Antibacterial Activity of Curcuma longa against Methicillin-resistant Staphylococcus aureus

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Methicillin-resistant Staphylococcus aureus (MRSA) has been emerging worldwide as one of the most important hospital and community pathogens. Therefore, new agents are needed to treat the MRSA. In the present study, we investigated antimicrobial activity of ethyl acetate, methanol, and water extracts of Curcuma longa L. (C. longa) aganist clinical isolates of MRSA. The ethyl acetate extract of C. longa demonstrated a higher antibacterial activity than the methanol extract or water extract. Since the ethyl acetate extract was more active than other extracts, we examined whether ethyl acetate extract may restore the antibacterial activity of β-lactams and alter the adhesion and invasion of MRSA to human mucosal fibroblasts (HMFs). In the checkerboard test, ethyl acetate extract of C. longa markedly lowered the MICs of ampicillin and oxacillin against MRSA. In the bacterial adhesion and invasion assay, MRSA intracellular invasion were notably decreased in the presence of 0.125 - 2 mg/ml of C. longa extract compared to the control group. These results suggest that ethyl acetate extract of C. longa may have antibacterial activity and the potential to restore the effectiveness of β-lactams against MRSA, and inhibit the MRSA adhesion and invasion to HMFs.

Key words : *Curcuma longa*, antibacterial activity, methicillin-resistant Staphylococcus aureus

Introduction

Staphylococcus aureus (S. aureus) is one of the most important pathogens that cause suppuration, abscess formation, a variety of pyogenic infection, and even fatal septicemia in human beings. S. aureus, which can induce bacteremia (associated with 80% mortality in the preantibiotic era), proved to be susceptible to the earliest antimicrobial substance; however, as antibiotic use increased, staphylococcal resistance rapidly developed[1,2]. Methicillin-resistant Staphylococcus aureus (MRSA), resistance of which was due to penicillin-binding protein (PBP) 2′ production, was isolated in the early 1960s[3]. MRSA is resistant to not only methicillin and other β -lactams but also to many other antibacterial agents. Since MRSA exhibits multidrug resistance, it has been emerging worldwide as one of the most important hospital and community pathogens. Therefore, new agents are needed to treat the MRSA. Some natural products are candidates of new antibiotic substances. In the course of screening for antibacterial activities of some natural products, we recently found that extracts of Curcuma longa L. (C. longa) has antibacterial activity against MRSA.

C. longa, popularly known as tumeric, has long been used as a spice in Southeast Asia. It has also been used in Oriental folk medicines to treat infectious diseases such as sinusitis, cough, cholecystitis, and cholangitis, and used as a therapy for hepatic disorders, rheumatism, and anorexia[4,5]. However, little is known about the antimicrobial effects of C. longa on MRSA. In the present study, we show that C. longa has antimicrobial activity against MRSA and lowers the MICs of β -lactams. In addition, we report that C. longa inhibits the adhesion and invasion of MRSA to human mucosal fibroblasts (HMFs).

Materials and Methods

1. Plant material

C. longa was obtained from the Korea Oriental Medical Herb Association (Seoul, South Korea). The identity was confirmed by Dr. Bong-Seop Kil at the Department of Natural Science, Wonkwang University. Voucher specimen (number 7-00-12) has been deposited at the Herbarium of Department of Oral Biochemistry in Wonkwang University. Dried rhizomes of C. longa (100 g) were grinded to powder and extracted with

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ethyl acetate, methanol, or water respectively. The powder was soaked separately in 1 L of ethyl acetate or methanol for 72 h at room temperature. For hot water extraction, 250 g of powdered sample were boiled in 2 L of water for 60 min. filtration of the extracted solution and evaporation under reduced pressure yielded ethyl acetate (8.16 g), methanol (8.26 g), and water extracts (4.28 g).

