Synergistic effects of silybin and curcumin on virulence and carbapenemase genes expression in multidrug resistant *Klebsiella oxytoca*

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**Abstract**

**Objective:** Silybin and curcumin have potential antimicrobial effects. This study aimed to evaluate the synergistic antimicrobial effects of silybin and curcumin on virulence and carbapenemase genes expression among multidrug-resistant (MDR) *Klebsiella oxytoca*.

**Results:** A total of 70 MDR *K. oxytoca* (carrying *bla*<sub>IMP</sub> and *bla*<sub>OXA-48-like</sub> genes) were included. The antibiotic susceptibility and biofilm production of isolates were determined. The silybin and curcumin at concentrations 10–500 mg/mL alone and in combination were exposed to bacterial isolates in Mueller Hinton broth medium for 24 h. The expression of *bla*<sub>IMP</sub>, *bla*<sub>OXA-48-like</sub>, *mrkA*, *pilQ*, *matB* and *fimA* genes was evaluated using quantitative real-time polymerase chain reaction (qRT-PCR). The mean minimum inhibitory concentration (MIC) of curcumin and silybin were 250 mg/mL and 500 mg/mL, respectively. The anti-virulent effect of 100 mg/mL of silybin and curcumin was shown by significant reduction in the expression of *fimA* (2.1-fold, *P* < 0.0001) and *mrkA* (2.1 fold, *P* < 0.0001) genes. Moreover, these compounds significantly decreased the expression of *bla*<sub>IMP</sub> (3.2-fold, *P* < 0.0001) gene. Notably, there was no significant effect on *pilQ*, *matB* and *bla*<sub>OXA-48-like</sub> genes. The results showed that silybin and curcumin can be candidate as natural way for control the MDR virulent strains of *K. oxytoca*.

**Keywords:** Silybin, Curcumin, Antimicrobial effects, Virulence genes, *Klebsiella oxytoca*

**Introduction**

Multidrug-resistant (MDR) *Enterobacteriaceae* members are causative agents of fatal nosocomial infections which have narrow or none therapeutic choices [1–3]. The evolution of carbapenemase-producing (CP) strains has limited last-line treatment resorts. These strains may develop the resistance through various mechanisms such as production of carbapenemase enzymes [2, 3]. Some of these enzymes include imipenemases (IMPs), OXA-type beta-lactamase, and New Delhi metallo-beta-lactamase 1 (NDM-1) [4].

MDR *Klebsiella oxytoca* with resistance to carbapenems has been reported from various areas which encode carbapenemase genes [5, 6]. *K. oxytoca* strains are mostly opportunistic pathogens among immunocompromised patients [7]. Biofilm formation is another strategy of *K.*
**Table 1** Biochemical test results for *Klebsiella oxytoca* isolates

| Test                        | Results                             |
|-----------------------------|-------------------------------------|
| Growth on MacConkey agar    | Pink mucoid colonies                |
| Gram staining               | Pink-red coccobacilli               |
| Growth on triple sugar iron agar | Acidic/acid, Gas positive; H2S negative |
| Methyl red                   | Negative                            |
| Voges-Proskauer             | Positive                            |
| Citrate utilization         | Positive                            |
| Urease                      | Positive                            |
| Lysine decarboxylase        | Positive                            |
| Arginine dehydrase          | Negative                            |
| Ornithine decarboxylase     | Negative                            |

**Curcumin and silybin antibacterial effects**

Curcumin and silybin compounds were purchased from Sigma Aldrich, USA. Curcumin is a polyphenol compound with antioxidant effects and showed antimicrobial activity against *K. oxytoca*. Silybin is a lipophilic compound that dissolves in acetone, dimethylsulfoxide, and ethanol but is insoluble in water, acidic, and neutral solutions. This compound is a major component of turmeric (*Curcuma longa*), which is used as an herbal remedy for treating a variety of diseases.

