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Synergistic Effect of *Artemisia scoparia* Extract and TiO_2 Nanoparticles on Antibiotic Resistance of *Klebsiella pnemoniae*

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ABSTRACT

Backgrounds: This study aimed to assess antibacterial properties of *Artemisia scoparia*, Titanium dioxide nanoparticles, and their synergistic effect on clinical isolates of *Klebsiella pneumonia*.

Materials & Methods: In this experimental study, 30 isolates of *K. pneumonia* were collected from patients' sputum in the microbiology lab of Masih Daneshvari hospital during 3 months. Then biochemical tests were performed for strain confirming. Moreover, genomic DNA was extracted from all the isolates, and *hly* gene was detected in the isolates via PCR method. The susceptibility of the isolates to 10 antibiotics was evaluated by the disk diffusion method. Then minimum inhibitory concentration (MIC) of all components (*Artemisia* extract, TiO_2, and their combination) was assessed using the microdilution method against the isolates.

Findings: The results indicated that simultaneous use of hydro-alcoholic extract of *A. scoparia* and titanium dioxide nanoparticles exhibited a significant synergistic antibacterial effect on 25 clinical isolates in comparison with the use of extract or nanoparticles alone.

Conclusion: It seems that simultaneous use of *Artemisia* herbal extracts and nanoparticles is beneficial in increasing their antibacterial effect and may decrease antibiotics consumption.

Keywords: *Klebsiella pneumonia*, Titanium dioxide, Nanoparticles, *Artemisia scoparia*..

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Introduction

Klebsiella is a Gram-negative bacterium of the large family *Enterobacteriaceae,* which is very common in different communities. This bacterium exists as a saprophyte microorganism in human nasopharynx and intestine and is considered as an opportunistic bacterium one of the main causes of pneumonia, nosocomial infections, and urinary tract infection (UTI). In recent years, *klebsiella* has been reported to cause many infections, and the importance of this group of organisms as a cause of serious infections in hospitalized patients has been accepted [1-2]. The ability of this organism to cause diseases due to weakened host defenses as a result of complex and lengthy surgeries as well as the use of various antibiotics has been well documented [3]. The rate of *Klebsiella* colonization is 2 to 4 times higher in hospitalized patients, especially in patients taking broad-spectrum antibiotics [4]. This strain is naturally resistant to penicillin and could acquire resistance to several antimicrobials [5-6]. Today, due to the drug resistance of *K. pneumoniae* to several antibiotics (Multiple Drug resistance) from different classes, including penicillin and carbapenem, there is a need to use new and combination therapies against this strain. In the last decade, the use of nanomaterials has become increasingly widespread so that they have entered all aspects of life. Meanwhile, the use of nanomaterials in medical processes has also increased [7-8]. Titanium dioxide (TiO_2) nanoparticles are an important product in nanotechnology, which are used as a pharmaceutical compound and an attractive candidate to deliver many small drug molecules or large biomolecules such as DNA, RNA, and proteins. TiO_2 nanoparticles are heat resistant and could emit O^{2-} and OH^- species due to light radiation. These compounds could affect membrane lipids and cause their degradation [9-11].

One of the popular treatment approaches for microbial infections is the use of plant extracts. *Artemisia* belongs to the *Asteraceae* (*Compositae*) family. This plant includes 200 to 500 species or subspecies and 5 subgenera. Iran has 34 species of *Artemisia* which is one of the most important plant species after *Astragalus* in terms of distribution and density. Therefore, *Artemisia* community has a 60% coverage [12]. This genus includes several types of medicinal plants. Different species of this genus are used not only in the pharmaceutical industry but also in the food and cosmetics industries. *Artemisia* is rich in substances that have various effects including anti-inflammatory, anti-tumor, anti-ulcer, diuretic, antioxidant, anti-malarial, indigestion relieving, anti-cell proliferation, and antimicrobial properties [13-14]. *A. scoparia* contains essential oils that have been reported to have antimicrobial and antioxidant effects [15-16].

