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Identification of the *Solanum nigrum* Extract Component Involved in Controlling Cabbage Black Leaf Spot Disease

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ABSTRACT: In this study, we discovered that an ethanol (EtOH) extract of *Solanum nigrum* inhibited spore germination of *Alternaria brassicicola*, the causative agent of cabbage black leaf spot disease. At a concentration of 500 mg/L, this ethanol extract also caused the germ tubes to become completely swollen. Detached cabbage leaves were then used to evaluate the effects of the extract in controlling the disease. It was observed that the extract-induced swelling of *A. brassicicola* germ-tube spores did not cause the symptoms of black spot disease on cabbage leaves. Furthermore, an *n*-butanol fraction of the EtOH extract exhibited strong antifungal activity; at a concentration of 25 mg/L, a derived subfraction (Bu-11−13) showed complete inhibition of spore germination. A white powder was collected from fraction Bu-11−13, and its minimum inhibitory concentration was determined to be 8 mg/L. Using NMR and LC-MS/MS analysis, this white powder compound was identified as degalactotigonin.

KEYWORDS: Cabbage, *Alternaria brassicicola*, *Solanum nigrum*, saponins, botanical pesticide, degalactotigonin

INTRODUCTION

Black leaf spot disease in cabbage (*Brassica oleracea* L.) is caused by *Alternaria brassicicola* (Schw.) Wiltshire and *Alternaria brassicae* (Berk) Sacc. Although this is a common disease of cruciferous vegetables worldwide, the disease caused by *A. brassicicola* is more common in Taiwan. The pathogen is easily spread through the contamination of seeds.[1] The leaves and stems of cabbages infected by *A. brassicicola* result in the formation of dark-brown, necrotic lesions with a yellow halo; this damage reduces both yield and economic value.[2] *A. brassicicola* invades the host via two different mechanisms; these include direct invasion through the stomata of leaves and the invasion of leaf tissue after formation of the fungal appressorium.[3] During the invasion of the host, *A. brassicicola* secretes a variety of cutinases,[4] esterases,[5] and lipases.[6] These enzymes facilitate penetration of the cuticle and waxes of the leaf surface and play important roles in the development of the lesions. Furthermore, *Alternaria* spp. also produce toxic substances associated with the pathogenicity and virulence of these pathogenic fungi.[7] These toxic substances are not usually produced when *Alternaria* spp. are cultured on glass slides, culture media, or the surfaces of nonhost plants.[8]

The use of synthetic chemical pesticides to control plant diseases has become less accepted in modern agriculture, as many of these pesticides are highly toxic and have long residual periods. Furthermore, these types of pesticides are harmful to nontarget species in the ecosystem and are hazardous to the environment.[9] Natural plant substances are considered to have less of an impact on the environment, as these chemical pesticides are readily broken down in nature and have short-lasting residual periods.[10] The ethanol extracts from dried roots of *Solanum nigrum* were reported to be useful for controlling black leaf spot disease in Chinese cabbage (*Brassica chinensis* L. and *Brassica pekinensis* Skeels) and other cabbage species (*B. oleracea* var. *Capitata*), reducing disease severity by 40%. These findings indicate that ethanol extracts of *S. nigrum* have the potential to be developed as plant protection agents.[11,12] However, the bioactive components of *S. nigrum* were not previously purified or identified. The aims of this study were to examine the effects of the extracts of *S. nigrum* on spore germination of *A. brassicicola*, to examine the effects of the extracts on the ability of *A. brassicicola* to infect cabbage leaves, and to identify the bioactive compounds that contribute to controlling black leaf spot disease of cabbage leaves.

