Thesis for the Degree of Master of Engineering

Isolation of an Anti-substance against a Tetracycline Resistant Gene, *tetB*, from *Poncirus trifoliata*

by

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Pukyong National University

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탱자로부터 테트라사이클린 내성 유전자(*tetB*) 에 대한 항균활성 물질 분리

Advisor : Dr. Young-Mog Kim

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탱자(*Poncirus trifoliata*)에서 테트라사이클린 내성 유전자(*tetB*) 에 대한 항균활성 물질 분리

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요 약

장염비브리오균은 식중독을 유발하는 병원성 세균으로 최근 조사에 의하면 어류 양식장에서 분리된 장염비브리오균의 97% 이상이 특정 항생제에 내성을 가지고 있을 정도로 내성이 심각한 수준으로 알려져 있다. 따라서 본 연구에서는 우선적으로 양식어장에서 많이 사용되고 있는 tetracycline계 항생제에 내성을 가지고 있는 장염비브리오균을 분리하고 이들 내성균에서 tetracycline에 내성을 부여하는 관련 유전자들의 클로닝을 시도하여 관련 유전자의 클로닝에 성공하였다.

Tetracycline 내성균주에 대한 대처치료제를 개발하기 위한 노력으로 몇몇 약재를 가지고 항균활성을 평가하였는데, 그 중 탱자의 MeOH 추출물이 가장 항균활성이 우수하였다. 탱자의 MeOH 추출물과 그 획분의 항균활성 실험 결과 EtOAc 획분> *n*-BuOH 획분 순으로 항균활성이 나타으며 *n*-hexane 획분, CH_2Cl_2 획분, H_2O 획분에서는 항균활성이 나타나지 않았다. EtOAc 획분을 silica gel column chromatography와 thin layer chromatography (TLC)에 의해 6개의 분획물을 얻었다.

EtOAc 획분 중 fraction PE1-F02의 항균활성이 가장 우수하였다 그들을 다시 실리카겔 컬럼 크로마토그래피를 수행하여 12개의 fraction을 얻었다. 그들 중 fraction PE2-F012이 tetracycline 내성 균주에 대해 항균활성을 가지고 있었으며, 다시 13개의 fraction으로 분획하였다. 다시 fractions PE3-F013을 12개의 fraction으로 분획하였다. 그들 중 fraction PE4-F02과 PE4-F04을 Sephadex LH-20 column chromatography을 실시하여 화합물 1과 2를 분리하였다. 화합물 1과 2는 ^{1}H-, ^{13}C-NMR 의 분광학적 분석방법에 의해 각각 poncirin과 naringin 임을 확인하였다.

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Introduction

Antibiotics are widely used in human, veterinary medicine and fish farming for increasing production or keeping them free from pathogens. As the result, the emergence and spreading of drug resistance bacteria is a serious clinical problem in many countries.

Also, contamination of the drug in aquatic environments is a growing concern due to increasing resistance genes which may affect on human health via food chain (Kim *et al*., 2007).

Tetracycline (TC) use in human and aquatic animals over the last 50 yr has influenced the appearance of TC resistance bacteria (Anderson & Sandra, 1994; Guardabassi *et al*., 2000). In bacteria, 3 mechanisms of TC resistance were known to be mediated by > 38 different TC resistance determinants (Roberts, 2005). Among them, many studies have examined the distribution of different *tet* genes encoding efflux pumps in various aquaculture environments (Depaola *et al*., 1993; DePaola & Reberts, 1995; Schmidt *et al*., 2001; Nonaka & Suzuki 2002; Teo *et al*., 2002). To date, however, there have been no unambiguous reports about the inactivation of the gene involved TC resistant to resolve serious problems caused by the antibiotic resistance bacteria.

Vibrio parahaemoliticus is one of the major food poisoning bacteria in Korea (Kim, 2001). It has been recently reported that over 97% of *V. parahaemoliticus* strains isolated from aquaculture farms or

cultivated fishes exhibits an antibiotic resistant (Kim, 2001). It has been also known that tetracyclines are frequently used in aquaculture farms as a feed additive not to treat fish diseases but to prevent fish diseases or to promote the growth of fishes (Kim *et al.*, 2007).

However, there have been no unambiguous reports about the mechanism of the antibiotic resistance. Therefore, studies related in the function of antibiotic resistant gene(s) or protein(s) is ungently necessary to elucidate the mechanism of tetracycline resistant in a molecular level. For the purpose, a tetracycline resistant *V. parahaemoliticus*, capable of growing on TCBS medium containing tetracycline, was isolated from cultivated fishes. A gene responsible for the tetracycline resistance was cloned from the chromosomal DNA of *V. parahaemolyticus*. The gene was used to design an *in vivo* screening assay to discover new drug(s) or specific anti-substance(s) against tetracycline resistant gene or protein. Several medicinal plants were evaluated for its inhibitory activity.

The results obtained in these studies will contribute to develope new drugs against antibiotic resistant bacteria or to diminish the occurrence of antibiotic resistant bacteria.

Materials and Methods

1. Materials

1.1. Plant materials, antibiotics and chemicals

The fruits of *Poncirus trifoliata* were purchased from a qualified oriental drug store, the Nulpuroon herb shop (Seoul, Korea). The fruits were collected in March 2008.

Tetracycline (TC) and chloramphenicol were purchased from Sigma (St. Louis, USA). All other reagents were of reagent grade and purchased from commercial sources.

1.2. Bacterial isolation and cultivation

TC resistant *Vibrio* spp. was isolated to culture on thiosulfate citrate bile salts sucrose (TCBS; Difco, USA) containing 20 ㎍/㎖ of TC. Isolates were further identified by an API 20E kit (BioMerieux, Hazelwood, USA).

2. Methods

2.1. Antibiotic sensitivity assay

Antibiotic-sensitivity of isolates was evaluated by an agar diffusion assay on Muller Hinton (MH) agar with disc containing 10 ㎍ ampicillin (AM), 5 ㎍ tetracycline (TC), 23.75 ㎍ trimethoprim-sulfamethoxazole (SXT), 1.25 ㎍ trimethoprim (TMP), 10 ㎍ streptomycin (S), 5 ㎍ chloramphenicol (C), 5 ㎍ rifampin (RA), and 5 ㎍ nalidixic acid (NA). Zones of growth inhibition were evaluated after overnight incubation according to NCCLS guidelines (National Committee for Clinical Laboratory Standards 2002).

2.2. Measurement of minimum inhibitory concentration

Measurement of minimum inhibitory concentration (MIC) of antibiotics against the isolates was determined by the two-fold serial dilution method in MH broth as described by the National Committee for Clinical Laboratory Standards (2002). MIC was defined as the lowest concentration of crude extract that inhibited the visual growth after incubation at 37°C for 24 h and was performed in triplicates.

2.3. Cloning of a gene responsible for the tetracycline resistance

Cells were cultured for 18 h at 30°C in Tryptic Soy Broth (TSB; Difco, USA) supplemented with 1% (w/v NaCl). Then, the culture was collected and chromosomal DNA was prepared using Accu-Prep

Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea). The DNA was partially digested with the restriction enzyme *Sau3A*I.

DNA fragments of about 2.5 kbp were separated and then ligated into the *BamH*I site of pBR322. Competent cells of *Escherichia coli* KAM3 (△*acrB*) were transformed with the ligated recombinant plasmids and then spread onto Luria-Bertani (LB) agar plates containing tetracycline (20 ㎍/㎖). The plates were incubated at 37℃ for 3 days. One colony was archived and plasmid was isolated from the candidate. Plasmid DNA was used for restriction mapping and sequencing. The nucleotide sequence of the inserted DNA was determined by the dideoxy chain termination method (Sanger *et al.*, 1977).

