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Antimicrobial Activity of *Elfvingia applanata* Extract Alone and in Combination with Naringenin

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Elfvingia applanata 엑스의 항균력 및 Naringenin과의 병용효과

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ABSTRACT: As part of our search for less toxic antimicrobial agents from natural resources, the antimicrobial activity of *Elfvingia applanata* ($P_{ers.}$) $K_{arst.}$ extract was examined alone and in combination with naringenin. EA, the aqueous extract from the carpophores of *E. applanata*, was lyophilized and a dark brownish powder was obtained. Antimicrobial activity of EA was tested *in vitro* against nineteen strains of bacteria and eleven strains of fungi by serial broth dilution method, and expressed by minimal inhibitory concentration (MIC). Among nineteen strains of bacteria tested, the antimicrobial activity of EA was the most potent against *Proteus vulgaris* showing MIC of 1.125 mg/ml. EA also inhibited the growth of the selected fungi at higher concentrations ranging from 7.5 mg/ml to 15.0 mg/ml. To investigate the effect of antimicrobial combinations of EA with naringenin, the fractional inhibitory concentration index (FICI) was determined by checkerboard assay for each strain. The antimicrobial combinations of EA with naringenin resulted in partial synergism against *Staphylococcus aureus* only, and showed additive effect in two strains including *Klebsiella pneumoniae* and *Salmonella typhi*. Antagonism was not found.

KEYWORDS: *Elfvingia applanata*, Carpophores, Antimicrobial activity, Minimal inhibitory concentration (MIC), Checkerboard assay, Fractional inhibitory concentration index (FICI), Naringenin, Antimicrobial combinations

Elfvingia applanata ($P_{ers.}$) $K_{arst.}$, which belongs to Polyporaceae of basidiomycetous fungi, grows spontaneously on a branch of latifoliate tree or coniferous tree, rarely. It forms carpophores on a branch in parallel with semicircular form. *E. applanata* is distributed all over the world including Korea, and has been used as a folk medicine to cure various human tumorigenic diseases (Kim *et al.*, 1990 and Lee *et al.*, 1983). Several investigators identified some kinds of biologically active components from *E. applanata*. Bitter triterpenoids such as methylganoderic acid AP, ganoderenic acid F and G, methylganoderate H and I, and furanoganoderic acid were isolated from *Ganoderma applanatum* (Nishitoba *et al.*, 1988). The isolation of alnusenone and friedelin, which are triterpenoid and steroid, respectively, were also reported (Protiva *et al.*, 1980). Ganoderan isolated from the cultured mycelia was shown to decrease and stabilize blood pressure, and was also shown to have antitumor activity (Misaki *et al.*, 1985). Polysaccharides such as α-D-glucan and β-D-glucan were also isolated from the cultured mycelia, and were shown to have antitumor activities (Mizuno *et al.*, 1982). The polysaccharides isolated from the carpophores of *G. applanatum* were shown to enha-

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nce the induction of delayed-type hypersensitivity in mice (Nakashima *et al.*, 1979). Fungal nucleic acids extracted from *E. applanata* were shown to induce the secretion of interferon-like materials in spleen cell of mice (Kandefer *et al.*, 1979). Other biologically active components from *E. applanata* include milk-clotting enzyme (Nerude *et al.*, 1989), coenzyme Q_9 (Yamada *et al.*, 1973), steroid (Protiva *et al.*, 1980; Ripperger *et al.*, 1975; Svridonov *et al.*, 1976) and mineral components such as germanium (Kondo *et al.*, 1987 and Han *et al.*, 1988).

Several investigators identified antimicrobial components from basidiomycetous fungi. Cinnabarin was isolated, and identified from *Coriolus sanguineus* grown in Korea (Chung *et al.*, 1978). Phlebiakauranol aldehyde and scorodonin were isolated from *Punctularia atropurascens* and *Marasmius scorodonius*, respectively, and their antimicrobial activities were investigated against bacteria, yeast and fungi (Anke *et al.*, 1980 and Anke *et al.*, 1986). 4-O-methylmelleolide and judeol, which showed strong antimicrobial activity against gram positive bacteria, were also isolated from *Armillaria mellea* (Donnelly *et al.*, 1985). The antimicrobial components isolated from basidiomycetous fungi appeared to be different from the existing antibiotics in structure, showing a possibility of development to a new class of antibiotics that may be used in the treatment of antibiotic-resistant strains. The antimicrobial activity of EA, the aqueous extract from the carpophores of *E. applanata*, was examined as a part of our search for less toxic antimicrobial agents from natural resources. The antimicrobial activity of EA was examined against bacteria and fungi, and then the effect of antimicrobial combinations with naringenin was also investigated in this study.

