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Original Article

Prevention of antibiotic resistance created by experimental evolutionary microbiology in *Staphylococcus aureus* and *Escherichia coli* with herbal substances

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ABSTRACT

Background and Aims: Recently, one of the biggest problems of the world is a bacterial antimicrobial resistance, that is developing against most of the existing antibiotics. In addition to conducting studies that continue to discover new antimicrobial agents for combating multidrug resistant bacteria, steps should be taken for the protection of existing antibiotics. With this in mind, many modern and classical strategies have been developed, and among them, using essential oils or extracts obtained from plants, which may be a practical and effective alternative.
Methods: We used the experimental evolutionary microbiology method to determine the effects of herbal substances, such as cinnamaldehyde from cinnamon, epigallocatechin gallate from greentea, curcumin from turmeric, punicalagin from pomegranate, and clove oil from clove, on the prevention or delay of antimicrobial resistance. In this study, *Staphylococcus aureus* and *Escherichia coli* standard and clinical strains were gradually exposed to increasing sub-inhibitory concentrations of meropenem and ciprofloxacin with or without the presence of herbal substances.
Results: Resistance was developed in the *E. coli* and *S. aureus* control groups which were exposed only to ciprofloxacin, but, when herbal substances were included to the test, there was no resistance development. When the control groups were exposed only to meropenem, there was only an increase in the minimum inhibitory concentrations (MIC), but they did not become resistant, and we observed similar MIC values when we added the herbal substances to the test.
Conclusion: These results showed that herbal substances might contribute to lowering MIC values of antibiotics and may help prevent the development of resistance in the studied bacteria.

Keywords: Antibiotic resistance, Escherichia coli, Evolutionary microbiology, Herbal substance, Staphylococcus aureus

INTRODUCTION

Bacterial resistance to antibiotics is steadily becoming a bigger problem, and it has reached a critical level both in our country (Türkiye) and around the world. There are major problems, such as increasing morbidity and mortality rates in the treatment of infections, especially the nosocomial infections caused by the multi drug resistant strains (Koksal, Ak, Kucukbasmaci & Samasti, 2009; Kunz & Brook, 2010). According to the World Health Organization (WHO), the most frequently reported multi drug resistant bacteria include *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterococcus* and *Salmonella* sp. (WHO, 2021). There are several resistance mechanisms which happen

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in the development of resistance, such as limiting the uptake of drugs, modification of drug targets, inactivation of drugs with enzymes, and active efflux pumps. Some situations, such as the irrational or misuse of antibiotics or using bacteriostatic antibiotics for immunosuppressive patients, facilitate the selection or development of antibiotic resistance in bacteria (Aslam et al., 2018).

There are many ongoing studies to combat antibiotic resistance. While some studies prioritize the discovery of new and effective antibiotics, some of them focus on the re-use of existing antibiotics. The experimental evolution studies are one prominent concept for re-use strategies (Allen, Popat, Diggle & Brown, 2014). Experimental evolution includes evolutionary dynamics through controlled field studies and/or laboratory experiments. In this technique, small organisms, such as bacteria that exhibit rapid growth, are used to examine changes that would normally take a long time in large living organisms. Evolution can be observed under laboratory conditions, with natural selection of individuals and/or populations under new environmental conditions. In experimental evolutionary studies, the adaptation can be observed in two ways: when organisms undergo a new and beneficial mutation, or when a trait predominates in a population that is observed in a very small number of individuals (Long, Liti, Luptak & Tenaillon, 2015).

Although there are studies that continue to examine new methods that might be used for treating resistant microorganisms, recently, the prevention of the emergence of antibiotic resistance has seemed like the most effective and safest way forward. Therefore, there are many ongoing studies about the use of various natural herbal substances that have been used in the community for many years, either alone or in combination with antibiotics (Yap, Yiap, Ping & Lim, 2014). In this study, we aimed to observe the stages of resistance development against meropenem and ciprofloxacin in *S. aureus* and *E. coli* using the experimental evolutionary microbiology method. In order to slow down or stop this resistance, we tested the effects of some herbal antimicrobial substances, including cinnamaldehyde, punicalagin, epigallocatechin gallate, curcumin, and clove oil combined with the antibiotics against *S. aureus* and *E. coli* standard and clinical strains.

