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Antibacterial Compound against *Pasturella multocida* and *Actinobacillus pleuropneumoniae* Causing Porcine Pneumonia

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Abstract Porcine pneumonia is caused by *Pasturella multocida* and *Actinobacillus pleuropneumoniae*. To identify a potent drug for antipneumonia therapy, several herbal compounds showing antibacterial effects were screened, and it was found that a methanol extract of *Coptidis rhizoma* root stem exhibited activity against both pneumonia-causing bacteria. Using an activity-guided fragmentation procedure, an isoquinoline alkaloid was isolated which would be responsible for the antibacterial activities against *P. multocida* and *A. pleuropneumoniae*.

Key words: Porcine pneumonia, *Pasturella multocida*, *Actinobacillus pleuropneumoniae*, *Coptidis rhizoma*

There are several porcine diseases, including alimentary diseases, respiratory ailments, and propagative diseases. Among these, respiratory ailments cause serious damage to pigs. One respiratory ailment, porcine pneumonia, is caused by *Pasturella multocida* and *Actinobacillus pleuropneumoniae*. This results in the retardation of an increase in weight, abiotrophy, and death. Sulfamethazine is currently used for pneumonia therapy and its permissible dose in pork is 0.1 ppm. However, its residual presence affects consumer confidence and sulfamethazine-resistant microorganisms have also appeared [5, 14]. Therefore, the development of a new drug is needed [9]. Accordingly, to identify a potent drug for antipneumonia therapy, several herbal medicines showing antibacterial effects against *P. multocida* and *A. pleuropneumoniae* were screened in this study.

The authors observed that a methanol extract of *Coptidis rhizoma* root stem exhibited activity against both bacteria. Using an activity-guided fragmentation procedure, a single compound known as palmatine was isolated, which would appear to be responsible for the antibacterial activity against *P. multocida* and *A. pleuropneumoniae*.

In order to obtain two test microorganisms, the following procedure was carried out. *A. pleuropneumoniae* was isolated from pigs with severe pneumonia. The bacterium tentatively causing pleuropneumonia was isolated on a blood agar and identified as *A. pleuropneumoniae* type 5 by a tube agglutination test using a panel of type specific antisera to corresponding bacteria. *P. multocida* was isolated from pigs and submitted to the immunopathological test at Konkuk University, Seoul, Korea. Briefly, the Gram-negative bacterium was isolated from a hepatized lung lesion using a blood agar and identified as *P. multocida* by a tube agglutination test using an antiserum specific to *P. multocida*. Drug sensitivity tests against *A. pleuropneumoniae* type 5 and *P. multocida* were performed using disks containing purified fractions of the *C. rhizoma* root stem. The disks were prepared by dropping the solubilized *C. rhizoma* root stem extract 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 then the prepared disk was 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.

Dried root stems of *C. rhizoma* were purchased from the Kyung-Dong Herb Market in Seoul, Korea in 1998. 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. The root stem was ground using a mixer and the powder was extracted with methanol. The extract was then freeze-dried and dissolved in a minimum amount of water. The

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resulting aqueous extract was successively extracted with methylenechloride and ethylacetate, and the extracts were chromatographed on a reversed phase C_{18} column by eluting with 2% HCl-methanol. After eluting, each fraction was collected by gravity (fractions I, II, III). Fraction I was purified using a Gilson prep-HPLC (Vydak C18 column; 22 mm×250 mm, acetonitrile-phosphate buffer; pH 4.0; UV detector, 254 nm). The purified fraction was freeze-dried.

The mass spectrum and NMR spectra were measured using a VG Micromass Autospec and Bruker ARX400 NMR spectrometer, respectively [8, 10, 11, 12, 13]. The final active compound (approx. 15 mg from 600 g of root stem) was isolated as a yellow crystal. The substance was dissolved in a minimal volume of dimethylsulfoxide for an activity test. The melting point ranged between 205°C and 210°C. Its molecular ion by ESI/MS was identified at m/z 388.9 (MH+). 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 all collected in DMSO-d_6 solvent systems. In the ^{13}C NMR spectrum, 21 signals were observed. Their multiplicities were determined by DEPT. There were four quartets, two triplets, six doublets, and nine singlets. Among these, four quartets were identified as methoxy groups based on the interpretation of the ^{1}H NMR data and HMQC. The ^{13}C peaks at 26.3 and 55.7 ppm were correlated to the ^{1}H peaks at 3.23 and 4.97 ppm in HMQC, respectively. The COSY

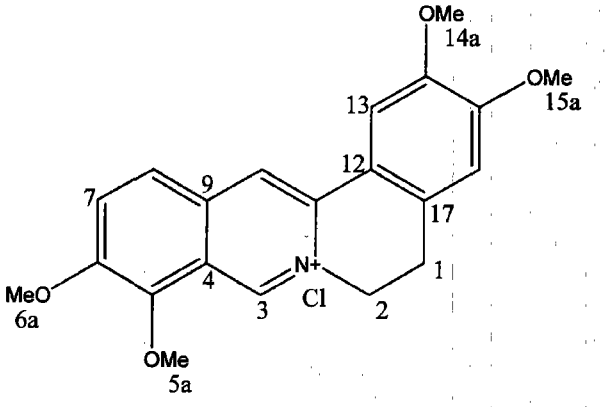


Fig. 1. Structure of the compound isolated from *Coptidis rhizoma* root stem and its numbering.

