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Research Article

IN VITRO INVESTIGATION ON SYNERGISTIC ANTIMICROBIAL EFFECTS OF DAIDZEIN, ROYAL JELLY AND BORIC ACID IN COMBINATION WITH CERTAIN ANTIBIOTICS AGAINST GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA STRAINS

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ABSTRACT. The prevalence of infections caused by multiple antibiotic resistant microorganisms has made it important to investigate substances that may have synergistic effects with antibiotics. The aim of this study is to investigate possible synergistic antimicrobial effects of daidzein (DZ), royal jelly (RJ) and boric acid (BA) in combination with certain antibiotics against different bacteria strains. *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Entercoccus faecium*, *Pseudomononas aeruginosa* and as a control strain, *S. aureus* ATCC 43300 were used in this study. Daidzein, royal jelly, boric acid, cefuroxime sodium (Cef), levofloxacin hemihydrate (Levo) and trimethoprim (Tri) MIC values were determined by the method of macrodilution. In vitro synergy tests were studied using the checkerboard method. The combination of Levo+BA against bacteria strains *S. aureus*, *E. coli* and *E. faecium*; and the combination of Tri+BA against *S. aureus*, *E. coli* and *K. pneumoniae* had a significant synergistic interaction (ΣFIC<0.5). The combinations of Cef+RJ against *K. pneumoniae*, Levo+RJ against *E. coli*, and Tri+RJ against *S. aureus*, *E. coli* and *K. pneumoniae* had also a significant synergistic interaction (ΣFIC<0.5). In addition, the Levo+DZ combination showed synergistic interaction against *E. coli* and *E. faecium* strains. Our study results showed that the effectiveness of some antibiotics against different bacterial strains can be increased, especially in combination with BA or RJ.

Keywords: *antimicrobial synergy, boric acid, checkerboard method, daidzein, royal jelly*

INTRODUCTION

When antibiotics first appeared, resistance was not a problem; however the widespread use of antibiotics in the treatment of bacterial infections has led to the emergence and spread of resistant strains [1]. In the last decade, a significant increase in the number of pathogens with multiple resistance to antibiotics was reported. Large-scale organizations, such as, the World Health Organization (WHO) and the US Centers for Disease Control and Prevention (CDC) have identified infections caused by multiple resistant bacteria as a major public health problem [2]. Multiple antibiotic-resistant pathogenic bacteria give rise to a significant issue: infections that are difficult to treat with conventional antibiotics. Thus, the increasing number of resistant pathogens has drastically raised the need for new antibiotics. As a result of this need, in recent years, several studies have been conducted

to develop new antimicrobials from different sources to combat resistant pathogens [1, 3-5].

Among the different approaches used to find the effective agents in overcoming bacterial resistance, plant products containing compounds with antimicrobial effects appear to be one of the most suitable strategies [6]. Isoflavones [such as genistin and daidzin as glucosides; genistein and daidzein (DZ) as aglycones] are phenolic compounds, which is a substance found in soybeans and other legumes [7-9]. The compounds have antioxidant and antimicrobial activities and also estrogenic properties. Their significant health benefits include reductions in the incidences of postmenopausal ailments, cardiovascular disease, and osteoporosis, chemoprevention of breast and prostate cancers, and protection from ionizing radiation-induced injury. The antimicrobial effect of isoflavones against some strains of potentially human pathogenic bacteria was demonstrated by previously published studies [6-8, 10, 11]. Unlike isoflavones, boron is a mineral element, which is present in the non-elemental form [boric acid (BA), borates etc.] in nature [12]. BA and its derivatives have been used for medicinal purposes since the 1860s, due to their bactericide, antiseptic and fungicide functions to control bacterial and fungal infections [13].

Royal Jelly (RJ), which is secreted from the hypopharyngeal and mandibular salivary glands of bees aged 5-14 days, is a white-yellowish gelatinous-viscous glandular secretion. RJ and its protein and lipid contents have been analyzed to determine their antimicrobial activities for probable medicinal use in the future. The antimicrobial activities of crude RJ, 10-hydroxy-2-decenoic acid, royalisin, jelleines, major RJ proteins on different bacteria (especially gram positive bacteria) have been documented [14].

