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| 단 보 |

Antimicrobial Effect of Ursolic Acid and Oleanolic Acid against Methicillin-Resistant *Staphylococcus aureus*

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Ursolic Acid와 Oleanolic Acid의 메티실린 저항성 *Staphylococcus aureus*에 대한 항균작용

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The antimicrobial activity of ursolic acid (UA) and oleanolic acid (OA), both triterpenoid compounds, against methicillin-resistant *Staphylococcus aureus* (MRSA) is controversial. We examined the antimicrobial effects of UA and OA against 19 strains of MRSA isolated from Koreans by determining minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC). The data showed that the methicillin-sensitive strain *S. aureus* KCTC 1621^T was more resistant to UA and OA than that of the MRSA strains. The MBC values of UA and OA against MRSA had broad ranges; 4 to 32 μg/ml and 16 to >256 μg/ml, respectively. It was difficult to understand the different antimicrobial activities of UA and OA among the MRSA strains, because UA and OA antimicrobial mechanisms are unknown. These results indicate that the antimicrobial effects of UA and OA against MRSA are dependent on resistance to UA and OA in each strain.

***Keywords*:** antimicrobial effect, methicillin-resistant *Staphylococcus aureus*, oleanolic acid, ursolic acid

Staphylococcus aureus is Gram-positive bacterium that is commonly found on the skin and nose but it is opportunistic in humans and animals (Morell and Balkin, 2010). *S. aureus* is a causative pathogen of endocarditis, septicemia, arthritis, osteomyelitis, abscesses, cysts, and skin necrosis (Morell and Balkin, 2010; Szczuka *et al*., 2010). Since the first report in 1961, the incidence of methicillin-resistant *S. aureus* (MRSA) and multidrug-resistant *S. aureus* infections has increased rapidly (Barber, 1961; Köck *et al*., 2010; Kreisel *et al*., 2010). Several studies on natural antimicrobial extracts against MRSA have been performed based on the increase in MRSA (Horiuchi *et al*., 2007; Jovel *et al*., 2007; Weckesser *et al*., 2007; Fontanay *et al*., 2008; Zheng *et al*., 2008).

Ursolic acid (UA, 3β-hydroxy-urs-12-en-28-oic acid) and oleanolic acid (OA, 3β-3-hydroxyolean-12-en-28-oic acid), which

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are derivatives of triterpenoid saponins (Liu, 1995), have a higher degree of anti-bacterial activity against pathogenic bacterial strains such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Streptococcus mutans*, and *Streptococcus sobrinus* (Fontanay *et al*., 2008; Kim *et al*., 2010, 2011). In contrast, the antimicrobial activity of UA and OA against MRSA is controversial (Horiuchi *et al*., 2007; Fontanay *et al*., 2008). We examined the antimicrobial effects of UA and OA against MRSA isolated from Koreans.

S. aureus KCTC 1621^T (=ATCC 25923^T) was purchased from the Korean Collection for Type Culture (KCTC, Korea). The *S. aureus* clinical strains (KCOM 1588, KCOM 1589, KCOM 1590, KCOM 1591, KCOM 1592, KCOM 1593, KCOM 1594, KCOM 1595, KCOM 1596, KCOM 1597, KCOM 1598, KCOM 1599, KCOM 1600, KCOM 1601, KCOM 1602, KCOM 1603, KCOM 1604, KCOM 1605, and KCOM 1606) were obtained from the Korean Collection for Oral Microbiology (KCOM, Korea).

S. aureus strains from Koreans were isolated using MRSA-selective media (Winstanley *et al*., 1993). All strains were cultured in Mueller Hinton (MH, Gellix^{TM}, Korea) broth or on agar plates in a 37℃ incubator.

Bacterial genomes were prepared using a G-spin^{TM} Genomic DNA Extraction kit (iNtRON Co., Korea), according to the manufacturer's instructions. DNA concentrations were determined by measuring optical density at 260 and 280 nm using UV-spectrophotometry (Ultrospec 2000, Pharmacia Biotech., UK).

