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Monira Abd El-Wahab Selim

*professor of parasitology department, faculty of medicine zagazig university, zagazig , al sharquia,
dr.monirseliem@hotmail.com*

Eman Magdy Fawzy

Medical Parasitology, Faculty of Medicine, Zagazig University, Zagazig, Egypt, e.magdy_83@hotmail.com

Reem Safaa Eldeen Abdelhady

medical parasitology,faculty of medicine, zagazig university, zagazig, Egypt, dr.reem_safaa@yahoo.com

Mohammad Samir Badr

obstetric and gynecology , faculty of medicine, zagazig university, Egypt, 2362044@gmail.com

Enas Fakhry Abdelhamed

Medical Parasitology Department, Faculty of Medicine, Zagazig University, Egypt, drenasfakhry@gmail.com

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ORIGINAL ARTICLE

Assesment of the effect of herbal medicine on cultured TrichomonasVaginalis

Monira A. Selim¹, Eman M. Fawzy¹, Reem S. Abdel Hady¹, Mohamed S. Badr², Enas F. Abdel Hamed¹

¹Department of Medical Parasitology, Faculty of Medicine, Zagazig University, Zagazig, Egypt

²Obstetrics and Gynecology, Faculty of Medicine, Zagazig University, Zagazig, Egypt



Corresponding author:

Enas fakhry abdelhamed

Lecturer at medical parasitology department faculty of medicine zagazig university

Dr.reem_safaa@yahoo.com

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ABSTRACT

Background: Trichomoniasis is a disease caused by the parasitic protozoan flagellate *Trichomonas vaginalis*. It is considered the most common non-viral sexually transmitted disease in the world. Metronidazole is the drug of choice in treatment of trichomoniasis. Side effects of Metronidazole and its resistance are the main causes for finding new alternatives for trichomoniasis treatment. The present study is concerned with evaluation of the effect of different concentrations of *Nigella sativa* oil at different durations in the treatment of *T. vaginalis* and comparison of their efficacy with Metronidazole under in vitro conditions of axenic culture of isolated *T. vaginalis* trophozoites.

Materials and Methods: Vaginal samples were collected from ninety female patients complaining of characteristic vaginal discharge or other manifestations of trichomoniasis. Vaginal swabs of infected females were examined microscopically for motile trophozoites. Then they were axenically cultured on modified Diamond's medium. The effect of *Nigella sativa* oil at different concentrations (500, 750, 1000 µg/ml) on *Trichomonas vaginalis* was measured by counting number of dead trophozoites using haemocytometer and trypan blue staining. The effect of *Nigella sativa* oil on ultrastructure of the parasite was determined by transmission electron microscope.

Results: *Nigella sativa* oil showed high toxic effect on the parasite as it causes severe cell damage with cytoplasmic and nuclear destruction.

Conclusions: *Nigella sativa* oil has antitrichomonal effect in vitro at different concentrations and different durations as well as it has low toxicity and low side effects. So, it can be a good alternative for metronidazole in treatment of Trichomoniasis.

Keywords: *Trichomonas vaginalis*, *Nigella sativa* oil, Transmission electron microscope.

INTRODUCTION

Trichomonas vaginalis (*T. vaginalis*) is a parasitic protozoan flagellate causing trichomoniasis. It is considered the most

common non-viral sexually transmitted disease in the world[1]. According to data collected by World Health Organization in (2012), 276.4 million new cases were recorded per year, or

143 million young women were infected each year [2].

T.vaginalis is a parasite of the urogenital tract. It affects male and female especially at reproductive age [3]. In men, infection causes urethritis and can be complicated with epididymitis, prostatitis and infertility. It may be associated with cancer prostate [4]. In women, it usually causes vaginal discharge characterized by foul smell and yellowish to greenish colour. It also causes itching sensation, urethritis, cystitis, dyspareunia and cervicitis [5]. In pregnant women it may cause serious complications as low birth weight infants and preterm rupture of membranes. Moreover, it may be associated with cervical cancer and infertility [6].

