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Full Length Research Paper

Feeding deterrents from the tubers of *Boschniakia himalaica* against the red flour beetle, *Tribolium castaneum*

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In our screening program for new agrochemicals from Chinese medicinal herbs and local wild plants, MeOH/$CHCl_3$ extract of the tubers of *Boschniakia himalaica* was found to possess strong feeding deterrent activity against the red flour beetle, *Tribolium castaneum*. From the MeOH/$CHCl_3$ extract, three feeding deterrents were isolated by bioassay-guided fractionation. The constituent compounds were isolated and identified as 3β-acetoxyurs-12-en-28-oic acid (1), 3β-acetoxyurs-28,13-olide (2) and (+)-pinoresinol monoglucoside (3) based on high-resolution electron impact mass spectrometry and nuclear magnetic resonance. Compounds 1, 2 and 3 exhibited feeding deterrent activity against *T. castaneum* adults with ED_{50} values of 378, 940 and 609 ppm, respectively.

Key words: *Boschniakia himalaica, Tribolium castaneum*, feeding deterrent.

INTRODUCTION

The red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) is one of the common pests found in indoor food storage facilities (Liu and Ho, 1999). Infestations not only cause significant losses due to the consumption of grains, they also result in elevated temperature and moisture conditions that lead to an accelerated growth of molds, including toxigenic species (Magan et al., 2003). Control of *T. castaneum* populations is primarily dependent on repeated applications of conventional insecticides or fumigants (Zettler and Arthur, 2000; Rajendran and Srianjini, 2008). Although effective, their repeated use fosters serious environmental and human health concerns (Isman, 2006, 2008). These problems have highlighted the need for development of selective stored product beetle-control alternatives. Plant secondary metabolites are known to have several biological activities against different insect species (Isman, 2006, 2008). The ecological importance of terpenoids in plant defense is well established; among them, some neo-clerodane diterpenes (Sosa et al., 1994; Enriz et al., 2000) and triterpenes (Argandona and Faini, 1993; Chandramu et al., 2003; Mallavadhani et al., 2003; Pungitore et al., 2005) have demonstrated antifeedant activity toward several insect species. In our screening program for new agrochemicals from local wild plants and Chinese medicinal herbs, MeOH/$CHCl_3$ extract of the tubers of *Boschniakia himalaica* Hook. f. et. Thoms (Family: Orobanchaceae) was found to possess strong feeding deterrent activity against the red flour beetle, *T. castaneum* (Liu et al., 2007).

Boschniakia is a small genus of three species of parasitic plant in the broomrape family. They are commonly known as groundcones and they are native to western North America and extreme northeastern Asia (Wu and Raven, 1998). *B. himalaica* is mainly distributed in Yunnan, Tibet, Shanxi, Sichuan, and Hubei provinces of China, and also in Bhutan, North India, Nepal, and Sikkim. It is parasitic on species of *Rhododendron* (Wu and Raven, 1998); it is a folk Tibetan medicine and a

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traditional Chinese medicinal herb. The whole plant is used medicinally for regulating vital energy, alleviating pain, relieving cough, and reducing sputum (Jiangsu New Medical College, 1977). A few studies on the chemical constituents of *B. himalaica* have been reported and a number of lignans and triterpenoids have been isolated from this plant (Chen and Shang, 1992; Liu et al., 2004; Jin et al., 2007, 2008). However, to date, there has been no report on the feeding deterrent activity of *B. himalaica* extracts and its active constituents. Accordingly, in this study, we attempted to isolate and identify three active constituents of the tubers of *B. himalaica* that showed feeding deterrent activity against the red flour beetle, *T. castaneum* using bioactivity directed fractionation.

MATERIALS AND METHODS

Insect

The red flour beetle (*T. castaneum*) was obtained from laboratory cultures maintained for the last 15 years in the dark, in incubators at 29 to 30°C and 70 to 80% relative humidity. The red f lour beetles were reared on wheat flour mixed with yeast (10:1, w/w) in glass jars (diameter 85 mm, height 130 mm) at 29 to 30°C and 70 to 80% relative humidity. Unsexed adult beetles used in all the experiments were about 2 weeks old. All containers housing insects and the glass vials used in experiments were made escape proof with a coating of polytetrafluoroethylene (Fluon).

