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ORIGINAL ARTICLE

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Effects of stilbenes from bark of *Picea glehnii* (Sieb. et Zucc) and their related compounds against feeding behaviour of *Reticulitermes speratus* (Kolbe)

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Abstract The effects of stilbene glucosides and related compounds on termite feeding behavior were investigated using paper disc methods against the subterranean termite *Reticulitermes speratus*. The stilbene-rich fraction and isorhapontin (3′-methoxy-3,4′, 5-trihydroxystilbene-3-β-D-glucoside) from bark extracts of *Picea glehnii* showed avoidance by termites in choice tests. In the no-choice tests using compounds purified from the stilbene-rich fraction, the largest feeding deterrent effect was observed for piceid (3,4′,5-trihydroxystilbene-3-β-D-glucoside), followed by isorhapontin, and astringin (3,3′,4′,5-tetrahydroxystilbene-3-β-D-glucoside), at the concentrations from 0.63 to 2.5μmol/disc. No change in activity was observed at retentions of more than 5.0μmol/disc. When the activities of isorhapontin and its aglycone derivative (isorhapontigenin: 3′-methoxy-3,4′,5-trihydroxystilbene) were compared with that of taxifolin (3,3′,4′,5,7-pentahydroxyflavanone) in the no-choice test, the stilbenes exhibited a larger antifeedant potential. Methylation of isorhapontigenin increased its termiticidal activity.

Key words Stilbenes · *Picea glehnii* · Bark · Termite · Antitermite activity

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Introduction

Although bark is a major waste product in wood industries, most waste bark is used as fuel or soil conditioner. It is expected that more effective uses of bark will develop from the viewpoint of saving natural resources. The bark of woody plants contains various extractives such as terpenoids, flavonoids, fatty acids, and waxes that have some biological activities. We have already reported that extractives comprosed of terpenoids, flavonoids, and other materials from the bark of several coniferous species inhibited the growth of wood-degradative microorganisms. Of these extractives, stilbenes that were present in appreciable levels in the bark of *Picea glehnii* (Sieb. et Zucc) showed strong inhibition against the growth of filamentous fungi.[1]

In addition to fungi, termites also play an important role in wood degradation. Many studies on the antitermite activity of extractives from plants have been conducted. Antifeedant substances in the heartwood of *Chamaecyparis obtusa* (Endl.) against *Coptotermes formosanus* Shiraki were identified as α-cadinol and T-murrorol.[2] Saponins such as barrigenol glycoside from *Ternstronea japonica* (Thunb.), exhibited large termiticidal activity against the same species.[3–5] *Adina racemosa* (Miq.), which is known to be termite-resistant, was found to contain scopolin and scopoletin.[6]

With regard to the antitermite activity of natural stilbenes, only pinosylvin (3,5-dihydroxystilbene) and related compounds reportedly had feeding deterrent activity against the West Indian dry wood termite *Crytotermes brevis* (Walker).[7] Stilbenes are well known as biologically active compounds, and have been shown to inhibit the growth of many filamentous fungi and bacteria.[8–12] However, there is no investigation on the activity of stilbenes, especially stilbene glucosides, against subterranean termites.

In this study, stilbenes and related compounds extracted from the bark of *P. glehnii* were investigated for antitermite activity against *Reticulitermes speratus* (Kolbe) by means of the paper disc tests.

Materials and methods

General analyses

High performance liquid chromatography (HPLC) analysis. Purity and yields of components were conducted by HPLC (LC-10; Shimadzu, Tokyo, Japan) with a Cosmosil 5C18AR column (5 × 150 mm, Nacalai Tesque, Kyoto, Japan) and an ultraviolet detector (SPD-10AV: Shimadzu, Tokyo, Japan). The solvent system used a linear gradient of H_2O up to 100% acetonitrile in a total of 50 ml at a flow rate of 1.0 ml/min and a detection wavelength of 280 nm.

Nuclear magnetic resonance (NMR) analysis. ^{13}C-NMR spectra were measured with a 400 MHz NMR spectrometer (Lambda 400, Jeol, Tokyo, Japan). Acetone-d_6 was used as the solvent.

Bark sample

Bark was obtained from logs of *Picea glehnii*, harvested in 1997 from the Hokkaido experimental forest. Bark was air-dried under atmospheric conditions for 2 weeks before extraction.