2. Bacterial strains

Staphylococcal stains listed in Table 1 were 13 clinical isolates (MRSA) from Wonkwang University Hospital and Seoul National University Hospital, and the standard strain of S. aureus ATCC 25923, which is methicillin-sensitive S. aureus (MSSA). Antibiotic susceptibility was determined from the size of the inhibition zone, in accord with guidelines of the National Committee for Clinical Laboratory Standards[6], and the used strains were defined as MRSA based on occurrence of the mecA gene and their resistance to oxacillin[7] β-Lactamase activity was also determined using the DrySlide Beta Lactamase test (Difco Laboratories, Detroit, MI, USA) according to manufacturer's specification. After culturing on Mueller-Hinton agar (Difco Laboratories,), the bacteria were suspended in Mueller-Hinton broth (Difco Laboratories) and used for inoculation.

Table 1. Bacterial strains used in the experiments

Strains	Class	mecA gene	β-lactamase activity	Antibiotic resistance pattern
S. aureus (ATCC 25923)	MSSA	-	-	-
S. aureus (OMS 1)	MRSA	+	+	AM, OX, ME, GE
S. aureus (OMS 2)	MRSA	+	+	AM, OX, ME, E, GE
S. aureus (OMS 3)	MRSA	+	+	AM, OX, ME, E, GE
S. aureus (OMS 4)	MRSA	+	+	AM, OX, ME, E, GE
S. aureus (OMS 5)	MRSA	+	+	AM, OX, ME, E, GE
S. aureus (OMS 6)	MRSA	+	+	AM, OX, ME, E, GE
S. aureus (OMS 7)	MRSA	+	+	AM, OX, ME, E, GE
S. aureus (OMS 8)	MRSA	+	+	AM, OX, ME, E, GE
S. aureus (OMS 9)	MRSA	+	+	AM, OX, ME, E, GE
S. aureus (OMS 10)	MRSA	+	-	AM, OX, ME, E, GE
S. aureus (OMS 11)	MRSA	+	+	AM, OX, ME, GE
S. aureus (OMS 12)	MRSA	+	+	AM, OX, ME, GE
S. aureus (OMS 13)	MRSA	+	-	AM, OX, ME, GE

+, positive; -, negative; AM, ampicillin; OX, oxacillin; ME, methicillin; E, erythromycin; C c hloramphenicol; VA, vancomycin; GE, gentamycin

3. Detection of mecA gene

Detection of the mecA gene in the strains of MRSA was performed by PCR amplication. Total genomic DNA was obtained from S. aureus by the phenol chloroform extraction method as described earlier in the previous report[8]. Bacteria collected from 5 ml of the 18 h culture in Mueller-Hinton broth were used for DNA extraction after treatment with lysostaphin and RNase (Sigma, St Louis, MO, USA). The PCR assay was performed in a DNA thermal cycler, GeneAmp PCR system 9700 (PE Applied Biosystems, Mississauga, Ontario, Canada), by using a Gene Taq Amplifying Kit (Wako Pure Chemicals Industries, Ltd., Japan), according to the manufacturer's recommendations. Synthetic oligonucleotides used as primers were 5′-ATGAGATTAGGCATCGTTCC-3′ and 5′-TGGATGACAGTACCTGAGCC-3′[9].

4. Disk diffusion method

The paper disc diffusion method was used to determine antibacterial activity, which is based on the method described previously[10]. Sterile paper discs (6 mm; Toyo Roshi Kaihsa, Japan) were loaded with 50 μl of different amount (0.25, 0.5, and 1 mg) of the extracts and were left to dry for 12 h at 37 ℃ in a sterile room. After Mueller-Hinton agar was poured into Petri dishes to give a solid plate and inoculated with bacteria, the discs treated with extracts were applied to Petri dishes. Ampicillin and oxacillin were used as positive controls and paper discs treated with vehicle were used as negative control. The plates were then incubated at 35 ℃ for 24 h in a incubator (Vision Co, Seoul, Korea). Inhibition zone diameters around each of the disc were measured and recorded at the end of the incubation time.

