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Cysticidal Activity of *Trachyspermum ammi* Essential Oil against *Acanthamoeba* Isolates from Dental Clinic and Hospital Water Networks

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Authors' Contribution
WMH conceived the study, performed the experiments, analyzed the data and wrote the article. HAHS performed GC-MS analyses, helped in data analysis and constructive discussion.

Key words
Amoebicidal, Cysticidal, *Acanthamoeba* spp., Water sources, Essential oil.

ABSTRACT

Acanthamoeba genus is widespread in the environment, including the hospital water sources and medical devices especially in dental units and dialysis machines. It poses a threat to public health as it is responsible for human infection. In inappropriate conditions, it forms cyst with high levels of resistance to disinfection methods. The aim of this study was to isolate the pathogen from the dental units and hospital water network and treatment of *Acanthamoeba* with *Trachyspermum ammi* essential oil that have the ability to kill cysts. The amoebicidal activity of *T. ammi* oil was determined *in vitro. Out of T. ammi* oil *showed that,* higher concentrations (90 µg/mL after 72 and 96 hrs and 100 µg/mL after 24 and 48 h) was more active, being able to kill 100% of the *Acanthamoeba* cysts. The results showed the cysticidal activity of *T. ammi* essential oil, LC_{50} values estimated to be 73.31, 61.69, 57.21 and 53.01 µg/mL with 24, 48, 72 and 96 h, respectively. This is the first report of in vitro cysticidal activity of *T. ammi* essential oil and the activity of this oil makes it a promising anti-*Acanthamoeba*.

INTRODUCTION

Globally, there has been a great interest and much attention among scientists in studying the occurrence and isolation of free-living amoebae from various water sources, because several species of *Acanthamoeba* (*e.g. A. castellanii, A. culbertsoni, A. hatchetti, A. healyi, A. polyphaga, A. rhysodes, A. astronyxis*, and *A. divionensis*) cause the insidious, chronic and mostly fatal disease granulomatous amoebic encephalitis (GAE), particularly in immune-compromised, patients with HIV/AIDS or who are chronically ill, diabetic, have undergone organ transplantation or are otherwise debilitated with no recent history of exposure to recreational freshwater (Marciano-Cabral and Cabral, 2003; Schuster and Visvesvara, 2004; Schuster, 2002) and *Acanthamoeba* keratitis causes a vision-threatening disease (Morlet *et al*., 1997). The risk of *Acanthamoeba* spp. became more dangerous after it has been shown that bacteria and virus survive and grow within *Acanthamoeba* and some of them are potential pathogens for humans, beside its resistance to water treatments, *Acanthamoeba* became important waterborne pathogens (Fritsche *et al*., 2000; Greub and Raoult, 2004; Berger *et al*., 2006).

The heightened concern of the *Acanthamoeba* on public health around the world has been recognized with the presence of pathogenic *Acanthamoeba* in the hospital water sources, dental unit waterlines during oral healthcare procedures and tap water and its seriousness of public health (Trabelsi *et al*., 2010; Lasjerdi *et al*., 2011; Hassan *et al*., 2012; Leduc *et al*., 2012; Hikal *et al*., 2015; Khurana *et al*., 2015). Previous studies have been demonstrated that essential oils and their constituents have antimicrobials, antiprotozoals and anti-*acanthamoeba* activity (Andrade *et al*., 2016; Lbouchi *et al*., 2017). *Trachyspermum ammi* seeds essential oil possess as insecticidal, antibacterial, antifungal, antimicrobial, nematicidal, anthelmintic, anti-inflammatory, anti-filarial, hepatoprotective and antioxidant (Bairwa *et al*., 2012; Chahal *et al*., 2017). Our study aimed to detect *Acanthamoeba* spp., in hospital water sources and in the dental unit water and test the cysticidal activity of *T. ammi* essential oil as a safe natural substance

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against *Acanthamoeba* cysts.

