Journal of Pharmacopuncture

ISSN 2093-6966 [Print], ISSN 2234-6856 [Online]
Journal of Pharmacopuncture 2016;19[4]:312-318
DOI: https://doi.org/10.3831/KPI.2016.19.032

Original article

Effect of Catechins, Green tea Extract and Methylxanthines in Combination with Gentamicin Against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

- Combination therapy against resistant bacteria -

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Key Words

antimicrobial resistant, catechins, gentamicin, methylxanthine, *Pseudomonas aeruginosa*, *Staphylococcus aureus*

Abstract

Objectives: Bacterial resistant infections have become a global health challenge and threaten the society's health. Thus, an urgent need exists to find ways to combat resistant pathogens. One promising approach to overcoming bacterial resistance is the use of herbal products. Green tea catechins, the major green tea polyphenols, show antimicrobial activity against resistant pathogens. The present study aimed to investigate the effect of catechins, green tea extract, and methylxanthines in combination with gentamicin against standard and clinical isolates of *Staphylococcus aureus* (*S. aureus*) and the standard strain of *Pseudomonas aeruginosa* (*P. aeruginosa*).

Methods: The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) values of different agents against bacterial strains were determined. The interactions of green tea extract, epigallate catechin, epigallocatechin gallate, two types of methylxanthine, caffeine, and theophylline with gentamicin were studied *in vitro* by using a checkerboard method and calculating the fraction inhibitory concentration index (FICI).

Results: The MICs of gentamicin against bacterial strains were in the range of 0.312 - 320 μg/mL. The MIC values of both types of catechins were 62.5 - 250 μg/mL. Green tea extract showed insufficient antibacterial activity when used alone. Methylxanthines had no intrinsic inhibitory activity against any of the bacterial strains tested. When green tea extract and catechins were combined with gentamicin, the MIC values of gentamicin against the standard strains and a clinical isolate were reduced, and synergistic activities were observed (FICI < 1). A combination of caffeine with gentamicin did not alter the MIC values of gentamicin.

Conclusion: The results of the present study revealed that green tea extract and catechins potentiated the antimicrobial action of gentamicin against some clinical isolates of *S. aureus* and standard *P. aeruginosa strains*. Therefore, combinations of gentamicin with these natural compounds might be a promising approach to combat microbial resistance.

Received: Jun 25, 2016 **Reviewed:** Oct 02, 2016 **Accepted:** Nov 28, 2016

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This paper meets the requirements of KS X ISO 9706, ISO 9706-1994 and ANSI/NISO Z39.48-1992 (Permanence of Paper).

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1. Introduction

Bacterial resistance to antibiotics has emerged as a major challenge in treating bacterial infections [1, 2]. The infectious diseases caused by resistant pathogens lead to serious negative effects, including the use of higher doses of antibiotics, the need for additional treatments and possibly prolonged hospitalizations, and increased mortality [3]. Gentamicin is a potent antibiotic and is traditionally used for the treatment of patients with a wide range of infections caused by Gram-positive and -negative bacteria [4, 5]. Despite all of its advantages, this antibiotic suffers from some shortcomings, such as its undesirable effects on health and bacteria's increased resistance to it.

These days, many strategies have been proposed to overcome bacterial resistances. New generations of antibiotics, combination therapies, natural compounds, and drug delivery systems are mentioned as common approaches in this field [6, 7]. Therapy involving a combination of antibiotics shows several advantages; it enhances the antibiotic activity, prevents the emergence of resistance, and reduces the risk of infection [8]. Consequently, combinations of gentamicin with other antibacterial agents in order to reduce the risk of increasing resistance and toxicity are important.

The aqueous extract of dried tea leaves (*Camellia sinensis* L.) has been widely used in traditional medicine because of its remarkable range of pharmacological activities [9, 10]. Aqueous extracts of *C. sinensis* were shown to be able to inhibit a wide range of pathogenic bacteria, such as methicillin-resistant *Staphylococcus aureus* (*S. aureus*). Tea extracts were also bactericidal to staphylococci and *Yersinia enterocolitica* [11]. Teas of *C. sinensis* prepared with different manufacturing processes have different contents based on the preparation method. Green tea is produced by steaming or panning, which prevents catechin oxidation by polyphenol oxidase. When this method is used, green tea leaves maintain their green color, and almost all of their original polyphenolic compounds are intact [12].