MATERIAL AND METHODS

Microorganisms

Clinical strains of *E. coli* (E1) and *S. aureus* (S1), which were isolated from different patients in Clinical Microbiology Laboratories at Group Florence Nightingale Hospitals in Türkiye, were used in the study. These isolates were identified with the VITEK 2 system and confirmed by routine biochemical tests. We also used *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 (Rockville, MD, USA) standard strains in the study.

Antimicrobial Substances

Two antibiotics, meropenem (activity 986 µg / mg) and ciprofloxacin (activity 90.6%), were provided by Astra Zeneca Turkiye and five herbal substances, cinnamaldehyde (activity ≥95%), punicalagin (activity ≥98%), clove oil, curcumin (activity ≥65%), and epigallocatechin gallate (activity ≥95%), were provided by Sigma - Aldrich. The stock solutions of ciprofloxacin and herbal substances, except curcumin, were prepared at a concentration of 5120 mg/L and stored at -80°C for up to 6 months. Meropenem and curcumin were prepared daily in each experiment. Cinnamaldehyde and punicalagin were dissolved in DMSO, epigallocatechin gallate in distilled water, clove oil in Tween 80, and curcumin in ethanol.

Media

Cation-adjusted Mueller-Hinton broth (CAMHB, Difco Laboratories) was used for determining the minimum inhibitory concentration (MIC) and Checkerboard experiments, and Tryptic Soy Agar (TSA, Difco Laboratories) was used for bacterial cultures and colony counts.

Experimental Evolution Method

In accordance with the evolutionary microbiology dynamics, each day, bacteria were exposed to increasing doses of antibiotics, starting from low/sub-MIC concentrations. In this process, the development of resistance in bacteria which survived after exposure to antibiotics, was examined by determining the rising MIC values. For each experiment, two-fold meropenem and ciprofloxacin solutions were added to the TSA medium, starting from 1/20 x MIC up to the concentration which was not allow any bacterial regrowth. We also prepared the sub-MIC concentrations of herbal substances (1/4 x MIC) and added them to the antibiotic including TSA plates. After the each passage of standard and clinical isolates, bacterial colonies that continued to grow in the presence of antimicrobials were collected and stored in -80°C for MIC determinations (McDonald, 2019). Experiments were performed twice.

Determination of Minimum inhibitory concentration (MIC)

MICs of antibiotics and herbal substances were determined by the microbroth dilution technique as described by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2003; EUCAST, 2021). Serial two-fold dilutions of antibiotics and herbal substances were prepared in CAMHB in 96-well, U bottom microtiter plates. Each well was inoculated with overnight bacterial cultures that gave a final concentration of 5 x 10^5cfu/mL. The plates were covered and placed in plastic bags to prevent evaporation and incubated at 37°C for 18-20 h. The MIC was defined as the lowest concentration of antimicrobials, producing complete inhibition of visible growth. Experiments were performed twice.

Determination of Fractional Inhibition Concentration (FIC) index

We conducted this experiment to determine whether the antibiotic and herbal substance combinations had any interaction besides the evolutionary microbiology results. The effects of combinations were tested using the microbroth checkerboard technique (Pillai, Moellering & Eliopoulos, 2005). For this purpose, different concentrations of meropenem or ciprofloxacin between 1/8 x MIC - 2 x MIC in the vertical plane and herbal substances in the horizontal plane were mixed and tested against *E. coli* and *S. aureus* strains. Each microplate well containing the mixture of antimicrobials was inoculated with overnight bacterial cultures to give a final concentration of approximately 5 × 10^5cfu/mL. After incubation at 37°C for 18-20 h, the FIC values of each antimicrobial

were calculated as combined inhibitory concentration divided by the single concentration, and the FIC index was defined as sum of the FIC values. The combination value was derived from the highest dilution of antibiotic combination permitting no visible growth. Synergy was defined as a FIC index as ≤ 0.5, additive as > 0.5-4, and antagonism as > 4.0 (Odds, 2003). Experiments were performed twice.

