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Chemical Composition, Genotoxicity and Antimicrobial Activities of *Dracocephalum kotschyi* Boiss against OXA-48 Producing *Klebsiella pneumoniae* Isolated from Major Hospitals of Kurdistan Province, Iran

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Authors' contributions

This work was carried out in collaboration between all authors. Author PS carried out the study and collected data. Author RR supervised the study, participated in designing and conducting it, and prepared the original version of the manuscript. Author MT participated in designing and conducting it, and revising of the manuscript. All authors studied and approved the content of the present manuscript and participated in revising the paper.

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ABSTRACT

Aims: *Dracocephalum kotschyi* Boiss is a flowering plant that the biological activities of the declared species *D. kotschyi* such as a warm herbal medicine for rheumatoid diseases, headaches, congestion, stomach disorders, liver treatment, and other usages. The current study aims to investigate the chemical composition, genotoxicity, and antimicrobial activity of *Dracocephalum kotschyi* Boiss against OXA-48 producing *K. pneumoniae* isolated in Kurdistan province, Iran.

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Methodology: Fourteen OXA-48 producing *K. pneumoniae* were isolated by the PCR assay from major hospitals in Kurdistan province, Iran. GC–MS examination, and *in vitro* antimicrobial and genotoxicity activities of this essential oil was studied. The data was analyzed using SPSS software and ANOVA.
Results: Out of 70 *K. pneumoniae* clinical strains, 14 OXA-48 producing *K. pneumoniae* were isolated by the PCR assay. The chemical composition of the oils contained Monoterpene Hydrocarbons, Oxygenated Monoterpenes, Sesquiterpene Hydrocarbons, and Oxygenated Sesquiterpenes. *D. kotschyi* essential oils showed antimicrobial activity against all of the tested strains, with inhibition values varying from >5000 to 1250 µg/ml for MIC and >5000 µg/ml for MBC in bacteria. In the Ames test, *D. kotschyi* had a strong anti-mutagenesis effect.
Conclusion: According to the findings of the current study, it can be concluded that *D. kotschyi* essential oils could be applied as a safe antibacterial agent for treatment of OXA-48 producing *K. pneumoniae.*

Keywords: Antibacterial effect; MIC; MBC; l Ames test; Dracocephalum kotschyi Boiss.

1. INTRODUCTION

Klebsiella pneumoniae is an opportunistic pathogen that causes infections in the urinary tract (UTI) and respiratory systems, and can result in systemic infections [1,2]. Carbapenems are one type of antibiotics that are used for treatment of *K. pneumoniae* infections [3]. Currently, carbapenem resistance in *K. pneumoniae* isolates is growing and is generating a great concern [2]. Carbapenem resistance is created by many mechanisms such as upregulation of efflux pumps or loss of porins, and existence of carbapenem-hydrolyzing enzymes (carbapenemases) [4]. Oxa-48-type carbapenemase class D β-lactamases are generally spread among *K. pneumoniae* [5]. Currently, different approaches are applied in order to control antibiotic-resistant pathogens, including medicinal plants that are being used for treatment of various pathogens [6].

Dracocephalum kotschyi Boiss is a flowering plant that belongs to the Labiateae family, floating fragments of *D. kotschyi* are original of essential oils and flavonoids [7]. *D. kotschyi* is native to Iran and grows in several provinces, including Lorestan, Golestan, Fars, Mazandaran, Hamadan, and Tehran. Some studies have conducted to research the biological activities of the declared species [8,9]. In traditional medicine, *D. kotschyi* is applied as a warm herbal medicine for rheumatoid diseases, headaches, congestion, stomach disorders, liver treatment, and other usages that are described for *D. kotschyi* [7,10,11]. There is no report of the antimicrobial activity of this plant against OXA-48 producing *K. pneumoniae* in Pub Med and Google Scholar. Therefore, the present study is aimed to investigate the chemical composition, genotoxicity, and antimicrobial activities of *D. kotschyi* against OXA-48 producing *K. pneumoniae* isolated from patients in Kurdistan province, Iran.