Green tea catechins (GTCs) are polyphenolic compounds found in green tea. These compounds provide health benefits and show remarkable antimicrobial activity against resistant pathogens [13, 14]. Tea extracts having high concentrations of epigallocatechin gallate (EGCG) and epigallocatechin (EGC) are more potent in antibacterial activity against bacterial pathogens [15]. EGCG at concentrations between 78 and 625 μg/mL was able to inhibit *Acinetobacter baumannii* [16]. Additionally, EGCG inhibits the growth of *S. aureus* by inhibiting the chromosomal penicillinase [17].

Methylxanthines are potent bronchodilator agents and are widely used as a treatment for patients with acute asthma. These therapeutic agents have shown antibacterial activities against some bacterial pathogens [18, 19]. For instance, aminophylline and caffeine increased the antimicrobial action of carbenicillin, ceftizoxime and gentamicin against *S. aureus* and *Pseudomonas aeruginosa* (*P. aeruginosa*). In other studies, caffeine was able to decrease the minimum inhibitory concentration (MIC) values of gentamicin against *S. aureus* and *P. aeruginosa* [20, 21]. Previously, the antimicrobial properties of caffeine against *Candida albicans* were demonstrated [22, 23]. Based on the above, the aim of the present study was to evaluate the antibacterial activities of caffeine, green tea extract, EGC, and EGCG alone and in combination with gentamicin against two clinically important bacteria, *S. aureus* and *P. aeruginosa*.

2. Material and Methods

C. sinensis leaves were collected from the surrounding areas of Lahijan city, Gorgan Province, Iran, were dried in the shade, and were ground to make powder. *C. sinensis* was properly identified by Professor Joharchi in the Department of Botany, Ferdowsi University of Mashhad (Mashhad, Iran), and voucher samples were preserved for reference in the Herbarium of the Department of Pharmacognosy, School of Pharmacy, Mashhad, Iran. EGCG and EGC were purchased from Sigma (ANHUI Minimetals Development, China). Caffeine, theophylline and gentamicin were obtained from Darou Pakhsh Pharmaceutical Company (Tehran, Iran). The aqueous extract of *C. sinensis* was prepared by adding 100 mL of boiling distilled water to 5 g of powdered plant material in a glass 2.5-L flask and then soaking the solution overnight at room temperature. The solution was subsequently filtered using Whatman No. 1 filter paper, and the residues obtained were stored in a freezer at -70°C and then freeze dried.

The MIC of gentamicin and methylxanthines against pathogens was determined as previously described [24]. Briefly, approximately 10^6 colony-forming units (CFU)/mL of cells from overnight bacterial cultures were used as inoculum. Serial dilutions of each tested compound were prepared in Muller Hinton Broth (MHB) (Difco) in 96-well microtiter plates. Then, the inoculum was added to each well to obtain 10^5 CFU/mL at the final bacterial concentration. The inoculated microplates were incubated at 37°C for 24 hours under aerobic conditions. The MIC was determined by adding triphenyl tetrazolium chloride (TTC, Merck) to each well at a concentration of 0.05% plus incubating at 37°C for 30 minutes. The MIC was defined as the lowest concentration of the tested compound at which the reduction of TTC to red formazan was not observed after a 30-minute incubation at 37°C.

In the cases of the aqueous extract and the catechins, different concentrations of the green tea extract (1 - 2000 μg/mL) were incorporated into MHB in different test tubes. In each test tube, 5 mL of green tea extract was added to 4.9 mL of nutrient broth and 0.1 mL of bacterial culture (1×10^8). A control tube containing the growth medium and the bacteria was set-up. The tubes were incubated at 37°C for 24 hours and then analyzed based on turbidity. The minimum concentration of green tea extract that inhibited the growth of bacteria was defined as the MIC [25]. For the minimum bactericidal concentration (MBC) determination, an aliquot of 10 μL from all wells and tubes without growth was seeded in Tryptone Soya Agar plates (TSA) (LabM). The plates were then incubated overnight at 37°C. The MBC is defined as the lowest concentration of antimicrobial agent that kills > 99.9% of the bacteria.