MATERIALS AND METHODS

Sample collection

A total of 50 samples, including 22 tap water samples and 10 tap swab samples were collected from different sections in hospitals as well as 13 tap water samples and 5 tap swab samples were collected from dental irrigation machines in dental centers, in Tabuk, Saudi Arabia during the period from March to June, 2017. Sterile cotton swabs were used to swipe the mouth of the taps before opening them and then dipped into 1 ml of sterile distilled water. The mouth of the tap was then flamed and water was allowed to run for 5-10 min, after which 500 ml of water was collected in sterile glass bottles (Senior, 2011).

Filtration of water and culture of FLA

Tap water

Five hundred milliliter of tap water was filtered through sterile 0.45 µm membrane filter within 2 h of collection (Hassan *et al*., 2012; Gradus *et al*., 1989). Filters were then inverted onto 1.5% non-nutrient agar (NNA) plates with an overlay of *E. coli*. All plates were sealed and incubated at 37°C (Khan and Paget, 2002). They were examined daily for growth of amoebae under inverted and then under 10 × magnification of light microscope for up to 2 weeks to obtain complete encystment (de Jonckheere *et al*., 1974). The *Acanthamoeba* spp. was confirmed by their cyst and trophozoite morphology using Pussard and Pons (1977) and Page (1988) keys for classification. Axenic cultures were obtained by transferring a piece of agar containing some amoeba to liquid culture medium peptone yeast-extract glucose (PYG) slightly modified and incubated at 30°C for subculture to obtain growth for molecular study (Schuster, 2002).

Swabs

The swabs dipped in 1 ml of distilled water during transportation, were vortexed for 30 sec followed by centrifugation at 800 g for 10 min (Raghavan, 2007). Pellet was resuspended in 100 µl of Pages saline which was spread onto the 1.5% NNA plate overlaid with *E. coli* and was incubated and processed in a similar manner as that for water samples.

Tolerant assays for pathogenicity of genus Acanthamoeba

Thermo and osmo-tolerance assays were performed as previously described (Khan *et al*., 2001; Chan *et al*., 2011).

Extraction of nuclear DNA

DNA is extracted manually by using the commercial kit QIAamp DNA mini kit (Qiagen, France) with the tissue protocol. A pretreatment with a proteinase K at 56°C for one night before using QIAamp DNA mini kit showed highest yield of DNA positivity (Yagi *et al*., 2008).

PCR amplification

To confirm the identity of *Acanthamoeba*, PCR reactions were performed using genus specific primers as previously described (Qvarnstrom *et al*., 2006). DNA was used as the template for PCR. Primer sequences were AcantF900 (5′-CCC AGA TCG TTT ACC GTG AA-3′) and AcantR1100 (5′-TAA ATA TTA ATG CCC CCA ACT ATC C-3′), were used to amplify a 18S ribosomal (r) RNA gene fragment of approximately 180 bp. PCR was performed in a volume of 50 µl containing1.25 U Taq polymerase (Qiagen), 0.1–1.0 ng DNA, 200 µM dNTPs, 4 mM MgCl2 and 0.5 µM primer. PCR reactions were performed at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min for 35 cycles, with a final elongation step of 10 min at 72°C. Amplified DNA was electrophoresed on a 1.5 % agarose gel.

Plant material and essential oil extraction

Seeds of *T. ammi* were obtained from the Richters Herbs-Goodwood, ON, Canada. *T. ammi* seeds essential oil was extracted by hydro distillation using Clevenger apparatus for 3 h according to Guenther (1961). The essential oils were collected and dehydrated over anhydrous sodium sulphate and kept in a refrigerator until GC/MS analysis.

