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Anti-microbial Effects of Rhizome Extracts of *Alpinia officinarum* Hance against VRE (vancomycin-resistant enterococci) and Other Pathogenic Microorganisms

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Abstract – The purpose of this investigation was to extract the bioactive agents from *Alpinia officinarum* Hance. The methanol with ethylacetate extracts alone and combined were examined for their activities against VRE (vancomycin-resistant enterococci) and pathogenic yeast *in vitro*. The incidence of infections caused by VRE and other pathogenic microorganisms and the importance of using novel synergistic drug combinations has become important. Previously, we reported the antimicrobial effects of the butanol extract from *Lonicera japonica* and have evaluated combinations of solvent extracts, with a focus on the MeOH and EtOAc extracts from *A. officinarum*. In the present study, enhanced inhibitory effects were achieved by employing a combination of the two solvent extracts. The MeOH and EtOAc combination was especially effective against four VRE strains: *E. faecalis* (K-10-22), *E. faecaium* (K-11-212), *E. faecalis* (K-10-57) and *E. faecalis* (K-10-361) with MIC values of 12.5, 12.5, 6.25 and 25 μg/mL, respectively. Thus, the combination was more effective than other antibiotics such as kanamycin, gentamicin or tetracycline against bacteria including *E. coli, Staphylococcus aureus*, and *Micrococcus luteus*. In addition, the combination was effective against yeasts such as *Candida albicans, Candida tropicalis* and *Cryptococcus neoformans*.
Keywords – *Alpinia officinarum* Hance, drug combination, methanol extract, ethylacetate extract, VRE (vancomycin-resistant enterococci)

Introduction

During the past 15 to 20 years, antimicrobial resistance among gram-negative and gram-positive bacteria has become an increasingly serious problem (Kim *et al*., 1999). At the same time, serious infections caused by gram positive bacteria have become more widespread. Particularly, VRE (vancomycin-resistant enterococci) have emerged as important noscomial pathogens during the past decade (Livornese *et al*., 1997; Shlaes *et al*., 1991). These bacteria are known to have intrinsic resistance to commonly used antimicrobial agents. VRE were first reported in 1988 and have since rapidly emerged as an important cause of hospital acquired infection in many parts of the world. The seriousness of the problem has warranted an increasing effort to develop new antimicrobials possessing activities against VRE. Therefore, the search for drugs against VRE is very important.

Alpinia officinarum Hance is a species in the family Zingiberaceae widely cultivated in southern China. There are about 46 species in this genus in China. *A. officinarum*, a pungent and aromatic rhizome, was used as a traditional Chinese medicine with anti-inflammatory, antioxidant, antiproliferative, anticancer and antiemetic activities (Claeson *et al*., 1996; Yang *et al*., 2002; Yokosuka *et al*., 2002). It has been reported that smaller galangal has biological activities including antitumor, antiulcer, antibacterial and antifungal properties (Itokawa *et al*., 1985; Matsuda *et al*., 2006; Newman *et al*., 2003). Previously, we have screened several antimicrobial agents from traditional Chinese medicine (Rhee and Lee, 2011). Use of an agent in combination with other antibiotics possessing activity against VRE and other pathogenic microorganisms is of potential interest since synergistic activities against highly resistant microorganisms may be obtained (Aeschlimann *et al*., 1998; Arvind *et al*., 2002; Grey *et al*., 1979; King *et al*., 2002; Lambert *et al*., 2003; Saito *et al*., 1994; Shlaes *et al*., 1991). Such a strategy may be especially useful in the treatment of infections caused by VRE and other resistant microorganisms. In this study, we evaluated the *in vitro* antimicrobial activities of a variety of antimicrobial agents alone and

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compared their activities against the activities of the methanol (MeOH) and ethylacetate EtOAc extracts from *A officinarum* alone and in combination. In particular, we employed the combination of the MeOH and EtOAc extracts in a 50 : 50 (wt/wt) ratio. The extract combination possessed potent antimicrobial activities against most VRE and fungus.

Materials and Methods

Preparation of plant material and antibiotics – The rhizomes of *A. officinarim* were purchased from Chungju Oriental drug store (Chungju, Korea). Vancomycin, teicoplanin, penicillin G, kanamycin, gentamicin, tetracycline, ketoconazole, amphotericin and nystatin were purchased from Sigma (Sigma Chemical Company, St. Louis, Mo. USA). Stock solution of each antibiotic was freshly prepared on the day of use. Also, to get the combination ratio that could show the strongest activity against microorganisms, we performed a series of experiments in which the extract ratios were 2 : 4, 3 : 7, 4 : 6, and 5 : 5 EtOAc : MeOH. Based on these results (data not shown), we confirmed that the 5 : 5 ratio was most potent. Thus, MeOH and EtOAc extracts were mixed at a 50 : 50 ratio [wt/wt].

