Mode of action of an insecticidal oleanane glycoside on digestive enzymes of *Crocidolomia pavonana*

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Abstract
Studies on insecticidal properties of methanolic extract of Barringtonia asiatica have been undertaken by Maharani et al[6] in 2009. The plant has been found to be potential as one of natural insecticides with an oleanane saponine as the active compound. In this current research, bioassay-guided isolation of insecticidal compound was re-undertaken with a modified method to get the most active compound towards Crocidolomia pavonana. The active isolate was obtained through chromatographic techniques, involving flash-column and preparative chromatography.

The characterization of active isolate was carried out by using HR-TOF-MS, ^{1}H-NMR and through a comparison with previously reported data. It was found that the obtained isolate was the same oleanane glycoside as was isolated by Herlt el al[3] and Maharani et al[6]. The compound was then investigated for its mode of action towards digestive enzymes of C. pavonana, namely amylase, invertase and protease. The isolated compound did not give any inhibitory effect on the enzyme assayed. Interestingly, the compound seems to increase protease activity assay.

Keywords*:* Barringtonia asiatica, insecticidal compound, natural insecticide, Crocidolomia pavonana.

Introduction

Barringtonia asiatica is a plant species with interesting bioactivities. It has been known as piscicide[1], moluscicide[2] and antifeedant[3]. Dono et al[4,5] reported that methanolic extract of seed of *B. asiatica* is potential as insecticide against *Spodoptera litura* and *Crocidolomia pavonana*. An attempt to isolate insecticidal compound from the seed of *B. asiatica* has been reported by Maharani et al[6] showing an oleanane glycoside as the responsible compound for the insecticidal activity. Previous study by Dono and Sudjana[7] indicated that the insecticidal activity of *B. asiatica* extract may be due to inhibitory effect on digestive enzyme.

In this current report, a bioassay-guided isolation of the insecticidal compound was re-carried out with different steps and instrument and its mode of action was assayed against some digestive enzymes.

Material and Methods

Material: Seeds of *B. asiatica* were collected from Kecamatan Jatinangor, West Java Province, Indonesia in September 2016.

Bioindicator used in this research was the first second instar larvae of *C. pavonana* Fabricius (age 2 hour after skin replacement). The larvae were taken from field colony developed in Propagation Room at Department of Plant Protection, Faculty of Agriculture, Universitas Padjadjaran.

General procedure: Separation steps were carried out on silica gel G60, Chromatorex ODS adsorbents and Buchi Sepachore® with UV photometer C-640, pump module C-605, control unit C-620, fraction collector C-660 and column sephacore C-18, 12 g. All chromatographic steps were monitored by thin layer chromatography (TLC) on silica gel GF_{254} (Merck, Darmstadt, Germany, 0.25 mm) or ODS-coated silica (Fujisylisia, Tokyo, Japan). The spot detection on TLC plate was achieved by spraying the plate with 10% H_2SO_4 in ethanol, that was followed by heating. HR-TOF-ESI-MS was obtained with Waters Xevo-QTOFMS. ^{1}H-NMR was performed on Agilent 500 MHz with CD_3OD used as solvent.

Isolation of insecticidal compound: Methanolic extract of seeds of *Barringtonia asiatica* (20 g) was chromatographed by using vaccuum liquid chromatography with gradual elution [a mixture of *n*-hexane and ethyl acetate (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80 and 0:100), followed by a combination of ethyl acetate and methanol (100:0, 90:10, 80:2)]. Fractions with similar spots on TLC were combined. The active fraction F8 was then subjected on Buchi Sepachore® with UV photometer C-640, pump module C-605, control unit C-620, fraction collector C-660 and column sephacore C-18, 12 g. The column was eluted by using a gradient mixture of water and methanol (100:0, 90:10, 80:20, 70:30 and 60:40). Fraction eluted by water:methanol (70:30), F84, was found to be active and was further separated on Buchi Sepachore® with UV photometer C-640, pump module C-605, control unit C-620, fraction collector C-660 and column sephacore C-18, 25 g.

