

Broad-spectrum antimicrobials from the essential oil of *Trachyspermum ammi*

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Abstract

Background: Skin diseases the common causes of morbidity in rural and urban areas of developing countries with a high proportion of visits to health facilities. The development of antimicrobial drug resistances challenges the management of skin infections. These prompted the search for new antimicrobials from plants for the management of skin infections.

Objective: To develop effective topical anti-microbial formulations from *Trachyspermum ammi* essential oil.

Method: The essential oil obtained by hydro distillation, subjected for GC-MS analysis and antimicrobial activity test. The essential oil was formulated into five different semisolid vehicles. The inhibition zones of the essential oil and the formulated products were determined by using agar dilution and well diffusion method. The skin sensitization potential of the formulations were evaluated by guinea pig maximization.

Results: The major constituents of the *Trachyspermum ammi* oil were γ -Terpinene (39.8%), thymole (34.1%) and p-cymene (24.8 %), and showed broad spectrum antimicrobial with MIC 0.125 to 1.25 μ g/ml. The formulations showed inhibition against the growth of microorganisms starting at a concentration of 0.5% (v/w). Bioactive compounds were released better from the hydrophilic preparations than from the hydrophobic ones. The release from macrogol blend was particularly better. The hydrophilic ointment formulation of the oil showed a mild skin sensitization potential while macrogol blend ointment was devoid of sensitization.

Conclusion: *Trachyspermum ammi* essential oil is potential medicinal plant source of broad-spectrum antimicrobial used for the management of skin infections. Therefore, it is suggested that the topical formulation of these oils can be used as alternative drugs for the treatment of skin disease. It is further recommended that, clinical trial study of the topical formulations must be performed before utilized as treatment of dermatological disorders.

Keywords: *Trachyspermum ammi*, antimicrobial activity, topical formulation, zone of inhibition, Guinea pig maximization test

Introduction

Skin diseases are amongst the most common causes of morbidity in rural and urban areas of developing countries accounting for a high proportion of visits to healthcare centers. In Ethiopia, skin diseases are among the ten top leading causes of morbidity; it ranges 2.4 - 4.6 % of the general population, 2.8 - 4% of females and 3.0 - 4.2% of children less than 5 years of age visited hospitals due to skin diseases (FDRE/MoH 2015). In the rural community of southwestern parts of Ethiopia, more than half of all the households and 80% of the selected children have manifested one or more skin diseases (Figueroa et al. 1996; Figueroa et al. 1998). The prominent skin diseases are bacterial infections include cellulitis, impetigo, Methicillin-resistant *Staphylococcus aureus* infections; dermatophyte related fungal infections of the hair, skin, nails, feet and groin (Dagnew and Erwin 1991; Figueroa et al. 1996; Figueroa et al. 1998; Hilete-work 1998; Shibeshi 2000). Skin Wounds, resulting from microbial infection are the most common public health problems. The common wound pathogens include *Streptococcus pyogenes*, *Staphylococcus aureus*,

Pseudomonas aeruginosa and fungal infections such as the dermatophytoses (*ring worm*, *tinea*), *ptyriasis versicolor* and candidiasis (Rezaei et al. 2011).

There are different conventional treatments for dermatological disorders. However, these medications are with adverse effects (such as hepatotoxicity, neurotoxicity, nephrotoxicity, hematologic reactions and Stevens-Johnson syndrome), and development of antimicrobial resistance. For example, resistance is reported for fluconazole (Fichtenbaum et al. 2000; Gupta and Cooper 2008) and for *C. albicans* (Maenza et al. 1997; Martins et al. 1997). *S. pyogenes* and *S. aureus* which commonly cause skin and soft tissue infections developed resistance for penicillin G; *Klebsiella spp.* (100%) ; *Pseudomonas spp.* (93.3%); and *Escherichia coli* (91.5%) for for ampicillin; and *Enterococcus spp.* (3.3%); *S. aureus* (4.0%); and *Streptococcus spp.* (16.7%) for vancomycin (Ten Hove et al. 2017). Due to the above mentioned reasons, treatment of dermatological infections is time consuming, costly and time consuming. Researchers due attention for evaluating the efficacy and safety of medicinal plants, to promote their

proper usage and to determine their potential as sources for new drugs.