5. Determination of minimum inhibitory concentrations (MICs)

MICs were determined by the agar dilution method, which is based on the method described previously[11]. MICs of ampicillin and oxacillin were also determined. A final inoculum of 10^4 CFU/ml was spotted with a multipoint inoculator (Denley Instruments, Sussex, UK) onto agar plates. The plates were then incubated at 35℃ for 24 h in the incubator (Vision Co, Seoul, Korea). The MICs was defined as the lowest concentration at which no visible growth was observed.

6. Checherboard dilution test

The antibacterial effects of combination of ethyl acetate extract of C. longa, which exhibited highest antimicrobial activity, and β-lactams were assessed by the checkerboard test as previously described[11]. The antimicrobial combinations assayed included C. longa extract plus amoxicillin and C. longa extract plus oxacillin. Serial dilutions of two different antimicrobial agents were mixed in cation-supplemented Mueller-Hinton broth. Inocula were prepared from colonies grown on Mueller-Hinton agar after overnight culture. The final bacterial concentration after inoculation was 5×10^5 CFU/ml. After 24 h of incubation at 35 ℃, the MIC was determined to be the minimal concentration at which there was no visible growth.

7. Bacterial adhesion and invasion assay

Bacterial adhesion and invasion to host cells and tissues are the one of the important pathogenic mechanisms in oral infection[12)]. To investigate the inhibitory effect of ethyl acetate extract of C. longa, which exhibited highest antimicrobial activity, on bacterial adhesion and invasion to cultured monolayers of HMFs, the previous methods were used with a slight modification[13)]. HMFs were obtained from the patients undergoing oral surgery. After 3 or 5 passages, the cells were used in this study. The HMFs were grown routinely in monolayers in α-minimum essential medium (α-MEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin G and 100 μg/ml streptomycin sulphate. Before use, cells were seeded at 5×10^4 cells/well in 24-well tissue culture plates (Costar, Cambridge, MA, USA) for adhesion and invasion assays and grown into confluent monolayers for 1 day in air at 37 ℃ in 5% CO_2 incubator (Vision Co, Seoul, Korea). The number of bacteria attached to HMFs was quantified by determining the number of recovered bacterial colonies after co-culture. Approximately 16 h prior to the experiments, HMFs were washed three times with invasion medium (growth medium without antibiotics) and held in this medium. Just before beginning the experiment, the medium was removed and the HMFs were washed once with invasion medium followed by a further addition of 1 ml of fresh invasion medium. Appropriate wells of HMFs were inoculated with 1 ml of invasion medium containing 0.125, 0.25, 0.5, or 1 mg/ml of C. longa extract and 10^5 CFU/ml/well of bacteria for the specified times at 37 ℃ in air with 5% CO_2. Subsequently, the medium was removed from the infected monolayers, before washing three times with sterile Ca^{2+}- and Mg^{2+}-free PBS to remove non-adherent bacteria. The HMFs were treated with 0.25% trypsin in Hanks' balanced salt solution (Gibco BRL) and further lysed with 0.025% Triton X-100 (Sigma, St. Louis, MO, USA) in sterile distilled water. Cell lysates were serially diluted 20-fold and plated in triplicate on blood agar plates; the plates were then incubated overnight at 37 ℃ and the CFU were counted. At this time, colonies of S. aureus were identified by Gram stain, catalase and coagulase tests.

8. Phytochemical screening

Phytochemical tests of extracts were performed as previously described[14,15)]. Mayer's reagent was used for alkaloids, Ferric chloride reagent for phenolics, Molish test for glycosides, Biuret reagent for proteins, Mg-HCl reagent for flavonoids, Libermann-Buchard reagent for steroids, silver nitrate reagent for organic acids.

9. Statistical analysis

Data analyzed using the statistical package for social sciences (SPSS). Differences between means of the experimental and control groups were evaluated by Student's t-test.

