SHORT REPORT

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A New Amide from the Stem Bark of *Illicium difengpi* and Its Anti-inflammatory Activity

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Abstract: A new amide, named (2*E*, 4*E*)-5-phenyl-*N*-(2-phenylethyl)-2,4-pentadienamide (**1**) , together with one known amide, *N*-2-phenylethylcinnamide (**2**) and two known ceramides, 2-hydroxy-*N*-[(1*S*,2*R*,3*E*)-2-hydroxy-1-(hydroxymethyl)-3-heptadecenyl]-pentadecanamide (**3**), 1-O-(*β*-D-glucopyranosyl)-(2*S*,3*R*,4*E*,8*E*)-2-[(2*R*)-2-hydroxypentadecanoylamino]-4,8-octadecadiene-1,3-diol (**4**) were isolated from the stem bark of *Illicium difengpi*. The structures of the isolated compounds were elucidated by analyses of their ^{1}H and ^{13}C NMR, COSY, HSQC, HMBC spectra and HR-ESI/MS mass spectrometric data. Anti-inflammatory assays with compounds **1**–**4** were carried out, compounds **1** and **2** showed significant inhibitory effect on TNF-α release in LPS stimulated RAW 264.7 macrophages.

Keywords: Amide; *Illicium difengpi*; anti-inflammatory.

1. Plant Source

Illicium difengpi K. I. B et K. I. M., belonging to family Illiciaceae, is a small shrub native to China growing in mountain area of Guangxi province [1, 2]. Its stem bark is listed in Chinese pharmacopoeia (2010 edition) for its traditional use of rheumatic arthritics (RA) treatment [3]. The alcoholature of stem bark of *I. difengpi* showed outstanding clinical efficacy and pharmacodynamics potency [4].

The stem bark of *I. difengpi* was purchased from Caitongde Pharmacy, Shanghai, China in January 2010 and identified by Dr. Lian-na Sun (Department of Pharmacognosy, School of Pharmacy, Second Military Medical University) based on morphological characters. Voucher specimen (NO.20100110) has been deposited in the Herbarium of the Department of Pharmacognosy, School of Pharmacy, Second Military Medical University, China.

2. Previous Studies

Previous investigations led to isolation of phenylpropanoids, lignans, neolignans and triterpenoids [5–11] from extract of *I. difengpi* stem bark and isolation of monoterpenoids and

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sesquiterpenes [12–14] from volatility oil of its stem bark and its fruit. *I. difengpi* stem bark has been used to treat rheumatic arthritics for years. However, the anti-inflammatory constituents were not clear.

3. Present Study

The 80% ethanol extract (1200 g) of stem bark of *I. difengpi* was suspended in water and extracted sequentially with petroleum ether, EtOAc and *n*-BuOH, affording petroleum ether fraction (40 g), EtOAc fraction (560 g) and *n*-BuOH fraction (300 g), respectively. The petroleum ether fraction ($Fr._1$) was subjected to silica gel column chromatography gradient with petroleum ether/EtOAc (100:0 to 0:100) to give 8 subfractions ($Fr._{1-1}$-$Fr._{1-8}$). Compounds **1** and **2** were isolated from $Fr._{1-4}$ through column silica gel chromatography gradient with petroleum ether/EtOAc (50:1 to 0:100). The EtOAc fraction ($Fr._2$) was chromatographed on silica gel column gradient with CH_2Cl_2/MeOH (300:1 to 0:100) to give four main fractions ($Fr._{2-1}$-$Fr._{2-4}$), among which $Fr._{2-1}$ gave compound **3** and $Fr._{2-4}$ provided compound **4** (Figure 1).