Materials and Methods

Materials and Apparatus

The carpophores of *Elfvingia applanata* ($P_{ers.}$) $K_{arst.}$ was purchased from Cheongju-city market. Nutrient broth and agar were purchased from Difco Co., and naringenin was isolated from peels of Citri fructus. UV-spectronic 21 from Milton Roy was used to adjust the density of bacterial inocula. The microorganisms were incubated in electric incubator from Astell Hearson Co. A vacuum evaporator from Tokyo Rikakika Co. and a freeze dryer from Edward Co. were also used.

Test strains

Test strains subcultured in our laboratory were used in this investigation. As seven gram positive bacterial strains, *Bacillus cereus* ATCC 27348, *B. subtilis* ATCC 6633, *B. anthracis* ORD-IUR, *Micrococcus luteus* ATCC 9341, *Staphylococcus aureus* ATCC 25923, *S. aureus* ATCC 29213 and *S. epidermidis* ATCC 12228 were used. Twelve gram negative bacterial strains were selected, and those were *Enterobacter aerogenes* ATCC 29751, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 10031, *K. oxytoca* ATCC 8724, *Proteus mirabilis* ATCC 25933, *P. vulgaris* 78615, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella tompson* ATCC 10256, *S. typhi* ATCC 6229, *S. typhimurium* ATCC 14028, *Serratia marcescens* ATCC 27117 and *Shigella sonnei* ATCC 11060. Eleven fungal strains tested were *Aspergillus ochraceus* NHL 5294, *A. parasititus* NHL 5243, *A. versicolor* ATCC 5877, *Candida albicans*, *C. pseudotropicalis*, *Fonsecaea compocta*, *Fusarium* spp. *Trichophyton mentagraphytes* and *T. chrysogenum*.

Preparation of EA

To prepare EA (the aqueous extract from the carpophores of *E. applanata*), the carpophores were dried, disrupted, and then extracted with distilled water in a water bath at 90~100℃ for 8 hours. After filtration, extraction was repeated in the same condition. The filtrates were combined, and concentrated in a vacuum evaporator. The semifluid material obtained was lyophilized in a freeze-dryer to yield a dark brownish powder.

Preparation of test solution

EA was dissolved with nutrient broth in a concentration of 5 or 15 mg/ml. Naringenin was dried in a vacuum desiccator to constant weight, and dissolved with dimethylsufoxide (DMSO) in con-

centration where DMSO did not inhibit the growth of bacteria and diluted with nutrient broth to a concentration of 1,000 μg/ml.

Antimicrobial activity

To prepare bacterial inocula, *S. marcescens* was incubated at 26℃, *B. cereus* and *M. luteus* at 30℃, and other strains at 37℃ for 18 hours. The cultured bacteria were adjusted with nutrient broth until transmission was about 30% at 540 nm by using UV-spectronic 21, and diluted 1 to 100 again. To prepare fungal inocula, *C. albicans* was incubated on a Sabouroud dextrose agar slant at 28℃ for two days, and colonies on the slant were suspended with Sabouroud dextorse broth to make fungal suspension. The fungal suspension was adjusted with Sabouroud dextrose broth until transmission was about 95% at 540 nm. Colonies of other fungal strains were formed by culturing them on the agar slant at 27℃ for ten days, and suspended with Sabouroud dextrose broth containing 0.05% Tween 80, and then the suspension was adjusted to transmission≒90% at 540 nm (Gary *et al.*, 1979).

The antimicrobial activity was determined by serial broth dilution method (Ericsson *et al.*, 1971). Initially, test materials were subjected to twofold step dilution with nutrient broth, and dispensed to each nine test tube. Fifty ul of bacterial inoculum was added to each test tube, and incubated for 18 hours. In the antifungal test, Sabouroud dextrose broth was used as a diluent, and each test tube inoculated with fungal suspension was incubated with 40~50 humidity at 27℃ for 14 or 20 days. Blank test as a control was performed in parallel. The MICs of tested materials were defined as the lowest concentration exhibiting no visual turbidity.

