ORIGINAL ARTICLE

A therapeutic effect of *Nigella sativa* extract on female Wistar rats vulvovaginal candidiasis model

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ABSTRACT

Aim Vulvovaginal candidiasis (VVC) is a disease mostly caused by *Candida albicans* and affects the quality of life of women especially in the form of chronic recurrent vulvovaginal candidiasis (RVVC). *Nigella sativa* is known to have several effects such as antimicrobial, anti-inflammatory, immune stimulation and anticancer properties. The aim of this study was to evaluate the effect of *Nigella sativa* on vulvovaginal candidiasis.

Methods This study is a true experimental design, we used 28 Wistar strain rats divided into 4 groups, all groups were conditioned in a pseudoestrus state. *Candida albicans* was inoculated into the rats' vagina for 3 consecutive days. All groups were observed every 24 hours, 48 hours and 72 hours to evaluate the number of *Candida albicans* colonies, IgG and IgM anti Candida.

Results After administration of intervention, there was a significant difference in the amount of fungal colonization after the treatment in each group (p<0.001). There was no significant difference in the effectiveness of *Nigella sativa* and fluconazole in supressing *Candida albicans* colonies after 72 hours (p=0.101). The administration of *Nigella sativa* showed a significant difference in the increase in IgM levels compared to the others group (p<0.001), while the IgG level did not show a significant difference (p=0.423).

Conclusion *Nigella sativa* provides a therapeutic effect by decreasing the number of fungal colonies and increasing IgM levels.

Key words: *Candida albicans,* fungal inoculation, pseudoestrus

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INTRODUCTION

Among human diseases caused by *Candida albicans*, vulvovaginal candidiasis (VVC), especially in chronic and recurrent form (RVVC), is arguably the most frequent candidiasis bacterial infection of humans. Epidemiological investigations provide a global estimate of the incidence of RVVC approaching 2% (1). It is important to deal with this disease immediately because of complications that can be caused. Complications that often occur in this disease, if it is not handled properly, are infertility in sexually active women due to pelvic inflammatory disease and chorioamnionitis that can cause abortion or premature birth in pregnant women (2).

Nigella sativa Linn is a member of the *Ranunculaceae* family, which grows in Asia and the Middle East and has been used as food and natural medicine. The ingredients contained by *Nigella sativa* are thymoquinone, thymohydroquinone, dithymoquinone, and thymol. The inflammatory process balances with lipo-oxygenizes and thymoquinone inhibits the oxygen cycle. Several pharmacological effects of *Nigella sativa* including antimicrobial, anti-inflammatory, immune stimulation, and anticancer properties have been reported (3).

Basically, this plant has been used in inflammation treatment since ancient times. Appearance of synthetic anti-fungal agents in the middle of the last century led to a reduced interest in usage of plants as a natural source of anti-fungal drugs. In recent years the situation has changed, and the field of ethnobotanical research has evolved (4).

Thymoquinone on *Nigella sativa* showed a selective anti-bacterial effect on seven bacteria, especially Gram-positive strains with a low minimal inhibitory concentration (MIC). Thymoquinone showed a potent growth inhibitory effect on Gram-positive bacteria, with MICs ranging from 8 to 64 μg/mL (5).

Zataria multiflora extract on *Candida albicans* has shown the MIC and minimum fungicidal concentration (MFC) value (50% and 90%) of 0.13, 0.38, 0.74, and 1.03 mg/mL, respectively (6).

Research to assess the MIC and MFC of *Nigella sativa* in *Candida albicans* has been conducted *in vitro* (6,7), but no *in vivo* studies have been found investigating vulvovaginitis in rats. The aim of this study was to compare *Candida albicans* colony count, anti-*Candida* IgG and IgM, before and after the treatment of vulvovaginal candidiasis by *Nigella sativa.*

MATERIALS AND METHODS

Materials and study design

This was experimental study to assess the effect of *Nigella sativa* extract on vulvovaginal candidiasis. This study was held between February and October 2019 in four institutions: Multidicipline Laboratory of Medical Faculty Universitas Sumatera Utara (USU) to assess anti-*Candida* IgG and IgM; Microbiology Laboratory of Universitas Sumatera Utara General Hospital; Faculty of Mathematics and Natural Sciences' Animal House of Universitas Sumatera Utara for acclimatization, intervention and blood sample collection of the animal; and Pharmacology Laboratory of Faculty of Pharmacy Universitas Sumatera Utara to produce the extract of *Nigella sativa.*

The sample included 28 female Wistar rats (*Rattus norvegicus* sp.) aged 2-3 months with a range of body weight between 101-241 grams, which met the inclusion and exclusion criteria. Before inoculation of *Candida albicans*, estradiol valerate 2 mg was given subcutaneously 3 days before inoculation and 4 days after inoculation to make the rat in the condition of pseudoestrus to maintain the *Candida albicans* and prevent self-healing of the rat.

