0971-4693/94 US $ 5.00
Allelopathy Journal 25 (2): 415-422 (2010) *International Allelopathy Foundation* 2010
Tables: 2, Fig : 1

Effects of water-soluble compounds of *Polygonatum cirrhifolium* rhizomes on plant pathogenic fungi

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(Received in revised form: February 28, 2009)

ABSTRACT

Three water-soluble compounds were isolated and characterized from the rhizomes of *Polygonatum cirrhifolium.* Their structures were determined as (25*R*)-spirost-5-ene-3*β*-ol-3-*O*-*α*-L-rhamnopyranosyl(1→2)-[*α*-L-rhamnopyranosyl (1→4)]-*β*-D- glucopyranoside (A), (25*R*)-spirost-5-ene-3*β*-ol-3-*O*-[*α*-L-rhamnopyranosyl (1→4)]-*β*-D- glucopyranoside (B) and n-butyl- *β*-D-fructopyranoside (C). These compounds were isolated first time from *P. cirrhifolium* and their antifungal activity was tested against growth of 11 plant pathogenic fungi. The compound A (3-sugars linked at C-3) drastically inhibited the growth of *Exserohilum turcicum* and *Botryosphaeria ribis* than compound B (2-sugars linked at C-3), with EC_{50} of 46.8 and 57.5 µg/ml, respectively. Diosgenin (the aglycone of compound A and B) was also more inhibitory to growth of *Fusarium oxysporum* with EC_{50} of 288.4 µg/ml.

Key words: Alternaria, antifungal activity, *Botryosphaeria ribis,* brassicae, *Colletotrichum lagenarium, Colletotrichum gloeosporioides, Cytospora chrysosperma, Exserohilum turcicum, Fusarium graminearum, Fusarium oxysporum,* isolation and identification*, Polygonatum cirrhifolium, Trichothecium roseum, Verticillium dahliae*, water-soluble compounds.

INTRODUCTION

Worldwide crop production is heavily dependent on use of agrochemicals, however, these synthetic agrochemicals pollute the environment and cause health hazards (5). Hence alternative methods which are less pesticide dependant or based on natural products, needs to be developed (1,4). Plant natural products are more environment-friendly than synthetic chemicals (3). Several antifungal compounds present in some plant species controls the pathogenic fungi (9,10,12). *Polygonatum cirrhifolium* (Liliaceae) plants are widely distributed in Qinling Mountains, China. Its rhizomes (called "tiger ginger") are used in folk medicine and healthy food (8,11,17). We found that the n-butanol extracts of *Polygonatum cirrhifolium* were most inhibitory to growth of plant pathogenic fungi than bacteria's (15.16). However, the water-soluble constituents with antifungal activity from these rhizomes were not studied. Therefore, further phytochemical analysis of water-soluble constituents led to the isolation of two steroidal saponins and one

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fructopyranoside. This paper describes the isolation and structure determination of three compounds and the aglycones of steroidal saponins by detailed analysis of their NMR spectra. The fungicidal activities of compounds are also described.

MATERIALS AND METHODS

Extraction and isolation of compounds

The *Polygonatum cirrhifolium* plants rhizomes were collected from Qinling Mountains in September 2005. The voucher specimen (No. 66) was deposited in Herbarium, Northwest Sci-Tech University of Agriculture and Forestry. The air-dried rhizomes of *Polygonatum cirrhifolium* (20 kg) were extracted with 95% ethanol at room temperature. The extract was concentrated under vacuum to give a brown waxy residue. The residue was partitioned between water and ethyl acetate (residues : water : ethyl acetate = 1 : 3 : 3). The water fraction was further extracted with n-butanol. The evaporated n-butanol extract (624.1 g) was chromatographically developed on a silica gel column eluted with a gradient chlorophorm/methenol (20:1, 15:1, 10:1, 5:1 and 1:1) to afford eight fractions. Further chromatography of fraction 3 (24 g) by silica gel , RP-18 and Sephadex LH-20 to yield compound A (14 g), compound B (100 mg) and compound C (120 mg) .