Objectives: Given the dangerous diseases caused by *K. pneumonia* and the prevalence of MDR strains, it is important to treat *K. pneumonia* infections by new compounds. The aim of this study was to evaluate the synergistic antibacterial effect of *A. scoparia* extract and TiO_2 nanoparticles on clinical isolates of *K. pneumoniae*.

Materials and Methods

In this experimental study, clinical samples were collected from patients with suspected respiratory infection, referring to Masih Daneshvari hospital during 3 months in autumn and winter of 2020. The best sample was morning sputum, which was collected through a deep cough. All ethical considerations were taken into account in the research, and informed written consent was obtained from the patients. Samples were cultured on Blood Agar, EMB Agar, and McKankey (Merck, Germany) plates. Subculturing and purification of colonies with similar properties

to *Klebsiella* were performed after 24-48 hours of incubation at 37 °C. Gram staining, differential culture medium, and biochemical tests including indole, Simmon citrate motion, triple-sugar iron agar (TSI), methyl red (MR), and urease (Merck, Germany) were used to identify *K. pneumoniae* isolates.

Molecular identification of *K. pneumoniae*: To confirm the phenotypic tests, PCR reaction was performed to amplify the 77 bp region of the *hly* gene encoding hemolysin which is specific for this strain. To extract DNA, a DNA purification kit (Biospin Bacteria Genomic DNA Extraction Kit, Bioer) was used. Materials used for PCR reaction were as follows: DNA 1µL (50 ng/µL), Master Mix 12.5 µL, and Primer Mix 2µL, and the final volume was increased to 25 µL by deionized water. Specific forward and reverse primers (PF: GATGAAACGACCTGATTGCATTC and PR: CCGGGCTGTCGGGATAAG) were used [17]. Temperature cycles included: primary denaturation at 95 °C for 5 min, secondary denaturation at 95 °C for 1 min, annealing at 60 °C for 30 s, extension at 72 °C for 30 s, and final extension at 72 °C for 5 min. The standard strain of *K. pneumoniae* (ATCC BAA-1705) was used as a positive control. The amplified DNA fragment was then sent to the Gene Fanavaran Company for DNA sequencing.

Antibiotic susceptibility test: Disc diffusion method was used to measure the isolates antibiotic resistance pattern against 10 antibiotics according to the Clinical and Laboratory Standards Institute (CLSI) 2018 guidelines, including gentamicin (10 µg), streptomycin (10 µg), ampicillin (10 µg), meropenem (10 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), cotrimoxazole (1.25-23.75 µg), chloramphenicol (30 µg), ceftriaxone (30 µg), and levofloxacin (5 µg), prepared from Padtan Teb Company (Iran). For each clinical specimen, a standard opacity equivalent to 0.5 McFarland was prepared, and after culturing the bacteria for 24 hours on Müeller-Hinton agar medium, antibiotic discs were placed on a plate and incubated for 24 hours at 37 °C. Then the diameter of the inhibition zone was measured with a ruler. Finally, antibiogram test report was recorded as susceptible, resistant, or semi-resistant. *K. pneumoniae* standard strain (ATCC BAA-1705) was used as the quality control.

Hydroalcoholic extract preparation: About 20 g of dried and grounded leaves of *A. scoparias* (Herbarium number IBRC P1000352) were prepared from Iran Genetic Resources Center and extracted using percolation method [18].

TiO_2 nanoparticles: TiO_2 nanoparticles were purchased from the American company US Nano with an average particle size of 20 nm and a purity of 99.99%.

