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Antimicrobial Activity of Lemongrass and Oregano essential oil against standard antibiotic resistant *Staphylococcus aureus* and field isolates from chronic mastitis cow

Jae-Young Choi[1], Dereje Damte[1], Seung-Jin Lee[1], Jong-Choon Kim[2], Seung-Chun Park[1*]

*Corresponding author:

Seung-Chun Park

[1]College of Veterinary Medicine, Kyungpook National University, Daegu 702-701, South Korea
[2] College of Veterinary Medicine, Chonnam National University, Gwangju 500-757, South Korea

Abstract

The study was carried out to investigate the antibacterial activity of lemongrass and Oregano essential oil against standard antibiotic resistant Staphylococcus aureus strains and field isolates from a chronic mastitic cow. Disc diffusion and minimum inhibitory concentration (MIC) assay were used to investigate antibacterial activity of lemongrass and Oregano essential oil while their combined effect with antibiotic were assessed using a microbroth chequerboard technique. For identification of the essential oil component Gas Chromatography Coupled Mass Spectroscopy (GC/MS) analysis was performed. Disc diffusion assay revealed dose-dependent inhibition zone with lemongrass oil showing consistently larger inhibition zone than oregano oil. Lemongrass essential oils were further evaluated for its antibacterial activity in combination with antibiotic which resulted both synergistic and additive interaction. Lemongrass oil-amoxicillin combination showed synergistic interactions against all tested organism while the combination for lemongrass oil-norfloxacin showed synergistic effect for two field isolates and an additive for the remaining strains. The current study indicated that both lemongrass and oregano essential oils exhibit antibacterial activity, and lemongrass oil in combination with antibiotics could be a potential agent for antibacterial treatments against antibiotic resistant bacterial infections.

Key Words: Lemongrass; Oregano essential oil, Resistant, S. aureus, antibiotics.

Introduction

Plant-derived essential oils have long been used as flavoring agents in food and beverages. Due to the presence of antimicrobial ingredients, they have potential use as natural agents for food preservation [1] and as antibiotic in various ways [2]. With the misuse and overuse of antibiotics to treat diseases, antibacterial resistance to the drugs has begun to appear and has become more serious because of selective pressure [3]. Staphylococcus aureus, one of the etiologic agents of bovine mastitis, has been reported several times as resistant pathogen against various antibiotics [4, 5, 6]. Antibiotic resistance of the methicillin-Resistant Staphylococcus aureus (MRSA) isolated from livestock against several antibiotics has been confirmed [5]. MRSA separated in many countries has wide-range of resistant against chloramphenicol, clindamycin, norfloxacin, ciprofloxacin, erythromycin, gentamicin, linezolid, oxacillin, penicillin, rifampin, trimethoprim/sulfamethoxazole, and tetracycline [7, 8].

Among the different options to tackle antimicrobial resistance, use of essential oil has been an alterative approach and an area of research concern. Many essential oils have been traditionally used by people for various purposes of which cinnamon, clove and rosemary oils had shown antibacterial and antifungal activity [9].

Lemongrass essential oil has been reported for its flavoring, antiseptic and deodorant properties [10] while oregano essential oil exhibit antifungal, antibacterial, and antimicrobial activity, respectively [11].

In the current study, lemongrass and oregano essential oil antibacterial activity against standard antibiotic resistant S. aureus type strains and field isolates S. aureus from chronic bovine mastitis were investigated. Furthermore, the effects of lemongrass essential oil in combination with antibiotics were also evaluated in order to seek effective combinations to treat infections caused by resistant S. aureus strains.

Materials and methods

Standard test microorganism

Standard antibiotic resistant strains of S. aureus (KCCM 11593, KCCM 11812, KCCM 40510, KCCM 40511, KCCM 40881, KCCM 41291, and KCCM 41294) were obtained from Korean Culture Center of Microorganisms (KCCM, http://www.kccm.or.kr). The selected test organisms characteristics are summarized in Table 1. These organisms were pre-incubated overnight with tryptic soy broth (DIFCO, USA) in shaking incubator at 37 .

