

## Efficacy of Thymoquinone against Vaginal Candidiasis in Prednisolone-induced Immunosuppressed Mice

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**Abstract:** Vaginal candidiasis is a disease that results from the over growth of *Candida* sp. Thymoquinone (TQ) is a major constitute of *Nigella sativa* oil shows wide spectrum antifungal activity. In the present study, the efficacy of TQ was tested against vaginal candidiasis in prednisolone-induced immunosuppressed mice. A cream containing different concentrations of TQ (from 1% to 10%) was used to treat the infected mice and the effectiveness was compared with that treated with miconazole nitrate (2%). The *C. albicans* cells disappeared from the mice treated with 10% TQ cream, while the miconazole treated mice showed heavy growth of both *C. albicans*. The histological analysis showed no difference between the mice that were treated with miconazole and TQ containing cream as compared with the normal mice. Through this study we strongly recommended the use of TQ as an active substance in the anti-candidiasis pharmaceutical formulas.

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### 1.Introduction:

*C. albicans* is known to be a member of natural flora in healthy humans. However, immunodeficiency, which could be resulted from the increased use of immunosuppressive agents in organ transplantations, aggressive anticancer chemotherapy, virus infection, antibiotic overuse and several medications intake, has led to a substantial increase in the occurrence of serious fungal infections (Lamagni *et al.*, 2001). Among these fungi, *Candida* sp. was more prominent. The over growth of *C. albicans* has caused pathogenic symptoms such as oral, intestinal and vaginal candidiasis (Ferrer, 2000; Yuuki *et al.*, 2005; Enoch *et al.*, 2006). Vaginal candidiasis (VC) is now recognized as a major health problem for women of childbearing age worldwide (Ferrer, 2000; Enoch, *et al.*, 2006).

Although fluconazole and miconazole compounds have been used against candidiasis, the development of new effective, cheap, safe and natural antifungal agents is required.

Thymoquinone (TQ) is a major constitute of *Nigella sativa* volatile oil. It has several applications as it acts as an antioxidant (Burits and Bucar, 2000), anti-tumor (Amara *et al.*, 2008), anti-parasitic (El-Wakil, 2007), anti-inflammatory (Salem, 2005), anti-diabetic agent (Rchid *et al.*, 2004) and offers protection against nephrotoxicity (Uz *et al.*, 2008) and hepatotoxicity (Nagi *et al.*, 1999) and has antibacterial (Ozmen *et al.*, 2007; Mariam, 2009) and anti-fungal (Taha *et al.*, 2010) actions.

In a previous study (Taha *et al.*, 2010) we found that TQ has antifungal activity against thirty fungal isolates that had been isolated from patients. Among the tested fungi was *C. albicans*, for which, the MIC was 0.75 mg/ml medium.

Since all of the previous studies were performed to test the antifungal activity of TQ in vitro, this study aimed to evaluate the efficacy of TQ in vivo against vaginal candidiasis in prednisolone-induced immunosuppressed mice comparing with miconazole nitrate.

### 2.Material and methods:

#### Isolation of *C. albicans*:

Swabs from six women with vaginal candidiasis were taken and suspended in 3-ml of sterilized saline solution. Taking swabs for the detection of *Candida* spp. from human subjects is a routine work in our hospital. A loop-full from this solution was streaked on chromogenic agar medium (Oxoid, England) for isolation of *C. albicans* (Murray *et al.*, 2005). The green colonies were picked, microscopically examined and maintained on PDA slants (Oxoid, England).

Each of these obtained isolates was primary tested for its virulence activity in a primary mice experiment. The most virulence isolate was grown in Sabouraud dextrose broth (Becton Dickinson, USA) for 24 hrs at 28°C for one day. The yeast cells were harvested by centrifugation at 1500rpm for 5 minutes and the pellet was re-suspended in 2-ml of fresh medium for mice infection.

**Animals:**

Female albino mice (4 weeks old) were purchased from and the animal experiment was performed in the Animal House, Faculty of medicine, Cairo University. Mice were given food and water *ad libitum* throughout the experiment.

