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Original Article

A Two-Year Comparative Study of the Anti-dermatophytic Activity of *Thuja orientalis* Essential Oil Stored at Various Temperatures

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ABSTRACT

Background: *Thuja orientalis* generally recognized as Chinese arborvitae, placed under the family Cupressaceae is a commonly available medicinal plant in India. **Objectives:** In present research work *Thuja* leaves essential oil stored in three temperatures for two-years storage periods was evaluated against fungi causing ring worm infection in man. **Materials and Methods:** *Thuja* oil was hydro-distilled and stored for 24 months in airtight amber glass bottles at three distinct temperatures. MIC was determined through semi solid agar diffusion method containing BHIA medium. Anti-dermtophytic activity of stored oil was examined in triplets after every fourth month of storage up to 2 years. **Results:** Oil stored in refrigerator was found to be more stable up to two year of storage time. No activity changes were observed in this condition against all test fungi. While variable temperature condition of room temperature was found less suitable for the storage of oil. MIC against *Candida albicans* was increased 40% after 2 years (>3.5 µL/mL) and 66% against *Microsporum fulvum.* Oil stored at 30°C temperature condition was found more stable upto IV testing. Slightly changes were observed during Vth testing against *C. albicans, M. fulvum* and *M. canis.* **Conclusion:** Present study concluded that essential oil of *Thuja* put away in chilliness condition can reserved their quality and properties of antifungal behaviour for long time. Accordingly, putting away of volatile oil ointment items in coolness (beneath 8°C) condition is best guidelines for long term use of therapeutic items.

Keywords: *Thuja*, Volatile oil, Trichophyton, Microsporum, MIC.

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INTRODUCTION

The incidence of dermatophytes and superficial candidal infections is very common worldwide. Contagious contaminations of fungi on epidermis are frequently reported worldwide and are estimated to affect 15-20% of the world's population. The allopathic preparation of azoles, allylamines, and polyenes are most frequently used against dermatophytes and *Candida*. The azoles are divided into two groups, the imidazoles (clotrimazole, ketoconazole, miconazole, and others) and triazoles (fluconazole, itraconazole, and others).[1] Regardless of the accessibility of novel fundamental dermatophytosis treatments, superficial fungal infection are challenging to destroy totally, with reoccurrence tendency in up to 20-40% of cases.[2] High cost of treatment, long duration of treatment course, resistant dermatophytic strains, disease reappearance, and negative consequences are some disadvantages related with these common medicines.[3] During last few decades there is progressive restoration of interest in the utilization of curative herbs against pathogenic microorganism due to safe and nontoxic properties of plant compounds.[4] Consequently, experiments and assessment of new medications with better and less expensive substitutes of natural sources assets are a natural choice.

Aromatic and medicinal florae have a complex and profoundly factor combinations of constituents that have a place with gatherings like flavonoids, steroids, terpenoids, phenolic, glycosides, alkaloids, aldehydes, essential oil and other related hydrocarbon and oxygenated compounds.[5] Aromatic oil of medicinal florae has been recognised from several years for pharmaceutical preparation, perfumery and for food additives.[6-8] Superiority and amount of essential oil and their constituents various with species to species and within the species, it is influenced by a several elements including the geographical state, climatical elements, the phase of development, oil production techniques and assortment period. These synthetic distinctions are straightforwardly interconnected to modifications to biological activities. Essential oils have tendency to undergo

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various changes in quality and quantity of components with aging and storage which can decline the effectivity of essential oil composition and might also negotiation user welfare. Only a few studies have been done to date to examine essential oil stability over time while taking into consideration the impact of different storage circumstances, despite the fact that quality monitoring and a complete understanding of essential oil properties upon storage are vital.[9]

Volatile oil degradation is influenced by a variety of biochemical and ecological factors that affect essential oil oxidation as well as the progression of the reaction. Oxidation and polymerization processes can induce losses of quality and pharmacological properties.[10] Therefore, it is important to precisely assess external conditions like temperature, light, and access to atmospheric oxygen. Additionally, stability may be influenced by the components of essential oils, the stability of compounds, their structure, and the frequency and extent of adulteration.

