Insecticidal Bufadienolides from The Leaves of *Kalanchoe daigremontiana* (Crassulaceae)

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ABSTRACT

Kalanchoe is the biggest genera of Crassulaceae family and distributed in tropical and subtropical regions. This genera is found to be a rich source of biologically active natural products such as triterpenes, flavonoids and steroids. As a part of our continuing search for novel insecticidal compounds from Indonesian *Kalanchoe* plants, we examined *Kalanchoe daigremontiana* collected from Bandung region, West Java, Indonesia. The methanolic extract of the dried leaves of *K. daigremontiana* was concentrated and extracted with methylene chloride The methylene chloride extract exhibited an insecticidal activity toward silkworms. The methylene chloride extract was partitioned between *n*-hexane and methanol containing 10% water. The active lower layer was extracted with ethyl acetate. By using the insecticidal activity to follow the separations, the ethyl acetate fraction was separated by combination of column chromatography on Kieselgel 60 and ODS to afford two insecticidal bufadienolides **1** and **2**. The structures of these compounds were elucidated based on spectroscopic analysis (UV, IR, NMR, 2D-NMR) and comparison with those related data previously reported. In this paper, the isolation, structural elucidation, and insecticidal activities against the third instar larvae of silkworm will be described.

Keywords: *Kalanchoe daigremontiana*, Crassulaceae, bufadienolides, insecticidal activity

INTRODUCTION

Insecticidal compounds are very important subtances in agricultural field, since they are needed in controlling insect pests. The unexpected effect of synthetic insecticidal on the environment has stimulated researches on finding new natural insecticidal compound that are more environmentally friendly.

One of the promising natural source of insecticidal compounds is *Kalanchoe* plants. *Kalanchoe* species are widely distributed in tropical and subtropical countries (Beckett 1990). The plant is used in Indonesian as a folk medicine for the treatment of infections, rheumatism, cough, fever, and inflammation (Hutapea 1994). Their uses as traditional drug are always related to constituents inside them. It makes *Kalanchoe* plants are interestingly investigated including for their insecticidal activity.

Previous studies have shown that *Kalanchoe pinnata* exhibited strong insecticidal activities against third instar larvae of silkworm (*Bombyx mori*). Its active compounds are bufadienolide derivatives, bryophyllin A and C (Supratman *et al*. 2000). Another *Kalanchoe* plant that showed insecticidal activity is *Kalanchoe daigremontiana x tubiflora* with daigremontianin, bersaldegenin-1,3,5-ortoasetat, and methyl daigremonate as its active compounds (Supratman *et al*. 2001). Screening for another *Kalanchoe* plant has showed that *Kalanchoe daigremontiana* showed strong insecticidal activity against the third larvae of silkworm. Their active compounds have not been investigated. This study deals with the isolation, structural determination and mode of action of insecticidal compounds from this plant.

METHODS

General

UV spectra were recorded on a Hitachi model U-3210 spectrophotometer; IR spectra on Perkin Elmer 1760X in KBr; ^{1}H and ^{13}C NMR spectra were obtained with a JEOL JNM A-500 spectrometer using TMS as internal standard. Chromatographic separations were carried out on Kieselgel 60 and Chromatorex ODS adsorbens.

Plant material

Leaves of K. daigremontiana were collected from plantation trees growing in Lembang district, West Java, Indonesia in August 2006 and identified by

staff of the Laboratory of Plant Taxonomy at Bandung Institute of Technology, Jalan Ganesha No. 10 Bandung, Indonesia.

Extraction and isolation

Dried leaves of K. daigremontiana were extracted with MeOH at room temp. The MeOH extract was evaporated in vacuo to yield a dark brown residue (520,44 g) that was then partitioned between CH_2Cl_2 and H_2O to afford an active CH_2Cl_2 extract (52,83 g). The extract was then partitioned between n-hexane and methanol containing 10% water. The active lower layer was then extracted with ethyl acetate to give the active ethyl acetate fraction.This fraction was subjected on Kieselgel 60 eluted with $CHCl_3$ and an increasing ratio of MeOH. The active fraction is then separated on ODS (MeOH) to obtain two active insecticidal compounds, **1** and **2**.