The gradual elution was applied starting from 50% methanol until 70% methanol in water. Fraction eluted with 50% methanol in water, F841, was the most active

fraction. This fraction was chromatographed on C-18 gradient chromatography with isocratic eluent of methanol:water (7:3) to obtain F8411 (9.7 mg).

Bioassay: Bioassay was tested on larvae of *Crocidolomia pavonana*. The procedure of the assay was based on the protocol applied in Maharani et al.[6]

3-*O*-{[*β*-D-galactopyranosyl(1→3)-*β*-D-glucopyranosyl (1→2)]-*β*-D-glucurono pyranosyloxy}-22-*O*-(2-methyl butyroyloxy)-15,16,28-trihydroxy-(3*β*,15*α*,16*α*,22*α*)-oleane-12-ene: White solid; HR-TOF-ESI-MS: *m/z* $[M-H]^-$ 1073.2826; ^{1}H-NMR (CD_3OD, 500 MHz) (table 1).

Preparation of digestive enzyme: Digestive enzyme was prepared from the whole *C. pavonana* larvae. Ten grams of clean larvae were weighed and 25 mL of phosphate buffer (pH 7; 50 mM) was added. The mixture was then homogenized using Potter-Elvehjem apparatus in cold temperature using ice bath. The crude extract was used as the enzymes source. The crude enzyme extract was then stored at -20°C for further use.

Amylase inhibition assay: Amylase inhibition assay was carried out as described by Hirashima et al[8] with some modification. 0.98 mL of sodium phosphate buffer (pH 7; 50 mM) was added to a micro tube. Then, 50 µL of crude enzyme extract was added onto the tube. The mixture was incubated for 5 minutes at 30°C. After incubation, 10 µL of F8411 isolate was added and the incubation continued for 20 minutes. To the mixture, 0.1 mL starch solution (0.1% w/v in sodium phosphate buffer 50 mM pH 7) was added. The mixture was then incubated for 30 minutes. The amylase activity was determined by measuring the decrease of blue color of starch-iodine complex[9].

Invertase inhibition assay: Invertase inhibition assay was carried out as described by Hirashima et al[8] with some modification. 0.98 mL of phosphate buffer (pH 7; 50 mM) was added to a micro tube. Then, 50 µL of crude enzyme extract was added onto the tube. The mixture was incubated for 5 minutes at 30°C. After incubated, 10 µL of F8411 isolate was added and the incubation was continued for 20 minutes. To the mixture, 0.5 mL sucrose solution (1 mg/mL in phosphate buffer pH 7; 50 mM) was added. The mixture was then incubated for 30 minutes. The reducing sugar was determined using alkaline ferry cyanide as described by Walker and Harmond[10].

Protease inhibition assay: Protease inhibition assay was carried out as described by Ishmayana et al[11] and Barret[12]. 0.98 mL of phosphate buffer (pH 7; 50 mM) was added to an Eppendorf tube. Then, 50 µL of crude enzyme extract was added onto the tube. The mixtures were incubated for 5 minutes in 30°C. After incubation, 10 µL of bioactive compound was added and the incubation continued for 20 minutes. To the mixture, 0,5 mL casein solution (20 mg/mL in phosphate buffer pH 7; 50 mM) was added. The mixture was then incubated for 30 minutes. The reaction was stopped by adding 0.25 mL 0.8% TCA solution. The resulting solution was then centrifuged for 30 minutes in the rate of 15.000 *g*. The absorbance of the supernatant is then measured using UV spectrometer in the wavelength of 280 nm.

Statistical Analysis: Data of inhibition assay were pooled and processed using Minitab 15. Two sample t-tests were performed to check significant differences between assay with isolated compound addition and control. Differences between means were considered significant when $p < 0.05$.

Results and Discussion

Isolation of Insecticidal Compound: Isolation of the most active insecticidal compound of *B. asiatica* was re-undertaken. It was done to further study its mode of action against digestive enzymes of the cluster cabbage caterpillar (*C. pavonana*).