MATERIALS AND METHODS

Fungal and Plant Materials. *A. brassicicola* ABB-16 was isolated from the leaves of cabbages (*B. oleracea* L.) showing symptoms of black leaf spot disease. *A. brassicicola* ABA-31 was obtained from the Plant Disease Management Laboratory (Department of Plant Pathology, National Chung Hsing University), which was isolated from diseased Chinese cabbage (*B. campestris*). The cultures of these two isolates were grown on a 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 third to fifth leaves of 1 month old cabbage seedlings (*B. oleracea* L. Capitata Group) were used for inoculation. The fresh, whole plant materials of *S. nigrum* were collected from Yangming Mountain (Taipei, Taiwan) in March 2007 and identified by Dr. Yen-Hsueh Tseng (National Chung Hsing University, Taiwan). A voucher specimen was deposited in the herbarium of the same university.

Preparation of Crude Extract from *S. nigrum*. The fresh, whole plant of *S. nigrum* was dried at 50 °C in an oven for 3 days, ground into powder (550 g), and then extracted with 70% ethanol at 50 °C for 7 days on a rotary shaker. The extracts were combined and filtered with Whatman #1 filter paper to remove plant debris. After removal of the ethanol by vacuum, the extracts were freeze-dried and dissolved with deionized water in 1/10 of the original volume.

Effect of Crude Extract on Toxin Production of *A. brassicicola*. *A. brassicicola* ABA-31 and ABB-16 isolates were cultured on

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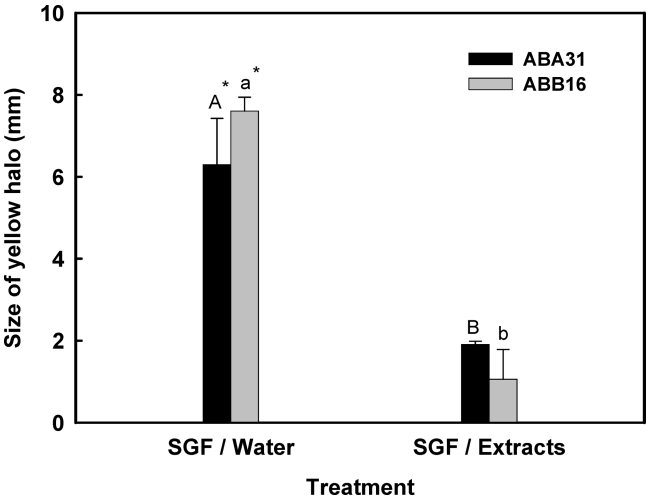

Figure 1. Effect of ethanol extracts from *S. nigrum* on the appearance of the yellow halo on cabbage leaf surfaces induced by SGFs of *A. brassicicola* isolates ABA-31 and ABB-16. *Means in each isolate showing the same letters are not significantly different ($P > 0.05$), according to Tukey's studentized range test.

