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Original Article

Investigation of in vitro efficacy of quercetin-meropenem combination in carbapenemase-producing *Klebsiella pneumoniae* isolates

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Abstract

Introduction: In recent years, the rapid spread of carbapenem-resistant *K. pneumoniae,* their higher mortality rates, and limited treatment alternatives cause difficulties in the treatment of these infections. New treatment alternatives are needed to cope with resistant strains. In recent years, natural products such as Quercetin have started to be preferred in combination studies due to their antimicrobial effects and low side-effect profiles. The aim of this study was to investigate the in vitro efficacy of the combination of Quercetin and Meropenem on carbapenemase-producing (blaKPC, blaNDM, blaVIM, blaOXA-48, and blaIMP), carbapenem-resistant *K.pneumoniae* isolates using the checkerboard method.
Methodology: Thirty Carbapenem-resistant *K.pneumoniae* strains in the culture collection of our laboratory were included in our study. Carbapenemase genes were determined using the Xpert® Carba-R (Cepheid, USA). Synergism with meropenem was assessed by checkerboard analysis, followed by FIC index, and combination index calculation.
Results: Twenty (66.6%) strains had OXA-48, 6 (20%) NDM, 1 (3.3%) KPC, 1 (3.3%) OXA-48+NDM genes, and 2 strains (6.6%) gene could not be detected. In the Quercetin and Meropenem combination study, synergy was found in 24 (80%) of the strains; an additive effect was found in 5 (16.6%) and an antagonist effect in 1 (3.3%). In 19 (63.3%) of the strains, meropenem MIC values were below the sensitive limit (MIC ≤ 2 μg/mL).
Conclusions: Although the combination of quercetin and meropenem has a high synergistic effect in carbapenem-resistant *K. pneumoniae* isolates, it seems that the carbapenemase species affects this situation. however, more work is needed on this subject.

Key words: Quercetin; carbapenem-resistant *Klebsiella pneumoniae*; checkerboard method; meropenem.

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Introduction

Klebsiella pneumoniae is an important pathogen that can cause community-acquired infections as well as complicated and difficult-to-treat hospital-acquired infections. Especially in recent years, the rapid spread of carbapenem-resistant strains, their higher mortality rates, and limited treatment alternatives cause difficulties in the treatment of these infections [1].

Carbapenem resistance was seen among *Enterobacterales* species; it usually develops due to the production of β-lactamases such as *K. pneumoniae* carbapenemase (KPC) and New Delhi metallo β-lactamase (NDM), extended-spectrum β-lactamase (GSBL), AmpC β-lactamase (AmpC) and rarely loss of outer membrane proteins. The genes that cause carbapenem resistance are usually found on plasmids. This resistance can be spread by clonal expansion or by horizontal transfer of genes to naive bacteria [2]. Carbapenemase-producing strains spread more easily than non-carbapenemase-producing strains. Carbapenem-resistant *K. pneumoniae* (CRKP) strains were initially only seen in hospital-acquired cases, but are now reported in community-acquired cases. Although the treatment options that can be used in the treatment of CRKP infections are very limited, nephrotoxic drugs such as colistin and tigecycline are generally used [3]. Combination therapies were thought to be hopeful due to the lack of an optimal treatment regimen, the inadequacy of single-agent antimicrobial treatments, or the presence of drugs with high side-effect profiles. Trial of new combined therapies will keep therapeutic options dynamic.

In recent years, natural products such as Quercetin have been reported to have antimicrobial effects as well as various therapeutic benefits. For this reason, it is seen that natural products such as quercetin are frequently preferred in combination studies. The disadvantages of natural agents such as quercetin, such as poor bioavailability, limit their oral use. However, Quercetin has advantages such as lower toxicity and low bacterial

resistance rates compared to other antibiotics [4,5]. Polyphenols represent a group of over 500 phytochemicals that are micronutrients naturally found in plants. Quercetin (3,3′,4′5,7-pentahydroxyflavone) is also a natural polyphenol. It is abundant in our foods such as fruits, vegetables, grains, nuts, and teas. Quercetin has been reported to have many health benefits such as antioxidant, anti-neoplastic, anti-hypertensive, and anti-cancer properties. In addition, it is one of the important polyphenols that attracts global attention due to its high antimicrobial activity [6]. It has also been reported that some polyphenols have synergistic effects when combined with other polyphenols or antibiotics [7]. In the literature, there are limited studies in which quercetin and carbapenems were combined and the synergistic effect was investigated. In some studies, it has been shown that Quercetin can increase the efficacy of antibiotics used in carbapenem-resistant *Escherichia coli* [8]. Studies investigating the synergistic effects of Quercetin and other antibiotics against multi-drug resistant *K. pneumoniae* isolates are rare in the literature.