MIC of *Artimisia scoparia* extract, TiO2 nanoparticles, and the synergistic effect: Minimum inhibitory concentration method was used to determine the MIC of TiO_2 nanoparticles and *Artemisia* extract. The MIC method was performed using the microdilution method in a microplate according to the CLSI 2018 guideline and repeated 3 times. For this purpose, at the first step, 90 µL of Mueller Hinton broth medium was poured into the wells, and then 10 µL of microbial suspension at a concentration of 0.5 McFarland was added (1.5×10^8 CFU/mL bacterial concentration), then it was diluted at a ratio of 1:100 to obtain a density of 10^6 CFU/mL. After mixing with liquid solvents, the final concentration of bacteria was 5×10^5 CFU/mL. In some wells, TiO_2 nanoparticles were poured at logarithmic concentrations of 2-32 µg/mL (5 wells for each isolate), and in some other wells, *Artemisia* extract was poured at logarithmic concentrations of 2-2048 µg/mL (11 wells for each isolate). In order to investigate their synergistic effect, logarithmic concentrations of nanoparticles and extract (5 wells for each

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isolate) were used simultaneously. One well was considered as negative control (without bacteria), and one well was considered as positive control (without extract and nanoparticles). After 24 hours, the amount of light absorption was read at 600 nm using an ELISA reader (AWARENESS, TechnilogyINC, Atat fax 2100), and the concentration of antimicrobial agent in the first well with no visible bacterial growth was reported as its MIC values.

The MIC numbers were analyzed using SPSS software Version 26 by employing independent t-test. The significance level of the results between the groups (treatment with extract, nanoparticles, and simultaneous treatment) was evaluated with a $p<.001$.

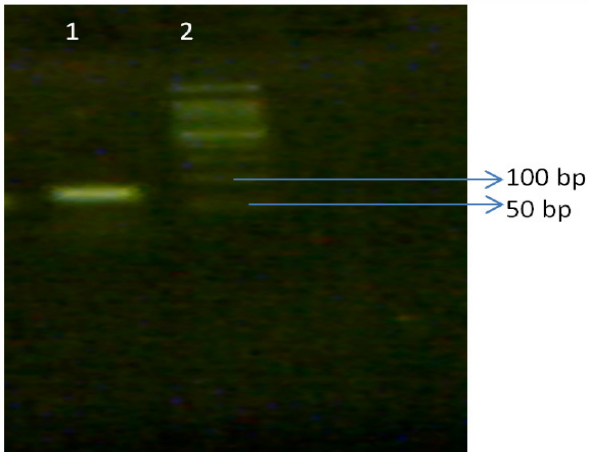

Figure 1) Amplified fragment of the *hly* gene encoding hemolysin by PCR reaction. 1: *K. pneumoniae*, 2: 50 bp size marker.

Findings

In this study, a total of 30 isolates of *K. pneumoniae* were studied and confirmed by phenotypic and genotypic methods. Then for final confirmation, the desired fragment of the *hly* gene was amplified by PCR and confirmed by comparing with a 50 bp DNA size marker. Figure 1 shows the result of electrophoresis of *hly* gene encoding hemolysin. The sequenced fragment was first checked by Chromas software, and then the sequence BLAST was performed on the NCBI site. The results indicated that the similarity of PCR product sequence with *hly* gene sequence in gene bank was more than 98%, indicating genetic confirmation of *K. pneumoniae* strain.

Antibiotic resistance of isolates: The antibiotic resistance of the isolates was evaluated by the disk diffusion method. The lowest resistance of the isolates was related to the levofloxacin (43%), and the highest antibiotic resistance was related to the streptomycin (81%) (Figure 2).

MIC determination: The results of MIC test demonstrated that the average MIC of the extract (896 µg/mL) was significantly (p <.001) higher than that of TiO_2 nanoparticles (14.13 µg/mL), indicating the greater antimicrobial effect of TiO_2 nanoparticles. Also, simultaneous use of both antimicrobials exhibited a synergistic effect against 25 out of 30 isolates while their

Table 1) Comparing the MIC values of all the treatment groups using the independent t-test.

