

Broad Spectrum Herbal Therapy against Superficial Fungal Infections

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Abstract

The objective of this paper is to develop some effective sources of chemotherapeutic agents that provide renewable sources of useful antifungal of biodegradable nature which are devoid of side effects and thermostable at high temperature with long expiry duration. The essential oil of *Trachyspermum ammi* against human pathogenic fungi in vitro was studied for thermostability, storage durability and their effect on human skin. Antifungal activity of the oil was compared with some synthetic antifungals. Finally, the essential oil was formulated for clinical trial in the form of ointment for clinical investigation. The essential oil of *Trachyspermum ammi* showed strong antifungal activity at 0.1 $\mu\text{l ml}^{-1}$ concentration against tested pathogens (dermatophytes) completely. The oil was found thermostable up to 100°C and did not expire up to 72 months of storage. On comparing the antifungal activity of the oil with synthetic antifungals, oil was found to be most effective and did not show any adverse effect on mammalian skin up to 1% concentration. The oil based ointment, was subjected to topical testing on patients attending out patient department (OPD) of M.L.N. Medical College, Allahabad. At the end of the medication, 70.0% of patients recovered complete cure, 20.0% showed significant improvement from the disease. No KOH negative cases of relapse were observed when patients were re-examined after two months following the end of treatment. Thus the oil based ointment (products) can be recommended for safe, thermostable and long duration effects against dermatophytoses without any adverse effect. The commercial viability of the oil can be determined after multicenter clinical trials which is in progress.

Keywords: Antifungal, dermatophytoses, dermatophytes, herbal therapy, thermostable, storage durability, plants

1.0 Introduction

Tinea infections or dermatophytoses is the disease caused by a group of fungi known as dermatophytes (*Epidermophyton*, *Microsporum* and *Trichophyton*). Dermatophytoses occur more frequently than subcutaneous and systemic mycoses and remain a therapeutic problem in tropical and subtropical countries, despite the availability of a number of ointments, lotion, paints and powders. Sources of these agents are largely petroproducts that are non-biodegradable and causes adverse effects and residual toxicity and other hand these products are thermo labile and expire in short duration (maximum 35 month). Naturally occurring antifungals described to date are mostly biodegradable [1] and are devoid of side effects [2] compared with commercially available antifungals. Recently, some products of higher plant origin have been shown to be

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effective source of chemotherapeutic agents without undesirable side effects with strong fungicidal activity [3-7]. These findings prompted us to explore other plant products (essential oil), which could be exploited as effective antifungals. We report here results of our investigation, the antifungal activity of the oil of *Trachyspermum ammi* at various temperature and storage durability against, *Epidermophyton floccosum* (Hartz) Langeron et Milochevitch, *Microsporum audouinii* Gruby, *M. canis* Bodin, *M. nanum*, Fuentes *M. gypseum* (Bodin) Guiart et Grigoraki, *T. mentagrophytes* (Robin) Blanchard, *T. rubrum* (Castellani) Sabouraud, *T. tonsurans* Malmsten, *T. violaceum* (Bodin) as well as clinical investigation in the form of ointment for making suitable drug. The plant *Trachyspermum ammi* (L.) Sprague is a small genus of herbs belonging to the family Apiaceae distributed in tropical Africa and from the Mediterranean region eastwards through southwestern Asian countries up to India and western China. Three species are found in India, of which *T. ammi* (Ajowan) is the most important and is cultivated for its fruits (Ajowan, Omum), which are commonly used as a spice.

2.0 Materials And Methods

2.1 Maintenance of human pathogenic fungi

The test pathogens, *Epidermophyton floccosum* (Hartz) Langeron et Mitochevitch, *Microsporum audouinii* Gruby, *M. canis* Bodin, *M. nanum*, Fuentes, *M. gypseum* (Bodin) Guiart et Grigoraki, *Trichophyton mentagrophytes* (Robin) Blanchard, *T. rubrum* (Castellani) Sabouraud, *T. tonsurans* Malmsten, *T. violaceum* (Bodin) were maintained on Sabouraud dextrose agar (SDA) medium. A seven day old culture of test pathogens was used for antifungal study.

