

IN VIVO AND IN VITRO EFFICACY OF NIGELLA SATIVA AQUEOUS EXTRACT ON BLASTOCYSTIS HOMINIS

By

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Abstract

Metronidazole (MTZ) was the most widely accepted treatment for *Blastocystis hominis* (*B. hominis*) with high treatment failure rate, resistance and potential mutagenic and carcinogenic effects so there is urgent need to find out new, effective and safe treatment against *B. hominis*. The present research aimed to evaluate the therapeutic effect of the aqueous extract of *Nigella sativa* (*NS*) at different doses on *B. hominis* *in vitro* and *in vivo* in comparison to MTZ as a control drug. Isolates of *B. hominis* were obtained from patients complaining of diarrhea and abdominal pain. Isolates were cultured in egg diphasic medium (LE) for *in vitro* study and to adjust proper inoculating dose for *in vivo* study. The aqueous extract of *NS* at concentrations of 100 & 500µg/ml showed a potent lethal effect on *B. hominis* isolates *in vitro*. Caecal tissue of experimentally infected and treated mice with two different doses of *NS* (250 & 500mg/kg/d) were examined histopathologically and compared with that of mice infected and treated by two doses of MTZ (62 & 125 mg/kg/d) as control drug and Infected untreated mice as negative control group. Histopathological examination of infected untreated group showed all pathological degrees in the caecal tissue while infected treated one showed remission of pathological changes especially with higher dose (500mg/kg). Present study proved that *N. sativa* had inhibitory effect on *B. hominis* *in vitro* and prevented cytopathic effect in infected mice inoculated orally with *B. hominis*.

Key words: *B. hominis*, *Nigella sativa*, *in vivo*, *in vitro*

Introduction

Blastocystis hominis are single-celled protozoan parasites belonging to a group of organisms known as the Stramenopiles. It includes algae, diatoms, and water molds. *Blastocystis* comprises several species inhabit the gastrointestinal tracts of species as diverse as humans, birds, farm animals, rodents, amphibians, fish, reptiles, and cockroaches (Yoshikawa et al, 2007). It exhibits low host specificity, and a wide range of *Blastocystis* species (Noël et al, 2005). Fecal-oral transmission is the most accepted pathway, and late studies have demonstrated that transmission involves only the parasite cyst form (Yoshikawa et al, 2004). This parasite can, however, be the cause of different gastrointestinal symptoms including diarrhea/constipation, watery or loose bowel

motions or abdominal pain, weight loss and Flatus. Many people are asymptomatic. *Blastocystis* has a widespread geographic distribution and are found at a rate of 5-10% in most developed countries, and a rate of up to 50% in less developed areas (Boroom et al, 2008) probably due to poor sanitation (Graczyk et al, 2005). In Africa, prevalence of *B. hominis* in Saudia Arabia was 17.5% (Zaki et al, 1991). High rates of disease are also found in people in developed countries who work with animals (Parkar et al, 2010). There have been several hypotheses and increasing researches in the last few years relating the incidence of *Blastocystis* infections with the prevalence of irritable bowel syndrome in patients (Stark et al, 2007). Due to lack of know-ledge about this parasite and the high failure rates of eradication using-

metronidazole (Flagyl[®]), in treating *B. hominis* infection, as well as the reports show that metronidazole is not ideal for children because of its mutagenicity and carcinogenic potential (Lemee et al, 2000), this has led to our development of a new cysticidal drug for radical treatment, to avoid relapse and to prevent transmission. *Nigella sativa* is a herbaceous annual plant that belongs to the Family Ranunculacea, and is commonly known as black seed, black cumin or habitual Barakah (Jansen, 1981). The seeds have been documented for medical purposes, since thousands of years ago (El Wakil, 2007). Emergency recognition of *B. hominis* has created a need for more knowledge about effective chemotherapy (Zierdt et al, 1983). In immunocompetent patients, infection is self-limiting (Kain et al, 1987). However, treatment is necessary in patients with resistant symptoms (Hoeprich et al, 1994). The metronidazole (MTZ) produces apoptosis like features in growing cultures of *B. hominis* as programmed cell death (Nasirudeen et al, 2004). Although MTZ is the drug of choice for treatment of *B. hominis* infection, it has been found that isolates of *B. hominis* organisms of different geographical origin have different level of resistance to MTZ (Michel et al, 2011). The need for discovery of new drug is necessary so we evaluated the *in vivo* and *in vitro* effect of *N. sativa* on *B. hominis* compared to MTZ as standard drug.

Subjects, Material and Methods

Fecal specimens from 10 gastrointestinal symptomatic patients having *B. hominis* and negative for other organisms were selected from the attendance, diagnosed by Jizan hospital laboratory as *B. hominis* infection.

Hot maceration was performed using one kilogram of seed waste with distilled water (4L x 4, 4h, with continuous stirring), filtered and concentrated to dryness under reduced pressure at 40°C to afford the aqueous extract (160 g). The dried extract was kept in desiccators for 4 weeks, pulverized into free powder and kept in tightly closed glass con-

tainers until use. One hundred grams were extracted by ultrasonication with distilled water at room temperature for 30 minutes. The extract was filtered, cleared up by centrifugation, transferred into 500 ml volumetric flask and the volume adjusted with distilled water to give 200 mg extract/ml (stock solution) (Hareesh et al, 1999). Final stock solution of 60 mg/ml was prepared by dissolving 600 mg MTZ (Amriya Pharm. Ind. Alexandria, Egypt) in 10 ml distilled water (Michel et al, 2011). All drugs were inoculated orally daily using a feeding tube after 24 hours postinfection for five days. Stool elutes from all groups were examined daily for *B. hominis* (cyst and/or trophozoite).

