

Repellent from Traditional Chinese Medicine, *Periploca sepium* Bunge

Jianxiao Shi^{a,b}, Tomoko Yamashita^a, Ayumi Todo^a, Teruhiko Nitoda^a,
Minoru Izumi^a, Naomichi Baba^a, and Shuhei Nakajima^{a,*}

^a Graduate School of Natural Science and Technology, Department of Applied Bioscience and Biotechnology, Laboratory of Natural Products Chemistry, Okayama University, Tsushima naka 3-1-1, Okayama 700-8530, Japan. Fax: +81-86-251-8302. E-mail: snaka24@cc.okayama-u.ac.jp

^b College of Life Science, Northwest University, Xi'an 710069, People's Republic of China

* Author for correspondence and reprint requests

Z. Naturforsch. **62c**, 821–825 (2007); received May 14, 2007

By using a new bioassay-guided method, 2-hydroxy-4-methoxybenzaldehyde isolated from the root bark of *Periploca sepium*, a traditional Chinese medicine, showed repellent activity against the olive weevil (*Dyscerus perforatus*) at 62.5, 125, 250 and 500 µg/disc, respectively. In addition, it also exhibited antinematodal activity against *Bursaphelenchus xylophilus* at a minimum effective dose of 200 µg/ball. The three related compounds obtained were also evaluated for the above-mentioned bioactivities.

Key words: New Method, Repellent and Antinematodal Activities, *Periploca sepium*

Introduction

The olive weevil [*Dyscerus perforatus* (ROE-LOFS); Coleoptera; Curculionidae], a native species in Japan, is of economic importance as a pest in agricultural situations, causing serious damage to its host plant, *Olea europaea*, the oil and fruit of which are very important products and its barks have been utilized as a remedy for some diseases (Ayres and Loike, 1990). Given a long survival time in a room (Matsuzawa *et al.*, 1958) and a long egg-laying period, the olive weevils have become a most devastating pest for its host plant, by weakening the infested parts of the plant and withering even the whole individuals. Furthermore, the few efficient strategies for controlling the pests have resulted in a further spread over to the infested area. In the efforts to search for the relationship between the olive weevils and its host plant, some compounds isolated from the host plant (Nakajima *et al.*, 1995; Kadowaki *et al.*, 2003a, b) have been found to possess the character of attractants toward the olive weevils.

The rootbark of *Periploca sepium* Bunge. (Asclepiadaceae), known as 'xiang jia pi' in China, is a traditional Chinese medicine and has been widely used as a remedy for expelling wind-dampness, promoting urination, alleviating swelling, and strengthening the heart (Pharmacopoeia Commission, 1995). As a part of our continuing program aimed toward the biological activity of traditional

Chinese medicinal plants and during the course of screening repellent-active substances from the plant, we found that the plant had repellent activity against the olive weevil, *D. perforatus*. The presence of steroidal glycosides A, B, C, D, E, neridienone A, periplogenin and periplocogenin in this plant has been reported by Itokawa *et al.* (1987, 1988a, b), showing the significant antitumour activity, but reports that the compounds from this plant have been applied to control the pests in the agriculture sector, have not been found in the literature so far.

In this study, under the bioassay-guided method, we isolated and purified biologically active substances from the rootbark of *P. sepium* and examined their repellent activity against the olive weevil and their antinematodal activity.

Materials and Methods

General experimental procedure

Melting points, specific rotation and IR (KBr) spectral data were taken on a Model MP-21 apparatus, on a Jasco DIP-360 polarimeter and on a Jasco FT/IR-5000 spectrometer, respectively. All the NMR experiments were performed on a Varian VX500 spectrometer (500 MHz for ¹H NMR and 125 MHz for ¹³C NMR). GC-MS (Automass 20, Jeol) analyses in the electron impact ionization

(EI, 70 eV) mode were conducted with a DB-1 column (0.25 mm \varnothing , 30 m), using a temperature program from 50 °C (3 min) to 250 °C (70 min) at 10 °C/min and an ion source temperature of 210 °C. Column chromatography (CC) was performed on silica gel 60 (Merck, 230–400 mesh) and thin layer chromatography (TLC) was carried out on silica gel 60 F₂₅₄ plates (Merck, 0.25 mm).