**Clinical isolates**

The flow chart of the employed procedures in the present study is shown in Additional file 1: Fig. S1 to the readers at one glance. Herein, 70 MDR *K. oxytoca* isolates that collected from stool samples during 2012–2019 were included. The isolation and identification of *K. oxytoca* were performed using standard bacteriology tests including MacConkey agar (Merck, Germany), triple sugar iron agar, methyl red/Voges-Proskauer, and citrate and urea utilization as mentioned in Table 1 [19]. The antibiotic susceptibility pattern was determined using Kirby-Bauer method according to the Clinical and Laboratory Standards Institute (CLSI) 2017 [20]. The antibiotic discs included cefepime (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), ciprofloxacin (5 μg), tetracycline (30 μg), amikacin (30 μg), piperacillin-tazobactam (100/10 μg), gentamicin (10 μg), imipenem (10 μg), meropenem (10 μg), and trimethoprim-sulfamethoxazole (1.25/23.75 μg) (Bioanalyse, Ankara, Turkey). *Escherichia coli* ATCC® 25922™ was used as quality control strain. Isolates that were resistant against three antibiotics in different classes were considered MDR [21]. All isolates carried the *blaIMP*, *blaOXA-48*-like, *mrkA*, *pilQ*, *matB*, and *fimA* genes.
the two units together to form one structure [23]. Various concentrations (10–500 mg/mL) of both compounds were prepared and their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were determined according to previously described method [24]. The Mueller Hinton broth (MHB) medium (Merk, Germany) was employed. A bacterial suspension equal to 0.5 MacFarland turbidity was prepared and added to each dilution and incubated at 37 °C for 24 h. The MIC of a concentration was defined following observation of no growth and the MBC was defined for each dilution without growth of 100 µL of suspension onto the MHA medium [24].

**Escherichia coli** ATCC® 25922™ was used as quality control strain.

**Biofilm formation**

The biofilm formation was conducted using microtiter plate assay. A bacterial suspension was cultured into each well of 96-well plate containing trypticase soy broth (TSB) medium (Merck, Germany) and incubated for 24 h. The plates were washed and dried. The well attachments were fixed using methanol. The crystal violet was added and left for 15 min and washed again. Using ethanol, the bacterial attachments were made soluble and the opacity was measured using ELISA reader at OD490. The biofilm formation was defined compared to the control wells. There were four categories of isolates: non-biofilm producers (ODT < ODc), weak-biofilm producers (ODc < ODT < 2 × ODc), moderate-biofilm producers (2 × ODc < ODT < 4 × ODc), and strong-biofilm producers (4 × ODc < ODT) [25].

**RNA extraction and real-time polymerase chain reaction**

The total RNA (Qiagen GmbH, Hilden, Germany) was extracted from 1 × 10⁶ cells suspension of isolates and cDNA (Takara, Japan) was synthesized according to the manufacturers’ instructions. The quantitative real-time polymerase chain reaction (qRT-PCR) was performed using specific primers represented in Table 2 and using CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). The gyrA gene was considered as the reference of expression analysis (Table 2) [26]. The *K. oxytoca* ATCC® 43165™ was used as control strain.

**Statistical analysis**

Chi Square and analysis of variance (ANOVA) tests were used with 95% confidence intervals to analyze the data using Statistical Package for the Social Sciences (SPSS) version 22.0 (IBM Corporation, Armonk, NY, USA). A P-value less than 0.05 was considered significant [8].

**Results**

**Antibiotic resistance**

As shown in Table 3 and Additional file 2: Fig. S2, all isolates were resistant to ceftazidime, cefotaxime, imipenem, tetracycline and trimethoprim-sulfamethoxazole and considered MDR. Moreover, 98.6%, 90.0%, 90.0%, 81.4%, 80.0% and 80.0% of them were resistant to meropenem, piperacillin-tazobactam, amikacin, ciprofloxacin, cefepime, and gentamicin, respectively. The highest rate of susceptibility was to cefepime (20.0%).