Results

Table 2 shows the antimicrobial activity of C. longa extracts determined by the disc diffusion method. The MICs for ethyl acetate extract, methanol extract, and water extract of C. longa against 13 strains of MRSA and 1 standard strain of MSSA are also determined (Table 3).

Table 2. Antimicrobial activity (mm inhibition zones diameter) of ethyl acetate extract, methanol extract, and water extract of C. longa against 13 MRSA and 1 standard MSSA.

Strains	Zone of inhibition (mm)					
	Ethyl acetate(mg)			Methanol (mg)		
	0.25	0.5	1	0.25	0.5	1
S. aureus (ATCC 25923)	ND	11	14	ND	ND	12
S. aureus (OMS 1)	ND	11	15	ND	10	12
S. aureus (OMS 2)	10	12	14	ND	10	12
S. aureus (OMS 3)	ND	13	15	ND	ND	10
S. aureus (OMS 4)	ND	13	16	ND	ND	13
S. aureus (OMS 5)	ND	11	14	ND	ND	11
S. aureus (OMS 6)	ND	12	15	ND	10	14
S. aureus (OMS 7)	ND	12	15	ND	ND	12
S. aureus (OMS 8)	11	12	14	ND	ND	10
S. aureus (OMS 9)	12	14	17	ND	11	15
S. aureus (OMS 10)	9	12	15	ND	9	14
S. aureus (OMS 11)	ND	10	13	ND	ND	12
S. aureus (OMS 12)	ND	10	13	ND	ND	10
S. aureus (OMS 13)	12	14	10	ND	12	15

Strains	Zone of inhibition (mm)				
	Water (mg)			Ampicillin	Oxacillin
	0.25	0.5	1	10 μg	1 μg
S. aureus (ATCC 25923)	ND	ND	7	34	24
S. aureus (OMS 1)	ND	ND	ND	12	9
S. aureus (OMS 2)	ND	ND	8	11	ND
S. aureus (OMS 3)	ND	ND	7	11	ND
S. aureus (OMS 4)	ND	ND	7	11	ND
S. aureus (OMS 5)	ND	ND	8	11	ND
S. aureus (OMS 6)	ND	ND	9	10	ND
S. aureus (OMS 7)	ND	ND	7	8	ND
S. aureus (OMS 8)	ND	ND	7	10	ND
S. aureus (OMS 9)	ND	ND	7	10	ND
S. aureus (OMS 10)	ND	ND	7	17	ND
S. aureus (OMS 11)	ND	ND	8	11	ND
S. aureus (OMS 12)	ND	ND	8	11	ND
S. aureus (OMS 13)	ND	ND	9	15	ND

ND, no detected activity at this concentration, Ampicillin: R ≤ 28 mm, Oxacillin: R ≤ 10 mm

Table 3. MICs of the ethyl acetate, methanol, and water extracts of C. longa against 13 MRSA and 1 standard MSSA.

Strains	Class	MIC				
		C. longa (mg/ml)			Ampicillin (µg/ml)	Oxacillin (µg/ml)
		Ethyl acetate	Methanol	Water		
S. aureus (ATCC 25923)	MSSA	2	8	64	0.125	0.031
S. aureus (OMS 1)	MRSA	2	4	64	32	8
S. aureus (OMS 2)	MRSA	4	4	64	32	4
S. aureus (OMS 3)	MRSA	4	4	64	64	4
S. aureus (OMS 4)	MRSA	2	4	64	64	4
S. aureus (OMS 5)	MRSA	4	4	64	32	4
S. aureus (OMS 6)	MRSA	4	4	64	64	16
S. aureus (OMS 7)	MRSA	4	4	64	64	16
S. aureus (OMS 8)	MRSA	2	8	64	32	8
S. aureus (OMS 9)	MRSA	1	4	64	64	8
S. aureus (OMS 10)	MRSA	1	4	64	4	4
S. aureus (OMS 11)	MRSA	4	4	32	64	16
S. aureus (OMS 12)	MRSA	4	4	16	64	4
S. aureus (OMS 13)	MRSA	1	2	16	4	4