In order to sub-clone a gene encoding tetracycline resistant protein (TetB) of *V. parahaemoliticus* 0854, a PCR was carried out using two synthetic oligonucleotides based on the sequences to generate a *BamH*I restriction enzyme site, respectively: (Forward) 5′-CGGGATCCCGTTTACCACTCCCTATCAGTG-3′, and (Reverse) 5′-CGGGATCCCGCGGAATAACATCATTTGGTG-3′ (Table 1). DNA was amplified through 30 cycles of denaturation (94℃, 30 sec), annealing (50℃, 30 sec), and polymerization (72℃, 60 sec). The PCR product amplified is expected to be about 1,200 bp. The PCR product was digested by *BamH*I restriction enzyme and then ligated into pSTV28 (Takara, Japan), which had been digested with *BamH*I. The

Table 1. Primer sets for PCR of *tet*B gene

Primer	Oligonucleotide sequence(5′-3′)
VP-tetBF	CGGGATCCCGTTTACCACTCCCTATCAGTG (*BamH* Ⅰ)
VP-tetBR	CGGGATCCCGCGGAATAACATCATTTGGTG (*BamH* Ⅰ)

resulting plasmid was designated as pSTVTetB. *E. coli* KAM3 cells harboring pSTVTetB were grown at 37℃ in MH medium in the presence of antibiotics indicated.

2.4. Preparation of methanolic extract

The powder of *P. trifoliata* fruits (6 ㎏) was extracted three times with 100% methanol (MeOH). The filtrate obtained under reduced pressure to yield a dark reside (2 ㎏).

2.5. Solvent fractionation of methanolic extract

The MeOH extracts (1.5 ㎏) were suspended in H_2O (10 L) and partitioned with *n*-hexane (300 g), dichloromethane (CH_2Cl_2) (240 g), ethyl acetate (EtOAc) (450 g), *n*-butanol (*n*-BuOH) (310 g), and H_2O (200 g) in the order of polarity and evaporated as described above (Mun *et al.*, 1994). The scheme of extraction and solvent fractionation were illustrated (Fig. 1).

2.6. Separation and isolation of an active compound

The active fractions or sub-fractions were further separated by silica gel column chromatography and Sephadex LH-20 column chromatography, eluting with corresponding eluent system, and

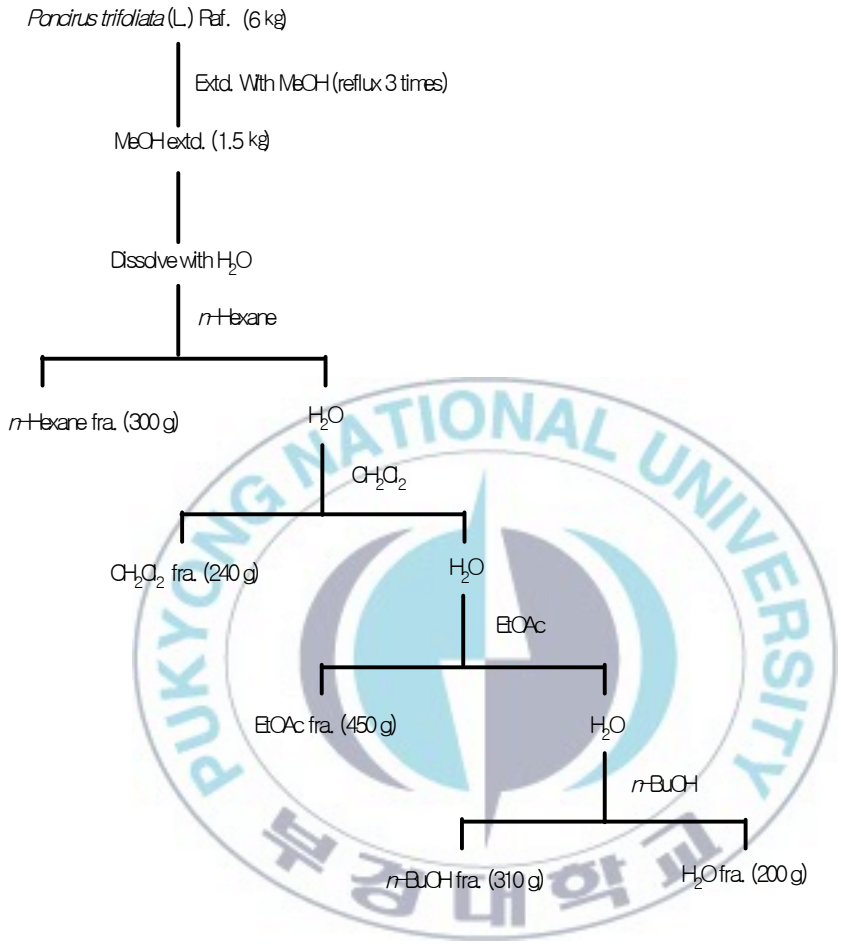


Fig. 1. Procedure for extraction and fractionation of *Poncirus trifoliata* fruits powder by various solvents.

monitored by thin-layer chromatography (TLC). Samples were dissolved in the appropriate organic solvent, applied to the silica gelG plates F_{254} (Merck, Germany) and developed with several different suitable solvent systems (Basile *et al.*, 2000).

The plates were dried in fume hood, photographed under an ultraviolet lamp (254 nm, 356 nm) and then were further sprayed by reagents to better display the components from the 20% H_2SO_4 reagent (Aldrich) and then heated at 110℃ for 5-10 min. The whole fractions were divided into R_f value (Ding *et al.*, 2008).

2.7. Determination of growth inhibitory activity against cells containing tetracycline resistance gene (*tetB*)

The antibacterial activity of the each fraction on the growth of test stain was determined using the paper disc method (National Committee for Clinical Laboratory Standards, 2002). Culture solutions of the test bacteria were prepared by growth in MH broth media containing TC (2 ㎍/㎖) at 37℃ for 18 h. Each fraction was dissolved in 1% dimethyl sulfoxide (DMSO) solvent to a final concentration of 100 ㎎/㎖ and sterilized through filtration by 0.45 ㎛ Millipore filters (Toyo, Japan). The 8 ㎜ diameter discs (Toyo, Japan) were impregnated with 50 ㎖ of the stock solution (final 5 ㎎/disc) and placed on the inoculated agar. Negative controls were prepared using the same solvents employed to dissolve each fraction. The inoculated

plates were incubated at 37℃ for 24 h. The growth-inhibitory activity was evaluated by measuring the zone of inhibition against the test organisms (Ahn *et al*., 1998).

2.8. Structural analysis

The structure of the compound **1** and **2** were identified and confirmed by ^{1}H-NMR (proton-nuclear magnetic resonance spectroscopy) and ^{13}C-NMR (carbon-nuclear magnetic resonance spectroscopy) experiments using a Bruker Avance 400 FT-NMR (^{1}H frequency = 400.13 MHz, ^{13}C frequency = 100.62 MHz). FT-NMR was instrument with tetramethylsilane (TMS) as a internal standard. The assignments by correlation spectroscopy, heteronuclear single quantum correlation, and heteronuclear multiple quantum coherence experiments were performed with standard Bruker software (XWINNMR 3.1) (Li *et al*., 2008).

Results and Discussion

1. Cloning and characterization of *tetB* gene from *V. parahaemolyticus*

1.1. Antibiotic resistance of *Vibrio* spp. isolates

The tetracycline resistant *V. parahaemolyticus*, capable of growing on TCBS medium containing tetracycline, was isolated from cultivated fishes and identified. Antibiotic resistant of each isolated strain was evaluated as described in Material and Methods. As shown in Table 2, all of isolates exhibited TC resistance. Among them, *V. parahaemolyticus* 0854 only showed antibiotic resistance against not only TC but also rifampin, indicating the isolate 0854 was to be multi drug resistance bacteria (Table 2). The MIC of TC resistant isolates against TC was over 64 ㎍/㎖ (Table 3).