The bioassay-guided isolation was carried out to obtain the most active compound against bioindicator of the first instar larvae of *C. pavonana*. The methanolic extract was purified through repetitive chromatographic techniques, involving flash-column and preparative chromatography with both normal- and reversed-phase. Even though the method to isolate the most active compound from the methanolic extract of *B. asiatica* was slightly different with the prior report[6], the isolated compound was exactly the same.

The isolated compound was characterised by HR-TOF-MS (Fig. 1) showing a correct molecular mass ion of *m/z* 1073.2826, representing the $[M-H]^-$ of previously reported oleanane glycoside, 3-*O*-{[*β*-D-galactopyranosyl(1→3)-*β*-D-glucopyranosyl(1→2)]-*β*-D-glucuronopyranosyloxy}-22-*O*-(2-methylbutyroyloxy)-15,16,28-trihydroxy-(3*β*,15α,16α,22α)-oleane-12-ene (fig. 2).[3,6]

The isolated compound was also characterised with ^{1}H-NMR spectroscopy (fig. 3). The proton NMR data showed overlapped chemical shifts with the ones reported by Maharani et al[6] (table 1). Both characterisations were undertaken in deuterated methanol. The ^{1}H-NMR data of oleanane glycoside reported by Herlt et al[3] was also compared with current isolate. Unfortunately, the later data gave some differences in the chemical shifts since the data were obtained in deuterated pyridine. The significant differences are particularly in the range of chemical shifts of all protons of the oxygenated carbons that appear at the range of 3.09-4.97 ppm in Maharani et al[6] and at the range of 3.80-5.66 ppm in Herlt et al.[3] Further characterisation of our present isolate was not attempted. The obtained isolate was ready for the mode of action assay against the digestive enzymes.

Enzyme Inhibition Assay: Based on previous study by Dono and Sudjana[7] which showed possibility that the active compound from *B. asiatica* inhibits digestive enzymes

indicated by decrease of body weight of larvae, we examine the inhibitory activity of the isolated active compounds, named F8411. The inhibitory effect was tested against amylase, invertase and protease from the larvae of *C. pavonana*. These enzymes are essential in digestive system of the larvae. The results of inhibitory assay are presented on fig. 4.

Our results suggested that F8411 does not have significant inhibitory effect against amylase ($p = 0.826$) and invertase ($p = 0.121$). On the contrary, assay on the protease showed relatively significant higher activity after addition of the active compound ($p = 0.032$). This result is similar with the finding of Ikedo et al[13] who found that soyasaponin slightly activates α-chymotrypsin, but the effect was not great. Further examination of the activation effect of protease by the present isolate needs further examination. In conclusion, the active insecticidal compound is not working towards inhibition of digestive enzymes.

As our results did not give a clear explanation on mechanism of insecticidal activity via digestive enzyme inhibition pathway, mode of action of insecticidal compound from *B. asiatica* remains to be elucidated. Therefore, investigations towards other targets still need to be performed. There are still many enzymes that should be tested against the active compound. Our ongoing research is trying to determine the inhibitory activity of the active compound against esterase, acetyl choline esterase and gluthation-S-transferase which has role in neurotransmitter process.