The *mecA* gene from *S. aureus* KCTC 1621^T and 19 strains of MRSA was detected by polymerase chain reaction (PCR) using the primers mecA1 (5′-AAA ATC GAT GGT AAA GGT TGG C-3′) and mecA2 (5′-AGT TCT GCA GTA CCG GAT TTG C-3′) (Murakami *et al*., 1991). PCR was carried out using an *AccuPower*® PCR PreMix (Bioneer, Korea) containing 5 nmole of deoxynucleoside triphosphate, 0.8 μmoles of KCl, 0.2 μmoles of Tris-HCl (pH 9.0), 0.03 μmoles of $MgCl_2$, and 1 unit of *Taq* DNA polymerase. Bacterial genomic DNA and 20 pmoles of each primer were added to a PCR PreMix tube. PCR was carried out in a final volume of 20 μl, and was run for 30 cycles on a Peltier thermal cycler (Model PTC-200 DNA Engine^{TM}, MJ Research Inc., USA) under the following conditions: denaturation at 94℃ for 30 sec, primer annealing at 55℃ for 30 sec, and extension at 72℃ for 30 sec. The final cycle included an additional 5 min extension at 72℃. A 4 μl aliquot of the reaction mixture was analyzed by 1.5% agarose gel electrophoresis in Tris-acetate buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) at 100 V for 30 min. The amplification products were stained with ethidium bromide and visualized by UV transillumination.

Minimum inhibitory concentration (MIC) was determined using a microdilution assay according to the Clinical and Laboratory Standards Institute standard (NCCLS, 2000). The bacterial strains were cultured in MH broth at 37℃ in an incubator for 24 h and added to a 96-well plate to a final concentration of 1×10^6 CFU/ml. UA (Sigma, USA) or OA (Sigma) solutions were added to each well to final concentrations of 1, 2, 4, 8, 16, 32, 64, 128, and 256 μg/ml. UA and OA were dissolved in dimethyl sulfoxide (DMSO, Sigma). The final DMSO concentration in each well was 1%. The 1% DMSO in the medium well was used as the negative control. Ampicillin (Sigma, final concentration of 100 μg/ml) was used as a positive control. After 24 h incubation under the appropriate conditions, the lowest concentration of UA that inhibited visible growth was considered the MIC value. A 10 μl aliquot of bacterial culture solution from each well was diluted 100- or 10,000-fold and plated onto an appropriate agar plate for each strain to obtain the minimum bactericidal concentration (MBC) values. The agar plate was incubated in a 37℃ incubator for 24 h, and the number of bacterial colonies was counted. The concentration when 99.9% of the colonies were killed was considered the MBC value.

The MRSA strains used in this study were isolated from MRSA-selective medium (Winstanley *et al*., 1993) and deposited at the KCOM without genetic information about the existence of the methicillin-resistance gene. MRSA is capable of producing a penicillin-binding protein, encoded by *mecA*, which has relatively low affinity for most 3-lactam antibiotics (Ubukata *et al*., 1989). Therefore, PCR was performed to determine if the MRSA strains had the *mecA* gene. The data showed that the *mecA* gene was detected from all MRSA strains (Fig. 1).

The data showed that the methicillin-sensitive strain *S.*

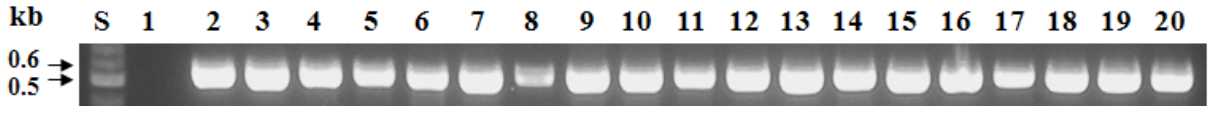

Fig. 1. Detection of the *mecA* gene from each methicillin-resistant *S. aureus* by PCR. The PCR reaction products were electrophoresed on 1.5% agarose gels. Lanes: S, size marker (100 bp ladder); 1, *S. aureus* KCTC 1621^T; 2, *S. aureus* KCOM 1588; 3, *S. aureus* KCOM 1589; 4, *S. aureus* KCOM 1590; 5, *S. aureus* KCOM 1591; 6, *S. aureus* KCOM 1592; 7, *S. aureus* KCOM 1593; 8, *S. aureus* KCOM 1594; 9, *S. aureus* KCOM 1595; 10, *S. aureus* KCOM 1596; 11, *S. aureus* KCOM 1597; 12, *S. aureus* KCOM 1598; 13, *S. aureus* KCOM 1599; 14, *S. aureus* KCOM 1600; 15, *S. aureus* KCOM 1601; 16, *S. aureus* KCOM 1602; 17, *S. aureus* KCOM 1603; 18, *S. aureus* KCOM 1604; 19, *S. aureus* KCOM 1605; 20, *S. aureus* KCOM 1606.

