DOI:10.1021/jf1017115

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Bioactive Saponin from Tea Seed Pomace with Inhibitory Effects against *Rhizoctonia solani*

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The present study was aimed to characterize the antifungal principles in methanol extract of tea (*Camellia oleifera*) seed pomace. Totally, two flavonoids, camelliasides A (**1**) and B (**2**), and one saponin mixture composed of camelliasaponin B_1 (**3**) were identified from the methanol extract. These constituents were tested for their ability to reduce the infection of cabbage seedlings by *Rhizoctonia solani* Kühn AG-4 and to inhibit growth of the pathogen on potato dextrose agar plates. The saponin mixture is a potential candidate as a new plant-derived pesticide to control *Rhizoctonia* damping-off of vegetable seedlings.

KEYWORDS: Saponin; tea seed pomace; *Rhizoctonia solani*

INTRODUCTION

Various fungal diseases cause severe losses of annual plants such as vegetables and flowers. These diseases have been successfully controlled by synthetic chemical fungicides. However, the indiscriminate utilization of these chemicals has also led to the development of fungicide resistance and environmental pollution, posing potential risk to animal and human health. The eco-friendly resolution of plant diseases is biological control, which is recognized as an environmentally sound method, such as the use of metabolites from natural resources such as microbes or plants. Many studies have examined crude plant extracts (*1*−*4*) as well as the active principles from medicinal plants (*5*−*8*) for control of several fungal diseases. Among the common plant pathogens, *Rhizoctonia solani* Kühn causes serious damping-off diseases of numerous crops in Taiwan; it is a major problem especially for the commercial production of vegetable seedlings grown in cell-plug systems (*9*, *10*). To control this pathogen effectively and try to reduce the risk of development of resistance with chemical fungicides, biological agents were more extensively studied.

Camellia oleifera was named trivially as tea-oil camellia because it is cultivated in its homeland of East Asia specifically for the seeds, which are extracted for commercial tea oil (*11*). There are already several reports regarding the bioactivities of the tea seed extracts. After the extraction of oil, the seed pomace is discarded as there are no further uses for it. Moreover, no research work related to the tea seed pomace had been published until now. However, these agricultural wastes have abundant contents of major triterpenoids such as camelliasaponins; thus, they possess potential for inhibiting fungi. In our preliminary screening, the methanol extract of tea seed pomace at 100 ppm markedly inhibited the mycelial growth of *R. solani* by 64.68%. For the purpose of discovering new plant-derived fungicides for controlling *Rhizoctonia* damping-off of cabbage seedlings, the seed pomace of *C. oleifera* was selected as the target. In the present study we explored the bioactive principles and determined the antifungal activities of these natural compounds.

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MATERIALS AND METHODS

General. Compounds were identified by the following spectrometric apparatus. Melting points were determined using a Fisher Scientific melting point measuring apparatus without corrections. The UV spectra were obtained on a GBC Cintra 101 UV−vis spectrophotometer. The IR spectra were obtained, as KBr disks, on a Varian Scimitar FTS-2000 FT-IR spectrometer. Optical rotations were measured with a Jasco P-1010 polarimeter. All of the NMR spectra were recorded with deuterated methanol on Bruker Avance-300 and AMX-400 NMR spectrometers using tetramethylsilane (TMS) as the internal standard. Standard pulse sequences and parameters were used for the NMR experiments, and all chemical shifts were reported in parts per million (ppm, δ). Mass spectrometry was analyzed on a Thermo Finnigan LCQ mass spectrometer (San Jose, CA) equipped with a quadrupole ion trap and electrospray ionization (ESI) source operated in the negative-ion modes. Samples were introduced into the ESI-MS by loop injection (5 μL injection loop) dissolved in distilled water or HPLC-grade methanol. GC analyses were performed on a Shimadzu GC-2014 gas chromatograph equipped with a flame ionization detector (FID). The sugars were identified by agreement of their retention times with the authentic compounds.