RESULTS

Susceptibility

The MIC values of antibiotics and herbal substances against *E. coli* and *S. aureus* strains are shown in Table 1.

Experimental Evolution Results

The changes in the MIC values of meropenem and ciprofloxacin with or without herbal substances against *E. coli* and *S. aureus* are shown in Figures 1 and 2, respectively.

According to these experimental evolution results, when bacteria were exposed to increasing doses of antibiotics, we observed the resistance development for ciprofloxacin, and the higher MIC values for meropenem. After the inclusion of herbal substances to the study, although there was a slight increase in MIC values, the resistance development was not observed. (Table 2, and 3).

Table 1. MIC values of meropenem, ciprofloxacin, and herbal substances against *S. aureus* and *E. coli* strains (mg/L).

Bacteria	Mer	Cip	Clo	Cur	Cin	Epi	Pun
***S. aureus* (Clinical strain)**	0.032-0.25	0.125-0.5	3.47	40.6	0.125	60.8	0.12
***S. aureus* (ATCC 25923)**	2	0.25	3.47	20,3	0.25	60.8	0.12
***E. coli* (Clinical strain)**	0.003-0.032	0.003-0.5	3.47	325	0.5	121.,6	0.12
***E. coli* (ATCC 25922)**	0.016-0.03	0.008	3.47	325	0.25	121.6	0.12

Mer: Meropenem, Cip: Ciprofloxacin, Clo: Clove Oil, Cur: Curcumin, Cin: Cinnamaldehyde, Epi: Epigallocatechingallate, Pun: Punicalagin

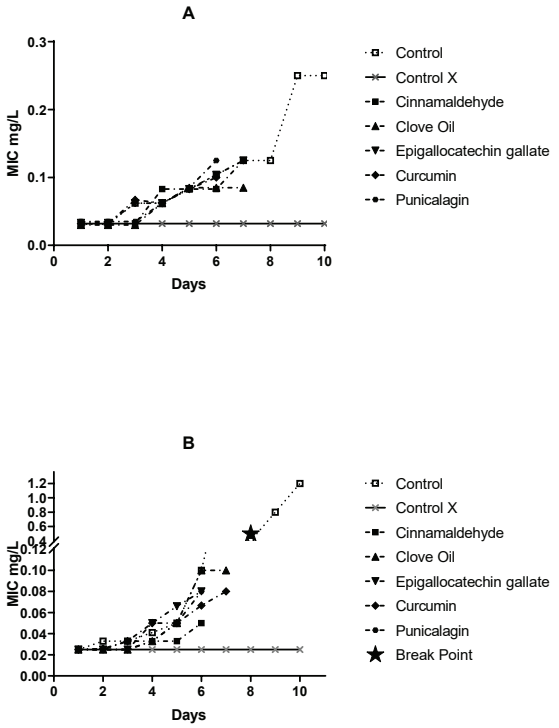


Figure 1. The changing MIC values of A: meropenem and B: ciprofloxacin with herbal substances against E. coli clinical strain. The X axis represents days that bacteria continue to grow; Y axis represents the MIC values after bacteria exposure to the antimicrobials. Break Point: EUCAST resistant breakpoint for E. coli (Meropenem: >8, Ciprofloxacin: >0.5). Control: Bacteria passage with only meropenem or ciprofloxacin. Control X: The bacteria passage without any antimicrobial substance.

