J. Microbiol. Biotechnol. (2001), **11**(6), 1102–1105

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Therapeutic Agents against Bacteria Causing Porcine Pneumonia

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Received: September 29, 2001
Accepted: November 7, 2001

Abstract In order to find therapeutic agents for porcine pneumonia, we screened for antibacterial activities of methanol extracts of 81 higher plants against four pathogenic microorganisms of *Heamophilus parasuis*, *Pasteurella multocida*, *Actinobacillus pleuropneumonia*, and *Bordetella bronchiseptica*, and found the bark of Cinnamomi cortex showed potent activities. Since this was inexpensive, we purified active compounds from it. The structures of the final active fractions were obtained through an activity-guided fractionation and their antibacterial activities are reported here.

Key words: Porcine pneumonia, *Heamophilus parasuis*, *Pasteurella multocida*, *Actinobacillus pleuropneumonia*, *Bordetella bronchiseptica*, *Cinnamomi cortex*

Among several porcine diseases such as alimentary diseases, respiratory ailments, and propagative diseases, one of the respiratory ailments, porcine pneumonia, is caused by many kinds of bacteria. Four pathogenic microorganisms, including *Heamophilus parasuis*, *Pasteurella multocida*, *Actinobacillus pleuropneumonia*, and *Bordetella bronchiseptica*, were isolated from pigs with severe pneumonia. In order to discover potent drugs to use against the above microorganisms, methanol extracts of 81 higher plants that have been used as traditional oriental medicine for antibacterial therapy were screened. In the case of drugs for animals, activities as well as costs should be considered. Among the 81 methanol extracts, several extracts showed potent antibacterial activities, however, the bark of *Cinnamomi cortex* showed the highest activity. Since its purchasing price was also inexpensive, it was decided to separate active compounds from it [9].

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Dried barks of *C. cortex* were purchased from the Kyung-Dong Herb Market in Seoul, Korea. The botanical identification was performed by Dr. Hee-Jae Cho (R&D Center, Cheiljedang) and a voucher specimen was deposited in the Department of Applied Biology and Chemistry, Konkuk University, Seoul, Korea. A fractionation procedure is shown in Fig. 1. Gilson prep-HPLC (Vydak C18 column; 22 mm× 250 mm, UV detector; 250 nm) was used for the final separation step. In order to confirm the purity of the final fraction, a Waters analytical-HPLC (Vydak C18 column; 5 mm×250 mm, Photodiodearray detector; 200–600 nm) was used. As listed in Table 1, three fractions, CH4-2, CH4-3, and CH4-5, showed potent activities, and they

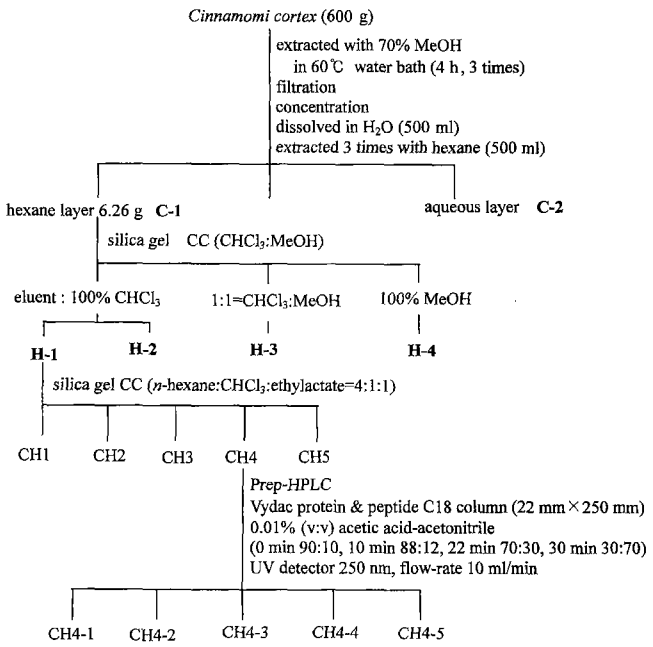


Fig. 1. A fractionation procedure.

Table 1. The antibacterial activities of the fractions separated through a fractionation against four test microorganisms.

