Thymoquinone inhibits germination of dermatophyte arthrospores

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Thymoquinone is an active principle of *Nigella sativa* (*N. sativa*), which is a member of the *Ranunculaceae* family of plants. Several active ingredients have been isolated from the seeds of *N. sativa*, and many pharmacological effects, such as anti-bacterial, anti-parasitic, anti-cancer, and anti-inflammatory have been attributed to them.[1] The dermatophytes are a group of fungi, which parasitize the stratum corneum of the skin, hair, and nail causing infections commonly called tinea or ringworm. In infected tissues, the dermatophytes grow in the form of hyphae breaking down by septation into arthrospores. The arthrospores are important morphological forms of dermatophytes for the spread of infection, particularly, via contact with scales exfoliated from infected sites.[2] The arthrospores are thus appropriate morphological forms of dermatophytes for the study of anti dermatophyte drugs. *In vitro* formation of arthrospores can be induced at conditions of increased carbon dioxide tension in the incubation atmosphere, temperature of 37°C, elimination of glucose from the growth medium, or presence of sub-lethal doses of antifungal drugs.[3,4] The objective of the present study was to investigate the inhibitory effect of thymoquinone on the germination of dermatophyte arthrospores.

The study was conducted in the departments of Dermatology, Pharmacology, and Microbiology, College of Medicine, King Faisal University, Dammam, Kingdom of Saudi Arabia (KSA) from 2005-2006. Approval for the study was obtained from the ethical committee of the King Fahd Hospital of the University (KFHU), Al-Khobar, KSA. Fourteen clinical isolates were obtained from patients attending the dermatology clinic of the KFHU, belonging to various genera of dermatophytes: 4 *Trichophyton rubrum*, 2 *T. violaceum*, one *T. mentagrophytes* var *interdigitale*, one *T. mentagrophytes* var *mentagrophytes*, 3 *Microsporum canis*, one *M. gypseum*, and 2 *Epidermophyton floccosum*, and tried for production of arthrospores. They were sub-cultured as surface lawns on petri dishes under the following conditions: Normal air at 37°C in Dermasel agar (Oxoid); 15% CO_2 and 85% normal air at 28°C in Dermasel agar; 15% CO_2 and 85% normal air at 37°C in Dermasel agar; 1% Peptone agar without glucose at 28°C and normal air; and 1% Peptone agar without glucose at 37°C and normal air. One isolate, *Trichophyton mentagrophytes* (var *interdigitale*), was selected for the present study because of the abundant formation of arthrospores at 15% CO_2 and 85% normal air (Abdullah Hashim Industrial gases and equipments Co. Ltd., Dammam, Saudi Arabia) and a temperature of 37°C after an incubation period of 10 days. Incubation was carried out in a modular incubator chamber (Flow laboratories, Irvine, UK) and gassing the chamber with CO_2 and air mixture on a daily basis. Stock cultures were maintained on glucose Peptone agar. The arthrospores suspension was prepared by harvesting surface growth from the petri dish with a scalpel blade, suspending in phosphate buffered saline (PBS) and shaking on a vortex mixer for 5 minutes to separate chains of arthrospores. The suspension was filtered through column type chromatography grade glass wool packed in a 10ml syringe barrel to a depth of 1cm to remove unbroken chains of arthrospores. The filtered arthrospores suspension was washed 3 times in PBS at 3000g for 3 minutes and adjusted to a concentration of $5x10^6$/ml. The viability of the arthrospores was checked before conducting anti-germination assay; 0.3 ml of arthrospores suspension was incubated in 0.9ml of 4% glucose plus 1% peptone broth (SDB) at 37°C for 12 hours on a rotary shaker (100rpm). An arthrospore was considered to have germinated when a visible germ tube had developed. Arthrospores germinated well in SDB, and the viability of arthrospores was 97.8%. Stock solutions of thymoquinone, clotrimazole and griseofulvin, were prepared by dissolving 64 mg of a drug in 50 ml dimethyl sulphoxide (DMSO), allowed to stand for 30 minutes to permit self-sterilization and stored at 4°C. From the stock solutions, the following concentrations of drugs were prepared: Thymoquinone 0.256 mg/ml, 0.128 mg/ml, and 0.064 mg/ml; clotrimazole 0.128 mg/ml; and griseofulvin 0.128 mg/ml. For the germination assay, to 0.3 ml of arthrospores suspension was added 0.3 ml SDB, and 0.6 ml of each concentration of the drugs. Thus, the final concentrations of the drugs in the germination assays were; 0.128, 0.064, and 0.032 mg/ml for thymoquinone, and 0.064 mg/ml for clotrimazole and griseofulvin. Similarly, for the preparation of DMSO control, to 0.3 ml of arthrospores suspension was added 0.3 ml SDB and 0.6 ml of DMSO 10%. Thus, the final concentration of DMSO in the germination assay was 5%, equal to its concentration in the solutions used to prepare 0.128 mg/ml of the drugs. The second control mixture containing 0.6ml of sterile distilled water, 0.3ml arthrospores suspension, and 0.3ml SDB was also prepared. Sets of 3 germination bottles were prepared for each concentration of the drugs and the controls. The

