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Monoterpene isolated from the essential oil of *Trachyspermum ammi* is cytotoxic to multidrug-resistant *Pseudomonas aeruginosa* and *Staphylococcus aureus* strains

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ABSTRACT

Introduction: The aim of this study was to determine whether an herbal extract containing monoterpene exhibited activity against multidrug-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolated from clinical infection samples. **Methods:** The essential oil of *Trachyspermum ammi* (L.) Sprague ex Turrill (Apiaceae) fruit was extracted by hydrodistillation. Fruit residues were treated with hydrochloric acid and re-hydrodistilled to obtain volatile compounds. Compounds in the distilled oil were identified using gas-chromatography (GC) and GC-mass spectrometry (MS). The antibiotic susceptibility of all bacterial isolates was analyzed using both the disc diffusion method and determination of the minimum inhibitory concentration (MIC). The sensitivity of antibiotic-resistant isolates to essential oil was also determined by using the disc diffusion method and MIC determination. **Results:** Of 26 clinical isolates, 92% were multidrug-resistant (MDR). Aromatic monoterpenes (thymol, paracymene, and gamma-terpinene) were the major (90%) components of the oil. Growth of *S. aureus* strains was successfully inhibited by the oil, with an inhibitory zone diameter (IZD) between 30-60mm and MIC <0.02µL/mL. The oil had no antimicrobial activity against clinical isolates of *P. aeruginosa;* rather, it prevented pigment production in these isolates. **Conclusions:** This study revealed that the essential oil of *Trachyspermum ammi*, which contains monoterpene, has good antibacterial potency. Monoterpenes could thus be incorporated into antimicrobial ointment formulas in order to treat highly drug-resistant *S. aureus* infections. Our findings also underscore the utility of research on natural products in order to combat bacterial multidrug resistance.

Keywords: Thymol. Monoterpenes. *Trachyspermum ammi*. *Staphylococcus aureus*. *Pseudomonas aeruginosa.*

INTRODUCTION

Antibiotic resistance is a hallmark among many pathogens that pose serious public health risks[1]. Patients colonized or infected with multi drug resistant (MDR) bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* are more at risk of death than patients infected with non-resistant strains[2] [3] [4]. Treatment of such infections at the local level to prevent systemic infections is thus required. Hence, finding suitable alternatives to conventional antibiotic therapies are is a prerequisite in the battle against infectious diseases.

Herbal extracts are increasingly being considered as alternative antimicrobial agents. There are many advantages of using natural products as antimicrobial compounds, including their generally low side effects, low costs, excellent biodegradability, and acceptance by patients due to their traditional applications and natural source[5] [6]. For thousands of years, *Trachyspermum ammi* (L.) Sprague ex Turrill (synonym: *T. copticum*) has been used as a medicinal plant in many parts of the world, ranging from Europe to Eastern Asia, and especially in Iran and India[7]. The brown seed-like fruit has traditionally been used for treatment of spasms, abdominal pains, and wound healing, but has also shown a positive effect on *Helicobacter pylori* infection[8]. Thymol, γ-terpinene, cymene, and limonene are the main components of the oil in this plant[9] [10].

The aim of this study was to determine whether *T. ammi* essential oil exhibited cytotoxicity toward two clinically relevant pathogens, *S. aureus* and *P. aeruginosa*. Our data suggest that this oil could be used in formulations designed to treat wound infections.

METHODS

Extraction

The seed, Ajowan, was collected in a region of Iran (Yazd- Central Plateau of Iran) and the plants were grown

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in the Tehran University herbarium following institutional approval. Seeds were dried at room temperature. Oil distillation was performed as described previously [5]. Subsequently, 10mL hydrochloric acid (Merck, Darmstadt, Germany) (1N) was added to the seed residue overnight at 25°C and hydrodistilled again for 4h. This acid treatment simplified the hydrolysis of glycosidic components.

Gas chromatography analysis

Gas chromatography (GC) of oil was performed using a Dani Master GC (Dani, Italy) with OV1 column (SE54CB, 25m × 0.25mm i.d., 0.25µm film thickness), with nitrogen as carrier gas, a split ratio of 1:20, and a flame ionization detector (FID). Temperature programming was performed from 75ºC (42 min) to 250ºC (14 min) at a ramping of 15ºC/min. Injector and detector temperatures were 250ºC and 260ºC respectively.