Fractions	*H. parasuis*	*P. multocida*	*A. pleuropneumonia*	*B. bronchiseptica*
1st fraction (Hexane:H_2O)				
C-1	20 mm	32 mm	36 mm	14 mm
C-2	-	10 mm	12 mm	-
2nd fraction (Silica Gel CC ($CHCl_3$:MeOH)				
H-1	16 mm	22 mm	22mm	20 mm
H-2	11 mm	12 mm	9 mm	10 mm
H-3	12 mm	8.5 mm	12 mm	-
H-4	18 mm	-	19 mm	-
3rd fraction (Silica Gel CC (*n*-hexane/$CHCl_3$/ethylacetate)				
CH1	-	-	-	-
CH2	-	-	-	-
CH3	22 mm	12 mm	22 mm	-
CH4	18 mm	28 mm	12 mm	11 mm
CH5	11 mm	20 mm	25 mm	14 mm
4th fraction (prep-HPLC (acetic acid/acetonitrile)				
CH4-1	-	8 mm	-	-
CH4-2	13 mm	25 mm	29 mm	14 mm
CH4-3	32 mm	43 mm	50 mm	33 mm
CH4-4	-	8 mm	-	-
CH4-5	13 mm	14 mm	36 mm	14 mm

Unit: mm (a diameter showing inhibition).

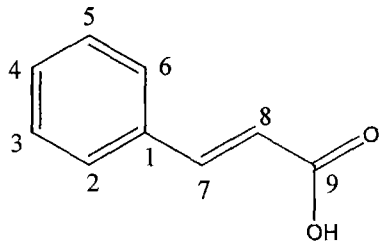
were then isolated and tested for their purity using a photodiodearray detector.

H. parasuis was isolated from pigs with typical Glassers disease signs, such as polyserositis. Isolated bacterium was identified with polyvalent positive serum specific to the *H. parasuis*. *B. bronchiseptica* was isolated from nasal swab of the pigs showing clinical atrophic rhinitis. Isolated bacterium was identified by tube agglutination test using antiserum specific to *B. broncheseptica*. Of the four test microorganisms, *A. pleuropneumoniae* and *P. multocida* were isolated as described in the previous paper [6].

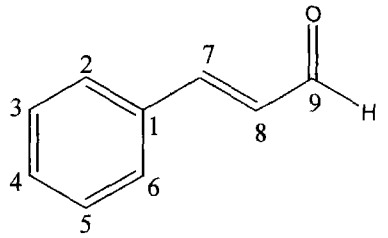
Drug sensitivity tests against the four test microorganisms were performed using disks containing purified fractions of the bark of *C. cortex*. The disks were prepared by dropping the solubilized fractions obtained according to an activity-guided fractionation procedure on 6 mm filter paper, followed by air-drying at room temperature. The bacterial culture solution was evenly spread out on a Muller Hinton agar using a sterilized cotton swab, and the prepared disk was then appropriately placed on the agar. The plate was placed in an incubator overnight and the antibacterial activity was evaluated by measuring the inhibition zone of the bacterial growth [6]. The antibacterial activities of the fractions against the four test microorganisms are listed in Table 1.

The substances were dissolved in a 0.4 ml of deuterated chloroform for NMR experiments. The NMR spectra, including ^{1}H NMR, ^{13}C NMR, Distortionless Enhancement of Polarization Transfer (DEPT) [4], Correlated Spectroscopy (COSY) [1], Heteronuclear Multiple Quantum Coherence (HMQC) [2], and Heteronuclear Multiple Bond Correlation (HMBC) [3] were collected on a Bruker Avance 400 NMR spectrometer (Karlsruhe, Germany) at room temperature [5, 7, 8]. ESI/MS was carried out on a VG Micromass Autospec (Angleton, U.S.A.).