T.vaginalis infection is routinely diagnosed by a wet mount through observation of the "corkscrew" motility of trophozoites [7]. However the gold standard method in diagnosis is culture as its sensitivity range is 85-95% [8]. There are other methods for diagnosis of this infection as staining methods [9], enzyme-linked immunosorbent assay (ELISA) [10], Latex agglutination [11], immunochromatography and nucleic acid amplification tests [9].

Metronidazole is the drug of choice in treatment of trichomoniasis. It is a nitroimidazole derivative and is used in treatment of *T.vaginalis* for more than 50 years. It is also used in treatment of other important intestinal protozoa as *Giardia lamblia* and *Entamoebahistolytica* [12]. There are many studies showing that metronidazole causes nausea, dizziness, hypersensitivity reactions and dermatological symptoms [13]. It also has teratogenic and carcinogenic effects on fetus. Beside, clinical cases showed some metronidazole resistance [14]. Therefore, it is necessary to find new drugs for treatment of *T.vaginalis* infection [15]. Medicinal plants may provide new therapeutic agents for treatment of some protozoal diseases as they have low cost, low toxicity and high activity [16].

Nigella sativa (*N.sativa*) grows annually in Mediterranean countries including Egypt. Traditionally it has been used in Europe, India and Arabic countries as a natural treatment for many diseases as headache, cough, fever, eczema, bronchitis, hypertension, diabetes and asthma [17]. The active principles extracted from the seeds of *N.sativa* are mostly from its essential volatile oil [18]. Recently it has been reported that *N.sativa* seeds have biological properties as antifungal, antimicrobial, antiparasitic, antioxidant and antiinflammatory [19]. Its alcoholic extract is effective against giardiasis [18]. On the other hand its aquatic extract was found to be potentially effective against *Blastocystishominis* [20] and *T.vaginalis* [21].

The present study is concerned with evaluation of the effect of different concentrations of *Nigella sativa* oil at different durations in the treatment of *T.vaginalis* and comparison of their efficacy with Metronidazole under in vitro conditions of axenic culture of isolated *T.vaginalis* trophozoites.

Methods

Parasites and culture

Vaginal samples were collected at Gynecology & Obstetric Department Faculty of Medicine, Zagazig University from ninety female patients complaining of characteristic vaginal discharge or other manifestations of trichomoniasis (as vaginitis, cervicitis). The swabs were taken from posterior fornix and vaginal wall by the help of Gynecology Department member. Wet mount examination was done to fresh vaginal swab within two hours [22]. The cotton-wool part of the swab was cut and put in the tube containing 9ml of Modified Diamond's medium culture (PH 6) at 37°C supplemented with 0.5 ml of fetal bovine serum, 0.5 ml of ready-made antibiotic mixture of penicillin (10000U/ml) and streptomycin (10000 µg/ml) and 10 units of antifungal (Diflucan). Isolates were sub-cultured every 48 h on modified Diamond's medium and maintained in Medical Parasitology Department and Scientific and Medical Research Center

(ZSMRC), Faculty of Medicine, Zagazig University[23].

Drugs and herbs

The drug and herbal extracts and their different concentrations were prepared at Pharmacognosy Department, Faculty of Pharmacy, Zagazig University.

○ Metronidazole

It was used in the form of tablets 250mg (Flagyl, Sanofi-Aventis, Egypt). Tablets were crushed and dissolved in distilled water. It was diluted in the culture medium to yield these concentrations 30 µg/ml, 60 µg/ml and 90µg/ml.[24]

○ Nigella sativa oil (NsO)

It was brought from herbs store. It was dissolved in dimethylsulfoxide (DMSO). Then it was diluted in the culture medium to give the concentration 500 µg/ml, 750 µg/ml and 1000 µg/ml[25].

Experimental design

Trophozoites of *T.vaginalis*, isolated from vaginal swabs of infected females, were examined microscopically for motile trophozoites. Then they were axenically in vitro cultured on Modified Diamond's medium. The ultrastructural changes of the cultured parasites were studied using Transmission Electron Microscope (TEM).

The studied groups were classified as follows:

Contol group:

- Group1: Cultured parasites without any drugs or remedies.

Drug control group:

- Group2: Cultured parasites with different concentrations of metronidazole and subdivided into:

- G2a: Metronidazole at concentration of (30µg/ml).