Plant material

The fresh tubers (5 kg) of *B. himalaica* were harvested in July 2009 from Lijiang City (Latitude: 26.86° N, Longitude: 100 .25°E), Yunnan Province 674800. The fresh tubers were air-dried for one week and ground to a powder using a grinding mill (Retsch Muhle, Germany). The species was identified by Dr. Liu, Q.R. and the voucher specimen (BNU-Liuzhilong-2009-07-18-008) was deposited at the Herbarium (BNU) of College of Life Sciences, Beijing Normal University.

Extraction and isolation

The ground powder was extracted with methanol/chloroform (10:1, 10 L) at room temperature over a period of three weeks. The extracts were concentrated using a vacuum rotary evaporator to afford a syrupy gum (177 g). This syrup was partitioned between methanol-water and *n*-hexane (3 × 1,000 ml). The *n*-hexane extracts were evaporated off to give a 29 g residue. The aqueous layer was re-partitioned with chloroform (3 × 1,000 ml) to provide a 30.2 g residue after evaporation of chloroform. Further partitioning with ethyl acetate (3 × 1,000 ml) gave a 23 g residue after evaporation of the solvent.

The chloroform residue (25 g) was applied to a silica gel column (160 to 200 mesh, Qingdao Marine Chemical Plant, China), eluting with chlroform containing increasing accounts of methanol (from 100:1 to 1:1) to give 6 combined fractions according to thin layer chromatography (TLC) detection. Based on bioassay, fractions 3 and 6 were chosen for further fractionation. 3β-Acetoxyurs-12-en-28-oic acid (1; 76 mg; Figure 1) and 3β-acetoxyurs-28,13-olide (2; 80 mg) were isolated from fraction 3 after being repeatedly purified on silica and preparative thin layer chromatography (PTLC, pre-coated G plates, Qingdao Marine Chemical Plant, China). Fraction 6 was further chromatographed on silica gel column and repeated PTLC to provide the bioactive compound which was determined to be (+)-pinoresinol monoglucoside (3; 1.09 g). The structures of the compounds were elucidated based on high-resolution electron impact mass spectrometry and nuclear magnetic resonance.

Instrumentation

Melting points were recorded using a Buchi 535 and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on Bruker Avance DRX 500 instruments using $CDCl_3$ and DMSO-d_6 as solvents with TMS as internal standard. EI-MS were determined on a ThermoQuest Trace 2000 mass spectrometer at 70 eV (probe); ESI-MS were determined on a Finnigan LCQ mass spectrometer.

TLC experiments were developed on readymade 0.25 mm thick layer of silica gel G (Qingdao Marine Chemical Plant, Shandong province, China) coated glass sheets and visualized by observation under UV light (254 and 365 nm). Normal column chromatography (CC) was conducted using different sizes of columns packed with silica gel (160 to 200, 200 to 300 mesh). PTLC was run on 0.5 mm thick layer silica gel G containing gypsum ($CaSO_4$ binder) coated on 20 × 20 cm glass plates (Qingdao Marine Chemical Plant, Shandong province, China).