Acid hydrolysis of compound A

Compound A (100 mg) was refluxed with 1M HCl/(dioxane:H_2O =1:1, 2 ml) in water bath at 100^oC for 6 h (6,20). The reaction mixture was evaporated to dryness. The dry residue was partitioned four times between chlorophorm and water (chlorophorm:water=1:1). The chlorophorm fraction was concentrated and subjected to silica gel CC to provide compound D (28 mg). The aqueous fraction was identified by direct comparison with standard sugar sample on TLC. D-glucose and L-rhamnose were detected in each compound as the common sugars.

Melting points of water soluble compounds of *P. cirrhifolium* plants rhizomes were determined on an XT-4 apparatus and are uncorrected. NMR spectra were recorded on Varian Unity 300 and Bruker DRX-500 spectrometers, using TMS as an internal standard. EI-MS data were obtained on a Finnegan DSQ spectrometer. FAB-MS data were obtained on a VG AutoSpec-3000 mass spectrometer. Sephadex LH-20 (Amersham Biosciences). Octadecylsilyl-A silica-gel (Yamamura Chemical). Detection was done by spraying the plates with 5% anisaldehyde-sulfuric acid, followed by heating.

Antifungal activity

The hyphae growth method was used to study the fungicidal effects of compounds against plant fungi (14). All the compounds were individually tested against the growth of 11 plant pathogenic fungi, which were obtained from Microbiology Laboratory, Northwest Sci-Tech of A & F University, and were identified by Li Xiao-Ming senior laboratory technician. They were *Alternaria brassicae* (Berk.) Sacc., *Botryosphaeria ribis* (Tode) Gross et Duss, *Colletotrichum lagenarium* (Pass.) Ellis & Halst., *Colletotrichum gloeosporioides* Penz, *Cytospora chrysosperma* (Pers) Fr., *Exserohilum turcicum* (Pass.) K.J. Leonard & Suggs, *Fusarium graminearum* Schwabe,

Fusarium oxysporum Schltdl., *Trichothecium roseum* (Pers.) Link, *Valsa mali* Miyabe & G. Yamada and *Verticillium dahliae* Kleb. Fungi were grown on potato dextrose agar (PDA) medium, stored in a refrigerator at 4°C and sub-cultured on fresh media whenever needed. The hyphae growth method was used to study the antifungal activity of compounds. Appropriate quantities of stock solution and distilled water were added to PDA medium to get 25, 50, 100, 200, 400 µg/ml concentrations of individual compound in the medium. Control received the same quantity of distilled water. The 20 ml media was poured into Petri plates (90 mm dia) and allowed to solidify. The plates were inoculated in the centre with 5 mm disc on PDA. Each treatment was replicated three times. The plates were incubated for 96 h at 28.8°C and colony diameter was measured. To calculate the inhibitory effects, the inoculum disc diameter (5 mm) was subtracted from the measured colony diameters. The EC_{50} (50% effect at conc) indicates the concentration of a compound which cause 50% growth inhibition) were also calculated from dose-response curves using regression analysis (Microsoft Excel) (9).

Growth inhibition (%) of fungal colonies was calculated by following formula (4):

Growth inhibition (%) = [(Growth in control - Growth in treatment) / Growth in control] × 100