**Mice experimental conditions:**

All animal experiments were performed according to the guidelines for use and care approved by Institutional Animal Care and Use Committee (IACUC) in accordance with Kasr Alainy policy. The average mouse weight at the start of the experiment was 25g. The mice were immunosuppressed by subcutaneous injection with methyl prednisolone on days 1 and 3 before infection. All mice were injected with 150 mg/kg body weight (Takakura *et al.*, 2003).

The infection via the vaginal route was induced by placing a swab saturated with *C. albicans* cell suspension at the fourth day into the vagina of the mice. After five days of infection the animals were divided into nine groups (five mice/ group) in addition to the control group. Group 1: normal mice (neither infected nor treated), group 2: infected with *C. albicans*, group 3: infected and treated with miconazole nitrate (2%) cream (sold from a pharmacy in a pharmaceutical formula prepared by an international pharmaceutical company), group 4: infected and treated with empty cream (without TQ), groups 5 to 10: infected and treated with TQ containing cream with different concentrations (1%, 2%, 4%, 6%, 8% and 10%). All cream treatments were administered once daily for 6 days.

**Preparation of TQ cream:**

Each 100 g of the cream composed of 15 g of stearic acid, 15g of glycerine, 0.7g of potassium hydroxide, 69 ml of distilled water. The appropriate amount of TQ (from 1 to 10g) was homogenized with an amount of the cream formula to prepare the different concentrations of the TQ containing cream.

**Assessment of the TQ efficacy:**

A swab from the vagina of each mouse in all groups was taken on day 3 and 6 after cream treatment and suspended in one ml of sterilized saline with vortex. This suspension was poured into a sterilized Petri dish 5-cm, followed by pouring of molten chromogenic medium and mixed well. The dishes were then incubated for 24-hrs at 37°C. The *C. albicans* colonies were counted for each mouse and the average was calculated for the replicates of each group.

**Histology:**

At the end of the experiment, longitudinal cut of vaginal tissue from two mice from each group was fixed in 10% formalin and embedded in paraffin wax. Sections of 4µm thickness were subjected to hematoxylin and eosin (H&E) staining for histological analysis. The examination was performed by using Olympus BX51 binocular.

**Statistical analysis:**

The data was statistically analyzed using the analysis of variance (Gomez and Gomez, 1984) using MSTATC program. The differences between means were compared using Duncan multiple test (Duncan, 1955).

**3.Results:****Isolation of *C. albicans*:**

Three isolates only out from the six collected samples showed green colonies, which is characteristic for *C. albicans*, while one isolate showed irregular pink-brown colonies, which is characteristic for *C. krusei*, on the chromogenic selective medium (Murray *et al.*, 2005). The microscopic examination of *C. albicans* isolates confirmed presence of the characteristic pseudohyphae. Each of the obtained three *C. albicans* isolates were tested for its virulence activity to induce candidiasis in a primary animal experiment and the most virulence isolate was selected for testing of mice infection.

**The animal experiment:**

The normal (not infected) mice showed presence of low number of *C. albicans* colonies (4 CFU/ mouse). All of the infected mice clearly showed the characteristic candidiasis symptoms especially the mucus growth on the vaginal surface. The number of *C. albicans* colonies raised to 32 CFU/mouse. The yeast colonies were covered with the mycelium of a *Rhizopus* sp. growth. *Rhizopus* sp. grow well on the chromogenic medium and showed also the green color, which is characteristic for *C. albicans*. The infected mice treated with miconazole nitrate cream showed presence of *C. albicans* as well as the *Rhizopus* sp. mycelium. The yeast colonies were reduced to be 27 CFU/mouse. The infected mice treated with the empty cream formula (cream without the TQ) showed also high number of *C. albicans* colonies approximately the same as the infected mice (30 CFU/mouse). In addition, it reduces the *Rhizopus* sp. growth (Fig.1 and Table 1).