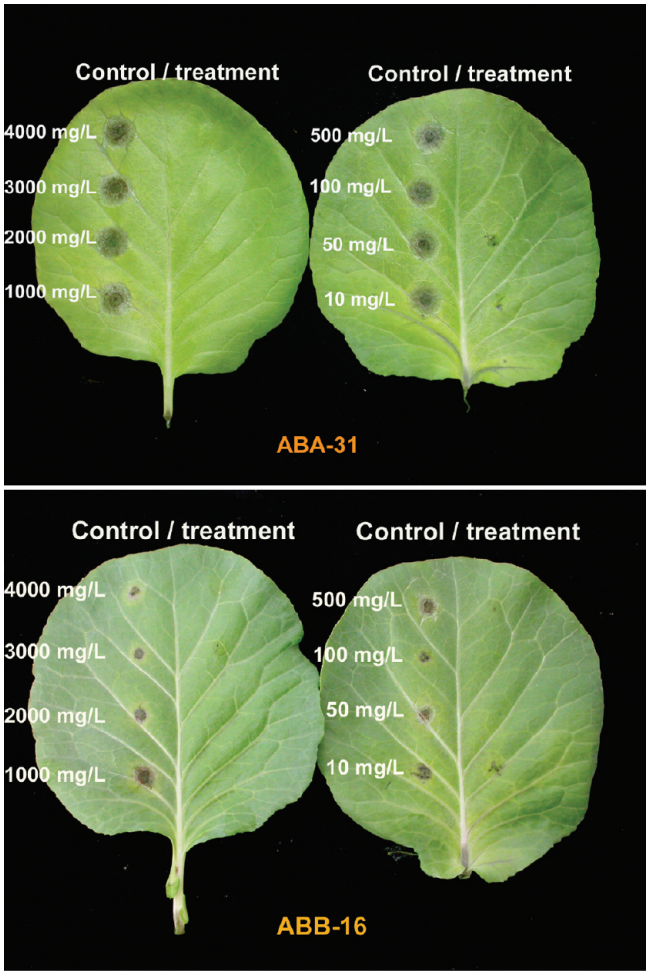

Figure 2. Effect of ethanol extracts from *S. nigrum* on infection of detached cabbage leaves by *A. brassicicola* ABA-31 and ABB-16. Treatment: Spore suspensions of *A. brassicicola* ABA-31 and ABB-16 were treated with different concentrations of ethanol extracts from *S. nigrum*. Control: DMSO at a concentration of 1% (v/v) was used as a control.

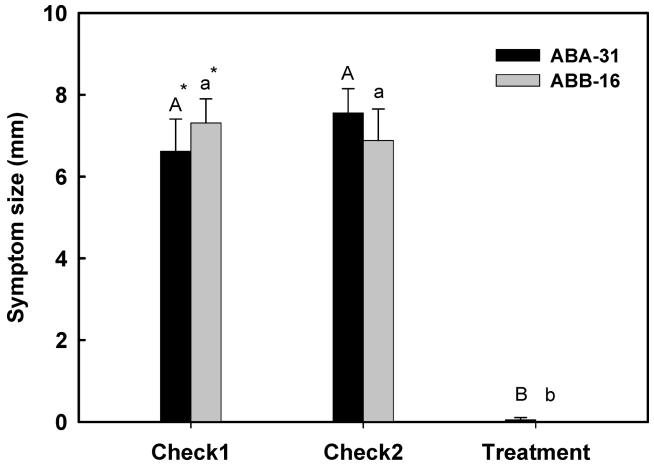

Figure 3. Effect of ethanol extracts from *S. nigrum* on infection of detached cabbage leaves by germinated spores of *A. brassicicola* ABA-31 and ABB-16. Treatment: Spores of *A. brassicicola* ABA-31 and ABB-16 were treated with 100 mg/L of extract from *S. nigrum* for 24 h, and then, the extract was removed by centrifugation at 900 rpm. Germinated swelling spores were washed 10 times with distilled water and then finally resuspended in distilled water for inoculation. Check 1: Spores of *A. brassicicola* ABA-31 and ABB-16 treated with distilled water as a control. Check 2: Extract from *S. nigrum* was removed with the same steps as the above treatment, and then, the material from its 10th wash was mixed with a spore suspension of *A. brassicicola* ABA-31 and ABB-16 for inoculation. *Means in each isolate showing the same letters are not significantly different ($P > 0.05$), according to Tukey's studentized range test.

PDA slants for 10 days; sterile, distilled water was then added to prepare the spore suspensions (2×10^5 spores/mL) for either inoculation or the spore germination test. The surfaces of cabbage leaves were sterilized with 70% (v/v) ethanol, rinsed twice with sterile distilled water, and then placed on sterile, moist filter paper in Petri dishes. Treatment 1: The spore germination fluids (SGFs) of *A. brassicicola* ABA-31 and ABB-16 were prepared as previously described.[12] An aliquot (10 μL) of SGF was dropped onto the detached cabbage leaves, and then, 10 μL of crude extract of *S. nigrum* (final concentration of 500 mg/L) was added. Equal volumes of sterile distilled water, crude extract of *S. nigrum* (500 mg/L), and SGF were used as controls. Treatment 2: Aliquots (10 μL) of spore suspensions of *A. brassicicola* ABA-31 or ABB-16 were dropped onto the detached cabbage leaves, and then, 10 μL of crude extract of *S. nigrum* (final concentration of 500 mg/L) was added. An equal volume of sterile distilled water was used as control. The SGFs were collected 2 days after incubation and then redropped onto sterilized cabbage leaves. The surfaces of the cabbage leaves beneath the treatment sites were stabbed lightly with needle tips. The detached cabbage leaves were then placed on wetted filter paper in Petri dishes and incubated in a moist chamber at 25 °C for 24 h. Each test contained five replicates. Lesions were examined after 3 days of incubation, and the diameters of the lesions were recorded.

