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Evaluation of Antibacterial Activity of *Withania somnifera* Leaf Extracts against Antibiotic-Resistant Isolates of *Klebsiella pneumoniae*

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Background: Herbal medicines has been a major remedy in traditional medical systems from thousands of years and made a great contribution in maintaining human health and in preventing many infectious diseases.
Objectives: The aim of this study was to evaluate antibacterial activity of leaf extracts of Winter Cheery (*Withania somnifera*) against antibiotic-resistant isolates of *Klebsiella pneumoniae* isolated from the urinary tract infection patients.
Materials and Methods: All 30 isolates of *K. pneumoniae* isolated from urine culture of hospitalized patients (Amir al-Mu'minin Hospital, Zabol, southeastern of Iran) suffered from urinary tract infection during the years 2011 and 2012. In this study, the extract of *W. somnifera* obtained by rotary evaporator. Then the minimum inhibitory concentrations were investigated to characterize the antibacterial activities of this extract.
Results: The isolates of *K. pneumoniae* were resistance to four of the agents including ceftazidime (60% of isolates) cefixime (60% of isolates), erythromycin (66.6% of isolates). The highest MIC values of extract were found to be 250 ppm against *K. pneumoniae* and MIC values for *K. pneumoniae* were 63 ppm.
Conclusions: This study confirmed the antimicrobial potential of investigated plants and their usefulness in treatment of resistant isolates of *K. pneumoniae*.

Keywords:Withania somnifera; Antibiotics; *Pneumoniae*

1. Background

Medicinal plants are very important in human health. It will act as an antibactericide, \followed from ancient times (1). *Withania somnifera* is an evergreen plant, native to the Indian subcontinent, successfully introduced worldwide, now extensively cultivated in many other countries including India. *W. somnifera* (solanacae) are gaining attentions in various field of research, as they are best suited to the present environmental conditions. *W. somnifera* is used for its anti-inflammatory (2), antioxidant (3), memory-improving (4) and analgesic effects (5). The leaves are an insect repellant (6). Development of microbial resistance to antibiotics is a global concern. Isolation of microbial agents less susceptible to regular antibiotics and appearance of increasing resistant isolates during antibacterial therapy is rising throughout the world. *Klebsiella pneumoniae* is a Gram-negative, nonmotile, encapsulated, lactose fermenting, facultative anaerobic, rod shaped bacteria, found in the normal flora of the mouth, skin, and intestines. *K. pneumoniae* is an important pathogen that causes urinary tract infections (UTIs), pneumonia, and intra-abdominal infections in hospitalized immunocompromised patients with severe underlying diseases (7).

2. Objectives

The aim of this study was to evaluate antimicrobial activity of leaf extracts of winter cheery (*W. somnifera*) against antibiotic-resistant *K. pneumoniae* isolated from the UTI patients by microtiterplate method.

3. Materials and Methods

3.1. Isolation of Bacteria

All 30 strains of *K. pneumoniae*, isolated from urine culture of hospitalized patients (Amir al-Mu'minin, Hospital, Zabol, southeastern Iran) suffered from urinary tract infection during years 2011 and2012 were evaluated. Isolated bacteria were identified by Gram's stain and standard biochemical tests (8).

3.2. Agar Disk Diffusion Assay

Resistance to tetracycline was tested by the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) protocols. Antibiotic disks were obtained from the Patan ted-Iran. *K. pneumoniae* isolates were evaluated based on the size of the zones of inhibition and classified as susceptible (S), intermediate resistant (I) or resistant (R) according to the CLSI criteria (9).

3.3. Plant Materials

The leafs *W. somnifera* were collected from Zabol, Southeastern of Iran) and dried at room temperature. Samples were crashed and transferred into glass container and preserved until extraction procedure was performed in the laboratory.

3.4. Preparation of Extracts

Twenty grams of selected fresh leaf materials were macerated with 60 mL of ethanol 95%, in a grinding machine for about 10 to 15 minutes for separating the extract phases for one day (shaking occasionally with a shaker). The supernatant was filtered through Whatman No. 1 filters paper. The extracts were preserved aseptically at 5°C for further use.

3.5. Minimum Inhibitory Concentration (MIC) of Plant Extracts

The broth microdilution method was used to determine MIC. All tests were performed in Mueller Hinton broth supplemented with Tween 80 at a final concentration of 0.5% (v/v). Briefly, serial doubling dilutions of the extract were prepared in a 96-well microtiter plate ranged from 500 ppm, 250 ppm, 126 ppm, 63 ppm and 31 ppm. In each well, 10 µL of indicator solution and 10 µL of Mueller Hinton Broth were added. Finally, 10 µL of bacterial suspension (10^6 CFU/mL) was added to each well to achieve a concentration of 10^4 CFU/mL. The plates were wrapped loosely with cling film to ensure that the bacteria did not get dehydrated. The plates were prepared in triplicates, and then they were placed in an incubator at 37°C for 18-24 hours. The color change was then assessed visually. The lowest concentration at which the color change occurred was considered as the MIC value. The average of three values was calculated providing the MIC values for the tested extract. The MIC is defined as the lowest concentration of the extract at which the microorganism does not demonstrate the visible growth. The microorganism growth was indicated by turbidity.