2.1.1 Isolation and characterization of essential oil

The essential oil was extracted from the fruits of *Trachyspermum ammi* by hydrodistillation using Clevenger's apparatus [8]. A colorless or brownish yellow colored oily layer was separated and dried with anhydrous sodium sulfate. The Physicochemical properties of the oil were determined by the technique described by Langenau [9]. Gas liquid chromatography (GLC) analysis of the oil was done using a Perkin-Elmer GC model 3920 equipped with TCD and an SS column (2 m / 0.125 in × 0.3175 cm) packed with 10% Carbowax 20 m on 80/100 Chromosorb WAW. The chromatography was run with column temperature from 60°C, with an initial hold of 8 min; to 180°C at the rate of 2 degree/min with a final hold time of 20 min. A hydrogen flow of 30 ml/min was maintained through the column. The relative retention and coinjection techniques were used to identify the constituents. A Hewlett-Packard integrator (HP 3390A) attached to the detector output was used to calculate percent area.

2.1.2 In vitro investigation

The minimum inhibitory concentrations (MIC) of the oil against test pathogens (dermatophytes) were determined following the poisoned food technique of Grover and Moore [10] with slight modification [6].

The nature of antifungal activity (fungistatic/fungicidal) of the oil at MIC was determined by the method of Garber and Houston [11]. The inhibited fungal discs (at MIC) were reinoculated up side down on plain SDA medium. Inoculated petri plate were incubated at $27 \pm 1^{\circ}\text{C}$, observations were recorded on seventh day. Fungal growth on seventh day indicated fungistatic action of the oil while absence of fungal growth denoted fungicidal action.

The minimum killing time of the oil was determined by mycelial disc killing technique of Shahi et al [6]. Two treatment sets were maintained one with pure oil (100 %) and other with the minimum fungicidal concentrations (MCC) of the oil. The treatment sets using MCC of the oil was prepared by dissolving the required quantity of the oil samples in 5 ml acetone and then added into 100 ml distilled water. Simultaneously, controls were maintained using sterilized water (in place of the oil) and acetone adding into the distilled water in appropriate quantities. Mycelial discs of 5 mm diameter cut out from the periphery of 7 day old culture of the test pathogens were aseptically placed in the culture tubes of different treatment and control sets. These mycelial discs were taken out from the tube at different time intervals and washed immediately in the washing solution (containing acetone and sterilized distilled water in 1: 2 ratio) to remove the treatment solution. These washed mycelial discs were aseptically transferred up side down to the SDA medium (pH - 5.6) in petri plates. The same procedure was followed with the control sets. The inoculated petri plates were incubated at $27 \pm 1^{\circ}\text{C}$ and the observations recorded on seventh day and calculate the percentage of mycelial growth inhibition according to Shahi et al [6].

The effects of inoculum potentiality at MIC of the oil were determined by the method of Shahi et al [7]. Effect of the temperature, on antifungal activity of the oil was evaluated according to Shahi et al [7]. Five lots of oil were kept in small vials, each containing 5 ml of oil; these were exposed to 40, 60, 80 and 100°C in incubator for an hour. Antifungal activity then tested at MIC by the poisoned food technique [10].

Expiry of toxicity of the oil was determined by storing the oil at room temperature ($32 \pm 4^{\circ}\text{C}$) and antifungal activity were tested at MIC by the regular intervals 60 days up to 72 months following the poisoned food technique [10].

To comparative study of the oil with some synthetic antifungals (miconazole, ketaconazole, ciclopirox alamine) was carried out by comparing minimum inhibitory concentrations and minimum fungicidal concentrations. Various concentrations 0.01 to $10 \mu\text{l ml}^{-1}$, of synthetic antifungal substances were prepared from their stock solutions by incorporating into adequately cooled autoclaved 100 ml SDA medium. In controls, sterilized water (in place of the antifungal substances) was used in the medium in appropriate amount. The MIC was assayed following the poisoned food technique of Grover and Moore [10]. The nature of toxicity at MIC was determined by the method of Garber and Houston [11]. All experiments were repeated twice and each contained five replicates; data represent mean values.

2.1.3 In vivo investigation

To find the maximum tolerable concentrations (MTC) and long term toxicity for irritant activity of the oil by their topical application on human skin, following the patch test method as described by Shahi et al [6].

People of different sex in the age group of 10 to 30 years were selected randomly and a group of 30 individuals of each sex was constituted. Circular areas of 5 cm² on upper hairy and lower glabrous surface of palms and 3 cm² of neck region of each individuals were first washed with distilled water followed by 70 % ethyl alcohol and then allowed to dry. 0.2 ml oil of various concentrations was applied to each individual separately twice in a day for three week. The qualitative observations were recorded after the interval of 24 hr up to three week.

2.1.4 Clinical response of the ointment prepared from the essential oil

The study was designed to see the activity of the essential oil based ointment (1% w/v) on patients for the control of tinea infections (dermatophytoses). Medication was administered twice a day for 3 week. The patients were not allowed to take any other systemic or topical therapy during the course of study.