RESULTS AND DISCUSSION

Identification of compounds A-D

Compound A was obtained as white needles, m.p. 189-193°C (methanol), reacted positively to Liebermann-Burchard and negatively to Ehrlich reagents, suggesting that A was a steroidal saponin. Compound A gave D-glucose and L-rhamnose as a result of acid hydrolysis, identified by comparision with an standard sample by TLC analysis. The FAB-MS (negative): *m/z* 867[M-H]$^+$. The 1H-NMR spectrum (pyridine-d5, 500MHz) of compound A showed two singlet methyl signals at δ 0.81and 1.03, and two doublet methyl signals at δ 1.03 and 1.06, which were recognized as typical steroidal methyls. The ^{1}H-NMR spectrum showed one olefinic protons at δ 5.31 were in accordance with the data obtained from ^{13}C -NMR (pyridine-d5). Signals for three anomeric protons at δ 4.94, 5.83 and 6.37 were in accordance with the three anomeric carbons at δ 100.36, 102.11 and 102.97(CH) obtained from ^{13}C-NMR spectrum, suggested the existence of three sugar units attached to the aglycone. Morever, a quaternary carbon signals at δ 109.37, four methyl groups signals at δ 16.40(CH3), 19.50(CH3), 17.50 (CH3) and 14.97(CH3), respectively. ^{1}H NMR(pyridine-d_5, 500MHz): *δ* 0.81(3H,s,CH_3-18), 1.03(3H,d,CH_3-27), 1.03(3H,s,CH_3- 19), 1.06 (3H,s, CH_3-21), 4.94(1H,d,H-1 of Glu), 5.31(1H,m,H-6), 5.83(1H,brs,H-1 of Rhaml), 6.37(1H, brs,H-1 of Rham2) ^{13}C NMR(pyridine-d_5,): 37.60(C-1), 30.24(C-2), 78.22(C-3), 39.06(C-4), 140.91(C-5), 121.89(C-6), 32.41(C-7), 31.79(C-8), 50.41(C-9), 37.23(C-10), 21.20(C-11), 39.96(C-12), 40.54(C-13), 56.74(C-14), 32.28(C-15), 81.28(C-16), 62.50(C17), 16.40(C-18), 19.50(C-19), 42.10(C-20), 14.97(C-21), 109.37(C-22), 31.90(C-23), 29.00(C-24), 30.00(C-25), 67.00(C-26), 17.50(C-27), 100.36(C-1 of Glu), 78.90(C-2 of Glu), 77.96(C-3 of Glu), 78.22(C-4 of Glu), 78.00(C-5 of Glu), 62.00(C-6 of Glu), 102.11(C-1 of Rham l), 72.30(C-2 of Rham l), 72.79(C-3 of Rham l), 73.96(C-4 of Rham l), 69.59(C-5 of Rham l),

18.56(C-6 of Rham l), 102.97(C-1 of Rham 2), 72.60(C-2 of Rham 2), 72.79(C-3 of Rham 2), 74.18(C-4 of Rham 2), 70.51(C-5 of Rham 2), 18.56(C-6 of Rham 2). Compound A was identified as (25*R*)-spirost-5-ene-3*β*-ol-3- *O*-*α*-L-rhamnopyranosyl (1→2)-[*α*-L-rhamnopyra-nosyl (1→4)]-*β*-D-glucopyranoside, which had been isolated from another plant of the same genus, *Polygonatum zanlanscianense* (18).

Compound B was obtained as white needles, m.p. 233-237°C (methanol), reacted positively to Liebermann-Burchard and negatively to Ehrlich reagents, suggesting that B was a steroidal saponin. Compound B gave D-glucose and L-rhamnose as a result of acid hydrolysis, identified by comparision with a standard sample by TLC analysis. FAB-MS (negative): *m/z* 721[M-H]$^+$. ^{13}C NMR(pyridine-d_5,): *δ* 37.60(C-1), 30.28(C-2), 78.22(C-3), 39.06(C-4), 140.91(C-5), 121.90(C-6), 32.41(C-7), 31.79(C-8), 50.41(C-9), 37.23(C-10), 21.20(C-11), 39.60(C-12), 40.54(C-13), 56.74(C-14), 32.28(C-15), 81.21(C-16), 62.80(C-17), 16.40(C-18), 19.50(C-19), 41.90(C-20), 15.00(C-21), 109.37(C-22), 30.00(C-23), 30.25(C-24), 30.50(C-25), 66.90(C-26), 17.50(C-27), 102.97(C-1 of Glu), 75.10(C-2 of Glu), 76.97(C-3 of Glu), 78.22(C-4 of Glu), 76.80(C-5 of Glu), 62.00(C-6 of Glu), 102.11(C-1 of Rham), 72.60(C-2 of Rham), 72.30(C-3 of Rham), 73.70(C-4 of Rham), 70.51(C-5 of Rham), 18.56(C-6 of Rham). Compound B was identified as (25*R*)-spirost-5-ene-3*β*-ol-3-*O*-[*α*-L-rhamnopyranosyl (1→ 4)]-*β*-D-glucopyranoside (2).

