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Antifungal Activities Against Plant Pathogenic Fungi of Flavonoids Isolated from Amboyna Wood

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Abstract: The activities of two flavanoids, liquiritigenin and isoliquiritigenin, from Amboyna wood against the pathogenic fungi *Corynespora cassiicola*, *Trichoderma harzianum*, *Fusarium oxysporum*, *Cochliobolus miyabeanus*, *Aspergillus niger* and *Penicillium italicum* were evaluated using the agar dilution and paper disk methods. Low concentrations of liquiritigenin (20-40 μM) and isoliquiritigenin (16-33 μM) satisfactorily inhibited the growth of all the test fungi. Antifungal activities of the two flavonoids were found to be concentration-dependent. This is the first report that flavonoids from Amboyna wood are active against fungi pathogenic to the plants.

Key words: Amboyna wood, antifungal compound, isoliquiritigenin, liquiritigenin, plant pathogenic fungi

INTRODUCTION

In the course of our research into biologically active compounds from trees and their production by tissue culture[1-7], two flavanoids which are active toward pathogenic fungi isolated from Amboyna wood.

Fungi are among the most important plant pathogens, causing disease in cultivated plants and reducing yields and quality in vegetable crops and other agricultural products. These pathogenic fungi cause considerable economic losses for producers[8] including at least 20% of the yield reduction of crops worldwide[9]. *Fusarium oxysporum* (vascular wilt, yellows, root rot and damping-off), *Corynespora cassiicola* (weeping fig, leaf spot and stem rot) and *Cochliobolus miyabeanus* (rice blight and dumping-off) are just a few of the species recognized as causal agents for agricultural diseases. Some of pathogenic fungi, such as *Aspergillus* and *Penicillium* species, are also responsible for the contamination and spoilage of foods and production of mycotoxins as well[10-12]. Although synthetic fungicides provide some control of plant diseases in agriculture[13,14], undesirable environmental effects and the occurrence of fungicide resistance limits their application.

In the previous study, two antifungal flavonoids, liquiritigenin and isoliquiritigenin, were isolated from Amboyna wood (*Pterocarpus indicus* Willd., leguminosae). In the present study, we report their ability to inhibit the growth of six pathogenic fungi, known to be causal agents of several agricultural diseases, soil-borne and post-harvest pathogens and producers of mycotoxin.

MATERIALS AND METHODS

Test fungi: Six fungi were used in this study, *Fusarium oxysporum* (NBRC 31630), *Corynespora cassiicola* (NBRC 30049), *Cochliobolus miyabeanus* (NBRC 6631), *Trichoderma harzianum* (NBRC 31292), *Aspergillus niger* (NBRC 31638) and *Penicillium italicum* (NBRC 9419). All fungi were purchased from the National Institute of Technology and Evaluation (NITE), Kisarazu, Chiba, Japan.

Fungal cultures: Cultures of *F. oxysporum, C. cassiicola, C. miyabeanus, A. niger* and *P. italicum* were grown on a PDA (potato dextrose agar) medium (200 g of potato, 20 g of dextrose and 20 g of agar powder per liter, pH 5.6) on a petri dish and a slant. The culture of *T. harzianum* was grown on a malt extract agar medium (20 g of malt extract powder, 20 g of dextrose, 1 g of peptone and 20 g of agar powder, pH 6.0) on a petri dish and a slant. All cultures were maintained at 25°C for a certain period to enable them to grow well. The subculture of all fungi was conducted every 30 days during the experiment.

Preparation of inoculum suspension: Cultures of fungi were grown on the PDA slant and incubated for 7 days at 25°C until spores had grown. Fungal spores were

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harvested from the slant cultures by adding sterile distilled water. The surface of the mycelium was gently scrubbed with an inoculating loop. Mycelical debris was removed by filtration through filter paper No. 2 (Advantec Toyo, Ltd., Japan) and the spore suspension was transferred to a beaker glass. The number of spores in the suspension was determined using a Direct Microscopic Counts[15].

Agar dilution method: The agar dilution method was conducted as described in our previous report[7].

Paper disk method: Ten-milliliter aliquots of sterile molten PDA were transferred to petri dishes and allowed to solidify. The PDA plates were inoculated with 10 μL of spore suspension [$1x10^5$ spore mL^{-1} (*A. niger*) or $2x10^5$ spore mL^{-1} (*P. italicum*)] spread uniformly on the surface of the plates. A sterilized paper disc (8 mm in diameter, Advantec Toyo, Ltd., Japan) containing 50 μL of extract was applied to the surface of each inoculated plate. The plates were incubated in the dark at 25°C for 48 h. Zones of inhibition around the discs were measured in mm.