**Biofilm formation**

Among 70 MDR *K. oxytoca* from stool samples, none of them produced strong-level biofilms and all were in moderate level (Additional file 3: Fig. S3).

### Table 2

| Primer | Sequence: 5’–3’ | Product size (bp) | Annealing (°C) | References |
|--------|-----------------|------------------|----------------|------------|
| *bla*<sub>IMP</sub> F: GGGTGGGGGCATTCTGTA<br>R: TCTATCCGGCGTGCTGC | 198 | 62 | [5] |
| *bla*<sub>OXA-48</sub> F: TGTTTTTGGTGGCGGATCGAT<br>R: GTAAMRATGGTTGTGGC | 177 | 45 | [5] |
| *mrkA* F: CTGGGCCGGCGCTACTGCTAAG<br>R: CAACCGGGATGATTGGTG | 172 | 61 | [5] |
| *fimA* F: GCACCGGCATTGACAG<br>R: CCAAGGGGTCGCTGCG | 132 | 61 | [5] |
| *matB* F: GTACATGGGGCGAACTTGG<br>R: GTGGGCCGTAGGTAGGAGAA | 98 | 61 | [5] |
| *pilQ* F: TCCCGACCGTCTCACC<br>R: GCCTGCCGGCGCTGGAG | 194 | 61 | [5] |
| *gyrA* F: CGCGTACTATAGCCCATG<br>R: ACCGTGATCAGTTCGTCAG | – | 57 | [26] |
Antibacterial effects

The mean MIC$_{90}$ and MBC$_{90}$ of curcumin were 250 mg/mL and > 250 mg/mL, respectively. Meanwhile, those of silybin included 500 mg/mL and > 500 mg/mL, respectively. All concentrations of both plants had antibacterial effects against MDR *K. oxytoca* isolates.

Gene expression

The calculation of expression levels was performed according to the $2^{-\Delta \Delta CT}$. We observed that 100 mg/mL of curcumin and silybin could singly decrease the expression of *fimA* and *mrkA* genes, respectively (data not shown). None of other genes were significantly affected. The anti-virulent effect of 100 mg/mL of silybin and curcumin in combination was shown by decrease in the expression of *fimA* (2.1-fold, $P < 0.0001$) and *mrkA* (2.1 fold, $P < 0.0001$) genes (Additional file 4: Fig. S4). Moreover, these compounds decreased the expression of *bla$_{IMP1}$* (3.2-fold, $P < 0.0001$) gene. Notably, there was no significant effect on expression of *pilQ*, *matB* and *bla$_{OXA-48}$-like* genes (Additional file 4: Fig. S4).

Discussion

In this study, 70 MDR *K. oxytoca* isolates collected from stool samples during 2012–2019 were included. All isolates were resistant to ampicillin, ceftazidime, trimethoprim-sulfamethoxazole, imipenem and tetracycline. All isolates carried the *bla$_{IMP}$*, *bla$_{OXA-48}$-like*, *mrkA*, *pilQ*, *matB* and *fimA* genes. We observed that all of them were moderate biofilm producers possibly mediated by adhesins including *mrkA*, *pilQ*, *matB* and *fimA* genes. In line with the current study, Ghasemian et al. [5] from Iran reported high rate of adhesins among MDR and non-MDR *K. oxytoca* isolates during 2016–2017.