Antimicrobial combinations

The antimicrobial combinations of EA with naringenin were tested by checkerboard assay (Lorian, 1991). The ten selected strains of bacteria were *B. subtilis*, *E. coli*, *K. pneumoniae*, *P. mirabilis*, *P. vulgaris*, *P. aeruginosa*, *S. aureus* ATCC 25923 and 29213, *S. epidermidis* and *S. typhi*. The concentrations tested for each antimicrobial agent typically ranged from 5 to 7 dilutions below the MIC to twice as much as the MIC (or higher if antagonism was suspected), using a twofold serial dilutions of each antimicrobial agent. Also included was a row of test tubes without any antimicrobial agent for each drug. Thus, the checkerboard was consisted of columns in which each test tube contained the same amount of EA being diluted along the x-axis, and rows in which each test tube contained the same amount of naringenin being diluted on the y-axis. Fifty ul of bacterial inoculum was added to each test tube, and incubated for 18 hours. The effect of antimicrobial combinations was evaluated by isobologram, fractional inhibitory concentration (FIC) and fractional inhibitory concentration index (FICI) (Elion *et al.*, 1954; Hallander *et al.*, 1982; Jadavji *et al.*, 1984). Synergism was assumed when the MIC of each component in one or more combinations was one-fourth or less of its original MIC (FICI≤0.5). Antagonism was assumed when the MIC of either component was increased twofold or more over its original MIC or when the MICs of both components were increased one or more compared to their respective MICs (FICI≥2.0). All other results were considered to be additive (0.5<FICI≤1.0) or indifferent effect (1.0<FICI<2.0).

Results and Discussion

Antimicrobial activity

The antimicrobial activity of EA against gram positive and negative bacteria is shown in Fig. 1 and Fig. 2, respectively. Among seven strains of gram positive bacteria tested, the antimicrobial activity of EA was the most prominent against *S. aureus* ATCC 25923 showing MIC of 1.75 mg/ml. Investigation of the effect of EA against the growth of gram negative bacteria showed that EA has relatively a strong antimicrobial activity against *P. vulgaris* (MIC=1.125 mg/ml) and *E. coli* (MIC=2.000 mg/ml). Two strains of gram negative bacteria tested were shown to have MIC values larger than 5 mg/ml. As indicated in Fig. 1 and Fig. 2, the antimicrobial activity of EA was

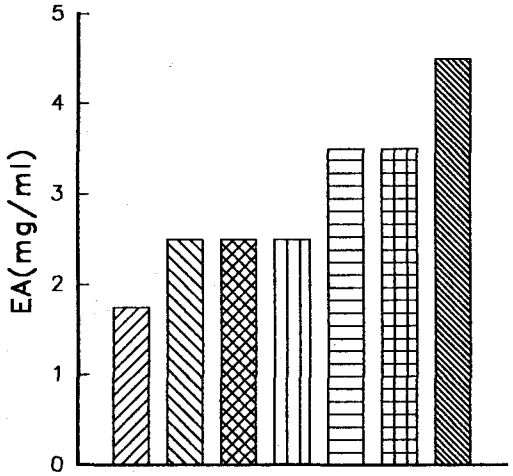

Fig. 1. MICs of EA against gram positive bacteria. EA: extract of *Elfvingia applanata*, □: *Staphylococcus aureus* ATCC 25933, □: *Bacillus cereus* ATCC 27348, □: *Bacillus anthracis* ORDIUR, □: *Micrococcus luteus* ATCC 9341, □: *Staphylococcus aureus* ATCC 12228, ▧: *Bacillus subtilis* ATCC 6633.