Extraction and isolation – Fresh rhizomes of galangal (1.00 kg) were chopped and homogenized in methanol and then successively extracted three times with hot MeOH for 3 h. After the solvent was removed, the MeOH extract (50 g) was subjected to chromatography on a 20 × 6.0 cm Amberlite XAD-2 resin column. The column was rinsed with distilled water (3 L) to eliminate water-soluble compounds and the retained material was eluted with MeOH (3 L) and then followed by EtOAc (3 L). The MeOH and EtOAc eluents were concentrated to dryness in vacuo and then dissolved in DMSO for use.

Test microorganisms and mediums – A panel of microorganisms that are potentially pathogenic for humans was used to assess the antimicrobial activities of the extracts. The medium used for the VRE was a brain/heart infusion agar (Difco,USA). The five clinical VRE strains were *E. faecium* K-11-212 (*vanA: vanA* phenotype), *E. faecium* K-11-361 (*vanA*), *E. faecalis* K-10-22 (*vanB*: *vanB* phenotype), *E. faecalis* K-10-57 (*vanA*) and *E. faecalis* K-10-115 (*vanB*), isolated at the Korea University hospital over a period of two years (2010-2011) and evaluated in the current year. The seven bacteria used were kept in our laboratory and included: *Escherichia coli* ATCC 10536, *Bacillus subtilis* IAM 1069 (NIHJ PCI 219P), *Micrococcus luteus* ATCC 4698, *Streptococcus faecalis* ATCC 10541, *Pseudomonas aerginosa* ATCC 14502, *Staphylococcus aureus* TK 784 and *Staphylococcus aureus* penicillin resistant ATCC 9144. The fungi belonged to the IAM, ATCC and CMPG and were: *Candida albicans* IAM, *C. glabrata* CMPG 681, *C. tropicalis* CMPG 678, *C. tropicalis* R2 (polyene-resistant strain from Pasteur Institute, France) CMPG 849, *Cryptococcus neoformans* 1 ATCC 13690 and *C. neoformans* 2 CMPG 682. The following media were used for each of the organisms: Bouillon agar (3 g dextrose, 10 g polypeptone, 5 g NaCl, 10 g meat extract, 10 g agar and 1 liter H_2O, adjusted to pH 7.0 before autoclaving) for *Bacillus subtilis*, *Micrococcus luteus*, *Streptococcus faecalis*, *Pseudomonas aerginosa*, *Staphylococcus aureus* and *Staphylococcus aureus* penicillin resistant; glucose bouillon (3 g dextrose, 10 g polypeptone, 5 g NaCl, 10 g meat extract, 10 g agar, and 1 liter H_2O, adjusted pH 7.0 before autoclaving) for *Escherichia coli*; Sabouraud medium (Polypeptone 5.0 g, Glucose 30.0 g, Agar 11.0 g, and 1 liter H_2O, adjusted pH 7.0 before autoclaving) for *Candida albicans* strains; Yeast-starch medium (Yeast extract 2.0 g, Soluble starch 10.0 g, Agar 10.0 g, and pH not adjusted) for *Cryptococcus neoformans* strains.

Antimicrobial activity – The vancomycin, teicoplanin and penicillin G mean inhibitory concentrations (MICs) were determined by the agar dilution method according to the National Committee for Clinical Laboratory Standards (NCCLS, 1997). In brief, the concentrations of the anti-VRE and anti-pathogens agents tested ranged from 6.25 to 512 μg/mL. Colony suspensions equal to a 0.5 McFarland standard were prepared and inoculated onto the antibiotic-containing medium using a Cathra Systems replicating device (MCT Medical Inc., St. Paul, MN) to yield a final inoculum of 10 CFU/spot. The plates were incubated in ambient air at 35 °C for 24 h. The MIC was defined as the lowest antibiotic concentration showing no growth.

Statistical analysis – All data are presented as the means ± SD. Statistical analysis were carried out using the Statistical Package for Social Science (SPSS; SPSS Inc., Chicago, IL, USA).