Methods

The *Nigella sativa* extract was produced by Pharmacology Laboratory using ethanol 96% solvent and sodium carboxymethyl cellulose (CMC Na) in order to obtain the extract of *Nigella sativa* 5 mg/mL.

The samples were divided into 4 groups: K1-7 rats as the control group, K2-7 rats were given a fluconazole 10 mg/kg of body weight, K3-7 rats were given *Nigella sativa* extract with the dose of 6.6 mL/kg of body weight, K4-7 rats were given a combination of *Nigella sativa* extract 6.6 mL/kg of body weight and fluconazole 10 mg/kg of body weight. All treatments were given orally with an oral-gastric tube. Twenty-four hours after inoculation, we gave the therapy for three days.

The propagation of *Candida albicans* was held in

the Microbiology Laboratory. *Candida albicans* (ATCC 14053, USA) inoculated on Sabouraud Chloramphenicol 2 Agar Media/ SAB CHL 2-D (Biomeruex, L'etoile, France) with 4 quadrants method and after 48 hours incubation in O_2 incubator at 25 ºC, *Candida albicans* suspension (3 McFarland) was prepared from grown colonies.

The colonies was identified macroscopically by observing the shape by Gram-staining, and confirmed with MALDI-TOF mass spectrometry and with Vitex 2 Compact (Biomeruex, L'etoile, France) in Microbiology Laboratory of USU Hospital.

Identification began by preparing a fungal suspension of 3 Mc-Farland turbidity using a nephelometer (DensiCHECK, Biomeruex, L'etoile, France). Then the tubes containing fungal suspension were arranged in a cassette and inserted into YST ID identification (Biomeruex, L'etoile, France) and AST-YS08 antifungal sensitivity test (Biomereux, L'etoile, France) cards. Barcode was given on each card to connect with the identity of the isolates that had been entered, then inserted into filler room and proceeded to the next room. The Vitek 2 Compact machine automatically identifies and gives antifungal sensitivity result.

Inoculation of the rat's vagina was done by swabbing the vagina with a cotton swab dipped in the fungal suspension. The colonies (colony forming unites, CFU) of *Candida albicans* on the rat's vagina were measured before inoculation, 24, 48 and 72 hours after. The rats underwent taking vaginal smear to take samples, which were incubated for 48 hours at 25 ºC.

The IgM and IgG level were measured by ELISA (Enzym Linked Immunosorbent Assay) method before the inoculation, 7 days and 14 days after the intervention. The blood sample was taken with 1mL disposable syringe from rat's tail as much 0.25 mL.

Statistical analysis

The data were analysed using Kruskal-Wallis and Mann-Whitney tests to assess differences of the colonies forming units (CFU) of *Candida albicans,* and Friedman test to assess the differences of IgM and IgG level.

RESULTS

There were differences in the average of colony forming units based on the time of observation (p<0.001). The number of colonies sharply increased on all groups after inoculation with the *Candida albicans*. The day after the treatment, the K2 group and K4 group showed the greatest decrease in the CFU and even showed a mean below the mean before inoculation. On the third day after the treatment, it appeared that the K2 group, K3 group, and K4 group showed the mean number of colonies below the number of colonies before inoculation (Table 1).

Table 1. The differences of *Candida albicans* colony forming units (CFU) based on the observation of each group

Group	*Candida albicans* mean (SD) of CFU					p
	Pre inoculation	Post inoculation	Day-1	Day-2	Day-3	
K1	0.14 (0.38)	237.86 (106.46)	229.29 (120.89)	110.29 (131.48)	130.43 (117.76)	<0.001
K2	10 (18.68)	136.86 (115.77)	4.29 (1.11)	0.14 (0.38)	0.43 (1.13)	<0.001
K3	2.14 (2.48)	164.57 (124.69)	88.29 (99.63)	6.43 (5.29)	1.14 (0.9)	<0.001
K4	0.57 (0.79)	210 (95.67)	2.86 (1.86)	0.57 (1.13)	0.14 (0.38)	<0.001

K1, control group; K2, fluconazole group; K3, *Nigella sativa* group; K4, fluconazole + *Nigella sativa* group

When observing each group, almost all groups showed an increase of IgM, but in the K1 group (control) the mean of IgM level decreased on the 7th day from 769.88 pg/mL to 547.72 pg/mL, then on the 14th day a sharp increase from 547.72 pg/mL to 1094.14 pg/mL. There were significant mean differences in each group for IgM level (p<0.05) (Table 2).