Plant material

The root bark of *P. sepium* was purchased from Xi'an Traditional Chinese Medicine Market in China and identified by Associate Professor Li Zhixuan and the type specimen is deposited at the College of Life Science of Northwest University, Xi'an, China.

Insect material

During the adult stage, the male and female olive weevils (*D. perforatus*) collected from infested olive trees (*O. europaea* L.) were separately brought up in plastic containers with pieces of young branches from olive trees and maintained at 25 °C for the repellent evaluation.

Chemicals

2-Hydroxy-5-methoxybenzaldehyde, vanillin, and *o*-vanillin were procured from Aldrich Chemical Co. (Japan).

Extraction and isolation

The dried rootbark of *P. sepium* (5.1 kg) was macerated with EtOH for 14 d at the room temperature. After filtration, evaporation to dryness yielded a residue of 377.26 g which was suspended in water; the suspension was separated with *n*-hexane and EtOAc. Based on the fractionation-directed method, the separated *n*-hexane layer showed the highest activity in three fractions. The active layer (169.14 g) was subjected to silica gel chromatography column eluted with a gradient of *n*-hexane/EtOAc to yield seven fractions, I–VII, tested for their bioactivity. The active fraction IV (53.13 g) obtained was subjected to silica gel CC eluted with *n*-hexane/EtOAc (85:15, v/v) to yield two fractions, IV-1 and IV-2. The higher active fraction IV-2 (29.486 g) was subjected to purification by silica gel CC eluted with a gradient of *n*-hexane and EtOAc to yield the active compound IV-2-2 (16.345 g).

Active compound: Colourless needles (hexane), m.p. 40.5–41.5 °C. – $[\alpha]_D^{25} -6.0^\circ$ (c 0.2, MeOH). – IR (KBr): $\nu_{\max} = 3026, 2960, 2854, 1647, 1578, 11518, 1479, 1241, 1166, 1139, 1021, 949, 849 \text{ cm}^{-1}$. – UV (EtOH): $\lambda_{\max} (\log \epsilon) = 211 (3.77), 230 (3.65), 277 (3.81), 315 \text{ nm} (3.40)$. – $^1\text{H NMR}$ (CDCl_3): $\delta = 3.84 (3\text{H, s, -OCH}_3), 6.41 (1\text{H, d, } J = 4.0 \text{ Hz, H-3}), 6.52 (1\text{H, dd, } J = 14.5, 4.0 \text{ Hz, H-5}), 7.41 (1\text{H, d, } J = 14.5 \text{ Hz, H-6}), 9.70 (1\text{H, s, CHO-}), 11.48 (1\text{H, s, -OH})$. – $^{13}\text{C NMR}$ (CDCl_3): $\delta = 55.6 (-\text{OCH}_3), 100.6 (\text{C-3}), 108.3 (\text{C-5}), 115.1 (\text{C-1}), 135.2 (\text{C-6}), 164.4 (\text{C-2}), 166.8 (\text{C-4}), 199.4 (\text{CHO-})$. – GCCIMS: m/z (rel. int.) = 152 $[\text{M}]^+$ (70), 108 (5), 95 (17), 81 (2).