Research regarding the interactions of antimicrobial compounds has gained popularity in recent years [15]. Today, the role of antibacterial synergism is crucial in the treatment of a wide range of infectious diseases [1, 2]. The aim of this study is to investigate possible synergistic antimicrobial effects of daidzein, royal jelly and boric acid in combination with certain antibiotics against bacteria such as *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Enterococcus faecium* using the checkerboard assay.

MATERIALS AND METHODS

Antibiotics and other compounds

The cefuroxime sodium, levofloxacin hemihydrate, trimethoprim used in this study were generous gifts from DEVA Holding A.Ş. (Turkey). The DZ and BA were purchased from LC Laboratories (USA) and Doğa İlaç Hammaddeler Tic. Ltd. Şti. (Turkey), respectively. The RJ was obtained from beekeepers in Erzurum, Turkey.

Bacterial strains

S. aureus, E. coli, K. pneumoniae, P. aeruginosa and *E. faecium* strains were obtained from Erzurum Regional Training and Research Hospital, Department of Microbiology. *S. aureus* ATCC 43300 was used as the control strain. The bacterial strains were cultured onto sheep-blood agar (BD Difco, USA) and eosin methylene blue agar (BD Difco, USA) and incubated at 37°C for 18 to 24 hours. Pure colonies of each strain were inoculated into cation-adjusted Mueller-Hinton broth (CAMHB) (Oxoid, Italy) and adjusted to turbidity to 0.5 McFarland standard.

Determination of minimum inhibitory concentration (MIC) by macro-dilution method

MIC values of known antibiotics and the compounds (DZ, RJ and BA) were determined by the macrodilution method according to the Clinical and Laboratory Standards Institute (CLSI) recommendations [16]. In accordance, stock dilutions of known antibiotics (3200 µg/mL for water-insoluble agents and 1024 µg/mL for the others) and the compounds were prepared (64 mg/mL for BA, 512 mg/mL RJ agents and 512 µg/mL for DZ). Stock solutions of water-insoluble antibiotics and daidzein were prepared using DMSO, and the DMSO concentration was adjusted to be 10% in the first tube. Moreover, as a control, the antimicrobial activity of 10% the DMSO solution was evaluated and found to be ineffective. The stock solutions were filtered using membrane filter (0.22 µm) in aseptic conditions. For macrodilution method, 15 tubes were prepared and 0.5 mL of CAMHB medium was placed in all tubes except for the first one. 0.5 mL of the main dilution of the antibiotic or compounds to be tested was placed in the first and second tube and serial two-fold dilutions were made starting from tube 2. Subsequently, 0.5 mL of microorganism was placed in a homogenous suspension (0.5 McFarland standard) in all tubes and incubated at 37 °C for 18-24 hours. The lowest concentration without visible growth of each strain was evaluated as MIC. Each experiment was repeated three times.

In vitro determination of possible synergistic effects of antibiotics and the compounds (DZ, RJ and BA) in combinations

In this study, the checkerboard assay was used for the determination of in vitro interactions between the compound (either DZ, RJ or BA) and certain antibiotics in several combinations. Therefore, a 96 well microplate panel was used to test the combinations at different concentrations against each bacterial strain. The range of tested concentrations of antibiotics and the compound (either DZ, RJ or BA) varies from two times the MIC to at least 1/4 of the MIC determined for each bacteria.