Due to volatile behaviour of low boiling temperature compounds, oxidation reduction procedures, and due to biological change, essential oil composition may alter during storage and processing.[11,12]

Thuja orientalis is an ornamental gymnosperm plant commonly found in parks and house gardens in Jaipur. It contains high amount of essential oil. In our previous work antifungal potential of *T. orientalis* was studied and found excellent results against fungi causing ring worm infection on human beings specially anthropophilic *Trichophyton* strains.[13] No scientific reports on longevity of *Thuja* essential oil have been found till date. These results encouraged us to investigate, before creating an ointment to treat superficial fungal infection on human, the impact of various storage temperatures and time periods on the activity of *Thuja* essential oil. Therefore, moisture-free oil from *T. orientalis* was held for six different storage durations (4,8,12,16,20 and 24 months) at three different stowage temperatures (Fridge temperature, RT, and 30ºC).

MATERIALS AND METHODS

Collection of plant material and extraction of oil

T. orientalis leaves were gathered from ground of Ram Niwas Park, Central Park, Jawahar circle gardens and Rajasthan University campus. When the sample was submitted to the herbarium department of botany at the University of Rajasthan, Jaipur, an authentic Voucher number RUBL 21183 was acquired. The leaves were divided into little pieces and dried in the shade. For 7 hr, Clevenger's apparatus hydro-distilled the dry, semi-squashed leaves and their powder. Anhydrous sodium sulphate was used to dry the volatile oil that was collected. The essential oil was kept dry and stored in an amber-colored sealed glass jar. For present study known amount of essential oil were than kept in three changed temperature condition like refrigerator temperature(2-8ºC), 30ºC temperature in incubator and room temperature condition for 2 years. Activities of stored oil was analyses after each forth month of stored up to two years for individually state.

Gas chromatography

Analyses using GC and GC-MS were performed in accordance with the established technique described in previous research articles.[14] Using a Shimadzu GC-2010, the constituents of essential oils were measured. Nitrogen was used as the carrier gas with an FID and an Omega SPTm column (30.0 m x 0.25 mm ID, film thickness 0.25 um) at an intake pressure of 10 psi. Temperature conditions for the injector and detector were kept at 270ºC and 280ºC, respectively. The temperature of the column was automatically adjusted to 80ºC for a 2 min hold, 80ºC to 180ºC at 4ºC/min, and 180ºC to 230ºC at 6ºC/min withhold times of six and 9 min, respectively. The split ratio of the carrier gas was kept at 1:80 while the flow rate was kept at 1.21 mL/min. For oil composition, the data were processed using GC solutions software.

GC-MS Analyses

Shimadzu GCMS-QP-2010 plus system with Omega SPTm column (30.0 m x 0.25 mm ID. film thickness 0.25 um) was used to collect GC-MS data. Injector, mass detector, ion source temperatures, and other parameters were maintained in the current investigation as specified in GC analysis while Helium was employed as the transport gas. The mass range and EI source were 40-850 amu and 70 eV, respectively. Utilising the libraries from NIST, Willey, and Perfumery, compounds were differentiated.

Qualitative analysis

Calibration curve of α-pinene, sabinene, β-pinene, myrcene, delta-3-carene, limonene, α-terpinene, bornyl acetate, α-terpinyl acetate, trans-caryophyllene, α-humulene, germacrene D and cedrol were created using five concentration points. The percentage rate of essential oil constitutes was quantified using these standardization arcs.

Micro-organisms used

Five dermatophytes and one yeast cultures namely *Trichophyton rubrum* (MTCC 296), *T. mentagrophytes* (MTCC 7687), *T. tonsurans* (MTCC8475), *Microsporum canis* (MTCC2820), *Candida albicans* (MTCC3018) and *Microsporum fulvum* (MTCC2837) were solicited from the Imtech Chandigarh. These dermatophytes were maintained on Sabouraud's Dextrose Agar medium (SDA) and Potato Dextrose Agar medium (PDA). Frequent subculturing and microscopic investigation were accomplished for the maintenance of test microbes.