MIC (µg/mL)	Strain Number	Mean ± SE	Significance
***Artemisia scoparia* extract**	30	896 ± 98	-
TiO_2	30	14.13 ± 1.5[a]	$P<.001$
***Artemisia scoparia* extract and TiO_2**	30	7.53 ± 0.78[b]	$P<.001$

a: Compared MIC of the TiO_2 with *A. scoparia* extract, b: Compared MIC of simultaneous treatment with TiO_2 or *A. scoparia* extract.

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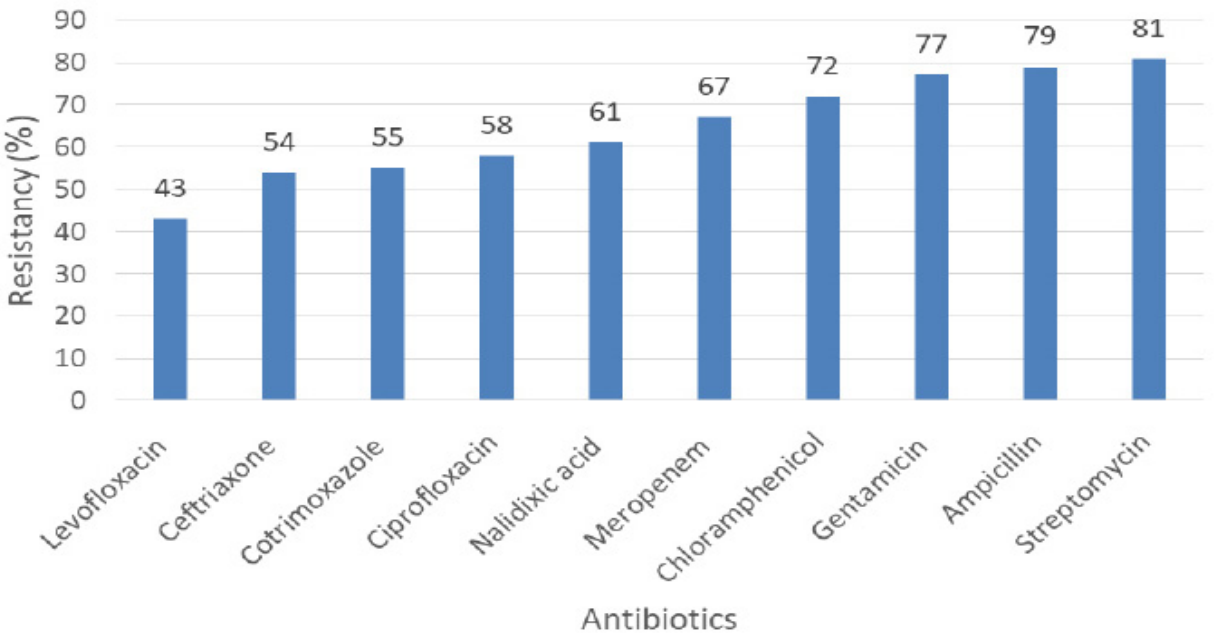

Figure 2) *K. pneumoniae* isolates resistance pattern to selected antibiotics.

MIC values were reduced by one logarithmic concentratio n . The number of isolates treated by the synergistic effect (25 isolates) was also significantly higher (p <.001) than the number o f isolates not treated by the synergistic e ffect (5 isolates). Therefore, it was found that simultaneous use of *Artemisia* ex t ract and TiO_2 nanoparticles exerts a syn e rgistic effect (Table 1).

Discussion

Most *K. pneumoniae* infections are either hospital-acquired or observed in people with debilitating diseases. *K. pneumonia* infection often occurs in hospitalized patients with impaired ciliary function and non-hospitalized patients with chronic obstructive pulmonary disease, diabetes, or alcoholism [19].

It has been reported that 30 µg of TiO_2 nanoparticles is able to produce a 9 mm inhibition zone diameter on *K. pneumoniae* strains using the disk diffusion method. This inhibition zone diameter for *Staphylococcus aureus* is 18 mm [20].