The aim of this study was to investigate the in vitro efficacy of the combination of Quercetin and Meropenem in carbapenem-resistant *K.pneumoniae* isolates producing carbapenemase (using the checkerboard method.

Methodology

Thirty Carbapenem-resistant *K.pneumoniae* strains in the culture collection of our laboratory were included in our study. Of the strains, 14 (46.6%) were urine, 8 (26.6%) blood, 4 (13.3%) wound, 2 (6.6%) tracheal aspirate, 1 (3.3%) catheter, and 1 strain (3.3%) isolated from a sputum sample. Identification of strains by mass spectrometry (VITEK MS®, Biomerieux, France); antibiotic susceptibility tests were performed with VITEK 2® (Biomerieux, France) automated system. Carbapenem resistance (Meropenem MIC > 8 mg/L) was confirmed by the broth microdilution method, which is the reference method in line with EUCAST guidelines, in strains determined to be carbapenem resistant by the automated system [9]

Determination of MIC values of Meropenem and Quercetin

Quercetin in 5% DMSO and Meropenem were dissolved in sterile distilled water. Stock solutions were prepared for Quercetin and Meropenem at a concentration of 512 µg/mL. and stored at -20 °C until the study. Serial dilution of drugs was performed using Müller Hinton Broth (MHB, HiMedia, Mumbai, India) medium in 96-well microplate wells customized for the microdilution method. The bacterial suspension prepared by the photometric method (Densichek plus, Biomerieux, France) at 0.5 McFarland turbidity was diluted to a final concentration of $5X10^5$ CFU/mL in the well and added to the wells. After 16-20 hours of incubation at 35-37°C, the results were evaluated visually. The lowest antibiotic concentration at which there was no visible turbidity, i.e., growth, was determined as the minimum inhibitory concentration (MIC).

Phenotypic and genotypic determination of carbapenemase production

A modified carbapenemase inactivation (CIM) test was applied to determine carbapenemase production phenotypically. A loopful was taken from the isolate in which carbapenemase enzyme production would be investigated, suspended in sterile distilled water and 10 µL Meropenem disc (BioMérieux, France) was thrown into it. After two hours of incubation, the Meropenem disc from the suspension was placed on *E. coli* ATCC 29522 inoculated Mueller-Hinton (Merck, USA) agar medium and incubated at 35 °C for six hours. If growth occured around the meropenem disc (no inhibition zone) at the end of the six-hour period, the result of the test was considered positive. Carbapenemase genes were determined using the Xpert® Carba-R kit (blaKPC, blaNDM, blaVIM, blaOXA-48, and blaIMP) and the GeneXpert instrument (Cepheid, USA).

Determination of Synergistic Effect with Checkerboard Method

The checkerboard test to investigate the synergistic activity of Quercetin and meropenem in CRE isolates was performed in 96-well microplates. In the checkerboard study, stock solutions of Quercetin and Meropenem were prepared with an initial concentration of 64 µg/mL in the microplate. Serial dilutions of Quercetin in the vertical direction and Meropenem in the horizontal direction were made in the wells. Concentrations of both active ingredients in the range of 64-0.5 µg/mL were obtained. The bacterial suspension was added to a final concentration of 5×10^5 CFU/mL in the well. At the end of 16-24 hours incubation at 35 ± 2°C, growths were evaluated visually and combination MIC values of antibiotics were determined. The following formula was used to calculate the Fractional Inhibitory Concentration (FIC) and FIC index (FICI) of the combınatıon: Quercetin FIC = quercetin MIC in combination/quercetin MIC alone; Meropenem FIC = meropenem MIC in

combination/meropenem MIC alone; that is, FICI = FIC of quercetin + FIC of meropenem. FICI ≤ 0.5 and FICI > 4.0 were considered synergistic and antagonistic, respectively, while FICI between 0.5 and 4.0 showed indifferent interaction between quercetin and meropenem [10].

