The infectious diseases caused by multidrug-resistant bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant *Streptococcus pneumoniae* (PRSP) and vancomycin-resistant enterococci (VRE), are very serious problems because these bacteria are resistant to a number of antimicrobials in many cases. They are Gram-positive cocci, and infections caused by them are becoming very serious problems throughout the world. MRSA was first reported in the U.K. in 1961 and then spread world-wide. From CDC reports, the rate of MRSA in *S. aureus* detected from intensive care unit (ICU) patients in 2003 in the United States was more than 60%, and it increased annually (http://www.cdc.gov/ncidod/hip/ARESIST/ICU_MRSA.pdf). In Japan, there are more than 20000 reported cases of MRSA infections per year (http://idscc.nih.go.jp/idwr/index.html). Vancomycin is one of a few effective drugs for MRSA infections. However, vancomycin-resistant *S. aureus* (VRSA) was first reported in 2002 in the United States.\(^3\) Thus, effective antimicrobials for treatment of patients infected with VRSA are very limited. *S. pneumoniae* is one of the most important pathogens for community acquired pneumonia, meningitis, and otitis media. PRSP was first reported in Australia in 1967,\(^2\) and drug-resistant *S. pneumoniae* (DRSP) which is resistant to several antimicrobials was first reported in South Africa in 1977.\(^3\) Cases of infection with *S. pneumoniae* that are resistant not only against β-lactams but also against other antimicrobials such as macrolides and fluoroquinolones have been reported to be increasing.\(^4\)

VRE has been first reported in U.K. and France in 1986.\(^5\) In the case of the United States, the percentage of VRE in enterococci isolated from ICUs was 0.4% in 1989, but it increased up to 28.5% in 2003.\(^6,7\) Of the strains belonging to the enterococcal species, *Enterococcus faecalis* and *E. faecium* are common isolates from human infections.\(^8\) Among the isolates of enterococcal species, *E. faecalis* accounts for 80—90% and the rest is mainly *E. faecium*.\(^9\) *E. faecium* is more resistant against many antimicrobials than any other enterococcal species.\(^10\)

There are many cases of infection by drug resistant bacteria whereas few drugs are available effective for the treatment of such patients. Thus, it is urgently necessary to discover or develop new drugs that are effective on such drug resistant bacteria. We have been trying to discover novel compounds, such as antimicrobial compounds and inhibitors of drug resistance systems in bacteria,\(^11—15\) that are effective against multidrug-resistant bacteria.

Though *Salvia officinalis* (sage) is known as one of the herbs that has antimicrobial activity, there are few papers that have showed its antibacterial activity. We found that a crude extract of sage leaves showed antimicrobial activity against VRE. Thus, we tried to purify and determine the structure of the effective compound.

### MATERIALS AND METHODS

#### Bacterial Strains

*E. faecium* FN-1, *E. faecium* BM4147, *E. faecalis* NCTC 12201 and *E. faecalis* FA2-2 were kindly provided by Dr. Y. Ike (Gunma University). *Serratia marcescens* NUSM8905 was kindly provided by Dr. Y. Iseki (Okayama University).

#### Oleanolic Acid

![Structure of Oleanolic Acid](image)

Fig. 1. Structures of Triterpenoids, Oleanolic Acid [1], Ursolic Acid [2], Uvaol [3], Betulinic Acid [4], and Betulin [5]
Arakawa (National Institute of Infectious Diseases). Clinical isolates of MRSA strains OM481 and OM584 were from Okayama University Hospital. *Streptococcus pneumoniae* R6 (ATCC BAA-255) was purchased from American Type Culture Collection (ATCC). *Escherichia coli* K12 and *Pseudomonas aeruginosa* PA01 were also used in this study.

**Drug Susceptibility Testing** Minimum inhibitory concentrations (MICs) of various drugs were determined as described previously with cells of VRE, MRSA, *P. aeruginosa*, *E. coli*, *S. marcescens* and *S. pneumoniae*. For cells of *S. pneumoniae*, Mueller–Hinton broth supplemented with strept-penho sample ‘Eiken’ was used.

**Isolation of Effective Compounds** Dried and ground leaves of *S. officinalis* (850 g) were extracted with 70% aqueous acetone at room temperature; the acetone extract was concentrated by using a rotary evaporator to give 300 g of dry sample. A half of the sample was dissolved with water and extracted with ethyl acetate (*AcOEt*). The *AcOEt* extract was concentrated and then dried by using *N*₂ gas, and 70 g of the sample was obtained. This *AcOEt* extract was subjected to column chromatography over DIAION HP-20 (Mitsubishi-Kagaku Co.) and eluted with aqueous methanol (70, 80, 90, 100%) in a stepwise manner, and with 100% acetone at the final step. Since the eluate with 100% methanol showed the highest antimicrobial activity against VRE, 5 g of the 100% methanol fraction was further subjected to column chromatography over Sephadex LH-20 (Amersham Biosciences Co.) by using 100% ethanol as a solvent. Fractions that showed antimicrobial activity against VRE, MRSA, *P. aeruginosa* and MRSA (MICs were >128 µg/ml), and chemicals used in this study were purchased from commercial sources.