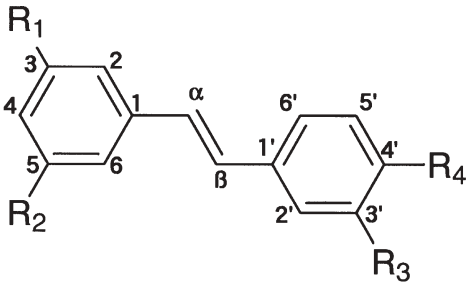
Extraction procedure

Air-dried bark was ground using a Wiley mill to pass through a 1-mm sieve. The bark meal (50 g) was subjected to extraction in *n*-hexane (500 ml) at room temperature for 24 h, replacing the solvent twice. The bark extracted with *n*-hexane was then subjected to extraction with ethyl acetate in the same manner.

Preparation of stilbene-rich fraction and purification

The ethyl acetate extract (3.0 g) was fractionated on a Sephadex LH-20 (Amersham Pharmacia Biotech, Uppsala, Sweden) column (25 × 300 mm) using ethanol as the eluent. The fraction having an Rf value of 0.3 on a thin dayer chromatography (TLC) plate (TLC aluminum sheets: silica gel 60 F_{254} 1.05554, Merck, Darmstadt, Germany) following separation in benzene/acetone/acetic acid (10/10/1, v/v) was kept as the stilbene-rich fraction.[1]

Isorhapontin (3′-methoxy-3,4′,5-trihydroxystilbene-3-β-D-glucoside: Fig. 1a), piceid (3,4′,5-trihydroxystilbene-3-β-D-glucoside: Fig. 1b), and astringin (3,3′,4′,5-tetrahydroxystilbene-3-β-D-glucoside: Fig. 1c) were purified from the stilbene-rich fraction using a preparative HPLC column: Cosmosil 5C18AR column (20 × 250 mm, Nacalai Tesque). A solvent mixture of acetonitrile and H_2O (15/85, v/v) was used as the eluent at a flow rate of 10 ml/min. Yields of the above compounds from the original stilbene-rich fraction were 48%, 9%, and 5%, respectively.



(a) Isorhapontin:
R1=glucoside, R2=OH, R3=OCH3, R4=OH
(b) Piceid:
R1=glucoside, R2=OH, R3=H, R4=OH
(c) Astringin:
R1=glucoside, R2=OH, R3=OH, R4=OH
(d) Isorhapontigenin:
R1=OH, R2=OH, R3=OCH3, R4=OH
(e) Trimethylastringin:
R1=glucoside, R2=OCH3, R3=OCH3, R4=OCH3
(f) Tetramethylastringenin:
R1=OCH3, R2=OCH3, R3=OCH3, R4=OCH3

Fig. 1. Chemical structures of the tested stilbenic compounds

Preparation of isorhapontigenin

Five-hundred milligrams of isorhapontin were dissolved in 20 ml of 5% methanol containing 100 mM sodium acetate adjusted to pH 5.0. The solution was incubated with 500 U almond β-glucosidase (Wako Chemical, Osaka, Japan) at 27°C for 3 days. The incubated mixture was extracted three times with 20 ml ethyl acetate. The combined ethyl acetate solution was evaporated in vacuo to give isorhapontigenin (3′-methoxy-3,4′,5-trihydroxystilbene: Fig. 1d). The yield of isorhapontigenin from its original glucoside was 52%.

Methylation of isorhapontin and isorhapontigenin

An amount of purified isorhapontin or isorhapontigenin was introduced into an ether solution of diazomethane prepared by reacting diethylene glycol monoethyl ether and *p*-tolylsulfonylmethylnitrosamide. Each methylated stilbene obtained by evaporation of ether was identified using ^{13}C-NMR analysis.

Trimethylastringin (3-hydroxy-3′,4′,5-trimethoxystilbene-3-β-D-glucoside). ^{13}C-NMR 100 MHz: δ 140.7 (C-1), 106.8 (C-2), 161.7 (C-3), 102.1 (C-4), 160.3 (C-5), 107.6 (C-6), 131.2 (C-1′), 110.4 (C-2′), 150.5 (C-3′), 150.5 (C-4′), 112.7 (C-5′), 107.6 (C-6′), 127.1 (Cα), 130.1 (Cβ); glucoside residue: 102.6 (C-1), 74.7 (C-2), 77.9 (C-3), 71.5 (C-4), 78.1 (C-5), 62.7 (C-6), 55.6 (-OCH_3: A ring), 56.1 (-OCH_3: B ring), (see Fig. 1e).

