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Antibacterial activity of *Marrubium vulgare* L. against antibiotic resistance *Klebsiella pneumoniae* strains

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ABSTRACT

Background and aims: Herbal medicines are the major remedy in traditional medical systems and have a great contribution in maintaining human health and preventing many infectious diseases. The aim of this study was to assay antibacterial potential of *Marrubium vulgare* L. extract against *Klebsiella pneumoniae* resistant strains to current antibiotics and also GC/MS analysis to better understanding the extract composition.
Methods: In this experimental research, 30 *K. pneumoniae* strains isolated from urine culture of hospitalized patients were used. The essential oil of *Marrubium vulgare L.* was obtained by hydro distillation for 2 hours using the Celevenger with yield of 75%. Methanolic extract from *M. vulgare L.* was prepared using Rotary apparatus. In order to determine chemical composition of essential oil, gas chromatography coupled with mass spectrometry (GC-MS) was performed. The minimum inhibitory concentrations and minimum bacterial concentrations were investigated to characterize the antimicrobial activities of this essential oil and its extract. Data were analyzed using analysis of variance (one-way) to determine the statistical differences between different tests.
Results: The results showed that *K. pneumoniae* strains were resistant to 4 or 3 agents including: Ampicillin (65%), Gentamicin (30%), Sulfamethoxazol (25%). The lowest and the highest MIC value of *M. vulgare L.* extract were 2.5 and 10 mg/mL, respectively. The highest and the lowest MIC value of *M. vulgare L.* essential oil was 5 and 1/25 mg/m respectively.
Conclusion: The present study confirmed that essential oil and extract of this plant could be served as an antibacterial agent in pharmaceutical industry.

Keywords: *Klebsiella pneumoniae*, Antibacterial effect, antibiotic resistance, Gas chromatography.

Original article

INTRODUCTION

The resistant of pathogenic bacteria to chemical drugs as current antibiotics is one of the most important problems in controlling and treatment of the microbial diseases. Klebsiella infections are mainly caused by *K. pneumoniae* and *K. oxytoca* strains. They are opportunistic pathogenic bacterial associated with nosocomial infections such as urinary tract infection,[1] pneumoniae and septicaemia.[2] Accordingly; natural plant products with antimicrobial properties have been recognized by their

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possibilities for applications in food in terms of preventive of bacterial and fungal growth. *Marrubium vulgare* L. *(Lamiaceae)* commonly is known as a white horehound that is a perennial and flowering plant.[3] About forty species belong to *Marrubium* genus have been identified which are distributed in Europe, Asia and Brazil.[4] Medicinal properties of horehound have been known for a long time. Chemical analysis of the essential oil of *M. vulgare* L. by GC and GC/MS showed that the composition consists twenty components so that β-bisabolenes (20.4%) were determined as the major constituent components.[5] In this work, firstly, it was attempted to assay antibacterial potential effects of *M. vulgare* L. extract against *K. pneumoniae* resistant strains to current antibiotic. Secondly, in order to better understanding the extract composition, GC/MS analysis was performed.

METHODS

The leaf of *M. vulgare* was collected in Kerman, south-eastern of Iran and then washed under running water to remove the dust. The plant samples were dried for few days in the room temperature. Finally, samples were powdered, and then transferred into glass containers and stored until extraction procedure.

Twenty gram grinded powders were soaked in 60 ml ethanol 95 %, for one day (shaking occasionally with a shaker). After one day of dissolving process, materials were filtered using a filter paper (Whatman No.1). Then, the filtrates were evaporated using rotary evaporator and were taken to be used for further use. Methanolic extract from *M. vulgare* was prepared using Rotary apparatus. The essential oil of *Marrubium vulgare* L. was obtained by hydro-distillation for 2hrs using the Celevenger with yield of 75%.

The essential oil was analyzed using an Agilent-Technologies 6890 N Network GC system equipped with a flame ionization detector and HP-5MS capillary column (30 m × 0.25 mm, film thickness 0.25 μm; Agilent-Technologies, Little Falls, CA, USA). The injector and detector temperatures were set at 250ºC and 280ºC, respectively. The column temperature was programmed from 35 to 250ºC at a rate of 5ºC/min, with the lower and upper temperatures which was held for 3 and 10 minutes, respectively. The flow rate of the carrier gas (Helium) was 1.0 ml/min. A sample of 1.0 μl was injected, by using a split mode (split ratio, 1:100). The identification of the essential oil constituents was based on a comparison of their retention times to N-Alkanes, compared to the published data and spectra of authentic compounds. Compounds were further identified and confirmed using their mass spectra compared to the Wiley version 7.0 library.