For evaluation of the results, the fractional inhibitory concentration (FIC) is calculated for each antibiotic and the compound using the following equation.

$$FIC \text{ of compound A } (FIC_A) = MIC \text{ of compound A in combination / } MIC \text{ of compound A alone}$$

$$FIC \text{ of compound B } (FIC_B) = MIC \text{ of compound B in combination / } MIC \text{ of compound B alone}$$

$$FIC \text{ index } (\Sigma FIC) = FIC_A + FIC_B$$

Eqn. 1

The interaction of substances in the combination was defined as synergistic if ΣFIC value was ≤0.5, as partial synergistic if ΣFIC value was between 0.5 and 1, as additive if ΣFIC value was 1, as indifference (no interaction) if ΣFIC value was between 1 and 4, and as antagonistic if ΣFIC value was ≥4 [15, 17].

RESULTS AND DISCUSSION

The obtained MIC values of the antibiotics and the compounds against gram-negative and gram-positive bacteria were given in Table 1. According to these results, *S. aureus* was resistant to trimethoprim and *E. faecium* was resistant to levofloxacin hemihydrate. The other bacteria were susceptible to all antibiotics.

The calculated ΣFIC values of the antibiotics and the compound (either DZ, RJ or BA) in combination with all the bacteria strains were given in Table 2. The results indicated that DZ, RJ and BA can interact synergistically with the antibiotics. Especially, for the *E. faecium* strain, a synergistic interaction (ΣFIC≤ 0.5) was observed in all combinations of levofloxacin hemihydrate with either DZ, RJ or BA. However, for the *S. aureus* strain, a synergistic interaction for the combination of trimethoprim with only BA or RJ was obtained. When the results of this study are to be evaluated in general, it can be deduced that positive results were obtained especially regarding the synergistic interactions of BA and RJ with antibiotics. However, it was found that the combination of trimethoprim with DZ showed antagonist effect (ΣFIC ≥4) for *S. auresus, E. coli* and *K. pneumoniae* strains.

Table 1. *The MIC values of the antibiotics and the compounds against gram-negative and gram-positive bacteria*

Antibiotics / Compounds	*S. aureus*	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*E. faecium*	*S. aureus* ATCC 43300
Cef	*	2 µg/mL	2 µg/mL	*	*	*
Levo	0.5 µg/mL	0.25 µg/mL	0.06 µg/mL	2 µg/mL	8 µg/mL	0.06 µg/mL
Tri	25 µg/mL	1.5625 µg/mL	3.13 µg/mL	*	*	6.25 µg/mL
BA	16 mg/mL	8 mg/mL	8 mg/mL	4 mg/mL	*	16 mg/mL
RJ	64 mg/mL	32 mg/mL	256 mg/mL	256 mg/mL	*	64 mg/mL
DZ	512 mg/mL	512 mg/mL	256 mg/mL	256 mg/mL	*	512 mg/mL

Cef:Cefuroxime sodium; Levo:Levofloxacin hemihydrate; Tri: Trimethoprim; BA: Boric acid; RJ: Royal Jelly; DZ: Daidzein. *:Antibiotics, not suitable for bacteria, were excluded from the study

Table 2. *ΣFIC values of the antibiotics and the compound (DZ or BA or RJ) in combination for all the bacteria strains*

Combinations	*S. aureus*	*E. coli*	*K.pneumoniae*	*P. aeruginosa*	*E. faecium*	*S. aureus* ATCC 43300
Cef+BA	**	0.56	1	**	**	**
Cef+RJ	**	0.56	0.16	**	**	**
Cef+DZ	**	0.56	1.5	**	**	**
Levo+BA	0.31	0.12	0.75	1	0.18	0.19
Levo+RJ	0.75	0.34	0.53	1.06	0.12	1.06
Levo+DZ	0.75	0.26	0.53	1.06	0.12	0.74
Tri+BA	0.16	0.12	0.38	**	**	0.31
Tri+RJ	0.31	0.25	0.26	**	**	0.18
Tri+DZ	≥4	≥4	≥4	**	**	0.37

Cef:Cefuroxime sodium; Levo:Levofloxacin hemihydrate; Tri: Trimethoprim; BA: Boric acid; RJ: Royal Jelly; DZ: Daidzein. **These combinations were not studied