Results and Discussion

Combination of MeOH and EtOAc extracts was more highly active against *E. faecium* K-11-212 (*vanA*), *E. faecium* K-11-361 (*vanA*), *E. faecalis* K-10-57 (*vanB*) and *E. faecalis* K-10-115 (*vanB*) than when each extract was tested alone. The MIC values for the combination of

Table 1. Minimal Inhibitory Concentrations (MIC) of the MeOH, EtOAc or combined MeOH/EtOAc extracts from *A. officinarum* Hance as well as vancomycin, teicoplanin and penicillin G toward anti-VRE strains

Pathogen	MeOH ext. + EtOAc ext.[a]	MeOH ext.[a]	EtOAc ext.[a]	Vancomycin [a]	Teicoplanin[a]	PenicillinG[a]
E. faecalis K-10-22	12.5±0.5[b]	64±1.5	128±1.5	25±0.5	>512±1.3	512±1.1
E. faecalis K-10-57	6.25±0.2	32±0.7	256±1.2	>512±2.6	512±0.5	>512±1.5
E. faecalis K-10-115	25±1.5	256±2.5	32±0.2	512±0.5	>512±1.7	>512±1.9
E. faecium K-11-212	12.5±0.5	128±1.5	26±0.5	256±0.7	512±1.5	>512±1.5
E. faecium K-11-361	12.5±0.6	128±1.5	128±1.5	>512±1.5	512±1.7	256±1.5

[a]Results are means of three determinations and are expressed as μg/ml; we adjust volume for each drug that used MeOH or EtOAc extract.
[b]The results represent the means ± SD from three measurements.

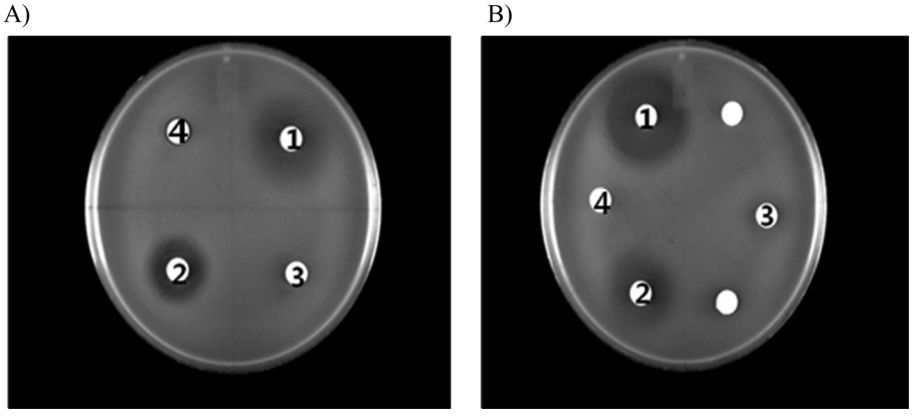

Fig. 1. Antimicrobial activities of MeOH and EtOAc extracts of *A. officinarum* Hance against *E. faecalis* and other pathogens. A) MeOH and EtOAc extract of *A. officinarum* Hance against *E. enterococci* [1: MeOH+EtOAc extract, 2: MeOH extract, 3: EtOAc extract, 4: DMSO only]. B) MeOH and EtOAc extracts combined against *Staphylococcus aureus R2* [1: MeOH + EtOAc extract, 2: MeOH extract, 3: EtOAc extract, 4: DMSO only].

extracts against these bacteria were 12.5 ± 0.5, 6.25 ± 0.2, 12.5 ± 0.5 and 12.5 ± 0.6 μg/ml, respectively (Table 1, Fig. 1A). Activity against *E. faecalis* K-10-22 (*vanA*) was moderate with an MIC value of 25 ± 0.5 μg/mL. In contrast, vancomycin, teicoplanin and penicillin G were inactive against all five VRE strains, with almost all MICs being 256 ± 0.7 and > 512 ± 1.5 μg/mL. A particularly strong antimicrobial activity, comparable to that of clinically used antibiotics, was observed for the MeOH : EtOAc combination extract (Table 2, Fig. 1B). It was highly active against *E.coli*, *Bacillus subtilus* and *Streptococcus faecalis* strains where the MICs were 12.5 ± 0.2 μg/mL; it also showed moderate activities against *Micrococcus luteus* and *Pseudomonas aeruginosa*, but was inactive against *Streptococcus faecalis* and *Staphylococcus aureus* strains. Also, gentamicin was active against *Pseudomonas aerginosa* and *Staphylococcus aureus* strains, with MICs of 12.5 ± 0.5 μg/mL for both strains. In contrast, kanamycin and tetracycline were practically inactive against almost all strains except for the case of *Pseudomonas aeruginosa*. The combination of MeOH : EtOAc extracts were also active against pathogenic yeast, as shown in Table 3. The most sensitive yeasts to the MeOH:EtOAc combination were *Candida albicans*, *C. tropicalis* (sensitive and resistant amphotericin) and one strain of *Cryptococcus neoformans* for which the MIC values were 12.5 ± 0.1, 12.5 ± 0.2 and 12.5 ± 0.3 μg/mL, respectively. On the whole, the combination extract was more active than ketoconazole and amphotericin, which had comparable potency with nystatin, except against *C. tropicalis* (less active) and *Candida albicans* (more active).