There is scarcity in data regarding effects of curcumin and silybin against expression of virulence and antibiotic resistance genes among *K. oxytoca*. Moreover, antimicrobial and anti-biofilm effects of curcumin and silymarin has been exhibited previously [15, 16, 27]. In this study, the mean MIC$_{90}$ and MBC$_{90}$ of curcumin were 250 mg/mL and > 250 mg/mL, respectively. Meanwhile, those of silybin were 500 mg/mL and > 500 mg/mL, respectively. In previous study by Adamczak et al. [15], *Acinetobacter lwoffii* (250 µg/mL), *Streptococcus pyogenes* (31.25 µg/mL), *Pseudomonas aeruginosa* and *Enterococcus faecalis* (62.5 µg/mL), and methicillin-sensitive *Staphylococcus aureus* (250 µg/mL) were found to be susceptible to curcumin. In another study by Evren et al. [27], MIC and MBC values were between 60 and > 241 µg/mL and greater than 241 µg/mL, respectively. The inhibition of bacteria may be due to this fact that compounds derived from *S. marianum* and *C. longa* are known to exert profound antibacterial effects, mostly by inhibiting RNA and protein production, quorum sensing (QS) system, and targeting the cellular components [28, 29].

In this study, 100 mg/mL of curcumin and silybin could singly decrease the expression of *fimA* and *mrkA* genes, respectively. None of other genes were significantly affected. The anti-virulent effect of 100 mg/mL of silybin and curcumin in combination was shown by 2.1 fold reduction ($P < 0.0001$) in the expression of *fimA* and *mrkA* genes of MDR *K. oxytoca* compared to control strain. Moreover, these compounds reduced the expression of *bla$_{IMP1}$* gene. Notably, there was no significant effect on *pilQ*, *matB* and *bla$_{OXA-48}$-like* genes. In an experiment by Eslami et al. [28], silymarin had no effect on *bla$_{IMP}$* and *bla$_{OXA-48}$* expression in MDR *E. coli*; however, curcumin down-expressed *bla$_{IMP}$*. In a previous study by Shariati et al. [30], synthesized nano-curcumin exhibited significant ($P < 0.0001$) downregulation of the transcription of some virulence genes in PAO1 (16-fold) and MDR (13-fold) strains of *P. aeruginosa*, Table 3: Antibiotic susceptibility of *Klebsiella oxytoca* isolates

| Antibiotics                  | Resistant N (%) | Intermediate N (%) | Susceptible N (%) |
|------------------------------|----------------|-------------------|-------------------|
| Ceftazidime                  | 70 (100.0)     | 0 (0.0)           | 0 (0.0)           |
| Cefotaxime                   | 70 (100.0)     | 0 (0.0)           | 0 (0.0)           |
| Imipenem                     | 70 (100.0)     | 0 (0.0)           | 0 (0.0)           |
| Tetracycline                 | 70 (100.0)     | 0 (0.0)           | 0 (0.0)           |
| Trimethoprim-sulfamethoxazole| 70 (100.0)     | 0 (0.0)           | 0 (0.0)           |
| Meropenem                    | 69 (98.6)      | 1 (1.43)          | 0 (0.0)           |
| Piperacillin-tazobactam      | 63 (90.0)      | 1 (1.43)          | 6 (8.6)           |
| Amikacin                     | 63 (90.0)      | 3 (4.3)           | 4 (5.7)           |
| Ciprofloxacin                | 57 (81.4)      | 2 (2.9)           | 11 (15.7)         |
| Cefepime                     | 56 (80.0)      | 0 (0.0)           | 14 (20.0)         |
| Gentamycin                   | 56 (80.0)      | 4 (5.7)           | 10 (14.3)         |
respectively. Another study by Kumbar et al. [31], showed that curcumin diminished the virulence of Porphyromonas gingivalis by reducing the expression of virulence factors genes. Also, Shen et al. [32], showed that silibinin, a flavonoid that is isolated from S. marianum, reduced the virulence of Streptococcus suis serotype 2. The decrease in the expression of virulence genes may be related to the effect of the tested compounds on QS genes, which play an important role in regulating other bacterial factors such as pathogenicity, biofilm production, and secretion systems [33]. In conclusion, this study showed the synergistic effects of curcumin and silybin against some of virulence factors and carbapenemase genes in MDR K. oxytoca. The results of this study provided a suitable platform for further investigations, especially in vivo experiments to verify these promising effects.