2.1.4 Patient's selection

Thirty patients of either sex within the age group of 8 to 40 years suffering from tinea corporis, tinea cruris or tinea pedis were selected randomly. Patients gave their informed consent to take part in this study. The diagnosis was confirmed mycological by KOH examination. To minimize the inconvenience to the patients additional thirty patients were selected as controls.

Patients were examined just before therapy was initiated and at the end of each week for 3 week of treatment. Where the cutaneous fungal disease occurred in more than one bodily area, all affected areas could be treated, but only one was selected and designated as the reference lesion. At each visit, the same reference lesion was scraped for fungal culture to identify the organism and for demonstration of hyphae by microscopic examination of the scrapings covered with 10% KOH preparations. Signs and symptoms of disease e.i. erythema, oedema, scaling, maceration, vesiculation, pustulation, crusting and itching were recorded as absent (-), mild (+), moderate (++) or severe (+++) and noted at every visit and scores were added. Less than 25% improvement was considered no improvement, 25-50% as mild, 50-75% as moderate, >75% as significant and complete cure when there was total clearance of signs and symptoms. Any adverse systemic or local reaction was noted at each visit. Satisfactory response with KOH negative cases after three weeks were reexamined after two month, to find out the relapse rates.

3.0 Results and Discussion

The fruits of *T. ammi* (Ajowan) on hydrodistillation yield 3.0% essential oil. The physiochemical properties of the oil were shown in table 1. The essential oil of *T. ammi* showed four minor and one major components. The percentage purity of the separated fractions was determined using analytical gas chromatography and was found to be as follows- fraction A - 0.71%, fraction B - 1.26 %, fraction C - 9.75 %, fraction D - 12.24 % and fraction E - 72.06 %. These components

were identified to be α -pinene, camphene, γ -terpene, *p*-cymene (minor) and thymol (major) respectively (Table 2), using physical constants and by comparison with authentic sample [12].

Table 1 Physiochemical properties of the oil of *T. ammi*.

| Properties studied | Observations |
|---------------------------------------|-------------------|
| Plant height (cm) | 158-180 |
| Oil yield (%) | 3.0 |
| Colour | Brownish yellow |
| pH | 5.8 |
| Specific gravity at 25 ⁰ C | 0.92231 |
| Refractive index at 20 ⁰ C | 1.5021 |
| Optical rotation | +3.0 ⁰ |
| Saponification value | 12.4 |
| Acid value | 3.2 |
| Ester value | 9.2 |
| Thymol (%) | 60-80 |
| Solubility | 90% alcohol |

Table 2 Chemical constituents above 0.7 % (vol/vol) detected in *T. ammi* by GLC

| Constituents [retention time (min)] | % composition |
|---------------------------------------|---------------|
| α -pinene ^a (2.29) | 0.71 |
| camphene ^a (3.77) | 1.26 |
| γ -cymene ^a (9.24) | 9.75 |
| <i>p</i> -cymene ^a (10.74) | 12.24 |
| thymol ^a (61.71) | 72.06 |

^aSeparated and confirmed on an SS column [2 mm by 1/8 in. (0.1375 cm)] packed with 10% OV-101 on 80/100 chromosorb WHP. The temperature changes and the flow of hydrogen were the same as those used for the carbowax 20M column.

3.1 *In vitro* test

The MIC of the oil of *T. ammi* were found to be 0.05 μ l ml⁻¹ for *Microsporum audouinii*, *Trichophyton rubrum*, *T. interdigitale*, *T. mentagrophytes*, *T. tonsurans* and 0.1 μ l ml⁻¹ for *E. floccosum*, *M. canis*, *M. nanum*, *T. tonsurans*. The minimum fungicidal concentration (MCC) were found 0.05 μ l ml⁻¹ for *M. audouinii*, 0.1 μ l ml⁻¹ for *M. canis*, *T. rubrum*, *T. interdigitale*, *T. mentagrophytes*, *T. tonsurans* 0.2 μ l ml⁻¹ for *M. nanum*, *T. violaceum* and 0.3 μ l ml⁻¹ for *M. gypseum* (Table 3). The oil inhibited heavy doses (30 mycelial discs, each of 5 mm in diameter) of fungal inoculum at 0.2 μ l ml⁻¹ concentration. The activity of the oil did not expire even up to 72 month of storage and persisted up to 100⁰C temperature. The pure oil (100 %) killed the fungi, *E. floccosum*, *M. gypseum*, *T. rubrum* in just 2 sec while at its minimum fungicidal concentrations it required 50, 80 and 40 minute respectively (Table 4).