spectrum showed a correlation between 3.23 ppm and 4.97 ppm. Likewise, the ^{13}C peaks at 123.8 and 127.0 ppm were correlated to the ^{1}H peaks at 8.04 and 8.17 ppm in HMQC, respectively, and a correlation between 8.04 ppm and 8.17 ppm was observed in COSY. Except for these peaks, no other correlations were observed in COSY, even though several ^{1}H peaks were correlated to ^{13}C peaks in HMQC. As a result, the compound is expected to include interconnected aromatic rings. The ^{13}C peak at 55.7 ppm was a triplet, yet it was downfield shifted, therefore, it would appear that the carbon is connected to nitrogen or oxygen. However, in HMBC, the ^{1}H peak at 9.89 ppm was long-range coupled to two ^{13}C signals at 55.7 ppm and 133.5 ppm such that the

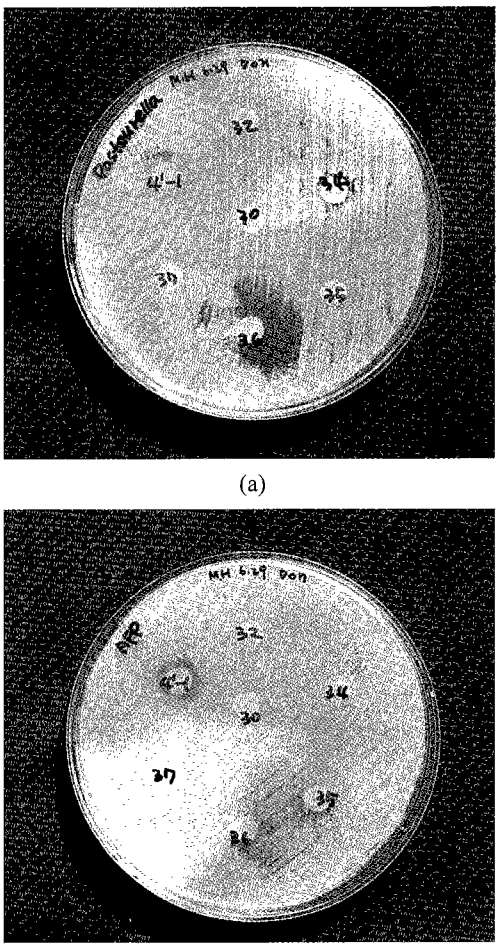
Table 1. Complete assignment of NMR data of the compound isolated from *Coptidis rhizoma* root stem.

δ of ^{13}C	Multiplicities by DEPT[a]	δ of ^{1}H by HMQC	HMBC	COSY	Assignments[b]
26.3	t	3.23(t, 6.2Hz)	C1/H13	H1/H2	1
55.7	t	4.97(t, 6.2Hz)	C2/H3	H2/H1	2
56.2	q	3.86(s)			14a
56.6	q	3.94(s)			15a
57.4	q	4.06(s)			5a
62.3	q	4.09(s)			6a
109.1	d	7.72(s)			16
111.6	d	7.08(s)			13
119.3	s		C17/H13		17
120.3	d	9.13(s)			10
121.7	s		C4/H3, H7, H10		4
123.8	d	8.04(d, 9.1Hz)		H7/H8	7
127.0	d	8.17(d, 9.1Hz)		H8/H7	8
128.9	s		C12/H1, H2, H16		12
133.5	s		C11/H3, H8		11
137.9	s		C9/H3, H10		9
143.9	s		C6/H6a, H8		6
145.7	d	9.89(s)			3
149.0	s		C15/H13; H15a		15
150.6	s		C5/H5a, H7		5
151.8	s		C14/H14a, H16		14

[a]q, quartet; t, triplet; d, doublet; s, singlet. [b]The numbering of the compound is shown in Fig. 1.

three carbons observed at 55.7, 133.5, and 145.7 ppm, which were directly connected to the ^1H peak at 9.89 ppm, were next to each other. Therefore, carbon would seem to be connected to nitrogen. The partial structures obtained are still being investigated using the Dictionary of Natural Products (Chapman & Hall, U.K., 2000). The closest matching structure is palmatine, however, its molecular weight is 352 which does not agree with the current result of 387.9. This difference indicates that the compound includes chloride. As a result, the compound appears to be palmatine chloride salt. The complete assignment of the NMR data of the compound is listed in Table 1, and the structure of the compound and its numbering are shown in Fig. 1.

The results of the activities against *P. multocida* and *A. pleuropneumoniae* are shown in Fig. 2. When the compound was soaked at 5 μg to the disk, the diameter size of the bacteriostatic zone against *P. multocida* was 20 mm, and that against *A. pleuropneumoniae* was 15 mm. When gentamicin was applied under the same conditions, the diameter sizes were 30 mm and 16 mm, respectively. In the case of *P. multocida*, the activity of the compound was lower than that of gentamicin, whereas *A. pleuropneumoniae* was able to stand the challenge of gentamicin.



(a)

(b)

Fig. 2. Results of activities against (a) *Pasturella multocida* (the disk marked as 4'-1 among disks shown in upper picture) and (b) *Actinobacillus pleuropneumoniae* (disk marked as 4'-1 among disks shown in lower picture).
The other disks marked as 30, 32, 34, 35, 36, and 37 indicate results of activities by crude extracts obtained from other plant sources.

Since the isolation of palmatine in 1925, it has been the focus of many studies [6, 7]. This study reports that palmatine exhibits antibacterial activities against *P. multocida* and *A. pleuropneumoniae* which cause porcine pneumonia. Palmatine is an isoquinoline alkaloid belonging to the same group as berberine which exhibits toxicities, such as reducing body temperature and death caused by central paralysis. Even though the toxicities of palmatine have not been extensively studied, toxicological studies on pigs are in need because of the relatedness between palmatine and berberine.

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