Effect of Crude Extracts on Infection Ability of Germinated Spores of *A. brassicicola*. Aliquots (250 μL) of the spore suspensions of *A. brassicicola* ABA-31 and ABB-16 were mixed with 250 μL of crude extract of *S. nigrum* (final concentration of 500 mg/L). Spore suspensions mixed with sterile distilled water were used as control. After aerobic incubation for 24 h, swelling of the crude extract-treated germ-tube spores was confirmed by optical microscopy. After centrifugation for 10 min at 900 rpm and removal of the supernatant (300 μL), 300 μL of a 1% (v/v) dimethyl sulfoxide (DMSO) solution was added. This step was repeated 10 times to remove inhibitors. The concentration of the germinated spore suspension was adjusted to 2×10^5 spores/mL after

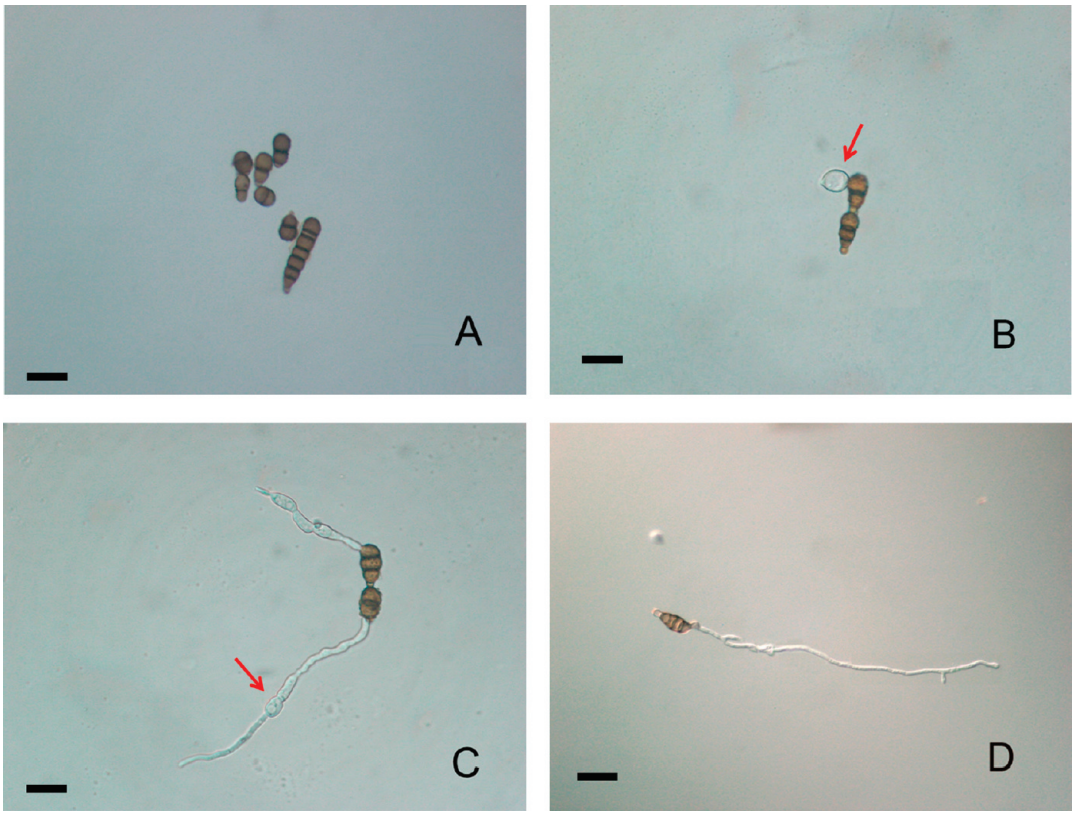

Figure 4. Effect of ethanol extracts from *S. nigrum* on conidial germination of *A. brassicicola* ABA-31. (A) Conidial germation of *A. brassicicola* ABA-31 was completely inhibited by ethanol extracts from *S. nigrum* (bar = 20 μm). (B) Germ-tube swelling of *A. brassicicola* ABA-31 was observed after treatment with ethanol extracts from *S. nigrum* (bar = 20 μm). (C) Germ-tube swelling showed the regrowth phenomenon after treatment with low concentrations of ethanol extracts from *S. nigrum* (bar = 20 μm). (D) Conidial germination of *A. brassicicola* ABA-31 treated with 1% (v/v) DMSO as a control (bar = 10 μm).

resuspension in sterile distilled water. The spore suspensions (20 μL) were then dropped onto sterile cabbage leaves. The crude extract of *S. nigrum* (250 μL, 500 mg/L), washed to remove inhibitors, was used as an additional control. Washed crude extract (10 μL) and 10 μL of spore suspension (final concentration of 2×10^5 spores/mL) were then dropped onto sterile cabbage leaves. The cabbage leaves were subsequently kept moist. Each test contained five replicates. The lesions were observed after a 3 day incubation, and the diameters of the lesions were recorded.

