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ANTIMICROBIAL COMPOUNDS FROM *ALNUS RUBRA*

Geeta Saxena[1], Susan Farmer[2], R. E. W. Hancock[2] and G. H. N. Towers[1] *

[1]Department of Botany, The University of British Columbia, Vancouver, B.C. V6T 1Z4. Canada [2]Department of Microbiology, The University of British Columbia, Vancouver, B. C. V6T 1Z3. Canada

ABSTRACT

The methanol extract of the bark of Alnus rubra *showed antimicrobial activity against Gram-positive and Gram-negative bacteria. Diarylheptanoid and its glycoside (oregonin) were identified as the two constituents responsible for this activity.*

INTRODUCTION

Although a variety of compounds are reported from species of *Alnus* (Lee *et al.*, 1992), these compounds have not been examined for their antimicrobial activity. On the basis of ethnobotanical information (Turner *et al.*, 1990), we have screened a number of medicinal plants of British Columbia (McCutcheon *et al.*, 1992) and found that the methanol extracts of *Alnus rubra* Bong. (Betulaceae), commonly known as 'red alder', show good activity against Gram-positive and Gram-negative bacteria. The isolation and characterization of two active components, diarylheptanoid and its glycoside, are reported here as antimicrobial agents, for the first time.

Keywords: Alnus rubra, antibiotics, diarylheptanoid, ethnopharmacology, British Columbia.

*All correspondence should be addressed to G.H.N. Towers, Department of Botany, The University of British Columbia, Vancouver, B.C. V6T 1Z4, Canada.

MATERIALS AND METHODS

General

^{1}H NMR spectra were recorded at 300 and 200 MHz on Varian XL-300 and Bruker AC-200 E spectrometers, respectively. ^{13}C NMR spectra was recorded at 75 MHz on Varian XL-300 spectrometer with TMS as internal standard. Low-resolution mass spectra were obtained on Kratos MS 50 mass spectrometer (70 eV). UV spectra were measured in MeOH on a PU 8007 UV/Vis.instrument. IR spectra were recorded in KBr discs on a Perkin Elmer 1710 FT-IR spectrometer. For CC, silica gel 60 (70-230 mesh), and for TLC, silica gel 60 F_{254} plates were used.

Plant Material

Bark of *Alnus rubra* Bong. (Betulaceae) was collected at Maple Ridge Forest, Vancouver, B.C. in June 1992. A specimen (no. V 206233) has been deposited in the Herbarium of the University of British Columbia.

Extraction and Isolation

Powdered bark (1.9 kg) was extracted successively at room temp. with MeOH. This extract was bioassayed against Gram-positive and Gram-negative bacteria. Different bioassay techniques *viz.* disc bioassay, thin layer chromatography (TLC) bioassay by contact process and bioautography using an overlay method were used. The crude extract was initially chromatographed on TLC (silica gel, $CHCl_3$-MeOH, 85:15). Further purification by CC and disk chromatotron using different proportions of $CHCl_3$-MeOH,

resulted in the isolation of the two active compounds, **1** (48 mg) and **2** (220 mg) as well as inactive triterpenes (Sheth *et al.*, 1973; Jain and Seshadri, 1971).

Microorganisms Used

Seven bacterial strains were used in the screening: *Bacillus subtilis*, *Escherichia coli* UB 1005, *Escherichia coli* DC2, *Pseudomonas aeruginosa* Z 61, *Pseudomonas aeruginosa* K 799, *Staphylococcus aureus* meth^S RN 4220, and *Staphylococcus aureus* meth ^{R} P00017.

Antimicrobial Testing

The antimicrobial activity of crude extracts was evaluated by the classic agar disc diffusion procedure using Mueller-Hinton agar (Difco). Paper discs (1/4") were impregnated with 20 µl of the methanol solution of each sample (1.6 g/ml), except pure compounds and allowed to evaporate at room temp. Gentamicin and methicillin were used as positive controls and MeOH as a negative control. The plates were incubated for 18 hr at 37° and the diameter of the zone of inhibition around each disc measured and recorded at the end of the incubation period. Simultaneously, TLC bioassay, using bioautography techniques (Rahalison *et al.*, 1991) were used to determine which component in the crude extract was active. As the sensitivity of the disc bioassay is low, final activity was performed using minimum inhibitory concentration (MIC) method.

MICs were determined by the standard broth microdilution method in Mueller-Hinton, with an inoculum of 10^4 CFU/ml. To ensure that the densities of the diluted cultures were all within the range, serial dilution plate counts were also made for each culture. All strains were grown at 37° in nutrient broth.