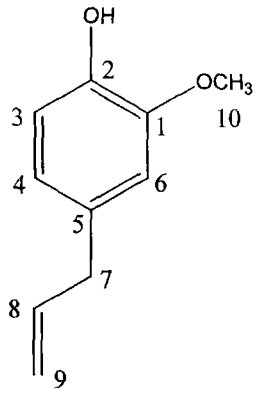
The molecular ion of CH4-2 was identified at *m/z* 149.2 (MH+). In the ^{13}C NMR spectrum, 7 signals were observed and their chemical shifts showed greater values than 110 ppm. Based on the multiplicities determined by DEPT, there were five doublets and two singlets. Because of two signals at 128.4 and 128.9 ppm showing double intensities and proton-proton connectivities by COSY, CH4-2 should contain a benzene ring. The ^{13}C signal at 172.6 ppm was caused by carboxylic acid. The existence of carboxylic acid was proved by the observation of the ^{1}H signal at 10.2 ppm. Two ^{13}C signals at 117.6 and 146.9 ppm were attached to corresponding ^{1}H signals at 6.45 and 7.70 ppm, respectively. The coupling constant of the two protons was determined to be 15.8 Hz, so they should be *trans* ethenyl groups. As a result, the structure of CH4-2 based on the long-ranged proton-carbon connectivities by HMBC could be drawn as shown in Fig. 2. The complete assignments of NMR data of CH4-2 are listed in Table 2. Using SciFinder Scholar 2000 (American Chemical Society), the structure shown in Fig. 2 was identified to be 3-phenyl-2-propenoic acid. In order to further confirm



CH4-2



CH4-3



CH4-5

Fig. 2. The structures of CH4-2 (top), CH4-3 (middle), and CH4-5 (bottom).

the result, the chromatogram of CH4-2 was compared to that of the standard sample of 3-phenyl-2-propenoic acid (data is not shown).

In the ^{13}C NMR spectrum of CH4-3, 7 signals were observed, which were similar to those of CH4-2, except for two ^{13}C signals at 128.6 and 193.8 ppm. The reason for the shift from 172.6 ppm to 193.8 ppm could be that carboxylic acid was exchanged with aldehyde. The data obtained by MS demonstrated that this explanation was correct, because the mass of CH4-3 was as small as 16. Based on proton-proton connectivities by COSY and proton-carbon connectivities by HMBC, the structure of the compound could be drawn as shown in Fig. 2. The complete assignments of NMR data of CH4-3 are listed in Table 3. The structure was identified to be 3-phenyl-2-propenal using SciFinder Scholar 2000. The result was confirmed by a comparison of the chromatogram of CH4-3 with that of the standard sample (data not shown).

The molecular ion of CH4-5 was identified at m/z 165.2 (MH+). In the ^{13}C NMR spectrum, 10 signals were observed. Their multiplicities were determined by DEPT. There were one quartet, two triplets, four doublets, and three singlets. Based on proton-proton connectivities by COSY and proton-carbon connectivities by HMBC, the structure of the compound could be drawn as shown in Fig. 2. The complete assignments of NMR data of CH4-5 are listed in Table 4. Using SciFinder Scholar 2000, the structure of CH4-5 was identified to be 2-methoxy-4-(2-propenyl)phenol. The result was confirmed by comparison of the chromatograms of CH4-3 and the standard sample (data not shown).

The structures of three active compounds have been known, however, the antibacterial activities of those compounds against the four pathogenic microorganisms *H. parasuis*, *P. multocida*, *A. pleuropneumonia*, and *B. bronchiseptica* have not previously been reported. In addition, their potencies were comparable to those of gentamycin. Since the original plant *C. cortex* with the active compounds has been used as a spice, it could easily be used as a therapeutic agent for porcine pneumonia.

Table 2. The complete assignments of NMR data of CH4-2.

δ of ^{13}C	CHn	δ of ^{1}H (J/Hz)	COSY	HMBC	Assignment
117.6	d	6.45(d, 15.8)	H8/H7	C8/H7	8
128.4	d	7.38(m)	H3,5/H2,6 H3,5/H4	C3, 5/H4	3,5
128.9	d	7.53(m)	H2,6/H3,5	C2, 6/H7 C2,6/H4	2,6
130.7	d	7.39(m)	H4/H3,5	C4/H2, 6	4
134.1	s	-	-	C1/H8 C1/H3, 5	1
146.9	d	7.70(d, 15.8)	H7/H8	C7/H2, 6	7
172.6	s	-	-	C9/H7 C9/H8	9
-	-	10.2(s)			-OH

Table 3. The complete assignments of NMR data of CH4-3.