Ampicillin: R ≥ 4µg/ml, Oxacillin: R ≥ 4µg/ml

The determination of the inhibition zones by the disc diffusion method revealed antimicrobial activity of the C. longa against MRSA as well as the standard MSSA, and these results were confirmed by the data expressed as MIC in the agar dilution method. The ethyl acetate extract of C. longa demonstrated a higher inhibitory activity (MIC: 2 mg/ml) than methanol extract (MIC: 8 mg/ml) and water extract (MIC: 64 mg/ml) in the standard MSSA. MICs of ampicillin and oxacillin against standard MSSA were 0.125 and 0.031 µg/ml, respectively. The MICs of MRSA were similar to the standard MSSA. MIC_{50} of ethyl acetate extract, methanol extract, and water extract were 2 mg/ml, 4 mg/ml, and 64 mg/ml respectively. The ethyl acetate extract of C. longa demonstrated a higher bacteriostatic activity than other extracts. The MIC_{90} and MIC range of ethyl acetate extract are 4 mg/ml and 1 - 4 mg/ml.

Table 4. MICs for β-lactams used in combination with ethyl acetate extract of C. longa against MRSA or the standard MSSA.

Strain	MIC of AM (µg/ml)				MIC of OX (µg/ml)			
	C. longa (mg/ml)				C. longa (mg/ml)			
	0	0.25	0.5	1	0	0.25	0.5	1
S. aureus (ATCC 25923)	0.125	0.031	0.016	0.008	0.031	0.031	0.016	0.008
S. aureus (OMS 1)	32	16	16	8	8	4	4	4
S. aureus (OMS 2)	32	16	16	16	4	2	2	2
S. aureus (OMS 3)	64	32	32	16	4	2	2	1
S. aureus (OMS 4)	64	32	32	16	4	2	2	1
S. aureus (OMS 5)	32	16	8	8	4	2	1	1
S. aureus (OMS 6)	64	64	32	32	16	8	8	8
S. aureus (OMS 7)	64	32	16	4	16	8	4	4
S. aureus (OMS 8)	32	16	16	8	8	4	4	2
S. aureus (OMS 9)	64	32	4	0	8	4	2	0
S. aureus (OMS 10)	4	2	1	0	4	2	1	0
S. aureus (OMS 11)	64	64	16	16	16	16	8	4
S. aureus (OMS 12)	64	64	32	32	4	4	2	2
S. aureus (OMS 13)	4	2	1	0	4	2	1	0

Following the determination of MIC values for MRSA or the standard MSSA, we examined whether ethyl acetate extract of C. longa, which exhibited highest antimicrobial activity, may lower the MICs of β-lactams by checkerboard dilution method. The results of the checkerboard dilution test for MRSA and the standard MSSA are shown in Table 4. ethyl acetate extract of C. longa markedly lowered the MICs of ampicillin and oxacillin against MRSA and a standard MSSA (Table 4).

To determine whether ethyl acetate extract of C. longa extract, which exhibited highest antimicrobial activity, inhibits the MRSA adhesion and invasion to HMFs, The cells were treated with various concentration of ethyl acetate extract of C. longa, and bacterial adhesion and invasion were assayed. The effect of various concentration of C. longa extract on MRSA adhesion and invasion to HMFs was presented in Fig. 1. MRSA adhesion and intracellular invasion were notably decreased in the presence of 0.125 - 1 mg/ml of ethyl acetate extract of C. longa extract compared to the control group. The effect of C. longa extract on MRSA adhesion and invasion appeared dose dependent. These finding suggest that ethyl acetate extract of C. longa may inhibit the MRSA adhesion and invasion to HMFs. The results of the phytochemical tests for ethyl acetate, methanol, and water extracts are shown in Table 5. The ethyl acetate and methanol extracts gave positive test for phenolics, flavonoids, glycosides, and steroids. The water extract gave positive test for proteins.