Results

Thirty strains that were found to be resistant to meropenem by the automated system were also found to be resistant in the broth microdilution method, and the strains were confirmed to be resistant to meropenem.

The modified carbapenemase inactivation test was positive in all of the strains. Twenty (66.6%) strains had OXA-48, 6 (20%) NDM, 1 (3.3%) KPC, 1 (3.3%) OXA-48+NDM genes, and 2 strains (% 6.6) gene could not be detected. Carbapenemase gene was not detected by molecular method in two isolates found positive for modified carbapenemase inactivation test.

In the Quercetin and Meropenem combination study, synergy was found in 24 (80%) of the strains; an additive effect was found in 5 (16.6%), and an antagonist effect in 1 (3.3%) (Table 1). In 19 (63.3%) of the strains, meropenem MIC values were below the sensitive limit (MIC ≤ 2 µg/mL).

It was observed that the MIC values of 79.8% of the carbapenem resistant strains decreased below 8 µg/mL, which is the sensitive limit of Meropenem, with the quercetin-Meropenem synergy combination. Meropenem MIC value was 0.5 µg/mL and below in 4 strains (16.6%) with synergistic effect; 1 µg/mL in 8 strains (33.3%); 2 µg/mL in 6 strains (25%); 4 µg/mL in 5 strains (20.83%); It was found to be 8 µg/mL in 1 strain (4.16%). Meropenem MIC value was found to be 32 µg/mL in 2 strains (40%) with additive effect, 8 µg/mL in 2 strains (40%), and 1 µg/mL in 1 strain (20%) (Table 1).

OXA-48 was found in 19 (79.1%) of the strains with synergistic effect; NDM in 2 (8.3%); While OXA-48 + NDM was detected in 1 (%) strain, no gene was detected in 2 (8.3%) strains. NDM gene was detected in 4 (80%) of the strains with additive effect, while OXA-48 gene was detected in 1 strain (20%). KPC gene was detected in 1 strain with an antagonistic effect (Table 1).

Table 1. MIC values of Meropenem and Quercetin alone and in combination against Carbapenem-resistant *K. pneumoniae* isolates.

Strain No.	Enzyme Type (Cepheid GeneXpert)	CIM	MIC values (µg/mL)				FIK index value	RESULTS
			Meropenem	Meropenem Combination	Quercetin	Quercetin Combination		
1	OXA-48	+	16	1	64	8	0.18	Synergy
2	NDM-1	+	64	32	64	8	0.62	Additive
3	OXA -48	+	16	2	64	2	0.15	Synergy
4	OXA-48	+	16	8	≥ 256	2	0.50	Synergy
5	NDM	+	32	0.5	≥ 256	64	0.26	Synergy
6	OXA-48	+	16	2	≥ 256	32	0.25	Synergy
7	OXA-48	+	16	0.5	64	16	0.28	Synergy
8	OXA-48	+	16	1	≥ 256	32	0.18	Synergy
9	OXA-48	+	16	1	64	4	0.12	Synergy
10	OXA-48	+	16	4	128	1	0.25	Synergy
11	OXA-48	+	16	8	≥ 256	8	0.53	Additive
12	NEG (-)	+	16	<0.5	64	< 0.5	< 0.03	Synergy
13	NDM	+	16	8	64	32	1	Additive
14	OXA-48	+	16	2	≥ 256	32	0.25	Synergy
15	NDM	+	64	32	64	1	0.51	Additive
16	KPC	+	16	64	64	64	5	Antagonist
17	OXA-48 + NDM	+	16	4	64	8	0.5	Synergy
18	OXA-48	+	16	1	32	2	0.12	Synergy
19	OXA-48	+	16	1	32	4	0.18	Synergy
20	OXA-48	+	16	4	32	8	0.50	Synergy
21	NEG (-)	+	16	2	≥ 256	32	0.25	Synergy
22	OXA-48	+	16	1	32	4	0.18	Synergy
23	NDM	+	16	2	32	32	1.5	Additive
24	OXA-48	+	16	0.5	32	8	0.28	Synergy
25	OXA-48	+	16	1	64	4	0.12	Synergy
26	NDM	+	16	4	64	2	0.28	Synergy
27	OXA-48	+	16	2	64	4	0.18	Synergy
28	OXA-48	+	16	2	64	8	0,25	Synergy
29	OXA-48	+	16	4	32	4	0.37	Synergy
30	OXA-48	+	16	1	64	4	0.12	Synergy