Fungal and Plant Materials. *R. solani* AG-4 isolate RST-04 was isolated from a seedling of Chinese kale (*Brassica alboglabra* Bailey) showing damping-off symptom. The culture of *R. solani* AG-4 RST-04 was grown on potato dextrose agar (PDA) (200 g of potato infusion, 20 g of glucose, and 20 g of agar in 1 L of distilled water) slant. The stems of 7-day-old cabbage seedlings (*Brassica oleracea* L. *capitata* group) were used for inoculation. The tea seed pomace (*C. oleifera*) was purchased from a shop in Ali Mountain, Chiayi, Taiwan.

 Published on Web 07/15/2010

Extraction and Purification. The tea seed pomace (250 g) was extracted with methanol (2.5 L × 7) at ambient temperature and concentrated to give a brown syrup (25 g). The methanol extract was subjected to Diaion HP-20 column chromatography with stepwise gradients of water and methanol (100:0, 80:20, 60:40, 40:60, 20:80, and 0:100, v/v) to afford five subfractions. The fourth subfraction (5.8 g) was further subjected to a Sephadex LH-20 column chromatography and eluted with a solvent mixture of water in a stepwise gradient with methanol (100:0, 80:20, 60:40, 40:60, 20:80, and 0:100, v/v) and led to the isolation of camelliasides A (**1**) (100 mg) and B (**2**) (500 mg) (*12*), respectively. Purification of subfraction 5 (12.6 g) by repeated column chromatography over Sephadex LH-20 and MCI yielded one saponin mixture composed of camelliasaponin B_1 (**3**) (5.0 g) (*13*).

Acid Hydrolysis of the Saponin Mixture. A solution of saponin mixture (5 mg) in 5% (v/v) aqueous H_2SO_4/1,4-dioxane (1:1, v/v, 1.0 mL) was heated under reflux for 1 h. After cooling, the reaction mixture was neutralized with 1% (v/v) aqueous $NaHCO_3$ solution. On removal of the solvent from the mixture under reduced pressure, the residue was passed through a Sep-Pak C_{18} cartridge by elution with water and then methanol. The water eluate was concentrated, and the residue was treated with L-cysteine methyl ester hydrochloride (0.01 mL) in pyridine (0.02 mL) at 60 °C for 1 h. After this reaction, the solution was treated with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (0.01 mL) at 60 °C for 1 h. The supernatant was then subjected to GC analysis to identify the derivatives of D-glucuronic acid, D-galactose, L-arabinose, and D-glucose, respectively, under similar analytical conditions as reported in the literature (*14*).

Assay of Antifungal Activity. The methanol extract, partially purified five subfractions, and purified flavonoids and one saponin mixture were dissolved in distilled water with the final concentration of 100 ppm. The assay of the antifungal activity against *R. solani* AG-4 RST-04 was carried out on 100 mm × 15 mm Petri dishes each containing 10 mL of PDA. After the mycelial colony had developed, sterile blank paper disks (6.5 mm in diameter) were placed at a distance of 5 mm away from the rim of the mycelial colony. An aliquot (10 μL) containing the tested sample was added to each paper disk. The Petri dishes were sealed with parafilm and incubated at 27 °C until the mycelial growth of *R. solani* AG-4 RST-04 had covered the surface of the paper disks containing the distilled water as the control, whereas the mycelial growth of *R. solani* AG-4 RST-04 had formed crescents of inhibition zone around the paper disks containing samples with antifungal activity (*15*). The standard fungicide for controlling *R. solani*, Pencycuron (Vietnam Pesticide Co., Ho Chi Minh City, Vietnam) at 0.25−250 ppm, was also examined for antifungal activity by using the same method as described above.

To determine the half-maximal inhibitory concentration (IC_{50}) for the antifungal activity, the tested samples were added separately to PDA at 45 °C, mixed immediately, and poured into small Petri dishes (90 mm × 15 mm). After the agar became solid, the mycelial disk (6.5 mm in diameter) of *R. solani* AG-4 RST-04 precultured on PDA plate for 3 days was placed on the center of each plate. The PDA plates containing distilled water served as the control, and all of the plates were incubated at 27 °C. When the mycelial growth of the control treatment had covered the entire surface of the plate, the areas of the mycelial colonies were measured, and the inhibition of fungal growth was determined by calculating the percentage of reduction in the area of the mycelial colony (*16*).