GC-MS analyses and identification of components

The essential oil of *T. ammi* was analyzed by gas chromatography–mass spectrometry (GC/MS), using a GC/MS system composed of a HP 5890 series II gas chromatograph and a HP 5973 mass detector. A TRFAME (Thermo 260 M142 P) capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness) was used with helium as the carrier gas, at a flow rate of 1.5 ml/min. GC oven temperature was programmed at an initial temperature of 40°C for 5 min, then heated up to 140°C at 5°C/min and held at 140°C for 5 min, then ramped up to 280°C at 10°C/min and held for 5 additional minutes. Injector and detector temperatures were 250°C. Diluted samples (1/100, v/v in heptane) of 1.0 µl were injected automatically. Mass spectrometry was run in the electron impact mode (EI) at 70 eV. Identification of components was based on the comparison of their GC retention times. Mass spectra interpretation was confirmed by mass spectral library search using the National Institute of Standards and Technology (NIST) database (Masada, 1976; Adams, 2007).

Assessment of amoebicidal (cysticidal) activity

Amoebicidal activity was performed according to Coulon *et al*. (2010) and Sauter *et al*. (2011). Briefly, the essential oils were solubilized with 1% Tween 20 and sterile water to a final concentration of 100 µg/ml and were tested at final concentrations of 10, 20, 30 and 40-100 µg/ml. For the assessment of amoebicidal activity, 100 µL of stock (10^3) cysts of *Acanthamoeba* (prepared by manual counting with a hemocytometer) were poured to adhere in plates for 15 min after that 100 µL of each concentration were inoculated into each well and incubated at 30°C with different contact time (24, 48, 72 and 96 h). Viability was assessed using trypan blue dye exclusion method. The control used was sterile water containing 1% Tween 20 plus parasite, also only the parasite in PAS saline and parasite plus reference drug control 0.02% chlorhexidine gluconate (prepared from a solution 20% in H_2O CHX, C-9394; Sigma) all were submitted according to above procedure. Experiment in all concentrations was performed in three repetitions.

Statistical analysis

The data in this study were analyzed with the analysis of variance (ANOVA) using JMP 10 program (SAS Institute, NC, USA). The mean values of treatments were compared using Tukey's HSD test. Values accompanied by different letters were significantly different at $p \leq 0.05$. Percentage of lethal concentration 50 (LC50) were calculated according to Finney (1971).

RESULTS AND DISCUSSION

Prevalence of heat-tolerant free-living amoebae

A total of 50 samples [32 (64%) from hospitals (22 Tap water (44%) and 10 tap swab (20%) and 18 (36%) samples from dental unites (13 tap water (26%) and 5 tap swab (10%)] were collected for the detection of heat and osmo-tolerant free-living amoebae of genus *Acanthamoeba* during the period from March to June, 2017. It was found that percentage of heat tolerant free-living amoeba was recorded in 11 from 15 swab samples (73.3%) and 16 of 35 (45.7%) isolated from tap water. A comparison of samples taken from dental clinics and in different sections of hospitals found that of 25 tap water (22 samples from hospital and 13 from dental units). *Acanthamoeba* was recorded from 10 of 16 (45.45%) from hospitals and 6 of 13 (46.15%) from dental units samples. With regard to 15 swabs water samples (10 samples from hospital and 5 from dental units). *Acanthamoeba* was recorded from 7 of 10 samples (70%) from hospitals and 4 of 5 samples (80%) from dental units samples. All samples gave growth at 1 mol manitol that means these isolates considered potentially pathogenic. Previous studies confirmed the isolation of heat-tolerant *Acanthamoeba* from dental unit waterlines, hemodialysis units and the different hospital water system (Trabelsi *et al*., 2010; Khurana *et al*., 2015; Ovrutsky *et al*., 2013). On the other hand all samples isolated give positive *Acanthamoeba* morphologically based on the characteristics morphology of cyst and trophozoite (Fig. 1) and confirmed by PCR using genus specific primers where 180bp specific amplification products were visualized in all isolated samples tested that were not evidenced in the negative control (Fig. 2).