In this study, 30 strains of *K. pneumoniae* were isolated from urine culture of hospitalized patients referred to Amir Al-Momenin Hospital (Zabol, Iran) who suffered from urinary tract infections. All strains were identified by Gram's stain and were confirmed using standard biochemical tests.

The susceptibility of isolated strain to tested antibiotics was carried out using standardized single disc diffusion method.[6] Briefly, a suspension of 10^7 cfu/mL was prepared using the sterile normal saline equivalent to a 0.5 McFarland standard and was spread over the plate containing Mueller Hinton Agar (Difco, Germany). Then, the antibiotic discs were aseptically transferred to the surface of agar plates seeded with tested strains. Antibiotics activity was performed using commercial antibiotic discs (Padtan Teb, Iran) containing Ampicillin 25 μg, Sulfamethoxazol 23.15 μg

and Gentamicin 10 µg. Plates were incubated at 37 for 24 hours and the zone of inhibited colony growth was recorded and then it was compared with control.

The broth micro-dilution method was used to MIC determination.[7] Briefly, serial doubling dilutions of the extract and the essential oil in Mueller Hinton broth containing 0.5 % (V/V) Tween 80 over the range 0.3 mg/ml to 10 mg/ml were prepared and added to a 96-well micro-titer plate. To 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 of the bacteria. 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 incubated at 37 °C for 18–24 hours. The MIC was regarded as the lowest concentration (highest dilution) of the essential oil or extract that visible growth of bacteria was inhibited. The average of three values was calculated to provide the MIC values for the tested extract. Following MIC assay, 10µl of bacterial solution in the determined MIC plate-well was transferred into extract free Mueller Hinton broth. After 18 incubation, a drop (100 µl) of bacterial suspension containing 10^8 Cfu/mL was aseptically transferred onto nutrient Agar plate. The plates were transferred onto incubator at 37 °C for 24 hours. The lowest concentration of the extract or the essential oil that reduces the viability of the initial bacterial inoculums by ≥99.9% was regarded as MBC. Data were analyzed using analysis of variance (one-way).

RESULTS

Antibiotic susceptibility of *K. pneumoniae* was evaluated for 3 antimicrobial agents. However, overall *K. pneumoniae* was resistant to three tested antibiotics. The highest resistance of strains to antibiotic was determined for Ampicillin antiobiotic (Table 1). The extract had preventive effect on the most isolates. The highest and the least MIC value for *M. vulgare* extract was 10 and 2.5 mg/mL, respectively (Table 2).

Table 1: Susceptibility of *Klebsiella pneumoniae* strains.to used antibiotic

Susceptibility	AM	GM	SXT
Susceptible	0	20	70
Intermediate	35	50	5
Resistance	65	30	25

Table 2: Antimicrobial activity of plant extract of *M. vulgare*

Concentration	10 mg/ml	5 mg/ml	2.5 mg/ml	1.25 mg/ml	0.62 mg/ml	0.3 mg/ml
*MIC	0	5.88	64.70	53.23	0	5.88
**MBC	100	94.11	30.41	5.88	5.88	0

**Minimal Inhibitory Concentration; **Minimum Bactericidal Concentration.*

The essential oil had preventive effect on the most isolates. The least and the highest MIC value of *M. vulgare* essential oil was 1.25 and 5 mg/mL respectively (Table 3). The GC-MS analysis of the essential oil showed that *M. vulgare* essential oil contains γ-Eudesmol (11%), Germaerene (10%), D-citronelly formaerene (10%), β-Citronellol (8%), which all of them were the percentages in the fairly good compounds (Table 4).

Table 3: Antimicrobial activity of the essential oil of *M. vulgare*

Concentration	10mg/ml	5mg/ml	2.5mg/ml	1.25mg/ml	0.62mg/ml	0.3mg/ml
*MIC	0	0	20.35	68.53	23.5	5.88
**MBC	100	94.11	80.59	29.35	5.88	0