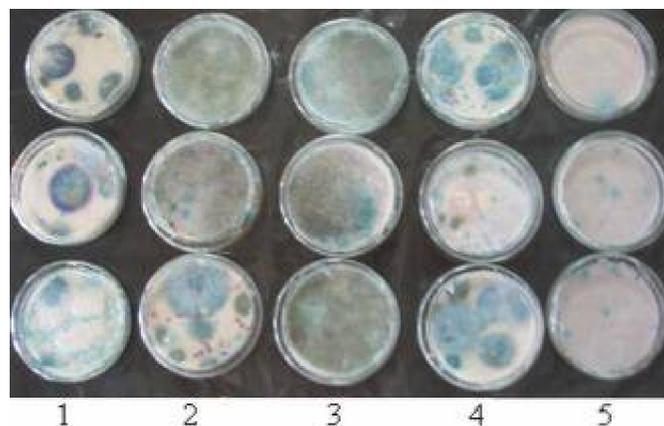


Fig. 1: The *C. albicans* colonies grown in chromogenic medium and isolated from the vagina of 1) normal mice, 2) infected, 3) treated with 2% miconazole nitrate, 4) treated with empty TQ cream and 5) treated with 10% TQ containing cream.

The groups infected and treated with TQ containing cream showed lower number of *C. albicans* colonies. The numbers of the yeast colonies decreases with the increase of TQ concentration. The number was reduced from 50 CFU/mouse with the TQ 1% cream to 5 CFU/mouse in case of treatment with 10% TQ cream. At this TQ concentration (10%) the cream was able to effectively kill most of *C. albicans* cells and all of the mold cells, where the *Rhizopus* sp. was completely eradicated. The average numbers of colonies for the other TQ concentrations were 55, 21, 21 and 8 CFU/mouse for the TQ concentrations 2%, 4%, 6% and 8%, respectively.

#### Statistical analysis:

The statistical analysis (Table I) of the animal experiment data showed significant variation between the control (normal mice) and the infected groups; where the number of yeast colonies was increased from 4.24 to 32.5 CFU/ mouse for each of these two groups, respectively. As unexpected result there is no significant variation was obtained from the infected

and the miconazole treated groups; where the colonies number were decreased only from 32.5 to 27 CFU/ mouse for the infected and miconazole treated groups, respectively. There is no significant variation between the mice group that has been treated with the empty TQ cream and both the infected and miconazole treated groups. This result reflects absence of any antifungal activity of the TQ empty cream. The mice groups that had been treated with either 1% or 2% TQ containing cream showed higher number of CFU than the infected group. The reason for increasing of the colonies number in these two groups is not clear, however, it reflects absence of the TQ antifungal efficiency at these two concentrations.

Significant variations were obtained from infected and all of the other TQ concentrations (from 4% to 10%) treated groups. The lowest number of yeast colonies (5CFU/mouse), that has been obtained from the 10% TQ treated group, is approximately the same as the normal mice.

**Table I: Mean values of number of *C. albicans* colonies in each group of the animal experiment:**

Treatment	Mean number of CFU/mouse
Control	4.25 <sup>F</sup>
Infected	32.50 <sup>BC</sup>
Miconazole	27.00 <sup>CDE</sup>
Empty TQ cream	30.00 <sup>BCD</sup>
1% TQ cream	50.00 <sup>B</sup>
2% TQ cream	55.00 <sup>A</sup>
4%TQ cream	21.00 <sup>C-F</sup>
6%TQ cream	21.00 <sup>C-F</sup>
8%TQ cream	8.25 <sup>DEF</sup>
10%TQ cream	5.00 <sup>EF</sup>

Means within rows and/or column, followed by the same letter(s) are not significantly different by Duncan's New Multiple Range Test ( $P < 0.05$ ).

**Histological analysis:**

The histological analysis of the mice vagina of the infected group showed presence of hyperkeratosis, para-keratosis and sub-epithelial mixed chronic inflammatory cellular infiltrate (Fig. 2B). The control group showed normal behavior where there was no hyper keratosis, no para keratosis and no sub-epithelial mixed chronic inflammatory cellular infiltrate (Fig. 2A).

The miconazole and TQ treated groups showed the same results. Both of them showed presence of mild and focal hyper keratosis, no para-keratosis and moderate sub-epithelial mixed chronic inflammatory cellular infiltrate (Fig. 2C and E). The same characters were also showed in the group treated with TQ empty cream in addition to high number of released epithelial cells (Fig. 2D). These results reflects the save effect of TQ on the vaginal tissue.