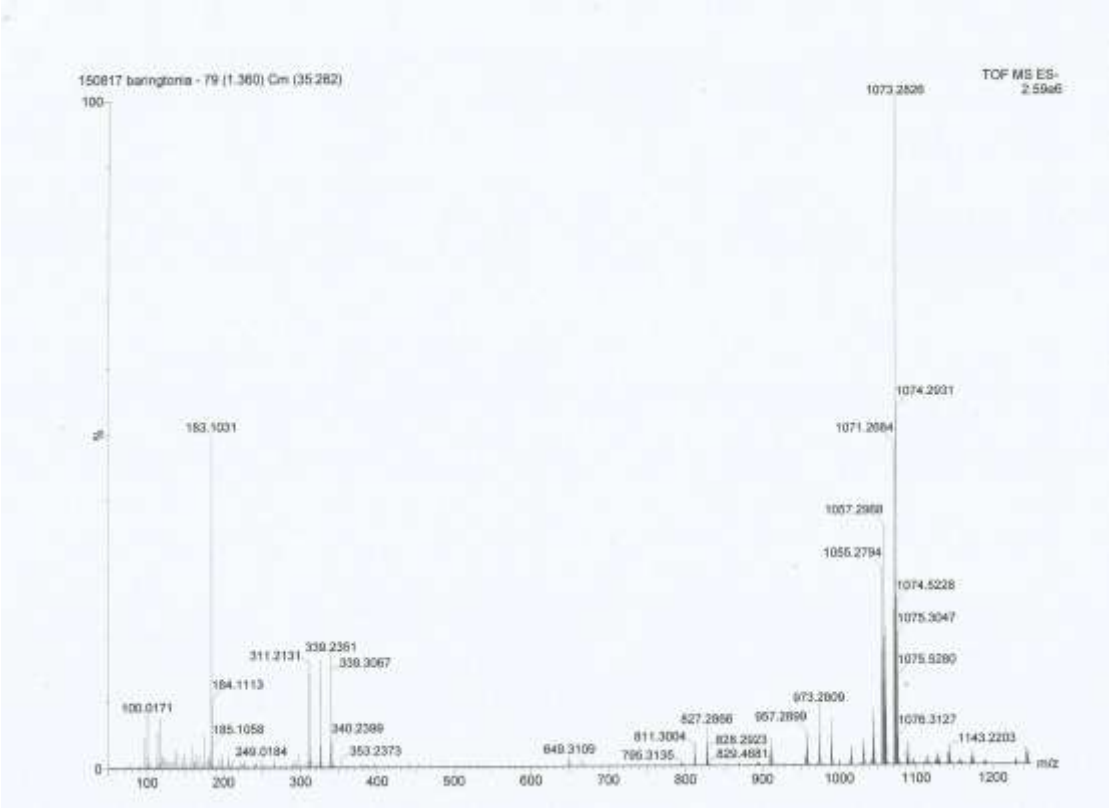

Fig. 1: HR-TOF-MS of F8411.

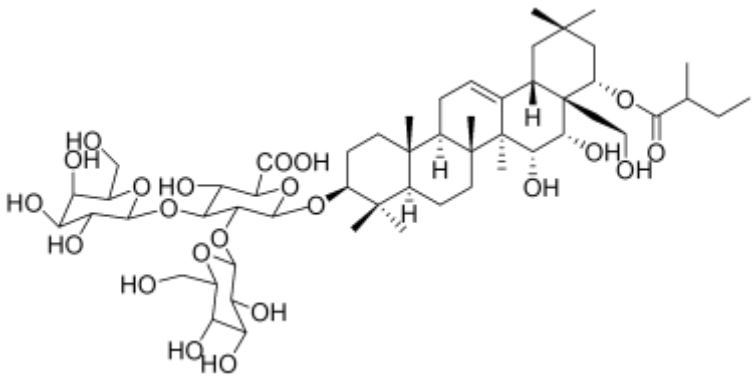

Fig. 2: Structure of an insecticidal oleanane glycoside isolated from *B. asiatica*.