Synthesis of liquiritigenin and isoliquiritigenin: Liquiritigenin and isoliquiritigenin were synthesized by the aldol condensation of resacetophenone and *p*-hydroxybenzaldehyde[16,17]. Purification was conducted by column chromatography over silica gel and repeated recrystallization from aqueous ethanol. The purity of the liquiritigenin and isoliquiritigenin obtained was over 95% in a HPLC analysis. Their identities were confirmed by means of instrumental analysis and a mixed-melting point test of the authentic samples. The spectral data (MS, UV, ^{1}H and ^{13}C-NMR) of the synthesized products were compared to those of liquiritigenin and isoliquiritigenin isolated from Amboyna wood.

Preparation of the extract solution: Various concentrations of liquiritigenin (20, 40, 80, 160 and 320 μM) and isoliquiritigenin (16, 33, 66, 132 and 265 μM) in acetone were prepared by two-fold serial dilution. The prepared concentrations were based on the exact amounts of liquiritigenin and isoliquiritigenin in the wood on a dry weight basis, which were determined by HPLC analysis.

Antifungal activity and statistical analysis: Antifungal activity was determined based on inhibition using the formula, percent inhibition = (1 – T/C) x 100, where, T is hyphal extension of the treated sample and C is hyphal extension of the control. An average value was calculated from triplicate experiments. The statistical analysis and graphic performance were done using GraphPad Statmate 2 and GraphPad Prism 4 (GraphPad Software, USA).

RESULTS

The inhibitory effects of two flavanoids from Amboyna wood, liquiritigenin and isoliquiritigenin, on the growth of six fungi pathogenic to plants were examined. Due to the lack of availability of these compounds in wood, liquiritigenin and isoliquiritigenin were prepared by aldol condensation of resacetophenone and *p*-hydroxybenzaldehyde. The identity of the synthesized products was confirmed based on their physicochemical properties and instrumental analysis. The melting points and spectral data (MS, UV, ^{1}H and ^{13}C-NMR) of the synthesized compounds completely matched those of liquiritigenin and isoliquiritigenin isolated from Amboyna wood. The chemical structures are shown in Fig. 1. Various concentrations of liquiritigenin (20-320 μM) and isoliquiritigenin (16-265 μM) were applied to the agar dilution and paper disks to examine their antifungal activities. An inhibitory effect on the growth of *F. oxysporum* was noted at all concentrations of liquiritigenin used in the experiment (Fig. 2). A large increase in activity was observed when the concentration of liquiritigenin in the medium was increased to 320 μM. The growth of *C. cassicola* also seemed to be inhibited at all concentrations of liquiritigenin (Fig. 2). Despite that liquiritigenin was relatively less active toward *C. cassiicola* than *F. oxysporum*, the smallest concentration tested (20 μM) still had a substantial inhibitory effect. Liquiritigenin demonstrated a moderate inhibitory effect on the growth of *C. miyabeanus*, especially at the low concentrations, 20-40 μM (Fig. 3). Nevertheless, increasing the concentration of liquiritigenin to 80 μM significantly strengthened the inhibitory effect. The effect of liquiritigenin on the growth of *C. miyabeanus* was enhanced slightly when higher concentrations (160-320 μM) were added to the medium. Similar trends were observed in the assay using *T. harzianum* as the test fungus (Fig. 3). However, at up to 80 μM of liquiritigenin in the medium, the activity was weaker in comparison with that toward the other test fungi. The activity of liquiritigenin was much stronger when the concentration was increased to 160-320 μM. From the results of antifungal assays with the four test fungi, the inhibitory effect of liquiritigenin was found to be concentration-dependent.

Similar to liquiritigenin, isoliquiritigenin inhibited fungal growth at most of the concentrations tested. It can be seen in Fig. 4 that the activity of isoliquiritigenin to inhibit the growth of *F. oxysporum* increased along with the concentration up to 132 μM. Furthermore, an increase in the concentration to 265 μM was found to be able to raise the level of activity greatly. In general, an inhibitory effect of isoliquiritigenin on the growth of *C. cassiicola*