Table 1 - Effects of thymoquinone, clotrimazole, griseofulvin, and dimethyl sulfoxide (DMSO) on the germination of *Trichophyton mentagrophytes* (var *interdigitale*) arthrospores.

Drug and control	Germination (%)	*P*-value compared to DMSO	*P*-value compared to Clotrimazole	*P*-value compared to Griseofulvin
Thymoquinone				
(0.128 mg/ml)	5.22 ± 1.68	<0.001	0.01	<0.001
(0.064 mg/ml)	10.9 ± 4.01	<0.001	>0.1	<0.01
(0.032 mg/ml)	42.22 ± 9.47	>0.1	>0.001	<0.01
Clotrimazole				
(0.064 mg/ml)	12.8 ± 3.46	<0.001	-	<0.01
Griseofulvin				
(0.064mg/ml)	26.22 ± 6.83	<0.001	<0.01	-
DMSO 5%	46 ± 6.32	-	<0.001	<0.001
Distilled water	95.9 ± 1.9	-	-	-
Viability	97.83 ± 0.41	-	-	-

incubation was carried out at 37°C on a rotary shaker (100 rpm) for 12 hours. At the end of the incubation period, mounts were made on glass slides and examined microscopically (40x) for germination (formation of visible germ tubes). The percentage of germination (mean with SD, n=9) was determined from triplicate readings per bottle.[5] The results of various drugs were compared with each other, and the controls containing DMSO by the Student's t-test (paired, 2 tailed), using 'Microsoft Excel 2003' and the level of significance of difference was P<0.05.

Thymoquinone inhibited the germination of arthrospores in a dose dependent manner. The inhibition of arthrospores germination by thymoquinone at the concentration of 0.064 mg/ml was similar to clotrimazole (0.064 mg/ml) and superior to griseofulvin (0.064 mg/ml). At the concentration of thymoquinone 0.128 mg/ml, the inhibition was statistically more significant than clotrimazole (0.064 mg/ml) and griseofulvin (0.064 mg/ml). At a concentration of 0.032 mg/ml of thymoquinone, the differences were in favor of clotrimazole (0.064 mg/ml), and griseofulvin (0.064 mg/ml) (**Table 1**).

Only a few studies have investigated antifungal drugs for anti-germination effects on arthrospores.[5] Germination of arthrospores is an essential step in the pathogenesis of dermatophytosis, since the penetration of the host tissue occurs by the emerging, elongating, branching, and multi-branching germ tubes.[2,4] Arthrospores can remain dormant in the exfoliated scales for long periods of time.[6] Thus, inhibition of germination of arthrospores is a mean of prophylaxis from dermatophytosis particularly in situations where exfoliation of infected scales is common; such as the floors of swimming pools, public bathing facilities, nursing houses, and so forth.[4] Although not all dermatophytes form arthrospores in vitro,[2] the simplicity and low cost of the above mentioned method of arthrospores production in vitro should encourage investigators to employ germination of arthrospores as a parameter in antifungal assays. Arthrospores can also be used as inoculums in the clinical and laboratory standards institute method (CLSI M38-A) or its modified version to investigate the growth inhibition by anti-fungal drugs. However, the procedure described in the present paper relies specifically on evaluating the germination of arthrospores, namely, formation of visible germ tubes, and requires shorter time of incubation. Thymoquinone has also been reported to inhibit the growth of dermatophytes in the agar diffusion method.[7] The mechanism of anti-germination action of thymoquinone on dermatophyte arthrospores has not been elucidated.

In conclusion, thymoquinone, which possesses antifungal activity against the colonial growth of dermatophytes and inhibits the germination of dermatophyte arthrospores, is a useful chemical for further clinical studies against dermatophytosis.

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