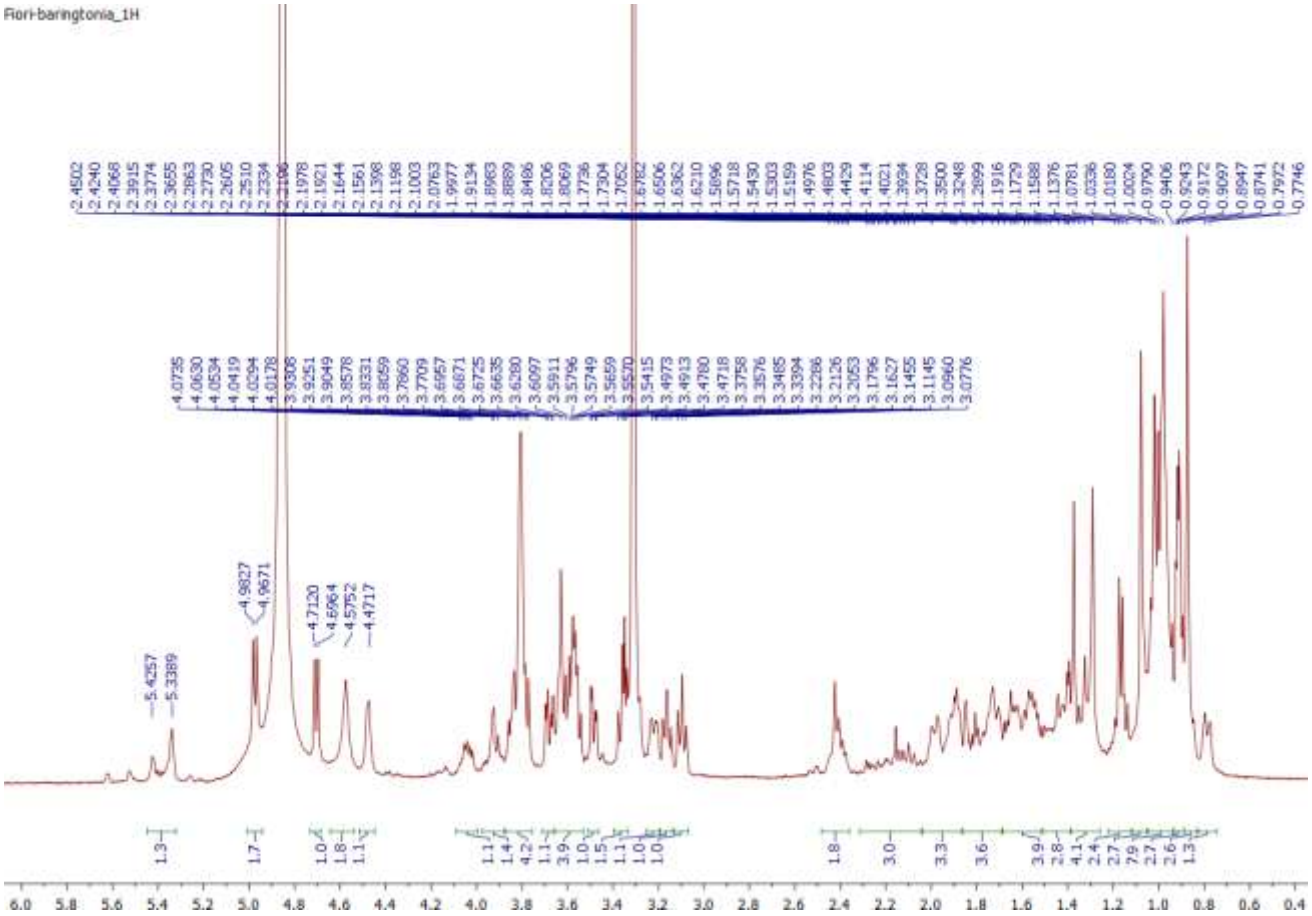

Fig. 3: ^{1}H-NMR spectra of F8411 (JEOL 500 MHz, CD_3OD)