4. Results

4.1. Antibiotic Susceptibility

Antibiotic susceptibility of *K. pneumoniae* isolates was evaluated by four antimicrobial agents. However, overall, *K. pneumonia* were resistance to four of the agent including ceftazidime (60% of isolates) cefixime (60% of isolates), erythromycin (66.6% of isolates).

4.2. Antibacterial Activity

Inhibitory effects of plant extract from *W. somnifera*, against *K. pneumoniae* were demonstrated in Table 1 showing that plant extracts from *W. somnifera* had inhibitory effect against *K. pneumoniae*. The MIC values were also determined against all the tested bacteria. The highest MIC values of extract were found to be 250 ppm against *K. pneumoniae* and two of MIC value for *K. pneumoniae* was 63 ppm.

Table 1. Antimicrobial susceptibility, Minimum Inhibitory Concentration Extract Plant for K. pneumonia [a,b]

Bacterial	MIC, µg/mL	Antibiotic-Resistant
1	250	$A_1, A_2,$
2	250	A_1, A_2
3	63	A_1
4	126	-
5	250	A
6	250	A_1, A_2, A_3
7	250	A_1, A_2, A_3
8	250	A_1, A_2, A_3
9	126	-
10	250	A_1, A_2, A_3
11	126	A_1
12	126	A_2
13	250	A_1, A_2
14	250	A_1, A_3
15	126	A_1, A_2, A_3
16	250	A_1, A_2, A_3
17	126	A_3
18	126	A_1, A_2, A_3
19	63	A_1, A_2, A_3
20	126	A_3
21	250	A_1, A_2, A_3
22	250	A_3
23	250	A_3
24	126	A_3
25	126	A_1, A_2, A_3
26	250	A_2
27	250	A_1, A_2, A_3
28	250	A_1, A_2
29	250	A_1, A_2, A_3
30	250	-

[a] Abbreviation: MIC, Minimum Inhibitory Concentration.
[b] A_1, Erythromycin; A_2, Cefixime; A_3, Ceftazidime.

5. Discussion

In this study, antibiotic susceptibility of *K. pneumoniae* isolates was evaluated for three antimicrobial agents. Generally, *K. pneumonia* clinical isolates were resistance to three of the agent including ceftazidime (60% of isolates), cefixime (60% of isolates), and erythromycin (66.6% of isolates). In another study, *K. pneumoniae* were resistance to six agents including ampicillin (65%), gentamicin (30%), trimethoprim-sulfamethoxazol (25%), ciprofloxacin (20%), nitrofurantoin (15%) and nalidixic acid (15%) (10). As discussed by Zamani et al. The most effective antibiotics against the isolates were tobramycin (79.05%), ceftazidime (79.05%), ceftizoxime (78.09%), ciprofloxacin (76.19%), ceftriaxone (76.24%) and amikacin (74.29%) (11). The MIC values were also determined against all the tested bacteria. The highest MIC values of extract were found to be 250 ppm against *K. pneumoniae* and two of MIC value for *K. pneumoniae* was 63 ppm.

Nowadays, plants are considered as one of the most important source of medicine and drugs and many secondary metabolites and essential oils come from medicinal plants (12). The use of medicinal plants proved to be economical and effective; in addition, they are easily available and safe to use. Highest antibacterial activity was recorded for glacial acetic acid extract - IZ (Inhibition zone) -25.83 ± 0.23 and AI (Activity index) -2.583 - and IZ -21.67 ± 0.21 and AI -1.084) against *P. mirabilis* and *K. pneumoniae*, respectively followed by toluene extract (IZ-20.33 ± 0.22 and AI-1.017) against *A. tumefaciens* (13). It was observed that aqueous extract of *W. somnifera* was more effective in inhibiting the growth of *Eschericha coli, Pseudomonas aeruginosa, Staphylococcus* mutans and *Candida albicans* with zone of inhibition ranging between 33 mm and 50 mm, as compared to methanol root extract (15 to 38 mm) (14). MIC obtained were 31 μg/mL and 62 μg/mL against both the pathogens *N. asteroids* and *S. pyogenes* with *W. somnifera* (15). However, antibacterial potentials were observed against *S. aureus* when treated with extracts of *W. somnifera* (leaves) with an inhibition zone 25 mm (16). Our result agree with other observations that the susceptibility of bacteria to *W. somnifera* was highly observed (17, 18). The results showed that pathogenic bacteria were significantly more susceptible to the extract of *W. somnifera* than bifidobacteria (average of diameters of inhibition zones 20.45 mm and 13.10 mm, respectively) at the concentration of 2 mg/disc (19). In general, flavonoids have been widely studied for their effective antibacterial activity (20). Since various flavonoids, including quercetin glycosides were also found in the leaves of *W. somnifera* (21), we suppose that flavonoid or other phenolic compounds may participate in selective inhibitory action of the tested extract. In vitro studies in this work showed that the plant extracts inhibited bacterial growth, though with various effectiveness. Ethanol extract of *W. somnifera* showed higher inhibition against *K. pneumoniae* at high concentration. The antibacterial activity has been attributed to the presence of some active constituents in the extract. The demonstration of broad spectrum of antimicrobial activities by the plant used in this study may help to discover new chemical classes of antibiotic substances that could serve as selective agents for infectious disease chemotherapy and control. The development of natural antimicrobial agents will help to decrease negative effects (pollution in environment and resistance) of synthetic chemicals and drugs. It can really contribute to medical and pharmaceutical practices. There are still many more activities waiting for screening the drugs.

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