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Antimicrobial synergistic effects of apigenin, (-)-epigallocatechin-3-gallate, myricetin and luteolin in combination with some antibiotics

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Abstract

Introduction and Objective. Antimicrobial resistance, which is considered one of the most important problems of the 21st century, brings many problems with it, such as increasing mortality rates and treatment costs. Difficulties in the treatment of infections caused by resistant microorganisms have led to the search and need for new antimicrobials or new molecules that interact synergistically with antimicrobials. The aim of this study is to investigate whether various flavonoids have synergistic effects with some antibiotics.

Materials and method. During this study, standard bacterial strains *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 29213 and *Staphylococcus aureus ATCC* 43300 were used. Minimal inhibitory concentrations of all antibiotics and flavonoids were found by the broth microdilution method. Interactions between antibiotics and flavonoids were then determined by using the checkerboard method. Interactions between antibiotics and flavonoids were evaluated according to the FIC index (ΣFIC) results.

Results. According to the results of the microdilution test, the bacterial strains used in this study (except for MRSA) were generally sensitive to antibiotics. Interaction study results showed promising results regarding the synergistic interactions of antibiotics with flavonoids. Epigallocatechin gallate and luteolin especially showed synergistic interaction with antibiotics in many microorganisms. It was found that myricetin showed synergistic interaction only with levofloxacin. Likewise, it was detected that apigenin had limited synergistic interaction with antibiotics.

Conclusions. The obtained results highlight that flavonoids may be a useful tool in overcoming antibiotic resistance.

Key words

(-)-epigallocatechin-3-gallate, apigenin, luteolin, myricetin, synergistic interaction

INTRODUCTION

Multi-drug-resistant bacteria, which attracts attention as a global health problem, has been a concern of the scientific world for many years. Considering their replication rate, microorganisms are thought to be extremely successful organisms in adapting to environmental conditions. Moreover, it is known that microorganisms have some mechanisms to resist antibiotics for more than 2 billion years. In order to prevent the spread of antibiotic resistance, suggestions, such as paying attention to the unnecessary use of antibiotics, not using antibiotics in viral infections, and not using antibiotics unnecessarily in animal husbandry, are offered [1, 2, 3]. Despite all these recommendations, antimicrobial resistance is accepted as one of the most important health issues of the 21st century. Moreover, it is estimated that up until 2050, around 10 million people will die due to this reason [4]. In November each year, the World Health Organization (WHO) organizes a World Antibiotic Awareness Week campaign to draw attention to this issue [5].

The increasing resistance against antibiotics continuously limits treatment options. Difficulties, such as death rates and treatment costs as inevitable results of high antimicrobial resistance, bring the need for new antimicrobial agents into discussion [6, 7, 8]. In recent years, combination treatments have become a preferred treatment method, especially for infection diseases due to multidrug-resistant microorganisms [9, 10].

Throughout human history, plants have been used for such basic purposes as nutrition, cosmetics, and health. Thanks to synergistic interactions, the therapeutic benefits of botanical sources have been known since ancient times. Many different cultures believed in the positive effects of combination treatments and have benefitted from this practice in the field of healthcare. This places the emphasis on how beneficial botanical sources could be, while antimicrobial resistance increasingly becomes a global health challenge. It is known that plants are widely used as therapeutic agents, especially in South America [9, 11].

Synergy is the effect of substances combining different components to produce a greater effect than expected [12]. In the light of knowledge since ancient times, studies on the synergistic interaction of plants or plant-based molecules with antibiotics have gained significance, considering that antibiotic resistance has become an important problem worldwide [8, 9, 13, 14]. Natural products are seen as a crucial source for the synthesis of new drug molecules. Plants consist of different natural components for the continuity of their

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vital activity [15], and one of these components, flavonoids, are plant pigments found in colourful flower petals and green plant cells. It is known that flavonoids, which play a role in plant growth and defence, have either an inhibiting or deadly effect on bacteria, viruses, and some protozoa, and could also increase the effect of antibiotics [16, 17].

OBJECTIVE

The purpose of the study is to investigate the antibacterial properties of apigenin, (-)-epigallocatechin-3-gallate, myricetin and luteolin – various flavonoid groups, and whether they have synergistic interactions with some antibiotics.

MATERIALS AND METHOD

Study materials. All of the antibiotics [gentamicin, levofloxacin, ampicillin, trimethoprim) and test compounds (apigenin, (-)-epigallocatechin-3-gallate, myricetin, luteolin)] were purchased from Sigma Aldrich (St. Louis, (MO), USA). Cation-adjusted Mueller-Hinton broth (CAMHB) was purchased from Oxoid (Thermo Scientific, UK) and standard bacterial strains [*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 29213 (methicillin sensitive-MSSA), *Staphylococcus aureus* ATCC 43300 (methicillin resistant MRSA)], were obtained from the bacterial library of the authors' faculty.