Fractionation and Separation of Crude Extract of *S. nigrum*. The crude extract of *S. nigrum* was successively partitioned with *n*-hexane, ethyl acetate, and *n*-butanol. The *n*-hexane-soluble fraction, ethyl acetate-soluble fraction, *n*-butanol-soluble fraction, and *n*-butanol-insoluble fraction were therefore obtained. The *n*-butanol-soluble fraction was further separated by chromatography over silica gel (60−80 mesh) and eluted stepwise with a gradient of 10−100% methanol (MeOH) in chloroform for separation (MeOH increased 10% every 1000 mL, with samples collected every 250 mL). Subfractions showing bands with equivalent R_f values by thin-layer chromatography (TLC) analysis were pooled together to yield 18 subfractions (Bu-01−Bu-18). The subfractions with bioactivity (Bu-11) were then subjected to C_{18} gel (Supelco Bellefonte, United States) column chromatography and then eluted stepwise with gradients of 10−100% MeOH in H_2O for further separation (MeOH increased 10% every 500 mL, with samples collected every 200 mL). Subfractions showing bands with similar equivalent R_f values in reverse-phase TLC (S RP-18 F254 Merck Co.) analysis were pooled together to yield 18 subfractions (Bu-11-01−Bu-11-18). For TLC analysis, the crude extract subfractions were dropped onto normal-phase TLC sheets (SIL G/UV254, ALUGRAM) and reverse-phase TLC sheets (RP-18 F254 Merck Co.) using a capillary; the TLC sheets were developed with different ratios of chloroform/MeOH and MeOH/water, respectively, based on the eluted mobile phases described above. The TLC sheets were observed under UV light and visualized with 15% sulfuric acid. The bioactive subfraction (Bu-11-13) was further purified with an HPLC system comprised of an Agilent 1200 Series high-performance liquid chromatograph equipped with an Agilent 1200 Series diode array detector (Agilent Technologies, Germany). The HPLC pumps, autosampler, column oven, and diode array system were monitored and controlled using the HP Chem Station computer program (Agilent) and fitted with a reverse-phase HPLC column (Cosmosil 5 μm, 250 mm × 10 mm). The mobile phase was a gradient of 30−100% acetonitrile (MeCN) in water, using the following parameters: time (min)/MeCN (%)/flow rate (mL/min) = 0/30/1.5, 5/30/1.5, and 40/100/1.5.