Table 3 Minimum inhibitory concentration of the oil of *T. ammi* against test pathogens

| Fungi | Per cent inhibition (MGI %) at various concentrations ($\mu\text{l ml}^{-1}$) | | | | | |
|----------------------------------|---|------------------|------------------|------------------|------------------|------------------|
| | 0.05 | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 |
| <i>Epidermophyton floccosum</i> | 81.0 | 100 ^s | 100 ^c | 100 ^c | 100 ^c | 100 ^c |
| <i>Microsporum audouinii</i> | 100 ^c | 100 ^c | 100 ^c | 100 ^c | 100 ^c | 100 ^c |
| <i>M. canis</i> | 94.1 | 100 ^c | 100 ^c | 100 ^c | 100 ^c | 100 ^c |
| <i>M. gypseum</i> | 53.1 | 67.0 | 100 ^s | 100 ^c | 100 ^c | 100 ^c |
| <i>M. nanum</i> | 80.1 | 100 ^s | 100 ^c | 100 ^c | 100 ^c | 100 ^c |
| <i>Tricophyton interdigitale</i> | 100 ^s | 100 ^c | 100 ^c | 100 ^c | 100 ^c | 100 ^c |
| <i>T. mentagrophytes</i> | 100 ^s | 100 ^c | 100 ^c | 100 ^c | 100 ^c | 100 ^c |
| <i>T. rubrum</i> | 100 ^s | 100 ^c | 100 ^c | 100 ^c | 100 ^c | 100 ^c |
| <i>T. tonsurans</i> | 100 ^s | 100 ^c | 100 ^c | 100 ^c | 100 ^c | 100 ^c |
| <i>T. violaceum</i> | 71.8 | 100 ^s | 100 ^c | 100 ^c | 100 ^c | 100 ^c |

^cFungicidal

^sFungistatic

MGI, Mycelial growth inhibition

Table 4 Minimum killing time of the oil of *T. ammi* against test pathogens

| Minimum killing time (MKT) | Percentage of mycelial growth inhibition (MGI) % | | | | | |
|-------------------------------|--|------------------|-------------------|------|------------------|------|
| | <i>E. floccosum</i> | | <i>M. gypseum</i> | | <i>T. rubrum</i> | |
| | PO ^a | MCC ^a | PO | MCC | PO | MCC |
| 120 min | 100 | 100 | 100 | 100 | 100 | 100 |
| 80 " | 100 | 100 | 100 | 100 | 100 | 100 |
| 70 " | 100 | 100 | 100 | 80.9 | 100 | 100 |
| 60 " | 100 | 100 | 100 | 76.9 | 100 | 100 |
| 50 " | 100 | 100 | 100 | 56.0 | 100 | 100 |
| 40 " | 100 | 93.0 | 100 | 41.8 | 100 | 100 |
| 30 " | 100 | 83.0 | 100 | 33.1 | 100 | 39.0 |
| 60 s | 100 | -- | 100 | -- | 100 | -- |
| 30 " | 100 | -- | 100 | -- | 100 | -- |
| 20 " | 100 | -- | 100 | -- | 100 | -- |
| 10 " | 100 | -- | 100 | -- | 100 | -- |
| 2 " | 100 | -- | 100 | -- | 100 | -- |
| 1 " | 63.0 | -- | 78.2 | -- | 83.0 | -- |

^aPO = Pure oil; MCC = minimum fungicidal concentration

On comparison the MIC as well as MCC of the oil with those of synthetic antifungal drugs, the MIC of the oil was found to be 60, 5, and 3 times more active than that of miconazole,

ketoconazole and ciclopirox alamine respectively against *E. floccosum*, 80, 80 and 6 times against *T. rubrum*, 25, 25 time more active against *M. gypseum* with that of miconazole and ketoconazole respectively. However, these synthetic antifungals were remained fungistatic action except ciclopirox alamine (Table 5).

Table 5 Comparative efficacy of the oil of *T. ammi* with commercial antifungal drugs

| Antifungals | Active ingredients | Minimum inhibitory and fungicidal concentration ($\mu\text{l ml}^{-1}$) | | | | | |
|-------------|--------------------|---|-----|-------------------|-----|------------------|-----|
| | | <i>E. floccosum</i> | | <i>M. gypseum</i> | | <i>T. rubrum</i> | |
| | | MIC | MCC | MIC | MCC | MIC | MCC |
| Ointment | Essential oil | 0.1 | 0.2 | 0.2 | 0.4 | 0.05 | 0.1 |
| Dactrine | Miconazole | 6.0 | * | 5.0 | * | 4.0 | * |
| Nizral | Ketoconazole | 0.5 | * | 5.0 | * | 4.0 | * |
| Batrafine | Ciclopirox alamine | 0.3 | 1.0 | 0.1 | 0.5 | 0.7 | 0.5 |