Table 2. IgM level changes according to inoculation time period

Group	Mean (SD) IgM level (pg/mL)			p
	Pre-Inoculation	Day-7	Day-14	
K1	769.88 (191.91)	547.72 (130.46)	1094.14 (136.24)	0.006
K2	682.03 (178.09)	866.79 (418.23)	1795.39 (318.01)	0.018
K3	982.57 (291.49)	1000.81 (660.05)	2295.23 (1165.42)	0.018
K4	448.97 (113.1)	1026.95 (843.94)	1106.49 (338.18)	0.028

K1, control group; K2, fluconazole group; K3, *Nigella sativa* group; K4, fluconazole + *Nigella sativa* group

There were significant difference in the mean value of IgG between the K1 (3553.67 pg/mL), K2 (3879.48 pg/mL) and K4 (4215.15 pg/mL) group based on the time of observation (p<0.05); the K3 (4021.07 pg/mL) group did not show significant difference in IgG level in relation to the time of observation (p=0.058) (Table 3).

Table 3. IgG level changes according to inoculation time period

Group	Mean (SD) IgG level (pg/mL)			p
	Pre inoculation	Day-7	Day-14	
K1	3588.05 (1037.50)	3553.67 (1139.07)	2209.72 (971.53)	<0.001
K2	4005.81 (974.02)	3879.48 (637.57)	3013.53 (918.45)	0.001
K3	4031.89 (570.05)	4021.07 (598.33)	3765.14 (614.55)	0.058
K4	4373.40 (492.20)	4215.15 (426.89)	2795.78 (835.41)	0.027

K1, control group; K2, fluconazole group; K3, *Nigella sativa* group; K4, fluconazole + *Nigella sativa* group

DISCUSSION

Creating, supporting and maintaining pathogens for vaginal candidiasis rat model need a special treat. The vaginal candidiasis condition in rats will quickly recover, so that the infection will heal quickly without creating a pseudoestrus situation. The estradiol valerate 2-5 mg was administered subcutaneously 3 days before inoculation and 4 days after to decrease rat immunity or creating a pseudoestrus situation. If needed, it can be repeated weekly (7).

The results of this study showed an increase of *Candida albicans* CFU in all rat groups after inoculation. IgM level showed an increase in all the study groups with an exception of group K1 (control) in which a decrease in IgM mean level on the 7th day after the treatment was found, and on the 14th day a sharp increase was noticed.

There were significant mean differences in groups K2, K4 and K1 for IgG level based on inoculation time. This is consistent with a trout fish study from Awad, et al., in which an increase of IgM level was found after *Nigella sativa* administration compared to the control group; they also found an increase in lysozyme, the amount of serum protein, antiprotease and antibacterial activity in the group that received *Nigella sativa* (8).

The role of humoral immunity against vaginitis is not very clear. In patients with recurrent vulvovaginal candidiasis (RVVC), elevated levels of IgA and specific IgG in serum or vaginal secretions show little or no protective role for antibodies against vaginitis. In fact, some women have elevated levels of IgA and IgG specific candida in serum or vaginal secretions (9). In rats, candida-specific IgM and IgG antibodies have shown to be protective against systemic and vaginal candida infections (10). In addition, in a mouse model of vaginal candidiasis, specific infection-induced aspartyl proteinase IgA antibodies contribute to protection against infection. Rats with primary vaginal infection showed resistance to vaginal rinse containing IgA antibodies directed against the secretion of aspartyl proteinase *Candida albicans* (11). These antibodies are able to provide protection to recipients who have not been infected (12).

Prince et al. stated that detection of candida IgG, IgM, and IgA by ELISA method showed excellent performance, as indicated by acceptable linearity, low intra-assay and inter-assay coefficient of variation (CV) values, and low specimen stability. Strong inter-laboratory reproducibility, as demonstrated by large panel sera testing at two different locations, was also very good, which confirms the strong nature of this ELISA. Seroprevalence level in a positive sera panel for antibodies to other fungal pathogens are similar to those observed in healthy individuals, suggesting that cross-reactivity due to other fungal infections continues to show consistent results (10,13). The number of fungal pathogens in secondary infection rats was less than in primary infections (a secondary infection is an infection that occurs during or after treatment for another infection, it may be caused by the first treatment or by changes in the immune system), and this is related to a higher level of anti-candida antibodies in secondary infections compared to primary infections (11).

In conclusion, *Nigella sativa* has therapeutic effect that reduces the number of *Candida albicans* colonies and increases the level of IgM. There is a need for further studies to asses the effect of *Nigella* sativa in the treatment of human vulvovaginal candidiasis.

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