1.2. Cloning of a gene responsible for tetracycline resistance

A gene responsible for the tetracycline resistance was cloned from the chromosomal DNA of *V. parahaemolyticus* 0854 using *E. coli* KAM3, which lacks major multidrug efflux pumps (*△acrB*) as a host

Table 2. Antibiotic resistance pattern of tetracycline resistant *Vibrio parahaemolyticus* strains

Strain	AM	TC	SXT	TMP	S	C	RA	NA
0854	28	AG	26	18	14	22	AG	28
1020	10	AG	28	22	14	22	22	30
1021	30	AG	30	18	14	22	22	30
9220	AG	AG	22	18	12	16	20	28

(AG; all growth, clear zone; mm)
AM, ampicillin (10㎍/disc); TC, tetracycline (5㎍/disc); SXT, trimethoprim-sulfamethoxazole (23.75㎍/disc); TMP, trimethoprim (1.25㎍/disc); S, streptomycin (10㎍/disc); C, chloramphenicol (5㎍/disc); RA, rifampin (5㎍/disc); NA, nalidixic acid (5㎍/disc).

Table 3. Minimum inhibitory concentration of tetracycline resistant strains

Strain	Tetracycline (㎍/㎖)
0854	<64
1020	<128
1021	<128
9220	<64

cell. It was obtained a candidate recombinant plasmid which enabled the KAM3 cells to grow in the presence of TC. The 2,018 bp DNA sequence analysis revealed that it contained an open reading frame to be an open reading frame (ORF) for tetracycline resistance protein, TetB (Fig. 2). The DNA sequences was translated in the reading frame (Fig 3) and the putative product was compared, using the BLAST algorithm, with all publicly available protein sequences contained in the nonredundant database. Comparison of the deduced primary structure of the TetB with those of proteins present in the GenBank database indicated that the greatest similarity was with bacterial TetBs (Table 4).

The putative TetB of *Photobacterium* sp. TC21 (Furushita *et al.*, 2003) showed the highest similarity (100% identity) throughout the entire sequence (Table 4). Many of the bacterial TetB registered in the GenBank database also showed similar levels of identity (99%) to the TetB of *V. parahaemolyticus* 0854 (Table 4).

In order to elucidate the mechanism of the antibiotic resistance caused by the TetB, the gene was sub-cloned into the plasmid pSTV28. The *tetB* gene was amplified by PCR using the VP-tetBF and VP-tetBR primer set (Fig. 4). The amplified DNA was digested with *BamH*I and then ligated into pSTV28 as described in Materials and Methods. The resulting plasmid was designated as pSTVTetB and transfomated into KAM3 cells. The KAM3 cells harboring the recombinant plasmid pSTVTetB are able to grow in MH medium containing TC and oxytetracycline but not doxycycline, indicating that

GATCTTCCAATACGCAACCTAAAGTAAAATGCCCCACAGCGCTGAGTGCATATAATGCATTCTCTA
GTGAAAAACCTTGTTGGCATAAAAAGGCTAATTGATTTTCGAGAGTTTCATACTGTTTTTCTGTAG
GCCGTGTACCTAAATGTACTTTTGCTCCATCGCGATGACTTAGTAAAGCACATCTAAAACTTTTAG
CGTTATTACGTAAAAAATCTTGCCAGCTTTCCCCTTCTAAAGGGCAAAAGTGAGTATGGTGCCTAT
CTAACATCTCAATGGCTAAGGCGTCGAGCAAAGCCCGCTTATTTTTTACATGCCAATACAATGTAG
GCTGCTCTACACCTAGCTTCTGGGCGAGTTTACGGGTTGTTAAACCTTCGATTCCGACCTCATTAA
GCAGCTCTAATGCGCTGTTAATCACTTTACTTTTATCTAATCTAGACATCATTAATTCCTAATTTT
TGTTGGCACTCTATCATTGATAGAGTTATTTTACCACTCCCTATCAGTGATAGAGAAAAGTGAA**AT**
GAATAGTTCGACAAAGATCGCATTGGTAATTACGTTACTCGATGCCATGGGGATTGGCCTTATCAT
GCCAGTCTTGCCAACGTTATTACGTGAATTTATTGCTTCGGAAGATATCGCTAACCACTTTGGCGT
ATTGCTTGCACTTTATGCGTTAATGCAGGTTATCTTTGCTCCTTGGCTTGGAAAAATGTCTGACCG
ATTTGGTCGGCGCCCAGTGCTGTTGTTGTCATTAATAGGCGCATCGCTGGATTACTTATTGCTGGC
TTTTTCAAGTGCGCTTTGGATGCTGTATTTAGGCCGTTTGCTTTCAGGGATCACAGGAGCTACTGG
GGCTGTCGCGGCATCGGTCATTGCCGATACCACCTCAGCTTCTCAACGCGTGAAGTGGTTCGGTTG
GTTAGGGGCAAGTTTTGGGCTTGGTTTAATAGCGGGGCCTATTATTGGTGGTTTTGCAGGAGAGAT
TTCACCGCATAGTCCCTTTTTTATCGCTGCGTTGCTAAATATTGTCACTTTCCTTGTGGTTATGTT
TTGGTTCCGTGAAACCAAAAATACACGTGATAATACAGATACCGAAGTAGGGGTTGAGACGCAATC
GAATTCGGTATACATCACTTTATTTAAAACGATGCCCATTTTGTTGATTATTTATTTTTCAGCGCA
ATTGATAGGCCAAATTCCCGCAACGGTGTGGGTGCTATTTACCGAAAATCGTTTTGGATGGAATAG
CATGATGGTTGGCTTTTCATTAGCGGGTCTTGGTCTTTTACACTCAGTATTCCAAGCCTTTGTGGC
AGGAAGAATAGCCACTAAATGGGGCGAAAAAACGGCAGTACTGCTCGGATTTATTGCAGATAGTAG
TGCATTTGCCTTTTTAGCGTTTATATCTGAAGGTTGGTTAGTTTTCCCTGTTTTAATTTTATTGGC
TGGTGGTGGGATCGCTTTACCTGCATTACAGGGAGTGATGTCTATCCAAACAAAGAGTCATCAGCA
AGGTGCTTTACAGGGATTATTGGTGAGCCTTAACAATGCAACCGGTGTTATTGGCCCATTACTGTT
TGCTGTTATTTATAATCATTCACTACCAATTTGGGATGGCTGGATTTGGATTATTGGTTTGGCGTT
TTACTGTATTATTATCCTGCTATCGATGACCTTCATGTTAACCCCTCAAGCTCAGGGGAGTAAACA
GGAGACAAGTGCT**TAG**TTATTTCGTCACCAAATGATGTTATTCCGCGAAATATAATGACCCTCTTG
ATAACCCAAGAGGGCATTTTTTACGATAAAGAAGATTTAGCTTCAAATAAAACCTATCTATTTTAT
TTATCTTTCAAGCTCAATAAAAAGCCGCGGTAAATAGCAATAAATTGGCCTTTTTTATCGGCAAGC
TCTTTTAGGTTTTTCGCATGTATTGCGATATGCATAAACCAGCCATTGAGTAAGTTTTTAAGCACA
TCATCATCATAAGCTTTAAGTTGGTTCTCTTGGATC