2. METHODOLOGY

2.1 Plant Material

Flowering fragments of *D. kotschyi* Boiss were collected from Ashrafi project [9]. *D. kotschyi* Boiss was collected from the Garin Mountain in Lorestan province, Iran, by Ashrafi et al.

2.2 Analysis of the Essential Oil

Gas chromatography - mass spectrometry (GC-MS) analysis was carried out in an Agilent 6890 gas chromatograph interfaced with an Agilent 5973 MSD, applying helium as the carrier gas (0.9 ml/min, and the same capillary column previously mentioned). The column temperature was raised from 50 (hold 3 min) to 180ºC at the rate of 5ºC/min and up to 240ºC at 10ºC/min rate; and then the temperature was kept stable for an additional 20 min at 280ºC. The injector and detector temperatures were 200 and 280ºC, respectively. A 0.3-µl sample was injected using the split mode [12].

2.3 Microbial Strains, DNA Extraction and PCR-Amplifications of OXA-48 Gene

Seventeen *K. pneumoniae* strains originating from clinical specimens were obtained from a general hospital in Kurdistan Province, Iran. *K. pneumoniae* clinical isolates were identified by standard microbiological tests including IMViC and oxidase tests and they underwent pure

culture [13]. DNA was extracted from 24 h cultures grown in LB (Lorea Broth) medium using SinaClon kit according to the manufacturer's instructions. The amplification of OXA-48 gene was performed by specific primers in conditions described by the authors. The amplification was done by the thermo cycler system (BioRad, Australia) in a 0.2 ml tubes in a total volume of 25 µL containing 12.5 µl of the master mix PCR (YT1553, Iran), and 200 pM of each primers were designed by Zowawi and 1 µl DNA. Positive Control was obtained from the Pasteur Institute of Iran. The PCR products were analyzed on a 2% TAE (Tris-Acetic Acid-EDTA) agarose gel, stained with DNA safe stain at 80 V for 45 min, imagined under ultraviolet light transillumination, and snapped with Gel Documentation [14].

2.4 Minimum Inhibition Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

By using 96-well microtiter plates and utilizing the broth microdilution method, the minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations were determined. In a typical procedure, the bacterial strains were cultured overnight at 37°C on Muller-Hinton agar adjusted to a final density of 8 log CFU/mL, which were used to inoculate (1-10) 96-well microtiter plates containing the serial dilutions of the essential oils (5120-20 µg/ml) on MHB. A positive control containing the bacterial culture and broth without the plant oils was included in each test. The contents of each well were incubated at 37°C for 24 h. Then, 2, 3, 5-triphenyltetrazolium chloride was used for visual indication of bacterial growth. The MIC of the essential oils was regarded as the lowest concentration that showed zero growth [15]. All the samples that showed no turbidity were subcultured; however, the lowest concentration, from which the microorganisms did not recover was the minimal bactericidal concentration (MBC). Each experiment was performed in triplicate [15].

2.5 Disc Diffusion Assay

Antibacterial activity of *D. kotschyi* was assessed by the well diffusion assay (NCCLS, 1993). Discs containing imipenem and DMSO 10% were used as positive and negative controls respectively; then, plates were incubated at 37°C for 24 h. Antimicrobial activity was determined by measuring the inhibition zone against the tested clinical isolates [16].

2.6 Ames Test, Media Preparation

The Ames test was carried out by glucose minimum agar (10% glucose), Top agar with 0.005 mM Biotin-Histidine and nutrient broth (Merck, Germany), and Salmonella typhimurium TA100 isolate. The strain had histidine mutation in the histidine operon [17].

2.7 Statistical Analysis

All the experiments were repeated three times. The data was analyzed by SPSS software program, version 18, using ANOVA with the least significant difference (LSD) at the 0.05 probability level.