Effect of Saponin Mixture from Tea Seed Pomace on Reducing Infection of Cabbage Seedlings by *R. solani*. The seeds of cabbage were sown on the wetted filter paper. The seedlings of cabbage were used for examination 7 days after sowing. The stems of seedlings were coated with carboxymethyl cellulose sodium salt (CMC, 0.5% w/v) mixed with 1% (w/v) methanol extract of tea seed pomace. The seedling was placed on a glass slide, and the mycelial disk (6.5 mm in diameter) of *R. solani* AG-4 RST-04 precultured on PDA plate for 3 days was placed on slide 1 cm away from the seedling. To retain the moisture, the slide was placed on a triangular glass rod in a Petri dish (9 cm in diameter) with wetted filter paper. The stem of cabbage seedling treated with 0.5% (w/v) CMC only and distilled water were used as controls. One hundred replicates were used for each treatment. The stems of seedlings were stained with cotton blue and observed with the optical microscope. The infection rate of *R. solani* AG-4 RST-04 on cabbage seedlings and the number of infection cushions of this fungus were recorded. Various concentrations from 0 to 100 ppm of saponin mixture from tea seed pomace were also examined for reduction of the infection rate of *R. solani* AG-4 RST-04 on cabbage seedlings by using the same method as described above.

Table 1. Antifungal Activity of Methanol Extract of Tea Seed Pomace and Its Partially Purified Subfractions on the Growth of *Rhizoctonia solani* AG-4 RST-04 on Potato Dextrose Agar Plates Incubated at 27 °C for 48 h

treatment[a]	colony size[b] (cm)	inhibition[c] (%)
methanol extract	2.43 ± 0.04 ab	64.68
subfraction 1	2.50 ± 0.04 b	63.66
subfraction 2	5.44 ± 0.08 d	20.93
subfraction 3	5.88 ± 0.04 e	14.53
subfraction 4	3.12 ± 0.06 c	54.65
subfraction 5	2.20 ± 0.06 a	68.02
check	6.88 ± 0.06 f	0.00

[a] Methanol extract of tea seed pomace and its partially purified subfractions were dissolved in distilled water (DW) with the final concentration of 100 ppm, and DW was used as a control. [b] Means (n = 10) ± standard error followed by the same letter do not differ significantly (p = 0.05) according to Duncan's multiple-range test. [c] Inhibition (%) = [(colony size of control − colony size of treatment)/colony size of control] × 100.

Statistical Analysis. Data were analyzed with Duncan's multiple-range test using a standard statistical package (SAS/STAT) (version 9.1, SAS Institute Inc., Cary, NC) to assess the statistical significance between each plot.

RESULTS AND DISCUSSION

Antifungal Activities of the Methanol Extract and Partially Purified Subfractions from Tea Seed Pomace. The methanol extract and partially purified subfractions were subjected to antifungal examinations with the procedures described in the literature (*15*, *16*). The methanol extract of tea seed pomace at 100 ppm showed 64.68% inhibition against the growth of *R. solani* AG-4 RST-04. The partially purified subfractions 1−5 were also examined for their antifungal activity, and the fifth subfraction exhibited the highest antifungal effect on *R. solani* AG-4 RST-04 with 68.02% inhibition at 100 ppm. In addition, the first and fourth subfractions at 100 ppm also showed antifungal activity, with inhibition rates of 63.66 and 54.65%, respectively. The inhibition percentages of all tested partially purified subfractions and the methanol extract are displayed in **Table 1**.