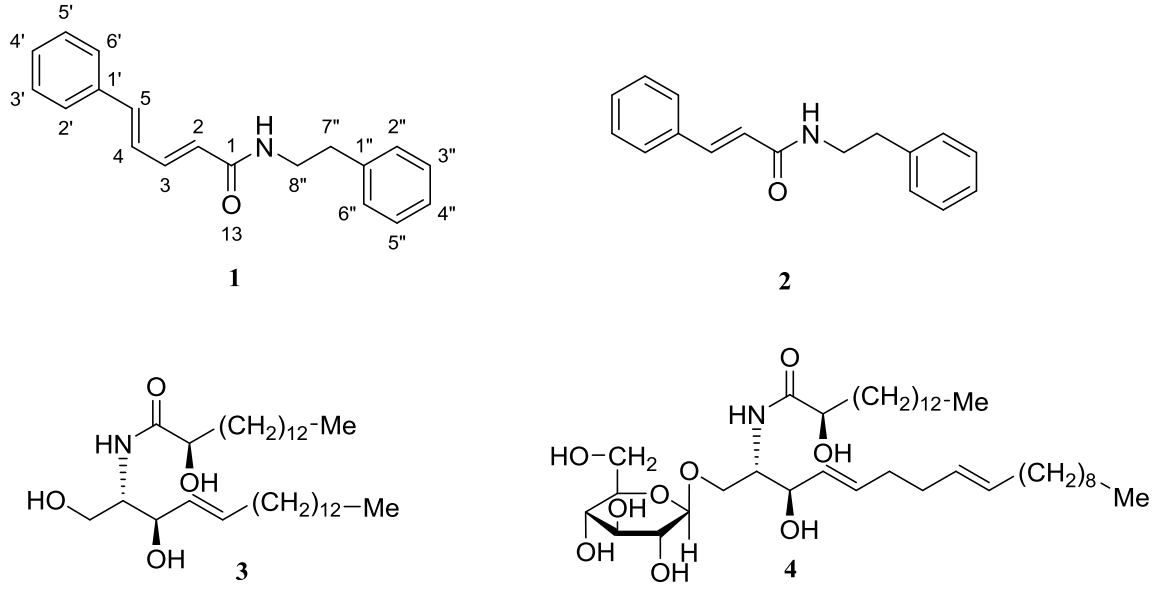

Figure 1. Structures of compounds isolated from *I. difengpi.*

Compound **1** was obtained as white powder with $[\alpha]_D^{25}$: +320.0° (c = 0.1, MeOH), and its molecular formula was determined to be $C_{19}H_{19}NO$ by HR ESI MS data (*m/z* 278.1477 $[M + H]^+$, calc. 278.1500), requiring 11 degrees of unsaturation. The structure of **1** was established on the basis of one- and two-dimensional NMR spectra (^1H, ^{13}C, DEPT, COSY, HSQC and HMBC) (Table 1). The ^{13}C NMR spectrum of **1** was very similar to the known amide (*N*-2-phenylethylcinnamide, **2**) [15], except that there is one addition double bond in **1**. The ^{13}C NMR spectrum of **1** showed particularly intense signals at δ_C 127.1, 128.8, 128.8 and 128.9 accounting for two magnetically equivalent carbons each (C-2' + C-6', C-2" + 6", C-3' + 5' and C-3" + 5"). These aromatic carbons together with the methine signal of 128.8 (CH, C-4') and 126.6 (CH, C-4"), and quaternary carbons 136.4 (C, C-1') and 139.0 (C, C-1") gave evidence of two monosubsititued benzenes. The carbonyl function C-1 (δ_C 166.2), protonyl function NH (δ_H 5.66) and the HMBC correlation (Figure 2) from NH to C-1 were observed, which were characteristic of amide group. Additionally, the ^{13}C NMR spectrum revealed the presence of two double bonds, C-2 (δ_C 124.0) and C-3 (δ_C 141.0), and C-4 (δ_C 126.4) and C-5 (δ_C 139.3), two methylene carbons C-7" (δ_C 35.7) and C-8" (δ_C 40.9). The ^1H NMR spectrum showed δ_H 2.88 (2H, t, J = 7.2 Hz, H-7"), δ_H 3.63 (2H, td, J = 7.2 Hz, 6.0 Hz, H-8") and δ_H 5.66 (1H, t, J = 6.0 Hz, NH), the coupling constants indicating the NH was conected to two consecutive methylene carbons C-7" (δ_C 35.7) and C-8" (δ_C 40.9), which can also be deduced from the COSY correlations of NH to the H_2-8" and H_2-8" to the H_2-7". The COSY spectrum also revealed the spin system from H-2 to H-5. The coupling constants between H-2 and H-3, H-3 and H-4, H-4 and H-5, which were 14.4 Hz, 9.6 Hz, 15.6 Hz respectely, indicated structure of 2*E*, 4*E*. The two monosubsititued benzenes were connected

to C-5 and C-7" as ending groups, which were confirmed from the HMBC correlations from H-4 to C-1' and from H-5 to C-2', 6', and correlations from H-8" to C-1", H-7" to C-2", 6". Therefore, the structure of compound **1** were elucidated as (2*E*, 4*E*)-5-phenyl-N-(2-phenylethyl)-2,4-pentadien-amide.

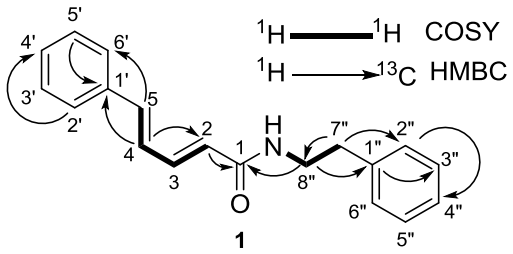

Figure 2. Important ^{1}H-^{1}H COSY and HMBC correlations for compound **1**

In addition to the new compound (**1**), three known compounds including one amide *N*-2-phenylethylcinnamide (**2**) [15] and two ceramides: 2-hydroxy-*N*-[(1*S*,2*R*,3*E*)-2-hydroxy-1-(hydroxymethyl)-3-heptadecenyl]-pentadecanamide (**3**) [16] and 1-O-(*β*-D-glucopyranosyl)-(2*S*,3*R*,4*E*,8*E*)-2-[(2*R*)-2-hydroxypentadecanoylamino]-4,8-octadecadiene-1,3-diol (**4**) [17] were isolated from *I. difengpi* extract. These compounds were identified by spectral analysis compared with spectroscopic data reported in the literatures.