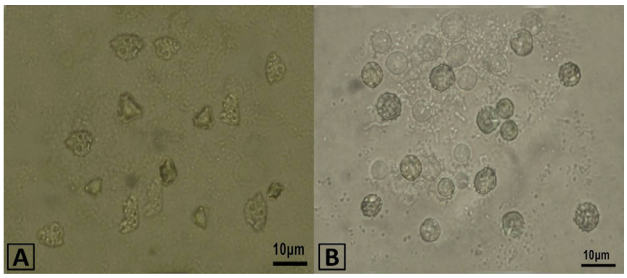

Fig. 1. *Acanthamoeba* morphology: A, trophozoite form; B, cyst form.

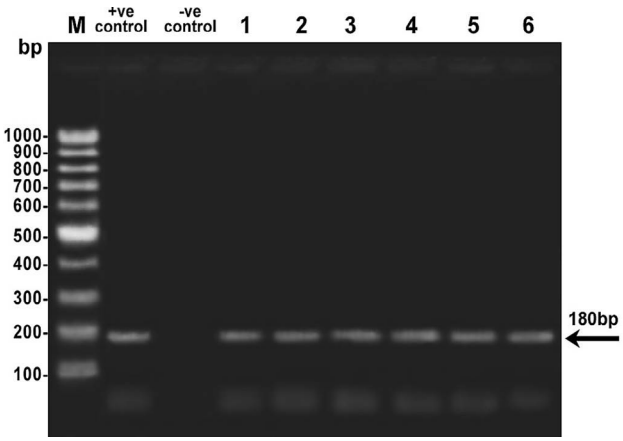

Fig. 2. Agarose gel electrophoresis showing amplification of 18S rDNA of different *Acanthamoeba* isolates were subjected to electrophoresis on 1.5% agarose gel parallel containing ethidium bromide to 100 bp DNA. M, 100 bp DNA ladder; 1-6, *Acanthamoeba* spp.

Chemical composition of T. ammi *seeds essential oil*

The essential oil yield obtained from *T. ammi* seeds by hydro-distillation was 3.50%. This result is consistent with previous studies (Hikal *et al*., 2015; Raghavan, 2007; Omer *et al*., 2014), that have demonstrated that the yield of essential oil was 2.0 to 5.2% and differences in essential oil yield is probably caused by difference in mode of extraction, harvesting time, drying conditions and

environmental conditions (Said-Al Ahl and Sabra, 2016; Said-Al Ahl *et al*., 2016).

T. ammi essential oil compositions were identified using gas chromatography-mass spectrometry (Table I). Sixteen compounds were identified, representing 98.09% of the essential oil. Thymol (40.20%) followed by γ-terpinene (32.39%) and ρ-cymene (19.45%) were the major constituents. Carvacrol (1.03%) was the minor constituent only. The other constituents were traces. These results are consistent with previous studies (Chahal *et al*., 2017; Razavizadeh and Adabavazeh, 2017; Singh and Ahmad, 2017; Torabi-Pour *et al*., 2017).

Essential oils have been used as treatments for ailments from headaches to parasite infections and demonstrated as antimicrobials, antiprotozoals and anti-*Acanthamoeba* activities (Andrade *et al*., 2016; Lbouchi *et al*., 2017; Jones, 1996). *T. ammi* essential oil has shown insecticidal, antibacterial, antifungal, antimicrobial, nematicidal, anthelmintic, anti-inflammatory, anti-filarial activities (Lbouchi *et al*., 2017; Chahal *et al*., 2017).

Table I.- Essential oil compounds (%) of *Trachyspermum ammi* seeds.

Compounds	%	Compounds	%
α-thujene	0.20	α-terpinene	0.33
α-pinene	0.48	γ-terpinene	32.39
β-pinene	0.60	β-Phellandrene	0.49
sabinene	0.23	sabinene hydrate	0.17
myrcene	0.87	terpinen-4-ol	0.45
ρ-cymene	19.45	α-terpineol	0.30
α-Phellandrene	0.37	thymol	40.20
limonene	0.53	carvacrol	1.03
Total identified compounds (%)		98.09	
Essential oil yield (%)		3.50	