Smaller galangal (*A. officinarum* Hance) is a pungent and aromatic rhizome that is a member of the ginger family (Zingiberaceae). The rhizome is cultivated in India, Vietnam, Southern China and Thailand because of its use as a spice and as a traditional medicine for several purposes such as treatment of pyogenic diseases (infectious acne, carbuncles, sty, pyoderma and pustular impetigo in Thailand), ring worm, venereal diseases and abdominal discomfort (Athamaprasangsa *et al*., 1994). In this study, we show marked activity of the MeOH and EtOAc

Table 2. Minimal Inhibitory Concentrations (MIC) of the MeOH, EtOAc or combined MeOH/EtOAc extracts from *A. officinarum* Hance as well as kanamycin, gentamicin and tetracycline against pathogenic bacteria

Pathogen	MeOH ext. + EtOAc ext.[a]	MeOH ext.[a]	EtOAc ext.[a]	Kanamycin[a]	Gentamicin[a]	Tetracycline[a]
E.coli	12.5±0.1[b]	64±0.3	32±0.1	64±0.3	32±0.3	64±0.8
Bacillus subtilus	25±0.5	12.5±0.1	>512±1.5	64±0.1	128±1.5	128±1.1
Micrococcus luteus	25±0.5	64±0.2	128±1.0	32±0.2	64±0.7	25±0.4
Streptococcus faecalis	12.5±0.2	128±1.2	128±1.0	512±1.4	>512±1.8	64±0.2
Pseudomonas aeruginosa	25±0.5	32±0.1	64±0.2	12.5±0.2	12.5±0.1	12.5±0.2
Staphylococcus aureus 1	25±0.4	64±0.5	12.5±0.3	256±1.2	12.5±0.1	512±1.5
Staphylococcus aureus R2[b]	25±0.5	12.5±0.5	>512±0.3	512±1.5	12.5±0.2	>512±1.7

[a]Results are means of three determinations and are expressed as μg/ml. [b]Resistant to tetracycline.
[b]The results represent the means ± SD from three measurements.

Table 3. Minimal Inhibitory Concentration (MIC) of the MeOH, EtOAc or combined MeOH/EtOAc extracts from *A. officinarum* Hance as well as ketoconazole[a], amphotericinB[a] and nystatin[a] toward pathogenic yeast

Pathogen	MeOH ext. + EtOAc ext.[a]	MeOH ext.[a]	EtOAc ext.[a]	Ketoconazole[a]	Amphotericin B[a]	Nystatin[a]
Candida albicans	12.5±0.1[b]	64±0.7	32±0.2	128±0.2	>512±1.2	32±0.1
C. glabrata	12.5±0.1	32±0.5	64±0.5	12.5±0.1	128±0.5	128±0.7
C. tropicalis	12.5±0.2	64±0.9	32±0.4	256±0.2	128±0.6	128±0.7
C.tropicalis R2[b]	25±0.5	32±0.4	64±0.4	12.5±0.	512±1.5	12.5±0.1
Cryptococcus neoformans 1	12.5±0.3	12.5±0.2	32±0.2	32±0.4	128±0.8	32±0.5
Cr. neoformans 2	128±1.2	256±1.2	>512±0.1	256±1.2	128±0.8	12.5±0.2

[a]Results are means of three determinations and are expressed as μg/ml. [b]Resistant to amphotericin.
[b]The results represent the means ± SD from three measurements.

extract combination from *A. officinarum* Hance against VRE and yeast. Accordingly, VRE and the pathogenic microbial susceptibility tests for the combined MeOH : EtOAc extracts were performed against a broad range of microorganisms. The current results demonstrate that the combination of extracts has an anti-VRE, anti-bacterial and anti-fungal activity that may have significant potential as a possible therapy for a broad range of microbial infections.

We found stronger activity with the MeOH:EtOAc extract combination than when the MeOH or EtOAC extracts were used alone. Combined use of antibiotics that act synergistically could reduce the dose and side effects as well as prevent the development of resistant bacteria (Frandberg *et al*., 2000; Niku-Paavola *et al*., 1999). In this study, we discovered that a combination of the antimicrobial MeOH and EtOAc extracts from *A. officinarum* Hance exhibited strong synergy against VRE and others pathogenic microorganisms. From this study, we may have discovered drug mixtures with potent synergism that support drug cocktail therapy providing better efficacy for the treatment of multi-resistant microorganisms.

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