Assay of Antifungal Activity. The crude extract, partially purified subfractions, and purified compound were dissolved in a 1% (v/v) DMSO solution to various final concentrations. The assays of the antifungal activity against *A. brassicicola* ABA-31 and ABB-16 isolates were carried out on slides. An aliquot (10 μL) of spore suspension (2×10^5 spores/mL) was dropped onto the slide, and then, 10 μL of the test extract was added. Each slide contained three replicates. The slide was placed on a triangular glass rod in a Petri dish (9 cm in diameter) with wetted filter paper and then incubated in the dark in an incubator (24 °C) for 12 h. These samples were then stained with lactophenol cotton blue and examined under an optical microscope. One hundred spores were randomly observed, and the percentages showing germination, swollen germ tubes, and regrowth were recorded.

Identification of the Bioactive Compound. The structure of a compound with bioactivity was elucidated and confirmed using spectroscopic analyses. UV spectra were recorded on a Jasco V-550 and IR spectra on a Bio-Rad FTS-40 spectrometer. Electron-impact mass spectrometry

Table 1. Effects of Different Concentrations of Ethanol Extracts from *S. nigrum* on Conidial Germination, Germ-Tube Swelling, and Regrowth of *A. brassicicola* ABA-31 and ABB-16

	germination[b] (%)		germ-tube swelling (%)		regrowth (%)	
concentrations of extract[a] (mg/L)	ABA-31	ABB-16	ABA-31	ABB-16	ABA-31	ABB-16
4000	20.3 a[c]	15.0 a	100.0 a	100.0 a	0.0 a	0.0 a
3000	33.0 ab	12.5 a	100.0 a	100.0 a	0.0 a	0.0 a
2000	44.0 bc	47.0 b	100.0 a	100.0 a	0.0 a	0.0 a
1000	58.8 cd	53.0 b	100.0 a	98.9 a	0.0 a	0.0 a
500	67.0 d	58.8 b	100.0 a	100.0 a	1.5 a	10.0 a
250	97.5 e	98.3 c	58.3 b	42.7 b	81.8 b	90.9 b
control[d]	99.0 e	98.5 c	0.0 c	0.0 c	0.0 a	0.0 a

[a] Extracts were obtained from 70% (v/v) ethanol extracts from *S. nigrum*. [b] Conidial germination, germ-tube swelling, and regrowth were recorded 12 h after treatment at 24 °C based on randomly scoring 100 conidia in each replicate. [c] Values ($n = 3$) in each column followed by the same letter do not differ significantly ($p = 0.05$) according to Tukey's studentized range test. [d] DMSO (1%, v/v) was used as a control.

(EIMS) and high-resolution electron-impact mass spectrometry (HREIMS) data were collected with a Finnigan MAT-958 mass spectrometer. NMR spectra were recorded with Bruker Avance 500 and 300 MHz FT-NMR spectrometers, at 500 (^{1}H) and 75 MHz (^{13}C), respectively.

Statistical Analysis. Data were analyzed with Tukey's studentized range test using a standard statistical package (SAS/STAT) (Version 9.1, SAS institute Inc., NC) to assess statistically significant differences among the various treatments.

RESULTS AND DISCUSSION

Effect of Crude Extracts on Toxin Production of *A. brassicicola*. The spore suspensions of ABA-31 and ABB-16 were treated with 500 mg/L of crude extracts and incubated on cabbage leaves for 2 days, and then, the SGFs were redropped onto needle-injured cabbage leaves. These SGFs caused necrotic lesions on cabbage leaves with smaller diameters of yellow halos than that caused with the control treatments (Figure 1). The yellow halos caused by the isolates ABA-16 and ABA-31 with the control treatments were approximately 7 and 3 times larger, respectively, than the crude extract treatment (Figure 1). However, the fresh SGFs mixed with the crude extracts and then dropped onto cabbage leaves caused necrotic lesions on the leaves with similar yellow halo diameters as the control treatments (data not shown).