In vitro *anti*-Acanthamoeba *assay*

This study is the first to test *T. ammi* essential oil as anti-*Acanthamoeba* cysts. Table II shows significant differences in the percentage of *Acanthamoeba* spp., cysts death due to various essential oil concentrations at different contact times. Also, from the Table II, it was noticed that, increasing essential oil concentrations was more effective as a cysticidal under the same time. Also, activity of essential oil as cysticidal was increased with increasing the contact times under the same concentration used. Increasing the concentration of essential oil or the contact times increased the death rate, that was concentrations 100 and 90 µg/ml (except after 24 h) caused significant high death rates compared to other concentrations and other various contact times. Concentrations of *T. ammi* essential oil (90 µg /ml after 72 and 96 h as well as 100 µg /ml after 24 and 48 h) was more active, being able to destroy 100% of the *Acanthamoeba* cysts. Once, no trophozoites formation was observed after the treatment and there is a shrinkage in the cyst form. Increasing essential oil concentrations demonstrated effective increase as a cysticidal activity, probably due to the increased percentage of monoterpenes, sesquterpens and aromatic compounds with known biological properties (Vunda *et al*., 2012). It can also be explained by the fact that increased exposure to volatile oils may increase the biological effectiveness of various components of the essential oil (Wang *et al*., 2017). The amoebicidal activity of essential oil could be occurring by the action of specific constituents within the oil or the synergistic action of several molecules (Escobar *et al*., 2010).

Table II.- The percentage of *Acanthamoeba* cyst mortality % under the influence of *Trachyspermum ammi* essential oil (EO).

EO concent. (µg/ml)	Time			
	24 h	48 h	72 h	96 h
Control	0±0^{m}	0±0^{m}	0±0^{m}	0±0^{m}
10	0±0^{m}	0±0^{m}	0±0^{m}	1.0±0.58^{m}
20	0±0^{m}	0±0^{m}	2.0±1.15^{m}	3.66±2.03^{m}
30	0±0^{m}	7.0±1.15^{lm}	15.0±1.15^{jk}	20.33±1.45^{ij}
40	0.66±0.33^{m}	14.0±2.65^{jkl}	24.0±1.15^{hi}	31.33±1.45^{h}
50	7.66±2.40^{klm}	27.33±2.19^{hi}	40.67±1.20^{g}	51.33±2.19^{ef}
60	29.67±1.45^{h}	45.33±2.03^{fg}	53.0±2.08^{ef}	64.33±2.19^{d}
70	40.0±0.58^{g}	58.0±1.0^{de}	64.0±2.31^{d}	73.0±1.00^{c}
80	57.0±0.58^{de}	73.0±2.08^{c}	74.33±2.60^{c}	84.33±2.33^{b}
90	72.67±2.33^{c}	93.0±1.15^{a}	100.0±0^{a}	100.0±0^{a}
100	100.0±0^{a}	100.0±0^{a}	100.0±0^{a}	100.0±0^{a}

*Means sharing the same letter are not significantly different.

Our study showed that *T. ammi* essential oil can kill the *Acanthamoeba* cysts. Essential oil analysis showed that thymol (40.20%) followed by γ-terpinene (39.32%) and ρ-cymene (19.45%) were the major constituents. Geranial, neral, limonene, nerol, carvacrol, p-cymene, γ-terpinene, carvone and thymol components in *Lippia alba* essential oil have activity against *Trypanosoma cruzi* and *Leishmania chagasi.* Also, γ-terpinene, thymol and ρ-cymene in *Origanum vulgare* and *Thymus vulgaris* essential oils exhibited against *Trypanosoma cruzi* (Santoro *et al*., 2007), however, thyme essential oil has possessed against *Entamoeba histolytica*, *Leishmania major* and *Trypanosoma brucei*a (Mikus *et al*., 2000; Behnia *et al*., 2008). Similarly, *Origanum vulgare* essential oil which contains γ-terpinene, thymol and ρ-cymene major components as thyme has anti-protozoal activity against

Blastocystis hominis, *Endolimax nana and Entamoeba hartmanni* (Force *et al*., 2000). *Lonicera japonica* essential oil had anti-encystment and amoebicidal activity against *Acanthamoeba triangularis* (Mahboob *et al*., 2016). Our data corroborate the study of Sauter *et al*. (2012), Ghazouani *et al*. (2017) and Saoudi *et al*. (2017), where the essential oils showed anti-amoebic activity against *Acanthamoeba*, however, amoebicial activity of the essential oil on *Acanthamoeba* cysts was not previously investigated.