Repellent bioassay with olive weevils

The experimental setup for determining the repellent activity against olive weevils was as follows. The upper plastic container (\varnothing 11 cm, height 8 cm) connected with a small pump (AS ONE, Japan) by a rubber tube was placed on the top of the lower plastic container (\varnothing 11 cm, height 8 cm), divided into the equivalent two parts with tiny nets fixed on its upper surface (Fig. 1). The filter papers (\varnothing 16 mm) with the test sample dissolved in EtOAc and with EtOAc alone as a control, after the solvent being evaporated, were put into the differently separated part of the lower container with a vent on the opposite side of the lower container near its bottom. The plastic tube connected each vent with one glass container full of the ap-

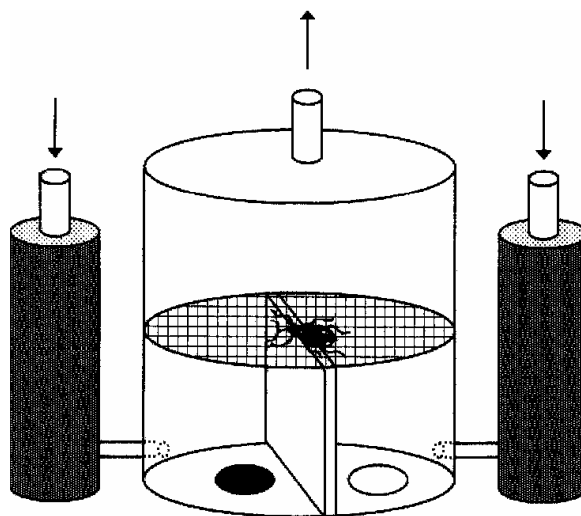


Fig. 1. Experimental setup used for the bioassay of olive weevils.

appropriate active carbon, each glass container with a small vent on its top was connected to a flow meter (LPM AIR) through a plastic tube, respectively.

The bioassay was conducted in the dark (air flow rate, 1 L/min; temperature, 28 °C). Five olive weevils were lined on the center of the net for each test, followed by starting the pump. After 10 min, the move position of the weevils was observed and recorded. If the weevils moved to the sample side, the sample was judged to possess attractant activity toward the weevils; if the weevils moved to the control side, the sample was judged as repellent against the weevils. The RAI (repellent activity index) was calculated as follows: $RAI = (C - S)/(C + S)$, where C is the number of control side weevils and S the number of sample side weevils.

Antinematodal bioassay with nematodes

The bioassay method by Kawazu *et al.* (1980) with some modification (Alen *et al.*, 2000) was carried out (each cotton ball with about 1500 head nematodes).

Results and Discussion

By bioassay-directed fractionation, the concentrated residue from the rootbark of *Periplora sepium*, after macerated with EtOH, was separated and purified to yield the active principle (16.345 g

The active compound was obtained as colourless needles, corresponding to the molecular formula $C_8H_8O_3$ indicated by 1H and ^{13}C NMR spectral data in combination with GCCIMS (m/z 152 [M^+]). The coupling pattern of 1H NMR at δ 6.41 (1H, d, $J = 4.0$ Hz), 6.52 (1H, dd, $J = 14.5, 4.0$ Hz) and 7.41 (1H, d, $J = 14.5$ Hz) revealed a 1,2,4-trisubstituted benzene ring. The NMR and GCCIMS spectral data suggested the presence of an aldehyde group (δ_H 9.70 s, δ_C 199.4) and of a methoxy group (δ_H 3.84 s, δ_C 56). A chemical shift (δ_H 11.48 s) downfield resulted from the formation of an internal hydrogen bonding between an aldehyde group and a hydroxy group, causing in the IR spectrum an absorption at lower wave numbers (1647 cm^{-1}). Thus, the active compound was identified as 2-hydroxy-4-methoxybenzaldehyde (Fig.

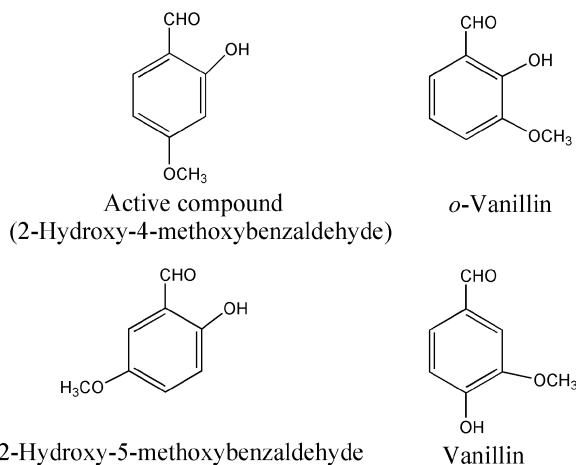


Fig. 2. Structures of the active compound and related compounds.