Tetramethylastringenin (3,3′,4′,5-tetramethoxystilbene). ^{13}C-NMR 100 MHz: δ 140.8 (C-1), 105.0 (C-2), 162.1 (C-3), 100.3 (C-4), 162.1 (C-5), 105.0 (C-6), 131.3 (C-1′), 110.3 (C-2′), 150.5 (C-3′), 150.6 (C-4′), 112.7 (C-5′), 120.9 (C-6′), 127.4 (Cα), 129.9 (Cβ), 56.1 (-OCH_3: A ring), 55.6 (-OCH_3: B ring), (see Fig. 1f).

The chemical shifts were assigned as above by comparison with the values for original compounds in a previous report.[13]

Tests of taxifolin

Taxifolin purified from Japanese larch (*Larix leptolepis* (Sieb. et Zucc.) Gord.) heart wood was kindly donated by Dr. Masakazu Aoyama of the Hokkaido Forest Products Research Institute.

Termite feeding tests

Termites were collected from an active wild colony of *Reticulitermes speratus* in a pine forest at Akita Prefectural University in July 2002. The colony was maintained in a dark room at 28°C and 80% relative humidity (RH) until use.

Choice and no-choice feeding tests were conducted. Paper discs (thickness, 1.5 mm; diameter, 8 mm; Advantec, Tokyo, Japan) were permeated with ethanol solution containing designated concentrations of the stilbene-rich fraction or of each purified stilbene. Paper discs treated only with solvent were used as controls. All paper discs were vacuum-dried and weighed before exposure to termites.

A container for a five-choice feeding test was made using a 300-ml plastic cup having a 15-mm hole in the bottom stoppered with absorbent cotton, upon which was layered 15 mm of plaster and covered with 10 mm of sand. Test containers were placed on a thick paper sheet that was moistened to supply water to the plaster and sandy layers. Five paper discs were independently placed on plastic saucers and set into the containers. Fifty workers were introduced into each container. Three replications were carried out per set.

No-choice feeding tests were conducted as described above, except that 100-ml plastic cups were used as test containers, with 50 workers being introduced into each.

Paper discs were removed and vacuum-dried after 6 days exposure in the choice test or after 12 days in the no-choice test to determine changes in mass due to termite feeding. Mortality was calculated based on the surviving number of termites after the no-choice test.

Results

Confirmation of avoidance from the stilbene-rich fraction and isorhapontin

Five choice feeding tests were conducted using paper discs treated with the stilbene-rich fraction or isorhapontin to evaluate the avoidance of *Reticulitermes sparatus*. Lower consumption of paper discs permeated with retentions of 1.7%, 3.8%, 7.5%, and 15% (w/w) of the stilbene-rich fraction was clearly observed in the choice test (data not shown). Isorhapontin was a major component of the stilbene-rich fraction. Avoidance from isorhapontin was the same as that of the stilbene-rich fraction in the five-choice tests (Fig. 2). As retention of isorhapontin increased, decreased paper disc consumption was observed.

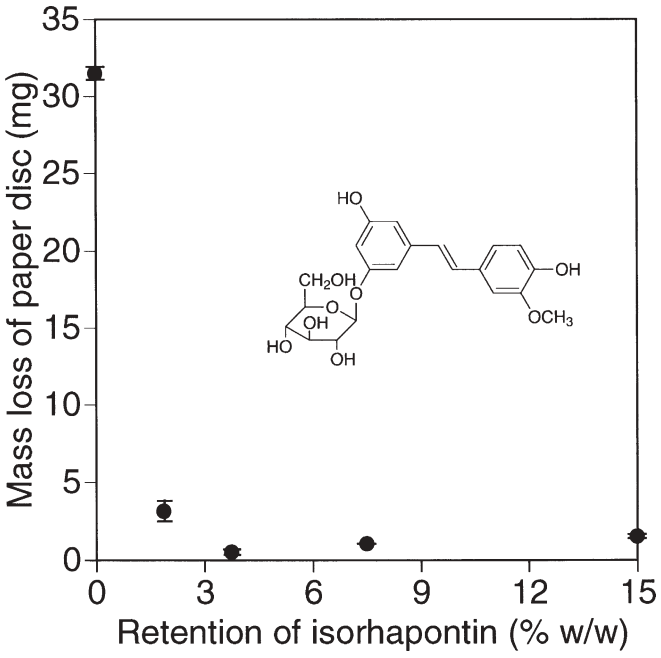


Fig. 2. Results of five-choice tests using the paper disks permeated with purified isorhapontin. Error bars represent standard deviations. The average total consumption of five untreated paper discs was 31.9 mg, standard deviation 1.4 mg

Comparison of antifeedant potentials between the three stilbene glucosides

Figure 3 shows the results of no-choice tests using the three stilbenes. There were significant differences in the antifeedant potentials of the three compounds at retentions of 0.63 μmol/disc. Astringin showed the weakest potential at retentions from 0.63 to 2.5 μmol/disc. No significant difference was observed among the three at retentions of more than 5.0 μmol/disc.