**Minimal Inhibitory Concentration; **Minimum Bactericidal Concentration.*

Table 4: The composition of essential oil of *M. vulgar*e by GC-MS analysis

Chemical composition	RI	%	Chemical composition	RI	%
α-Humulene	1462	0.52	Camphene	790	3.15
α-Muurolene	1458	0.63	N,N-bistrimethylsily	862	0.77
α-Amorphene	1492	0.88	α-pinene	935	2.16
D-Germacrene	1510	10	1-vinylcyclohexane	960	0.50
Neryl acetate	1515	3.41	Geraniol	1022	3.70
δ-Eudesmol	1530	11	α-Thujone	1131	2.45
Lenden	1539	5.15	1,8-cineole	1151	3.75
β-Lisabdene	1544	0.77	Camphor	1188	1.03
Candinene	1565	3.35	Iso menthon	1192	0.67
α-Agarofurane	1589	0.42	Borneol	1212	0.62
Furane-2-one, 4-phenyl tetrahydro	1623	1.44	β-citronellol	1256	8
Geranyl tiglate	1639	7.1	Trans-caryophyllene	1290	2.34
β-Cubebene	1662	3.30	Citronellyl formate	1343	10
Cyclononasiloxane octadecamethyl	1689	4.3	Geranyl formate	1367	6.02
α-Humulene	1462	0.52	α-Copaene	1422	1.35
α-Muurolene	1458	0.63	β-Bourbonene	1456	1.80
α-Amorphene	1492	0.88	α-Humulene	1462	0.52
D-Germacrene	1510	10	α-Muurolene	1458	0.63
Neryl acetate	1515	3.41	α-Amorphene	1492	0.88
δ-Eudesmol	1530	11	D-Germacrene	1510	10
Lenden	1539	5.15	Neryl acetate	1515	3.41
β-Lisabdene	1544	0.77	δ-Eudesmol	1530	11
Candinene	1565	3.35	Lenden	1539	5.15
α-Agarofurane	1589	0.42	β-Lisabdene	1544	0.77
Furane-2-one, 4-phenyl tetrahydro	1623	1.44	Candinene	1565	3.35
Geranyl tiglate	1639	7.1	α-Agarofurane	1589	0.42
β-Cubebene	1662	3.30	Furane-2-one, 4-phenyl tetrahydro	1623	1.44
Cyclononasiloxane octadecamethyl	1689	4.3	Geranyl tiglate	1639	7.1
α-Humulene	1462	0.52	β-Cubebene	1662	3.30
α-Muurolene	1458	0.63	Cyclononasiloxane octadecamethyl	1689	4.3

DISCUSSION

In this study, the antibacterial effect of plant extract and the essential oil of *Marrubium vulgare* L. on the resistant antibiotics such as *K. pneumoniae* strains were investigated. To illustrate better of this aspect, firstly this essential oil was analyzed by GC mass chromatography. Sixty components in the essential oil of *M. vulgare* were identified. The data analysis of these components showed that Eudesmol (11%), and Geranyl formate (6.02%) have the highest and the lowest percentage, respectively. Our results showed that there are no significant

differences in the chemical composition when it is compared to the same essential oil of plant from other country,[2] but in the most of studies, Eudesmol has been detected in large amount.[9] In other study, chemical analysis of the essential oil of *M. vulgare* L. the main component was E-caryophyllene,[10] but these results are not in agreement with our results which can be related to several factors including species of the plant and growth conditions. Antibacterial activity of methanolic and aqueous extract of this plant revealed that methanolic extract of the crude drug was more effective than the aqueous extract on gram positive *B. subtilis*, *Staphilucoccus epidermidis* and *S. aureus* strains and moderately is effective on the *P. vulgaris* and *E. coli* strains.[11] According to this observation, it was used the antibacterial activity of *M. vulgare* essential oil against *K. pneumoniae* strains. The lowest and the highest MIC values of *M. vulgare* essential oil were 0.3 mg/mL and 5 mg/mL, respectively. The effect of aqueous extract of *M. vulgare* from Syria on *Mycobacterium tuberculosis* showed a significant antimicrobial activity which was represented by the decrease in the degree of bacterial growth.[12] In the current study, it was not found a correlation between the concentration of the essential oil and MIC activity while the MBC activity showed a direct relation to the used concentration. This finding is in accordance with some related studies.[12] The methanolic extract of *M. vulgare* exhibited moderate to significant antibacterial activity against five out of six tested bacterial organisms, as it was compared to the standard ciprofloxacin (10 µg/mL).[10]

High antibiotic activity of the crude methanolic extract of *M. vulgare* L. against *B. subtilis, S. epidermidis, S. aureus* (Gr+) and *C. albicans* and moderately effectiveness of *P. vulgaris* and *E. coli* has been revealed.[12] The antifungal activity of this plant against four plants pathogenic fungi showed that this essential oil has the highest activity on *Botrytis cinerea* with inhibition zones of 12.6 mm, while *Fusarium solani, Penicillium digitatum* and *Aspergillus niger* were less sensitive to the essential oil.[13]

CONCLUSION

Regarding these results, it is concluded that the essential oil of *M. vulgare* had strong antimicrobial activity on tested strains. The obtained results suggest that further studies for the isolation and identification of the more active component of extract are required for assessing their antimicrobial activity. For this purpose the degree of toxicity of these extracts should be determined to introduce this plant to the pharmacological industry.

CONFLICT OF INTEREST

All authors disclose any financial and personal relationships with other people or organizations and the authors declare that there are not any potential conflicts of interest. I indicate here that any color photo in print is required.

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