Gas chromatography-mass spectrometry analysis

The essential oils were also analyzed using the Gas chromatography-mass spectrometry (GC-MS) method on an Agilent 6890 with MS instrument (Agilent, U.S.) equipped with a BPX5 fused silica column (30m × 0.25mm i.d., film thickness 0.25µm). The oven temperature was raised from 50ºC through 300ºC at a rate of 3ºC/min for 75 min. The oven temperature was held for 5 min at 50ºC and the transfer line temperature was 290ºC. Helium was used as a carrier gas at a flow rate of 0.8mL/min with a split ratio equal to 1/30. The quadrupole mass spectrometer was scanned over an ionizing voltage of 70 electron volts (eV) and an ionization current of 150µA. The retention indices for all components were calculated using retention times of n-alkanes (C8-C25) injected after the essential oils under the same temperature and conditions. The compounds were identified by comparison of retention indices with those reported in the literature, together with comparison of their mass spectra with the Wiley, Adams, and National Institute of Standards and Technology (NIST) libraries.

Bacterial strains

Antibacterial efficacy of the oils was determined against 16 unique clinical isolates of *S. aureus* and 10 unique clinical isolates of *P. aeruginosa, S. aureus* ATCC 29213, and *P. aeruginosa* ATCC 27853. Clinical isolates were collected from burn wound infections in Tehran, Iran. The antibiotic susceptibility of all isolates (8 different classes for *S. aureus* isolates and 4 different classes for *P. aeruginosa* isolates) was analyzed using the disc diffusion method on Muller-Hinton agar (MHA) (Merck Co., Germany), as recommended by Clinical and Laboratory Standards Institute (CLSI) guidelines [11]. The tested antibiotics were purchased from Mast Group Ltd. (Merseyside, UK). Based on their resistance to antibiotics, clinical isolates were divided into different groups. Strains resistant to more than three different classes of antibiotic were defined as MDR. The study was approved by the Ethics Committee of Tehran University of Medical Sciences.

Analysis of antimicrobial efficacy

Disk diffusion assay: the disc agar diffusion (DAD) method was performed as described previously[13]. The bacterial suspension was adjusted to a density of 0.5McFarland to obtain an inoculum of approximately 1.0×10^8 CFU/mL. A sterile swab immersed with this bacterial suspension was used to inoculate the entire MHA. Sterile filter paper discs (Whatman, 6mm in diameter) were impregnated with 10µL of the oil and were placed on the inoculated agar plates (one disc per plate). Subsequently, agar plates were incubated for 24h at 37°C. The diameters of the inhibition zone were measured in millimeters and were compared with the results obtained from the reference strains[12] [13].

Minimum inhibitory concentration **(MIC):** the MIC was determined using the broth micro dilution method as recommended Clinical and Laboratory Standards Institute [CLSI (2013, M100-S23)]. The sample was subjected to a ten-fold dilution series in order to give final amounts of the original suspension ranging from 0.02-100µL/well. Thymol ($C_{10}H_{14}O$) was used as the positive control. All tests were performed in triplicate, and data are presented as mean ± standard deviation (SD).

RESULTS

The results of the oil analysis by GC and GC-MS are shown in **Table 1.** Thirteen compounds were identified in the oil of the *T. ammi* before acid treatment, of which thymol (74%), para-cymene (16%), and γ-terpinene (7%) were the major components. After acid treatment, thymol (68%), para-cymene (14%), and γ-terpinene (7%) remained the major components.