Infections caused by multidrug-resistant (MDR) bacteria are considered as an emergent global disease and a major public health problem. The present global threat of antimicrobial resistance prompts the urgent need to find new antibacterial products [2]. Besides, the use of antimicrobial agents in combinations may produce synergistic effects in the treatment of bacterial infection, reduce their adverse effects, and provide broad antimicrobial spectrum and also, delay the emergence of antimicrobial resistance [1, 5]. Plants and other natural products are still major sources of new antimicrobial agents. These sources may provide many complex and structurally different active compounds [4]. The interaction of already known, certain antibiotics with natural antimicrobial products in combinations may raise useful (additive/synergistic) or harmful (antagonistic/toxic) outcomes [1, 3]. Recently, studies focusing on the investigation of antimicrobial activities of natural products and new-synthesized molecules are being performed [4]. Soy isoflavones mostly prevent nucleic acid synthesis by decreasing of RNA and DNA of bacterial cells [7]. In this context, the effect of DZ, an isoflavone, on microorganisms is associated with its inhibitory activity against topoisomerase 1. Isoflavones generally show inhibitory activity against *S. aureus* but not *E. coli* [8, 18].

Mbaveng et al. [19]. investigated the antimicrobial activity of 19 natural products (terpenoids, alkaloids, thiophenes and phenolics compounds) against 14 gram-negative multidrug-resistant bacteria strains (*E. coli, K.pneumoniae, P. aeruginosa* etc.). They found that the MIC values for DZ were in range of 64-256 µg/mL.

In our study, the MIC values obtained for DZ were 256 µg/mL against *K.pneumoniae*, *P. aeruginosa* and 512 µg/mL against *S. aureus* and *E. coli.* However, we evaluated the interaction of DZ with certain known antibiotics in different combinations. The Levo+DZ combination showed synergistic interaction against *E. coli* and *E. faecium* strains. On the other hand, the Tri+DZ combination showed antagonistic interaction against *S. aureus, E. coli* and *K. pneumonia*e strains.

BA shows low-toxicity, herbicidal properties and fungicidal effect. Therefore, BA and its salts have been used in medicine as an antiseptic, a bactericide and a fungicide for many years. (13). Zan et al. [13]. investigated the antimicrobial effects of BA (at concentration of 2-6%) in root canals infected with *E. faecalis* biofilms and they found that especially the 6% concentration of BA demonstrated the highest antibacterial effect. In addition, Reichman et al. [20]. evaluated the effectiveness of the combination of BA and nitroimidazole in the treatment of recurrent bacterial vaginosis, which is the most common vaginal infection worldwide. The use of BA and nitroimidazole in a combination demonstrated reasonable cure results. They reported that the combination has a low cost, a good safety profile and hence, may be beneficial for the treatment of bacterial vaginosis. In our study, the MIC values for BA against *S. aureus, E. coli, K. pneumoniae, P. aeruginosa* strains were found to be in the range of 4-16 mg/mL. Besides, the Levo+BA combination against *S. aureus, E. coli, E. faecium* and the Tri+BA combination against *S. aureus, E. coli, K. pneumoniae* had a significant synergistic interaction ($\Sigma FIC<0.5$).

Since ancient times, RJ, which has been used in traditional medicine in Asia, especially in China and is thought to be the reason for the longevity of ancient dynasties of China, is notable for its antibacterial and antifungal properties [14, 21].

Susilowati et al. [22]. reported the anti-adherent, antibacterial, and anti-inflammatory effects of RJ against *P. aeruginosa* and found that RJ inhibits adherence and prevents excessive inflammatory responses against *P. aeruginosa* infection. In another study, it was reported that major RJ protein 2, which is a major protein in RJ, is responsible for the antioxidant and antimicrobial activities of RJ [23].

In our study, the MIC values for RJ against *S. aureus, E. coli, K. pneumoniae, P. aeruginosa* strains were found to be in the range of 32-256 mg/mL. Besides, the Cef+RJ combination against *K. pneumoniae,* the Levo+RJ combination against *E. coli,* and the Tri+RJ combination against *S. aureus, E. coli, K. pneumoniae* had a significant synergistic interaction (ΣFIC<0.5).