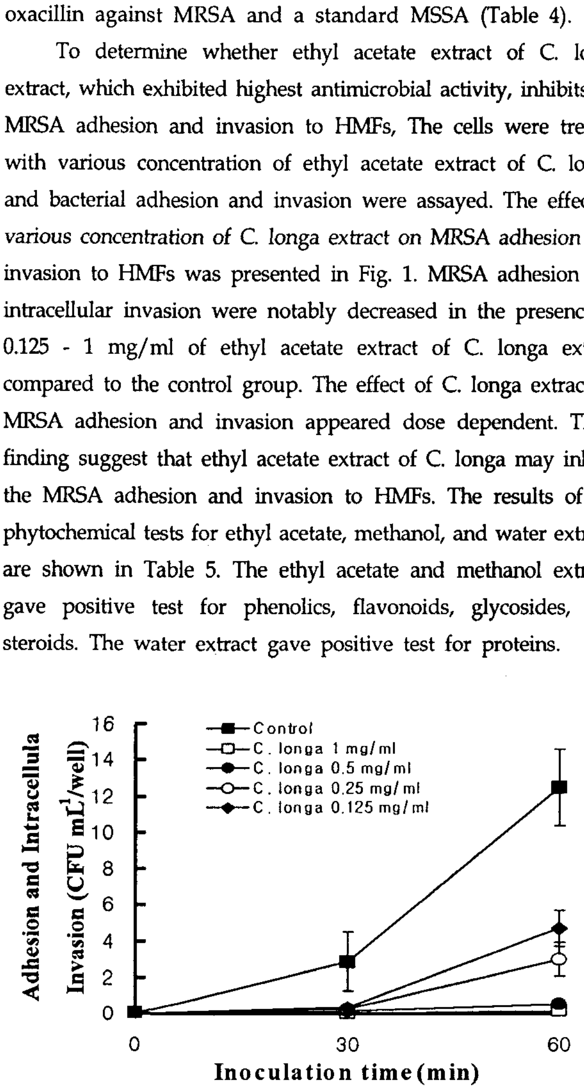

Fig. 1. Effect of C. longa extract on adhesion and invasion MRSA to HMFs. Cells were infected with S. aureus (OMS 7) in the different sub-MIC concentrations of C. longa extract for several time periods, followed by measurement of CFU recovered from cell monolayers. Each point represents means±standard errors.

Table 5. Phytochemical analysis of Curcuma longa extracts

Plant constituents	Ethyl acetate	Methanol	Water
Alkaloids	-	-	-
Phenolics	+ + +	+ +	-
Flavonoids	+ + +	+ +	-
Glycosides	+ +	+ + +	-
Proteins	-	-	+ + +
Steroids	+ +	+ + +	-
Organic acids	-	-	-

+ + + strong, + + medium, + poor presence, - absence.

Discussion

In the present study, we investigated antimicrobial activity of C. longa extract aganist clinical isolates of MRSA and effect of C. longa extract on the adhesion and invasion of MRSA to HMFs. Our results indicate that C. longa extract showed antimicrobial activity against all tested strains of MRSA and a standard MSSA (Table 2). The fact that the extracts of C. longa inhibited growth of S. aureus provides some scientific rationales that the local inhabitants used the extracts for antimicrobial agents. Although all fraction of C. longa produced some inhibitory activities against MRSA and standard MSSA, ethyl acetate extract exerted more inhibitory activity than methanol extract and water extract. These results suggest that ethyl acetate would be a better solvent than methanol or water in an attempt to isolate the antibacterial principles. In the previous studies curcumin, demethoxycurcumin, bisdemethoxycurcumin, oleoresin and essental oils were isolated in the C. longa[16,17]. The essential oil(5.8%), obtainable by steam distillation of the rhizomes, has the following constituents ; α-phellandrene 1%, sabinene 0.6%, cineol 1%, borneol 0.5%, zingiberene 25%, and sesquiterpenes 53%5). In our investigation, we found that ethyl acetate and methanol extracts contains flavonoids. Since several reports have shown that some compounds belonging to flavonoids have antibacterial activity[18], we consider that flavonoids in the C. longa may, in part, be related to the antibacterial effects in the present study. However further studies are needed to elucidate the antimicrobial mechanism in the extracts of C. longa.