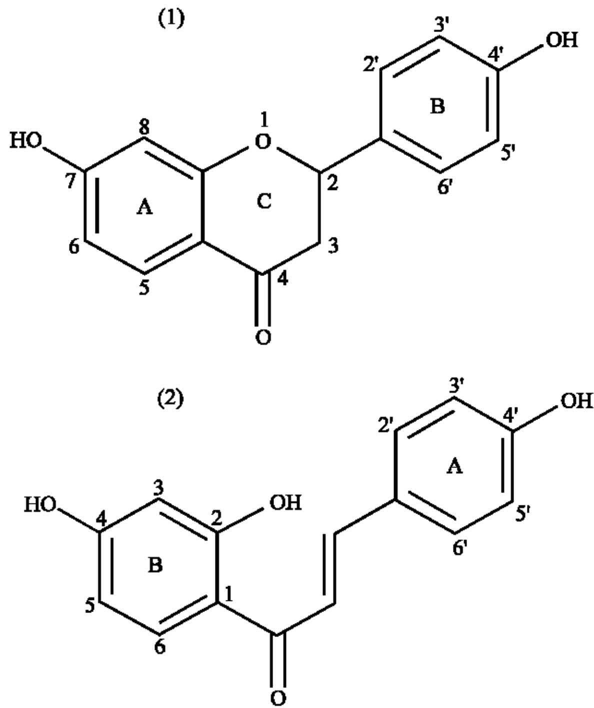

Fig. 1: Chemical structures of liquiritigenin (1) and isoliquiritigenin (2)

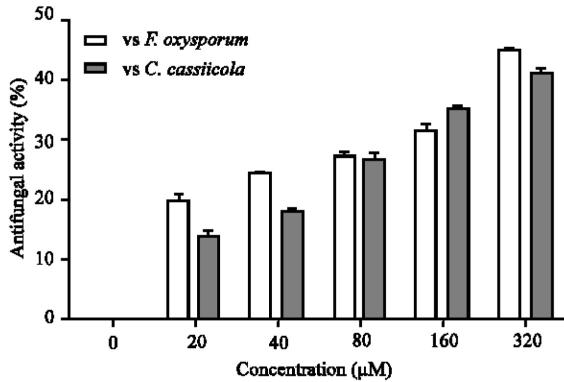

Fig. 2: Antifungal activity of liquiritigenin against *F. oxysporum* and *C. cassiicola*. Each data point represents the mean for triplicate samples of a representative experiment. Bars indicate the standard errors

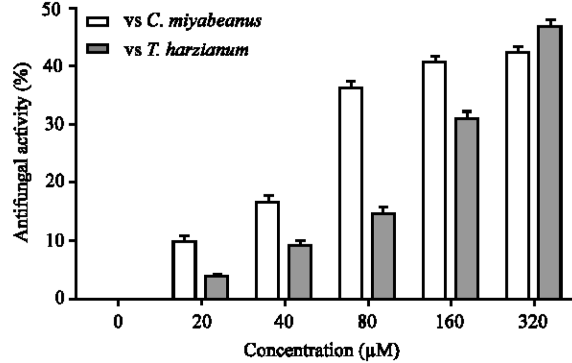

Fig. 3: Antifungal activity of liquiritigenin against *C. miyabeanus* and *T. harzianum*. Each data point represents the mean for triplicate samples of a representative experiment. Bars indicate the standard errors

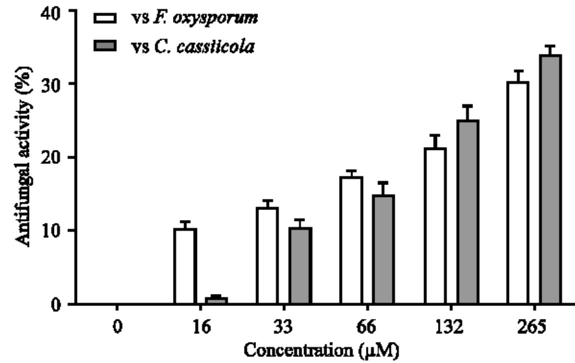

Fig. 4: Antifungal activity of isoliquiritigenin against *F. oxysporum* and *C. cassiicola* Each data point represents the mean for triplicate samples of a representative experiment. Bars indicate the standard errors

was observed at various concentrations (Fig. 4). However, at the lowest concentration, 16 μM, isoliquiritigenin seemed not to be active. The effect of isoliquiritigenin on the growth of *C. cassiicola* appeared increase considerably when the concentration was raised to 132 μM. Isoliquiritigenin had an inhibitory effect on the growth of *C. miyabeanus* at all concentrations tested. From Fig. 5, it can be observed that the inhibitory effect at between 33 and 66 μM of isoliquiritigenin was distinctly different. In spite of this, adding more isoliquiritigenin to the medium only slightly improved the inhibitory effect. The inhibitory effect of isoliquiritigenin on the growth of *T. harzianum* was relatively weak compared to that on *F. oxysporum*, *C. cassiicola* or *C. miyabeanus*. Increasing the concentration of isoliquiritigenin up to 166 μM seemed to result in a constant improvement of the inhibitory effect. A considerable improvement was obtained when the concentration was optimized to 265 μM. Increasing the concentration of isoliquiritigenin raised the inhibitory effect on fungal growth suggesting a concentration-dependent antifungal activity (Fig. 4 and 5).

