Synergistic antibacterial effect of ciprofloxacin and green tea extract on methicillin-resistant *Staphylococcus aureus* (MRSA) strains isolated from clinical cases

Klinik vakalardan izole edilen metisilin dirençli *Staphylococcus aureus* (MRSA) suşlarına siprofloksasin ve yeşil çay ekstraktının sinerjik antibakteriyel etkisinin araştırılması

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ABSTRACT

Objective: The aim of this study was to investigate the synergistic effect of ciprofloxacin and green tea extract combinations against methicillin resistant *Staphylococcus aureus* (MRSA) strains isolated from various clinical registries.

Methods: Presumptive 90 MRSA strains isolated from various clinical specimens were identified by the biochemical tests followed by an automated bacterial recognition system. Resistance of isolates to ciprofloxacin was determined using a disk diffusion test. Minimum inhibitory concentration (MIC) values of green tea extract and ciprofloxacin were determined for 54 MRSA isolates resistant to ciprofloxacin by microdilution method. Subsequently, the synergistic effect of the green tea + ciprofloxacin combinations against the isolates were determined by the checkboard dilution method to calculate fractional inhibitory concentration values.

Results: MRSA strains were isolated from three clinical sample types including wounds (54.4%), blood (30%) and urine (15.6%). MIC values of green tea extract, ciprofloxacin and their tested combinations ranged

ÖZET

Amaç: Bu çalışmanın amacı, siprofloksasin ve yeşil çay ekstraktı kombinasyonunun çeşitli klinik örneklerden izole edilen metisiline dirençli *Staphylococcus aureus* (MRSA) suşlarına karşı sinerjik etkisinin araştırılmasıdır.

Yöntem: Farklı klinik örneklerden izole edilen muhtemel 90 adet MRSA suşu biyokimyasal testlerden sonra otomatize bakteri tanımlama sistemiyle tür düzeyinde tanımlanmıştır. İzolatların siprofloksasine karşı dirençleri disk difüzyon testi kullanılarak belirlenmiştir. Siprofloksasine karşı dirençli bulunan 54 MRSA izolatının yeşil çay ekstraktı ve siprofloksasin için minimum inhibisyon konsantrasyon (MİK) değerleri mikrodilüsyon yöntemiyle tespit edilmiş ve izolatlara karşı yeşil çay + siprofloksasin kombinasyonun sinerjik etkisi dama tahtası yöntemi ile hesaplanan fraksiyonel inhibisyon konsantrasyon değerleri kullanılarak hesaplanmıştır.

Bulgular: MRSA izolatlarının %54,4'ü yara, %30'u kan ve %15,6'sı idrar olmak üzere üç farklı klinik örnek türünden izole edilmiştir. MRSA izolatlarının yeşil çay ekstraktı, siprofloksasin ve farklı kombinasyonlarının

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between 8 and 256 µg/ml. The synergetic effect of green tea extract on ciprofloxacin for 54 MRSA isolates were determined as ineffective for 18 (33.3%), aditive for 17 (31.5%), and synergic for 19 (35.2%) strains based on fractional inhibitory concentration values.

Conclusion: The results of this study showed that green tea extract and ciprofloxacin had a synergetic antibacterial effect on MRSA strains isolated from various clinical specimens.

Key Words: Checkerboard dilution method, MRSA, synergistic effect, ciprofloxacin, green tea, *Camellia sinensis*

MIK değerleri 8-256 µg/ml arasında belirlenmiştir. Yeşil çay ekstraktının siprofloksasin üzerine sinerjik etkisi, kombinasyonunun franksiyonel inhibitör konsantrasyonu indeksi değerlerine göre 54 MRSA izolatında için 18 suş (%33,3) için etkisiz, 17 suş (%31,5) için aditif, 19 suş (%35,2) için sinerjik etki olarak saptanmıştır.

Sonuç: Bu çalışma sonucunda göre yeşil çay ekstraktı ve siprofloksasin kombinasyonunun klinik örneklerden izole edilen MRSA suşları üzerinde sinerjik antibakteriyel etkisi olduğu görülmüştür.