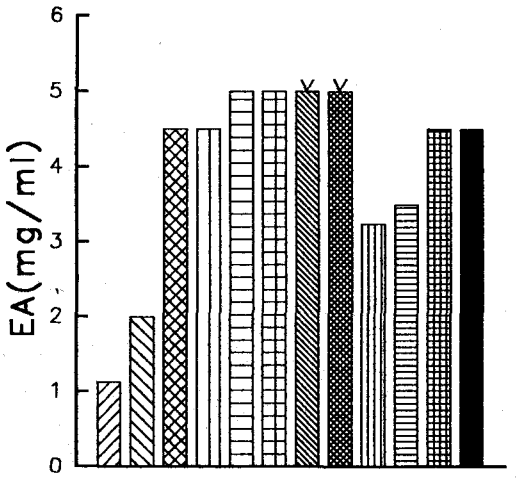

Fig. 2. MICs of EA against gram negative bacteria. EA: extract of *Elfvingia applanata*, □: *Proteus vulgaris* 78615, □: *Escherichia coli* ATCC 25922, □: *Salmonella tompson* ATCC 10256, □: *Salmonella typhi* ATCC 6229, □: *Klebsiella pneumoniae* ATCC 10031, □: *Pseudomonas aeruginosa* ATCC 27853, ▧: *Salmonella typhimurium* ATCC 14028, ▩: *Serratia marcescens* ATCC 27117, ▥: *Shigella sonnei* ATCC 11060, ▤: *Proteus mirabilis* ATCC 25933, ▦: *Klebsiella oxytoca* ATCC 8724, ■: *Enterobacter aerogenes* ATCC 29751

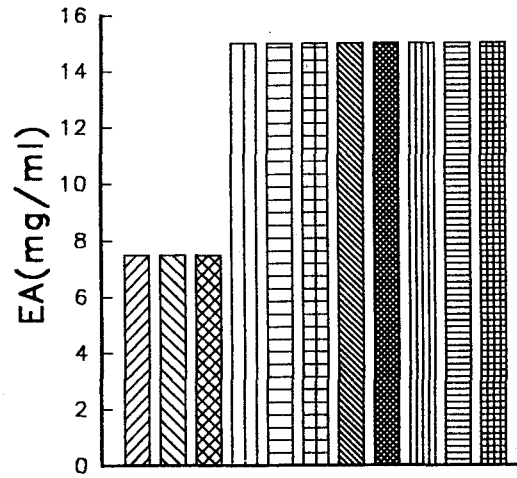

Fig. 3. MICs of EA against fungi. EA: extract of *Elfvingia applanata*, □: *Candida albicans*, □: *Trichophyton mentagraphytes*, □: *Fusarium* spp., □: *Aspergillus flavus* ATCC 15517, □: *Aspergillus funigatus*, □: *Aspergillus ochraceus* NHL 5294, ▧: *Aspergillus parasititus* NHL 5243, ▩: *Aspergillus versicolor* ATCC 5877, ▥: *Candida pseudotropicalis*, ▨: *Fonsecaea compocta*, ▦: *Trichophyton chrysogenum*.

generally more potent against gram positive bacteria than against gram negative bacteria. Several researchers proposed that most antimicrobial components of basidiomycetous fungi were potent against gram positive bacteria only (Robbins *et al.*, 1947 and Lee *et al.*, 1982), but the growth of *P. vulgaris*, which belongs to gram negative bacteria, was inhibited by EA at lower concentration compared to other gram positive bacteria. The antifungal activity of EA is shown in Fig. 3, and its MICs were generally high as compared with that of the antimicrobial activity. Among eleven strains of fungi tested, the antifungal activity of EA was relatively strong against *C. albicans*, *Fusarium* spp. and *T. mentagraphytes*. The antimicrobial activity of naringenin is also shown in Fig. 4, and the antimicrobial activity of naringenin was predominent against five strains of bacteria, i.e., *B. subtilis*, *E. coli*, *P. vulgaris*, *S. aureus* and *S. epidermidis*.

Antimicrobial combinations

The antimicrobial combinations of EA with na-

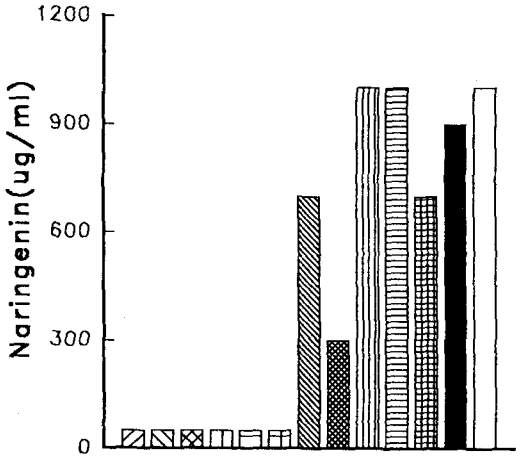

Fig. 4. MICs of naringenin against the bacteia. □: *Proteus vulgaris* 78615, □: *Staphylococcus aureus* ATCC 25923, □: *Escherichia coli* ATCC 25922, □: *Staphylococcus aureus* ATCC 29213, □: *Staphylococcus epidermidis* ATCC 1228, □: *Bacillus subtilis* ATCC 6633, ▨: *Salmonella typhi* ATCC 6229, ▩: *Klebsiella pneumoniae* ATCC 10031, ▥: *Pseudomonas aeruginosa* ATCC 27853, ▤: *Shigella sonnei* ATCC 11060, ▦: *Proteus mirabilis* ATCC 25933, ■: *Klebsiella oxytoca* ATCC 8724, □: *Enterobacter aerogenes* ATCC 29751