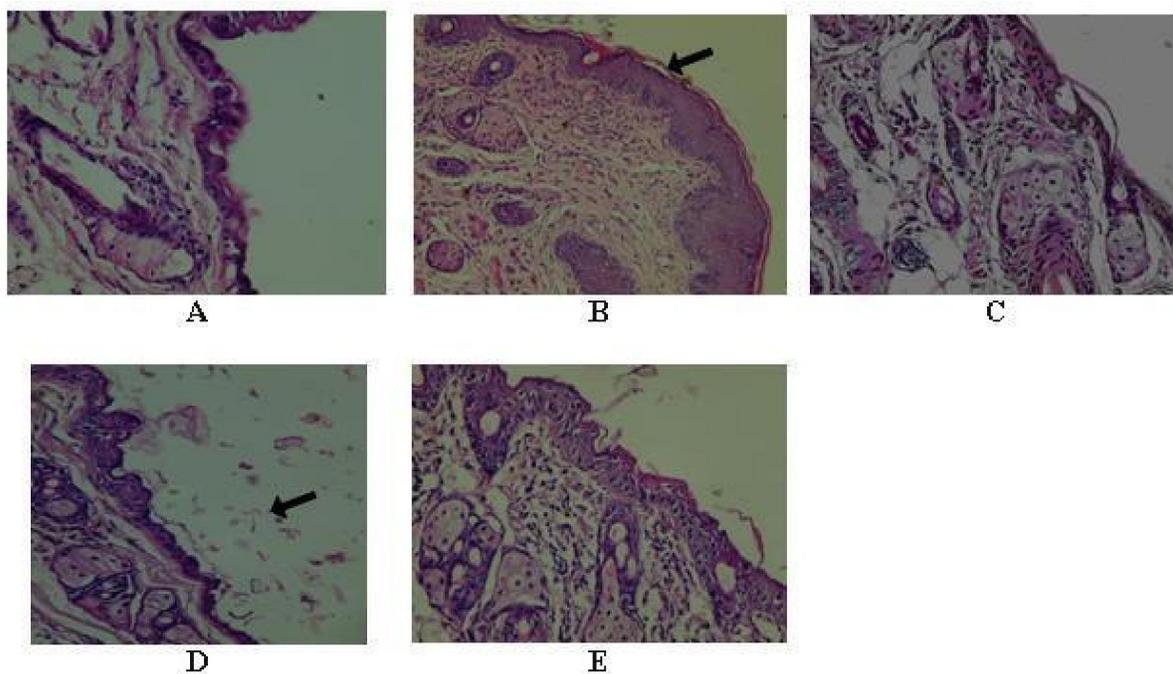


Fig. 2: The hisopathological sections of vaginal mice: A) normal mice, B) infected, C) infected and treated with 2% miconazole nitrate, D) infected and treated with empty TQ cream and E) infected and treated with 10%TQ containing cream.

**4. Discussion:**

Thymoquinone (TQ) is a major constitute of *Nigella sativa* volatile oil. Several *in vitro* studies had been conducted to test the antifungal activity of TQ, however, there was no *in vivo* experiment has been conducted for this purpose. Therefore, the present study has been performed to test the efficacy of TQ against vaginal candidiasis in prednisolone-induced immunosuppressed mice, to be the first study that tests this effect *in vivo*. Vaginal candidiasis is now considered as a major health problem for women of childbearing age worldwide (Ferrer *et al.*, 2000 and Enoch *et al.*, 2006). It results from the over growth of *C. albicans*. Among the tested concentrations of TQ, the best anti-candidiasis activity was obtained from the highest TQ concentration (10%). Quinones are known to complex irreversibly with nucleophilic

amino acids in proteins, which may lead to inactivation of the proteins in the plasma membrane of microorganisms and loss of its function (Stern, 1996). Therefore, we strongly suggested that TQ bind with the nucleophilic amino acids of the *C. albicans* plasma membrane causing loss of its function followed by yeast cells death. However, TQ showed no effect on the vaginal cells, as obtained from the histology analysis. This result is in agreement with Worthen *et al.*, 1998 and Rooney and Ryan, 2005 who reported that TQ has no significant effects on the growth of normal cell lines. Through this research we strongly recommended that TQ can be safely and effectively used in the antifungal pharmaceutical formulas for diseased human treatments.

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