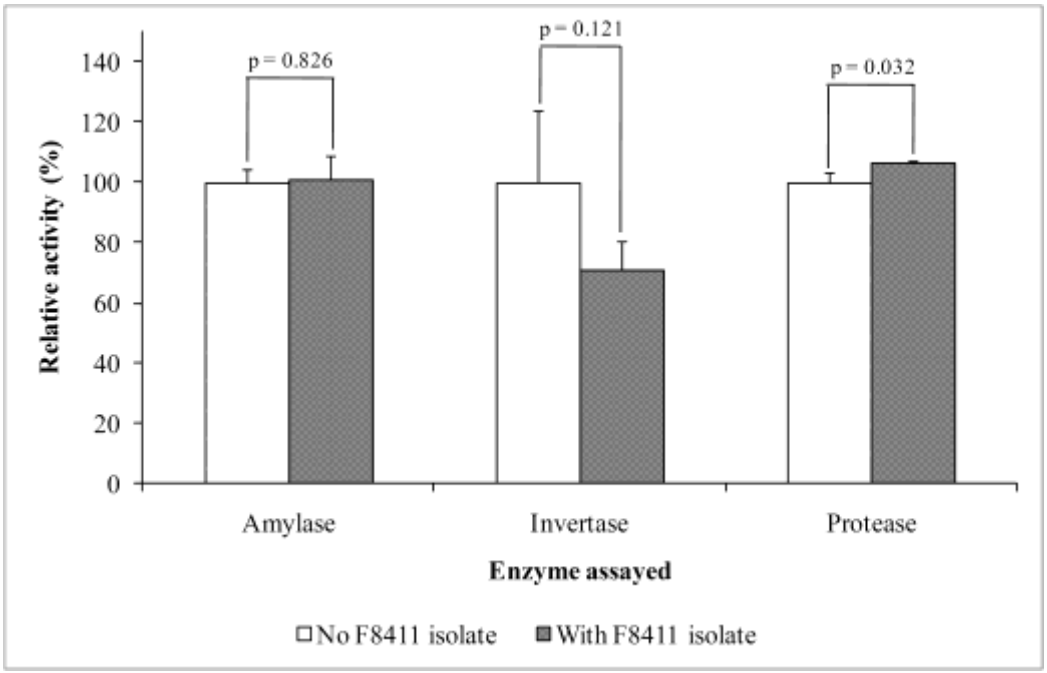

Fig. 4: Inhibition assays of F8411 from *B. asiatica* against amylase, invertase and protease. p-value above the graphs was obtained from two-sample t-test between control and assay with active compound addition. Differences between means were considered significant when $p < 0.05$.

Table 1
Comparison ^{1}H-NMR data of oleanane glycoside of Herlt et al[3], Maharani et al[6] and current isolate