***In-vitro* susceptibility tests.** Before starting the susceptibility studies, stock solutions were prepared from all the antibiotics and flavonoids. For the stock solutions of gentamicin and trimethoprim CAMHB, and for the stock solutions of other antibiotics and flavonoids, 10% dimethyl sulfoxide (DMSO) were used. After the stock solutions were prepared, the minimum inhibitory concentration (MIC) values of all antibiotics and flavonoids were found by broth microdilution technique. In this technique, using a 96 well microplate, all antibiotics and flavonoids were diluted within the range of 1,024 µg/mL- 0.5 µg/mL via 2-fold serial dilution. After the bacteria addition (5×10^5 CFU/mL), the MIC value was determined as the lowest concentration which had no visible turbidity in the wells of plates incubated for 18–24 hours at 37 °C. The interactions between antibiotics and flavonoids were then determined using the checkerboard method. In the checkerboard method, the effectiveness of antibiotic and flavonoid combinations was tested, with dilutions prepared two times above and four times below the MIC values of antibiotics and flavonoids, simultaneously on 96 well microplate for each bacterium.

The type of interaction between antibiotics and flavonoids, the fractional inhibition concentration index (ΣFIC) values of the combinations were calculated and defined using the formula below. Calculated FIC index values were evaluated as follows: ≤ 0.5 – 'synergy', 0.50–1 – 'partial synergy', =1 – 'additive', 1–4 – 'indifferent', and > 4.00 – 'antagonism' [18, 19].

$$\text{FIC A } (FIC_A) = \text{MIC of A in combination / MIC of A alone}$$
$$\text{FIC B } (FIC_B) = \text{MIC of B in combination / MIC of B alone}$$
$$\text{FIC index } (\Sigma FIC) = FIC_A + FIC_B$$

RESULTS

According to the results of the microdilution test, the bacterial strains used in the study (except for MRSA) were generally sensitive to antibiotics. MIC values of antibiotics are shown in Table 1 and MIC values of flavonoids in Table 2.

Table 1. MIC values of antibiotics (µg/mL)

	E. coli	*K. pneumoniae*	*P. aeruginosa*	MSSA	MRSA
Gentamicin	0.5	0.5	0.5	0.5	32
Levofloxacin	0.5	0.5	16	0.5	32
Ampicillin	2	4	*	8	512
Trimethoprim	1	2	*	2	8

* antibiotics not studied

Table 2. MIC values of flavonoids (µg/mL)

	E. coli	*K. pneumoniae*	*P. aeruginosa*	MSSA	MRSA
Apigenin	128	64	128	64	512
EGCG	32	32	64	128	256
Myricetin	64	64	64	256	1024
Luteolin	128	64	128	64	256

EGCG – (-)-epigallocatechin-3-gallate

The interaction study results using the checkerboard method proved to be promising regarding the synergistic interactions of antibiotics with flavonoids. Epigallocatechin gallate and luteolin especially showed synergistic interaction with antibiotics in many microorganisms. It was found that myricetin showed synergistic interaction only with levofloxacin. Likewise, it was detected that apigenin had limited synergistic interaction with antibiotics (Tab. 3).

DISCUSSION

The fact that antimicrobial resistance has become an important problem affecting human health and the economy worldwide has resulted in the urgent need to develop new antimicrobials [16]. In general, microorganisms can easily develop resistance to most of the available antimicrobials. In order to overcome this resistance, it is necessary either to synthesize new molecules that do not contain substitutions on the known drug, or to discover a new target for existing antimicrobials [15]. It is a known fact that plants have antimicrobial effects, and a prominent option for dealing with antimicrobial resistance is to combine antibiotics with various plant components [8]. Flavonoids are secondary metabolites that are ubiquitous in various parts of plants, from their leaves to their flowers and fruits. These compounds, which are important for the plant's defence system, also have beneficial effects for human health [15]. It is reported that flavonoids show their effects on plant pathogens as well on human pathogens [20]. Bacteria can acquire resistance to antibiotics by various mechanisms, such as changes in the target site of the drug, excretion of the antibiotic from the cell, and modification of the drug. However, they cannot develop resistance to flavonoids, which act by mechanisms different from antibiotics [15, 21]. Hence, this highlights the potential role of flavonoids in research against antibiotic resistance