The antifungal activities of liquiritigenin and isoliquiritigenin against two food contaminants and mycotoxin-producing fungi, *Aspergillus niger* and *Penicillium italicum*, were also evaluated. Based on the inhibitory effect of various concentrations of liquiritigenin and isoliquiritigenin added to the media, a small amount of

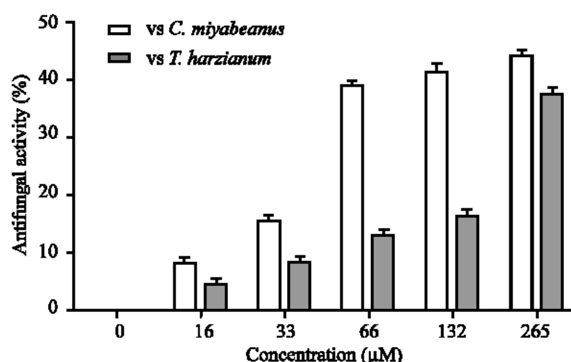

Fig. 5: Antifungal activity of isoliquiritigenin against *C. miyabeanus* and *T. harzianum*. Each data point represents the mean for triplicate samples of a representative experiment. Bars indicate the standard errors

Table 1: Minimum inhibitory amounts of liquiritigenin and isoliquiritigenin against six pathogenic fungi

	Minimum inhibitory amounts (μM)					
Flavonoids	FO	CC	CM	TH	AN	PI
Liquiritigenin	20	40	20	40	320	40
Isoliquiritigenin	16	33	16	33	265	33

Remarks: FO= *Fusarium oxysporum*, CC=*Corynespora cassiicola*, CM=*Cochliobolus miyabeanus*, TH=*Trichoderma harzianum*, AN=*Aspergillus niger*, PI=*Penicillium italicum*.

extract (40 μM of liquiritigenin, 33 μM of isoliquiritigenin) was required to generate a visible zone of inhibition zone for the growth of *P. italicum* (Table 1). Nevertheless, a larger amount of extract (320 μM of liquiritigenin, 265 μM of isoliquiritigenin) was needed to produce a visible zone of inhibition for the growth of *A. niger* (Table 1).

DISCUSSION

New agents are needed to control pathogenic fungi in plants. The findings presented here show that liquiritigenin and isoliquiritigenin isolated from Amboyna wood had a significant inhibitory effect on the mycelial growth of fungal pathogens. A low concentration of either compound was enough to inhibit the growth of *F. oxysporum* and *C. miyabeanus*, while higher concentrations were needed for the other fungi. A high concentration (320 μM for liquiritigenin and 265 μM for isoliquiritigenin) was required to inhibit the growth of *A. niger*, *F. oxysporum* and *C. miyabeanus* appeared to be most sensitive to liquiritigenin and isoliquiritigenin. *C. cassicola,* which seemed to be sensitive to liquiritigenin, possesses more resistance to isoliquiritigenin. Furthermore, *T. harzianum*, a fungus found in soil, had low levels of sensitivity to both liquiritigenin and isoliquiritigenin.

The fungi used in this study are common causal agents of various agricultural diseases, spoilage and contamination of foods and mycotoxin production. The results of this study suggest that liquiritigenin and isoliquiritigenin have the potential to control agricultural diseases and prevent spoilage and contamination. Even so, further analysis of the effect of this compounds on the sensory aspects of food is needed.

Flavonoids, a huge group of phenolic compounds, have been reported to possess various biological activities, including: anti-inflammatory, antihepatotoxic, anti-ulcer actions[18,19], antiallergic, antiviral and antitumoral effects[20,21]. Among naturally occuring flavonoids, liquiritigenin and isoliquiritigenin are considered characteristic constituents of the leguminosae family[22]. These compounds have been proven to act as monoamine oxidase inhibitors[23], inhibitors of cancer cells[24], anti-inflammatory[25] and xanthine oxidase inhibitors[26]. To the best of our knowledge, this is the first study to show that liquiritigenin and isoliquiritigenin possess antifungal activity. Present results provide new information on the biological activities of liquiritigenin and isoliquiritigenin, which at low concentrations inhibit the growth of six fungi pathogenic to plants.

Other plant species have also been reported to be sources of liquiritigenin and isoliquiritigenin, such as *Dalbergia sericea*[27], *Glycyrrhiza inflata*[28], *Crinum bulbispermum*[29] and *Sinofranchetia chinensis*[26]. This is an advantage in that having various sources will increase the possibility of producing these potentially useful compounds with biotechnological methods.

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