3. RESULTS

3.1 Microbial Strains, DNA Extraction and PCR-Amplifications of OXA-48 Gene

Out of 70 *K. pneumoniae* clinical isolates, 14 OXA-48 producing *K. pneumoniae* were isolated by the PCR assay from different general hospitals of Kurdistan Province of Iran.

Fig. 1. Results of PCR assay for OXA-48 gene, PCR products were separated in a 2% agarose gel. Lane 1, molecular size marker 100 bp; Lane 2, negative control; Lane 3, *K. pneumoniae* isolates non-OXA-48 gene; Lane 4, positive control; lane 5,6, *K. pneumoniae* isolates with OXA-48 gene

3.2 Analyses of the Essential Oils

The Mass chromatogram of *D. kotschyi* essential oil is shown in Fig. 2 and chemical composition of the oils is listed in Table 1. The retention indices for each compound are also presented in Table 1.

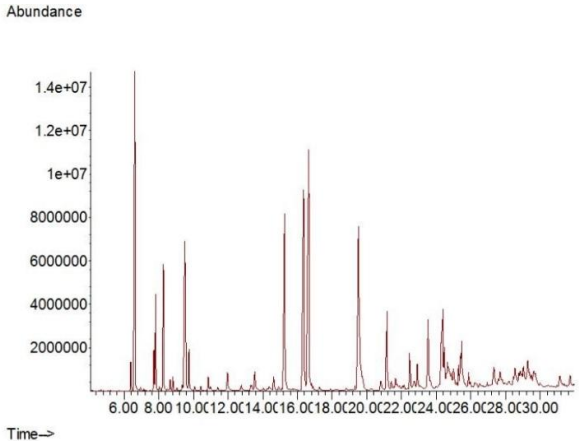

Fig. 2. *Dracocephalum kotschyi* essential oil chromatogram, carried out using a gas chromatography mass spectrometry Agilent 6890 with a capillary column of HP-5MS

Table 1. Chemical composition of the essential oils from the aerial parts of *D. kotschyi*

No.	Compounds		RI (retention indices)	Relative content (%)
1	Monoterpene	α-Thujene	929	0.71
2	Hydrocarbons	α-Pinene	935	10.34
3		Sabinene	975	1.01
4		β-Pinene	978	2.18
5		β-Myrcene	993	3.42
6		α-Phellandrene	1006	0.31
7		Δ-3-Carene		0.34
8		p-Cymene	1024	0.18
9		Limonene	1032	6.95
10		(Z)- β-ocimene	1038	1.13
11		ϒ-Terpinene	1057	0.15
12	Oxygenated	trans-Sabinene hydrate	1093	0.41
13	Monoterpenes	Linalool	1100	0.81
14		α-Terpineol	1192	0.54
15		Neral (citral b)	1231	8.90
26		Geranial (Citral a)	1236	12.08
17		Geraniol	1258	9.55
18		Carvacrol	1297	0.72
19		Geranyl acetate	1365	10.27
20	Sesquiterpene	α-Cubebene	1375	0.17
21	Hydrocarbons	α-Copaene	1377	3.61
22		β- Bourbonene	1387	0.37
23		β- Elemene	1411	0.43
24		trans-Caryophyllene	1415	2.04
25		β-selinene	1436	0.76
26		trans-β-Farnesene	1452	3.46
27		Zingiberene	1476	0.93
28		Aromadendrene	1479	1.50
29		Germacrene-D	1481	3.38
30		β-Bisabolene	1504	1.16
31		δ-Cadinene	1537	2.33
32		α-Gurjunene	1557	1.20
33		valencene	1483	0.66
34		ϒ-Gurjunene	1587	0.43
35	Oxygenated Sesquiterpenes	Caryophyllene oxide	1595	0.36

3.3 Antimicrobial Activity of the Essential Oil

The antimicrobial activities of *D. kotschyi* against oxa-48 producing *K. pneumoniae* are investigated in the present study by in vitro microbiological techniques including the minimum inhibitory concentrations (MICs), minimum bactericidal concentration (MBC), and disc diffusion assay. MIC and MBC were determined by the broth micro-dilution method and the results are listed in Table 2. *D. kotschyi* showed antimicrobial activities against 14 oxa-48 producing isolates, with inhibition values varying from >5000 to 1250 µg/ml for MIC and >5000 µg/ml for MBC in bacteria [18].