Effect of Crude Extracts on Infection Ability of Germinated Spores of *A. brassicicola*. The effect of crude extracts of *S. nigrum* against *A. brassicicola* isolates ABA-31 and ABB-16 were examined in vivo on detached cabbage leaves. The crude extracts effectively prohibited the development of symptoms of cabbage black leaf spot caused by *A. brassicicola* isolates ABA-31 and ABB-16, at concentrations of 50 and 100 mg/L, respectively (Figure 2). Furthermore, the spore suspensions of ABA-31 and ABB-16 were treated with 500 mg/L of crude extract, and then, the inhibitory substances were removed 24 h after incubation. The crude extract-treated germ tubes became swollen and lost the ability to develop the symptoms of infection on cabbage leaves; in contrast, the control-treated germ tubes were normal and retained the ability to develop the symptoms of infection on cabbage leaves (Figure 3).

Antifungal Activities of the Extracts from *S. nigrum* and Identification of the Bioactive Compound. The germination rates of *A. brassicicola* isolates ABA-31 and ABB-16 were 15.0 and 20.3%, respectively, when treated with crude extract of *S. nigrum* (4000 mg/L). All of the germ tubes of *A. brassicicola* ABA-31 and ABB-16 appeared swollen (Figure 4) when treated with concentrations of crude extract above 500 mg/L. Furthermore, the regrowth rates were less than 10% (Table 1).

Table 2. MIC of Extracts Obtained from *S. nigrum* on Conidial Germination of *A. brassicicola* ABA-31 and ABB-16

	MIC (mg/L)[a]		MSC (mg/L)[b]	
extracts[c]	ABA-31	ABB-16	ABA-31	ABB-16
ethanol	4000	4000	500	500
n-hexane	7000	7000	1000	1000
ethyl acetate	400	700	350	500
n-butanol	75	80	20	25

[a] MIC was assessed by conidial germination of *A. brassicicola* isolates ABA-31 and ABB-16. Conidial germination was recorded based on random scoring of 100 conidia in each treatment. All conidial germination data were the means of three replicates per treatment. [b] Minimum swelling concentration (MSC) was assessed by conidial germ tube swelling of *A. brassicicola* isolates ABA-31 and ABB-16. Conidial germ tube swelling without regrowth was recorded based on random scoring of 100 conidia in each treatment. All conidial germination data were the means of three replicates per treatment. [c] Freeze-dried 70% (v/v) ethanol extracts from *S. nigrum* were dissolved in distilled water and then sequentially extracted with *n*-hexane, ethyl acetate, and *n*-butanol. The *n*-butanol-insoluble material was also tested for antifungal activity; however, it did not show inhibitory efficacy at concentrations of up to 10000 mg/L.

The crude extract of *S. nigrum* was successively partitioned with *n*-hexane, ethyl acetate, and *n*-butanol, to give 1.75 g of an *n*-hexane-soluble fraction, 9.39 g of an ethyl acetate-soluble fraction, and 32.46 g of an *n*-butanol-soluble fraction. At a concentration of 80 mg/L, the *n*-butanol partition revealed the highest antifungal activity, completely inhibiting spore germination of both tested isolates; at a concentration of 25 mg/L, this fraction caused all germ tubes to become swollen and prevented regrowth of treated spores. However, the *n*-butanol-insoluble fraction did not have inhibitory activity (Table 2). Furthermore, the active fractions were fungistatic but not fungicidal. Because the pathogen spores were treated with the minimum inhibitory concentration (MIC) of each fraction (Table 2), the inhibitory substances were removed 24 h after incubation so that these spores could regrow on the PDA medium (data not shown).