Daigremontianin (1)

Colorless prisms; mp 262-271 ^oC; $[\alpha]^{20}_D$ +12.6 ^oC (c 0.095, MeOH); [lit. (Wagner *et al.* 1985), mp 270-280 ^oC, $[\alpha]^{20}_D$ +10 ^oC (c 0.237, $CHCl_3$)]; UV λ_{max} (MeOH) nm (ε): 300 (6100); IR ν_{max} (KBr): 3445, 2882, 1709, and 1122 cm^{-1}; ^{1}H- and ^{13}C-NMR (Table 1).

Bersaldegenin-1,3,5-orthoacetate (2)

Colorless plates; mp 286-288 ^oC; $[\alpha]^{20}_D$ -31.0 ^oC (c 0.087, CHCl3); [lit. (Kupchan et al., 1969) mp 288-295 ^oC, $[\alpha]^{20}_D$ -24 ^oC (c 0.85, $CHCl_3$)]; UV λ_{max} (MeOH) nm (ε): 299 (5800); IR ν_{max} (KBr): 3464, 2877, 1714, and 1122 cm^{-1}; ^{1}H-NMR (270 MHz, $CDCl_3$): δ 0.66 (3H, s, Me-18), 1.50 (3H, s, Me-26), 1.26-2.50 (19H, m), 4.35 (1H, br.s, H-3), 4.61 (1H, br.s, H-1), 6.26 (1H, d, J=9.8 Hz, H-23), 7.26 (1H, d, J=2.7 Hz, H-21), 7.78 (1H, dd, J=2.7, 9.8 Hz, H-22), 10.18 (1H, s, H-19); ^{13}C-NMR (67.5 MHz, $CDCl_3$): δ 16.2 (q, Me-18), 20.6 (t, C-11), 22.2 (t, C-7), 25.7 (q, Me-26), 27.2 (t, C-2), 28.4 (t, C-16), 31.7 (t, C-15), 32.5 (t, C-6), 33.4 (t, C-4), 40.1 (t, C-12), 40.9 (d, C-9), 42.5 (d, C-8), 48.4 (s, C-13), 50.8 (d, C-17), 52.7 (s, C-10), 66.8 (d, C-3), 70.8 (d, C-1), 74.4 (s, C-5), 84.3 (s, C-14), 110.7 (s, C-25), 115.4 (d, C-23), 122.2 (s, C-20), 146.5 (d, C-22), 148.7 (d, C-21), 162.3 (s, C-24), and 206.6 (d, C-19).

Silkworm bioassay (Hayashi *et al.* 1989)

Larvae of Bombyx mori were used for the bioassay, and were cultured on an artificial diet purchased from Nippon Nosan Kogyo Co., Ltd. One Hundred microlitres of MeOH extract or a certain amount of the sample to be tested were added to one gram of the diet in a Petri dish. After removing the solvent, ten larvae of the third instar stage were introduced into the petri dish.

Thirty larvae (three petri dishes) were treated at each dosage, and the mortality rate of the silkworm was observed after 24 h. MeOH was used as a control treatment. The insecticidal activities of tested compounds at each dose were examined by triplicate experiments. The average mortality rate of each dose were determined and used for determining of LD_{50} values by using logarithmic increment.