Screening for anti-dermatophytic activity

Semisolid agar anti-fungal susceptibility testing method of Provine and Hadley[15] with slightly modification was applied for

determination of Minimum Inhibitory Concentration (MIC). Brain Heart Infusion Agar (BHIA) was used for experimental purposes. For inoculum preparation uncontaminated colony of test fungi was pick with a disinfected gauze dipped into tween 80 and then added in 3-4 mL of sterile normal saline and vigorously swirled. The turbidity of inoculum suspension was refurbished to ~0.5 McFarland standard. The semisolid agar tubes were inoculated with known amount of test microbes' suspension by implanting the loop bottomless within the test tube. Three replicates kept made in each experiment. The purity and viability of test pathogen was also examined by streaked onto Sabouraud dextrose agar. The tubes were kept at 37°C for 72 hr. Result was obtained as per guidelines of NCCLS/CLSI M27-A and M38-A. By comparing fungal growth to that of an oil-free control, the following growth scores were determined: growth identical to control ++++; slight growth reduction +++; notable growth reduction (80% in yeast and 50% in filamentous) ++; negligible growth/rarely detectable hyphal fragments +; no growth 0.

Statistical analysis

Each framework was verified in three-fold. Traditional measurable strategies were utilized to determined means and standard deviations. *t*-test was used to the data to govern differences ($p<0.05$).

RESULTS

The chemical constituents of the essential oil can be affected on storage conditions of essential oil. In present experiment the biological potential of hydrodistilled *T. orientalis* oil was determined against dermatophytes at different storage temperatures and times.

T. orientalis composed of 38 major and minor components belonging to monoterpene (34.210%), monoterpene alcohol (13.157%), oxygenated monoterpene (2.631%), alcoholic ester (2.631%), monoterpene ester, sesquiterpene (29.315%), sesquiterpenoids (15.789%), and sesquiterpenoids oxide (2.631%) (Table 1). Delta-3-carene (30.37) was found to be key component of GC and GC-MS studies, followed by alpha-pinene (13.10%), limonene (10.07%), alpha-terpinene (8.44%), α-terpinyl acetate (2.96%), trans-caryophyllene (4.48%), α-humulene (3.98%), cedrol (5.65%), myrcene (5.80%), β pinene (2.67%), sabinene (1.40%), germacrene D (1. 08%) and bornyl acetate (1.00%) (Figure 1).

For the present experiments volatile oil acquired from *Thuja* leaves was stored in three different temperature condition like refrigerator, 30°C and variable room temperature for 24 months of study. Minimum inhibitory concentration of stored oil was checked after forth month of storage in triplet against selected dermatophytic fungi.

During Ist testing after forth month of storing, maximum anti-fungal activity of *Thuja* oil was observed against *T. mentagrophytes* (0.7 µL/mL) followed by *M. canis* and *T. rubrum* (0.9 µL/mL), *M. fulvum* (1.1 µL/mL) and *C. albicans* (>2.5 µL/mL) at all three storage temperature conditions. No activity changes of oil stored in refrigerator was observed against all test fungi up to 24 months of storage. At room temperature condition MIC value of *Thuja* oil was increased against *C. albicans* (>3 µL/mL), *M. fulvum* (1.2 µL/mL) and *M. canis* (1.0 µL/mL). Similarly, MIC value of oil stored in 30°C found to be change against *M. canis* (1.0 µL/mL). During third testing MIC value was future changed against *C. albicans* (3.5 µL/mL), *M. fulvum* (1.3 µL/mL) and *M. canis* (1.0 µL/mL) (Table 2).