Comparison of antifeedant potential between isorhapontin and its aglycone

In comparing the antifeedant potentials between isorhapontin and isorhapontigenin (Fig. 4), no significant difference was observed at retentions of less than 1.3 μmol/disc. The aglycone had stronger potential than isorhapontin at retentions of more than 2.5 μmol/disc. Mass losses of paper discs permeated with taxifolin were twice as large as

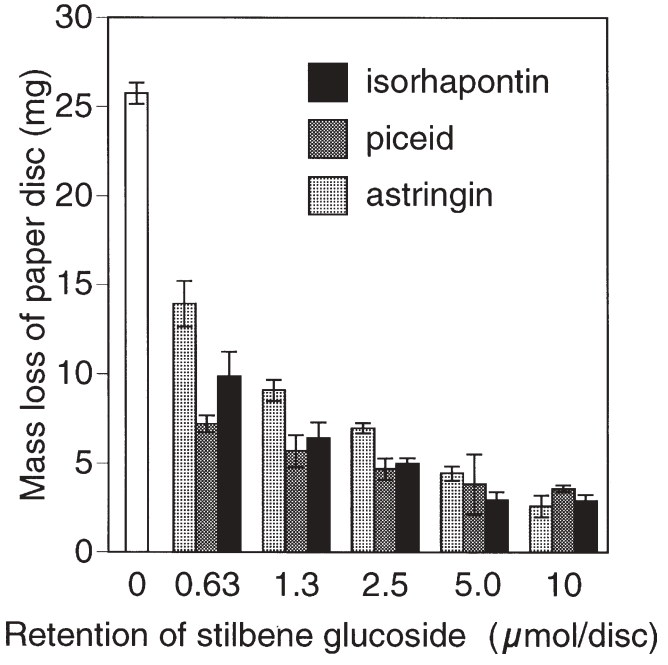


Fig. 3. Comparison of antitermite potential against *Reticulitermes speratus* between three stilbene glucosides

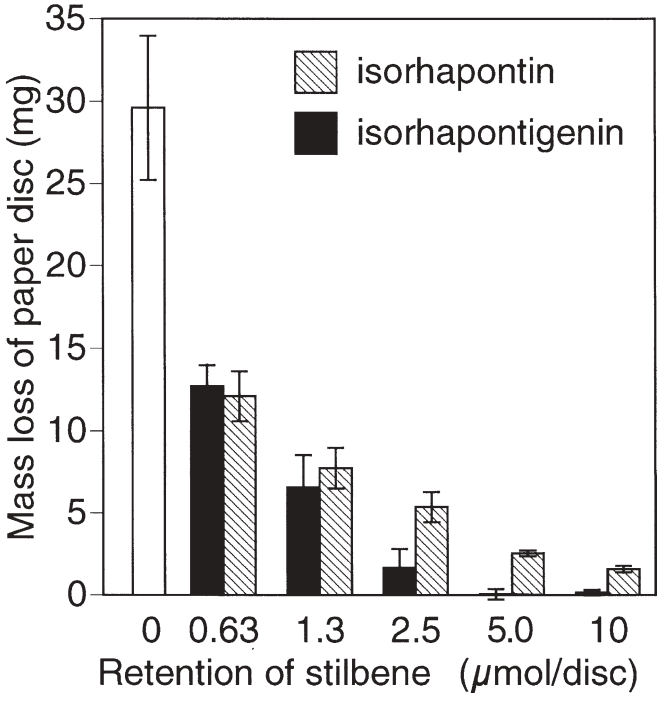


Fig. 4. Comparison of the antitermite potential against *R. speratus* between isorhapontin and isorhapontigenin

those of isorhapontin or isorhapontigenin at the same retentions of 0.63 to 10 μmol/disc. The average weight losses for taxifolin-treated discs were 4.2, 8.1, 17, 22, and 26 mg per disc at 10, 5, 2.5, 1.3, 0.63 μmol/disc, respectively, with the standard deviations being less than 2.5 mg for each.