Table 3. ΣFIC values of combinations of antibiotics and flavonoids

	E. coli		*K. pneumoniae*		*P. aeruginosa*		MSSA		MRSA	
	ΣFIC	int	ΣFIC	int	ΣFIC	int	ΣFIC	int	ΣFIC	int
GN+AP	0.56	PS	0.56	PS	0.37	SN	0.31	SN	0.5	SN
GN+EGCG	0.5	SN	0.37	SN	1	AD	1.25	IN	2.5	IN
GN+MYR	2.5	IN	2.25	IN	2.5	IN	1	AD	2.25	IN
GN+LUT	0.31	SN	0.5	SN	0.37	SN	0.31	SN	0.75	PS
LEVO+AP	0.75	PS	0.56	PS	0.37	SN	0.31	SN	0.31	SN
LEVO+EGCG	0.28	SN	0.31	SN	0.5	SN	0.12	SN	0.26	SN
LEVO+MYR	0.75	PS	0.5	SN	0.12	SN	0.5	SN	0.5	SN
LEVO+LUT	0.37	SN	0.31	SN	0.56	PS	0.5	SN	0.5	SN
AM+AP	1	AD	1.25	IN	**	**	1.5	IN	1.5	IN
AM+EGCG	0.25	SN	0.28	SN	**	**	0.31	SN	0.37	SN
AM+MYR	2.5	IN	2	IN	**	**	1.25	IN	1.5	IN
AM+LUT	0.5	SN	0.5	SN	**	**	0.75	PS	1.25	IN
TRI+AP	1	AD	0.75	PS	**	**	2.5	IN	2	IN
TRI+EGCG	0.75	PS	0.62	PS	**	**	0.62	PS	0.75	PS
TRI+MYR	1	AD	0.75	PS	**	**	1.5	IN	2.5	IN
TRI+LUT	0.31	SN	0.5	SN	**	**	0.75	PS	0.75	PS

Int – Interaction; SN – Synergy; PS – Partial synergy; AD – Additive; IN – Indifference; GN – Gentamicin; LEVO – Levofloxacin; AM – Ampicillin; TRI – Trimethoprim; AP – Apigenin; EGCG – (-)-epigallocatechin-3-gallate; MYR – Myricetin; LUT – Luteolin.
** combinations not studied

as they stand out as exceptional compared to traditional antibacterial treatment methods. The potential of flavonoids to increase the effectiveness of known antibiotics has been a feature that stands out more than their antibacterial effects [16]. This suggests that even though they might not be an adequate treatment option alone, flavonoids could be contributors to ongoing treatment methods.

The presented study, which was designed in consideration of the above-information, investigated whether 4 different flavonoids have synergistic interactions with various antibiotics. Apigenin (4,5,7-trihydroxyflavone) is a flavonoid of the flavone group commonly found in vegetables and fruits. It is known that apigenin, which has an important place in the human diet, has many positive properties such as anticancer, antibacterial, anti-inflammatory and antioxidant properties [22]. It has been reported that apigenin interacts synergistically with ceftazidime on ceftazidime-resistant *Enterobacter cloacae* [23], while another study reported that apigenin has reverse antibiotic activity against quinolone resistant bacteria [13]. Cha et al. reported that apigenin showed significant antibacterial and synergistic activity with antibiotics against oral pathogens [22]. The current study determined that apigenin interacts synergistically with gentamicin and levofloxacin, especially in MSSA, MRSA strains and *P. aeruginosa*. In general, partial synergistic interactions were observed in *E. coli* and *K. pneumoniae*. In the light of all these data, it can be concluded that apigenin shows promise in dealing with drug resistance.

In this study, the interactions between (-)-epigallocatechin-3-gallate, another popular flavonoid in the scientific world, and antibiotics were also evaluated, and extremely positive results were obtained. (-)-epigallocatechin-3-gallate is a flavonoid name of which is identified with green tea and has come to the fore with its health benefits, having antifungal, antibacterial, antiviral properties [24]. Hu et. al. found that epigallocatechin gallate decreased ampicillin-sulbactam MIC values in MRSA strains [14]. In the presented study, (-)-epigallocatechin-3-gallate also showed synergistic interactions with other antibiotics in *Staphylococcus* strains, with the exception of gentamicin. Myricetin, obtained from such sources as vegetables, fruits and tea in our daily diet, is a flavone that provides many medical benefits [25]. Although myricetin was effective in bacteria (except for MRSA) at MIC values, which would not be considered very high, in this study it did not give positive results in terms of synergistic interaction (except in combination with levofloxacin). Although data on the antimicrobial activity of myricetin in the literature are limited compared to other flavonoids, Lin et al. reported that myricetin showed synergistic interaction with various antibiotics in ESBL-producing *K. pneumoniae* strains [26]. In this case, it is necessary to conduct more studies on myricetin.

Another flavonoid that stands out thanks to its pharmacological properties is luteolin. Guo et al. concluded that luteolin has antibacterial activity against *Trueperella pyogenes*, thanks to its effectiveness on the cell wall and cell membrane, on protein synthesis, nucleic acid synthesis and cell metabolism [27]. Another study shows that luteolin potentiates the effects of β-lactam and aminoglycoside antibiotics against MRSA [28]. In the current study, it was also found that luteolin had a synergistic or partial synergistic effect with antibiotics in almost all bacteria included in the study.

As a result of evaluating all the data from the current study with data from previous studies, it became obvious that flavonoids can help in overcoming antibiotic resistance, which is one of the leading health problems worldwide. The addition of more data and the encouragement of researchers to continue with studies may open new doors for the future.

CONCLUSIONS

The combination of natural components with existing antibiotics, and the realization and fruition of studies on the transformation of these ideas into pharmaceutical products, might be our biggest weapon against antibiotic resistance under current circumstances. When combined with traditional antibacterial treatment methods, flavonoids could be a strong contributor to overcoming antibiotic resistance.

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