Components from Tea Seed Pomace. In the present study, bioactive principles were explored according to the concept of bioassay-guided purification. Repeated column chromatography purification of the fourth subfraction with the assistance of Sephadex LH-20 gels yielded two flavonoid glycosides, **1** and **2**. Compound **1** was purified as a colorless powder with mp 210−211 °C and pseudomolecular ion peak at m/z 755 ($[M - H]^-$) in ESI-MS analysis. The UV absorption maxima at 344 and 269 nm were characteristic of a flavone skeleton (*17*). The IR absorption bands at 3399, 1644, and 1554 cm^{-1} displayed the presence of a hydroxyl group, a carbon−carbon double bond, and a hydrogen-bonded carbonyl group, respectively. In the ^1H NMR spectrum, a typical set of A_2B_2 signals at δ 8.03 (2H, d, J = 7.6 Hz) and 6.91 (2H, d, J = 7.6 Hz) was attributed to H-2′, -6′ and H-3′, -5′ of the para-substituted B-ring. Two singlets at δ 6.41 (1H, s) and 6.22 (1H, s) were assumed to be H-8 and H-6. In addition, there is one upfield doublet at δ 1.09 (3H, d, J = 5.8 Hz), which was a characteristic proton signal of the rhamnose. Moreover, in the ^{13}C NMR spectrum, three sugar moieties including rhamnose (δ 102.2, 73.8, 72.3, 71.4, 69.7, and 17.9), galactose (δ 104.4, 77.9, 76.9, 75.4, 71.3, and 62.6), and glucose (δ 101.1, 82.0, 78.3, 77.9, 72.1, and 68.2) by comparison with literature values and with the assistance of comprehensive COSY, NOESY, HMQC, and HMBC spectroscopic examinations could establish the structure of **1** as camelliaside A (*12*). Compound **2** was also isolated as a colorless powder

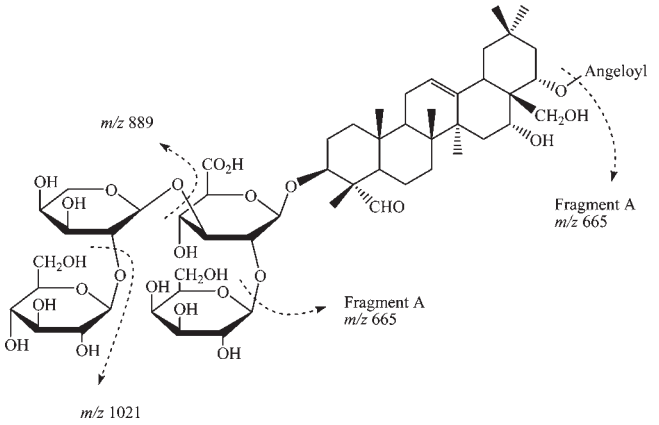

Figure 1. MS/MS fragmentation of camelliasaponin B_1.

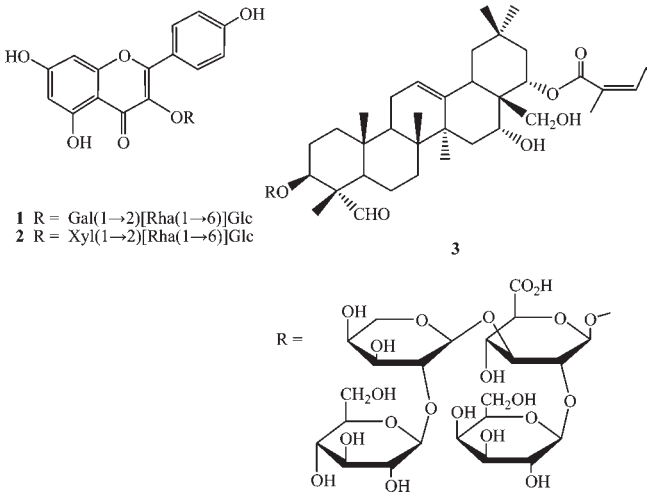

Figure 2. Chemical structures of isolated compounds **1**−**3**.

with mp 200−201 °C and a pseudomolecular ion peak at *m*/*z* 725 ($[M - H]^-$) in ESI-MS analysis. The only spectral difference between camelliasides A (**1**) and B (**2**) was that the ^{13}C NMR spectrum of **2** displayed the presence of three sugar moieties including rhamnose, glucose, and xylose (*δ* 105.2, 77.1, 74.7, 71.0, and 66.6) instead of the galactose in **1**. Therefore, compound **2** was identified as camelliaside B with comprehensive studies of their physical and spectral data and comparison with those reported in the literature (*12*).