Compound C was obtained as a white amorphous powder. ^{1}H NMR(Pyridine-d_5,300MHz): 4.18(1H,d,*J*=11.35Hz), 3.73(2H,m), 1.56(2H,m), 1.35(2H,m), 0.81(3H,t). ^{13}C NMR (pyridine-d_5,): δ 60.63(C-1), 32.68(C-2), 19.95(C-3), 14.19(C-4), 64.42(C-1′), 101.33(C-2′), 71.45(C-3′), 70.81(C-4′), 72.58(C-5′), 65.14(C-6′). Compound C was identified as n-butyl-*β*-D- fructopyranoside. This compound has been isolated from another plant of the same genus, *Polygonatum kingianum* (19).

Compound D (the aglycone of compound A and B) was obtained as white needles. EI-MS: *m/z* 414[M]$^+$(4), 355(6), 342(10), 300(16), 282(42), 139(100), 69(24) ^{1}H NMR ($CDCl_3$, 300MHz): 1.60(3 H,s), 1.03(3 H,s), 0.97(3 H,d , *J*=6.9Hz), 0.80(3 H,d, *J*=4.5Hz). ^{13}C NMR (pyridine-d_5,): *δ* 37.44 C-1), 30.52(C-2), 71.97(C-3), 40.01(C-4), 141.01(C-5), 121.67 (C-6), 32.07(C-7), 31.65(C-8), 50.26(C-9), 36.87(C-10), 21.10(C-11), 40.49(C-12), 41.82(C-13), 56.73(C-14), 32.27(C-15), 81.06(C-16), 62.29(C-17), 16.53(C-18), 19.66(C-19) ,42.49(C-20), 14.76(C-21), 109.54(C-22), 31.84(C-23), 29.02(C-24), 31.60(C-25), 67.07(C-26), 17.37(C-27). The aglycone was identified as diosgenin by comparison with standard sample (13).

Antifungal activity of compounds

All test compounds proved inhibitory to fungal colony growth except compound C (Table1). Compound A and B (400 μg/ml) were most inhibitory to *Exserohilum turcicum* (76.0 and 74.3% inhibition, respectively) and caused 73.7 and 72.8% inhibition in growth of *Botryosphaeria ribis*. These were less inhibitory to *Fusarium oxysporum* (40.1 and 39.6% inhibition) and *Colletotrichum coffearum* (41.7 and 35.2 % inhibition) (Table 1). Compound D inhibited (62.4%) only the growth of *Fusarium oxysporum* (Table 1). None of the test compounds affected the *Fusarium graminearum*, *Cytospora chrysosperma* and *Trichothecium roseum* growth (Table 1). Compound A was most inhibitory to growth of *Exserohilum turcicum* and *Botryosphaeria ribis*, with EC_{50}

Table 2. Toxicity of tested compounds to the hyphae growth of several plant pathogens

