ALPHA-GLUCOSIDASE INHIBITORY LIMONOIDS FROM THE LEAVES OF *AZADIRACHTA INDICA* A. JUSS GROWN IN NINH THUAN PROVINCE

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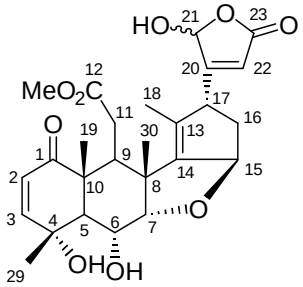
***ABSTRACT**: Two new limonoids, named nimbandiol A (1) and azadirachtolid E (2) were isolated from the leaves of Azadirachta indica, along with deoxyazadirachtolid (3), a known compound. Their structures were determined by spectroscopic methods and compared with literatures. Three compounds (1-3) showed moderate α-glucosidase inhibitory activities against Saccharomyces cerevisiae α-glucosidase with IC_{50} values of 38.7 μM, 85.76 μM and 48.24 μM, respectively.*

INTRODUCTION

α-Glucosidase inhibitors are oral anti-diabetic drugs used for diabetes mellitus type 2 that work by preventing the digestive hydrolysis of carbohydrates into monosaccharides such as D-glucose, which can be absorbed through the intestine. So, α-glucosidase inhibitors reduce the impact of carbohydrates on blood sugar[1,2]. The leaves of neem tree (*Azadirachta indica*) has been used in traditional medicine both for treating and preventing diabetes. As a part of our continuing efforts in the discovery of effective α-glucosidase inhibitors from natural sources, we have isolated three limonoids (**1**-**3**) from the leaves of neem tree, *Azadirachta indica*, grown in Ninh Thuan Province, Vietnam, and test for their inhibitory effect on α-glucosidase acitivity. This paper reports their structure elucidation and α-glucosidase inhibitory activities.



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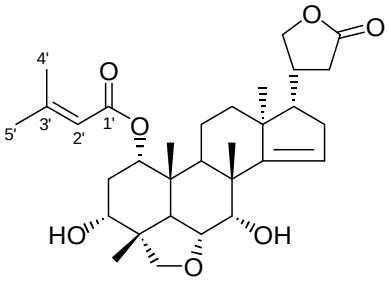

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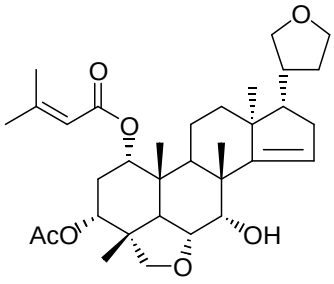

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EXPERIMENTAL

General

Optical rotations were measured on a A. Krüss Optronic. Melting points were determined on a Polytherm A hot stage microscope. UV spectra were on

NMR spectra were recorded on Bruker Avance at 500 MHz (^{1}H) and 125 MHz (^{13}C) at the Institue of Chemistry, Vietnamese Academy of Science and Technology, Cau Giay Dist., Ha Noi, Vietnam.

HR-ESI-MS spectra were recorded on Bruker MicrOTOF-Q II, at Central Laboratory of Analysis, University of Science, HCM City.

Saccharomyces cerevisiae α-glucosidase, p-nitrophenyl-α-D-glucopyranosid (PNP-G) and glutathione were purchased from Sigma Aldrich. The other chemicals used in this study were of analytical grade.

Plant material

The leaves of *A. indica* were collected in Ninh Thuan province, Vietnam.

Extraction and isolation

The air-dried leaves (7.5 kg) was extracted with MeOH to give 1.30 kg residue after removal of the solvent. This residue was suspended in H_2O and then extracted with petroleum ether, ethyl acetate and n-butanol, respectively. The petroleum ether, ethyl acetate and n-butanol layer were concentrated after filtration and evaporation of solvent under reduced pressure to give 470 g, 125 g and 138 g of respective extracts. The ethyl acetate extract was repeatedly chromatographed over silica gel eluted with $CHCl_3$-MeOH in order of increasing polarity to give 19 fractions (A1-A19). Compounds **1** (94 mg, fraction A16), **2** (230 mg, fraction A12) and **3** (239 mg, fraction A12) were obtained as white needles, after purifying by silica gel chromatography methods.