Carbon	δH[a] (ppm)	δH[b] (ppm)	δH F8411 (ppm)
aglycon			
1	1.38, dm, 12.5; 2.07, dm, 12.5	δ 0.93 (1H, d, *J*=7.35, H-1); 1.63 (1H, d, *J*=12.25, H-1)	0.91; 1.63
2	1.80, dm, 12.0; 2.15, dm, 12.0	1.72 (1H, m, H-2), 1.95 (1H, m, H-2)	1.72; 1.91
3	3.30, dd, 3.5, 11.0	3.23 (1H, dd, *J*=4.3; 11.6, H-3),	3.21
4			
5	0.80	0.79 (1H, bd, *J*=11.6, H-5)	0.78
6	1.39, br.d, 12.5; 1.60, br.d, 12.5	1.44 (1H, m, H-6), 1.54 (1H, m, H-6)	1.44; 1.54
7	2.13, m	1.73 (2H, m, H-7)	1.73
8			
9		1.59 (1H, m, H-9)	1.62
10			
11	1.74, dm, 11.0; 1.87, dm, 11.0	1.84 (1H, s, H-11), 1.95 (1H, m, H-11)	1.84; 1.99
12	5.46, t, 3.2	5.41 (1H, bt, H-12)	5.42
13			
14			
15	4.24, d, 5.5	3.77 (1H, d, *J*=4.9, H-15)	3.77
16	4.49, m	3.89 (1H, d, *J*=4.9, H-16)	4.01
17			
18		2.52 (1H, dd, *J*=14.05;4.05, H-18)	2.45
19	2.87, dd, 12.5, 14.0; 3.03, dd, 4.0, 14.0	1.16 (1H, m, H-19), 2.19 (1H, m, H-19)	1.17; 2.19
20			
21	1.95, dd, 6.0, 11.0; 2.78, dd, 11.5, 12.0	1.30 (1H, d, *J*=19.55, H-21), 1.92 (1H, s, H-21)	1.30; 1.91
22	6.15, dd, 6, 12	5.38 (1H, t, *J*=6.1, H-22)	5.34
23	1.24, s	1.08 (3H, s, H-23)	1.08
24	1.10, s	0.88 (3H, s, H-24)	0.87
25	0.83, s	0.99 (3H, s, H-25)	0.98
26	1.03, s	1.02 (3H, s, H-26)	1.03
27	1.87, s	1.39 (3H, s, H-27)	1.40
28	3.59, d, 10.5; 3.78, d, 10.5	3.74 (1H, m, H-28), 3.82 (1H, bm, H-28)	
29	1.07, s	0.92 (3H, s, *J*=3.05, H-29)	1.00
30	1.26, s	1.02 (3H, s, H-30)	1.29
glucoronic acid			
1’	4.93, d, 7.5	4.49 (1H, bd, *J*=6.7, H-1’)	4.47
2’	4.43, m	3.35 (1H, d, *J*=9.15, H-2’)	3.35
3’	4.36, m	3.82 (1H, bm, H-3’)	3.83
4’	4.51, m	3.82 (1H, bs, H-4’)	3.83
5’	4.55, m	3.69 (1H, d, *J*=7.3 H-5’)	3.69
6’			
Glucose			
1”	5.66, d, 8.0	4.67 (1H, d, *J*=7.95, H-1”)	4.70
2”	4.09, dd, 8.0, 9.0	3.63 (1H, t, *J*=7.95, H-2”)	3.63
3”	4.23, m	3.49 (1H, dd, *J*=3.05, 9.8, H-3”)	3.49
4”	4.15, m	3.82 (1H, bm, H-4”)	3.83
5”	3.81, ddd, 4.0, 5.0, 5.0	3.44 (1H, m, H-5”)	3.54
6”	4.33, m; 4.45, m	3.30 (1H, m, H-6”), 3.56 (1H, q, *J*=7.35, H-6”)	3.35; 3.54

Galactose			
1'''	5.31, d, 7.5	4.97 (1H, d, *J*=7.95, H-1''')	4.97
2'''	4.48, m	3.15 (1H, t, *J*=6.8, H-2''')	3.15
3'''	4.14, m	3.82 (1H, bm, H-3''')	3.82
4'''	4.46, m	3.09 (1H, t, *J*=7,95, H-4''')	3.09
5'''	4.16, m	3.61 (1H, m, H-5''')	3.59
6'''	4.33, m; 4.45, m	3.09 (1H, t, *J*=7.95, H-6'''); 3.88 (1H, d, *J*=4.3, H-6''')	3.20; 3.92
Ester			
1''''			
2''''	2.29, dq, 7.0	2.41 (1H, sept., *J*=7.35, H-2'''')	2.41
3''''	1.36, ddt, 6.0, 7.0, 15.0; 1.68, ddt, 6.0, 7.0, 15.0	1.49 (2H, m, H-3'''')	1.51
4''''	0.84, s	0.96 (3H, m, H-4'''')	0.92
5''''	1.10, d, 7.0	1.12 (3H, d, *J*=6.75, H-5'''')	1.15

Conclusion

One oleanane glycoside, 3-*O*-{[*β*-D-galactopyranosyl(1→3)-*β*-D-glucopyranosyl (1→2)]-*β*-D-glucuronopyranosyloxy}-22-*O*-(2-methylbutyroyloxy)-15, 16,28-trihydroxy-(3*β*,15α,16α,22α)-oleane-12-ene was re-isolated. This compound was known as an active compound responsible for the insecticidal activity of methanolic extract of seeds of *B.asiatica* towards *C.pavonana*.

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