- G2b: Metronidazole at concentration of (60µg/ml).

- G2c: Metronidazole at concentration of (90µg/ml).

- Group3: Cultured parasites with different concentrations of Nigella sativa oil extract and subdivided into:

- G3a: Nigella sativa oil extract at concentration of (500 µg/ml).

- G3b: Nigella sativa oil extract at concentration of (750µg/ml).

- G3c: Nigella sativa oil extract at concentration of (1000µg/ml).

After preparation of subcultures, they were examined by light microscope to make sure that the parasite was presented alive and the number of trophozoites was counted by using haemocytometer. Each tube in each group was examined by light microscope with objectives x10, x40 and x100 after 24hrs, 48hrs and 72hrs of addition of drug or herb. The numbers of dead trophozoites were counted in each tube after 24hrs, 48hrs, and 72 hrs by using haemocytometer and trypan blue staining[26]. In addition, culture tubes containing *T.vaginalis* trophozoites without adding any herbs was used as control tubes.

Electron microscopy for *T.vaginalis*

Transmission electron microscope (TEM) was used to detect the ultrastructural changes of the parasite in culture. The tubes of *T.vaginalis* culture without adding drugs and after adding drugs were chilled in ice and centrifuged at 1000g for 10 min. Trophozoites were fixed with 2.5 % (v/v) glutaraldehyde in 0.1, phosphate buffers (PBS), pH 7.2 for one h. Then the fixed samples were washed twice in PBS and post-fixed for 30 minutes with 1 % (v/v) osmium tetroxide in 0.1 M cacodylate buffer, pH 7.2, at room temperature. They were dehydrated in increasing concentrations of ethanol. Finally they were dehydrated in 100 % propylene oxide. Samples were embedded in Araldite 502/ EMBED – 812. Thin sections were cut in the Reichert ultra-microtome, mounted on copper grids, stained with uranyl acetate and lead citrate and examined in a JOEL JEM - 2100 electron microscope [27].

Ethical consideration

Before taking vaginal samples an informed consent was taken from all patients. The work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans. Approval of the

study design was obtained from the Institutional Review Board (IRB) unit, Faculty of Medicine, Zagazig University.

Statistical analysis

The collected data were computerized and statistically analyzed using SPSS program (Statistical Package for Social Science) version 25.0. Quantitative data were expressed as mean \pm SD (Standard deviation).

RESULTS

Regarding the use of different concentrations of Metronidazole (Table 1), it was reported that there were statistical significant difference in the mean number of dead *T.vaginalis* trophozoites between Metronidazole 30 μ g/ml, 60 μ g/ml and 90 μ g/ml at 24 hrs (0.04), but no difference was found between different concentrations at 48hrs (0.61 NS) and at 72hrs (0.09 NS). Moreover, differences between mean number of dead trophozoites at different follow up periods for each Metronidazole concentration showed very highly significant difference (<0.001).

As regards NsO treated culture of *T.vaginalis* at different concentrations (500 μ g/ml, 750 μ g/ml and 1000 μ g/ml)(Table 2), there were statistical significant difference between NsO 1000 μ g/ml and Metronidazole 90 μ g/ml at 24 hrs and 48 hrs and statistical significant difference between NsO 500 μ g/ml, 750 μ g/ml and Metronidazole

90 μ g/ml at 72 hrs. As regards the negative control, there were difference between all concentrations and modified Diamond's medium results at all follow up periods. Furthermore, there were statistical significance difference between NsO 500 μ g/ml and 750 μ g/ml concentrations at different follow up periods and very highly statistical significant difference at NsO 1000 μ g/ml at the same durations.

The fine structure of the untreated *T.vaginalis* trophozoites (control group) showed one nucleus (N), hydrogenosomes (H) and few vacuoles (V). The cell membrane (C) is obvious and intact (Fig.1).

T.vaginalis trophozoites treated with Metronidazole after 72hrs were swollen compared to non-treated group and lost much of the material within cytoplasm. The remaining part of organelles (nucleus, some vesicles and few hydrogenosomes) showed centripetal displacement (Fig. 2). TEM of *T.vaginalis* trophozoites treated with NsO after 72hrs showed severe cell damage, nucleus and cytoplasm were severely destructed with large vacuolization and cell membrane defects. Moreover, in treated culture some *T.vaginalis* trophozoites showed completely destructed cell membrane (Fig.3).