Assay for α-glucosidase inhibitory activities

The assay was performed according to the Sigma Quality Control Test Procedure[3]. The enzyme inhibition studies were carried out in test-tube. A reaction mixture containing 500 µl of 67 mM phosphate buffer (pH 6.8), 20 µl of 3 mM glutathione, 20 µl of 0.3 U/ml α-glucosidase in cold deionized water and 20 µl of sample was pre-incubated in thermoregulator for 5 minute at 37^{o}C, and then 50 µl of 5 mM PNP-G solution was added to the mixture. After further incubation at 37^{o}C for 30 min, the reaction was stopped by adding 2440 µl of 100 mM Na_2CO_3 (pH 9.6). The released PNP was monitored spectrophotometrically by measuring the absorbance at 400 nm. Acarbose were used as positive control. The percentage of α-glucosidase enzyme inhibition by the sample was calculated by the following formula: % inhibition = [AC – AS]/AC×100, where AC is the absorbance of the control and AS is the absorbance of the tested sample. In order to evaluate the type of inhibition using the Lineweaver-Burk plot, this enzyme reaction was carried out with many concentrations of the tested sample.

RESULTS AND DISCUSSION

Isolation of Chemical Constituents

The molecular formula of **1** was established to be $C_{26}H_{32}O_9$ by (+)-HR-ESI-MS

with an $[M+H]^+$ ion signal at *m/z* 489.2157 (the theoretical ion $C_{26}H_{33}O_9^+$ is at *m/z* 489.2119), mp. 192-195°C, $[\alpha]^{25}_D$ +452° (*c* 0.2, MeOH). The ^{1}H-NMR data were indicative of the terpenoidal nature of **1** with the presence of four tertiary methyl singlets at δ 1.77 (3H, s, H-18); 1.22 (3H, s, H-19); 1.59 (3H, s, H-29); 1.32 (3H, s, H-30), an –OMe singlet at δ 3.75 (3H, s, 12-OMe); a pair of doublets of an AB system at δ 5.75 (1H, d, J=10.5Hz, H-2) and 6.54 (1H, d, J=10.5Hz, H-3) could be assigned to the olefinic protons of the enone system in ring A**.** The ^{1}H-NMR further showed the presence of signals at δ 5.38 (1H, brs, H-15), 4.01 (1H, d, H-7) along with signals of carbon in ^{13}C-NMR at δ 86.6 and 88.0 attributable to C-15 and C-7, respectively. These signals indicated the presence of the ether bridge between C-15 and C-7 in **1**. Moreover, the signals of a furan ring, the characteristic feature of limonoids were missing in the NMR spectra (^{1}H and ^{13}C-NMR, Table 1) and, instead, the signals of a hydroxybutenolide ring were observed. A critical comparison of the spectral data of **1** with those of two C-seco nortriterpenes nimbanal[4] and isomargosinolide[5] isolated from *A. indica* suggested that **1** is a C-seco nortriterpene with hydroxybutenolide ring. However, the signal at C-28 were missing in the NMR spectra (^{1}H and ^{13}C-NMR, Table 1), instead, HMBC spectrum indicated the presence of a hydroxyl function at C-4 in compound **1** which has been confirmed by signal at δ 71.2 (C-4). Its ^{1}H and ^{13}C-NMR assignments were made through 2D-NMR studies including HMBC, HSQC and ^{1}H -^{1}H COSY data. This is enabled its identification as a C-seco limonoid with γ-hydroxybutenolide ring, According to SciFinder, this compound had not been reported before, so it is a new natural compound and named nimbandiol A.