Table 1. FICs and FICIs of EA with naringenin

Strains	FiC[a]		$FICI_b$
	EA[c]	naringenin	
Bacillus subtilis ATCC 6633	0.063	1.000	1.063
Escherichia coli ATCC 26922	0.063	1.000	1.063
Klebsiella pneumoniae ATCC 10031	0.500	0.500	1.000
Proteus mirabilis ATCC 25933	0.500	1.000	1.500
Proteus vulgaris ATCC 78615	0.063	1.000	1.063
Pseudomonas aeruginosa ATCC 27853	0.063	1.000	1.063
Salmonella typhi ATCC 6229	0.500	0.500	1.000
Staphylococcus aureus ATCC 29213	0.063	1.000	1.063
Staphylococcus aureus ATCC 25923	0.250	0.500	0.750
Staphylococcus epidermidis ATCC 12228	0.063	1.000	1.063

ringenin were performed by checkerboard assay, and the results are summarized in Table 1. In antimicrobial combination with naringenin, partial synergism, FICI=0.75, was shown in *S. aureus* only. Additive effects were shown in two strains of bacteria, i.e., *K. pneumoniae* and *S. typhi*. Indifferent effect was shown in seven strains. Isobolograms corresponding to the FICI obtained with nine bacterial strains except *P. mirabilis* are presented in Fig. 5. Partial synergism are found in *S. aureus* ATCC 25923, and additive effect in two strains including *K. pneumoniae* and *S. typhi* with antimicrobial combinations of EA plus naringenin.

The advantages of antimicrobial combinations have been demonstrated, and antimicrobial combinations have been shown to have a variety of merits such as prevention or delay of the *in vivo* emergence of drug-resistant subpopulations, and reduction of toxicity as a result of reduced dosage. With the simultaneous use of two or more antimicrobial agents against bacteria which develop resistance by different mechanisms, the possibility that pathogenic bacteria will emerge resistant to all of antimicrobials employed is theoretically very low (Selkon *et al.*, 1964; Weinstein, L., 1975; Chamovitz *et al.*, 1985). The antimicrobial combinations of two or more antibiotics thus would prevent or delay the development of bacterial resistance to an antibiotic. Other important uses of antimicrobial combinations are the treatment of documented or suspected polymicrobial infections (Louie *et al.*, 1977), ability to use nontoxic amounts of two antibiotics when toxic doses of a single antibiotic would be required (Rodriguez *et al.*, 1985) and that combination therapy may be more effective against a single organism than the use of one antimicrobial agent alone (Krogsted *et al.*,