RESULTS AND DISCUSSION

Repeated column chromatography of the methanolic extract of dried leaves of *K. daigremontiana* by biassay-guided fractionation resulted in the isolation of two bufadienolides. Compound **1** was isolated as colorless prisms; mp 262-271 ^oC. The UV spectrum showed an absorption maximum at 300 nm (ε 6100), indicating the presence of a dienone system. The IR spectrum showed bands which were ascribed to hydroxyl (ν_{max} 3445 cm^{-1}), an aldehyde (ν_{max} 1709 cm^{-1}), and an ether group (ν_{max} 1122 cm^{-1}). The ^{1}H- and ^{13}C-NMR revealed the characteristic due to a γ-substituted α, β, γ, δ-unsaturated δ-lactone (α-pyrone) [δ_H 7.65 (1H, dd, *J*=2.4, 9.8 Hz, H-22), 7.38 (1H, dd, *J*= 0.9, 2.4 Hz, H-21), and 6.29 (1H, dd, *J*=0.9, 9.8 Hz, H-23; δc 115.5, 124.4, 149.1, 150.8, and 164.7]. The spectra also indicated the presence of an orthoester [δ_H 5.10 (1H, d, *J*=4.3 Hz, H-1), 4.36 (1H, d, *J*=1.2 Hz, H-3), 1.46 (3H, s, Me-26; δc 25.6 (q), 67.2 (d), 73.2 (d), 74.6 (s), and 110.6 (s)], an aldehyde [δ_H 10.41 (1H, d, *J*=1.2 Hz, H-19); δc 208.7 (d)], and two singlet methyl [δ_H 0.92 (3H, s, H-18); δc 16.9). It can be inferred that **1** contained a tetracyclic steroidal structure considering the degree of unsaturation on the α-pyrone and other substituents.

The ^{1}H- and ^{13}C-NMR spectra of **1** were quite similar to those of bryophyllin A (Supratman *et al*. 2000), except the absence of a methylene signal at [δ_H 1.41 (1H, t, J=12.5 Hz), and 1.67 (1H, m) (δc 51.1) as observed for it, and the presence of a ketone signal at δc 211.7, suggesting that **1** is a keto analogue of bryophyllin A. In order to determine the location of the keto group, HMBC experiments were carried out. The signals of Me-18 (δH 0.92) and H-11 (δH 4.98) were correlated with C-12 (δc 211.7), indicating that **1** is a 12 keto-analogue of bryophyllin A. These observations along with the similarity of spectral data and physicochemical properties between **1** and previously reported daigremontianin (Wagner *et al*. 1985), let us to identify **1** as daigremontianin (Figure 1).

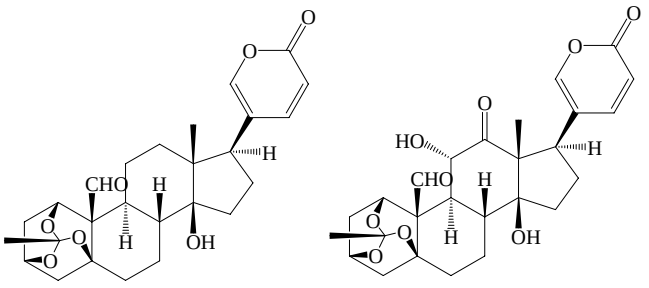

Figure 1. Structure of compounds **1** and **2**.

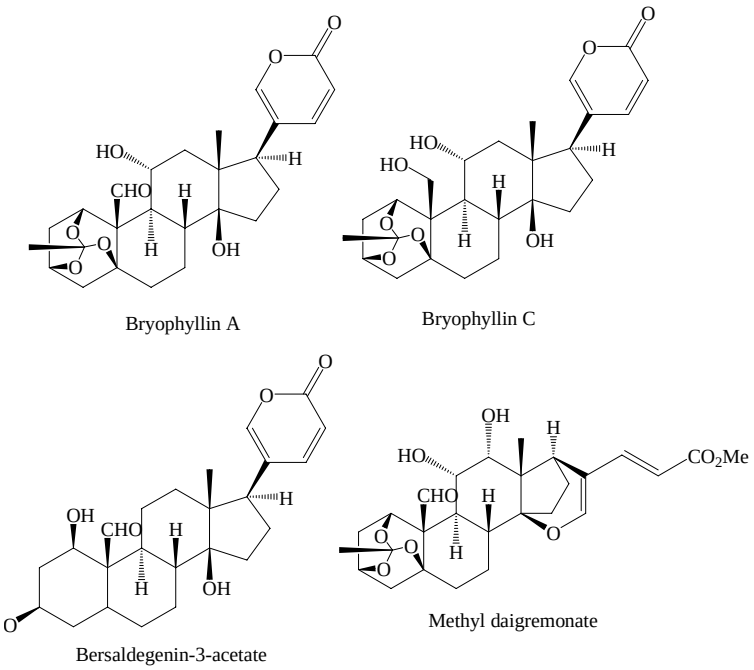

Figure 2. Structure of insecticidal bufadienolides (Supratman *et al*. 2000, 2001).