The amount of aromatic monoterpenes in the oil before hydrolysis was higher than after acid treatment (90% and 82%, respectively). In contrast, after acid hydrolysis, the amount of monoterpene hydrocarbons and oxygenated monoterpene components was higher in the oil. The antibiotic susceptibility patterns and antibacterial efficacy of *T. ammi* are outlined in **Table 2**. The DAD method revealed that *T. ammi* essential oil was highly active against all *S. aureus* strains tested. There was no growth inhibition observed for the *P. aeruginosa* clinical isolates. The highest inhibition zone diameter (IZD) was achieved upon treatment of *S. aureus* ATCC 29213 and two clinical isolates (almost the whole plate was free of bacterial growth). In combination with the MIC results, the results from the disc diffusion method showed that all *S. aureus* strains tested were susceptible to the oil, and no growth was observed in any of the wells, with the exception of the negative control no extract well. None of the *P. aeruginosa* clinical strains were susceptible to the oil. Although reference strain *P. aeruginosa* ATCC 27853 had an average IZD before acid (25mm) and after acid treatment (10mm), it did not grow in any of wells and was thus classified as a susceptible strain. The highest MIC was measured in the *P. aeruginosa* standard isolate (50µL/mL). To examine the major compounds within the oil, thymol was crystallized, and its antibacterial activity was tested at final concentrations of 1, 10, and 100mg/mL. Activity was only observed at the highest concentration 100mg/mL of thymol, a dose that dramatically inhibited the growth of *S. aureus* strains.

TABLE 1 - The most important chemical components of essential oils of *Trachyspermum ammi* before and after acid treatment.

Number	Compound	KI[1]	RT[2]	Percentage	
				BA[3]	AA[4]
1	thymol	30.19	1,306	74	68
2	para-cymene	16.63	1,031	16	14
3	gamma-terpinene	18.32	1,063	7	7
4	carvacrol	30.46	1,312	0.5	0.6
5	beta-pinene	14.06	981	0.3	0.05
6	terpinen-4-ol	24.71	1,190	0.2	0.4
7	carvone	28.04	1,260	0.2	0.1
8	alpha-terpinene	16.11	1,021	0.1	1.1
9	1-terpineole	22.43	1,144	-	1.5
10	1,4- cineole	16.02	1,019	-	1.1
11	alpha-terpinolene	19.71	1,090	-	0.5
12	para-cymenene	20.14	1,098	-	0.8
13	1,8-cineole	16.97	1,037	-	0.6

1: kovats index; **2:** retention time; **3:** before acid treatment; **4:** after acid treatment.

DISCUSSION

In this study, we investigated the antimicrobial efficacy of *T. ammi* essential oil against two relevant healthcare-associated bacteria, *S. aureus* and *P. aeruginosa*. Our data suggest that this oil could be suitable for the treatment of wound infections. Despite the variation in the proportion of minor oil compounds, the fraction of each of the major oil compounds before and after acid treatment was similar. Thymol was the most predominant component in oil both before and after acid treatment. With regard to the relative amounts of each essential oil component, our results are concordant with those of previous studies[14] [15] [16] [17]. The minor differences in results between several studies are due to the different extraction methods that were used. For example, some compounds identified in the oil after acid treatment, such as 1-terpineole, 1, 4 -cineole, paracymenene, and 1, 8-cineole, were absent prior to acid hydrolysis. Antibacterial effects of these minor compounds, an in particular 1, 4- cineole, have been previously reported[6]. Such compounds may be derived following acid hydrolysis of major hydrocarbons in the fruit cell walls[18].

In general, the outer membrane of Gram-negative bacteria is less sensitive to the antimicrobial effects of essential oils[19]. The outer layer of the Gram-negative outer membrane is composed primarily of lipopolysaccharide molecules, and forms a hydrophilic permeability barrier that provides protection against highly hydrophobic drugs[6] [19]. This may explain the low sensitivity of Gram-negative bacteria to the cytotoxic effect of the lipophilic monoterpenes. In our study, essential oil did not exhibit antimicrobial activity against clinical isolates of *P. aeruginosa*. However, the oil had a high bacteriostatic effect against all *S. aureus* clinical isolates, and elicited a relatively large IZD. For all *P. aeruginosa* strains, pigment production was inhibited near the disk. According to previous studies, the MIC values of plant oil against Gram-positive and Gram-negative bacteria ranged from 0.06 to 6μL/mL[19]. In this survey, the MIC values for all *S. aureus* strains were consistently <0.02μL/ml.