(5*S*)-1,7-*bis*(3,4-Dihydroxyphenyl)-5-hydroxyheptan-3-one (1)

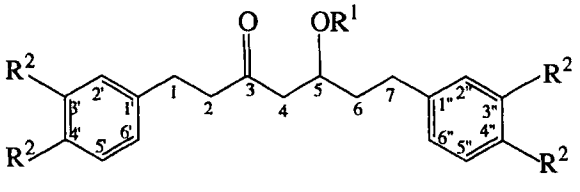
Amorphous, pale yellow in color, $[\alpha]_D$ -2.0° ± 1° (MeOH, *c* =0.1). UV λ^{max}(MeOH) (log ε): 210 (2.55), 236.8 (2.82), 284 (2.57)nm. IR ν^{max}(KBr) cm^{-1} : 3360 (OH), 1700 (C=O), 1610 and 1510 (aromatic ring). ^{1}H NMR (300 MHz, CD_3OD) : δ 6.65-6.75 (4H, m, H-2', H-2", H-6', H-6"), 6.5 (2H, dd, *J*= 2,8 Hz, H-5', H-5"), 4.03 (1H, q, *J*= 6 Hz, H-5), 2.4-2.6 (8H, m, H-1, H-2, H-4, H-7), 1.55-1.70 (2H, m, H-6). ^{13}C NMR (75 MHz,C_5D_5N) : δ 204.5 (C-3), 149.2, 149.0, 148.8, 147.3, 134.2, 133.7, 120.6, 112.9, 112.7 (aromatic C), 67.5 (C-5), 51.6 (C-4), 45.6 (C-2), 41.0 (C-6), 38.8 (C-7), 29.2 (C-1). EIMS *m/z* (rel. int.): 348 $[M]^+$ (0.5), 330 (1.5), 258 (0.1), 180 (2.8), 168 (1.9), 138 (5.4), 123 (100), 91 (15.9).

Oregonin (2)

Brown amorphous powder, mp 155°, $[\alpha]_D$ -27.0° ± 1° (MeOH, *c* =1.6). UV λ^{max}(MeOH) (log ε) : 211.6 (2.52), 229.8 (2.69), 283.1 (2.47)nm. IR ν^{max}(KBr) cm^{-1} : 3360- 3500 (OH), 1700 (C=O), 1605 and 1512 (aromatic ring). ^{1}H NMR (300 MHz, CD_3OD) : δ 6.54 -6.63 (4H, m, H-2', H-2", H-6', H-6"), 6.42 (2H, dd, J= 2,8 Hz, H-5', H-5"), 4.15 (1H, d, *J*=7.6 Hz, xyl-1), 4.05 (1H, m, H-5), 3.8 (1H, dd, *J*= 5,11 Hz, xyl-5), 3.45 (1H, m, xyl-4), 3.24 (1H, xyl-2 overlapped with HOD), 3.1 (1H, t, *J*= 8 Hz, xyl-3), 2.35-2.50 (8H, m, H-1, H-2, H-4, H-7), 1.65-1.78 (2H, m, H-6). ^{13}C NMR (75 MHz, C_5D_5N): δ 209.0 (s, C-3), 146.9 (C-3', 3"), 145.1 (C-4', 4"), 134.2 (C-1"), 133.1 (C-1'), 119.8 (C-6"), 119.7 (C-6'), 116.8 (C-5', 5"), 116.7 (C-2"), 116.4 (C-2'), 104.18 (anomeric C), 78.2 (xyl C-3), 75.4 (C-5), 74.8 (xyl C-2), 70.9 (xyl C-4), 67.0 (xyl C-5), 48.1 (C-4), 45.9 (C-2), 38.3 (C-6), 31.4 (C-7), 29.3 (C-1). EIMS *m/z* (rel. int.): 330 [M^+-xyl-H_2O] (1.0), 180 (0.5), 168 (0.5), 150 (2.4), 138 (1.2), 132 (0.8), 123 (100), 114 (0.4), 96 (0.8), 91(11.0).

Tetramethyl Ether of 2

Methylation of **2** with diazomethane (Ohta *et al.*, 1984) gave amorphous yellow powder **3**, mp 54°, $[\alpha]_D$ -19.5 ± 1 (MeOH, *c* =0.58). UV λ^{max} (MeOH) (log ε) : 228.3 (2.76), 280 (2.24)nm. IR ν^{max}(KBr) cm^{-1} : 3365 (OH), 1710(C=O), 1608 (aromatic ring). ^{1}H NMR (200 MHz, $CDCl_3$) : δ 6.68-6.95 (4H, m,



1. R^1 = H; R^2 = OH

2. R^1 = -β-D-xylopyranose; R^2 = OH

3. R^1 = β-D-xylopyranose; R^2 = OMe

Fig. 1.

H-2', H-2", H-6', H-6"), 4.52 (2H, m, H-5', H-5"), 4.20 (1H, d, *J*=7 Hz, xyl-1), 4.05 (1H, m, H-5), 3.68 (12H, s, 4 x OMe), 2.43-3.10 (8H, m, H-1, H-2, H-4, H-7), 1.65-1.72 (2H, m, H-6).