Anahtar Kelimeler: Dama tahtası duyarlılık yöntemi, MRSA, sinerjik etki, siprofloksasin, yeşil çay, *Camellia sinensis*

INTRODUCTION

MRSA has been found with high prevalence in hospitals and intensive care units since the first isolate was recovered in the early 1960s in Denmark (1). Community-acquired MRSA isolates were also started to be reported towards the end of the twentieth century (2, 3). MRSA isolates have been identified even in countries where the use of antibiotics is not common, suggesting that this form of resistance have existed in staphylococci for a long time (4). When MRSA bacteria are isolated due to nosocomial infection, both the difficulties in the eradication of disease factors and the resistance of strains to antibiotics lead to serious therapeutic problems and high care costs (5, 6).

Antibiotics are the gold standard for treatment of MRSA infections. However, long-term antibiotic treatments can cause numerous side effects and bacterial resistance. Ciprofloxacin is a broad-spectrum synthetic antibiotic (chemotherapeutic agent) of the fluoroquinolone class used orally or intravenously in the clinical treatment of severe and life-threatening infections. Ciprofloxacin interferes with DNA replication by inhibiting DNA gyrase in both Gram-positive and Gram-negative bacteria (7). To avoid development of resistance, ciprofloxacin should be combined with a suitable antibiotic (8). For example, quinolones combined with rifampicin helps eradicate MRSA strains completely (9). Therefore, researchers seek alternative treatments to conventional antibiotic therapy.

Antimicrobial effects of some herbal extracts on MRSA have been reported previously (10, 11). Green tea (*Camellia sinensis*) leaves are known for their antibacterial activity against various pathogens, including MRSA (12, 13). Research also focuses on the synergistic effect of antibiotics with plant extracts as green tea on numerous types of microorganisms (14-18). Green tea extracts combined with other antibiotics (chloramphenicol, gentamicin, methicillin, and nalidixic) have been resulted in a synergistic effect on enteropathogenic bacteria with in vitro assays (18). Any in vitro susceptibility procedure designed to understand the antibacterial activity of combined antibiotics is referred to as synergy

testing. Two-dimensional dilution (checkerboard, checkerboard dilution method), time-kill kinetic assays, and Epsilometer test (E-test) are some of common methodologies used in synergy testing (19, 20). The aim of this study was to determine the in-vitro synergistic effect of ciprofloxacin + green tea extract on MRSA strains isolated from the clinical samples of patients in different units of the hospital.

MATERIAL and METHOD

Identification of Bacterial Isolates

Strains were isolated from clinical samples (wounds, blood, and urine) obtained from clinics of different hospitals between 01 January 2010 and 12 December 2014. This study was approved by the Atatürk University Faculty of Medicine Clinical Research Ethics Committee at 08 December 2016 with meeting number 7 and decision number 10. Isolation was conducted at the research and application center medical bacteriology laboratory of the microbiology department of the faculty of medicine at Atatürk University. Stock MRSA strains (-80 °C) were cultured on nutrient agar (Merck, Germany) and incubated at 35 ± 2 °C for activation. A series of preliminary identification tests (Gram staining, motility, oxidase, catalase, and coagulase) were conducted on the activated cultures. Cefoxitin-resistant isolates were also evaluated for further identification. These isolates were identified at the species level using an automated bacterial identification system (VITEK 2®, BioMérieux, USA). The identified MRSA isolates were kept at -80 ºC until study in a nutrient broth medium (Merck, Germany) containing 16% glycerol. *S. aureus* (ATCC 43300) was used as control.

Determination of Ciprofloxacin Resistance of Isolates

Resistance to ciprofloxacin was determined using Kirby-Bauer disk diffusion test. For activation, frozen isolates were streaked on 5% sheep blood agar (Merck, Germany) and incubated overnight at 35 ± 2 °C. After incubation, one colony was suspended in a Nutrient Broth (Merck, Germany) with a 0.5 McFarland turbidity (10^8 CFU/ml). A lawn of bacteria was cultured on a Mueller Hinton Agar (MHA; Merck, Germany, 4-mm thickness) plate by spreading the suspension. Afterwards, ciprofloxacin (CIP, 0.5 µg; Oxoid, UK) disks were placed and incubated at 35 ± 2 °C for 18-24 h. After incubation, disk diffusion susceptibility was interpreted based on zone diameters (mm) according to the CLSI M100-S24 breakpoint guidelines. Disk diffusion susceptibility was considered as sensitive (≥ 21 mm), moderately sensitive (16-20 mm), and resistant (≤ 15 mm). Fifty-four ciprofloxacin-resistant MRSA strains were included in the study for minimum inhibitory concentration values of ciprofloxacin, green tea extracts and ciprofloxacin + green tea extract synergy testing.