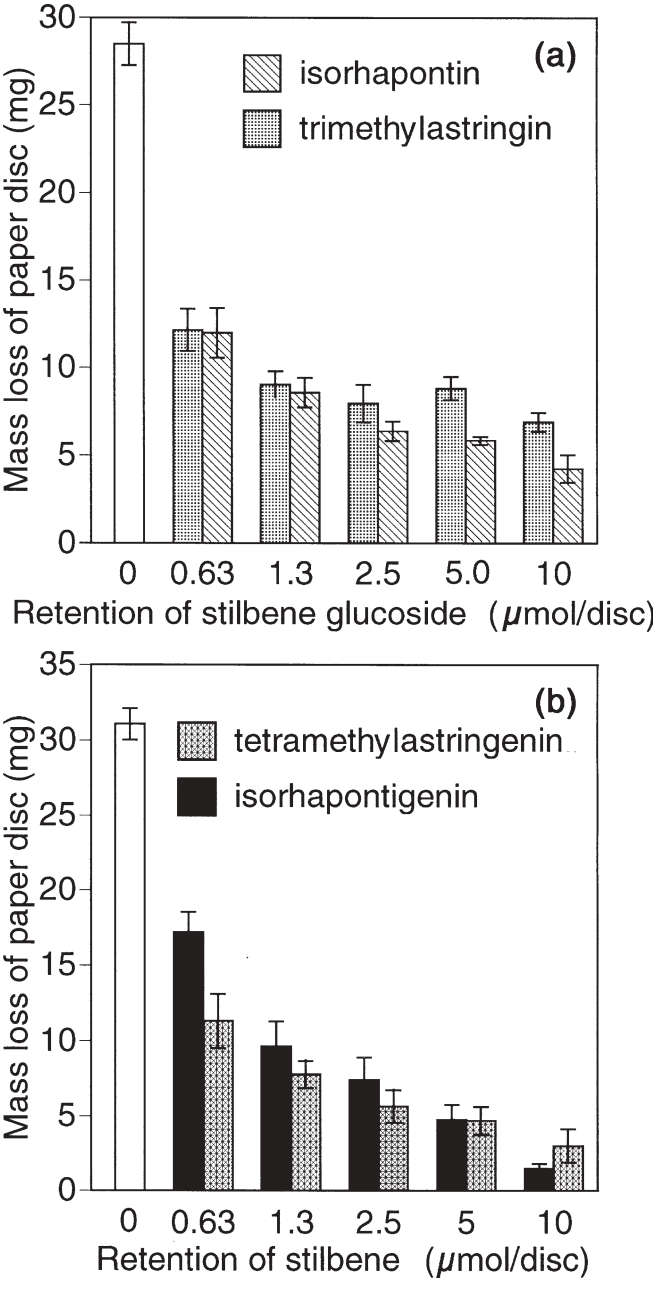


Fig. 5. Comparison of the antitermite potential against *R. speratus* between trimethylastringin and isorhapontin (**a**) and between isorhapontigenin and tetramethylastringenin (**b**)

Antitermite potential of methylated stilbenes

The effects of methylation of stilbenes on their antifeedant activities are shown in Fig. 5. Methylated isorhapontin showed the same antifeedant activity as the original compound up to 2.5 μmol/disc. At more than 5.0 μmol/disc, the methylated compound exhibited larger activities than the original one. This distinct effect of methylation was observed only at 0.63 μmol/disc for aglycone (isorhapontigenin). There was no significant difference in antifeedant activity between the original aglycone and methylated isorhapontigenin at more than 1.3 μmol/disc.