Fig. 2. Nucleotide sequences of the inserted DNA containing a gene responsible for tetracycline resistance.

```
   1 ATG/AAT/AGT/TCG/ACA/AAG/ATC/GCA/TTG/GTA/ATT/ACG/TTA/CTC/GAT/GCC/ATG/GGG/ATT/GGC/
       M   N   S   S   T   K   I   A   L   V   I   T   L   L   D   A   M   G   I   G

  61 CTT/ATC/ATG/CCA/GTC/TTG/CCA/ACG/TTA/TTA/CGT/GAA/TTT/ATT/GCT/TCG/GAA/GAT/ATC/GCT/
       L   I   M   P   V   L   P   T   L   L   R   E   F   I   A   S   E   D   I   A

 121 AAC/CAC/TTT/GGC/GTA/TTG/CTT/GCA/CTT/TAT/GCG/TTA/ATG/CAG/GTT/ATC/TTT/GCT/CCT/TGG/
       N   H   F   G   V   L   L   A   L   Y   A   L   M   Q   V   I   F   A   P   W

 181 CTT/GGA/AAA/ATG/TCT/GAC/CGA/GGT/CGG/CGC/CCA/GTG/CTG/TTG/TTG/TCA/TTA/ATA/GGC/GCA/
       L   G   K   M   S   D   R   F   G   R   R   P   V   L   L   L   S   L   I   G

 241 TTT/ TCG/CTG/GAT/TAC/TTA/TTG/CTG/GCT/TTT/TCA/AGT/GCG/CTT/TGG/ATG/CTG/TAT/TTA/GGC/
       A   S   L   D   Y   L   L   L   A   F   S   S   A   L   W   M   L   Y   L   G

 301 CGT/TTG/CTT/TCA/GGG/ATC/ACA/GGA/GCT/ACT/GGG/GCT/GTC/GCG/GCA/TCG/GTC/ATT/GCC/GAT/
       R   L   L   S   G   I   T   G   A   T   G   A   V   A   A   S   V   I   A   D

 361 ACT/ACC/TCA/GCT/TCT/CAA/CGC/GTG/AAG/TGG/TTC/GGT/TGG/TTA/GGG/GCA/AGT/TTT/GGG/CTT/
       T   T   S   A   S   Q   R   V   K   W   F   G   W   L   G   A   S   F   G   L

 421 GGT/TTA/ATA/GCG/GGG/CCT/ATT/ATT/GGT/GGT/TTT/GCA/GGA/GAG/ATT/TCA/CCG/CAT/AGT/CCC/
       G   L   I   A   G   P   I   I   G   G   F   A   G   E   I   S   P   H   S   P

 481 TTT/TTT/ATC/GCT/GCG/TTG/CTA/AAT/ATT/GTC/ACT/TTC/CTT/GTG/GTT/ATG/TTT/TGG/TTC/CGT/
       F   F   I   A   A   L   L   N   I   V   T   F   L   V   V   M   F   W   F   R

 541 GAA/ACC/AAA/AAT/ACA/CGT/GAT/AAT/ACA/GAT/ACC/GAA/GTA/GGG/GTT/GAG/ACG/CAA/TCG/AAT/
       E   T   K   N   T   R   D   N   T   D   T   E   V   G   V   E   T   Q   S   N

 601 TCG/GTA/TAC/ATC/ACT/TTA/TTT/AAA/ACG/ATG/CCC/ATT/TTG/TTG/ATT/ATT/TAT/TTT/TCA/GCG/
       S   V   Y   I   T   L   F   K   T   M   P   I   L   L   I   I   Y   F   S   A

 661 CAA/TTG/ATA/GGC/CAA/ATT/CCC/GCA/ACG/GTG/TGG/GTG/CTA/TTT/ACC/GAA/AAT/CGT/TTT/GGA/
       Q   L   I   G   Q   I   P   A   T   V   W   V   L   F   T   E   N   R   F   G

 721 TGG/AAT/AGC/ATG/ATG/GTT/GGC/TTT/TCA/TTA/GCG/GGT/CTT/GGT/CTT/TTA/CAC/TCA/GTA/TTC/
       W   N   S   M   M   V   G   F   S   L   A   G   L   G   L   L   H   S   V   F

 781 CAA/GCC/TTT/GTG/GCA/GGA/AGA/ATA/GCC/ACT/AAA/TGG/GGC/GAA/AAA/ACG/GCA/GTA/CTG/CTC/
       Q   A   F   V   A   G   R   I   A   T   K   W   G   E   K   T   A   V   L   L

 841 GGA/TTT/ATT/GCA/GAT/AGT/AGT/GCA/TTT/GCC/TTT/TTA/GCG/TTT/ATA/TCT/GAA/GGT/TGG/TTA/
       G   F   I   A   D   S   S   A   F   A   F   L   A   F   I   S   E   G   W   L

 901 GTT/TTC/CCT/GTT/TTA/ATT/TTA/TTG/GCT/GGT/GGT/GGG/ATC/GCT/TTA/CCT/GCA/TTA/CAG/GGA/
       V   F   P   V   L   I   L   L   A   G   G   G   I   A   L   P   A   L   Q   G

 961 GTG/ATG/TCT/ATC/CAA/ACA/AAG/AGT/CAT/CAG/CAA/GGT/GCT/TTA/CAG/GGA/TTA/TTG/GTG/AGC/
       V   M   S   I   Q   T   K   S   H   Q   Q   G   A   L   Q   G   L   L   V   S

1021 CTT/AAC/AAT/GCA/ACC/GGT/GTT/ATT/GGC/CCA/TTA/CTG/TTT/GCT/GTT/ATT/TAT/AAT/CAT/TCA/
       L   N   N   A   T   G   V   I   G   P   L   L   F   A   V   I   Y   N   H   S

1081 CTA/CCA/ATT/TGG/GAT/GGC/TGG/ATT/TGG/ATT/ATT/GGT/TTA/GCG/TTT/TAC/TGT/ATT/ATT/ATC/
       L   P   I   W   D   G   W   I   W   I   I   G   L   A   F   Y   C   I   I   I

1141 CTG/CTA/TCG/ATG/ACC/TTC/ATG/TTA/ACC/CCT/CAA/GCT/CAG/GGG/AGT/AAA/CAG/GAG/ACA/AGT/
       L   L   S   M   T   F   M   L   T   P   Q   A   Q   G   S   K   Q   E   T   S

1201 GCT/TAG
       A
```

Fig. 3. DNA sequences and deduced amino acid sequences of a tetracycline resistant gene, *tetB*, cloned from *Vibrio parahaemolyticus* 0854. The DNA sequence is numbered on the left.

Table 4. Homology analysis of the TetB of *Vibrio parahaemolyticus* 0854

Strain	Protein	Homology (%)	Accession no.
Photobacterium sp. TC21	tetracycline resistance protein	99	BAC67134
Photobacterium sp. TC33	tetracycline resistance protein	99	BAC67137
Vibrio sp. TC68	tetracycline resistance protein	99	BAC67141
Serratia marcescens	tetracycline resistance protein	99	NP_941291
Escherichia coli	tetracycline resistance protein	99	YP_001096450
Haemophilus parasuis	tetracycline resistance protein	99	YP_195816

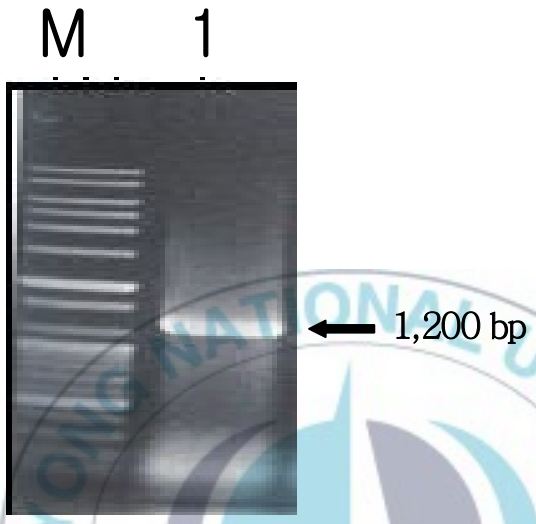


Fig. 4. PCR products of the *tet*B gene containing *Bam*H I endonuclease sites. M, 100 bp plus DNA Ladder (Bioneer, Korea); lane 1, *tet*B of *Vibrio parahaemolyticus* 0854.

the TetB gene originated from *V. parahaemolyticus* 0854 confer the tetracycline- and oxytetracycline-resistance to the host cells (Table 5).