Limitations
Major limitations of this study included lack of in vivo experiment, low number of samples and no investigation of curcumin and silybin effects on virulence gene expression of other nosocomial pathogens.

Abbreviations
CP: Carbapenemase-producing; MBC: Minimum bactericidal concentration; MDR: Multidrug-resistant; MIC: Minimum inhibitory concentration; MHB: Mueller Hinton broth.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s13104-022-06172-3.

References
1. Yazdansetad S, Alkhudhairy MK, Najafpour R, Farajtabrizi E, Al-Mosawi RM, Saki M, et al. Preliminary survey of extended-spectrum β-lactamases (ESBLs) in nosocomial uropathogen Klebsiella pneumoniae in north-central Iran. Helyson. 2019;5:e02349.
2. Sheu CC, Chang YT, Lin SY, Chen YH, Hsueh PR. Infections caused by carbapenem-resistant Enterobacteriaceae: an update on therapeutic options. Front Microbiol. 2019;10:1080.
3. Abbas AF, Al-Saadi AG, Alkhudhairy MK. Biofilm formation and virulence determinants of Klebsiella oxytoca clinical isolates from patients with colorectal cancer. J Gastrointest Cancer. 2020;51:855–60.
4. Behzadi P, García-Perdomo HA, Karpiński TM, Issakhanian L. Metallo-β-lactamases: a review. Mol Biol Rep. 2020;47:6281–94.
5. Ghaseimian A, Mobarez AM, Peerayeh SN, Abadi ATB, Khodaparast S, Nojoomi F. Report of plasmid-mediated colistin resistance in Klebsiella oxytoca from Iran. Rev Med Microbiol. 2018;29:59–63.
6. Tsilipounidaki K, Athanasakopoulou Z, Müller E, Burgold-Voigt S, Florou Z, Braun SD, et al. Plethora of resistance genes in carbapenem-resistant gramm-negative bacteria in Greece: no end to a continuous genetic evolution. Microorganisms. 2022;10:159.
7. Gómez M, Valverde A, Del Campo R, Rodríguez JM, Maldonado-Barragán A. Phenotypic and molecular characterization of commensal, community-acquired and nosocomial Klebsiella spp. Microorganisms. 2021;9:2344.
8. Alkhudhairy MK, Alshadeddi SM, Mahmood SS, Al-Bustan SA, Ghaseimian A. Comparison of adhesin genes expression among Klebsiella oxytoca ESBL-non-producers in planktonic and biofilm mode of growth, and imipenem sublethal exposure. Microb Pathog. 2019;134:103558.
9. Behzadi P. Classical chaperone-usher (CU) adhesive fimbriae: uropathogenic Escherichia coli (UPEC) and urinary tract infections (UTIs). Folia Microbiol. 2020;65:45–65.
10. Khonsari MS, Behzadi P, Foroozi F. The prevalence of type 3 fimbriae in Uropathogenic Escherichia coli isolated from clinical urine samples. Meta Gene. 2021;28:100881.
11. Lehti TA, Bauchart P, Heikkinnen J, Hacker J, Korhonen TK, Dobrindt U, et al. Mat fimbiae promote biofilm formation by meningitis-associated Escherichia coli. Microbiology. 2010;156:2408–17.

12. Ligthart K, Belzer C, De Vos WM, Tytgat HL. Bridging bacteria and the gut: functional aspects of type IV pilus. Trends Microbiol. 2020;28:340–8.

13. Bessam F, Mehdadi Z. Evaluation of the antibacterial and antifungal activity of different extract of Flavoniques Silybum marianum L. Adv Environ Biol. 2014;8:1–9.

14. Rahaman M, Rakib A, Mitra S, Tareq AM, Shahid-Ud-daula AF, et al. The genus curcuma and inflammation: overview of the pharmacological perspectives. Plants. 2021;10:63.

15. Adamczak A, Ozarowski M, Karpinski TM. Curcumin, a natural antimicrobial agent with strain-specific activity. Pharmaceuticals (Basel). 2020;13:153.