It should be noted that RJ cannot show antimicrobial effect by ingestion, because of the destruction of its active substances as a result of digestion and neutralization through pH changes [24]. Therefore, it is important to develop suitable pharmaceutical dosage forms for the administration of either RJ or its synergistic combinations with known antibiotics.

CONCLUSION

Consequently, it is a fact that resistant microorganisms are a serious threat to the World's health and thus, either new antimicrobial compounds or their combinations with certain, already known antibiotics are needed to overcome this situation. Our study results showed that the effectiveness of certain antibiotics against different bacteria strains could be increased and enhanced by especially combining them with BA or RJ. However, further studies should be conducted to identify new antimicrobial compounds or the combinations with synergistic effect against more bacteria strains with different resistance profiles and to develop appropriate pharmaceutical dosage forms containing the antimicrobial compounds combinations.

REFERENCES

[1] Aktaş, G. (2014): Antibiyotik Kombinasyonları ve Sinerjistik Etkileşimleri. Türk Mikrobiyoloji Cemiyeti Dergisi 44: 47-55.

[2] Roca, I., Akova, M., Baquero, F., Carlet, J., Cavaleri, M., Coenen, S., Cohen, J., Findlay, D., Gyssens, I., Heure, O. E., Kahlmeter, G., Kruse, H., Laxminarayan, R., Liebana, E., Lopez-Cerero, L., MacGowan, A., Martins, M., Rodriguez-Bano, J. (2015): The Global Threat of Antimicrobial Resistance: Science For İntervention. New Microbes and New Infections 6: 22-29.

[3] Adwan, G., Mhanna, M. (2008): Synergistic Effects of Plant Extracts and Antibiotics on *Staphylococcus aureus* Strains Isolated From Clinical Specimens. Middle-East Journal of Scientific Research 3: 134-139.

[4] Balouiri, M., Sadiki, M., Ibnsouda, S. K. (2016): Methods For In Vitro Evaluating Antimicrobial Activity : A Review. Journal of Pharmaceutical Analysis 6: 71-79.

[5] Chung, P. Y., Navaratnam, P., Chung, L. Y. (2011): Synergistic Antimicrobial Activity Between Pentacyclic Triterpenoids and Antibiotics Against *Staphylococcus aureus* Strains. Annals of Clinical Microbiology and Antimicrobials 10(1): 1-6.

[6] Hummelova, J., Rondevaldova, J., Balastikova, A., Lapcik, O., Kokoska, L. (2015): The Relationship Between Structure and In Vitro Antibacterial Activity of Selected Isoflavones and Their Metabolites with Special Focus On Antistaphylococcal Effect of Demethyltexasin. Letters in Applied Microbiology 60: 242-247.

[7] Dhayakaran, R. P. A., Neethirajan, S., Xue, J., Shi, J. (2015): Characterization of antimicrobial efficacy of soy isoflavones against pathogenic biofilms. LWT-Food science and technology, 63(2): 859-865.

[8] Hong, H., Landauer, M. R., Foriska, M. A., Ledney, G. D. (2006): Antibacterial Activity of The Soy Isoflavone Genistein. Journal of Basic Microbiology 46: 329-335.

[9] Liang, Y. S., Qi, W. T., Guo, W., Wang, C. L., Hu, Z. B., Li, A. K. (2018): Genistein and Daidzein Induce Apoptosis of Colon Cancer Cells By Inhibiting The Accumulation of Lipid Droplets. Food & Nutrition Research 62: 1384.

[10] Berakdar, N., Al-Kayali, R., Sabbagh, G. (2016): In vitro antibacterial activity of Genistein and Quercetin Against Escherichia coli Isolated From Clinical Samples. Innovare Journal of Life Sciences 4: 5-8.