To evaluate the antibacterial activities of two antibacteri-

al agents, we used the checkerboard method. In brief, serial 2-fold dilutions of gentamicin and other agents were mixed in each well of a 96-well microtiter plate. Consequently, each row and column contained a fixed amount of one antibacterial agent and increasing amounts of the second one. The MIC was assessed as mentioned above, and finally the fraction inhibitory concentration index (FICI) value was used to assess whether synergism, indifference, or antagonism had occurred between the agents.

The FICI of the antibacterial agent combination was the FIC of drug A + the FIC of drug B, where the FIC of drug A is equal to (MIC of drug A in combination)/(MIC of drug A alone) and the FIC of drug B is equal to (MIC of drug B in combination)/(MIC of drug B alone). The combination effects were evaluated based on the following criteria: < 0.5 denotes synergy, 0.5 - 0.75 denotes partial synergy, 0.76 - 1 denotes an additive effect, 1 - 4 denotes indifference, and > 4 denotes antagonism [24].

3. Results

The MIC and the MBC values of gentamicin, green tea extract, and catechins for each clinical isolate and reference bacteria are presented in Table 1. The MIC and the MBC values of caffeine were more than 1000 μg/mL. The combined effects of gentamicin and the different compounds are shown in Tables 2-5. These results indicate that combinations of gentamicin and methylxanthines were not effective against ant bacterial strains, except for one clinical isolate (*S. aureus* No.2).

From the results in Tables 3-5, one can conclude that for combinations of gentamicin with natural compounds, partial synergistic effects can be expected. For instance, a combination of gentamicin with green tea extract was effective against *P. aeruginosa* and *S. aureus*. In combination with a herbal extract, the MIC values of gentamicin against one isolate and standard strains of *S. aureus* were reduced twofold. One should also note that a combination of gentamicin with natural products such as EGCG was more effective than herbal extracts alone. The MIC values of gentamicin against standard stains of *P. aeruginosa* and *S. aureus* were reduced up to twofold when the antibiotic was combined with EGCG.

4. Discussion

Clinically isolated bacteria are the main causes of nosocomial infections. The spread of these types of infections leads to public health problems. Consequently, finding the proper way to prevent infections that seems to be important. Combination therapy is a promising approach to combating bacterial resistance. Therefore, in the present study, combinations of gentamicin with other therapeutic agents were evaluated. The MIC and the MBC values of gentamicin against bacterial species (Table 1) indicated that with respect to the standard strain (*S. aureus* ATCC 6538p), isolated strains showed more resistant to antimicrobial agents. This evidence suggests that the bacterial strains used in this study showed high levels of resistances to antibacterial agents. To enhance the antibacterial activity of gentamicin, we combined it with different compounds.

Methylxanthines are useful therapeutic agents for treating patients with acute asthma. These agents are also used to treat patients suffering from bacterial infections [18, 21]. In the present study, the combinatorial effect of caffeine and gentamicin against bacterial species was investigated (Table 2). According to the data, the synergistic effects were only observed in one strain of a clinical isolate of *S. aureus*. These findings support the supposition that caffeine might be more efficient against Gram-positive bacteria than it is against Gram-negative ones. These results are in line with previously published data, which showed that some derivatives of methylxanthines exert antibacterial activities and that they are more potent against Gram-positive species [18].