2. Isolation and characterization of anti-substance against tetracycline resistance gene

2.1. Growth-inhibitory activity of *Poncirus trifoliata* against *E. coli* KAM3/pSTVTetB

It was expected that a fraction exhibiting an growth inhibitory activity against KAM3/pSTVTetB cells will contain an anti-substance to inhibit the mechanism of TC resistance since the cells are able to grow in the presence of TC. The methanolic extract of *P. trifoliata* showed an inhibitory activity against KAM3/pSTVTetB cells in the presence of TC (2 ㎍/㎖). The extract was partitioned using *n*-hexane, CH_2Cl_2, EtOAc and *n*-BuOH, and obtained yields were presented (Table 6). The soluble fraction of EtOAc showed the highest yield content (30%) and that of water was the lowest yield content (13.3%).

Among them, the EtOAc fractions showed strong antibacterial activity with inhibitory zone of 12 to 14 ㎜ diameters on the growth of KAM3/pSTVTetB cells in the presence of TC (2 ㎍/㎖) and chloramphenicol (1 ㎍/㎖), which was stronger than other fractions

Table 5. Antibiotic resistance profiles of the tetracycline resistance protein (TetB) of *Vibrio parahaemolyticus* 0854

Strain	Minium inhibitory concentration (㎍/㎖)		
	Tetracycline	Oxytetracycline	Doxycycline
V. parahaemolyticus 0854	128	32	16
KAM3/pSTV28	<4	<4	<4
KAM3/pSTVTetB	64	32	<4

Table 6. Yield of the *Poncirus trifoliata* solvent fractions

Solvents	*n*-hexane	CH_2Cl_2	EtOAc	*n*-BuOH	H_2O
Yields (%)	20.0	16.0	30.0	20.7	13.3

MeOH : methanol
EtOAc : ethyl acetate
CH_2Cl_2 : dichloromethane
n-BuOH : buthanol.

(Table 7). The BuOH fraction had a comparatively weak activity producing clear zones with 7 to 8 ㎜ diameters. However, *n*-hexane, CH_2Cl_2 and H_2O fractions did not exhibit the growth inhibitory activity against KAM3/pSTVTetB cells.

2.2. Isolation of an active compound exhibiting growth inhibitory activity

As described above, the EtOAc souble fraction of *P. trifoliata* fruit exhibited strong inhibitory activity on the growth of bacteria cells containing the tetracycline resistant gene, *tetB*, indicating that the fraction contain an anti-substance against the gene or the drug resistant protein.

In an attempting to isolated and identify an active compound, the EtOAc fraction (100 g) was impregnated with a silica gel (particle size 0.063-0.2 mm, Merck, Germary) and purified by chromatographyon a silica column (80 × 1,200 ㎜). Solvents for gradient elution were *n*-hexane : EtOAc (from 1:1 to 1:5 v/v) in a gradient mode to yield 6 fractions (PE1-F01~06). The fractions exhibiting growth inhibitory activity against KAM3/pSTVTetB in the presence of TC (2 ㎎/㎖) were assayed using the paper disc method. Among them, fraction PE1-F02 showed significant inhibitory activity against KAM3/pSTVTetB cells in the presence of TC (Table 8).

The fraction PE1-F02 (6 g) was further chromatographed on a

Table 7. Antibacteral activity of various solvent fraction from 100% methanol extract against *Vibrio parahaemolyticus* 0854

Poncirus trifoliata MeOH Ex.	Inhibition Zone Diameter (mm)[a]			
	KAM3/pSTV28 (with chloramphenicol)	KAM3/pSTVTetB (with chloramphenicol)	KAM3/pSTVTetB (with tetracycline)	KAM3/pSTVTetB (with chloramphenico and tetracycline)
n-hexane	–[b]	–	–	–
CH_2Cl_2	–	–	–	–
EtOAc	–	12	12	14
n-BuOH	–	–	7	8
H_2O	–	–	–	–

Escherichia coli cells were cultivated on Muller-Hinton agar in the presence or absence of antibiotics (2 ㎍/㎖ of tetracycline, 1 ㎍/㎖ of chloramphenicol). A disc containing 5 mg of each fraction placed on the inoculated agar. [a] Disc diameter (6 mm) was included. [b] – : No inhibition. CH_2Cl_2, dichloromethane; EtOAc, ethyl acetate; *n*-BuOH, buthanol.

Table 8. Growth inhibitory activity of 6 sub-fractions of the ethyl acetate

PE1	*P.trifoliata* EtOAc Fr.	Weight (g)	Inhibition Zone Diameter (mm)[a]			
			KAM3/pSTV28 (with chloramphenicol)	KAM3/pSTVTetB (with chloramphenicol)	KAM3/pSTVTetB (with tetracycline)	KAM3/pSTVTetB (with chloramphenico and tetracycline)
PE1-F01	Fr. 14~22	9.98	-[b]	-	7	6.5
PE1-F02	Fr. 23~33	6.19	-	-	9	7
PE1-F03	Fr. 34~35	1.50	-	-	7.5	7
PE1-F04	Fr. 36~54	19.11	-	-	6.5	7
PE1-F05	Fr. 55	0.73	-	-	7	7
PE1-F06	Fr. 56	0.76	-	-	7	7

Escherichia coli cells were cultivated on Muller-Hinton agar in the presence or absence of antibiotics (2 ㎍/㎖ of tetracycline and 1 ㎍/㎖ of chloramphenicol). Ethyl acetate soluble fraction was subjected for a silica gel column chromatography. Solvents for gradient elution were *n*-hexane : ethyl acetate (from 1:1 to 1:5 v/v). A disc containing 1 mg of each fraction placed on the inoculated agar.

[a] Disc diameter (6 mm) was included. [b] - : No inhibition.

silica gel column (40 × 600 ㎜) and eluted with *n*-hexane : EtOAc (from 2:1 to 1:1 v/v) to yield 12 fractions (PE2-F01～012). Among them, fraction PE2-F011 and PE2-F012 exhibited strong growth inhibitory activity against KAM3/pSTVTetB in the presence of TC (Table 9). Specially, PE2-F012 fraction only exhibited growth inhibitory activity against KAM3/pSTVTetB not control cells, KAM3/pSTV (Table 9). In order to isolate a specific anti-substance inhibiting the function of TC resistance, PE2-F012 fraction wasused for further separation.

The fraction PE2-F012 (3.3 g) was re-chromatographed on a silica gel column (30 × 500 ㎜) and eluted with CH_2Cl_2 : MeOH (from 10:1 to 7:1 v/v) in a gradient mode to yield 13 fractions (PE3-F01～013). However, the growth inhibitory activity of the 13 fractions was disappeared after running third silica gel column chromatography (Table 10). This kind of phenomena often occurs during the purification procedures of natural compounds due to the loss, the conformational change, or the degradation of an active compound (Jung, 2007).

As the other idea, it was also supposed that the growth inhibitory activity exhibited in the PE2-F012 would originated from a synergistic effect between compounds existing in the fraction. Although, the 13 fractions did not exhibited the growth inhibitory activity, further separation procedures were conducted to obtain single pure compound for future study. Among the 13 fractions, the content of fraction PE3-F013 (1 g) was higher than others.

