Antidermatophyte Activity of the Essential oil of *Hypericum perforatum* of North of Iran

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Abstract

Background: Dermatophytes are the main cause of human superficial mycosis that is still a public health problem especially in tropical countries such as Iran.

Objective: The aim of this study was determining the antifungal effect of *Hypercom perforatum* essential oil.

Methods: The minimum inhibitory concentration (*MIC*) and minimum fungicidal concentration (MFC) for the essential oil of the plant *Hypericum perforatum* against various dermatophytes were determined. The essential oil of *Hypericum perforatum* was obtained by hydro distillation of the dried plant. Clinical isolates of dermatophytes (*Epidermophyton floccosum, Microsporum canis, Microsporum gypseum, T. mentagrophytes var. interdigital, T. mentagrophytes var. mentagrophytes., T. rubrum* and *Trichophyton tonsurans*) were used for determining antifungal activity of this essential oil by *in vitro* tube dilution technique.

Results: MIC90 and MFC90 values were remarkable. *T. mentagrophytes var. interdigital* showed a>1 log10 difference in viable count between treatment and control within the first hour, whereas *E. floccosum* did not. The essential oil of *H. perforatum* sufficiently inhibited and killed all tested dermatophytes in all different dilutions. The changes in growth curve of the treated dermatophytes were significant compared with the untreated dermatophytes.

Conclusion: Terpinen-4-ol is the main component of the essential oil of *H. perforatum*, and perhaps could play the important role in antidermatophyte activity among the other components. It is suggested trying the in-vivo effects of *Hypericum perforatum* ointment or its other medicinal forms in the treatment and controlling of dermatophytes infections.

Keywords: ***Hypericum perforatum*, Antidermatophyte activity**

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Introduction

Dermatophytes are the major cause of superficial mycosis of man and remain a public health problem especially in tropical countries such as Iran. The humid weather, over population and poor hygienic conditions are conducive to the growth of dermatophytes. Even though it responds to treatment with conventional antifungal, the disease has a tendency to recur at the same or at different sites. In recent years, there has been growing interest in the usage of medicinal plants. A medicinal plant is any plant used in order to relieve, prevent or cure a disease or to alter physiological and pathological process, or any plant employed as a source of drugs or their precursors. [4, 9, 11, 12, 17] Exact function of essential oils has not been known but several investigations have demonstrated antimicrobial activity of these compounds [13, 14, 15, 16]

H. perforatum is one of the medicinal plants that grows in Europe, Western Asia and Northern Africa and Iran and is distinguished by its golden yellow flowers. From the time of the ancient Greeks it has been used widely to heal wounds, remedy kidney troubles, and alleviate nervous disorders, even insanity. *H.Perforatum* that is growing in north of Iran, have been advocated for use in complementary medicine as antibacterial and antifungal, anti-viral and infections that it was curd including boils, acne, gingivitis, even have been researched as antidepressant and anticancer. [5] By steam distillation, essential oils of *H. perforatum* are separated and yielded compounds such as: *Terpinene, Myrecene, Limonene, Cymene* and *Alcohols*. The in vitro activity of *H. perforatum* oil against dermatophytes *E. floccosum, M. canis, M. gypseum, T.m.v.interdigital, T.m.v. mentagrophytes. T. rubrum* and *T. tonsurans* was determined.

Material and Methods

Preparation of *Hypericum perforatum* essential oil to GCM

H. perforatum is a member of the genus Hypericum that has 400 species worldwide. The plant is native to Europe, North Africa, Middle east notably Iran and is naturalized in many parts of the world. *H. perforatum* spreads rapidly and willingly in the sides of roads and sparse woods and the mount.

The spices were collected from the mount of Alborz of north of iran, and is dried to prevent the enzymatic hydrolysis of biologically active components. Drying can be made in an oven under vacuum or by lyophilization. It is important that the plant should be avoided from the sunlight during the drying process. The plant should be stored in a dark place with little or no humidity. The plant dried, was blended in a Waring blender to obtain the highest surface interaction between the plant material and the extracting solvent. After filtration of the plant from the solvent the solvent is evaporated in a rotary evaporator or by lyophilization. It is important that the solvent is finely removed from the solid extract. The most common method for the analysis is steam distillation. By steam distillation essential oils can be separated from the extract [6, 7]. The oils were analyzed on a Hewlett-Packard GCD system. Innowax FSC column (60 m × 0.25 mm with film thickness) by using Helium as the carrier gas. Injector temperature was 250^0C. Split flow was 1 ml/min. The GC oven temperature was kept at 60^0C for 10 mins and programmed to 220^0C at a rate of 4^0C/min and then kept constant at 220^0C for 10 mins to 240^0C at a rate of 1^0C/min. MS were taken at 70 eV and a mass range of 35to425 [1, 5, 8]. Essential Oil Constituents were also determined.