16. Krausz AE, Adler BL, Cabral V, Navati M, Doerner J, Charafeddine RA, et al. Curcumin encapsulated nanoparticles as innovative antimicrobial and wound healing agent. Nanomedicine. 2015;11:195–206.

17. Kong WY, Ngi SC, Goh BH, Lee LH, Htar TT, Chuah LH. Is curcumin the answer to future chemotherapy cocktail? Molecules. 2021;26:4329.

18. Behzadi P, Gajdacs M. Writing a strong scientific paper in medicine and the biomedical sciences: a checklist and recommendations for early career researchers. Biol Futur. 2021;7:239–47.

19. Collee JG, Miles RS, Watt B. Tests for the Identification of Bacteria. In: Collee JG, Fraser AG, Marmion BP, Simmons A, editors. Mackie and McCartney Practical Microbiology. 4th ed. Livingstone: Churchill; 1996. p. 131–49.

20. Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing. 27th ed. CLSI supplement M100. Wayne, PA. 2017.

21. Mapiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect. 2012;18:268–81.

22. Urošević M, Nikolić L, Gajić I, Nikolić V, Dinić A, Milićković V. Curcumin: biological activities and modern pharmaceutical forms. Antibiotics (Basel). 2022;11:135.

23. Bijak M. Silybin a major bioactive component of milk thistle (Silybum marianum L. Gaernt.)—chemistry, bioavailability, and metabolism. Molecules. 2017;22:1942.

24. Saki M, Seyed-Mohammadi S, Montazeri EA, Siahpoosh A, Moosavian M, et al. The genus curcuma and inflammation: overview of the pharmacological perspectives. Plants. 2021;10:63.

25. Khalil OA, Enbaawy MI, Taher Salah HM, Ragab E. In vitro investigation of antiadherent activities of silymarin. Folia Microbiol. 2015;60:351–6.

26. Kamali E, Jamali A, Izanloo A, Ardebili A. In vitro activities of cellulase and the antibacterial effect of silver nanoparticles on ESBL-producing strains of Escherichia coli. J Microbiol. 2018;29:177–81.

27. Eslami M, Ghasemian A, Najafiolya Z, Mirforughi SA, Nojoomi F. Silymarin essential oil against clinical extensively drug-resistant bacteria. Eur J Clin Microbiol Infect Dis. 2021;40:2327–34.

28. Evren E, Yurtcu E. In vitro effects on biofilm viability and antibacterial and antifungal activity of different extract of Flavoniques Silybum marianum L. Adv Environ Biol. 2014;8:1–9.

29. Zheng D, Huang C, Huang H, Zhao Y, Khan MR, Zhao H, et al. Antibacterial mechanism of curcumin: a review. Chem Biodivers. 2020;17:e2000171.

30. Shariati A, Asadian E, Fallah F, Azimi T, Hashemi A, Yasbolaghi Sharahi J, et al. Evaluation of nano-curcumin effects on expression levels of virulence genes and biofilm formation of multidrug-resistant Pseudomonas aeruginosa isolated from burn wound infection in Tehran. Iran Infect Drug Resist. 2019;12:223–35.

31. Kumbar VM, Peram MR, Kugaji MS, Shah T, Patil SP, Muddapur UM, et al. Effect of curcumin on growth, biofilm formation and virulence factor gene expression of Pseudomonas gingivalis. Odontology. 2021;109:18–28.

32. Shen X, Liu H, Li G, Deng X, Wang J. Silibinin attenuates Streptococcus suis serotype 2 virulence by targeting sulycin. J App Microbiol. 2019;126:435–42.

33. Tanhay Mangoudehi H, Zamani H, Shahangian SS, Mitzanejad L. Effect of curcumin on the expression of ahv3/R quorum sensing genes and some associated phenotypes in pathogenic Aeromonas hydrophila fish isolates. World J Microbiol Biotechnol. 2020;36:70.

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