Several mechanisms are known by which microorganisms can overcome the toxicity of antimicrobial agents. These include the production of drug insensitive enzymes, modification of targets for drug, and extrusion of drugs from bacterial cells by multidrug resistance (MDR) pump. It seems that genes responsible for MDR are present mainly in the mec region of the MRSA chromosome and several other genes like fem, llm and sigB are also involved[19]. In the present study, the MICs of C. longa extract againt MRSA were not higher than standard MSSA. These data show that the tested stains of MRSA in this experiment may not have resistance against C. longa extract, although a lot of MRSA have the multidrug resistance[19]. Since recent reports showed that some natural products may lower the MIC of β-lactams[19,20], we examined whether ethyl acetate extract of C. longa, which exhibited highest antimicrobial activity, may lower the MICs of β-lactams by the checkerboard dilution method. As expected, C. longa extract markedly lowered the MICs of ampicillin and oxacillin against MRSA. To our knowledge, this is the first report that C. longa extract lowered the MICs of β-lactam antibiotics. However, it is not clear yet how C. longa extract enhances the antibacterial activity of β-lactam antibiotics against MRSA. Recent reports show that some medical plants have MDR pump inhibitors, which may lower the MIC of antimicrobial agents[21]. Futher studies are needed to elucidate whether C. longa extract may have the MDR pump inhibitors.

Since bacterial adhesion and invasion into cells and tissues are the one of the important pathogenic mechanisms in oral infection[11], we examined whether C. longa extract could affect the adhesion and intracellular invasion of MRSA to HMFs. Surprisingly, C. longa extract inhibited the MRSA adhesion and invasion to HMFs. In the adhesion and invasion mechanism of Staphylococcus aureus, Staphylococcal protein A (SPA) may be important roles[12]. Additional experiments are required to determine the possibility of inhibition of SPA role by C. longa extract.

In conclusion, the results obtained suggest that C. longa extract may have antimicrobial activity and lowered the MICs of β-lactam antibiotics against MRSA, and inhibit the MRSA adhesion and invasion to HMFs.

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References

1. Bramley, A.J., Patel, A.H., O'Reilly, M., Foster, R., Foster, T.J. Roles of alpha-toxin and beta-toxin in virulence of Staphylococcus aureus for the mouse mammary gland. Infect Immun 57, 2489-2494, 1989.
2. You, Y.O., Kim, K.J., Min, B.M., Chung, C.P. Staphylococcus lugdunensis- a potential pathogen in oral infection. Oral Surg Oral Medicine Oral Pathol Oral Radiol Endod 88, 297-302, 1999.
3. Tsuchiya, H., Sato, M., Miyazaki, T., Fujiwara, S., Tanigaki, S,, Ohyama, M., Tanaka, T., Iinuma, M. Comparative study on the antibact erial activity of phytochemical flavanones against methicillin-resistant Staphylococcus aureus. J Ethnopharmacol 50, 27-34, 1996.
4. Kim, J.K. Illustrated natural drugs encyclopedia. p 387, Namsandang Publishers, Seoul, Korea, 1989.
5. Eigner, D., Scholz, D. Ferula asa-foetida and Curcuma longa in traditional medical treatment and diet in Nepal. J Ethnopharmacol 67, 1-6, 1999.