Table (1): Comparison between the effect of different Metronidazole concentrations on the mean number of dead *T.vaginalis* trophozoites at different follow up periods:

Group	24 h	48 h	72 h	P [^]
	No. of trophozoites $\times 10^4$	No. of trophozoites $\times 10^4$	No. of trophozoites $\times 10^4$	
	Mean \pm SD	Mean \pm SD	Mean \pm SD	
Met 30 μ g/ml	6.66 ^a \pm 0.57	23.3 ^a \pm 10.0	38.3 ^a \pm 13.5	<0.001***
Met 60 μ g/ml	11.6 ^a \pm 2.88	25 ^a \pm 5.29	42.6 ^a \pm 7.5	<0.001***
Met 90 μ g/ml	15 ^a \pm 4.35	29 ^a \pm 4	74.6 ^b \pm 25.50	<0.001***
F	5.72	0.525	3.28	
P	0.04 S	0.61 NS	0.09 NS	

SD: Standard deviation.

t: Independent t test

[^]: Repeated measure ANOVA test

*: Significant (P<0.05)

**: Highly significant (P<0.01)

***: Very highly significant (P<0.001)

Table (2): Comparison between the effects of different concentrations of N.sativa oil (NsO) on the mean number of dead T.vaginalistrophozoites at different follow up periods:

Group	24 h	48 h	72 h	P [^]
	No. of trophozoites ×10 ⁴ Mean±SD	No. of trophozoites ×10 ⁴ Mean±SD	No. of trophozoites ×10 ⁴ Mean±SD	
NsO 500µg/ml	78.3 ^a ±5.5	103.00 ^a ±14.5	137.33 ^a ±39.01	0.02*
NsO 750µg/ml	82.00 ^a ±4.00	113.00 ^a ±4.5	137.6 ^a ±6.5	0.02*
NsO 1000µg/ml	191.3 ^b ±30.02	243.60 ^b ±38.00	266 ^b ±43.5	<0.001***
Met 90µg/ml	15 ^a ±4.35	29 ^a ±4	74.6 ^b ±25.50	<0.001***
Diamond	20 ^d ±5	22 ^c ±2	40 ^d ±2	0.01*
F	39.15	56.49	18.89	
P	<0.001***	<0.001***	0.001	

Diamond media =negative control, Met 100 µg/ml =positive control

SD:Standard deviation. t: Independent t test ^: Repeated measure ANOVA test

*: Significant (P<0.05) **: Highly significant (P<0.01) ***: Very highly significant (P<0.001)

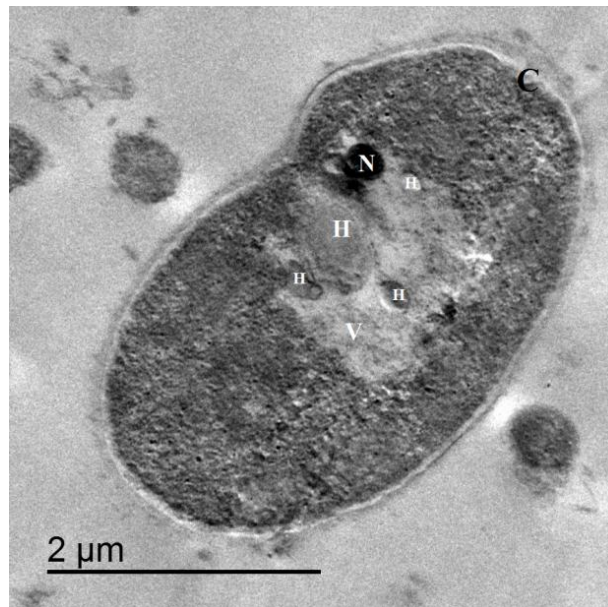


Figure 1: TEM of T.vaginalis trophozoite without adding any herb (control tube) showing one nucleus (N), hydrogenosomes (H) and few vacuoles (V). The cell membrane (C) is obvious and intact