In addition, one saponin mixture was isolated from the most active fifth subfraction as a white powder. The solids were visualized by spraying with 1% (w/v) $Ce(SO_4)_2$ in 10% (v/v) aqueous H_2SO_4 followed by heating at 120 °C and displayed purplish black spots on TLC plate. This suggested that these solids possessed the triterpenoid basic skeleton (*18*). ^1H NMR spectral analysis of this mixture displayed several characteristics to evidence this compound as tritepenoid saponin. In the upfield region of the ^1H NMR spectrum, one set of five singlets at *δ* 0.88 (3H), 0.95 (6H), 1.03 (3H), 1.16 (3H), and 1.30 (3H) was ascribable to the methyl groups of the oleanane-type triterpenoids in these compounds. The resonances located in the region of *δ* 3.0−5.5 suggested the presence of sugar moieties in this mixture. Acid hydrolysis followed by GC analysis of the trimethylsilyl thiazolidine derivatives determined the sugar moieties as D-glucuronic acid, D-galactose, L-arabinose, and D-glucose (*14*). The downfield proton singlet at *δ* 9.44 (1H) also indicated the presence of a formyl group. This was further confirmed with the assistance of the ^{13}C NMR spectrum, which displayed a characteristic carbon signal at *δ* 210.9. The spectral characteristics were very similar to those of camelliasaponin B_1 (**3**) reported in the literature (*13*). The ESI-MS analysis of these mixtures displayed one major $[M - H]^-$ peak at *m*/*z* 1201 corresponding to the pseudomolecular formula reported for the camelliasaponin B_1 (**3**) ($C_{58}H_{89}O_{26}$). The MS/MS fragmentation patterns (**Figure 1**) of the parent ion at *m*/*z* 1201 confirmed the loss of a hexose (*m*/*z* 1021 $[M - H - C_6H_{11}O_6]^-$) and a pentose (*m*/*z* 889 $[M - H - C_{11}H_{19}O_{10}]^-$), successively (*14*). A prominent peak at *m*/*z* 665 corresponded with the triterpenoid moiety fragmentized from the parent molecule (fragment A). On the basis of the above NMR and mass spectroscopic evidence, the major component of the saponin mixture was established as camelliasaponin B_1 (**3**) (*13*). The chemical structures of the isolated compounds **1**−**3** are displayed in the **Figure 2**.

Table 2. Antifungal Activity of Camelliasides A (**1**) and B (**2**) and Saponin Mixture (**3**) from Tea Seed Pomace on the Growth of *Rhizoctonia solani* AG-4 RST-04 on Potato Dextrose Agar Plates Incubated at 27 °C for 48 h

treatment[a]	colony size[b] (cm)	inhibition[c] (%)
camelliaside A (125 ppm)	8.70 ± 0.12 h	−14.66
camelliaside B (125 ppm)	8.69 ± 0.12 h	−14.50
saponin mixture[d] (100 ppm)	2.78 ± 0.04 c	63.43
saponin mixture (60 ppm)	3.30 ± 0.11 d	56.51
saponin mixture (30 ppm)	4.04 ± 0.07 e	46.79
saponin mixture (10 ppm)	7.10 ± 0.04 f	6.43
pencycuron (250 ppm)	1.38 ± 0.14 a	81.88
pencycuron (25 ppm)	1.23 ± 0.12 a	83.86
pencycuron (2.5 ppm)	2.00 ± 0.13 b	73.64
pencycuron (0.25 ppm)	2.54 ± 0.10 c	66.56
check	7.59 ± 0.14 g	0.00