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Dermatophyte isolates

Dermatophytes were obtained from the culture collections of the Department of Mycology at The University of Tarbiat modarres. Inocula were prepared by growing isolates on (SDA) slopes as described by the NCCLS with the following exceptions dermatophytes [18]. Slopes were flooded with 0.85% saline. Dermatophyte growth was gently probed and the resulting suspension was removed and mixed thoroughly with the use of a vortex mixer. After the settling of the larger particles, suspensions were adjusted by nephelometry and diluted as necessary to correspond to final inoculum concentrations ofc: 2.5×10³-2.5×104 cfu/ml for dermatophytes [4, 12, 18].

Broth micro dilution method

Broth micro dilution testing was based on reference method M38-P recommended by the NCCLS [18]. A series of doubling dilution of *H. perforatum* ranging from 8% to 0.004% was prepared in a 96 well micro dilution tray, with a final concentration of 0.001% (v/v) Tween 80 to enhance *H. perforatum* oil solubility. After the addition of inocula, trays were incubated for 96 h at 30°C for MICs were determined visually with the aid of a reading mirror, according to NCCLS guidelines [18]. Minimum fungicidal concentrations (MFCs) of *H. perforatum* oil were determined by sub culturing 10 µL from wells not visibly turbid and spot inoculating on to Sabouraud dextrose agar (SDA) plates. MFCs were determined as the lowest concentration resulting in no growth on subculture. Isolates were tested on at least two separate occasions, and were retested if resultant MIC or MFC values differed. Modal values were then selected Griseofulvin (sigma) was supplied as a powder and stock solution were prepared in dimethylsulphoxide.

Antidermatophyte activity in vitro

Dermatophytes inocula were prepared as described above except that dermatophyte inocula were suspended and diluted in PBS with 0.02% (v/v) Tween 80. Starting inocula concentrations were **c:** 106 cfu/mL for dermatophytes. After preliminary experiments, *H. perforatum* oil concentrations were chosen that corresponded to 4× MFC for dermatophytes. *Hypericum perfratum* oil treatments were prepared in 1 mL volumes at twice the desired final concentrations in PBS, with final concentrations of 0.001% Tween 80. Controls contained PBS with the relevant concentration of Tween 80. Test solutions and controls were inoculated with 1 mL volumes of inoculum and a 100 µL sample was taken immediately from the controls for viability counts. Test solutions were incubated at 35°C with shaking. Further samples were taken at 2, 4, 6, 8 and 24 h for viable counting. Viable counts were carried out by serially diluting samples 10-fold in sterile distilled water (SDW) and plating these dilutions on to SDA. Limits of detection were calculated based on a minimum of 30 cfu from the 10^{-3} dilution, and were 7.5×10^3 cfu/mL. Assays was carried out two to six times. Colony count for each experiment were converted into values relative to the colony count at time zero to normalize data and correct for slight variations in starting inocula concentrations between experiments. Mean and standard error values for each isolate at each time point were calculated and plotted against time on a log scale [2, 3]. Time–kill studies were carried out against *E. floccosum* and *T. m. v.interdigitale*.

Results

Essential Oil Constituents

Compounds of terpinen-4-ol, terpinolene, 1,8-cineole, c-terpinene, a-terpinene, q-cymene, a-terpineol and b-myrcene were also

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determined.The essential oil of *H. perforatum* of North Iran origin consists of %82 terpinen-4-ol, 74% of monoterpene hydrocarbons, 7% of oxygenated monoterpenes, 10% of sesquiterpene hydrocarbons and only 1.5% of oxygenated sesquiterpenes. α-cuprenene was isolated (besides some well-known monoterpene acetates and other sesquiterpenes) from the high boiling fraction of the oil. The low boiling fraction contained 5- and 6-methylheptan-2,4-dione as trace consistuents, while from the residue two new dimethylchromene derivatives 7-*sec*-butyl- and 7-isobutyl-2,2-dimethyl-*2H,5H*-pyranopyran-5-one were isolated. Terpinen-4-ol is the most of component of the essential oil of *H. perforatum*.