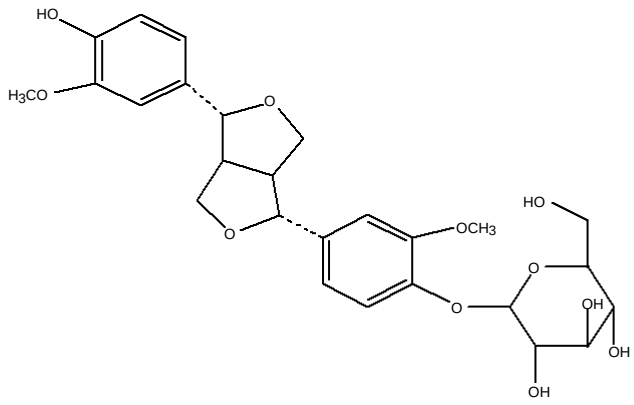
Feeding deterrent test

A flour disk bioassay was used to direct the isolation of active compounds from *B. himalaica* tubers according to the method of Xie et al. (1996) with some modifications (Liu et al., 2009). Wheat flour (0.8 g) was ultrasonically stirred in 4 ml of distilled water, and 50 µl ethanol containing a fraction/compound was added. Pure compounds were first dissolved in 500 µl ethanol and two drops of Tween-20 (approximately 50 µg) were added to the wheat flour suspension. Aliquots of 200 µl of this stirred suspension were placed on the bottom of a polystyrene Petri dish to form disks. The pipette was fitted with a disposable tip that had an opening enlarged to about 2 mm internal diameter by cutting about 1 cm from the bottom of the tip with a razor blade. The same amounts of ethanol and Tween-20 were applied to produce the control flour disks. The flour disks were left in the fume-hood overnight to air dry. The flour disks were then transferred to an incubator to equilibrate at 28 to 30°C and 70 to 80% R.H. for 48 h. Each flo ur disk weighed between 36 and 39 mg. The moisture content of the disk was determined to be 13.5±0.1% using the Kett's Grain moisture tester (Model PB-1D2, Japan). The disks were placed in glass vials (diameter 2.5 cm, height 5.5 cm) for weighing. Twenty group-weighed, unsexed insects were then added to each vial prior to further weighing. All the insects were starved for 24 h before use. The experimental set-up was left in the incubator for 3 days. Finally, insects and the uneaten parts of the flour disks were weighed. The insect consumption for the different test substances was compared to the control group. Glass vials containing treated flour disks but without insects were prepared to determine any decrease in weights that might have occurred due to evaporation of solvents. Analysis of variance (ANOVA) and Tukey's test were conducted by using SPSS 10 for Windows 98. Percentage was subjected to an arcsine square-root transformation before ANOVA and Tukey's tests. The EC_{50} (the concentration needed to inhibit insect feeding by 50% relative to controls) was determined by linear regression (Sakuma, 1998).

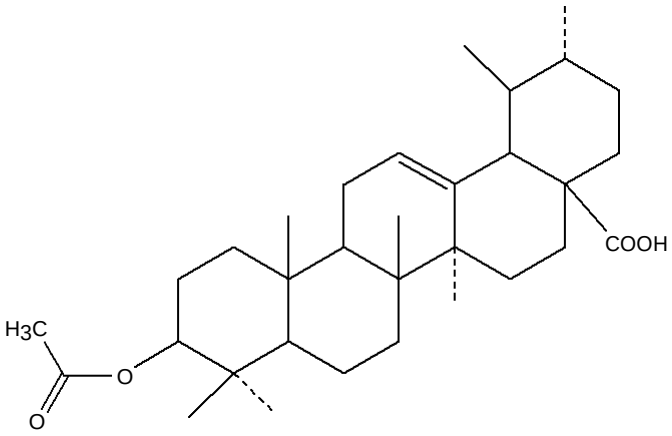
RESULTS AND DISCUSSION

Bioactive compounds isolation data

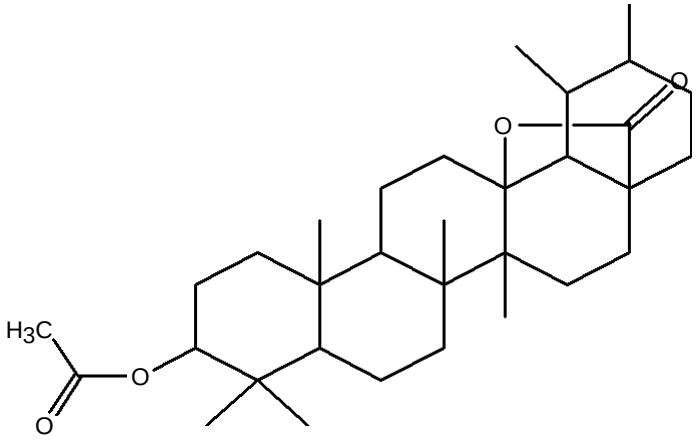
Based on bioassay-guided fractionation, six compounds