Preparation of Green Tea Extract

In-season, green tea with 2.5 leaves was collected in Rize and dried at room temperature in a sun-free environment. A 100 gram of powdered green tea was then extracted using a Soxhlet extractor (Sigma-Aldrich, 322415) in a liter of methanol for 72 h at a temperature below the boiling point. The extracts were filtered through Whatman filter No.1 (Dassel, Germany) and then concentrated using a rotary evaporator under vacuum at 40 °C. The extract was lyophilized and kept at + 4 °C until testing.

Determination of MIC Values of Ciprofloxacin-Resistant Isolates

A ciprofloxacin (Sigma Aldrich, USA) stock solution with a final concentration of 1024 µg/ml was prepared using sterile distilled water as recommended by CLSI. Green tea stock solution with a final concentration of 1024 µg/ml was prepared using saline (physiological serum) of tea extract previously dissolved in dimethyl sulfoxide (DMSO; Sigma Aldrich, USA). A cation-adjusted (containing 20-25 mg/l Ca^{++} and 10-12.5 mg/l Mg^{++}) Mueller Hinton Broth (CAMHB; Merck, Germany) medium was prepared for liquid microdilution (21). 100 µl

of CAMHB medium was placed to sterile 96 well round bottom (U-base) plates. 100 µl of the solution with the initial concentration of the test antibiotic (1024 µg/ml) was placed in the first wells. Two-fold ciprofloxacin concentrations were prepared for testing (dilution range: 1-512 µg/ml). The last two wells on the plate were used for reproduction and broth control. Bacterial suspensions with 0.5 McFarland turbidity were diluted 1:10 in CAMHB, and 10 µl of the final bacteria with an inoculum concentration of 5×10^5 CFU/ml was added to each well, including the reproduction control well. After 18-24 hours of incubation at 35 °C ± 2 °C, the minimum inhibitory concentration (MIC) values of the isolates were determined for ciprofloxacin and green tea extracts depending on the growth in the wells.

Synergy Testing of Ciprofloxacin-Resistant Isolates

For 54 MRSA isolates, the synergistic effect of ciprofloxacin + green tea extracts (CIP + GTE) was determined using the checkerboard dilution method. A two-fold dilutions were performed in separate tubes for the stock green tea extract and ciprofloxacin solutions. Serially diluted tubes were prepared for ciprofloxacin and green tea extracts based on the MIC of each isolate. A separate 96 well round bottom (U-base) ELISA plate was then prepared for each MRSA isolate and test combination. Bacterial suspensions with 0.5 McFarland turbidity (1.5×10^8 CFU/ml) for the isolates were diluted 1:30 in CAMHB (5×10^6 CFU/ml). For MIC testing, 10 µl of the suspensions was inoculated to all wells, except for the sterility control well. The green tea extract and ciprofloxacin solutions were mixed and added to the serial wells from the serial dilution tubes according to the checkerboard dilution method in order to prepare 50 µl of solutions with desired concentrations. Dilution effect was also calculated. After the checkerboard panel was prepared, the lids of the ELISA plate were closed and incubated at 35 ± 2 °C for 16-20 h. The plates were monitored for reproduction and medium sterility control.

Fractional Inhibitory Concentration Index (FIC)

FIC index was used (equation below) to determine the synergistic effect of the combinations (21). FIC indices of the 54 MRSA clinical isolates were calculated, and interaction effects were recorded as synergistic, additive, ineffective, and antagonistic.

A: Antibiotic in the combination

B: Green tea extract in the combination

$$FIC_A = \frac{\text{MIC values of A in the presence of B}}{\text{MIC of A alone}}$$

$$FIC_B = \frac{\text{MIC values of B in the presence of A}}{\text{MIC of B alone}}$$

Σ FIC index = $FIC_A + FIC_B$

Σ FIC index ≤ 0.5: synergistic,

>0.5 and <1: additive,

≥1 and 4 ≤ : ineffective,

>4: antagonistic.

RESULTS

Fractional Inhibitory Concentration Index (FIC)

The MRSA (n = 90) isolates were mostly isolated from patients in internal medicine, orthopedics and traumatology and neurosurgery clinics. Table 1 shows the distribution of the MRSA isolates by clinics. The MRSA isolates were isolated from three types of clinical samples; wounds (54.4%), blood (30%), and urine (15.6%). According to the antibiogram results, 54 MRSA isolates (60%) were ciprofloxacin-resistant, while the remainder were ciprofloxacin-susceptible.