**RESULTS AND DISCUSSION**

**MICs of Triterpenoids** The acetone extract from sage obtained at the first step showed very weak antimicrobial activity, and the MIC of the extract against VRE was 256—512 µg/ml. We purified the active compound and identified it as oleanolic acid. The MICs of oleanolic acid, and other triterpenoids which have been reported to be contained in *S. officinalis*15) against various bacteria, are shown in Table 1. The MIC values of oleanolic acid and ursolic acid against VRE were 8 µg/ml and 4 µg/ml, respectively. These MIC values are fairly low as constituents of plants. In other words, antimicrobial activity of these compounds is fairly high among compounds derived from plants. Both of them also showed antimicrobial activity against other Gram-positive cocci such as MRSA and *S. pneumoniae* (Table 1). The MIC of oleanolic acid was 16 µg/ml for *S. pneumoniae* R6 and *S. aureus* OM481 (MRSA), and 32 µg/ml for *S. aureus* OM584 (MRSA). The MIC of ursolic acid was 8 µg/ml for *S. pneumoniae* R6 and *S. aureus* OM481, and 16 µg/ml for *S. aureus* OM584. On the other hand, neither compound showed antimicrobial activity against Gram-negative bacteria tested (MICs were >128 µg/ml). The antimicrobial activity of ursolic acid on VRE, *S. pneumoniae* and MRSA strains were two times stronger than those of oleanolic acid judging from the MIC values. Uvaol, betulinic acid and betulin did not show significant antimicrobial activity against VRE, *S. pneumoniae* and MRSA (MICs were >128 µg/ml). We compared the antibacterial activities between these compounds and ampicillin, a β-lactam antibiotic. Both ursolic acid and uvaol showed stronger activity than ampicillin on *E. faecium* and MRSA strains used in this study, and similar activity on *E. faecalis* strains used (Table 1).

Although Woldemichael et al. previously described the antimicrobial properties of oleanolic acid against *S. aureus* and *Bacillus subtilis*, our paper is the first report on its anti-

<table>
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<th>Oleanolic acid</th>
<th>Ursolic acid</th>
<th>Uvaol</th>
<th>Betulinic acid</th>
<th>Betulin</th>
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n.t.: not tested.
crobial activity against *E. faecium*, *E. faecalis* (especially VRE) and *S. pneumoniae*. Similarly, Kowalewski et al. investigated the antimicrobial activity of ursoic acid against *E. faecalis* but neither against *E. faecium* nor *S. pneumoniae*. According to their results, since the MIC against *E. faecalis* was 75—500 µg/ml, the antibacterial effect was weak. Those MIC values are different from our data (4—8 µg/ml) by 2 to 3 orders of magnitude. Their antibacterial activity was specific to enterococci and *S. pneumoniae*. It seems that such differences are due to differences in assay conditions and in strains used. Other activities of both compounds have also been reported, such as, for example, anti-inflammatory activity, anti-hyperlipidemic activity, anti-tumor-promotion activity and hepatoprotective effects, and so on. One group has reported that betulinic acid showed antimicrobial activity against *S. aureus*, whereas another group reported that it did not.

Our data support the latter result.

We tested the effects of oleanolic acid and ursoic acid on *E. coli*, *P. aeruginosa* and *S. marcescens*, a Gram-negative bacterium. However, these two compounds did not show significant antibacterial activity (Table 1). Multidrug efflux pumps play an important role in intrinsic drug resistance in *E. coli* and *P. aeruginosa*. Therefore, we also tested the effects of oleanolic acid and ursoic acid on mutants of *E. coli* and *P. aeruginosa*, both mutants of which are drug hyper-susceptible due to their lack of major multidrug efflux pumps. MICs of oleanolic acid and ursoic acid were higher than 128 µg/ml with such hyper-susceptible mutants (data not shown). Although we tested concentrations higher than 256 µg/ml, a precipitate was formed at such higher concentrations. Thus, it is not clear whether these compounds become substrates for multidrug efflux pumps. Anyway, we conclude that oleanolic acid and ursoic acid are not effective on Gram-negative bacteria, perhaps due to the presence of the outer membrane. We also tested antifungal activity with *Candida albicans*, and found that the MICs of oleanolic acid and ursoic acid were higher than 128 µg/ml (data not shown).

Since oleanolic acid and ursoic acid showed fairly high antimicrobial activity on VRE, we investigated whether activity of oleanolic acid and ursoic acid was bactericidal or bacteriostatic (Fig. 2). Oleanolic acid showed bactericidal activity on *E. faecium* BM4147 when added to the culture medium at concentrations higher than 16 µg/ml (2 times MIC) for 48 h. However, when 8 µg/ml of oleanolic acid (same as MIC) was added, bactericidal activity continued for 12 h, and cells started to grow after 24 h. Ursoic acid showed almost the same effects on *E. faecium* BM4147 (data not shown). Similar results were obtained with oleanolic acid and *E. faecalis*, but it seemed that the bactericidal activity was slightly weaker compared with that on *E. faecium* (data not shown). On the other hand, ursoic acid showed bacteriostatic activity on *E. faecalis* at concentrations higher than 8 µg/ml (2 times MIC) (data not shown).

The antimicrobial activity of oleanolic acid or ursoic acid is not so strong as compared with antimicrobial drugs that are clinically used, although oleanolic acid and ursoic acid showed fairly high activity. Nonetheless, it seems that both compounds are not so toxic. In fact, oleanolic acid has been successfully used as an orally administered drug to treat human liver diseases in China. Thus, they might be used for the treatment of infections by VRE.

Acknowledgments We thank Dr. Manuel Varela of Eastern New Mexico University for critically reading the manuscript. This research was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Fig. 2. Effect of Oleanolic Acid on Viable Cell Number of VRE

*E. faecium* BM4147 cells grown in Brain-Heart Infusion broth were diluted with fresh medium and incubated at 37 °C under aerobic conditions in the absence (C) or presence of oleanolic acid at 8 µg/ml ( ), 16 µg/ml ( ), or 32 µg/ml ( ). Samples were taken at the indicated time points, and viable cell numbers (colony forming unit; CFU) were counted after growth on plates.