2), unequivocally confirmed by comparison with NMR spectral data of the authentic sample.

Given its volatile and smell, the active compound assayed for its effects on the olive weevils in the range from $62.5\text{ }\mu\text{g/disc}$ to $500\text{ }\mu\text{g/disc}$ displayed an evidently repellent activity against the olive weevils (Table I).

At $62.5\text{ }\mu\text{g/disc}$, the active compound had the comparatively highest RAI value (0.35) against the female weevils, followed by 2-hydroxy-5-methoxybenzaldehyde ($RAI = 0.26$) and the least was vanillin ($RAI = -0.32$). The active compound also revealed the highest repellent activity against the male weevils ($RAI = 0.44$) in comparison with the other compounds. In addition, under the condition of the equivalent dose the RAI values of vanillin (-0.32) and of o-vanillin (-0.29) indicated attractant activity toward the weevils.

At $125\text{ }\mu\text{g/disc}$ for testing repellent activity against the female weevils, the RAI value of the active compound was lower ($RAI = 0.40$) than that of o-vanillin but higher than that of the other two compounds, and the highest averaged value of RAI ($RAI = 0.49$) in the compounds was obtained examining the activity against the male weevils, showing a most repellent activity.

At 250 and $500\text{ }\mu\text{g/disc}$, respectively, the active compound displayed notably repellent activity against the female weevils with a maximum average RAI value of 0.38 and 0.64, respectively, and against the male olive weevils 0.65 and 0.39, respectively. At $500\text{ }\mu\text{g/disc}$, the active compound, o-vanillin, 2-hydroxy-5-methoxybenzaldehyde and

Treatment	Dose [$\mu\text{g}/\text{disc}$]	RAI ^a (\pm SD)	
		♀	♂
2-Hydroxy-4-methoxybenzaldehyde	500	0.64 (\pm 0.09)	0.39 (\pm 0.22)
<i>o</i> -Vanillin		0.38 (\pm 0.21)	0.11 (\pm 0.16)
2-Hydroxy-5-methoxybenzaldehyde		0.29 (\pm 0.21)	0.25 (\pm 0.16)
Vanillin		0.2 (\pm 0.16)	-0.02 (\pm 0.17)
2-Hydroxy-4-methoxybenzaldehyde	250	0.38 (\pm 0.04)	0.64 (\pm 0.09)
<i>o</i> -Vanillin		0.06 (\pm 0.22)	0.17 (\pm 0.22)
2-Hydroxy-5-methoxybenzaldehyde		0.15 (\pm 0.13)	0.17 (\pm 0.27)
Vanillin		0.12 (\pm 0.21)	0.04 (\pm 0.32)
2-Hydroxy-4-methoxybenzaldehyde	125	0.40 (\pm 0.17)	0.49 (\pm 0.29)
<i>o</i> -Vanillin		0.57 (\pm 0.05)	0.18 (\pm 0.18)
2-Hydroxy-5-methoxybenzaldehyde		0.30 (\pm 0.26)	0.11 (\pm 0.43)
Vanillin		-0.04 (\pm 0.28)	0.12 (\pm 0.38)
2-Hydroxy-4-methoxybenzaldehyde	62.5	0.35 (\pm 0.10)	0.44 (\pm 0.24)
<i>o</i> -Vanillin		0.02 (\pm 0.21)	-0.29 (\pm 0.24)
2-Hydroxy-5-methoxybenzaldehyde		0.26 (\pm 0.15)	0.08 (\pm 0.34)
Vanillin		-0.32 (\pm 0.11)	-0.17 (\pm 0.13)

Table I. Effect of the dose of the repellent 2-hydroxy-4-methoxybenzaldehyde from *P. sepium* against the weevils as compared to that of 2-hydroxy-5-methoxybenzaldehyde, *o*-vanillin and vanillin.