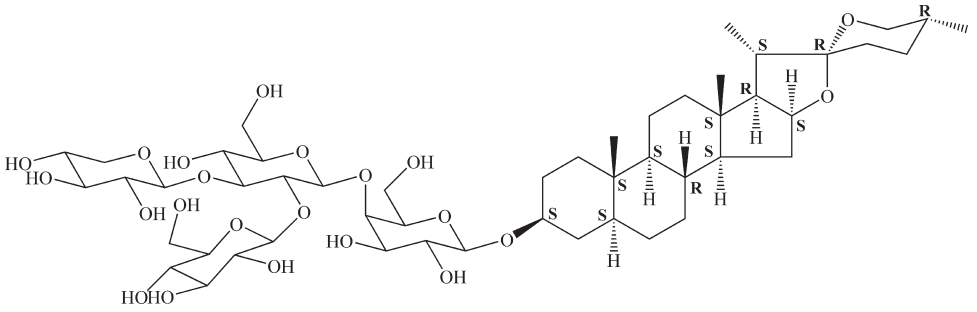

Figure 5. Chemical structure of degalactotigonin.

The bioactive subfraction Bu-11-13 (33.6 mg) was separated by RP-HPLC to yield compound **1** (R_t = 18.0 min, 21 mg, 62.5% yield). Compound **1** was a white amorphous powder and showed a quasi-molecular ion peak of $[M + Na]^+$ at m/z 1057. The ^1H and ^{13}C NMR data of compound **1** were identical to those of degalactotigonin (Figure 5).[13] Degalactotigonin completely inhibited spore germination of both tested pathogen isolates at a concentration of 8 mg/L. It was the principle antifungal isolated from *S. nigrum* by the bioactivity-guided fractionation procedure.

Treatment with 10 mg/L of crude extract was able to reduce the size of lesions on the detached cabbage leaves caused by these pathogens (Figure 2), while fungal spores treated with 10 mg/L of crude extracts still retained the ability to regrow after treatment. These results indicate that mechanisms other than inhibition of spore germination exist that contribute to preventing the successful penetration of these pathogens. The SGFs from fungal spore suspensions treated with crude extracts caused smaller yellow halos as observed with treatment with distilled water (Figure 1). However, the fresh SGFs mixed with the crude extract caused similar yellow halo diameters as the control treatments. These results indicate that instead of detoxification, crude extracts prohibit the secretion of toxic substances of the pathogens during germination. The SGF treatment showed similar symptoms as those caused by pathogens on wounded cabbage leaves but not on intact surfaces of cabbage leaves. Once the germ tubes lack the mechanical force needed to damage the cabbage leaf surface, the toxic substances produced by these pathogens are not able to cause lesions. According to the results of this study, the crude extracts of *S. nigrum* caused the germ tubes of these pathogens to swell and to lose the ability to form the appressoria; therefore, these pathogens were not able to successfully penetrate the host plants.

Most reports of degalactotigonin have been studied in human diseases. Degalactotigonin has been reported to exhibit considerable cytotoxicity in vitro against cultured solid tumor cell lines isolated from the human colon, prostate, liver, lung, breast, and glia.[13,14] It also showed antifungal activity toward *Candida* spp., *Cryptococcus neoformans*, and *Aspergillus fumigatus*, the causative agents of candidiasis, cryptococcosis, and aspergillosis, respectively, in humans.[15,16] Only the plant pathogen *Pyricularia oryzae* has previously been tested with degalactotigonin for antifungal activity as a bioassay material in vitro.[13] To our knowledge, this is the first report of the involvement of degalactotigonin from the extracts of *S nigrum* in controlling the plant disease black leaf spot of cabbage. The results presented in this study suggest that extracts of *S. nigrum* have the potential to be developed and applied as a natural product in the management of crop diseases.

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Author Contributions

§This author made the same contribution as the first author.

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