6. National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 4th ed. Approved Standard. NCCLS document M7-A4. Villanova (PA): National Committee for Clinical Laboratory Standards. 1997.
7. Wallet, F., Roussel-Delvallez, M., Courcol, R.J. Choice of a routine method for detecting methicillin-resistance in staphylococci. J Antimicrob Chemother 37, 901-909, 1996.
8. Tsen, H.Y., Chen, T.R. Use of the polymerase chain reaction for specific detection of type A, D and E enterotoxigenic Staphylococcus aureus in foods. Applied Microbiol Biotechnol 37, 685-690, 1992.
9. Ryffel, C., Tesch, W., Birch-Machin, I., Reynolds, P.E., Barberis-Maino, L., Kayser, F.H., Berger-Bachi, B. Sequence comparison of mecA genes isolated from methicillin-resistant Staphylococcus aureus and Staphylococcus epidermidis. Gene 94, 137-138, 1990.
10. Ali, N.A., Julich, W.D., Kusnick, C., Lindequist, U. Screening of Yemeni medicinal plants for antibacterial and cytotoxic activities. Journal of Ethnopharmacology 74, 173-179. 2001.
11. Chang, S.C., Chen, Y.C., Luh, K.T. Hsieh, W.C. In vitro activities of antimicrobial agents, alone and in combination, against Acinetobacter baumannii isolated from blood. Diagnostic Microbiol Infect Dis 23, 105-110, 1995.
12. Schuster, G.S., Burnett, G.W. The microbiology of oral and maxillofacial infections. In: Management of infections of the oral and maxillofacial regions. p 457-472, Saunders, Philadelphia, U.S.A., 1981.
13. Jung, K.Y., Cha, J.D., Lee, S.H., Woo, W.H., Lim, D.S., Choi, B.K., Kim, K.J. Involvement of staphylococcal protein A and cytoskeletal actin in Staphylococcus aureus invasion of cultured human oral epithelial cells. J Med Microbiol 50, 35-41, 2001
14. Houghton, P.J., Raman, A. Laboratory handbook for the fractionation of natural extracts. p 154-185, Chapman & Hall, London, U.K., 1998.
15. Woo, W.S. Experimental methods for phytochemistry, p 1-62, Seoul National University Press, Seoul, Korea, 2001.
16. Apisariyakul, A., Vanittanakom, N., Buddhasukh, D. Antifungal activity of turmeric oil extracted from Curcuma longa (Zingiberaceae). J Ethnopharmacol 49, 163-169, 1995.
17. Song, E.K., Cho, H., Kim, J.S., Kim, N.Y., An, N.H., Kim, J.A., Lee, S.H., Kim, Y.C. Diarylheptanoids with free radical scavenging and hepatoprotective activity in vitro from curcuma longa. Planta Med 67, 876-877, 2001.
18. Sato, Y., Suzaki, S., Nishikawa, T., Kihara, M., Shibata, H., Higuti, T., Phytochemical flavones isolated from Scutellaria barbata and antibacterial activity against methicillin-resistant Staphylococcus a ureus. Journal of Ethnopharmacology 72, 484-488. 2000.
19. Shiota, S., Shimizu, M., Mizushima, T., Ito, H., Hatano, T., Yoshida, T., Tsuchiya, T. Marked reduction in the minimum inhibitory concentration (MIC) of beta-lactams in methicillin-resistant Staphylococcus aureus produced by epicatechin gallate, an ingredient of green tea (Camellia sinensis). Biol Pharm Bull 22, 1388-1390, 1999.
20. Liu, I.X., Durham, D.G., Michael, E., Richards, E. Baicalin synergy with β-lactam antibiotics against methicillin-resistant Staphylococcus aureus and other β-lactam-resistant strain of S. aureus. J Pharm Pharmacol 52, 361-366, 2000.
21. Stermitz, F.R., Lorenz, P., Tawara, J.N., Zenewicz, L.A., Lewis, K. Synergy in a medicinal plant: antimicrobial action of berberine potentiated by 5′-methoxyhydnocarpin, a multidrug pump inhibitor. Proc Natl Acad Sci U S A 97, 1433-1437, 2000.