^a Data are expressed as means of repellent activity index \pm SD ($N = 100$, 4 replicates).

vanillin showed decreasingly repellent activity against the female weevils, and the repellent activity against the male weevils was highest for the active compound, the next were 2-hydroxy-5-methoxybenzaldehyde, *o*-vanillin and vanillin.

At 62.5, 125 and 250 $\mu\text{g}/\text{disc}$, respectively, the active compound showed similar repellent activity against the female weevils, and the most potent was at the dose of 500 $\mu\text{g}/\text{disc}$. At 62.5, 125 and 500 $\mu\text{g}/\text{disc}$, respectively, obtaining nearly the same RAI values demonstrated that there was no significant difference in the ranges of the dose-dependent study against the male weevils, and at 250 $\mu\text{g}/\text{disc}$ the strongest repellent activity was observed.

It is interesting to note that with the increase of the dose from 250 $\mu\text{g}/\text{disc}$ to 500 $\mu\text{g}/\text{disc}$ the repellent activity against the female and male weevils appeared to have the opposite tendency, the former on the decrease and the latter on the increase, and so did some similar results for the related compounds (Table I).

In the meantime, we made a choice of the dose of 400, 200, 100, 50, 40, 20, and 10 $\mu\text{g}/\text{cotton ball}$ to measure the active compound and the related compounds for their antinematodal activity against *Bursaphelenchus xylophilus*. The results showed that there was no difference between the active compound and the authentic sample (data not shown), displaying that its antinematodal activity at a minimum effective dose (MED) of 200 $\mu\text{g}/\text{cotton ball}$ was stronger than that of both 2-hydroxy-5-methoxybenzaldehyde and *o*-vanillin at a MED of 400 $\mu\text{g}/\text{cotton ball}$. Vanillin (Table II) showed the least antinematodal activity against nematodes. In addition, the active compound still showed antinematodal activity at 20 $\mu\text{g}/\text{cotton ball}$ and appeared to be not antinematodal at 10 $\mu\text{g}/\text{cotton ball}$ (Table II).

The aldehyde is an important factor for the biological inhibitory activity mentioned above, which easily reacts with biologically important nucleophilic groups. The positions of the hydroxy group

Table II. Antinematodal activity of 2-hydroxy-4-methoxybenzaldehyde (2,4) against *B. xylophilus* in comparison with that of 2-hydroxy-5-methoxybenzaldehyde (2,5), *o*-vanillin and vanillin ($\mu\text{g}/\text{cotton ball}$).

Compound	400	200	100	50	40	20	10
2,4	+	+	\pm	\pm	\pm	\pm	-
2,5	+	\pm	\pm	-	-	-	-
<i>o</i> -Vanillin	+	\pm	-	-	-	-	-
Vanillin	\pm	\pm	-	-	-	-	-

The sign + stands for the mycelia not being consumed, the sign - stands for the mycelia being completely consumed, the sign \pm stands for the mycelia being partly consumed. Each compound test was evaluated in triplicate.

and the methoxy group on the ring of these compounds produce important effects on biologically active inhibition. The *o*-hydroxylaldehyde moiety, together with the biologically nucleophilic groups, forms a stable six-membered ring and increases its inhibitory activity, and the *p*-methoxy group donates electrons to further stabilize the structure (Kubo and Kinst, 1998). In the tested compounds, the active compound, *o*-vanillin and 2-hydroxy-5-methoxybenzaldehyde, owing to different positions of the methoxy group on their ring, compounds with the *para*-orientating methoxy group exhibited more potential activities than the other compounds. The active compound exhibited the most potent tyrosinase inhibitory activity in the related compounds (Anderson, 1979); tyrosinase is one of the significant enzymes in the insects molting process (Neville, 1975). Because vanillin may not form a stable six-membered ring by reacting with the biologically nucleophilic groups, in general, it showed the least biological activity of all tested compounds mentioned above.