Considering the radius of the inhibition zones, essential oils exhibited weak antibacterial activity against OXA-48 producing isolates, with a 10.40 ± 0.05 mm zone of inhibition. The detected antibacterial properties of the essential oils against various bacteria were different, ranging from strong antibacterial activity (inhibition zone > 20 mm) to moderate activity (inhibition zone < 12–20 mm) and even no inhibition (zone < 12 mm) [19].

3.4 Genotoxicity Activity of the Essential Oil

Results of the Ames test in this study, showed that inhibition percentage of *D. kotschyi* was 65, 37, and 19% in 5000, 2500, and 1250 (µg/ml) respectively (Table 3). Inhibition percentages were evaluated by the formula presented by Ong et al. in 1986 [20]. According to the analyses, significant differences were observed between all the treatments (S9-) compared to the control group ($P<0.05$). A significant difference was observed between 5000(µg/ml) in comparison with the control group (S9+) ($P<0.05$). A significant difference was observed between 5000 (µg/ml) compared to 2500 (µg/ml) treatment (S9+) ($P<0.05$). A significant difference was observed between 2500 (µg/ml) in comparison with the control group (S9+) ($P<0.05$). A significant difference was observed between 2500(µg/ml) compared to 1250(µg/ml) treatment (S9+) ($P<0.05$). No significant difference was observed between 1250(µg/ml) compared to the control group (S9+) ($P>0.05$).

4. DISCUSSION

OXA-48 carbapenemase in *Klebsiella pneumoniae* was first defined in Turkey, then in several European countries such as France, and then in Middle Eastern countries including Lebanon and Kuwait, and afterwards in India and North Africa [21-24]. In this study, 14 OXA-48 positive clinical strains were isolated from different general hospitals of Kurdistan province of Iran. Carbapenemase strains are one of the main concerns in treatments because they are resistant to most antibiotics that are currently being used [5]. In recent decades, essential oils are used as an alternative to antibiotics for treatment of many drug-resistant bacterial infections [6]. Accordingly to this, the aim of our study was evaluate antimicrobial activities of *Dracocephalum kotschyi* Boiss against OXA-48

Table 2. Antimicrobial activity of *D. kotschyi* essential oils

Bacterial strains	Essential oil			Imipenem		
	DD	MIC	MBC	DD	MIC	MBC
Kp 1	8	>5000	>5000	43	2	>128
Kp 2	-	5000	>5000	44	2	>128
Kp 3	-	5000	>5000	39	2	>128
Kp 4	-	5000	>5000	31	2	>128
Kp 5	-	2500	>5000	31	2	>128
Kp 6	-	>5000	>5000	28	0.5	>128
Kp 7	-	2500	>5000	29	>128	>128
Kp 8	-	5000	>5000	34	2	128
Kp 9	10	>5000	>5000	40	0.5	>128
Kp 10	-	>5000	>5000	31	2	>128
Kp 11	-	1250	>5000	34	2	>128
Kp 12	-	5000	>5000	33	2	>128
Kp 13	-	2500	>5000	22	32	>128
Kp 14	-	5000	>5000	35	2	>128

**DD: diameter of inhibition zone (mm) including disk diameter of 6 mm. MIC, MBC: values given as µg/ml (for the essential oils and antibiotics). KP: Klebsiella pneumoniae*

Table 3. Anti-mutagenesis effect of *D. kotschyi* using *S. typhimurium* strain TA100, in the absence (-S) or in the presence (+S) of liver microsoma