Sampling and field Isolates microorganisms

Mastitis milk samples were collected from chronic mastitic cows in Kyungsangpook-Do province and immediately transported to the laboratory in ice-cooled containers. Mastitic milk samples of 500 μL were inoculated into 5 mL of Trypticase soy broth (Difco) with NaCl and incubated at 37°C for 20 h with shaking. The inoculums were spread onto agar plates and incubated at 37°C for 24 h. The colonies were tested for S. aureus levels using conventional methods that included Gram staining, colonial morphology, and coagulase assays. Phenotypic methicillin-Resistant of S. aureus was determined by an agar screen test performed according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS) (20, 34) with Mueller-Hinton agar (Difco) containing 4% NaCl and 2, 4, or 8 μg/mL of oxacillin (Sigma, St. Louis, Mo.). Isolated methicillin-Resistant colonies are listed in Table 1. The isolates were stored at -70°C in freezer vials till further analysis.

Table 1. Antibiotic resistance Staphylococcus aureus type strains and field isolates.

Strain	KCCM No.	ATCC No.	Antibiotic resistance
Staphylococcus aureus (Type strains)	11593	12692	Novobiocin
	11812	13301	Penicillin
	40510	33591	Methicillin
	40511	33593	Methicillin and Gentamicin
	40881	29213	(Susceptibility testing organism)
	41291	27659	Tetracycline, novobiocin, streptomycin, macrolides. constitutive resistance to macrolides.
	41294	27660	Resistant to tetracycline and streptomycin. (Sensitive to novobiocin) inducible resistance to macrolides.
	Code No		**Antibiotic resistance**
Isolates*	9-NA-3		Norfloxacin, Amoxicillin
	6-N-2		Norfloxacin
	2-20-2		Amoxicillin
	3-NA-1		Norfloxacin, Amoxicillin
	6-N-1		Norfloxacin
	9-NA-1		Norfloxacin, Amoxicillin

* : Isolates are from bovine mastitis milk samples of Kyungsanpook-Do province. Samples were collected and diagnosted by the inspector, Eun-young Kim of Kyungsangpook-Do Veterinary Service Laboratory.

Essential oils and antibiotics

Lemongrass essential oil, oregano essential oil and solvents (Polysorbate 80 and Polysorbate 20) were supplied from Biomist, Co. Ltd. Korea. The oils were stored in an amber bottle at 4°C until use. Norfloxacin, and amoxicillin were purchased from Becton (Becton Dickinson, Heidelberg, Germany).

Gas Chromatography Coupled Mass Spectroscopy analysis

Gas Chromatography Coupled Mass Spectroscopy (GC/MS) analysis was performed by Kyungpook National University Center for Scientific Instruments. GC/MS was carried out using a HP (Hewlett-Packard) 6890 Plus GC gas chromatograph with (MSD) – HP 5973 MSD mass selective detector. Samples were diluted with HPLC-grade methanol to 1:1000(v/v). Sample portions (1μL) were injected into the HP-5 column. GC oven program was set at 50 °C for 4 min and then raised to 280 °C at a rate of 4 °C/min and held at the final temperature for 2 min. The velocity of Helium carrier gas (99.99%) was 0.7 mL/min. Quantitative analysis is performed with area normalization method.

Disk diffusion assay

To test antimicrobial activity against selected standard antibiotic resistant S. aureus type strains and field isolates from chronic mastitis cow, disk diffusion susceptibility test was performed according to the guideline of NCCLS. Antibiotic resistance S. aureus type strains and field isolates were seeded in 5 mL of Muller-Hinton broth (MHB) (DIFCO, USA) and incubated in 37 shaking incubator overnight. Incubated S. aureus (10^5CFU/mL) were mixed with warmed Muller-Hinton agar (MHA) (DIFCO, USA) and poured into Petri dish and cooled to room temperature. 1%, 5% and 10% of 60 μL lemongrass or oregano essential oil were loaded on sterilized paper discs (8 mm) and placed upside-down on MHA surface and incubated at 37 for 24h. After incubation, the diameter (mm) of hollow zone was measured.

Minimal Inhibitory Concentration (MIC)

The MICs were determined by a broth microdilution method in Muller-Hinton broth [4]. A series of two fold dilution of antibiotics (Amoxicillin, AM and norfloxacin, NF) and essential oil (lemongrass and oregano ranging from 0.2 to 2.6 mg/ml) were inoculated with an overnight culture at a final inoculum of ~$5x10^5$ CFU/mL. The plates were incubated at 37°C overnight and MIC was determined. The MIC was defined as the lowest concentration of the antibiotic that prevented visible growth. Inhibition of bacterial growth in the plates containing test oil was judged by comparison with growth in blank controls.