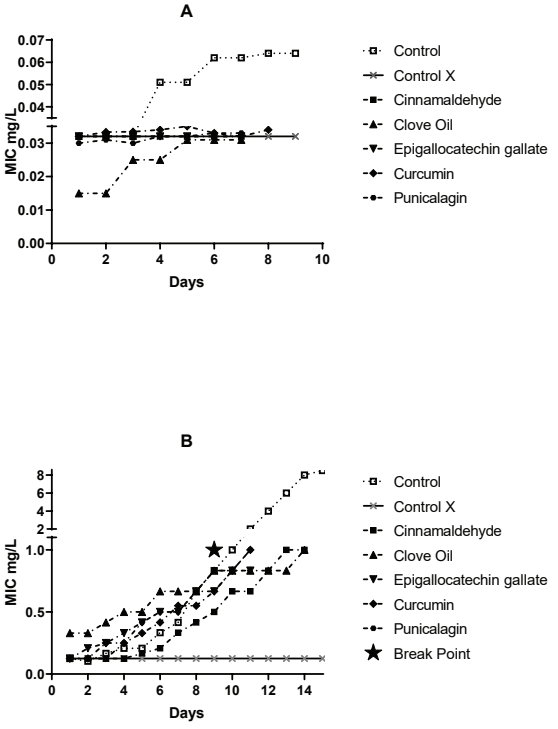


Figure 2. The changing MIC values of A: meropenem and B: ciprofloxacin with herbal substances against S. aureus clinical strain. The X axis represents days that bacteria continue to grow; Y axis represents the MIC values after bacteria exposure to the antimicrobials. Break Point: EUCAST resistant breakpoint for E. coli (Meropenem: >4, Ciprofloxacin: >1). Control: Bacteria passage with only meropenem or ciprofloxacin. Control X: The bacteria passage without any antimicrobial substance.

Table 2. FIC indexes of meropenem and ciprofloxacin combinations with herbal substances against *S. aureus* and *E. coli*.

	Staphylococcus aureus		*Escherichia coli*	
Herbal substances	**Meropenem**	**Ciprofloxacin**	**Meropenem**	**Ciprofloxacin**
Cinnamaldehyde	0.625	**0.5**	0.625	0.75
Curcumin	0.625	0.625	0.625	0.625
Epigallocatechin gallate	0.625	0.625	0.625	0.625
Clove Oil	**0.375**	**0.375**	0.75	0.75
Punicalagin	0.625	0.625	0.625	0.625

According to the FIC index, ≤0.5 as synergistic, 0.5 - 4.0 as additive, and ≥ 4.0 as antagonist

Table 3. The influence of herbal substances on antibiotic activities against *E. coli* and *S. aureus* in evolutionary microbiology assays.

	Escherichia coli		*Escherichia coli*		*Staphylococcus aureus*		*Staphylococcus aureus*	
	Resistance development		**Increase in MIC values**		**Resistance development**		**Increase in MIC values**	
Herbal substances	**Mer**	**Cip**	**Mer**	**Cip**	**Mer**	**Cip**	**Mer**	**Cip**
None	-	**+**	-	**+**	-	**+**	+	+
Cin	-	**-**	-	**-**	-	**-**	-	+
Cur	-	-	-	-	-	-	-	+
Ep	-	-	-	-	-	-	-	+
Clo	**-**	**-**	**-**	**-**	**-**	**-**	-	+
Pun	-	-	-	-	-	-	-	+

Mer: Meropenem, Cip: Ciprofloxacin, Clo: Clove Oil, Cur: Curcumin, Cin: Cinnamaldehyde, Epi: Epigallocatechin gallate, Pun: Punicalagin.

Combination studies

When we tested the combined effects of antibiotics and herbal substances, the synergistic interaction was observed against *S. aureus* with meropenem-clove oil, ciprofloxacin-clove oil, and ciprofloxacin-cinnamaldehyde combinations by the checkerboard method (Table 2).

DISCUSSION

In recent years, in parallel with the increasing number of antibiotic resistant microorganisms, the importance of infectious diseases caused by resistant pathogens has increased at an alarming rates. Due to the inadequacy of existing antibiotics for infection control, finding alternative treatment strategies has become very crucial. One novel therapeutic strategy involves using the herbal antimicrobial substances either alone or in combination with antibiotics. Since ancient times, plants have been the main resource that people administer for pain relief and treatment of many kind of diseases. A great deal of evidence suggests that the use of herbs for medicinal purposes dates back thousands of years, and today, the active ingredients of more than 50% of current drugs originate from plants. Considering the various studies about the treatment effects of herbs, used in traditional medicine, it could be said that traditional medicine guides modern medicine (Pan et al., 2013).