Trachyspermum ammi (*T. ammi*): belongs to family Apiaceae is a highly valued medicinally important seed spice. It is widely used for curing various diseases in humans and animals. The fruit is used for flatulence, atonic dyspepsia and diarrhea (Bentely and Trimen 1999), anthelmintic, carminative, laxative, and stomach ache, to treat abdominal tumors, abdominal pains and piles (Krishnamoorthy and Madalageri 1999). The essential oil of the seed contains thymol which is a strong germicide, anti-spasmodic and fungicide. Therefore, this study was designed to develop effective topical anti-microbial formulations from *T. ammi*.

Materials and methods

Chemicals: Cetostearyl alcohol, liquid paraffin, hard paraffin, propylene glycol, stearyl alcohol, and wool fat (Sigma-Aldrich, Steinheim, Germany), polyethylene glycol (PEG) 4000 and PEG 600 (BDH, Poole, England), sodium lauryl sulfate (Labort Fine Chemical, India), white petrolatum USP (EPHARM, Addis Ababa, Ethiopia), and anhydrous sodium sulphate (Sigma-Aldrich, GMBH, Germany)

Culture media and test organisms: Muller-Hinton agar (Oxoid, UK), sabouraud dextrose agar (Oxoid, UK), yeast extract (LABORT, India), tryptone soya broth (Oxoid, UK), Sabouraud dextrose broth (Oxoid, UK), Muller-Hinton broth (IDG, UK), nutrient broth (Oxoid, UK) were used. *Staphylococcus aureus* (ATCC 25923 and clinical isolate), *Pseudomonas aeruginosa* (ATCC 27853 and clinical isolate) and *Streptococcus pyogen* (ATCC 19615 and clinical isolate), *Aspergillus niger* (ATCC 10535 and clinical isolate), *Aspergillus flavus* (ATCC 13697 and clinical isolate), *Trichophyton mentagraphyte* (American Type Culture Collection /ATCC/ 18748 and clinical isolate), *Trichophyton rubrum* (ATCC 28188 and clinical isolate) and *Candida albican* (clinical isolate).

Commercial topical antimicrobial products: Tetracycline hydrochloride ointment (USP tetracycline 3%) Galentic Pharma, Batch No: 529, India), Mycoril® cream (1% clotrimazole, Remedica Limassol Industrial State, Lot No: 33989, Cyprus), were obtained from local drug retails in Addis Ababa.

Isolation and GC-MS Analysis of essential oil: The essential oil extracted from the dried seeds of *T. ammi* by hydrodistillation using Clevenger type apparatus. The oil collected and dehydrated with anhydrous sodium sulphate and stored in a clean, amber brown bottle and subjected to GC-MS analysis using a Hewlett-Packard GCD system.

Innowax FSC column (60 m × 0.25 mm i.d., 0.25 µm film thickness) was used with helium as carrier gas at a flow rate of 1 ml/min. GC oven temperature was kept at 60 °C for 10 min and programmed to 220 °C at a rate of 4°C/min, and then kept constant at 220 °C for 10 min and programmed to 240 °C at a rate of 1 °C/min. The injector temperature was at 250 °C. MS were taken at 70 eV. Mass range was m/z 35–425.

Preparation of topical formulations: Five different dermatological formulations: Macrogol cream base, Hydrophilic ointment, Macrogol blends ointment base, Simple ointment and White petrolatum alone were prepared by the fusion methods (Tadele et al. 2008). A different concentration v/w of the essential oil topical formulations were prepared by incorporating the oil into the soft mass of the different dermatological bases. The bases without the essential oil were used as negative controls while tetracycline hydrochloride ointment and Mycoril® cream were employed as positive controls.