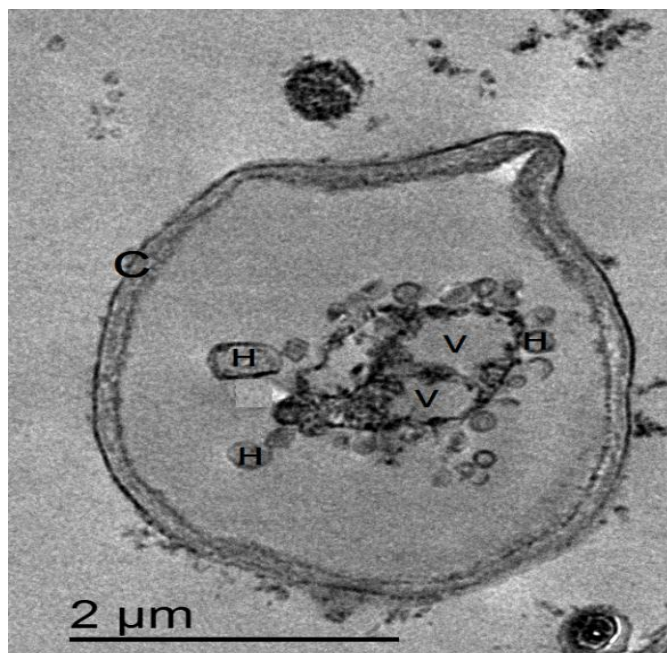


Figure2: TEM of *T.vaginalis* trophozoite after 72hrs incubation with Metronidazole showing centripetal displacement to all the remaining part of organelles (nucleus, some vesicles and few hydrogenosomes).

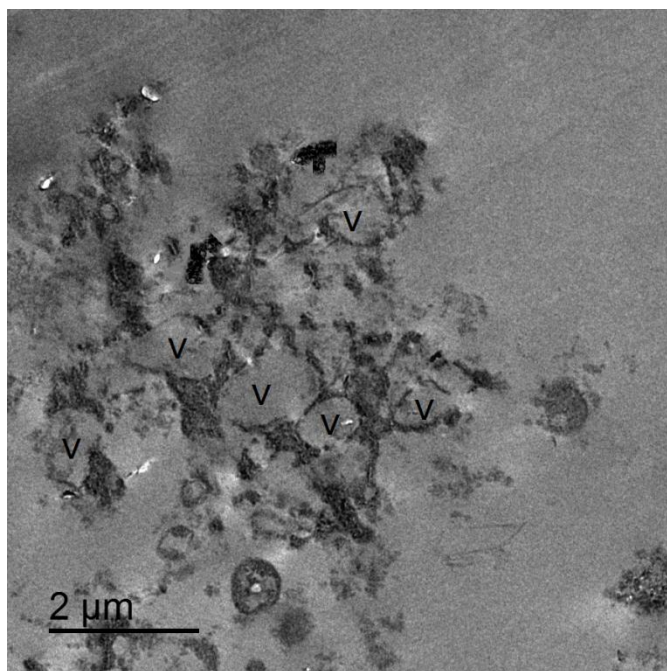


Figure 3: TEM of *T.vaginalis* trophozoite after 72 hrs incubation with NsO showing severe cell damage, nucleus and cytoplasm were severely destructed with large vacuolization and cell membrane defects.

DISCUSSION

Trichomoniasis is a cosmopolitan sexually transmitted disease caused by a protozoan parasite called *T.vaginalis*[2]. It can affect both male and female at reproductive age with

dangerous sequelae[3]. Metronidazole is the standard treatment for Trichomoniasis[28]. Unfortunately there is increased prevalence of metronidazole-resistant strains in addition to its side effects. This drew

the attention to find new antiprotozoal drugs of good effectiveness and low toxicity. So herbal medicine seems to be a good choice due to low side effects and low cost.

The present work is an attempt to evaluate the efficacy of *N.Sativa* oil in the treatment for *T.vaginalis* and compare its efficacy with metronidazole (traditionally used drug in treatment for the trichomoniasis). Various concentrations of *NsO* were tested on the culture of *T. vaginalis* in comparison to various concentrations of metronidazole at different follow up periods.