Table III.- LC_{50} values of *Trachyspermum ammi* essential oil against on *Acanthamoeba*.

Time (h)	LC_{50} (µg/ml)
24	72.31
48	61.69
72	57.21
96	53.01

From Table III, we see that *T. ammi* essential oil was potent to kill the cysts of *Acanthamoeba* spp., with 24, 48, 72 and 96 h LC_{50} were 72.31, 61.69, 57.21 and 53.01 µg/mL, respectively. The activity of essential oil is probably due to the presence of three main components are thymol (40.20%), γ-terpinene (32.39%) and p-cymene (19.45%), which makes up 92.04% of the total compounds of *T. ammi* essential oil with known biological activities. Our study is similar to the results of the basil plant where leaves, flowers and aerials parts of *Ammoides pusilla* essential oils have amoebicidal activity against *Acanthamoeba castellanii* Neff. The main components of leaves and flowers and aerials parts in *Ammoides pusilla* (Apiaceae) were thymol (39.6% and 33.05%), γ-terpinene (28.97% and 28.19%), p-cymene (13.69% and 15.31%) and thymol methyl ether (7.33% and 8.91%), respectively. Furthermore, amoebicidal activity from leaves and flowers essential oil (LC_{50} = 65.32 ± 5.43 µg/mL) was more important than those of aerial parts essential oil (LC_{50} = 97.18 ± 1.43 µg/ml) (Souhaiel *et al*., 2017).

Our result are compatible with a previous report conducted (Souhaiel *et al*., 2017; Martín-Navarro *et al*., 2010; Leal *et al*., 2013; Hajaji *et al*., 2017) on *Matricaria recutita*, *Pterocaulon polystachyum*, *Piper* spp., *Ammoides pusilla* and *Teucrium ramosissimum* essential oils, respectively, which had demonstrated that these essential oils had the amoebicidal activity against *Acanthamoeba* spp. The anti-parasitic property of essential oil could be occurring by the action of several individual molecules within the oil or the synergistic action between them (Ovrutsky *et al*., 2013).

Khan (2006) has previously stated, *Acanthamoeba* cysts are highly resistant to the usual anti-protozoal drugs. To the best of our knowledge, this is the first report of the amoebicidal activity of *T. ammi* essential oil against *Acanthamoeba* spp. Our findings revealed that *T. ammi* essential oil may be useful as novel therapeutic agent for the prevention or treatment of *Acanthamoeba* infections. Thus, more efforts are necessary in order to identify the phyto-compounds responsible for this biological activity, their mechanisms of action and to determine their possible therapeutic potential. Knowing that previous studies have shown that *T. ammi* essential oil save and not toxicity and can use directly on mammalian cells (Bairwa *et al*., 2012; Chahal *et al*., 2017).

CONCLUSION

Essential oil of *T. ammi* showed amoebicidal activity against *Acanthamoeba* cysts. *T. ammi* essential oil was able to kill the cysts that is the major problem for re-infection and caused their mortality. So, this essential oil is very important for cysts treatment. Importantly, *T. ammi* essential oil is capable of killing the cysts, and also able to eliminate the trophozoites. Thus, the use of *T. ammi* essential oil would be as a great alternative therapy or complemented with the treatments used. However, further studies are necessary to evaluate the possible active compound from *T. ammi* essential oil, as well as identify the molecular targets of the essential oil and thus determine its possible therapeutic use. Further in vivo studies would be needed to confirm the potential role of *T. ammi* oil in the treatment of *Acanthamoeba* genotypes.

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Statement of conflict of interest

The authors declare no conflict of interest.

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