Concentration of *D. kotschy* (µg/ml)	Average number of reverting colonies S9-(µg/ml)	Percent inhibition of mutation	Average number of reverting colonies S9+ (µg/ml)	Percent inhibition of mutation
5000	-	-	4.00 ± 1	65
2500	-	-	7.00 ± 1	37
1250	-	-	9.00 ± 1.527	19
NaNo3	11.67 ± 1.527	-	11.15 ± 1	-
SEM	1.534	-	0.842	-
P-Value	<0.0001	-	<0.0001	-
LSD P≤0.05	2.074	-	1.372	-

producing *Klebsiella pneumoniae* isolates. Zhang et al. suggested that *D. heterophyllum* Benth is a medicinal resource to be used against methicillin-resistant Staphylococcus [25]. According to table 2, the highest sensitivity contributed to OXA-48 producing *K. peneumoniae* number 11 and 4 isolates had the highest resistance in this study. Ashrafi et al. reported that the antimicrobial effects of *D. kotschyi* essential oils were stronger against Gram-positive bacteria in contrast to Gram-negative bacteria [9]. Aligiannis et al. described an organization for plant materials based on MIC results, as follows: strong inhibitors, MIC up to 0.5 mg/ mL, moderate inhibitors, MIC between 0.6 and 1.5 mg/ mL, and weak inhibitors, MIC above 1.6 mg/ml; therefore, *D. kotschyi* essential oils were strong inhibitors of OXA-48 positive *K. peneumoniae* strains. In addition, according to the radius of the inhibition zone, essential oils exhibited weak antibacterial activity against OXA-48 producing isolates, with a 10.40 ± 0.05 mm zone of inhibition. The detected antibacterial properties of the essential oils against various bacteria were different, ranging from strong antibacterial activity (inhibition zone > 20 mm) to moderate activity (inhibition zone < 12–20 mm) and to even no inhibition (zone < 12 mm) [19].

Based on the results of the analyses of the essential oils, their chemical composition was composed of: Monoterpene Hydrocarbons, Oxygenated Monoterpenes, Sesquiterpene Hydrocarbons, and Oxygenated Sesquiterpenes. Previous studies have described the chemical composition of essential oils of *D. kotschyi* in various locations that had certain similarities compared to the current work's results [26].

In the present study, TA100 *S. typhimurium* strain was used; and results of the Ames test showed that inhibition percentage of *D. kotschyi* was 65, 37, and 19% in 5000, 2500, and 1250 (µg/ml) respectively. The anti-mutagenesis effect had three levels of weak, moderate, and strong, which were considered to be less than 25%, between 25% and 40%, and above 40%, respectively. According to the results, *D. kotschyi* had strong anti-mutagenesis effect, and also Ashrafi et al. described that *D. kotschyi* had a high anti-oxidant activity [9].

We showed for the first time antimicrobial and anti-mutagenesis activities of *D. kotschyi* against OXA-48 producing *Klebsiella pneumoniae* clinical isolates.

Yahiaoui et al. showed that mentha pulegium essential oil effect on ESBL producing bacteria and described prospective synergistic effects with conventional antibiotics such as amoxicillin [27], also we demonstrated *D. kotschyi* effect on OXA-48 producing isolates.

5. CONCLUSION

In conclusion, result presented here may suggest that *D. kotschyi* can be used as an alternative antibiotic drug to treat infections caused by OXA-48 producing *K. pneumoniae* isolates. Recently, OXA-48 producing *K. pneumoniae* isolates have been prevalent in hospitals and treatment centers, and they are resistant to many antibiotics. In addition, another feature of *D. kotschyi* to be mentioned is that it is safe for humans due to its anti-mutagenesis effect against TA100 *S. typhimurium*.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The study was approved by Kurdistan University of medical science, Iran. All the members were

fully informed of the purpose of the investigation, and were informed.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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