MIC Values of Ciprofloxacin-Resistant Isolates

MIC values of the 54 MRSA isolates for ciprofloxacin resistance were determined by using disc diffusion and liquid microdilution methods, respectively. The MIC values of the MRSA isolates for ciprofloxacin were

256 µg/ml for 19 isolates, 128 µg/ml for 17 isolates, 64 µg/ml for eight isolates, 32 µg/ml for two isolates, 16 µg/ml for three isolates, and 8 µg/ml for five isolates (Figure 1). The ciprofloxacin-resistant MRSA isolates had a MIC value of 8 to 256 µg/ml. MIC50 and MIC90 were 128 and 256 µg/ml, respectively. Methicillin-resistant S. *aureus* (ATCC 43300, standard strain) had a ciprofloxacin MIC value of 128 µg/ml similar to tested isolates.

The MIC values of the ciprofloxacin-resistant MRSA isolates against GTE were 256 µg/ml for two isolates, 128 µg/ml for 10 isolates, 64 µg/ml for 27 isolates, 32 µg/ml for 12 isolates, and 16 µg/ml for three isolates (Figure 1). The MIC values of the ciprofloxacin-resistant MRSA isolates against GTE ranged from 16 to 256 µg/ml. MIC50 and MIC90 were 64 and 128 µg/ml, respectively. The MIC value of the MRSA (ATCC 43300) against GTE was 64 µg/ml.

Table 1. Clinical distribution of MRSA isolates (n = 90)

Unit	Number of Isolates	%
Internal Medicine	14	15.6
Orthopedics and Traumatology	14	15.6
Neurosurgery	12	13.3
Pediatrics	11	12.2
Anesthesia and Reanimation	10	11.1
Infectious Diseases	6	6.7
General Surgery	6	6.7
Neurology	5	5.6
Urology	5	5.6
Thoracic Surgery	3	3.3
Cardiology	2	2.2
Otorhinolaryngology	1	1.1
Plastic and Reconstructive Surgery	1	1.1
Total	**90**	**100**

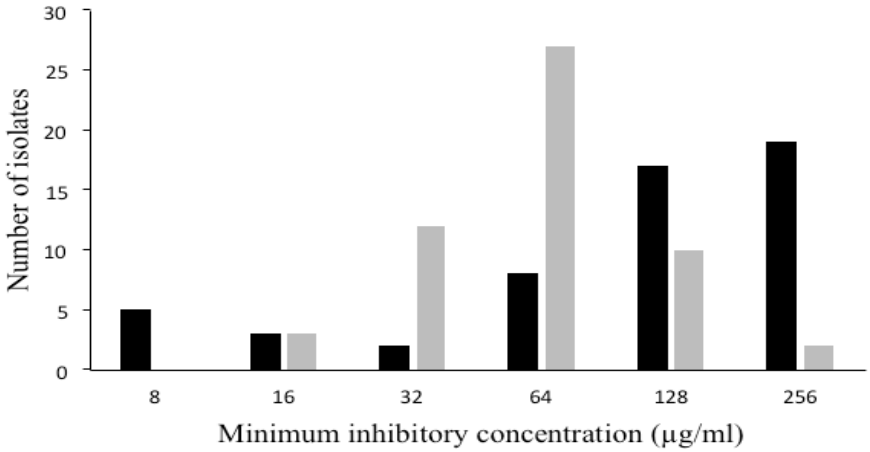

Figure 1. Minimum inhibitory concentrations of ciprofloxacin (■) and green tea extract (■) on isolated MRSA strains (n = 54)

CIP + GTE Synergy Testing of Ciprofloxacin-Resistant Isolates

Figure 2 shows the MIC values of CIP + GTE combinations for the ciprofloxacin-resistant MRSA isolates (checkerboard dilution method). The MIC values of the ciprofloxacin-resistant MRSA isolates against ciprofloxacin and GTE combinations were 1 to 4 µg/ml, 1 to 8 µg/ml, and 16 to 64 µg/ml for one isolate each, 2 to 8 µg/ml for five isolates, 4 to 6 µg/ml for nine isolates, 8 to 32 µg/ml for 22 isolates, 32 to 64 µg/ml for 13 isolates, and 64 to 128 µg/ml for two isolates (Figure 2).