Tested compounds	Plant pathogenic fungi	Test compounds conc. (μg/ ml)	Colony diameter (mm)	Toxicity regression equation	R^2	EC_{50} (μg/ ml)
(25*R*)-spirost-5-ene-3*β*-ol-3-*O*-*α*-L-rhamnopyranosyl (1→2)-[*α*-L-rhamnopyra-nosyl (1→4)]-*β*-D-glucopyranoside	*Botryosphaeria ribis*	400	18.0	*Y*=0.262 4*x*+ 0.038	0.9702*	57.5
		200	26.2			
		100	28.9			
		50	46.4			
		25	53.3			
		Control	68.3			
	Exserohilum turcicum	400	9.9	*Y*=0.288 2*x*+ 0.018	0.9939*	46.8
		200	15.7			
		100	18.4			
		50	20.0			
		25	24.3			
		Control	41.3			
(25*R*)-spirost-5-ene-3*β*-ol-3-*O*-[*α*-L-rhamnopyranosyl (1→ 4)]-*β*- D-glucopyranoside	*Botryosphaeria ribis*	400	18.6	*Y*= 0.487 5*x*+0.358	0.9788*	57.7
		200	27.3			
		100	29.7			
		50	47.3			
		25	54.2			
		Control	68.3			
	Exserohilum turcicum	400	10.6	*Y* =0.375 5*x*+0.169	0.9808*	60.5
		200	14.3			
		100	19.7			
		50	21.8			
		25	24.6			
		Control	41.3			
Diosgenin	*Fusarium oxysporum*	400	33.8	*Y*=.237 5*x*+0.086	0.9907*	288.4
		200	51.4			
		100	54.7			
		50	63.8			
		25	70.0			
		Control	90.0			

Note: Compound 'C' stimulated the fungi growth.