MIC = minimum inhibitory concentration

MCC = minimum fungicidal concentration

* = remained static

3.2 In vivo test

The oil when tested for the irritant activity and long-term toxicity on human skin, the oil didn't show any irritation or adverse effect at 1% concentration up to 3 weeks. Formulation was prepared using 1% oil in ointment. After topical application of the ointment, improvements were observed right from the first week; 30.0% showed moderate improvement and 45.0% mild improvement, while 15.0% showed significant improvement. After second week, 25% and 35% patients exhibit significant and moderate improvement respectively. However, at the end of medication (i.e. after 3rd week), 70.0% of the patients were classed as complete clear and 20.0% as significant improvement while 10.0% as moderate improvement (Table 6). Majority of the controls showed no improvement and none of them showed more than a mild improvement.

During cost-benefit analysis [6,7] of the oil based ointment compared with synthetic antifungal drugs, the ointment was found to be most effective, less expensive (INR. 1.0/gm), long shelf life (72 month), thermostable 100°C and absence of any adverse effects.

Gentles [13] was to revolutionize the therapy of ringworm infections. He reported that the oral administration of griseofulvin cured experimental dermatophytoses in a guinea pig. Clinical trials were carried out in many parts of the world and included the whole gamut of dermatophytic disease. These trials conducted by Neves [14], have been reviewed by Blank [15] and are the standards for therapy. Recently several new topical drugs have been introduced. Of these, tolnaftate (Tinactin) has gained wide popularity but is of limited value in some types of infection. More recently a few of the imidazoles- clotrimazole, ketoconazole, miconazole, and econazole-

have been used as topical agents in the treatment of certain dermatophyte infections, however, these agents are associated with skin sensitization and drug induced eruptions. Treatment failures and relapses occur with all presently available drugs. The need for better therapeutic agent is apparent.

Table 6 Patients showing clinical response of *T. ammi* oil based ointment

| Parameter | Percentage of patients showing clinical response for three weeks | | | | | |
|-------------------------|--|-----|------|----|------|----|
| | 1 | | 2 | | 3 | |
| | T | C | T | C | T | C |
| No improvement | 10 | 100 | 00 | 90 | 00 | 80 |
| Mild improvement | 45 | 00 | 10 | 10 | 00 | 20 |
| Moderate improvement | 30 | 00 | 35 | 00 | 10 | 00 |
| Significant improvement | 15 | 00 | 25 | 00 | 20 | 00 |
| Complete cure | 00 | 00 | 30 | 00 | 70 | 00 |
| T test paired deference | | | | | | |
| SD | 53.0 | | 52.0 | | 55.2 | |
| SE of mean | 23.7 | | 23.2 | | 24.6 | |

Discovery of essential oils exhibiting narrow/broad spectrum antifungal activity, as suggested in the present investigations may prove useful in the development of effective antifungal substances.

According to Wellman (16) a fungicide must be retain its antifungal activity at the extremes of temperature and must be stable in storage for a long period. In the present study the antifungal activity of the oil of *T. ammi* was found thermostable upto 100⁰C and activity persisted even after 72 month of storage. Hence, the fresh oil can easily be used for developing the ointment with out any fear of expiry and did not need for refrigeration in comparison to synthetic fungicides. A chemical should be tested under both in vitro and in vivo conditions in order to prove its potential as promising antifungal for the control of the disease. Since, detail in vitro studies of the essential oil of *T. ammi* indicate their potentiality as ideal antifungal agents against dermatophytes; these were further subjected to in vivo investigation so as to confirm their efficacy as therapeutant for the control of dermatophytoses.

According to Polak [17] and Pierard et al [18] the evaluation of antifungal by in vitro and animal experiments can not predict clinical efficacy with certainty. Hence the clinical trials conducted by Shah et al [6,7] were followed in the present study, to prove the efficacy of the ointment, beyond any doubt.

In the present investigation the essential oil of *T. ammi* interestingly did not show any irritation or adverse effect at 1 % concentration on human skin up to three week. Hence, the oil of *T. ammi* due to its strong antifungal activity, inhibiting heavy doses of inoculum with long shelf life, thermostable and absence of adverse effect on human skin, this can be used successfully in the

form of broad spectrum antifungal treatment against dermatophytoses in human beings. The commercial viability of the same can be determined after successful multicenter clinical trial.

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