RESULTS AND DISCUSSION

Preliminary screening of methanol extracts of the bark of *Alnus rubra* by thin layer chromatography (TLC) and by bioautography with different microorganisms (Rehalison *et al.*, 1991) revealed the presence of two antimicrobial compounds. In addition, several non-polar, inactive compounds were also detected and identified as β-sitosterol, betulin, lupeol and taraxerol by direct comparison with authentic samples. Isolation of the two antimicrobial compounds was achieved using column chromatography (CC) and the disk chromatotron (Materials and Methods).

Both compounds **1** and **2** gave a blue color with $FeCl_3$ reagent on TLC (Lee *et al.*, 1992) and reacted with $NaBH_4$ reagent indicating the presence of a phenolic moiety and a carbonyl group, respectively, in both molecules.

Compound **1** was stable to mild acid hydrolysis while **2** gave two products, one of which was identical to **1** and the other was found to be D-xylose, identified by paper chromatography (Hough and Jones, 1962), which indicated that **2** is a glycoside of **1**.

On the evidence of the ^{13}C NMR chemical shifts and the coupling constant of the anomeric proton (Materials and Methods), the C-1' position of D-xylopyranose in **2** was determined to be bound to the 5-position of the aglycone via a β-glycosidic linkage. On comparison of the ^{13}C NMR chemical shifts of **2**

Table 1. Antimicrobial activity of extracts and pure compounds from *Alnus rubra*.

Extracts and compounds	Minimum Inhibitory Concentration (μg/ml)						
	Pseudomonas aeruginosa		*Escherichia coli*		*Staphylococcus aureus*		*Bacillus subtilis*[d]
	Z61[a]	K799[a]	UB1005[a]	DC2[a]	RN4220[b]	SAP00017[c]	
Methanol	125	250	1000	125	62.5	125	250
Hexane	500	2000	>2000	>2000	500	1000	125
Pet-ether	500	2000	>2000	>2000	500	1000	250
Ether	125	250	500	250	62.5	62.5	250
Butanol	125	250	500	250	62.5	125	500
Water	125	500	500	250	62.5	62.5	500
Compound **1**[e]	125	250	>500	250	31.2	62.5	125
Compound **2**[e]	125	250	500	125	31.2	62.5	250
Methicillin[f]	≤1	128	≥1024	≤1	1	512	48
Gentamicin[f]	0.25	1	0.25	0.5	0.25	32	<0.2

[a] Strain K799 (Angus *et al.*, 1987) and UB1005 (Clark, 1984) are wild type, and Z61 (Angus *et al.*, 1987) and DC2 (Clark, 1984) their respective supersusceptible mutants.
[b] Strain RN4220 (Kreiswirth *et al.*, 1983) is a wild type strain described previously.
[c] Strain SAP00017 is a methicillin resistant clinical isolate kindly provided by Dr. T. Chow, UBC.
[d] General wild type strain.
[e] Isolated in this study, see Fig. 1 for structure of compounds **1** and **2**.
[f] Control antibiotics.

with those of **1**, it was found that the glycosylation shift at C-4 (-3.4 ppm) was larger than that at C-6 (-2.7 ppm) (Materials and Methods). By application of the glycosylation shift rule (Seo *et al.*,1978) to these shifts, the configuration at C-5 of the glycoside (**2**) was assigned as *S*.

Compound **2** was identified as (5*S*)-1,7-*bis* (3,4-dihydroxyphenyl)-5-(β-D-xylopyranosyloxy) heptan-3-one (oregonin) on the basis of its physical and spectral data and of its tetramethyl ether (**3**), and the aglycone (**1**) was proven to be (5*S*)-1,7-*bis* (3,4-dihydroxyphenyl)-5-hydroxyheptan-3-one. These physical and chemical data were found to be similar to those reported for oregonin and its aglycone (Suga *et al.*, 1982; Ohta *et al.*, 1984).

The methanol extract had a MIC of 62.5 μg/ml against *Staphylococcus aureus* and 125 μg/ml against *Pseudomonas aeruginosa* and *Escherichia coli* and 250 μg/ml against *Bacillus subtilis*. After fractionation with solvents of different polarities, the lowest MIC was found in the ether, butanol and water fractions, respectively, which were showing similar spots on TLC. This was confirmed by disc bioassay and bioautography using an overlay technique showing maximum inhibition zone. Both compounds **1** and **2** were isolated from the ether fraction only. The results obtained are summarized in Table 1.

This study has indicated that the bark of *Alnus rubra* contains two antimicrobial compounds, **1** and **2**, with MICs of 31.2 μg/ml and 125 μg/ml, respectively, against both *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and 250 μg/ml and 125 μg/ml respectively, against *Escherichia coli* strains and 125 μg/ml and 250 μg/ml against *Bacillus subtilis*. This activity is considerably inferior to commercial antibiotics like gentamicin and methicillin but nevertheless indicated a basis for the ethnopharmacological use of *Alnus rubra* (Turner *et al.*, 1990).

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