Table 9. Growth inhibitory activity of 12 sub-fractions of PE1-F02 fraction

PE2	PE1-F02	Weight (mg)	Inhibition Zone Diameter (mm)[a]			
			KAM3/pSTV28 (with chloramphenicol)	KAM3/pSTVTetB (with chloramphenicol)	KAM3/pSTVTetB (with tetracycline)	KAM3/pSTVTetB (with chloramphenico and tetracycline)
PE2-F01	Fr. 10	8.3	–[b]	–	–	–
PE2-F02	Fr. 12~14	9.3	–	–	–	–
PE2-F03	Fr. 20~24	159.6	–	–	–	–
PE2-F04	Fr. 28~30	97.5	–	–	–	–
PE2-F05	Fr. 34~36	46.7	–	–	–	–
PE2-F06	Fr. 38~40	30.3	–	–	–	–
PE2-F07	Fr. 48~50	87.8	–	–	–	–
PE2-F08	Fr. 52~54	97.1	–	–	–	–
PE2-F09	Fr. 64~66	237.5	–	–	–	–
PE2-F010	Fr. 76~80	295.8	–	–	–	–
PE2-F011	Fr. 81	505.7	7.5	7	8	8.5
PE2-F012	Fr. 82	3286.0	–	–	6.5	7.5

Escherichia coli cells were cultivated on Muller-Hinton agar in the presence or absence of antibiotics (2 ㎍/㎖ of tetracycline and 1 ㎍/㎖ of chloramphenicol). PE1-F02 was subjected for a silica gel column chromatography. Solvents for gradient elution were *n*-hexane : ethyl acetate (from 2:1 to 1:1 v/v). A disc containing 0.5 mg of each fraction placed on the inoculated agar. [a] Disc diameter (6 mm) was included. [b] – : No inhibition.

Table 10. Growth inhibitory activity of 13 sub-fractions of PE2-F012

PE3	PE2-F012	Weight (mg)	Inhibition Zone Diameter(mm)[a]			
			KAM3/pSTV28 (with chloramphenicol)	KAM3/pSTVTetB (with chloramphenicol)	KAM3/pSTVTetB (with tetracycline)	KAM3/pSTVTetB (with chloramphenicol and tetracycline)
PE3-F01	Fr. 1~2	201.2	-[b]	-	-	-
PE3-F02	Fr. 3~7	225.7	-	-	-	-
PE3-F03	Fr. 8~11	238.1	-	-	-	-
PE3-F04	Fr. 12~14	154.4	-	-	-	-
PE3-F05	Fr. 15~16	217.5	-	-	-	-
PE3-F06	Fr. 17~18	391.1	-	-	-	-
PE3-F07	Fr. 19~20	73.0	-	-	-	-
PE3-F08	Fr. 21~24	241.6	-	-	-	-
PE3-F09	Fr. 25~30	247.1	-	-	-	-
PE3-F010	Fr. 31~35	65.0	-	-	-	-
PE3-F011	Fr. 36~37	189.2	-	-	-	-
PE3-F012	Fr. 38	19.4	-	-	-	-
PE3-F013	Fr. 39	1022.7	-	-	-	-

Escherichia coli cells were cultivated on Muller-Hinton agar in the presence or absence of antibiotics (2 ㎍/㎖ of tetracycline and 1 ㎍/㎖ of chloramphenicol). PE2-F12 fraction was subjected for a silica gel column chromatography. Solvents for gradient elution were dichloromethane : methanol (from 10:1 to 7:1 v/v). A disc containing 0.5 mg of each fraction placed on the inoculated agar. [a] Disc diameter (6 mm) was included. [b] - : No inhibition.

The PE3-F013was rechromatographed on a silica gel column (30 × 500 ㎜) and eluted with CH_2Cl_2 : MeOH : H_2O (from 10:1:0.1 to 3:1:0.1 v/v) in a gradient mode to yield 12 fractions (PE4-F1～12) (Fig. 5).

Finally, compound **1** (21.1 ㎎) was obtained from fraction PE4-F02 (0.8 g) after further purification over Sephadex LH-20 column chromatography (Amersham Pharmacia Biotech, 30 × 400 ㎜) eluted with MeOH and recrystallisation in MeOH. Compound **2** (19.8 ㎎) was obtained from fraction PE4-F04 (0.5 g) after further purification over Sephadex LH-20 column chromatography eluted with MeOH and recrystallisation in MeOH (Fig. 5).

2.3. Structure determination of compound 1 and 2

Compound **1** (Fig. 6 and 7) and **2** (Fig. 8 and 9) were identified to be poncirin and naringin by ^{1}H-NMR and ^{13}C-NMR, respectively (Fig. 10). The nuclear magnetic resonance (NMR) spectrum of compound **1** and **2** was as follows.

Compound **1**. Yellow powder; ^{1}H-NMR (CD_3OD, 250 MHz) δ: 7.31 (2H, d, J = 8.7 Hz, H-2′, H-6′), 6.86 (2H, d, J = 8.7 Hz, H-3′, H-5′), 6.09 (1H, d, J = 2.2 Hz, H-8), 6.07 (1H, d, J = 2.2 Hz, H-6), 5.31 (1H, dd, J = 12.7 Hz, 2.9 Hz, H-2), 5.16 (1H, d, J = 1.3 Hz, H-1′), 4.98 (1H, d, J = 7.2 Hz, H-1′), 3.79 (3H, s, OCH3), 3.08 (1H,