Dose–response and time course experiments showed that the degree of growth inhibition was dependent upon the concentration of the drug and incubation time. The current results proved that *N.sativa* oil (500µg/ml) after 24hrs was as efficient as the high concentration of metronidazole after 72hrs with the additional advantage of being natural products. Moreover *NsO* gave the highest effect at concentration of 1000µg/ml after 72hrs as it gave the highest mean count of dead *T.vaginalistrophozoites*.

These results agree with the study done by **Mahmoud et al [25]** which proved that *NsO* is a valuable agent in treating *T.vaginalis* infection and has higher anti *Trichomonas* activity over that of the alcoholic extract. **Aminou et al [29]** also proved a clear evidence of the toxic effect of *NsO* that could offer an effective, cheaper and safer alternative for metronidazole. They also reported that ultrastructural alterations in *T.vaginalistrophozoites* provide an evidence of the toxic effect of *NsO* and to a lesser extent *N.sativa* alcoholic extract.

This remarkable effect of *NsO* may be due to the fact that essential oils are mostly the active principles extracted from *N.sativa* seeds. Thirteen fatty acids were found in *N.sativa* fixed oil, which represented 94.49 % of the total fatty acids while eleven fatty acids were found in the alcoholic extract of *N.sativa* (represented 92.03 % of the total fatty acid) [25].

Fatty acids of *N.sativa* have the ability to interact with cell membranes of *T.vaginalis* and

create transient or permanent pores of variable size. These pores lead to leakage, reduction of nutrient uptake and inhibition of cellular respiration [25]. Another explanation might be that *N.sativa* affects the lipidic metabolic processes in the parasite. When *T.vaginalistrophozoites* are exposed to high fatty acids concentrations of *N.sativa* (mainly oleic acid and linoleic acid), these fatty acids are inhibited in the parasite. It is suggested that the antitrichomonal effects of *N.sativa* may be due to its power as anti-adhesion agent [30].

Purified proteins of *N.sativa* have immunomodulatory effect as they increase the ratio of helper to suppressor T cells. They also cause enhancement of natural killer cell activity [31]. The production of interleukin-3 by human lymphocytes is induced by *N.sativa*. Moreover it has stimulatory effect on macrophages [32]. *N.sativa* has the ability to minimize the immunopathological changes of different parasites as it enhances interleukin-1b and tumor necrosis factor- α production which induce production of reactive nitrogen intermediates including nitric oxide [33].

Regarding the results of TEM It was found that the fine structure of the untreated *T.vaginalistrophozoites* (control group) showed one nucleus, hydrogenosomes and few vacuoles. The cell membrane is obvious and intact (Fig. 1). These findings are in accordance with **Costamagna and Figueroa [34]** who studied the ultrastructure of *T.vaginalis* in liquid cultures. They found that glycogen granules, vacuoles and hydrogenosomes were easily distinguishable following a longitudinal ordering and the nucleus is prominent.

T.vaginalistrophozoites treated with Metronidazole after 72hrs were swollen compared to non-treated group and lost much of the material within cytoplasm. The remaining part of organelles (nucleus, some vesicles and few hydrogenosomes) showed centripetal displacement (Fig. 2). These results are in the same line with **Oxberry et al. [35]** who found that after usage of Metronidazole there were loss of the cytoplasmic material,

vacuolization and disruption of cytoplasmic membrane.

As for NsO treated culture, some *T.vaginalistrophozoites* showed completely destructed cell membrane (Fig. 3). These results agree with **Aminou et al. [29]** who proved the toxic effect of *N.sativa* oil and crude extract on *T.vaginalistrophozoites* through their ability to cause severe cell damage with cytoplasmic and nuclear destruction detected by TEM.

In conclusion, the results in the present study support that *N.sativa* oil may be a promising valuable agent as efficient as Metronidazole in the treatment of Trichomoniasis, and will form the basis for further complementary studies to fractionate and evaluate the efficacy of its bioactive components against *T.vaginalis*. Moreover more researches are recommended to evaluate and standardize the efficient doses of these natural herbs to be safe and efficient.

Conflict of interest: The authors declare that they have no conflict of interest.

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