Broth micro dilution assay

The aim of this study was to isolate essential oil Constituents, of *H. perforatum* by using a simple chromatography methods and evaluation Antidermatophyte activity of the essential oil of Iranian *H. perforatum* oils. MICs for all dermatophytes ranged from 0.004%-0.25%, and MFCs ranged from 0.1- 3. (Table 1) Generally, MIC90 and MFC90 values were low. T.m.v.interdigital showed a>1 $\log_{10}$ difference in viable count between treatment and control within the first hour, whereas *E. floccosum* did not (Figure 1). The viable counts for *E. floccosum* did not differ from controls by >1 log10 until between 6 and 8 h of incubation with *H. perforatum* oil. By 24 h, none of the test dermatophytes was detected in the *H. perforatum* oil treatments .After 8 hour relative viable count about T.m.v.i approximately reached to zero but to *E. floccosum* did not (Figure 1).

Discussion

Nowadays, about 25 percent of the drugs prescribed worldwide come from plants and 252 of them are considered as basic and essential by the World Health Organization. The World Health Organization considers phytotherapy in its health programs and suggests basic procedures for the validation of

Table 1- In vitro susceptibilities of dermatophyte to *Hypericum perforatum* oil (%v/v) and griseofulvin determined by the broth microdilution method, *Hypericum perforatum* oil (%v/v), Griseofulvin (mg/L)

Name of Dermatophyte	Hypericum Perforatum oil (%v/v)				Griseofulvin	
	MIC		MFC		MIC	
	Rang	90%	Rang	90%	Rang	90%
E. floccosum	0.06-0.1	0.06	0.25-3	3	0.25-1	1
Microsporum gypseum	0.004-0.03	0.03	0.25-1.5	1. 5	0.5-2	1
Microsporum canis	0.01-0.03	0.03	0.25-1.6	1. 6	0.25-1	1
T. rubrum	0.004-0.06	0.03	0.3-0.9	0.9	0.5-2	1
T. tonsurans	0.004-0.03	0.01	0.12-1	1	0.5-1	1
T. mentagrophytes var. interdigital	0.008-0.03	0.03	0.12-0.25	0.25	0.25-1	0.5
T. mentagrophytes var. *mentagrophytes*	0.004-0.06	0.03	0.12-0.5	0.7	0.5-2	1

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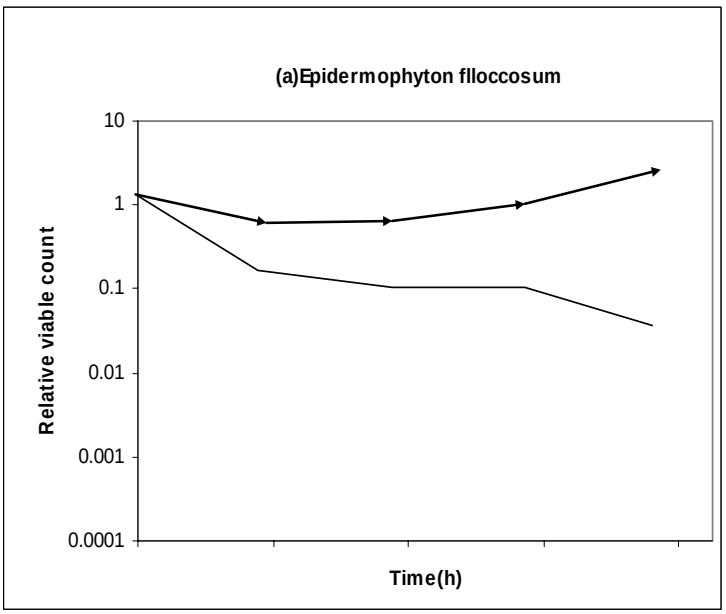