Combination effect of lemongrass and antibiotics

S. aureus (KCCM 11812, KCCM 40511) and isolates (3-NA-1, 6-N-1, 9-NA-1) were selected as antibiotic resistant model. The effects of lemongrass oil, amoxicillin and norfloxacin in combination were assessed by using a microbroth chequerboard technique [3]. Each well containing the mixture of antibiotic and citral was inoculated with a 4-6 h broth culture diluted to give a final concentration of ~$5x10^5$ CFU/mL and incubated at 37°C for 18-20 h. Fractional inhibitory concentration (FIC) was calculated by dividing the MIC of the combination of antibacterial and essential oil by the MIC of antibacterial or essential oil alone. The FIC index, obtained by adding both FICs, was interpreted as synergistic when it was ≤ 0.5, as additive (indifferent) when it was > 0.5 and ≤ 2.0, and as antagonistic when it was >2.0. The combination value was derived from the highest dilution of antibiotic combination permitting no visible growth.

Result and discussion

In the GC/MS analysis of lemongrass essential oil, geranial (50.04%) and neral (36.20%) were mainly identified. These are the isomeric citral (the trans-isomer, geranial, and the cis-isomer, neral), making 86.24% of lemongrass essential oil consisted of citral. The retention times of the two resulting peaks were 20.2 min and 21.2 min. The remaining trace compounds identified are summarized in Table 2. For case of oregano essential oil, the major components identified was carvacrol (68.78%). All the compounds identified oregano essential oil is also summarized in Table 2.

As Table 3 shows, gradient-dependent inhibition zone were revealed by disk diffusion assays. In almost every test group, lemongrass oil showed larger inhibition zone than oregano oil. Inhibition zone of 5% lemongrass oil was two times larger than that of 5% oregano oil. In case of oregano oil, the diameter of 1% and 5% showed great differences but 5% and 10% shows little differences except S. aureus KCCM 40881.

The result for the MIC value of lemongrass oil, oregano oil, amoxicillin, & norfloxacin against standard antibiotic resistant bacterial strains and field isolates are summarized in Table 4. MIC for lemongrass oil ranged from 100 to 1000 μg/mL and for oregano oil from 100 to 4000 μg/mL. For both standard antibiotic resistant bacterial strains and field isolates, lemongrass oil has shown equal or higher MIC levels than oregano essential oil. The antibiotics amoxicillin and norfloxacin had also shown MIC values ranging from 0.5 to 128 μg/mL and 1 to 64μg/mL, respectively. Norfloxacin had shown more stable antibacterial activity with smaller range than amoxicillin. Lemongrass oil showing consistent higher antibacterial activity in both disc diffusion and MIC tests, hence it was further tested for its antibacterial effect in combination with antibiotics.

The results of combination studies are shown in Table 4. The effect of lemongrass oil-antibiotics combination against both selected standard antibiotic resistant bacterial strain and field isolates represented both synergistic and additive interaction. Lemongrass oil-amoxicillin combination showed synergistic interactions against all tested organism while the combination for lemongrass oil-norfloxacin showed synergistic effect for two field isolates and an additive for the remaining strains.

In improving the quality of healthcare which could be obtained from essential oil, it is necessary to investigate the plant extracts scientifically for their optimum usage. In the current study, the potential use of essential oils antibacterial activity against standard and field isolates antibiotic resistant strain was reported.

Both disk diffusion assay and minimal inhibitory concentration (MIC) revealed antibacterial activities by the essential oils. Lemongrass oil showed higher or equal antimicrobial activity than oregano oil in both assay and it was further studied for its combined effect with antibiotics. However, comparison between these results and previously issued data were difficult or otherwise impossible due to variations in plants essential oil composition which is affected by their chemotypes, and/or extraction procedure, and also differences in antimicrobial analysis methods [12]. The combination effect of lemongrass oil-amoxicillin had shown the highest potential antibacterial activity showing synergistic effect in all tested organisms which was not expected due to the higher range of MIC values of amoxicillin observed when tested alone. Contrary to this, norfloxacin which has shown stable MIC values when tested alone and had a mode of action that suppresses the DNA gyrase (site for mutation for resistant) of bacteria [13] had shown synergistic effect in two of the isolates. The composition of lemongrass oil and oregano oil assessed using GC-MS revealed citral and carvacrol-thymol as the most abundant compound, respectively. Both of these compounds could be associated to the antibacterial activity observed by these essential oils which had been also reported previously [14].

Table 2. GC/MS analysis of lemongrass and oregano essential oil.