MIC value of *Thuja* oil was found to be unchanged in 30°C temperature conditions. At IVth testing of oil stored at room temperature no activity changes were found. While at 30°C temperature condition MIC had increased against *C. albicans* (2.6 µL/mL). During Vth testing MIC was found to be increased against *T. rubrum* (1.2 µL/mL) and *M. fulvum* (1.4 µL/mL) at

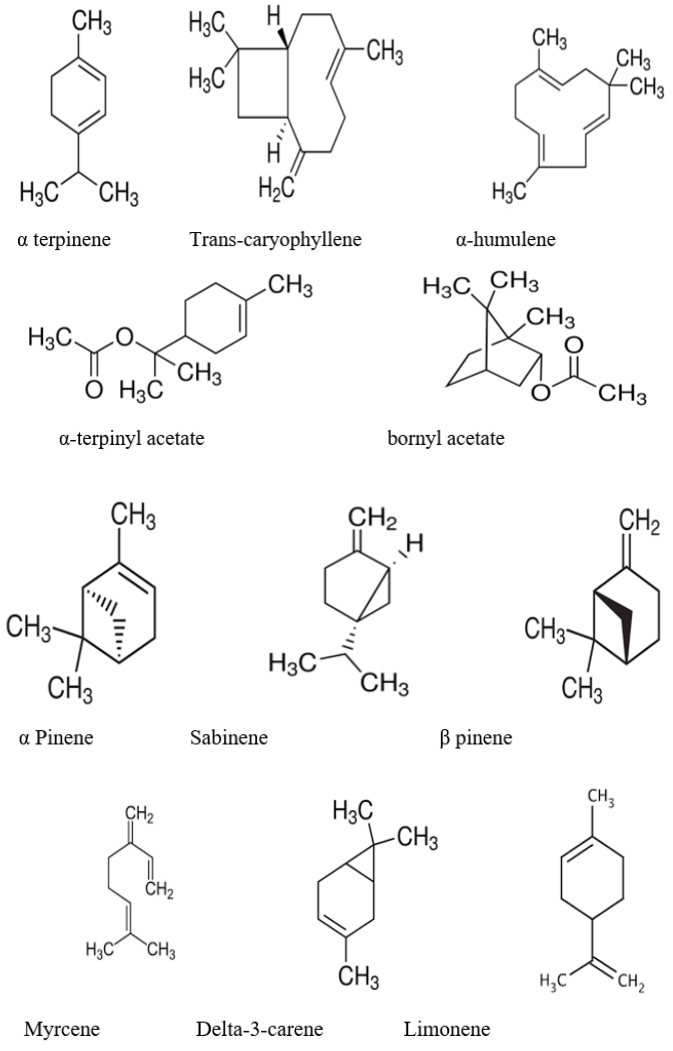

Figure 1: Chemical structure of some major compound of *Thuja* essential oil.

Table 1: **Chemical compositions of *Thuja orientalis*.**

Major groups	Percentage
Alpha-pinene	13.10
Sabinene	1.40
Beta-pinene	2.69
Myrcene	5.80
Delta-3-carene	30.37
Limonene	10.07
Alpha-terpinene	8.44
Bornyl acetate	1.00
Alpha-terpinyl acetate	2.96
Trans-caryophyllene	4.48
Alpha-humulene	3.98
Germacrene D	1.08
Cedrol	5.65
Major groups Monoterpene	34.210%
Monoterpene alcohol	13.157%
Oxygenated monoterpene	2.631%
Alcohol ester	2.631%
Monoterpene ester	2.631%
Sesquiterpene	29.315%
Sesquiterpenoid	15.789%
Sesquiterpenoid oxide	2.631%

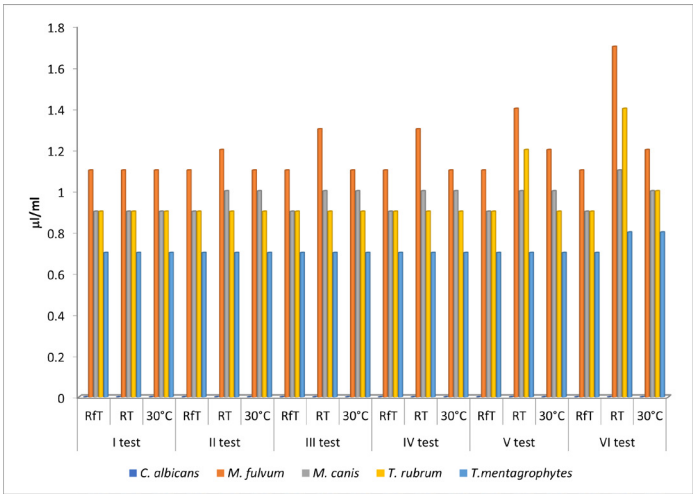

Figure 2: Longevity testing of *Thuja orientalis*.

room temperature condition and against *M. fulvum* (1.2 µL/mL) at 30°C. *Thuja* oil's antifungal activity was observed to diminish after 24 months of storage at room temperature and 30°C (Figure 2).