[a] The flavonoids and saponin mixture from tea seed pomace were dissolved in distilled water (DW) with various concertrations, and DW was used as a control. [b] Means (n = 8) ± standard error followed by the same letter do not differ significantly (p = 0.05) according to Duncan's multiple range test. [c] Inhibition (%) = [(colony size of control − colony size of treatment)/colony size of control] × 100. [d] The major component of saponin mixture was camelliasaponin B_1. The IC_{50} values were calculated using the Microsoft Excel program. The IC_{50} of saponin mixture was 55.70 ppm and the regression equation was $y = -0.0102x^2 + 1.6706x - 2.3253$ (R^2 = 0.9587). The IC_{50} of camelliaside A and camelliaside B were more than 125 ppm while the IC_{50} of pencycuron was less than 0.25 ppm.

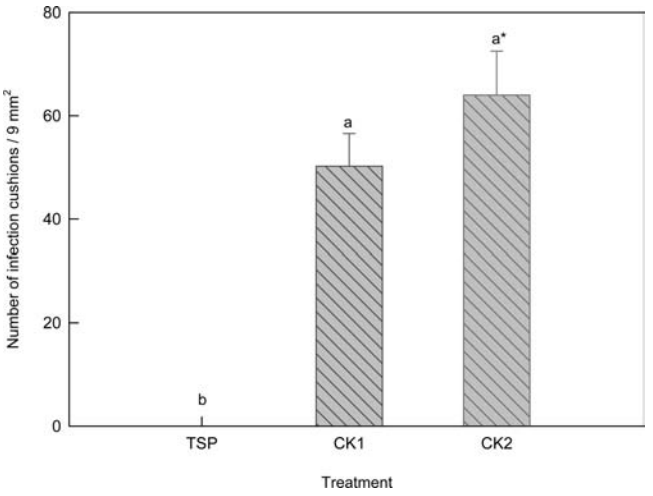

Figure 3. Effect of methanol extract of tea seed pomace (TSP) on the formation of infection cushions of *Rhizoctonia solani* AG-4 RST-04 on stem surfaces of cabbage seedlings [CK 1, distilled water only; CK 2, 0.5% (w/v) carboxymethyl cellulose sodium salt (CMC) only; TSP, 0.5% (w/v) CMC mixed with 1% (w/v) methanol extract]. Values in each column topped by the same letter do not differ significantly (p = 0.05) according to Duncan's multiple-range test.

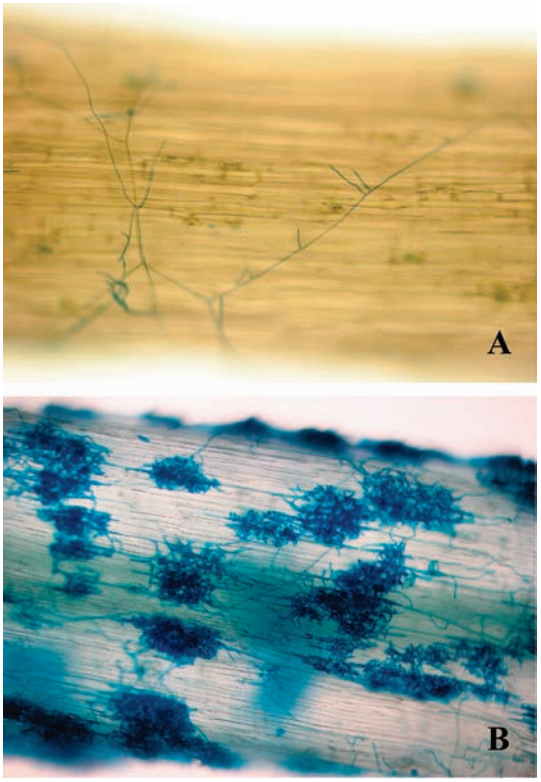

Figure 4. Infection cushions of *Rhizoctonia solani* AG-4 RST-04 formed on stem surface of a cabbage seedling treated with or without 1% (w/v) methanol extract of tea seed pomace under microscope: (**A**) 0.5% (w/v) carboxymethyl cellulose sodium salt (CMC) mixed with 1% (w/v) methanol extract (100×); (**B**) 0.5% (w/v) CMC only (100×).