Essential oil	Compounds	Retention Time	Area (%)
Lemongrass	N-Methylguanidine	2.036	0.81
	Dimethyl-2,8 nonadiene-1,8	10.785	1.47
	1-dodecyne	17.515	0.89
	1-dodecyne	18.178	1.60
	Neral	20.233	36.20
	2-pentene, 4,4'-oxybis-	20.715	2.37
	Geranial	21.272	50.04
	Nerol	25.067	5.15
	Cyclopropane, 1,1'-ethenylidenebis-	26.234	1.46
Oregano	α-pinene	8.443	0.21
	α-pinene	8.662	1.54
	Camphene	9.204	0.48
	Mycrene	10.89	1.67
	Terpinolene	11.831	1.37
	o-cymene	12.14	9.80
	Terpan	12.396	0.13
	γ-terpinene	13.472	7.61
	L-Linalool	15.069	1.96
	Linalool	17.952	0.14
	Thymol	22.04	4.03
	Carvacrol	22.439	68.78
	β-caryophyllene	26.242	2.05
	β-caryophyllene	28.982	0.14
	Trans-bicyclo[5.2.0]non-8-ene	31.218	0.10

Table 3. Antimicrobial activity assay with disc diffusion method

Compound	Concentration	KCCM (Diameter, mm)					Isolates(Diameter, mm)					
		40881	41291	41294	11593	40510	9-NA-3	6-N-2	2-20-2	3-NA-1	6-N-1	9-NA-1
Lemongrass	10%	28±2	26±6	25±2	25±6	34.5±2.5	26.5±2	29±1	17±3.4	24±1.5	25.5±1.3	21±1.25
	5%	19±1	18±0.5	19.5±0.5	18±2.5	28±1.5	13±1.5	12.5±1	8±2	14±0.5	11.5±1	10±1
	1%	8.5±0.5	6.5±2.5	6±0.1	8.5±0.5	11±1	5.5±1	6±2.5	1.4±1	6±2.5	8.5±1	7±2
Oregano	10%	14±0	5.5±1.5	5±0.5	6±0	10±1	9.5±1	11±2	8±1.5	6±2	7.5±1	5.5±1
	5%	9±2	5.5±0.5	5±0.2	4±0.2	9.5±1.5	5.5±1	5.5±2	3.5±1	3±2	4±1.5	2.5±0.5
	1%	3.5±0.5	1.5±0.5	3.5±0.5	1.5±0.5	1±1	2±1	1±0.5	1.5±0.5	1±1	1.5±0.5	2±1.5
Control		8	8	8	8	8	8	8	8	8	8	8

* : positive control. (Diameter of paper disc (8 mm) is included.

Table 4. Comparison of MIC of oregano oil, lemongrass oil, amoxicillin, and norfloxacin and combination effect of Lemongrass essential oil and antibiotics.

Antibiotic resistant strains	MIC (μg/mL)			MIC (μg/mL)	
	C.-Thymol		Citral	Amoxicillin	Norfloxacin
KCCM 11593	500		500	0.5	1
KCCM 11812	100		500	32	4
KCCM 40510	100		500	32	4
KCCM 40511	100		100	64	1
KCCM 40881	100		500	2	2
KCCM 41291	100		100	4	1
KCCM 41294	500		100	8	2
9-NA-3	1000		500	128	64
6-N-2	4000		1000	128	64
2-20-2	2000		1000	128	64
3-NA-1	4000		1000	64	32
6-N-1	4000		500	128	8
9-NA-1	1000		500	128	8
	LEO+ NF			LEO+AM	
	MIC	FIC	Interpretation	MIC	FIC	Interpretation
KCCM 11812	200, 1	0.065	Additive	50, 2	0.1625	Synergism
9-NA-3	200, 8	0.525	Additive	25, 8	0.1125	Synergism
6-N-2	50, 8	0.175	Synergism	25, 8	0.0875	Synergism
2-20-2	25, 8	0.15	Synergism	25, 4	0.0563	Synergism

LEO: Lemongrass essential oil,

Conclusion

The current study indicated that both lemongrass and oregano essential oils exhibit antibacterial activity, and lemongrass oil in combination with antibiotics could be a potential source for antibacterial treatments against antibiotic resistant bacterial infections. Further in vivo studies and clinical trials are recommended to justify and further evaluate for the potential use in clinical and preventive applications.

Authors Contribution

Jae-Young Choi,
Experimental designing and doing experiment
Dereje Damte
Experimental design, result interpretation and writing manuscript
Seung-Jin Lee
Result interpretation and data analysis
Jong-Choon Kim
Result interpretation and data analysis editing manuscript
Seung-Chun Park
Supervisor role, Experimental design, reviewing manuscript

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