Natural product-based pesticides could offer more advantages in that they have possessed the less risk to the human health and the environment in application for the pest control as alternatives in agricultural sectors compared with the synthetic pesticide agents. The compound, although the simple structure, together with the related compounds, discovered in the present study using the new method appears to play a significant role in the repellent activity against the olive weevils, in the meantime, and it also displayed the antinematodal activity against the nematode, *B. xylophilus*. The natural product based on pesticides occurring in this plant may provide the potential and commercial application for the pest control in that the compound not only has a characteristic of the simple structure in it and of the easiness to be synthesized but shows no apparently active discrepancy mentioned above compared with that of the authentic sample (data not shown).

- Alen Y., Nakajima S., Nitoda H., Baba N., Kanzaki H., and Kawazu K. (2000), Antinematodal activity of some tropical rainforest plants against the pinewood nematodes, *Bursaphelenchus xylophilus*. *Z. Naturforsch.* **55c**, 295–299.
- Anderson S. O. (1979), Biochemistry of the insect cuticle. *Annu. Rev. Entomol.* **24**, 29–61.
- Ayres D. C. and Loike J. D. (1990), Lignans. Cambridge University Press, Cambridge.
- Itokawa H., Xu J. P., and Takeya K. (1987), Study on chemical constituents of antitumor fraction from *Periploca sepium* Bge. I. *Chem. Pharm. Bull.* **35**, 4524–4529.
- Itokawa H., Xu J. P., Takeya K., Watanabe K., and Shoji J. (1988a), Study on chemical constituents of antitumor fraction from *Periploca sepium*. II. Structures of new pregnane glycosides, periplocosides A, B and C. *Chem. Pharm. Bull.* **36**, 982–987.
- Itokawa H., Xu, J. P., and Takeya K. (1988b), Study on chemical constituents of antitumor fraction from *Periploca sepium*. IV. Structures of new pregnane glycosides, periplocosides D, E, L and M. *Chem. Pharm. Bull.* **36**, 2084–2089.
- Kadowaki E., Yoshida Y., Nitoda T., Baba N., and Nakajima S. (2003a), (–)-Olivil and (+)-1-acetoxypinoresinol from the olive tree (*Olea europaea* Linne; Oleaceae) as feeding stimulants of the olive weevils (*Dyscerus perforatus*). *Biosci. Biotechnol. Biochem.* **67**, 415–419.
- Kadowaki E., Yoshida Y., Baba N., and Nakajima S. (2003b), Feeding stimulant activity of steroidal and secoiridoid glucosides and their hydrolysed derivatives toward the olive weevils (*Dyscerus perforatus*). *Z. Naturforsch.* **55c**, 441–445.
- Kawazu K., Nishii K., Ishii K., and Tada, M. (1980), A convenient screening method for nematocidal activity. *Agric. Biol. Chem.* **44**, 631–635.
- Kubo I. and Kinst H. I. (1998), 2-Hydroxy-4-methoxybenzaldehyde: A potent tyrosinase inhibitor from African medicinal plants. *Planta Med.* **65**, 19–22.
- Matsuzawa H., Miyamoto Y., Okamoto H., and Kawahara S. (1958), Studies on the control of the olive weevils *Hylobius desbrochersi*. *Tech. Bull. Faculty of Agriculture, Kagawa University* **8**, 172–188.
- Nakajima S., Kitamura T., Baba N., Iwasa J., and Ichikawa T. (1995), Oleuropein, a secoiridoid glucoside from olive, as a feeding stimulant to the olive weevil (*Dyscerus perforatus*). *Biosci. Biotechnol. Biochem.* **59**, 769–770.
- Neville A. C. (1975), *Zoophysiology and Ecology*, 4/5. Springer-Verlag, New York.
- Pharmacopoeia Commission (1995), Chinese Pharmacopoeia (I). Guangdong Science & Technology Publ., China, pp. 344–345.