(+)-Pinoresinol monoglucoside



3β-Acetoxyurs-12-en-28-oic acid



3β-Acetoxyurs-28,13-olide

Figure 1. Constituent compounds isolated from the tubers of *B. himalaica*.

were separated and purified by column chromatography and preparative thin layer chromatography. The identifications were supported by the following data:

1) 3β-Acetoxyurs-12-en-28-oic acid, white needle, m.p. 252-254°C, EI-MS m/z(%): 498[M^+] (10), 248 (100), 203 (50), 190 (30), 133 (60), and 43 (90). $C_{32}H_{50}O_4$. ^{1}H-NMR (500 MHz, $CDC1_3$) δ: 5.26 (1H, brs, H-12), 4.52 (1H, dd, *J* = 9.5, 6.0 Hz, H-3), 2.21 (1H, d, *J* = 11.0 Hz, H-18), 2.07 (3H, s, -CO-CH_3), 1.10 (3H, s, H-27), 0.98 (3H, s, H-25), 0.98 (3H, d, H-30) 0.88 (3H, d, H-29),0.89 (3H, s, H-23), 0.87 (3H, s, H-24), 0.79 (3H, s, H-26). ^{13}C-NMR(125MHz, CD_3Cl) δ: 183.04 (C-28), 171.02 (C-32, -$\underline{C}$O-CH_3), 137.96 (C-13), 125.76 (C-12), 80.94 (C-3), 55.3 (C-5), 52.56 (C-18), 47.95 (C-17), 47.47 (C-9), 41.92 (C-14), 39.5 (C-8), 39.03 (C-19), 38.83 (C-20), 38.27 (C-1), 37.7 (C-4), 36.92 (C-10), 36.72 (C-22), 32.85 (C-7), 30.6 (C-21), 28.08 (C-23), 27.99 (C-15), 24.08 (C-16), 23.57 (C-2), 23.57 (C-27), 23.29 (C-11), 21.31 (C-31, -CO-$\underline{C}$$H_3$), 21.18 (C-30), 18.16 (C-6), 17.11 (C-29), 17.02 (C-26),16.71 (C-24), 15.54 (C-25). The ^{1}H and ^{13}C NMR data were in agreement with the reported data (Tkachev and Denisov, 1994).

2) 3β-Acetoxyurs-28,13-olide, white needle, m.p. 248-250°C, EI-MS m/z(%): 498[M^+](9), 248(80) 234(100), 203(40), 189(60), 133(30), 119(35), and 43(50). $C_{32}H_{50}O_4$. ^{1}H-NMR (500 MHz, $CDC1_3$) δ: 4.51 (1H, d, *J* = 10.0, 6.2 Hz, H-3), 2.07 (3H, s, -CO-CH_3), l.20 (3H, s, H-26), 1.20 (3H, s, H-27), 1.12 (3H, d, H-29), 0.97 (3H, s, H-23), 0.92 (3H, s, H-25), 0.86 (3H, s, H-24), 0.86 (3H, d, H-30). ^{13}C-NMR (125 MHz, $CDCl_3$) δ:180 (C-28), 170.03 (-$\underline{C}$O-CH_3), 93.2 (C-13), 80.79 (C-3), 61.18 (C-18), 55.3 (C-5), 51.2 (C-9), 45.68 (C-17), 43.24 (C-14), 42.43 (C-8), 39.8 (C-20), 38.7 (C-1), 38.69 (C-19), 37.8 (C-4), 36.99 (C-10), 34.66 (C-12), 34.08 (C-7), 31.59 (C-22), 30.84 (C-21), 27.94 (C-23), 27.03 (C-15), 23.66 (C-2), 22.87 (C-16), 21.28 (-CO-$\underline{C}$$H_3$), 19.55 (C-30), 18.86 (C-11), 18.45 (C-26),17.68 (C-6), 17.59 (C-29), 17.4 (C-27),

Table 1. Feeding deterrents of the constituent compounds isolated from *Boschniakia himalaica* tubers against *T. castaneum* adults.