Based on the results in Table 3, green tea extract when combined with gentamicin shows sufficient antibacterial

Table 1 MIC values of gentamicin against *S. aureus* and *P. aeruginosa* strains

Bacterial strains	Strain	Gentamicin		Green tea extract		EGC		EGCG	
		MIC/MBC (μg/mL)		MIC/MBC (μg/mL)		MIC/MBC (μg/mL)		MIC/MBC (μg/mL)	
S. aureus	1	> 80	> 80	250	< 1,000	62.5	250	62.5	125
S. aureus	2	20	40	1,000	< 1,000	125	500	62.5	250
S. aureus	3	> 80	> 80	500	< 1,000	125	500	62.5	500
S. aureus	4	> 80	> 80	500	< 1,000	62.5	500	62.5	500
S. aureus (ATCC 6538p)	5	0.312	0.625	500	< 1,000	62.5	250	125	500
P. aeruginosa (ATCC 9027)	1	0.312	0.625	1,000	< 1,000	125	500	250	500

MIC, minimum inhibitory concentration; *S. aureus*, *Staphylococcus aureus*; *P. aeruginosa*, *Pseudomonas aeruginosa*; EGC, epigallocatechin; EGCG, epigallocatechin gallate; MBC, minimum bactericidal concentration.

Table 2 Results of the combination of gentamicin and methylxanthines, caffeine and theophylline against *S. aureus* and *P. aeruginosa*

Bacterial strains	Strain	Agent	MIC (μg/mL)		FIC	FICI	Outcome
			Alone	Gentamicin + Caffeine			
S. aureus	1	gentamicin	> 80	> 80	1	2	Indifference
S. aureus	2	methylxanthines	> 200	> 80	1	0.56	Partial synergy
		gentamicin	12.5	6.25	0.5		
S. aureus	3	methylxanthines	> 200	12.5	0.06	2	Indifference
		gentamicin	> 80	> 80	1		
S. aureus	4	methylxanthines	> 200	> 80	1	2	Indifference
		gentamicin	> 80	> 80	1		
S. aureus (ATCC 6538p)	5	methylxanthines	> 200	> 80	1	2	Indifference
		gentamicin	0.312	0.312	1		
P. aeruginosa (ATCC 9027)	1	methylxanthines	> 200	200	1	2	Indifference
		gentamicin	0.312	0.312	1		

S. aureus, Staphylococcus aureus; *P. aeruginosa, Pseudomonas aeruginosa*; MIC, minimum inhibitory concentration; FICI, fraction inhibitory concentration index.

Table 3 Results of the combination of gentamicin and green tea extract against *S. aureus* and *P. aeruginosa*

Bacterial strains	Strain	Agent	MIC (μg/mL)		FIC	FICI	Outcome
			Alone	Gentamicin + Extract			
S. aureus	1	gentamicin	> 80	> 80	1	2	Indifference
		green tea extract	250	250	1		
S. aureus	2	gentamicin	20	10	0.5	0.75	Partial synergy
		green tea extract	1,000	250	0.25		
S. aureus	3	gentamicin	> 80	80	1	2	Indifference
		green tea extract	500	500	1		
S. aureus	4	gentamicin	> 80	> 80	1	2	Indifference
		green tea extract	500	500	1		
S. aureus (ATCC 6538p)	5	gentamicin	0.312	0.156	0.5	0.56	Partial synergy
		green tea extract	500	31.25	0.06		
P. aeruginosa (ATCC 9027)	1	gentamicin	0.312	0.156	0.5	1	Additive
		green tea extract	1,000	500	0.5		

S. aureus, Staphylococcus aureus; *P. aeruginosa, Pseudomonas aeruginosa*; MIC, minimum inhibitory concentration; FICI, fraction inhibitory concentration index.

activities. The antibacterial activity of green tea extract was previously described, and the finding that it was related to the catechin content was noteworthy [14, 15]. Additionally, green tea extract was shown to be able to kill *S. aureus* and other harmful bacteria [26]. Various hypotheses have tried to explain the possible antimicrobial mechanism of action of *C. sinensis*. One suggestion was that *C. sinensis* irreversibly damaged the bacterial cytoplasmic membrane [27]. Other possible mechanisms for the antibacterial activity of *C. sinensis* are related to the activity of dihydrofolate reductase (DHFR) [28]. The results of the present study clearly show that Gram-negative bacteria are less susceptible to green tea extract. These results were previously confirmed [14].