Antimicrobial testing: The Antimicrobial activity of the essential oil was determined by using Das et al. 2010 and Rodheaver et al. 1980 techniques using agar dilution and agar diffusion methods.

1. Agar dilution method: two-fold serial dilutions of essential oils solution in 0.2% (v/v) tween-80 was made in Sabouraud Dextrose Agar (for fungi) and Muller-Hinton agar (for bacteria) to have a final concentration of essential oil ranging from 5% (v/v) to 0.0625 % (v/v). The assays were done in triplicates and control plates with no essential oils were run concurrently. The agar surface of the plates containing the dilution of essential oils and the control plate were inoculated by 5mm discs of the test fungi taken from advancing edge of 7 days fungal cultures and 24 hours bacterial growth. Then inoculated plates were incubated at 25°C for 7 days (for fungi) and at 37 °C for 24 hours (for bacteria) before being read. End-points for the essential oils are determined by placing plates on a dark background and observing the lowest concentration that inhibits visible growth, which is recorded as the minimum inhibitory concentration (MIC).

2. Agar diffusion methods: A volume of 0.2 ml suspension of overnight microbial having standard turbidity of the respective fungus and bacteria were added to a test tube containing 20 ml of molten and cooled (45°C) Sabouraud Dextrose Agar (for fungi) and Muller-Hinton agar (for bacteria). The agar suspension of organisms was thoroughly mixed by vortex mixer and poured onto the respective sterile petri dish and allowed to solidify. Four equidistant holes 10mm apart from the edge of the plate were

formed in the agar by removing the plug made with sterile cork borer (10mm). Using of micropipette and disposable syringe, 150µl of essential oil solutions and approximately 0.2 gm formulated and commercial products, respectively were added to each well. The plates were allowed to stand for 2 hour at room temperature in order to allow diffusion and incubated 25°C for 7days (for fungi) and 37 °C for 24 h (for bacteria). After incubation the diameter of zones of inhibition was measured.

Skin Sensitization study of the Formulated Products:

This test was conducted by the Guinea Pig Maximization Test (GPMT) using the BUEHLER TEST method (OECD 1992). The animals were randomized to treatment and control groups. The treatment groups were further randomized into 5% preparation of the hydrophilic and macrogol blend ointment formulations. The positive test group provided 1% sodium lauryl ointment and negative control was free of any medication. Different results of the study were analyzed by using excel.

Results

Phytochemical and Antimicrobial activity of the selected medicinal plants: Essential oil extracted

from *T. ammi* yield of 2.2 % (v/w) and the constituents of the oil were *Thujene* 0.088 %, *α-Pinene* 0.058 %, *β-Pinene* 1.011%, *Unknown* 0.149 %, *p-Cymene* 24.812%, *γ-Terpinene* 39.798% and *Thymole* 34.079%, with 11.32, 11.61, 13.81, 14.50, 16.87, 18.88 and 30.79 RT (in min), respectively.

Antimicrobial activity of the *T. ammi* essential oil: *T. ammi* essential oil as indicated in Table 1 and 2 showed a promising antimicrobial activity on different fungal and bacterial strains. At 0.5% of the oil, it showed a comparable activity to the commercial drug on *A niger* and *A flavous* strains, while 1% of the oil showed better antifungal activity than the commercial drug and more activities were observed as concentration increased (Table 1). The oil also showed a promising antibacterial activity at 1.25% and more antibacterial activities were observed at 2.5% of the oil (Table 2).

The MIC also varies with the different types of the bacterial and fungal strains (Table 3). For clinical isolates of *T mentagraphyte*, the MIC is 0.125µg/ml and *A niger clinical* isolate 1 µg/ml while for bacterial strains: *Staphylococcus aureus* 0.625 µg/ml and *P. aeruginosa* 1.25 µg/ml.