At room temperature, the MIC against *M. fulvum* was determined to be 1.7 µL/mL, followed by 1.4 µL/mL against *T. rubrum*, 1.1 µL/mL against *M. canis*, and 0.8 µL/mL against *T. mentagrophytes*.

Table 2: **Longevity testing of *Thuja orientalis*.**

	I test			II test			III test			IV test			V test			VI test		
	R_f	T	30°C	R_f	T	30°C	R_f	T	30°C	R_f	T	30°C	R_f	T	30°C	R_f	T	30°C
C. albicans	>2.5	>2.5	>2.5	>2.5	>3	>2.5	>2.5	>3.5	>2.5	>2.5	>3.5	>2.6	>2.5	>3.5	>2.6	>2.5	>3.5	>2.7
M. fulvum	1.1	1.1	1.1	1.1	1.2	1.1	1.1	1.3	1.1	1.1	1.3	1.1	1.1	1.4	1.2	1.1	1.7	1.2
M. canis	0.9	0.9	0.9	0.9	1.0	1.0	0.9	1.0	1.0	0.9	1.0	1.0	0.9	1.0	1.0	0.9	1.1	1.0
T. rubrum	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	1.2	0.9	0.9	1.4	1.0
T. mentagrophytes	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.8	0.8

At 30°C, *T. mentagrophytes* had the greatest impact (0.8 µL/mL), followed by *T. rubrum* (1.0 µL/mL), *M. canis* (1.0 µL/mL), *M. fulvum* (1.2 µL/mL), and *C. albicans* (2.7 µL/mL).

DISCUSSION

Essential oil composition may change during storage due to oxidation, evaporation of low boiling components, chemical changes during storage conditions etc. Present investigation exhibited the changes of antidermatophytic potential of *T. orientalis* essential oil during three different storage conditions up to 24 months. *T. orientalis* is a monotypic evergreen tree of gymnosperm belonging to the family Cupressaceae. Lots of work of *Thuja* regarding antifungal, antibacterial and antimicrobial potential has been done by various workers.[16,17] No report on quality changes during storage has not been reported till date.

Most changes were reported in room temperature conditions. Room temperature conditions varies from 4°C-45°C as summer temperature condition exceed from 45°C-47°C while winter conditions fluctuate from 4°C to 22°C in Jaipur. Maximum increase in MIC was reported against *C. albicans* during six testing at room temperature conditions. MIC exceed >3.5 µL/mL as compared to initial MIC >2.5. µL/mL. Similar changes were also reported against *M. fulvum* 1.7 µL/mL (initial 1.1 µL/mL). Very mild changes were seen at 30°C condition against all test microbes up to 24 months of storage. While no anti-fungal changes were seen in refrigeration condition.

Similar outcomes from storage experiments of *Cymbopogon citratus* essential oil were reported by Jain and Sharma.[18] Up to two years into the experiment, it was discovered that the MIC of *C. citratus* essential oil was constant against *Trichophyton rubrum* (0.1 µL/mL) and *T. mentagrophytes* (0.1 µL/mL). It was also found that the temperature at 30°C remained fairly constant. It was revealed that the MIC against *Candida albicans* and *M. fulvum* had somewhat increased at 24 months (the sixth testing) of storage. Oil stored at room temperature had a modification in its primary MIC values.

Kazaz, *et al.*[19] explained the effects of diverse stowage temperatures (0°C and 3°C) and times on the production and constituents of *Rosa damascena* Mill. oil. The influence of stowage time was significant, as opposed to the importance of storing temperatures on oil composition.