[11] Guo, J. M., Xiao, B. X., Liu, D. H., Grant, M., Zhang, S., Lai, Y. F., Guo, Y. B., Liu, Q. (2004): Biphasic Effect of Daidzein On Cell Growth of Human Colon Cancer Cells. Food and Chemical Toxicology 42: 1641-1646.

[12] Bakirdere, S., Örenay, S., Korkmaz, M. (2010): Effect of Boron on Human Health. The Open Mineral Processing Journal 3: 54-59.

[13] Zan, R., Hubbezoglu, I., Ozdemir, A. K., Tunc, T., Sumer, Z., Alıcı, O. (2013): Antibacterial Effect of Different Concentration of Boric Acid Against Enterococcus faecalis Biofilms In Root Canal. Marmara Dental Journal 2: 76-80.

[14] Fratini, F., Cilia, G., Mancini, S., Felicioli, A. (2016): Royal Jelly: An ancient Remedy With Remarkable Antibacterial Properties. Microbiological Research 192: 130-141.

[15] Laishram, S., Pragasam, A. K., Bakthavatchalam, Y. D., Veeraraghavan, B. (2017): An Update On Technical, Interpretative and Clinical Relevance of Antimicrobial Synergy Testing Methodologies. Indian Journal of Medical Microbiology 35: 445-468.

[16] Clinical and Laboratory Standards Institute. (2014): Performance Standards for Antimicrobial Susceptibility Testing. 24th Informational Supplement. Document M100-S24, CLSI, Wayne, PA.

[17] Kus, H., Arslan, U., Tuncer, I. (2014): In vitro synergistic activity of sulbactam in combination with imipenem, meropenem and cefoperazone against carbapenem-resistant Acinetobacter baumannii isolates. Mikrobiyoloji bulteni 48(2): 311-315.

[18] Ulanowska, K., Tkaczyk, A., Konopa, G., Węgrzyn, G. (2006): Differential Antibacterial Activity of Genistein Arising From Global Inhibition of DNA, RNA and Protein Synthesis In Some Bacterial Strains. Archives of Microbiology184: 271-278.

[19] Mbaveng, A. T., Sandjo, L. P., Tankeo, S. B., Ndifor, A. R., Pantaleon, A., Nagdjui, B. T., Kuete, V. (2005): Antibacterial Activity of Nineteen Selected Natural Products Against Multi - Drug Resistant Gram - Negative Phenotypes. Springerplus, 4: 823.

[20] Reichman, O., Akins, R., Sobel, J. D. (2009): Boric Acid Addition To Suppressive Antimicrobial Therapy For Recurrent Bacterial Vaginosis. Sexually Transmitted Diseases 36: 732-734.

[21] Dinkov, D., Stratev, D., Balkanska, R., Sergelidis, D. (2016): Antibacterial Activity of Royal Jelly and Rape Honey Against Methicillin-Resistant *Staphylococcus aureus* Strains. Journal of Food and Health Science 2: 67-73.

[22] Susilowati, H., Murakami, K., Yumoto, H., Amoh, T., Hirao, K., Hirota, K., Matsuo, T., Miyake, Y. (2017): Royal Jelly Inhibits Pseudomonas aeruginosa Adherence and Reduces Excessive Inflammatory Responses In Human Epithelial Cells. BioMed Research International, 2017: 3191752.

[23] Park, M. J., Kim, B. Y., Park, H. G., Deng, Y., Yoon, H. J., Choi, Y. S., Lee, K. S., Jin, B. R. (2019): Major Royal Jelly Protein 2 Acts As An Antimicrobial Agent and Antioxidant In Royal Jelly. Journal of Asia-Pacific Entomology 22: 684-689.

[24] Pavel, C. I., Mărghitaş, L. A., Bobiş, O., Dezmirean, D. S., Sapcaliu, A., Rodoi, I., Mădaş, M. N. (2011): Biological Activities of Royal Jelly – Review. Scientific Papers: Animal Science and Biotechnologies 44: 108-118.