Discussion

In recent years, the rapid spread of CRE strains has become a concern due to limited treatment alternatives and high mortality rates. In infections caused by resistant pathogens such as CRE, treatment with only a single antibiotic may be insufficient and combination treatments are needed [10] Today, natural products used in combination therapy have been widely used due to a number of advantages such as low toxicity and low antibiotic resistance stimulation [5]. Polyphenols are among the natural products used for this purpose. Polyphenols are responsible for the color formation of many plants that produce them and are thought to give plants an evolutionary advantage for survival. In addition to these properties, it is known that polyphenols have anti-inflammatory and antioxidant effects. In recent years, the presence of antimicrobial activity of polyphenols, including Quercetin, has been reported [11]. Quercetin is thought to exert its antibacterial activity against bacteria by cytoplasmic membrane damage, inhibition of nucleic acid synthesis, inhibition of biofilm formation, changes in cell permeability, or inhibition of bacterial energy metabolism [12,13]. Studies have reported that when given in combination with other polyphenols or antibiotics, the therapeutic activity of the other polyphenol or the combined antibiotic is enhanced by synergistic activity[12,13]. It also made it possible to reduce the dose of antibiotics used thanks to the combination [12,13]. Synergy has been observed in most antibiotic combination studies with quercetin. However, synergy rates differ between the antibiotics and strains used. The reason for this may be the existence of different mechanisms of action and resistance among bacteria and the different mechanisms of action of Quercetin [14]. Vipin *et al.* obtained higher synergistic activity on resistant *P.aeruginosa* isolates in the combination of quercetin tobramycin and amikacin compared to the combination of levofloxacin, ceftriaxone, gentamycin. They suggested that this result may be due to the difference in the mechanism of action of drugs [14]. The first studies on the antimicrobial activity of quercetin showed that quercetin may have antibacterial activity against Gram-positive bacteria isolated from the oral cavity and intestine [11,12]. However, few subsequent studies have revealed that Quercetin may show synergistic activity in combined treatments on ESBL-producing *K. pneumoniae,* multi-drug-resistant *Mycobacterium tuberculosis, Campylobacter jejuni*, and resistant *P.aeruginosa* strains [15,16]. However, studies on this subject are not enough. There is insufficient information about the synergistic activity with carbapenems, especially in carbapenem resistant strains. In this respect, the results of our study will contribute to the existing information in the literature regarding the antibacterial and synergistic activity of Quercetin. In this study, where we aimed to investigate the efficacy of quercetin + meropenem combination in 30 carbapenem-resistant *K. pneumoniae* strains, we found synergistic activity in 80% of the strains. In our study, it was determined that 91.6% of the strains with synergistic effects (FIKI range: 0.003-05) produced one and/or more than one carbapenemase gene. Carbapenemase gene could not be detected in only 2 strains. In our study, it was thought that there might be an enzyme type other than carbapenemase genes (blaKPC, blaNDM, blaVIM, blaOXA-48, and blaIMP), which are within the scope of the automated multiplex PCR system (Cepheid, GeneExpert) in which carbapenemase genes were analyzed. Pal *et al.* They reported that the quercetin-meropenem combination showed synergistic activity in 89.25% of carbapenem-resistant *K. pneumoniae* and *E. coli* isolates with NDM, OXA-48, and KPC genes [8] In our study, antagonism was observed in 1 strain in which we detected the KPC gene; additive effect in 66.6% of NDM detected genes (7/4), synergistic effect in 42.8%; in 95% of the strains (20/19) in which we detected the OXA-48 gene, a synergistic effect was detected, while an additive effect was observed in only 1 strain. It can be said that the carbapenemase species produced by the bacteria affect the in vitro synergistic activity of the Meropenem-Quercetin combination. However, there is not enough data in the literature showing the relationship between synergistic activity and carbapenemase types of bacteria. The low number of isolates examined in our study can be counted among the limitations of the study.

Conclusions

As a result, it has been determined that the combined use of Quercetin and Meropenem against carbapenem-resistant *K. pneumoniae* isolates has a high synergistic effect. However, carbapenemase types were found to affect the outcome. More comprehensive studies are needed on this subject.

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