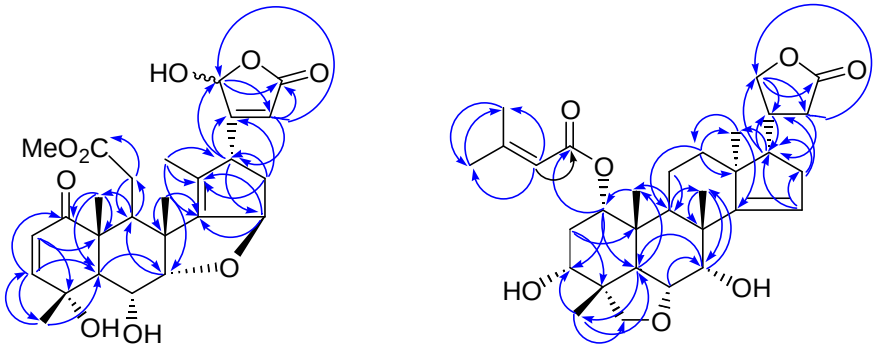


Figure 1. HMBC correlation of **1** and **2**

The molecular formula of **2** was determined to be $C_{31}H_{44}O_7$ by (+)-HR-ESI-MS with a $[M+H]^+$ signal at *m/z* 529.3210 (the theoretical ion $C_{31}H_{44}O_7^+$ is at *m/z* 529.3159), mp. 168.6-172.0°C, $[\alpha]^{25}_D$ -359.4° (*c* 0.22, MeOH). The ^{1}H-NMR data were indicative of the terpenoidal nature of **2** with the presence of four tertiary methyl singlets at δ_H 0.97 (3H, s,

H-18); 0.97 (3H, s, H-19); 1.07 (3H, s, H-30) and 1.14 (3H, s, H-29) and a senecioyl substituent is present at C-1 [δ_H 4.95 (1H, t, H-1); 5.72 (1H, s, H-2′); 1.92 (3H, s, H-4′); 2.22 (3H, s, H-5′); δ_C 72.6 (C-1), 164.7 (C-1′), 115.1 (C-2′), 159.5 (C-3′), 27.5 (C-4′), 20.4 (C-5′)] and the oxygen at C-6 now forms an ether linkage between C-6 and C-28 [δ_H 4.13 (1H, m, H-6), 3.61 (1H, d, J=7.5, H-28a), 4.08 (1H, d, J=7.5, H-28b); δ_C 73.9 (C-6), 78.1 (C-28)]. Furthermore, the ^{1}H-NMR spectrum showed resonances for a olefinic hydrogens δ_H 5.50 (1H, d, H-15); methylene hydrogens bonded to oxygenated carbons [δ_H 3.91 (1H, t, J=9.5 Hz, H-21a), 4.40 (1H, t, J=8.0 Hz, H-21b)] and methine hydrogens bonded to oxygenated carbons [δ_H 3.84 (1H, H-3), 4.15 (1H, H-7)] (Tabale 1). The ^{13}C- and DEPT-NMR spectra gave the following other functionalities, a carbonyl of a lactone at δ_C 176.6 (C-23), a carbonyl of a conjugated ester at δ_C 164.7 (C-1′), two oxygenated methine carbons at δ_C 71.3 (C-3), 73.1 (C-7), a oxygenated methylene carbons δ_C 72.4 (C-21), a non-protonated olefins at δ_C 159.6 (C-14). The interactions of H-1 with C-1′ in the HMBC plot displayed the senecioyl moiety at C-1 (Fig 1). The foregoing account of the spectral data led to elucidate the structure of azadirachtolid E as **2**.

Table 1. ^{1}H- and ^{13}C-NMR Spectral Data of Compounds 1 and 2

No.	**1** ($CDCl_3$ and CD_3OD)		**2** ($CDCl_3$)	
	δ_H	δ_C	δ_H	δ_C
1	−	203.4	4.95 (1H, t, J= 2.5)	72.6
2	5.75 (1H, d, J = 10.0)	124.8	2.00 (1H, dt, J= 3.0; 16.0) 2.28 (1H, dt, J= 2.5; 16.0)	30.4
3	6.54 (1H, d, J = 10.0)	152.5	3.84 (1H, m)	71.3
4	−	71.2	−	43.8
5	2.65 (1H, d, J = 11.5)	49.7	2.55 (1H, m)	38.6
6	4.26 (1H, dd, J = 3.0; 12.0)	66.7	4.13 (1H, m)	73.9
7	4.01 (1H, d, J = 2.50)	88.0	4.15 (1H, m)	73.1
8	−	49.6	−	45.4
9	2.62 (1H, brs)	38.9	2.44 (1H, dd, J = 5.0; 11.5)	33.6
10	−	48.3	−	39.7
11	2.19 (1H, dd, J = 4.0; 16.5) 2.88 (1H, dd, J = 5.5; 17.0)	34.5	1.34 (1H, m) 1.51 (1H, m)	15.4
12	−	174.8	1.43-1.50 (2H, m)	34.2
13	−	132.1	−	46.6