Treatments	Concentration (ppm)	Consumption of diet* (% control ± SD)	EC_{50} (95% FL)	Slope±SD	Chi square (χ^2)
Control	-	100.00±4.45[a]	-	-	-
Toosendanin	-	-	94.3 (87.3-103.4)	-	-
Compound 1	2000	25.52±1.87[f]	379.9 (337.2-430.2)	2.78±0.11	24.82
	600	38.37±2.56[e]			
	200	57.18±3.28[d]			
	60	83.25±4.54[c]			
	20	92.61±2.43[b]			
Compound 2	2000	42.52±1.78[e]	940.1 (804.1-1118.7)	2.90±0.12	33.75
	600	58.38±2.48[d]			
	200	68.37±3.29[c]			
	60	89.45±3.34[b]			
	20	97.60±3.67[a]			
Compound 3	2000	33.52±2.34[e]	609.0 (537.1-696.8)	3.08±0.12	30.59
	600	43.58±3.26[d]			
	200	69.38±4.02[c]			
	60	87.45±4.43[b]			
	20	96.41±3.21[a]			

* Multiple range test using Tukey's test (P<0.05). The same letters denote treatments not significantly different from each other.

were in agreement with the reported data (Masaaki et al., 1983).

3) (+)-Pinoresinol monoglucoside, yellowish powder, m.p. 180-182°C, EI-MS m/z: 520.19[M^+]. $C_{26}H_{32}O_{11}$. ^{1}H-NMR (500 MHz, DMSO-d_6) δ:8.92 (1H, H-4'), 7.04 (1H, H-5'), 6.96 (1H, s, H-2), 6.89 (1H, H-2'), 6.89 (1H, H-6'), 6.74 (1H, H-5), 6.74 (1H, H-6), 5.21 (1H, s, H-1'', Glu-H)4.67 (1H, H-7), 4.67 (1H, H-7'), 4.48 (1H, H-9), 4.48 (1H, H-9'), 4.14 (1H, H-9), 4.14 (1H, H-9'), 3.77 (3H, s, H-3-OCH_3), 3.77 (3H，s，H-3'-OCH_3), 3.05 (1H, H-8), 3.05 (1H, H-8'). ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 149.4 (C-4'), 148.0 (C-4), 146.4 (C-3'), 146.4 (C-3), 135.7 (C-1'), 132.7 (C-1), 119.1 (C-6'), 118.6 (C-6), 115.7 (C-5'), 115.6 (C-5), 110.9 (C-2), 111.0 (C-2'), 100.7 (C-1'',Glu-C), 85.3 (C-7), 85.6 (C-7'), 77.5 (C-5''),77.3 (C-3''), 73.7 (C-2''), 71.5 (C-9'), 71.4 (C-9), 70.2 (C-4''), 61.1 (C-6'')56.1 (C-3-OCH_3), 56.0 (C-3'-OCH_3), 54.2 (C-8'), 54.0 (C-8). The ^{1}H and ^{13}C NMR data were in agreement with the reported data (Ouyang et al., 2007).

Feeding deterrent activity

The feeding deterrent activity of three isolated compounds against adults of the red flour beetle is shown in Table 1. Incorporation of 3β-acetoxyurs-12-en-28-oic acid into diets at concentrations of 20 ppm and above significantly (P<0.05) reduced food consumption of *T. castaneum* adults compared to the control (Table 1). The consumption of diet (percentage of the control) at 20 to 2000 ppm of 3β-acetoxyurs-12-en-28-oic acid ranged from 92.61 to 25.52% and EC_{50} value was calculated to be 379.9 ppm. The other two isolated compounds also significantly inhibited food consumption of *T. castaneum* adults at concentrations of 60 ppm and above in a concentration-dependent manner (Table 1). Dietary (+)-pinoresinol monoglucoside and 3β-acetoxyurs-28,13-olide also exhibited feeding deterrent activity with EC_{50} values of 609.0 and 940.1 ppm, respectively (Table 1). When compared with the commercial feeding deterrent, toosendanin, the six isolated compounds were 4 to 10 times less active against *T. castaneum* adults (EC_{50} value of toosendanin was determined as 94.3 ppm).