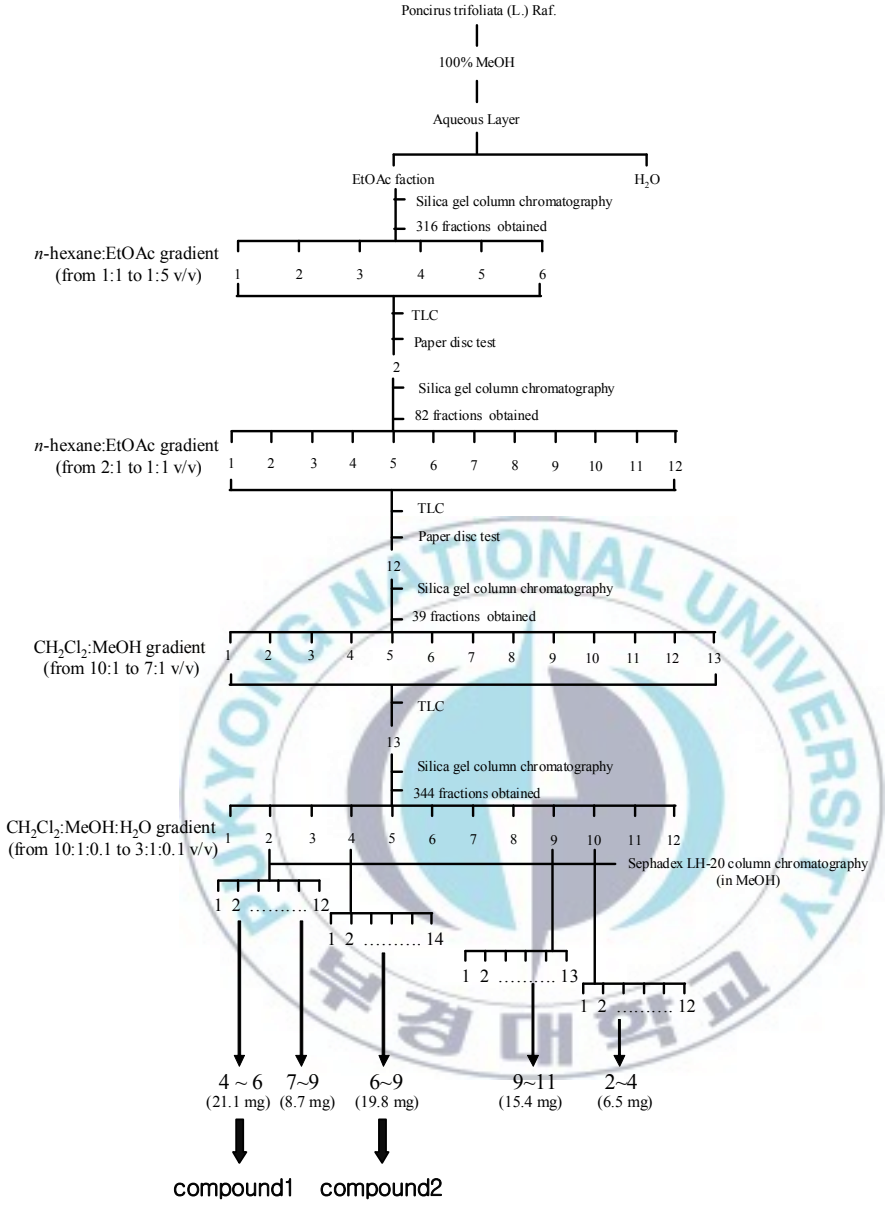


Fig. 5. Isolation of active compound responsible for antimicrobial activity in ethyl acetate fraction of *Poncirus trifoliata*.

Fig. 6. ^{1}H-NMR spectrum of compound 1.

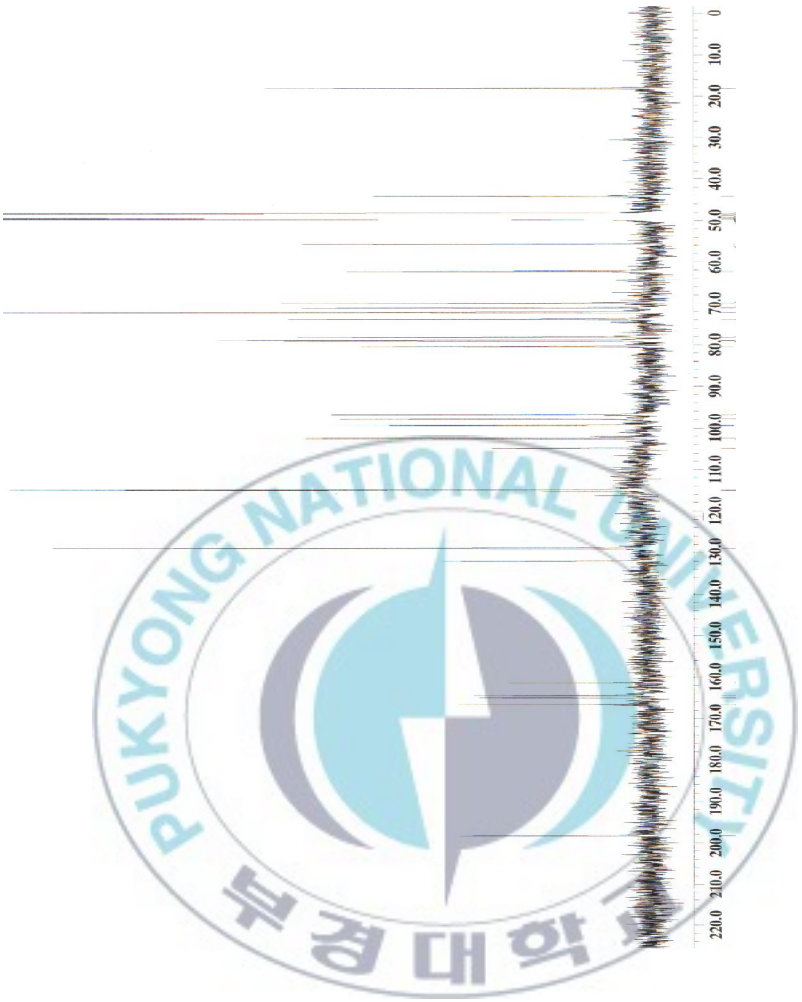


Fig. 7. ^{13}C-NMR spectrum of compound 1.

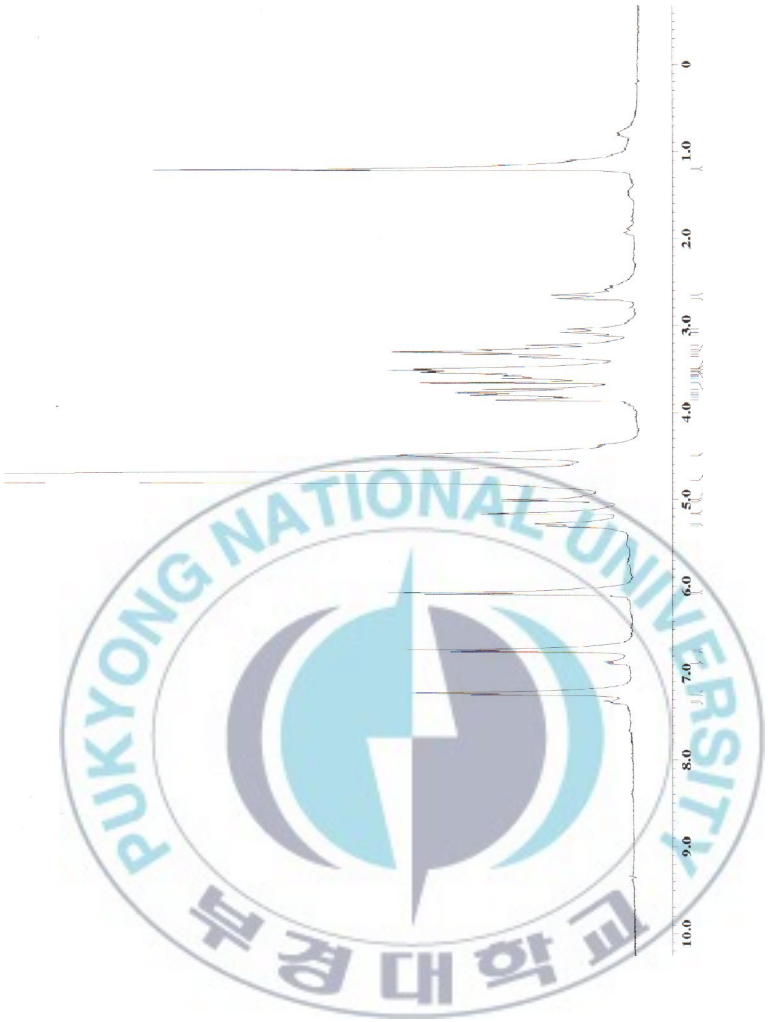


Fig. 8. ^{1}H-NMR spectrum of compound 2.