Verdier et al. (2014) indicated that TiO_2 nanoparticles could show an antibacterial effect within two hours [21]. Their study was conducted on *Escherichia coli* strains by employing two types of experiments. These researchers attributed the mechanism of antimicrobial action of TiO_2 nanoparticles to their photocatalytic properties. In the present study, it was found that TiO_2nanoparticles with MIC values of 8-32 µg/mL could exhibit antimicrobial properties.

Tambekar and Dahikar (2011) selected a number of plants native to India and tested their antibacterial properties against a number of bacteria, including *K. pneumoniae*. The results showed that most plants had antibacterial effect [22]. In the present study, it was also observed that *A. scoparia* extract exhibited antimicrobial properties on clinical isolates of *K. pneumoniae* at concentrations between 512 and 1024 µg/mL.

Winnett et al. (2017) prepared 106 extracts from 40 native Australian plants and studied their antimicrobial effects on *K. pneumoniae* isolates [23]. According to the results, 81.1% of the extracts had the ability to inhibit the growth of this bacterium [16, 22]. Furthermore, in the current study, it was found that *A, scoparia* extract possesses antimicrobial

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properties.

Gavanji and colleagues (2014) studied the antibacterial effect of three plant essential oils on *K. pneumoniae*, *Pseudomonas aeruginosa,* and *S. aureus*. The results indicated that all three essential oils had a significant effect on the studied bacteria and among them, *Zataria multiflora* essential oil showed a greater antibacterial effect, and *S. aureus* indicated the highest susceptibility [24]. They also reported the antibacterial effect of *Artemisia* plant against the studied bacteria, indicating that this family species own a strong antibacterial effect. Indeed, the strength point of the present study is that it was performed on clinical isolates, but standard strains were studied in Gavanji's study. However, the effective concentration of *Artemisia* essential oil in Gavanji's report was 100 µg, while in the present study, the average MIC of *Artemisia* hydroalcoholic extract was 896 µg/mL.

Haroun and Al-Kayali (2016) reported the synergistic effect of *Thymbra spicata* L. crude extract with antibiotics against MDR strains of *K. pneumonia* and *S. aureus*. The fractional inhibitory concentration (FIC) index was measured between 0.25-2 for *K. pneumonia* [25].

Hacioglu et al. (2017) studied the antimicrobial activity of 31 widely consumed herbal teas alone and in combination with antibiotics on the bacteria such as *P. aeruginosa*, *Acinetobacter baumannii*, *E. coli*, *K. pneumoniae*, *Enterococcus faecalis*, methicillin-resistant *S. aureus*, and *Candida albicans* yeast through the disc diffusion method [26]. None of the herbal teas showed significant bactericidal properties alone, but their simultaneous use with antibiotics increased their bactericidal properties. There was a significant synergistic property between herbal teas and ampicillin, ampicillin-sulbactam, and nystatin antibiotics. In the present study, it was observed that *Artemisia* extract in the presence of TiO_2 nanoparticles exhibited a synergistic effect (with 7.53 µg/mL mean) against 25 out of 30 isolates of *K. pneumoniae*, this antimicrobial property was significant ($p<.001$).

Conclusion

Considering the importance of controlling infections caused by MDR strains of *K. pneumoniae* and their antibiotic resistance as well as the effect of simultaneous use of herbal compounds and nanoparticles on this bacterium to overcome antibiotic resistance, in this study, the synergistic effect of *Artemisia* extract and TiO_2 nanoparticles on *K. pneumoniae* isolates was studied. The obtained results indicated that this approach could be considered as a combination therapy for MDR strains of *K. pneumoniae* in the future.

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Conflicts of interests: None declared by authors.

Authors' contribution: Conceptualization: MR, SH; Data curation and formal analysis: MR; Investigation: MR; Methodology and project administration: All authors; Supervision: MR; Validation: MR, SH; Writing of original draft: SS; Writing, reviewing, and editing: MR, SH.

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