Feeding deterrents/antifeedants, compounds that reduce feeding on plants by insect pests, are gaining importance as potential components of integrated pest management (IPM) strategies for agricultural insect control. There are numerous reports on the feeding deterrent, post-ingestive, and toxic effects as well as repellency of different classes of triterpenoids/lignans against stored product insects (Garcia and Azambuja, 2004; Taylor et al., 2004; Christodoulopoulou et al., 2005; Broussalis et al., 2010; Du et al., 2011; Geng et al., 2011; Liu et al., 2011). To date, there has been no report on the feeding deterrent activity of the three isolated constituents [(+)-pinoresinol monoglucoside, 3β- acetoxyurs-12-en-28-oic acid and 3β-acetoxyurs- 28, 13- olide] against insects.

However, in the previous studies, pinoresinol was shown to be a feeding deterrent to ants (*Crematogaster scutellaris*) and termites (*Reticulitermes balkanensis*) (Christodoulopoulou et al., 2005) as well as other ants (*Formica exsectoides*) (Schroeder et al., 2006). Pinoresinol maybe an inhibitor of ecdysis of insects because oral treatment with pinoresinol reduces ecdysis in fourth-instar larvae of *Rhodnius prolixus* (Cabral et al., 2000). Moreover, many pentacyclic triterpenoids have been demonstrated to feeding deterrent activity against insects. For example, ursolic acid exhibited antifeedant activity against *Spilosomu obliqua* and *Spodoptera litura* with effective dose (ED_{50}) of 1730 and 1986 ppm, respectively (Shukla et al., 1996). Ursolic acid and betulinic acid also exhibit strong antifeedant activity against the larvae of the castor semilooper (*Achoea janata*) (Chandramu et al., 2003). Oleanolic acid has been reported as an antifeedant to *Heliothis zea* (Argandona et al., 1993) and stored products insects, *Sitophilus oryzae* adults (Pungitore et al., 2005). Moreover, the 3-O-fatty acid ester derivatives (C_{12}-C_{18}) of two pentacyclic triterpenic acids, ursolic acid and oleanolic acid had been synthesized and evaluated for antifeedant activity against tobacco caterpillar larvae (*S. litura*) (Pungitore et al., 2003). Urs-12-ene-28-carboxy-3α-octadecanoate and olean-12-ene-28-carboxy-3α-hexadecanoate were found to exhibit exceptionally potent antifeedant activities at a concentration of 50 μg/cm^2, even after 48 h. These findings suggested that pentacyclic triterpenoids may provide us useful models for the development of potent feeding deterrents.

3β-Acetoxyurs-12-en-28-oic acid exhibited 2.5 times stronger feeding deterrent activity against the red flour beetles than 3β-acetoxyurs-28,13-olide (Table 1). It seems that the acid moiety at C_{17} is very important for increased feeding deterrent activity. It was reported in the literature that the acid moiety at C_{17} and the ester functionality at C_3 are essential for enhanced biological activities (for example, anti-HIV) of pentacyclic triterpenes (Kashiwada et al., 2000; Ma et al., 2000). In traditional Chinese medicine, *B. himalaica* is used for regulating vital energy, alleviating pain, relieving cough, and reducing sputum (Jiangsu New Medical College, 1977). However, no experimental data about the safety of extracts of this medicinal herb and the three isolated constituents is available so far. Therefore, any attempt to develop a triterpenoid-derived agrochemical must be carefully evaluated for harmful effects.

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