Table 1: Comparative Antifungal efficacy of the essential oil of *T. ammi*

	Concentration (%)	Mean Inhibition Zone Diameter (mm)± standard deviation					
		Standard			Clinical isolate		
		<i>Tm</i>	<i>Tr</i>	<i>Af</i>	<i>Tm</i>	<i>Tr</i>	<i>An</i>
<i>T ammi</i> in 2% Tween	0.5	18.40(±7.47)	34.80(±5.72)	15.00±0.63	18.40(±7.47)	34.80(±5.72)	14.67±0.52
	1	37.40(±6.43)	57.20(±13.99)	22.17±0.75	37.40(±6.43)	57.20(±13.99)	18.50±2.35
	2	78 (± 2.0)	76(±4.33)	24.83±3.92	73.6(± 2.68)	>71(± 1.91)	27.00±0.63
	4	90 (±0.78)	89(0.89)	33.00±1.10	90 (±3.23)	90(±8.06)	32.67±2.73
Tween 80	2	0.0	0.0	0.0	0.0	0.0	0.0
Mycoril cream	1	37.17(±3.60)	37.17(±3.60)	17±1.00	37.17(±3.60)	37.17(±3.60)	11±0.87

Af = *Aspergillus flavus*; *An* = *Aspergillus niger*; *Tm* = *Trichophyton mentagraphyte*; *Tr* = *Trichophyton rubrum*;

Table 2: Comparative Antibacterial efficacy of the essential oil of *T. ammi*

	Concentration (%)	Mean Inhibition Zone Diameter (mm)± standard deviation					
		Standard			Clinical Isolate		
		<i>Sp</i>	<i>Sa</i>	<i>Pa</i>	<i>Sp</i>	<i>Sa</i>	<i>Pa</i>
<i>T ammi</i> in 2% Tween	5	30.67±2.08	35.33±0.58	27.00±1.00	30.67±2.52	31.67±1.53	27.00±2.00
	2.5	28.33±2.08	28.33±1.53	27.67±2.31	25.00±1.00	27.67±2.08	19.33±1.53
	1.25	20.67±2.08	22.33±1.53	14.67±1.53	20.33±0.58	21.00±0.00	13.67±1.54
TTC	3	23.25±1.50	29.00±1.83	25.00±1.83	23.75±2.06	25.75±2.99	17.50±1.91
Tween 80	2	0	0	0	0	0	0

Sp = *Streptococcus pyogen*; *Sa* = *Staphylococcus aureus*; *Pa* = *Pseudomonas aeruginosa*; *TTC* = Tetracycline hydrochloride ointment

Antifungal activity of the topical formulations of *T. ammi* essential oil: As shown in Tables 4, only Hydrophilic and Macrogol blend ointment formulations showed activities against fungal strains

employed in this study. Macrogol cream base, simple ointment and white petrolatum base formulations of the *T. ammi* essential oil did not show activities up to 2% of the formulations. Hydrophilic ointment

formulation was visibly better than the commercial drug (Mycoril® creams) at 1% concentration against selected fungal strains *T. mentagraphyte* and *T. rubrum*. Similarly, Macroglol blend ointment showed comparable antifungal activity at 1% concentration and better antifungal activity at 1.5% formulation than the commercial drug (Mycoril® cream). In both formulations, the antifungal activity was increased as the concentration increased (Table 4).

Comparative Antifungal study of the formulations:

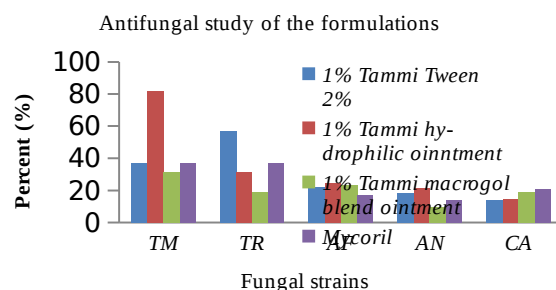
Figure 1 showed the broad spectrum antifungal activity of *T. ammi* essential oil ointment formulations on different fungal strains. All the formulations showed comparable and better antifungal activity than the commercial antifungal cream (Mycoril® cream). *T. mentagraphyte* was more sensitive to the hydrophilic ointment while *T. rubrum* was more sensitive to the tween 80 emulsion preparation of the oil (Figure 1).