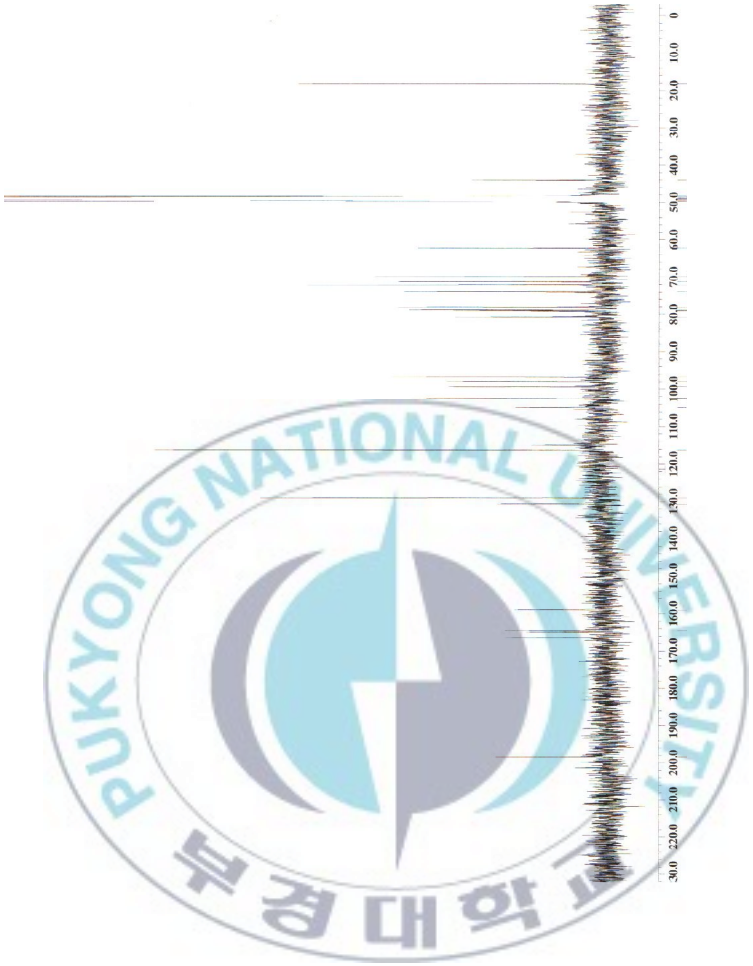
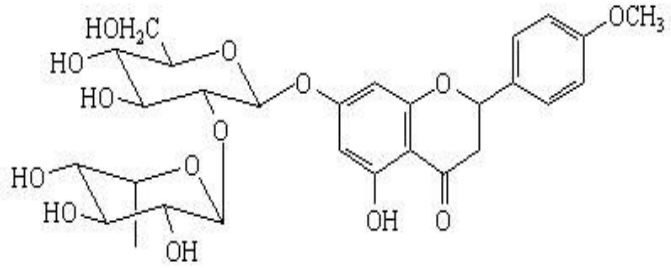


Fig. 9. ^{13}C-NMR spectrum of compound 2.

(1)



(2)

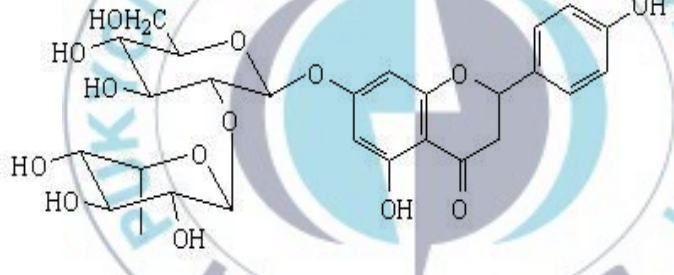


Fig. 10. Structures of poncirin (1) and naringin (2).

m, H-3a), 2.66 (1H, dd, J = 17.2 Hz, 3.1 Hz, H-3b), 1.20 (3H, d, J = 6.2 Hz, H-6′); ^{13}C-NMR (CD_3OD, 63 MHz) δ: 198.4 (C-4), 166.6 (C-7), 165.0 (C-5), 164.5 (C-9), 161.5 (C-4′), 132.1 (C-1′), 129.0 (C-2′, C-6′), 115.0 (C-3′, C-5′), 104.9 (C-10), 102.5(C-1′), 99.4 (C-1′), 97.9 (C-6), 96.8 (C-8), 79.5 (C-2), 79.0 (C-2′), 78.9 (C-5′), 78.1 (C-3′), 73.9 (C-4′), 72.2 (C-2′, C-3′), 71.2 (C-4′), 70.0 (C-5′), 62.2 (C-6′), 55.8 (OCH3), 44.1 (C-3), 18.2 (C-6′).

Compound **2**. Yellow powder; ^{1}H-NMR (CD_3OD, 250 MHz) δ: 7.20 (2H, d, J = 8.6 Hz, H-2′, H-6′), 6.70 (2H, d, J = 8.6 Hz, H-3′, H-5′), 6.05 (1H, d, J = 2.2 Hz, H-8), 6.03 (1H, d, J = 2.2 Hz, H-6), 5.25 (1H, dd, J = 12.9 Hz, 2.8 Hz, H-2), 5.13 (1H, d, J = 1.5 Hz, H-1′), 4.97 (1H, d, J = 7.2 Hz, H-1′), 3.07 (1H, m, H-3a), 2.63 (1H, dd, J = 17.2 Hz, 2.9 Hz, H-3b), 1.17 (3H, d, J = 6.2 Hz, H-6′); ^{13}C-NMR (CD_3OD, 63 MHz) δ: 198.5 (C-4), 166.6 (C-7), 165.0 (C-5), 164.6 (C-9), 159.1 (C-4′), 130.8 (C-1′), 129.1 (C-2′, C-6′), 116.3 (C-3′, C-5′), 104.9 (C-10), 102.5(C-1′), 99.3 (C-1′), 97.8 (C-6), 96.7 (C-8), 80.7 (C-2), 79.0 (C-2′), 78.9 (C-5′), 78.1 (C-3′), 73.9 (C-4′), 72.2 (C-2′, C-3′), 71.2 (C-4′), 70.0 (C-5′), 62.2 (C-6′), 44.1 (C-3), 18.2 (C-6′).

Summary

A tetracycline resistant *V. parahaemoliticus*, capable of growing on TCBS medium containing tetracycline, was isolated from cultivated fishes. A gene responsible for the tetracycline resistance was cloned from the chromosomal DNA of *V. parahaemolyticus* 0854 using *E. coli* KAM3, which lacks major multidrug efflux pumps (*△acrB*) as host cells. It was determined to be an open reading frame (ORF) for tetracycline resistance protein (TetB) by the nucleotide sequence. In order to characterize the antibiotic resistance of TetB originated from *V. parahaemolyticus* 0854, the *tetB* gene was sub-cloned into the plasmid pSTV28. The resulting plasmid was designated as pSTVTetB and transfomated into KAM3. KAM3 cells harboring the recombinant plasmid pSTVTetB is able to grow on plates containing tetracycline and oxytetracycline but doxycycline, indicating that the tetB gene originated from *V. parahaemolyticus* 0854 confer the tetracycline- and oxytetracycline-resistance to the host cells.

Next, it was designed an *in vivo* screening assay using KAM3/pSTVTetB to discover new drug(s) or specific anti-substance(s) against tetracycline resistant gene or protein. Several medicinal plants were evaluated for its inhibitory activity against KAM3/pSTVTetB in the presence of tetracycline and chloramphenicol. The methanolic extract of *Poncirus trifoliata* (L.) Raf. exhibited significant inhibitory activity against the cells.

To perform more detail investigation on the inhibitory activity, the

methanolic extract was further participated with several organic solvents to yield *n*-hexane, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and *n*-butanol fractions. The inhibitory activity of each fraction against KAM3/pSTV TetB was assayed by using a paper disc method in the presence of tetracycline. Among them, EtOAc fraction showed the highest inhibitory activity. The EtOAc fraction was subjected to column chromatography over a silica gel with *n*-hexane : EtOAc (from 1:1 to 1:5 v/v) gradually, yielding 6 subfractions. PE1-F02 fraction showed the strongest growth inhibitory activity against KAM3/pSTVTetB cells and were further separated into thirteen fraction. Among them, fractions PE3-F013 were re-separated into twelve fraction. Among twelve fraction, PE4-F02 (compound **1**) and PE4-F04 (compound **2**) fractions were obtained by Sephadex LH-20 column chromatography. In the fraction, two major compounds were observed by thin layer chromatography (TLC). Compound **1** and **2** were identified to be poncirin and naringin by ^{1}H-NMR and ^{13}C-NMR, respectively.

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