δ of ^{13}C	CHn	δ of ^{1}H (J/Hz)	COSY	HMBC	Assignment
128.5	d	7.52(m)	H2, 6/H3, 5	-	2, 6
128.6	d	6.68(dd, 7.7, 15.8)	H8/H9 H8/H7	C8/H7 C8/H9	8
129.1	d	7.37(m)	H3, 5/H2, 6 H3, 5/H4	-	3, 5
131.3	d	7.38(m)	H4/H3, 5	C4/H2, 6	4
134.0	s	-	-	C1/H8 C1/H3, 5	1
152.9	d	7.41(s)	H7/H8	-	7
193.8	d	9.69(d, 7.7)	H9/H8	C9/H7	9

Table 4. The complete assignments of NMR data of CH4-5.

δ of ^{13}C	CHn	δ of ^{1}H (J/Hz)	COSY	HMBC	Assignment
39.9	t	3.31(d, 10.2)	C7/H8	C7/H4 C7/H6 C7/H9	7
55.9	q	3.86(s)	-	-	10
111.1	d	5.49(m)	-	C6/H7 C6/H4	6
114.3	d	5.05(d, 8.5)	H3/H4	-	3
115.5	t	6.83(m)	H9/H8	C9/H7	9
121.2	d	6.68(m)	H4/H3	C4/H7 C4/H6	4
131.9	s	-	-	C5/H7 C5/H3	5
137.8	d	5.94(dd 10.2, 17.1)	H8/H7, H8/H9	C8/H7	8
143.9	s	-	-	C2/H6 C2/H3	2
146.5	s	-	-	C1/H10 C1/H3 C1/H6	1

Acknowledgments

This work was supported by a grant from the Korea Ministry of Agriculture and Forest in 1999. All NMR measurements by a Bruker Avance 400 spectrometer system were partially supported by Shared Research Equipment Assistance Program 2001 (grant R23-2001-00002) of Korea Basic Science Institute.

REFERENCES

1. Bax, A. and R. Freeman. 1981. A simple method for suppressing dispersion-mode contributions in nmr spectra: the "pseudo echo". *J. Magn. Reson.* **43:** 333–338.
2. Bax, A., R. Griffey, and B. Hawkins. 1983. Correlation of proton and nitrogen-15 chemical shifts by multiple quantum nmr. *J. Magn. Reson.* **55:** 301–315.
3. Bax, A. and M. Summers. 1986. ^{1}H and ^{13}C assignments from sensitivity-enhanced detection of heteronuclear multiple-bond connectivity by 2D multiple quantum nmr. *J. Am. Chem. Soc.* **108:** 2093–2094.
4. Dodrell, D., D. Pegg, and M. Bendall. 1982. Distortionless enhancement of nmr signals by polarization transfer. *J. Magn. Reson.* **48:** 323–327.
5. Lee, C.-H., H. Kyung, and Y. Lim. 2000. Comparison of the solution structure of vancomycin with its x-ray crystallographic structure. *J. Microbiol. Biotechnol.* **10:** 733–736.
6. Lyoo, Y., D. Park, Y. Choi, J. Jung, T. Jun, and Y. Lim. 2001. Anti-bacterial compound against *Pasturella multocida* and *Actinobacillus pleuropneumoniae* causing porcine pneumonia, *J. Microbiol. Biotechnol.* **11:** 350–353.
7. Park, H., Y. Lim, Y. Kim, and K. Kyung. 1999. 10-Hydroxyoctadecanoic acid produced by *Lactococcus lactis* subsp. Lactis as a part of flocculent aggregate. *J. Microbiol. Biotechnol.* **9:** 39–43.
8. Park, J., J. Suh, J. Kim, C. Kim, K. Kim, and Y. Lim. 2000 Determination of the substituted position of piperazine in substitution reaction of oxazolidinones. *Agric. Chem. Biotechnol.* **43:** 115–116.
9. Ryu, J., S. Lee, S. Lee, D. Kim, S. Cho, S. Park, D. Bae, K. Park, and H. Yun. 2000. Screening and identification of an antifungal *Pseudomonas* sp. that suppresses balloon flower root rot caused by *Rhyzoctonia solani*. *J. Microbiol. Biotechnol.* **10:** 435–440.