NMR spectra showed two doublets of aromatic protons (δ (ppm): 6.42 (1H, d, *J* = 2.0 Hz, H-8), 6.26 (1H, d, *J* = 2.0 Hz, H-6) four symmetric-coupled aromatic protons (δ (ppm): 8.07 (2H, d, *J* = 8.0 Hz, H-2', H-6'), 6.90 (2H, d, *J* = 8.0 Hz, H-3', H-5'). The ^{13}C-NMR spectrum displayed six aromatic methine carbons at δ 132.28 (C-2', C-6'), 116.32 (C-3', C-5'), 99.97 (C-6), 94.80 (C-8), eight aromatic quaternary carbons at δ 166.26 (C-7), 163.11 (C-5), 161.59 (C-4'), 159.07 (C-8a), 158.56 (C-2), 135.47 (C-3), 122.83 (C-1'), 105.70 (C-4a), and one carbonyl carbon at δ 179.53 (C-4). The molecular formula was established as a glucose group connected to flavonoid which was supported by ^{1}H-NMR and ^{13}C-NMR spectra. It showed that six carbons of a glucose group at δ 104.11 (C-1''), 78.44 (C-5''), 78.07 (C-3''), 75.75 (C-2''), 71.38 (C-4''), 62.65 (C-6''). The compound **4** is identified as kaempferol-3-*O*-glucoside [11].



1



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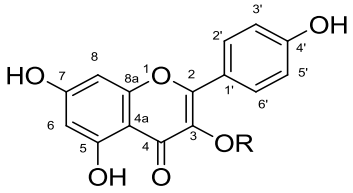

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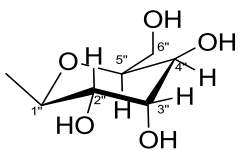
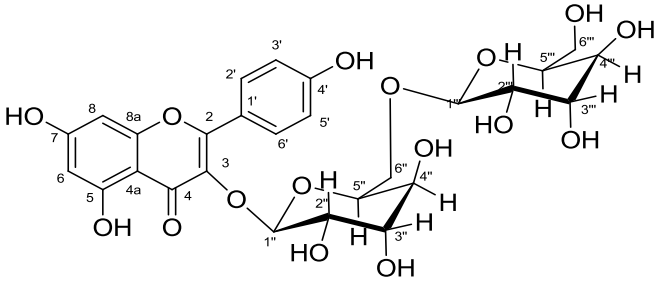
4 R =





5

The ^{1}H- and ^{13}C-NMR spectra of **5** expressed a very similar pattern to those of compound **4**, except for additional signals due to a glucopyranosyl moiety. The ^{13}C-NMR spectrum of **5** displayed 27 carbon signals, at which the signal at δ 69.53 was assigned to the C-6 of the 3-*O*-β-D-glucosyl moiety to which an additional glucose was attached. The aglycone moiety of compound **5** was deduced to be kaempferol based on aglycone carbon signals at δ 179.41 (C-4), 166.20 (C-7), 161.56 (C-5), 161.56 (C-4'), 158.99 (C-2), 158.55 (C-8a), 135.52 (C-3), 132.37 (C-2', C-6'), 122.70 (C-1'), 116.23 (C-3', C-5'), 105.75 (C-4a), 100.05 (C-6), 94.93 (C-8) in ^{13}C-NMR spectrum and signals of aromatic protons as two doublet signals for H-6 and H-8 at δ 6.21 (1H, d, *J* = 2.0 Hz) and 6.42 (1H, d, *J* = 2.0 Hz) together

with two doublets at δ 6.99 (2H, d, *J* = 8.5 Hz, H-3', H-5'), 8.11 (2H, d, *J* = 8.5 Hz, H-2', H-6') in ^{1}H-NMR spectrum. Based on ^{1}H- and ^{13}C-NMR data and compared with the data given in references, compound **5** was established as kaempferol-3-*O*-gentiobioside [12, 13].

In our study, the MeOH leaf extract of *C. alata* exhibited a significant antibacterial activity against human pathogenic bacteria strains *S. aureus* and *B. cereus* with the same MIC value of 63 µg/ml (data not shown). The phytochemical constituents in the leaves of *C. alata* were also determined to be as methyl 2,4,6-trihydroxybenzoat (**1**), kaempferol (**2**), **(-)** epiafzelechin **(3)**, kaempferol-3-O-glucoside (**4**) and kaempferol-3-O- gentiobioside (**5**). The phenolic compounds were observed to occur in *C. alata* leaf in previous papers [7-16]. These phenolic compounds were also reported to have antimutagenic, antifungal, analgesic, anti-inflammatory, and hypoglycaemic activities properties [5]. Wang et al. evaluated the antioxidant activity of several flavonoids and found that kaempferol was one of the strongest scavengers for the Fenton-generated hydroxyl radical, with an IC_{50} of 0.5 µM [17]. Kaempferol and its major glycoside kaempferol-3-O-gentiobioside from the ethyl acetate and *n*-butanol fractions respectively displayed moderate anti-α-glucose-inhibitory activity. The half maximal inhibitory concentrations of kaempferol (IC_{50} = 16.2 µg/ml) and kaempferol-3-O-gentiobioside (IC_{50} = 82.5 µg/ml) were also reported previously [4].

The antibacterial activity of kaempferol was tested against six types of bacteria include *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *S. aureus*. It exhibited antibacterial activity against all of the test bacteria with MIC value of 128 (µg/ml) [18]. The property of kaempferol may response for potently antibacterial activity against *S. aureus* and *B. cereus* of MeOH leaf extract of *C. alata*. The further study of the other compounds **1**, **3**-**5** in testing against human pathogenic bacteria strains *S. aureus* and *B. cereus* should be conducted to confirm a synergistic or additive interaction between these constituents for the antibacterial efficacy of extracts from the leaves of *C. alata*.

4. CONCLUSION

From the ethyl acetate extract of the leaf of *C. alata* there were isolated and determined structure five compounds: methyl 2,4,6-trihydroxybenzoate (**1**), kaempferol (**2**), (-)epiafzelechin (**3**), kaempferol-3-O-glucoside (**4**) and kaempferol-3-O- gentiobioside (**5**). The chemical structures of the isolates were identified by analysis of MS and NMR data and compared with references.

Acknowledgments. *This research was financially supported by Ministry of Industry and Trade with project code 136.16.ĐTKHCN.*

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