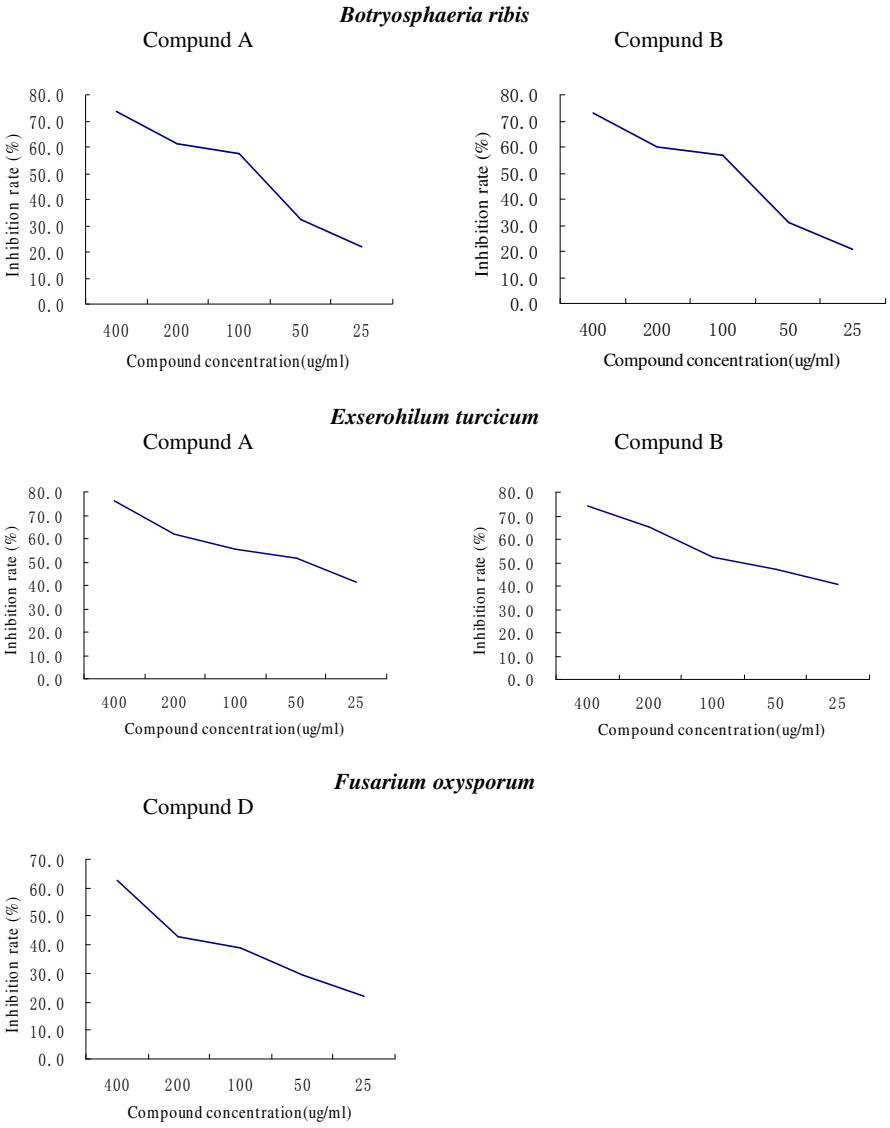

Figure 1. Inhibitory effects of compounds A, B and D on the *Botryosphaeria ribis*, *Exserohilum turcicum* and *Fusarium oxysporum* hyphal growth.

Note: Compound 'D' had no inhibitory effects on the *Botryosphaeria ribis* and *Exserohilum turcicum* growth.

Table 1. Effects of four compounds (at 400 μg/ ml) on the Inhibition circles diameter (mm) over control growth of plant pathogenic fungi

Plant pathogenic fungi	Inhibition circles diameter (mm) of control and four test compounds				
	Control	A	B	C	D
Alternaria brassicae (Berk.) Sacc.	52.0	44.9	43.9	52.0	48.9
Botryosphaeria ribis (Tode) Gross et Duss	68.3	18.0	18.6	+69.4	63.1
Colletotrichum lagenarium (Pass.) Ellis & Halst.	56.0	44.0	44.5	+56.2	45.6
Colletotrichum gloeosporioides Penz	47.2	27.5	30.6	+48.1	43.7
Cytospora chrysosperma (Pers) Fr.	33.4	+36.8	33.5	+33.9	+42.1
Exserohilum turcicum (Pass.) K.J. Leonard & Suggs	41.3	9.9	10.6	+42.5	35.8
Fusarium graminearum Schwabe	38.6	+42.7	+39.4	+40.6	+38.9
Fusarium oxysporum Schltdl.	90.0	35.1	54.3	90.0	33.8
Trichothecium roseum (Pers.) Link	39.5	+40.2	+39.6	+40.0	38.0
Valsa mali Miyabe & G. Yamada	32.5	27.5	27.9	+33.6	27.9
Verticillium dahliae Kleb.	52.5	41.6	41.1	+53.6	42.1

+ Stimulation in fungi growth.

respectively. (Table 2). Compound D was also inhibitory to growth of *Fusarium oxysporum*, with EC_{50} of 288.4 μg/ml (Table 2).

Comparing the compound A, B and D (the aglycone of compound A and B), the compound A had three sugars (one glucose and two rhamnoses) linked at C-3 position, compound B had two sugars (glucose and rhamnoses) linked at C-3 position, while compound D had no sugar linked at C-3 position. The compound A was more inhibitory than compound B (Figure 1), this effect might due to the changes of sugar number and chains. Many examples in literature support our findings. Liu (7) reported that the antifungal activity of saponins was decreased with decrease in sugar chains. Similar results of inhibitory effects of various saponins from *Medicago arabica* against growth of *Geumannomyces graminis* have been reported (9). But the mechanism, how the sugar chains affects the antifungal activity (such as altering the physical properties of compounds or affecting the recognition and combination of receptor molecules etc.), should be deeply researched.

ACKNOWLEDGEMENTS

We acknowledge the financial support from the Forestry Research Foundation for the Public Service Industry of China(200904004) ,the Program for Youth Foundation of Northwest A&F University (07ZR013), the Program for Youth Barebone Talents of Northwest A&F University, National Key Technologies R & D Program of Shaanxi province (2009K19-06) and Science Research Foundation for Doctor Program of Northwest A&F University (01140505).

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