Similar report was given by Ahmed Abdulhakeem Al-Sammarraie.[20] Authors found that all plant extracts showed excellent anti-microbial potential but activity found to be declined during storage at different temperature and moisture. Low cooling temperature condition maintained the quality of plant extract for long time as compared to high temperature storage.

Storing and temperature conditions effect on anti-fungal potential of *C. martinii* was also studied by Jain and Sharma.[21] Cooling temperature condition was suggested for quality storage of essential oil and their formulations. Monohydrocarbon components of essential oils tend to vaporise at lower boiling temperatures when they are stored. Usually, low temperatures (freeze temperature) prevent the centralizations of the oil parts from forming or dropping and helps to maintain the pharmaceutical value of oil with the minute changes.[22] Vahid *et al.*[23] The study of storage temperature condition on essential oil composition of *Thymus daenensis* Celak also showed similar results.[23] Researchers discovered that storing *T. daenensis* essential oil at low temperatures helps to maintain the oil's primary quality with the fewest changes and prevents concentrations of its components from rising or falling.

During present investigation *T. mentagrophytes* was found most susceptible fungi followed by *T. rubrum*. These fungi are most causative agent of superficial mycoses in human and animals. *Thuja* essential oil was found very effective against both fungi up to 24 months storage conditions. Dermatophytoses is a serious fungal infection commonly reported in India. For the treatment of dermatophytosis topical application of essential oil has been found effective.[4,24] Depending on the sort of oil, different essential oils have different shelf lives. Storage conditions may have an effect on the essential oil's chemical composition. The interplay and reactivity of the volatile oil components with air, evaporation, and other unfavourable changes during storage could all contribute to this situation.[25]

Therefore, the effect of storage period and storage condition on the quality of essential oil should be evaluated before application of oil for the preparation of ointment.

CONCLUSION

During storage low boiling point constituents of essential oil like Monohydrocarbons primarily get evaporate. Essential oil of *T. orientalis* stored in the freezer, compared to the other two storage conditions, maintained its primary quality for up to 24 months, according to the conclusions of the current investigation. Generally speaking, keeping *Thuja* essential oil at low temperatures helps maintain the essential oil's primary quality with the least number of modifications and avoids concentrations of the oil's components from rising or falling. This experiment provides assurance that an ointment made to treat dermatophytosis will be secure and efficient when kept at a low temperature. Low boiling-point compounds, primarily monoterpene hydrocarbons, evaporate during essential oil preservation. As a result, significant activity changes were seen under ambient temperature circumstances. Thus, we deduced that products containing *T. orientalis* essential oil can be used for up to two years without experiencing any activity changes when kept at moderate temperatures, such as those found in a refrigerator or freezer.

These findings suggested that producers and consumers, who used this essential oil formulation in pharmacological and cosmetic trades, should apply this spectacle benefits.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest. The authors are solely responsible for the paper's accuracy and integrity.

ABBREVIATIONS

MIC: Minimum Inhibitory Concentration; **hr:** Hour; **GC:** Gas Chromatography; **µL:** Microlitre; **mL:** Millilitre.

AUTHOR'S CONTRIBUTION

Neetu Jain: Conceptualization, experimental studies, data handling, manuscript preparation and editing, **Seema Bhadauria:** Conceptualization, data analysis, review, editing.

SUMMARY

T. orientalis essential oil obtained through hydro-distillation were stored for 24 months in airtight glass bottles at three distinct temperatures conditions for longevity testing. MIC was determined after every fourth month of storage through semisolid agar antifungal susceptibility testing method. During present investigation *T. mentagrophytes* was found most susceptible fungi followed by *T. rubrum*. The current findings indicate that *T. orientalis* essential oil stored in the freezer preserved its primary quality for up to 24 months longer than essential oil stored in the other two storage settings. Present finding suggested that formulation of *T. orientalis* essential oil against superficial fungal infection can be used for up to two years without experiencing any activity changes when kept in refrigerator or freezer.

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