Table 1. ^{1}H and ^{13}C NMR Data of **1** (150 and 600 MHz, resp., in $CDCl_3$), δ in ppm, J in Hz

Position	^{1}H	^{13}C
1	-	166.2 (C=O)
2	5.90 (d, 14.4)	124.0 (CH)
3	7.39 (dd, 14.4, 9.6)	141.0 (CH)
4	6.82 (dd, 15.6, 9.6)	126.4 (CH)
5	6.86 (d, 15.6)	139.3 (CH)
1'	-	136.4 (C)
2', 6'	7.43 (m)	127.1 (CH)
3', 5'	7.34 (m)	128.8 (CH)
4'	7.28 (m)	128.8 (CH)
1"	-	139.0 (C)
2", 6"	7.22 (m)	128.8 (CH)
3", 5"	7.31 (m)	128.9 (CH)
4"	7.25 (m)	126.6 (CH)
7"	2.88 (t, 7.2)	35.7 (CH_2)
8"	3.63 (td, 7.2, 6.0)	40.9 (CH_2)
NH	5.66 (t, 6.0)	-

Anti-inflammatory activity test: The anti-inflammatory activities of compounds **1**–**4** were assessed by determining the inhibitory ratio of TNF-α release in lipopolysaccharide (LPS) stimulated RAW 264.7 macrophages in *vitro*. Tripterygium tablets (TT) and total glucosides of paenia (TGP) were used as positive controls. As shown in Figure 3, the concentrations of TNF-α in the RAW 264.7 cells pretreated with compounds **1** and **2** were reduced by 92% and 85%, respectively, compared to LPS stimulated RAW 264.7 cells, while the IR of two positive controls TT and TGP were 59% and 49%, respectively. These results demonstrated that compounds **1** and **2** had a significant inhibitory effect on TNF-α release from macrophages.

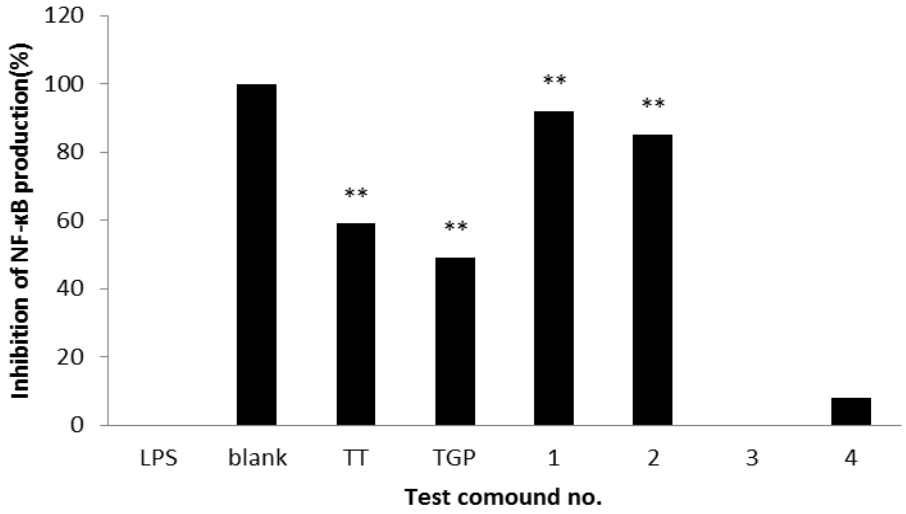

Figure 3. Inhibitory rate of TNF-α production from LPS stimulated RAW 264.7 cells by compounds **1**–**4** at a concentration of 0.25 mg/mL. **, $p < 0.01$ for TNF-α levels in RAW 264.7 cells treated with LPS in the presence of the test compounds versus that in the absence of the test compounds.

Based on the results obtained from TNF-α release experiments, compounds **1**–**4** were further assessed for their possible effect on NF-κB production from RAW 264.7 cells stimulated with LPS. The cytotoxic effects of tested compounds on LPS-stimulated RAW 264.7 cells were determined initially. The results showed that compounds **1**–**4** did not affect cell viability at concentrations up to 25 μg/mL. Compound **1** and **2** reduced the NF-κB production stimulated by LPS ($p < 0.01$). It was observed that compounds **1** and **2** showed a dose dependent inhibition of NF-κB release in LPS-stimulated RAW 264.7 cells. Compounds **3** and **4** showed no activities against NF-κB release (Table 2).

Table 2. Inhibitory rate (IR) of NF-κB production (%) from LPS stimulated RAW 264.7 cells by compounds **1**–**4**.

Compound	IR at 20μg/mL(%)	IR at 10μg/mL(%)	IR at 5μg/mL(%)
1	31.1	21.4	20.9
2	35.6	31.5	27.7
3	-	-	-
4	-	-	14.3
TPG	64.4	54.0	49.0
TT	93.6	83.1	74.7
LPS	0	0	0
Normal	90.6	84.8	92.8

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