The biological properties of green tea have been mainly attributed to the catechin constituents. EGCG and EGC are the most abundant catechins [29]. Thus, in this study, the antibacterial activities of these two pure compounds were investigated separately. According to the results in Tables 4-5, EGCG and EGC when combined with gen-

Table 4 Results of the combination of gentamicin and EGC against *S. aureus* and *P. aeruginosa*

Bacterial strains	Strain	Agent	MIC (μg/mL)		FIC	FICI	Outcome
			Alone	Gentamicin + EGC			
S. aureus	1	gentamicin	> 80	20	0.25	0.75	Partial synergy
		EGC	62.5	31.25	0.5		
S. aureus	2	gentamicin	20	10	0.5	1.5	Indifference
		EGC	125	125	1		
S. aureus	3	gentamicin	> 80	80	1	1.5	Indifference
		EGC	125	62.5	0.5		
S. aureus	4	gentamicin	> 80	80	1	2	Indifference
		EGC	62.5	62.5	1		
S. aureus (ATCC 6538p)	5	gentamicin	0.312	0.156	0.5	0.56	Partial synergy
		EGC	62.5	3.90	0.06		
P. aeruginosa (ATCC 9027)	1	gentamicin	0.312	0.312	0.5	2.5	Indifference
		EGC	125	250	2		

EGC, epigallocatechin; *S. aureus*, *Staphylococcus aureus*; *P. aeruginosa*, *Pseudomonas aeruginosa*; MIC, minimum inhibitory concentration; FICI, fraction inhibitory concentration index.

Table 5 Results of the combination of gentamicin and EGCG against *S. aureus* and *P. aeruginosa*

Bacterial strains	Strain	Agent	MIC (μg/mL)		FIC	FICI	Outcome
			Alone	Gentamicin + EGCG			
S. aureus	1	gentamicin	> 80	2.5	0.03	0.53	Partial synergy
		EGCG	62.5	31.25	0.5		
S. aureus	2	gentamicin	20	10	0.5	0.75	Partial synergy
		EGCG	62.5	15.62	0.25		
S. aureus	3	gentamicin	> 80	80	1	2	Indifference
		EGCG	62.5	62.5	1		
S. aureus	4	gentamicin	> 80	80	1	2	Indifference
		EGCG	62.5	62.5	1		
S. aureus (ATCC 6538p)	5	gentamicin	0.312	0.156	0.5	1.5	Indifference
		EGCG	125	125	1		
P. aeruginosa (ATCC 9027)	1	gentamicin	0.312	0.312	0.5	1	Additive
		EGCG	250	125	0.5		

EGCG, epigallocatechin gallate; *S. aureus*, *Staphylococcus aureus*; *P. aeruginosa*, *Pseudomonas aeruginosa*; MIC, minimum inhibitory concentration; FICI, fraction inhibitory concentration index.

tamicin were effective in inhibiting the growth of bacteria. As mentioned above, these compounds as a part of green tea extract can damage the cell membranes of bacteria and, thereby, enhance the penetration of antibiotics into bacterial cells. This interaction is similar to the interaction of various types of penicillins with gentamicin. Penicillins inhibit the cell wall synthesis by bacteria, which leads to enhanced penetration of other antibiotics such as gentamicin. The anti-staphylococcal activities of penicillins are also increased by the addition of gentamicin as an aminoglycoside antibiotic [30]. The slight differences between the antibacterial activities of these compounds might be related to their structures, which affect cell penetration. EGCG differs from EGC only by the presence of an additional hydroxyl group on the aromatic ring.

5. Conculsion

The findings of the present study highlight the advantages of combinations of antibiotics with green tea extract and catechins against Gram-positive and Gram-negative bacteria and merit further investigations and complementary studies.

Acknowledgment

The authors are thankful to the Vice Chancellor of Research, Mashhad University of Medical Sciences for financial support. The results described in this paper are part of a Pharm. D. (Doctor of Pharmacy) thesis.

Conflict of interest

The authors declare that there are no conflicts of interest.

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