According to the FIC values, ciprofloxacin and green tea extract combinations had no effect on 18 MRSA isolates (33.3%), an additive effect on MRSA isolates (31.5%), and a synergistic effect on 19 MRSA isolates (35.2%) (Table 2). According to the mean ΣFIC value (0.74), green tea extract had an additive ciprofloxacin effect on the isolates.

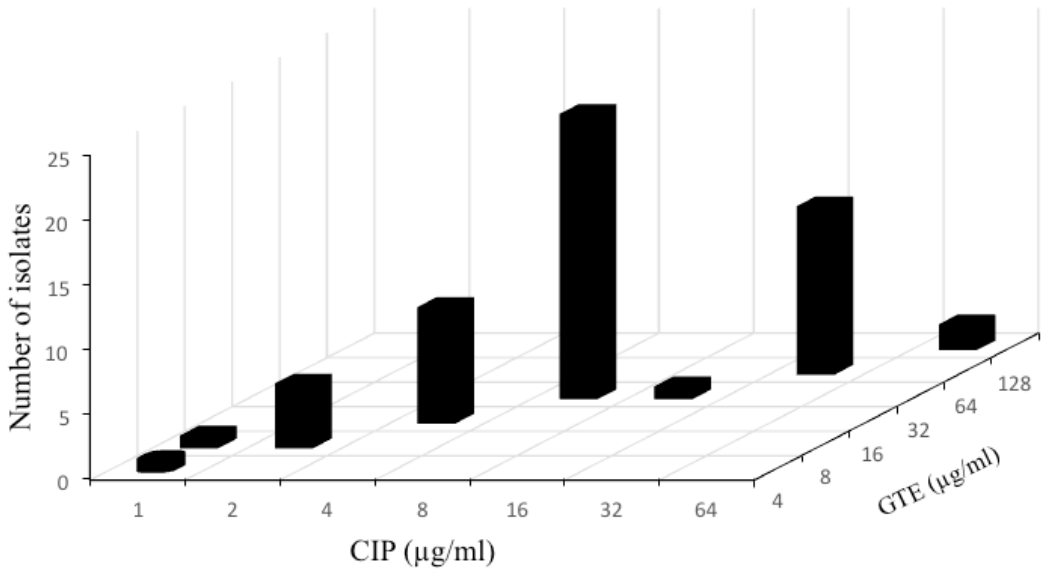

Figure 2. Minimum inhibitory concentrations of ciprofloxacin (CIP) and green tea extract (GTE) combinations on isolated MRSA strains (n = 54)

Table 2. Antimicrobial effect of ciprofloxacin and green tea extract combination on MRSA strains based on calculated sum of fractional inhibitory concentration indices

Interaction	Isolate Number	Total
Ineffective	4, 10, 11, 12, 16, 22, 26, 32, 33, 38, 65, 66, 69, 71, 73, 74, 75, 86	18 (33.3%)
Additive	1, 13, 14, 17, 19, 25, 29, 36, 39, 40, 41, 61, 67, 70, 77, 81, 84	17 (31.5%)
Synergistic	2, 6, 7, 8, 18, 27, 28, 35, 37, 42, 43, 58, 60, 68, 72, 80, 83, 87, 88	19 (35.2%)

DISCUSSION and CONCLUSION

Ciprofloxacin has an antibacterial effect on some MRSA strains, however, resistance can be developed over time. MRSA strains isolated from some regions of Mexico had almost 100% ciprofloxacin-resistance (22, 23). Altoparlak et al. (24) reported a 48% ciprofloxacin-resistance of 83 MRSA strains isolated from the Eastern Anatolia region of Turkey. In this study, samples had a 60% of ciprofloxacin-resistant MRSA isolates, indicating a clear increase in ciprofloxacin resistance. This may be because ciprofloxacin has a broad spectrum and is available both orally and intravenously.