Table 1. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) value of each methicillin-resistant *S. aureus* strain used in this study for oleanolic acid and ursolic acid

Strains	Oleanolic acid (μg/ml)	Ursolic acid (μg/ml)
	MIC/ MBC	MIC/ MBC
S. aureus KCTC[a] 1621[T]	32/ >256	8/ 32
S. aureus KCOM 1588	128/ >256	4/ 16
S. aureus KCOM 1589	64/ 128	8/ 32
S. aureus KCOM 1590	16/ 32	4/ 4
S. aureus KCOM 1591	32/ 64	4/ 8
S. aureus KCOM 1592	32/ 64	4/ 32
S. aureus KCOM 1593	16/ 16	4/ 8
S. aureus KCOM 1594	16/ 32	4/ 4
S. aureus KCOM 1595	16/ 16	4/ 4
S. aureus KCOM 1596	32/ >256	8/ 16
S. aureus KCOM 1597	16/ 64	4/ 4
S. aureus KCOM 1598	16/ 128	4/ 16
S. aureus KCOM 1599	16/ 32	8/ 8
S. aureus KCOM 1600	16/ 64	8/ 8
S. aureus KCOM 1601	16/ 64	4/ 8
S. aureus KCOM 1602	16/ 32	4/ 8
S. aureus KCOM 1603	16/ 128	4/ 16
S. aureus KCOM 1604	64/ >256	4/ 8
S. aureus KCOM 1605	64/ >256	4/ 16
S. aureus KCOM 1606	16/ 64	4/ 16

[a] Korean Collection for Oral Microbiology

aureus KCTC 1621[T] had more resistance than that of the MRSA strains used in this study (Table 1), which agreed with a previous report (Horiuchi *et al*., 2007). In addition, vancomycin-resistant enterococci and MRSA strains are sensitive to UA and OA (Horiuchi *et al*., 2007). In particular, UA had a bactericidal effect against all *S. aureus* strains used in this study. In contrast, methicillin-sensitive *S. aureus* strains are sensitive to UA and OA but MRSA strains are resistant to UA and OA (Fontanay *et al*., 2008). Our data showed that the antimicrobial effect of UA against the MRSA strains was stronger than that of OA (Table 1). This result coincided with previous reports that UA has a stronger antimicrobial effect against mutans streptococci (Kim *et al*., 2010, 2011). The MIC_{90} values of UA and OA against 55 strains of mutans streptococci are 8 μg/ml and 2 μg/ml (Kim *et al*., 2010, 2011). It was difficult to understand the strain-dependent antimicrobial activity of UA and OA, because the antimicrobial UA and OA mechanisms are unknown. These results indicate that the antimicrobial effects of UA and OA against MRSA are dependent on UA and OA resistance of each strain.

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적 요

Ursolic acid (UA)와 oleanolic acid (OA)들의 메티실린 저항성 *Staphylococcus aureus* (MRSA)에 대한 항균 활성에는 상반된 의견들이 있다. 본 연구는 한국인으로부터 분리된 19개의 MRSA에 대한 UA와 OA의 항균 활성을 최소성장억제농도 및 최소살균농도를 측정하여 조사하였다. 연구 결과, 메티실린 감수성 균주인 *S. aureus* KCTC 1621[T]가 MRSA 균주들보다 UA와 OA에 대한 저항성이 컸다. UA와 OA 각각의 MRSA 19 균주에 대한 최소살균농도는 4–32 μg/ml와 16->256 μg/ml로 넓은 범위를 보였다. UA와 OA에 대한 균주에 따른 항균 작용의 차이는 UA와 OA의 항균 기전이 밝혀져 있지 않기 때문에 이해하기 힘들다. 이러한 결과들은 MRSA에 대한 UA와 OA의 항균 효과는 균주들 간의 UA와 OA에 대한 저항 능력에 의한 것임을 시사한다.

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