In order to slow down or stop the development of resistance and restore the effects of some antibiotics, of which clinical use has become very limited, we investigated the effects of some herbal substances. For this purpose, we studied the resistance development in clinical *S. aureus* and *E. coli* strains against meropenem or ciprofloxacin, and determined the effects of cinnamaldehyde, curcumin, epigallocatechin gallate, clove oil, and punicalagin in this process.

The selected herbal substances have been used by people for thousands of years and their antimicrobial activities have been demonstrated in various studies. Among them, cinnamaldehyde shows its antimicrobial activities by damaging the cell membrane of bacteria, inhibiting ATPases, or damaging cell division, motility, and biofilm formation (Vasconcelos, Croda & Simionatto, 2018). Punicalagin, obtained from pomegranate peel, causes morphological damages on the cell membranes and prevents biofilm formation (Xu et al., 2017). Green tea catechins inhibit the virulence factors and intracellular enzymes of bacteria, damage the cell membrane and cell wall, and cause oxidative stress, DNA damage, and iron chelation (Renzetti, Betts, Fukumoto & Rutherford, 2020). Curcumin, obtained from turmeric, has an antibacterial effect by inhibiting the virulence factors and biofilm formation mechanisms of bacteria, and also by preventing the adhesion to host tissues (Zheng et al., 2020). Clove oil, obtained from

the clove plant, affects the movement of bacteria and their adhesion forces. According to in vitro studies such as cytotoxicity on fibroblasts and other cells, these substances are considered as safe (Hu, Zhou & Wei, 2018). Additionally, the presence of these substances in food such as cinnamon, pomegranate, green tea, turmeric, and cloves, which are frequently consumed in daily life, provides the correlation with those studies.

As shown in Figure 1 and Table 3, when *E. coli* was exposed to increasingly larger ciprofloxacin doses, we observed the rising MIC values, resistance development, and surviving bacteria even reaching 0.8 mg/L concentration. On the other hand, when *E. coli* was exposed to increasingly larger concentrations of meropenem, although we observed an increase in MIC values, the resistance breakpoints were not exceeded, and all bacteria died at a concentration of 0.25 mg/L at the end of ten days. There were no changes in the MICs of the *E. coli* control group, which were passage without being exposed any antimicrobial agent, and the MIC values were 0.025 and 0.032 mg/L for ciprofloxacin and meropenem, respectively. Similarly, Fröhlich and friends showed that the sub-MICs of ceftazidime could affect the development of resistance in *E. coli* by evolutionary microbiology, and these results indicates that the exposure to β-lactams at very low concentrations drives the evolution of β-lactamases. On the other hand, Mantage and friends indicated that, when *E. coli* was exposed to low concentrations of rifampicin, there was no development of resistance, and instead their population adapted to the antimicrobial environment via evolving small colony variants. These results indicated that some lifestyle changes have the potential to affect the ultimate fate of populations through mutations and altering genetic make up (Matange, Hegde & Bodkhe, 2019; Fröhlich et al., 2021).

When we included the herbal substances in the experiment, although there was a slight increase in MIC values, we did not observe any resistance for ciprofloxacin or meropenem in *E. coli*. Also, the MIC values were always below the MICs without herbal substances. However, these results might be explained by the meropenem resistance gene that is effective in *E. coli* (Hong et al., 2005; Hitzenbichler et al., 2018); the changes in the MIC values might also results from the deterioration in colony morphology due to the long-term passages. These results suggested that during the antibiotic treatment, consuming the studied herbal substances, could be effective to prevent or delay the development of antibiotic resistance.