Compound **2** was shown to have two less oxygen than **1** and showed spectral data similar to those of **1**. In the ^{1}H- and ^{13}C-NMR spectra of **2**, two oxygenated carbons observed in **1** were lacking, and two signals assignable to methylene were newly observed at [δC 20.6 and 40.1]. Since the ^{1}H- and ^{13}C-NMR spectral data and physicochemical properties of **2** was identical to those of previously reported bersaldegenin-1,3,5-orthoacetate (Kupchan *et al*. 1969), **2** was identified as bersaldegenin-1,3,5-orthoacetate. Compound **1** and **2** were evaluated for insecticidal activity against the third instars larvae of silkworm. The insecticidal activities of compound **1** and **2** and four previously isolated bufadienolides (Supratman *et al*. 2000, 2001) are shown in Table 2. All bufadienolides having an orthoacetate and α-pyrone moiety showed strong activity, while non-orthoacetate bufadienolide, bersaldegenin-3-acetate, and a non α-pyrone moiety bufadienolide, methyl daigremonate, showed no activity. In addition, compound **1** having oxygenated substituents at C-11 and C-12 showed the strongest activity among these compounds, indicating that oxygenated substituents at C-11 and C-12 on C-ring enhanced the insecticidal activity.

Table 1. NMR data for compound **1.**

Posisi	NMR-^{13}C δ_C (mult.)	NMR-^1_H δ_H (Integral, mult, *J* Hz)
1	73.2 (d)	5.10 (1H, d, 4,3)
2	27.6 (t)	1.92 (1H, dt, 1,5, 14,0)
		2.31 (1H, ddd, 4,3, 6,1, 14,0)
3	67.2 (d)	4.36 (1H, d, 1,5)
4	33.3 (t)	2.04 (1H, m)
		2.14 (1H, m)
5	74.6 (s)	
6	33.2 (t)	1.74 (1H, m)
		2.02 (1H, m)
7	22.1 (t)	1.42 (1H, m)
		2.17 (1H, m)
8	39.8 (d)	2.22 (1H, m)
9	45.1 (d)	1.58 (1H, dd, 1,5, 12,5)
10	52.4 (s)	
11	72.0 (d)	4.98 (1H, dd, 3,0, 12,5)
12	211.7 (s)	
13	61.9 (s)	
14	85.0 (s)	
15	32.0 (t)	1.42 (1H, m)
		1.73 (1H, m)
16	27.7 (t)	2.09 (1H, m)
		2.45 (1H, m)
17	40.4 (d)	4.07 (1H, dd, 6,7, 9,8)
18	16.9 (q)	0.92 (3H, s)
19	208.7 (d)	10.41 (1H, d, 2,1)
20	120.1 (s)	
21	150.2 (d)	7.38 (1H, dd, 0,9, 2,4)
22	146.2 (d)	7.65 (1H, dd, 2,4, 9,8)
23	115.9 (d)	6.29 (1H, dd, 0,9, 9,8)
24	161.9 (s)	
25	110.6 (s)	
26	25.6 (q)	1.46 (3H, s)

Table 2. Insecticidal activity of compound **1** and **2.**

Compounds	**LD_{50} μg/g of diet**
Bryophyllin A	3
Bryophyllin C	5
Bersaldegenin-3-acetate	>100
Methyl daigremonate	82
1	0.9
2	20

CONCLUSIONS

Two insecticidal bufadienolides, daigremontianin (1) and bersaldegenin-1,3,5-orthoacetate (2), have been successfully isolated from the leaves of K. daigremontiana. Their structures were elucidated by spectral data and comparison with standards. Insecticidal activity studies showed that bufadienolides with orthoacetate and α-pyrone moiety showed strong activity and the present of oxygenated substituents at C-11 and C-12 on C-ring enhanced the insecticidal activity.

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