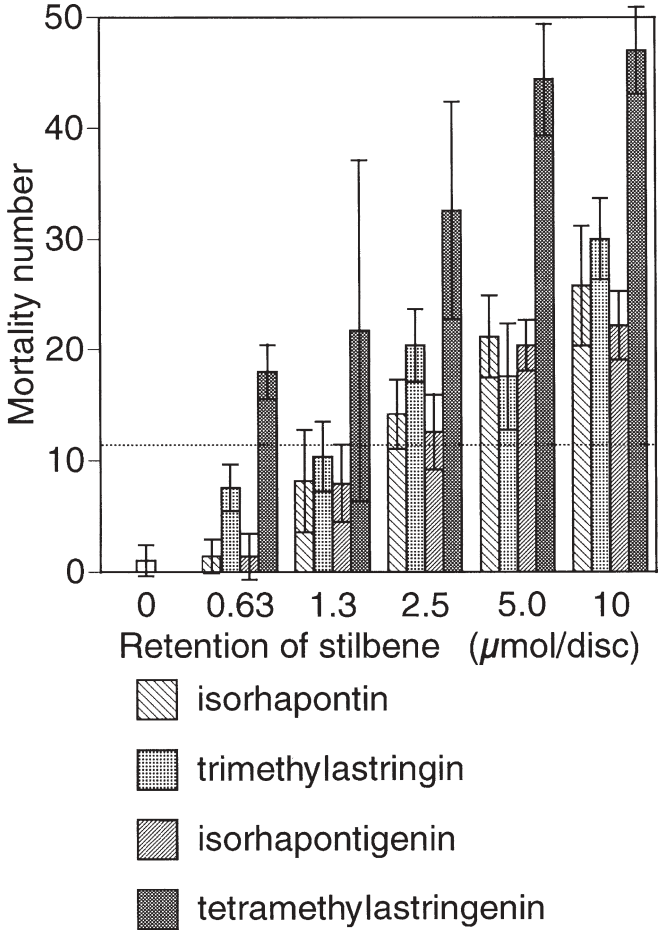


Fig. 6. Comparison of *R. speratus* mortality after 20 days exposure to isorhapontin, trimethylastringin, isorhapontigenin, and tetramethylastringenin. *Dotted line* indicates the average mortality in starvation tests, standard deviation 1.5 mg

Figure 6 shows the termite mortality after the no-choice tests. Tetramethylastringenin exhibited the largest the termiticidal activity among the compounds tested at retentions of 5.0 and 10 μmol/disc. No significant difference in termiticidal activity of the other three compounds was observed.

Discussion

Antitermite potentials of stilbene glucosides from the bark of *Picea glehnii* against *Reticulitermes speratus* were investigated. Isorhapontin, the main stilbene glucoside constituent, was recognized to have comparatively lower antifeedant potential than its aglycone derivative against *R. speratus*. Although the stilbene glucosides from the bark of *P. glehnii* also showed weaker antifungal activities, their activity increased when the glucosidic bond was hydrolyzed with β-glucosidase from fungi.[14,15] Taxifolin, which was tested for comparison, is well known as an antitermite compound[16] and showed the same ability as pinosylvin dimethyl ether against *Crytotermes brevis*.[7] This compound also showed about the half of the antifeedant activities of isorhapontin and isorhapontigenin at 0.63 to 10 μmol/disc. Stilbenes showed stronger antifeedant activity than taxifolin against *R. speratus.* At 5 μmol/disc, stilbenes reduced termite feeding activity by 90% in comparison with control consumption while the same retention of taxifolin gave a 70% reduction in feeding.

Mortality by isorhapontigenin and tetramethylastringenin were larger than those of starvations over the range of retentions tested. Other compounds also showed larger mortalities at retentions of 5–10 μmol/disc. These observations indicated that the stilbenes acted as toxicants against termites rather than as mere feeding deterrents. When the phenolic hydroxyl groups in isorhapontin and its aglycone derivative were methylated, termite mortality increased when compared with those of the precursor compounds. It was reported that the moderate hydrophobicity of stilbenes increased the suppression effects of microbial growth.[17,18] Improved hydrophobicity of components generally enhances absorption, i.e., passive diffusion or endocytosis.[19,20] In this study, increases in the termiticidal potential of tetramethylastringenin and trimethylastringin may be attributed to the hydrophobic properties, which were improved by methylation because of improvement of absorption of the compounds. On the other hand, oxidative enzymes play an important role in detoxification of phenolics. The methylated compounds may also have reduced reactivity against the oxidative enzymes because the original dissociative functional groups, the hydroxy groups, were converted. As a result, the toxicities of methylated compounds were possibly maintained for the longer period and caused higher mortality of the termites than that of the original compounds. These points should be investigated in future studies.

This experiment revealed the large feeding deterrent ability of stilbene glucosides and methylated stilbene against *R. speratus*. These compounds may be used as agents for preventing termite attacks in place of ordinary chemicals, such as pyrethroids and carbamates. Such an application may be worthwhile because stilbene glucosides account for as much as 15% (w/w, dry base) of *P. glehnii* bark,[21] which is a waste material of the wood industry.

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