As shown in Figure 2 and Table 3, when *S. aureus* was exposed to increasingly larger ciprofloxacin doses, similar to *E. coli*, we observed the rising MIC values, resistance development, and surviving bacteria even reaching 8 mg/L concentration. When *S. aureus* was exposed to increasing concentrations of meropenem, we didn't observe any increase in MIC values and the resistance breakpoints were not exceeded, and also all bacteria died at a concentration of 0.064 mg/L, at the end of nine days. There were no changes in the MICs of the *S. aureus* control group which were passage without any antimicrobial agent, and the MIC values were 0.125 and 0.032 mg/L for ciprofloxacin and meropenem, respectively. Similarly, Johnson and Levin showed that, sub-MIC concentrations of ciprofloxacin and gentamicin resulted in increased surviving bacterial fraction (level of persistence), and they postulated that persistence is an inevitable consequence of a metabolic disruption, similar to mutation in cell replication (Johnson & Levin, 2013).

When we included the herbal substances in the experiment, although there was a slight increase in MIC values, we did not observe any resistance for ciprofloxacin in *S. aureus*. For meropenem, no resistance development was observed, and also the MIC values were not increased, after we added the herbal substances, except cinnamaldehyde. These results indicated that there are different mechanisms required for the development of meropenem resistance in *S. aureus* (Lemaire, Van Bambeke, Mingeot-Leclercq, Glupczynski & Tulkens, 2007; Hassanzadeh et al., 2017). These results also suggested that consuming herbs during the antibiotic course could be effective for preventing the development of antibiotic resistance.

In this study, considering the possible other interactions between studied antibiotics and herbal substances, we perform the microdilution checkerboard assay, which is the most popular and prevalent in-vitro combination test. According to our results, synergy was observed with meropenem-clove oil, ciprofloxacin-clove oil, and ciprofloxacin-cinnamaldehyde combinations against *S. aureus*. When these results are considered together, in evolutionary microbiology studies performed with clove oil, the MIC values of meropenem and ciprofloxacin against *S. aureus* were decreased, and found lower than the antibiotic free control group, as expected.

Considering the differences in *E. coli* and *S. aureus* results, the morphological differences between Gram positive and Gram negative bacteria seems to be an important factor, in addition to the possibilities related to the action mechanisms, mentioned above. Meropenem, is a beta lactam antibiotic that acts on a peptidoglycan layer of the cell wall, while ciprofloxacin belongs to the fluoroquinolone group, which targets the DNA gyrase enzymes of bacteria. The development of resistance against ciprofloxacin is generally acquired spontaneous chromosomal mutations in genes, while beta lactams generally disrupted by beta lactamases enzymes. As a result of mutations, changes in DNA gyrase enzymes or decreases in membrane permeability may occur in bacteria (Hooper, 2001). We thought that the serial passage procedures in the evolutionary microbiology may hasten, and support the mutations that allows the fluoroquinolone resistance in bacteria. On the other hand, the studied herbal substances generally act against the bacterial cell wall or cytoplasmic membrane, and we thought that the prevention of ciprofloxacin resistance by herbal substances might be related to those combinations of different targets.

CONCLUSION

In this study, it was shown that, very low levels of antimicrobials can direct the development of antimicrobial resistance. We also showed that the combination of sub-MIC herbal substances and antibiotics prevents the development of resistance or

reduces MICs against *E. coli* and *S. aureus*. According to these results, we thought that the use of herbs such as cinnamon, cloves, turmeric, green tea, and pomegranate, together with antibiotics, might be beneficial in avoiding or delaying resistance, and this should be investigated against different types of bacteria.

Ethics: This study was approved by Istanbul University, Faculty of Medicine, Clinical research ethics committee (07.03.2017-91678).

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- C.Ö., S.D.; Data Acquisition- C.Ö.; Data Analysis/Interpretation- C.Ö., S.D.; Drafting Manuscript- C.Ö.; Critical Revision of Manuscript- S.D.; Final Approval and Accountability- C.Ö., S.D.

Conflict of Interest: The authors have no conflict of interest to declare.

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