14	−	150.1	−	159.6
15	5.38 (1H**,** br.s)	86.6	5.50 (1H, d, J = 1.5)	120.2
16	2.05 (1H, dt, J = 3.0; 8.5; 12.0) 2.36 (1H, dd, J = 6.5; 12.0)	38.6	2.1-2.2 (2H, m)	34.7
17	3.68 (1H, s)	51.8	1.71 (1H, m)	58.1
18	1.77 (3H, s)	12.9	0.97 (3H, s)	20.5
19	1.22 (3H, s)	15.9	0.97 (3H, s)	15.3
20	−	170.2	2.69 (1H, m)	37.5
21	6.00 (1H, s)	98.8	3.91 (1H, t, J= 9,5) 4.40 (1H, t, J= 8.0)	72.4
22	5.87 (1H, s)	118.1	2.51 (1H, t, J= 9.5) 2.24 (1H, m)	34.0
23	−	171.0	−	176.6
28	−	−	4.08 (1H, d, J= 7.5) 3.61 (1H, d, J= 7.5)	78.1
29	1.59 (3H, s)	22.9	1.14 (3H, s)	19.8
30	1.32 (3H, s)	17.4	1.07 (3H, s)	26.1
12-OMe	3.75 (3H, s)	52.1	−	−
1’	−	−	−	164.7
2’	−	−	5.72 (1H, s)	115.1
3’	−	−	−	159.5
4’	−	−	1.92 (3H, s)	27.5
5’	−	−	2.22 (3H, s)	20.4

Compounds **3** were identified as deoxyazadirachtolide[6] on the basis of extensive spectroscopic studies including 1D (^{1}H-, ^{13}C-NMR) and 2D (COSY, HSQC, HMBC) NMR and comparison with the literatures.

Assay for α-glucosidase inhibitory activity

Three compounds **1**, **2** and **3** showed *in vitro* α-glucosidase inhibitory activities with IC_{50} of 38.7, 85.76 and 48.24 μM, respectively, comparable to that of acarbose (IC_{50} 360.0 μM), a clinically used drug for type-2 diabetes. The significant activity of **1** is probably due to the C-seco structure and it may the presence of two hydroxyl groups at C-4 and C-6. However, the activity of compounds **2** and **3** showed that the hydroxyl group at C-3 may reduce the α-glucosidase inhibitory activities of compound **2.**

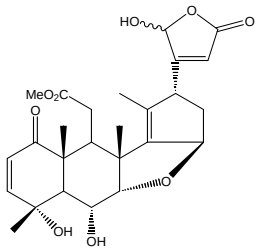
Table 2. Inhibitory activity of compounds 1-3 and acarbose against α-glucosidase

Compounds	**IC_{50} (μM)**
Acarbose	360.0
Compound 1	38.7
Compound 2	85.76
Compound 3	48.24

CÁC HỢP CHẤT ỨC CHẾ ENZYME α-GLUCOSIDASE ĐƯỢC CÔ LẬP TỪ LÁ *AZADIRACHTA INDICA* A.JUSS TRỒNG Ở TỈNH NINH THUẬN.

Nguyễn Thị Ý Nhi, Trần Lê Quan, Trần Kim Qui
Trường Đại học Khoa học Tự Nhiên, ĐHQG-HCM

TÓM TẮT: *Hai hợp chất limonoid mới, azadirachtolid D (1) và azadirachtolid E (2) cùng với một hợp chất đã biết là azdirachtolid (3) đã được cô lập từ lá cây azadirachta indica A.Juss. Ba hợp chất 1, 2 và 3 cho thấy có khả năng ức chế enzyme α-glucosidase. Cấu trúc của các hợp chất được xác định bằng các phương pháp phổ nghiệm và so sánh với các tài liệu tham khảo.*



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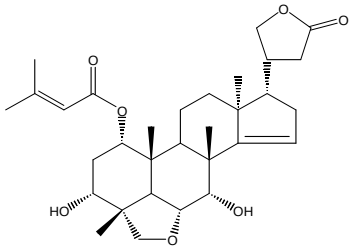

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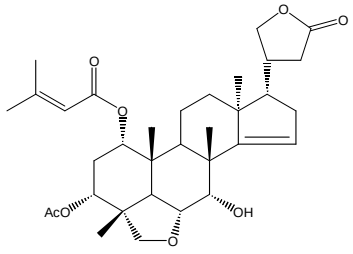

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