Thymol, a member of a natural class of phenolic monoterpene compounds known as biocides, was the predominant compound in *T. ammi* oil. Thymol has a strong antimicrobial effect either when used alone or in combination with other biocides such as carvacrol[6] [20]. According to previous studies of Gram-positive test strains, these compounds strongly interfere with physiological characteristics such as cytoplasmic membrane permeability, coagulase activity, salt tolerance, and enterotoxin production[6] [21]. The oil may also influence the signal transduction in the *agr* system, a global regulator of virulence factor expression in staphylococci[21]. A previous study indicated that these types of mechanism of action were responsible for growth inhibition in *P. aeruginosa* reference strains[22] [23]. In contrast, our current data indicate that the oil and its derivatives were ineffective in highly resistance clinical isolates. The discrepancy in results may indicate that there are other molecular and cellular targets beyond outer membrane proteins that determine the sensitivity of Gram-negative bacteria, such *P. aeruginosa*, to monoterpene compounds. A previous study showed that monoterpene concentration determined the degree of resistance *P. aeruginosa*[22]. The MexAB-OprM efflux pump has an important role in the protection of *P. aeruginosa* against cyclic monoterpenes[24]. Considering that aromatic monoterpene compounds were the most predominant components both before

TABLE 2 - Antibacterial activity of the essential oil of *Trachyspermum ammi* against clinical isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Isolates	Number	BA[1] IZD[3] (mm)	AA[2] IZD (mm)	MIC V/V	Resistance pattern
S. aureus ATCC 29253	1	WP†	WP	<0.02	
Clinical isolates of *S. aureus*	2	35	30	‘’	E, GM, RP
	3	30	27	‘’	C, GM
	4	35	27	‘’	C, CIP, RP
	5	50	40	‘’	CD, E, GM, MUO, OX, T
	6	45	28	‘’	OX
	7	60	45	‘’	CD, E, GM, OX, T
	8	48	30	‘’	E, GM, T
	9	55	46	‘’	E, OX, T
	10	50	40	‘’	CD, E, T
	11	45	30	‘’	CD, E, GM, OX, RP, T
	12	30	27	‘’	CD, E, GM, OX, T
	13	45	30	‘’	CD, E, OX, RP
	14	40	28	‘’	CD, E, RP
	15	WP	WP	‘’	CD, E, OX
	16	WP	WP	‘’	CD, OX, T
P. aeruginosa ATCC 27853	17	25	10	50	
Clinical isolates of *P. aeruginosa*	18	0	0	> 100	AK, CAZ, CPM, GM, IMI, MEM
	19	0	0	‘’	AK, CAZ, CPM, GM, IMI, MEM
	20	0	0	‘’	AK, CAZ, CPM, GM, IMI, MEM
	21	0	0	‘’	AK, CAZ, CPM, GM, IMI, MEM
	22	0	0	‘’	AK, CPM, GM, IMI, MEM
	23	0	0	‘’	CAZ, GM, IMI, MEM
	24	0	0	‘’	AK, CPM, GM, IMI, MEM
	25	0	0	‘’	AK, CPM, GM, IMI, MEM
	26	0	0	‘’	AK, CPM, GM, MEM
	27	0	0	‘’	AK, CAZ, CPM, GM, IMI, MEM
	28	0	0	‘’	AK, CAZ, CPM, GM, IMI, MEM

BA: before acid treatment; **AA:** after acid treatment; **IZD:** inhibition zone diameter; **†WP:** growth inhibition in the whole plate. **AK:** amikacin; **MIC:** minimum inhibitory concentration; **V/V:** volume/volume. **C:** chloramphenicol; **CAZ:** ceftazidime; **CD:** clindamycin; **CIP:** ciprofloxacin; **CPM:** cefpiramide; **E:** erythromycin; **GM:** gentamicin; **IMI:** imipenem; **MEM:** meropenem; **MUO:** mupirocin; **OX:** oxacillin; **RP:** rifampin; **T:** tetracycline.

and after acid treatment of the oil, we suggest that resistance of clinical isolates of *P. aeruginosa* is due to the export of these compounds via efflux pumps present in these strains. Although the principle active ingredient appears to be thymol, we infer that other minor components in oil may synergize or antagonize with this monoterpene in a context-dependent manner.

In conclusion, our study revealed the effect of natural plant products on antimicrobial activity and highlights their potential utility as components of new antibiotics. Multidrug resistance among bacteria is a worldwide burden, and further research in natural products is thus needed.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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