Table 3: MIC of the *T. ammi* on different bacteria and fungi

Organisms	MIC µg/ml
Fungal strains	
<i>Trichophyton mentagraphyte</i>	0.125
<i>Trichophyton verrucosum</i>	0.125
<i>Mycrosporum canis</i>	1.00
<i>Aspergillus niger</i> standard	1.00
<i>Aspergillus niger</i> clinical Isolate	1.00
Bacterial strain	
<i>Staphylococcus aureus</i> standard	0.625
<i>Staphylococcus aureus</i> clinical isolate	0.625
<i>Streptococcus pyogen</i> standard	0.625
<i>Streptococcus pyogen</i> clinical isolate	0.625
<i>Pseudomonas aeruginosa</i> standard	1.250
<i>Pseudomonas aeruginosa</i> clinical isolate	1.250
<i>Klebsilla pneumonia</i> standard	0.625
<i>Klebsilla pneumonia</i> clinical isolate	0.325

Table 4: Comparative antifungal efficacy of the topical formulation of the essential oil of *T. ammi* with commercial antifungal drug; Mycoril 1% cream (Remedica Ltd, Lot: IMAPT 33989).

Formulations	Concentr ation (%)	Mean Inhibition Zone (mm) ± standard deviation	
		<i>T. mentagraphyte</i>	<i>T. rubrum</i>
<i>T. ammi</i> in Macroglol cream base	2	0	0
<i>T. ammi</i> in Hydrophilic ointment	1	81.67(±2.94)	31.5(±1.05)
	1.5	90(3.56)	51.5(±2.51)
	2	140± 6.87	90(1.63)
<i>T. ammi</i> Macroglol blend ointment	1	31.33 (±1.15)	18.83 (±1.60)
	1.5	90(.87)	69.83(±15.5)
	2	140± 2.88	90(1.34)
<i>T. ammi</i> in simple ointment base	2	0	0
<i>T. ammi</i> in white petrolatum base	2	0	0
Macroglol cream base	0	0	0
Hydrophilic ointment	0	13.0(±1.78)	8.0(±0.78)
Macroglolblend ointment	0	0	0
Simple ointment base	0	0	0
White petrolatum base	0	0	0
Mycoril cream	1	37.17(±3.60)	37.17(±3.60)



AF = *Aspergillus flavus*; AN = *Aspergillus niger*; TM = *Trichophyton mentagraphyte*; TR = *Trichophyton rubrum*; CA = *Candida albicans*

Figure 1: Comparative antifungal activity of *T. ammi* topical formulations against different fungal strains.

Skin sensitization and irritation: A 5% topical formulation on hydrophilic ointment produced 10 % sensitization rate and described as mild sensitization class by producing discrete or patchy erythema as compared to the standard sensitizer sodium lauryl sulphate 70% sensitization rate by producing intense erythema and swelling in *Magnusson and Kligman Grading Scale*. The macroglol blend ointment preparation of the *T. ammi* didn't produce skin sensitization and irritation on guinea-pigs.

Discussion

The hydrodistillation of the dry seeds of *T. ammi* yielded pale yellow colored oil with the major compounds of γ -terpinene 39.8%, thymole 34.1% and p-cymene 24.8 %. Other studies indicated that thymol, p-cymene, γ -terpine and o-cymene as major components of the oil 32 - 72.3 %, 15.7 - 33.7%, 13 - 48 % and 11 - 19 %, respectively (Gemedat et al. 2014; Kazemi et al. 2011; Mahboubi and Kazempour 2011; Singh et al. 2004). The variation may be attributed to the different growing environment, differences in genetic and climatic, temperature and altitude.