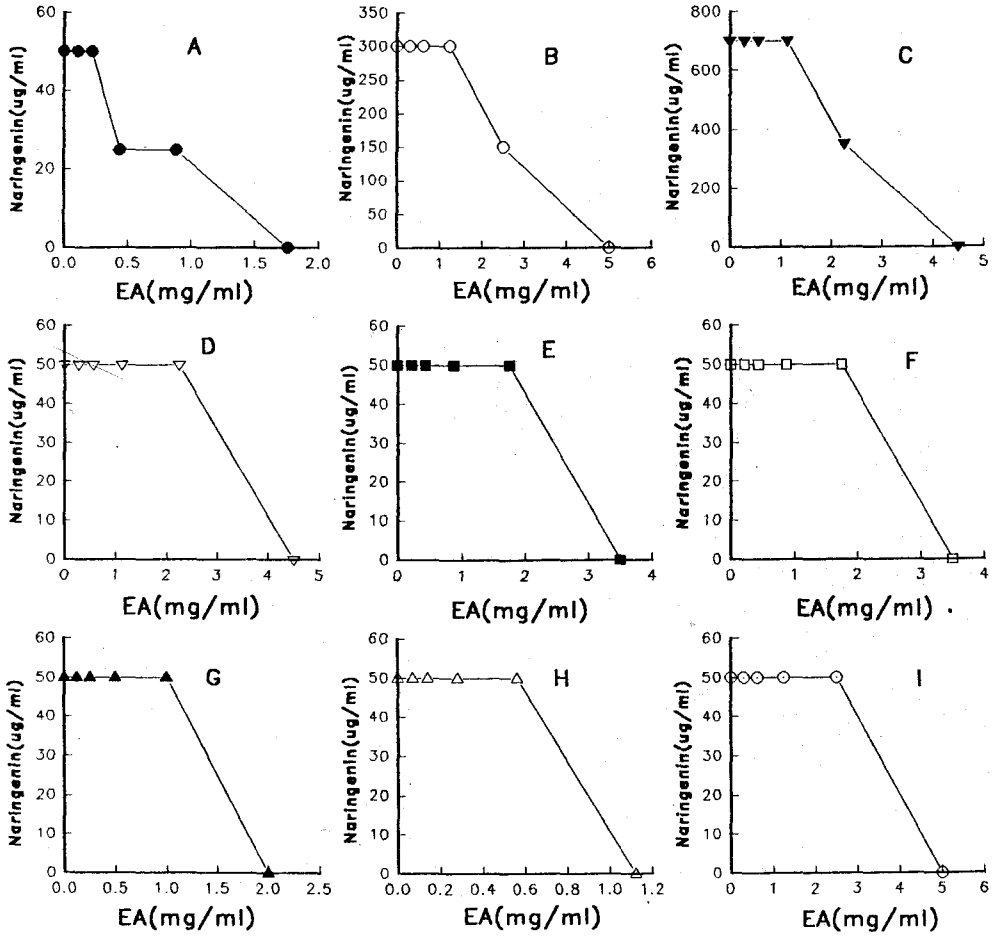

Fig. 5. Isobolograms constructed with the FICI values obtained by the checkerboard assay for *S. aureus* ATCC 25923 (A), *K. pneumoniae* ATCC 10031 (B), *S. typhi* ATCC 6229 (C), *B. subtilis* ATCC 6633 (D), *S. aureus* ATCC 29213 (E), *S. epidermidis* ATCC 12228 (F), *E. coli* ATCC 25922 (G), *P. vulgaris* 78615 (H) and *P. aeruginosa* ATCC 27853 (I).

1985). As an example of antimicrobial combinations, trimethoprim and sulfonamide block a different reaction step of folic acid metabolism respectively, and thus their antimicrobial combinations are shown synergism and markedly prevent the development of bacterial resistance (Poe, 1976). Also penicillin enhances the influx of aminoglycoside antibiotics and thus antimicrobial combinations with penicillin plus aminoglycoside decrease the nephrotoxicity and increase the bactericidal effect of aminoglycosides against pathogenic bacteria (Jawetz *et al.*, 1950). The antimicrobial combinations of EA with naringenin showed partial synergism or additive effects in several pathogenic bacteria as described in the results of checkerboard assay, and thus EA may be able to used as restorative drug that can permit a significant reduction in the dosage of the toxic antimicrobial agents without the loss of antimicrobial activity.

적 요

담자균류의 하나인 잔나비걸상 자실체로부터 얻은 수성엑스의 항세균 시험과 항진균 시험을 액체배지 희석법으로 실시하고 naringenin과의 병용시험을 checkerboard assay 법으로 평가하였다. 항세균 시

험결과 전반적으로 그람음성균보다 그람양성균에 대해 비교적 양호한 항균력을 나타내었으나 사용된 균주중 그람음성균인 *Proteus vulgaris* 78615는 MIC가 1.125 mg/ml로 가장 양호한 항균효과를 나타내었다. 11종의 균주에 대해 실시된 항진균 시험결과 *Candida albicans*, *Fusarium* spp. 그리고 *Trichophyton mentagraphytes*의 MIC가 7.5 mg/ml로 다른 균주에 대한 항진균 효과보다 두배 더 강한 효과를 나타내었다. naringenin과의 병용시험에서 *Staphylococcus aureus* ATCC 25923에 대하여 부분적 상승효과를 나타내었고, *Klebsiella pneumoniae* ATCC 10031과 *Salmonella typhi* ATCC 6229 두균주에서 상가효과가 관찰되었다. 그리고 *Bacillus subtilis* ATCC 6633을 비롯한 7종의 균주에서 무관효과를 나타내었으며 길항효과는 관찰되지 않았다.

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