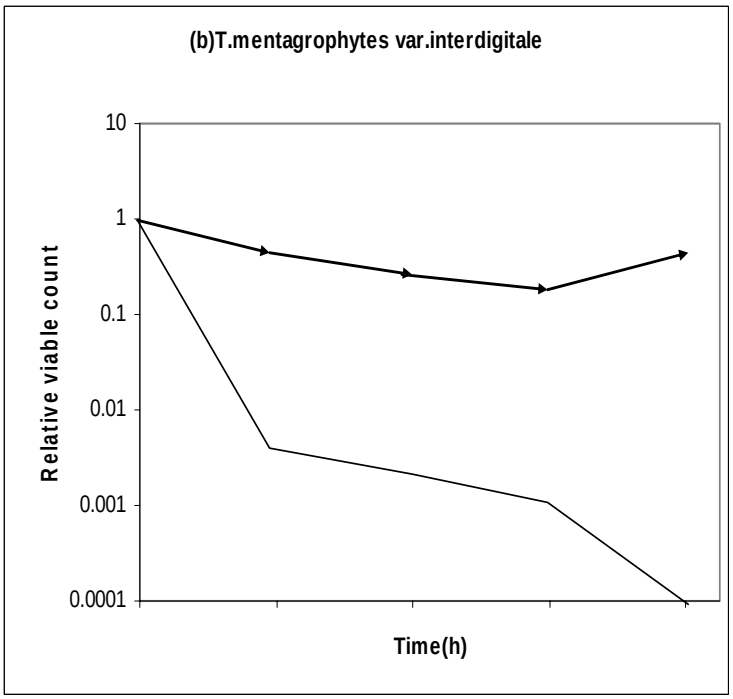

Fig. 1- Time – kill curves for two sample dermatophytes: (a) Epidermophyton floccosum (b) T. mentagrophytes var. interdigitale.viable count values were determined by dividing all viable count values by the cfu/mL count for the control at time zero. Mean ± S.E.M. plotted against time. Time considered from 0 to 8 hours.Symbols: arrowed squares control (a–b); (a); circles, 2% Hypericum perforatum oil

drugs in developing countries. Stabilization is usually made by drying the material in a shady place in a controlled airflow and temperature. The dried materials should then be powdered

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and subjected to a suitable extraction process. This is due to several reasons, namely, conventional medicine can have side effects, abusive or incorrect usage of synthetic drugs result in complications, and the large percentage of world's population do not have access to conventional pharmacological treatment, and folk medicine and ecological awareness suggest that natural products are harmless. Among the many medicinal herbs used throughout the long history of Occidental culture, *H. perforatum*, has always been and still is of great interest. From the time of the ancient Greeks down through the middle Ages, the plant was considered to be imbued with magical powers and was used to ward off evil and protect against disease. *H. perforatum* is known as a potent spice and a medicine with broad therapeutic properties ranging from antibacterial to anticancer and anticoagulant also. Since the discovery of Allicine, much progress has been made in identifying the various unusual organosulfur compounds that are formed when garlic extract is prepared [4, 5, 9, 11, 12, 18]. Despite this progress, many other compounds remain to be identified in *H. perforatum* oil. In this study we attempt to patial purify the *H. perforatum* oil too to identify which Essential Oil Constituents can inhibit ane killing the growth of dermatophytes. The second was the pharmacokinetic of partially purified Essential Oil as an anti biotic on the dermatophytes in vitro. Few previous studies have comprehensively investigated the activity of *H. perforatum* oil against some of fungal, and limited data suggest MICs. The MICs published previously have largely been obtained using the agar dilution method, and the differences between the agar dilution and broth micro dilution assay may explain the difference between these results and those of the present study [4, 5, 11]. Only one previous study has used NCCLS methods [18], and although NCCLS methods are widely accepted for determining in vitro susceptibilities of dermatophytes to antimicrobial agents, how well essential oils, or indeed fungi, fit within these protocols is questionable. It was important to establish whether *H. perforatum* oil was dermatocidal as well as inhibitory as an indication of the potential usefulness of the oil as an antidermatophyte treatment. Most isolates showed a difference of several concentrations between inhibitory and cidal values, indicating that although *H. perforatum* oil does have fungicidal activity, at particular concentrations it is fungistatic only (Table 1). However *H. perforatum* oil may be a useful agent for treating dermatophyte infections. However, the mechanism of inhibitory effect of *H. perforatum* oil on dermatophytes growth is unclear. Superficial mycoses such as onychomycosis rarely responds to topical therapy and is therefore usually treated systemically, it is very well that the topical application of *H. perforatum* oil be useful effectiveness in cure clinical trials. This emphasizes the need for more clinical trial data, particularly in relation to tinea pedis, which can often be treated successfully topically. Although *H. perforatum* is still, to a large extent, grouped together with many other therapies as 'alternative' medicine, invitro and in vivo studies are increasingly showing that some of the anecdotal claims made about the oil have a scientific basis. In particular, data from the present study have begun to illustrate the ways in which *H. perforatum* oil inhibits and kills dermatophyte, which may ultimately be useful in developing *H. perforatum* oil therapies and in the search for novel antifungal agents. Thus these results suggest that they may be useful in the treatment of various microbial infections. The results of this study showed that essential

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oils of *H. perforatum* have a very broad spectrum of antidermatophyte activity. Effects against any of the seven dermatophytes tested and a potential antidermatophyte effect was comparable to that of griseofulvin, the standard antidermatophyte agent.

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