The essential oil of *T. ammi* showed a promising antimicrobial activity with MIC value of 0.625 - 1.250 µg/ml for bacterial and 0.125 - 1 µg/ml for different fungal strains. Other studies indicated that the antibacterial activities with MIC values ranges from 0.087 - 162.5 mg/ml for different bacterial strains (Hassanshahian et al. 2014; Mayaud et al. 2008; Paul et al. 2011) and 0.025 - 6 µL/ml for fungal strains (Gemedat et al. 2014; Pirbalouti et al. 2009; Sharifzadeh et al. 2015; Singh et al. 2004). In this study, the MIC is within alignment to the different study despite the different growing environment

which leads to the different phytochemical composition of the essential oil may lead to the variation in antimicrobial activity (Farag et al. 1989).

From the five dermatological bases tested in this study, the activity of the hydrophilic and macrogol blend ointment formulations showed activities against fungal strains employed in this study. This indicated that the bioactive component(s) of the essential oil may be released more easily from hydrophilic bases than from hydrophobic preparations. The preparation was effective as that of the commercial drugs at 1% of preparation and greater as concentration increased in both hydrophilic and macrogol blend ointment preparations.

Hydrophilic and Macrogol blend ointment formulations of *T. ammi* have a remarkable antifungal activity. Similar results were also reported that hydrophilic ointment formulations of the essential oil show better release properties (Tadele et al. 2008; Tesfaye et al. 2006). The increased activity of the hydrophilic ointment formulation is expected and could be attributed to the presence of sodium lauryl sulphate, which is used as a surfactant, has bacteriostatic and fungistatic actions and is also used pharmaceutically as a protective skin cleaner as well as in medicated shampoos (Florence and Attwood 1998). While the better antimicrobial activities of the macrogol blend ointment formulation may be the skin penetration enhancing abilities of glycols (Reynolds 1982) and may be partly ascribed to the fact that polyethylene glycols have an excellent solubility in water, and hence, have a solvent power over numerous compounds that are sparingly soluble in water.

The major components of *T. ammi* such as γ -terpinene and thymol, which are hydrophobic in nature known to have antimicrobial activities (Didry et al. 1993; Farag et al. 1989; Guo et al 2009; Nostro et al 2007). The active components easily diffuse into the surrounding area from the hydrophilic bases (hydrophilic and macrogol blend ointments) than the hydrophobic nature of bases and inhibit the growth of the microorganisms.

A 5% topical formulation on hydrophilic ointment produces 10 % sensitization rate. This could be attributed to the surfactant sodium lauryl sulphate produce sensitization and irritation in the base only and common irritant. This study is in line with Gilani et al. (2013), 5% cream preparation did not cause edema or erythema in rabbits. This variation may be due to the nature of the bases in which the drug was formulated (Omotosho et al. 1986), the viscosity of the preparation (Florence and Attwood 1998;

Oyedele et al. 2000), and the different animal species tested; the different growth conditions of the plants from which the oil was obtained and different chemical composition which leads to the different biological effects.

Conclusions and Recommendations

The *in vitro* antimicrobial activities of the hydrophilic and PEG-based topical formulation of *T. ammi* essential oil were found to be comparable with those of the commercially available antimicrobial products. The PEG based formulation of *T. ammi* did not shown skin sensitization potential up 5%. Therefore, it is suggested that the topical formulation of these oils can be used as alternative drugs for the management of skin diseases even though more empirical studies on effects of *T. ammi* on humans are needed.

Acknowledgements

We are thankful to the Directorate of Traditional and Modern Medicine Research, EPHI for the support to conduct the research smoothly Clinical Microbiology research case Team, EPHI for providing all the clinical isolate microorganisms used in the present study.

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