Studies have focused on the antimicrobial activity of green tea extract alone or in combination with various antibiotics against MRSA isolates. Abaza et al. (25) isolated 81 MRSA strains from nasal carriers and investigated the antimicrobial activity of green tea extract with the disc diffusion method. Green tea extract was reported as strongly effective (55% with a zone diameter > 20mm) or moderately effective (45 % with a zone diameter > 16-20mm) on the isolates. Mahmoud et al. (26) looked into the synergistic effect of green tea catechins + antibiotics on MRSA isolates. 57 (83%) out of 90 MRSA isolates resulted in MIC values of 64 to 256 µg/ml for epigallocatechin-3-gallate (EGCG). Mahmoud et al. (26), therefore, concluded that ciprofloxacin and EGCG had a synergistic effect on MRSA isolates. Radji et al. (12) used the disk diffusion method and the broth microdilution (BMD) method to investigate the effect of green tea extract on an MRSA isolate and a methicillin-susceptible *Staphylococcus aureus* (MSSA) standard isolate. Two isolates showed similar green tea extract zone diameters and MIC values. In this study, the MIC values of the MRSA isolates were analyzed using BMD method, and MIC_{50}, MIC_{90} were found to be 64 mg/ml and 128 mg/ml, respectively. Abascal and Yarnell (27) reported that green tea exposed potential benefits and synergistic effect in terms of antimicrobial activity against many pathogenic microorganisms, including MRSA when combined with ß-lactam antibiotics. In this study, the synergistic effect of green tea extract and ciprofloxacin on MRSA isolates was tested by using the checkerboard dilution method. According to the MIC values, green tea extract had no synergistic effect on 18 MRSA isolates (33.3%), whereas it had an additive effect on MRSA isolates (31.5%), and a synergistic effect on 19 MRSA isolates (35.2%). According to the mean ΣFIC value (0.74), green tea extract had an additive ciprofloxacin effect on the isolates.

Green tea has an anti-staphylococcal effect by inhibiting the PBP2 synthesis and beta lactamase release in anti-staphylococcal effect. Green tea can also be regarded as a new approach in the treatment of MRSA (28). Peng et al. (29) measured the amount of catechin in green tea using high-performance liquid chromatography (HPLC) to detect the development of resistance of catechin against ß-lactam antibiotics in staphylococci. They focused on the antibacterial effect of combined with tea extract. They found that tea extract combined with tested antibiotics (ampicillin, cefazolin, and oxacillin) had a synergistic effect on MRSA with the exception of amoxicillin. In fact, amoxicillin, when combined with tea extract, had higher MIC values. Therefore, Peng et al. (29) recommended that patients who are on amoxicillin treatment not consume green tea. Research in general shows that green tea causes a decrease in the MIC values of beta lactam antibiotics in MRSA isolates. Cho et al. (30) observed that green tea polyphenols resulted in a reduction in the MIC value of oxacillin on MRSA isolates oxacillin. They analyzed the susceptibility of 13 strains of MRSA with green tea extract and oxacillin combination and found that the combination lowered MIC values at ratios between 1/8 and 1/128. Polyphenols stimulated the expression of various proteins in the bacterium was concluded as reason for a synergistic effect on the bactericidal activity of oxacillin.

Many studies explain the reasons for the differences in synergy rates. For example, although the checkerboard dilution method may seem like

a standard method, limit values for determining the interaction type after selecting the wells and calculating FIC values index differ. Most researchers in Turkey use the E-test because of its easy use (31). Few researchers using the checkerboard dilution method do not clearly explain which method is used to interpret the checkerboard plate despite limited values used for synergy identification. Synergistic activity rates depend not only on the existing methods but also on the isolates to be tested. Combinations of antibiotics with green tea extracts recommended by earlier in-vitro studies can be empirically used to treat *S. aureus* infections in patients with multiple drug resistance. However, isolates with high resistance or susceptibility to an antimicrobial agent may alter synergistic activity. Paramount importance of synergy testing is to determine the activity of a potential combination against strains isolated from a patient. In addition, in vitro studies do not always represent actual in vivo interactions.

In conclusion, the results of present study indicated that ciprofloxacin is a common antibacterial agent used to treat infections caused by MRSA strains. Green tea has reportedly good antibacterial activity against numerous microorganisms with a synergistic effect in combination with antibiotics. Rates of resistance in MRSAs vary, and therefore, researchers focus on developing new treatment protocols, new antibiotics, and new combination therapies. Antimicrobial drug combinations are used to achieve a broad spectrum, to prevent the development of resistant isolates, to minimize toxicity, and to increase the synergistic effect between two drugs. Green tea extract alone and in combination with antibiotics (green tea extract + ciprofloxacin) has a synergetic effect in the treatment of MRSA infections and may also help to reduce potential ciprofloxacin-related side effects. Further research should be performed on more strains and different antibiotic combinations for the treatment of MRSA infections.

ETHICS COMITTEE APPROVAL

* The study was approved by the Atatürk University Faculty of Medicine Clinical Research Ethics Committee (Date: 08.12.2016 and Number: Meeting number 7 and decision Number 10).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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