Antifungal Activity of the Components from Tea Seed Pomace. The isolated compounds **1**−**3** were examined for their antifungal activity as described above, and the data are displayed in **Table 2**. The flavonoids camelliasides A (**1**) and B (**2**) purified from the fourth subfraction did not exhibit any inhibition effects at the tested concentrations ranging from 30 to 125 ppm. The saponin mixture (**3**) isolated from the fifth subfraction at 100 ppm inhibited the growth of the mycelial colony of *R. solani* AG-4 RST-04 by 63.43%. In addition, the antifungal effects of the saponin mixture (**3**) also showed the concentration-dependent tendency. Consequently, the isolated saponin mixture (**3**) displayed significant antifungal acitivity with the IC_{50} of 55.70 ppm, compared to the reference pesticide Pencycuron (IC_{50} < 0.25 ppm) (**Table 2**). Although the first and fourth subfractions also showed significant antifungal activity, there were no antifungal principles identified from these subfractions in the present study.

Effect of Saponin Mixture from Tea Seed Pomace on Reducing Infection of Cabbage Seedlings by *R. solani*. The cabbage seedling stems coated with 0.5% CMC (w/v) mixed with 1% (w/v) methanol extract of tea seed pomace showed low infection rate of *R. solani* AG-4 RST-04 (11.33%), whereas the infection rates on the seedlings treated with 0.5% (w/v) CMC only or distilled water were 88.67 and 77.67%, respectively. In addition, abundant infection cushions of this fungus were formed when the seedlings were treated with 0.5% (w/v) CMC only or distilled water, whereas only a few or no infection cushions were formed when the seedlings were treated with 0.5% CMC (w/v) mixed with 1% (w/v) methanol extract of tea seed pomace (**Figures 3** and **4**). Furthermore, the stems of cabbage seedlings treated with the saponin mixture extracted from tea seed pomace showed the dosage response on reducing the infection rate of *R. solani* AG-4 RST-04

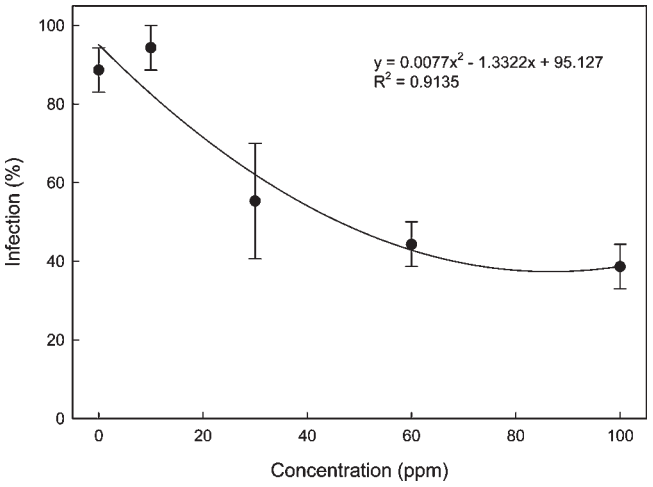

Figure 5. Effect of various concentrations of saponin mixture (**3**) from tea seed pomace on reducing infection of cabbage seedlings by *Rhizoctonia solani* AG-4 RST-04.

(**Figure 5**). The infection rate was markedly reduced from 88.67 to 38.67% when the stems of cabbage seedlings were treated with 100 ppm saponin mixture (**Figure 5**). The results of the present study suggest the bioactive saponin mixture was involved in the effect of tea seed pomace on control of cabbage seedling damping-off caused by *R. solani* AG-4 RST-04 (*19*).

ACKNOWLEDGMENT

We are grateful to the National Center for High-performance Computing, Taiwan, for computer time and facilities.

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Received for review May 5, 2010. Revised manuscript received June 28, 2010. Accepted July 5, 2010. We are grateful for financial support from the National Science Council, Taiwan, Republic of China (NSC 098-2811-B-005-010) awarded to J.-W.H.