In the present study, the culture of *B. hominis* was isolated from the diarrheic stool of a patient from Jizan Hospital, Saudi Arabia. Each positive stool sample for *B. hominis* was examined for other parasite by formalin Ethyl acetate (FEA) sedimentation method to exclude helminthes infection. Also, modified acid fast, trichrome and modified trichrome stains were used to exclude other protozoan infection (Garcia, 2001). The untreated fecal specimens were collected after diagnosis as *B. hominis* infection and cultured in Egg Diphasic medium (LE) supplemented with Horse serum (10%) and antibiotic mixture for axenization before inoculation (Zierdt et al, 1974). The culture tubes were incubated at 37°C in an anaerobic gas Jar for one week before use. For *in vitro* assessment of *Nigella sativa* aqueous extract and metronidazole effects on *B. hominis*: aqueous extract was dissolved in distilled water and sterilized by filtration using Acrodisc (Gelman, 0.22µm size).

Nigella sativa effect on *B. hominis* growth was assayed. Nine similar experiments were used. Each experiment includes 4 groups and each group represented by 3 cultures tubes; GI (negative control) included *B. hominis* inoculated free Egg Diphasic medium (LE; Sigma, St. Louis, Missouri, USA.); groups (II-IV) included *B. hominis* inoculated Egg Diphasic medium (LE) add-

ed with *N. sativa* and metronidazole with different concentrations (10µg/ml, 100µg/ml & 500 µg/ml). Initial inoculation for experiment was 4×10^6 *B. hominis* per ml, taken from one-week-old culture incubated at 37°C. After inoculation with *B. hominis* and incubation of culture tubes at 37°C with 5% CO₂, different concentration of NS and MTZ were added to different cultured tubes then examination were done on day 1, day 3 and day 6. The effect of *N. sativa* different concentrations on *B. hominis* was determined by counting of number of *B. hominis* isolates and MTZ by haemocytometer. The number of *B. hominis* in each group was determined from the representative 3 culture tubes/group and was used to calculate the main parasite counts and standard deviation for each group.

An experimental study: Mice were divided into 4 groups: GA, 6 mice sub-divided into GA nc; 3 uninfected untreated mice as negative control and GA pc; 3 infected untreated mice as positive control. GB, 8 mice infected and treated with MTZ as standard drug. They were subdivided into GBI given (62mg/kg/day) and GBII given (125mg/kg/day). GC composed of 8 mice were infected and treated with aqueous extracts of *N. sativa*. They were subdivided into GCI (4 mice) given (250 mg/kg/day), GCII (4 mice) given (500 mg/kg/day). Proper inoculating dose of *B. hominis* (0.5ml culture containing 4×10^6) was orally inoculated in 2- 4 weeks albino mice daily (Yoshikawa *et al*, 2004). After the first and second week post infection, mice were sacrificed. Cecum and ileum were collected from infected mice after confirmed diagnosis by stool elutes examination. Grasp the abdominal end of the esophagus and began to disembowel the gastrointestinal tract until the rectum was reached. Cecum was perfused with 150 PBS, and then fixed with 10% formalin as soon as possible. Cecum was cut up into smaller sections for fixative to penetrate mucosa, and submitted for embedding, processing and

sectioning. Dehydration for embedding tissues into paraffin wax was done with ascending of ethanol and xylene for 60 min. Tissues were put into paraffin wax for 60 min at 58°C for two consecutive changes and into embed molds and arranged into position (desired tissue down). Cassettes were placed onto cold plate until wax solidified and cut at 5µm thickness, and stained in Hematoxylin and Eosin.

Statistical analysis: Data were expressed as M±SD and were compared by Chi-square test. Chi square test was used for association between two qualitatively expressed relations. Significant = P <0.05 by SPSS, V. 16.

Ethical considerations: Informed consents were taken from patients. Mice were provided from the Faculty of Medicine, Jazan University, and they were housed (five/cage) in proper room temperature and were given drinking water and regular mouse feed ad libitum.

Results

Ten out of sixty examined patients (16.6%) were infected by *B. hominis* with variable symptoms (Tab. 1). *In vitro* study showed that count of *B. hominis* isolates decreased gradually in cultured tubes with the *N. sativa* ascending concentrations (Tab. 2, Fig 1). Metronidazole at 500µg/ml concentration was more effective *in vitro* than *N. sativa* as it inhibited growth of *B. hominis* isolates in cultured tubes after 1, 3 and 6 days respectively while *N. sativa* with the same concentration inhibited growth only on 6th day (Tab. 2, Fig 1).

For *In vivo* study, the cure rate of low dose of MTZ and *N. sativa* were 75% and 50% respectively but both was equal in higher dose; 100% cure rate for each (Tab. 3).

In a comparison between MTZ and *N. sativa* to treat *B. hominis*, *N. sativa* proved effective (100%) in mice with high dose (500mg /kg/day) while in low dose (250mg/kg/day) it decreased severity of infection with 50% cure rate (Tab. 3, Fig. 3).

Table 1: Complaints among 10/ 60 patients infected with *B. hominis*

Complaints	Number (n=10)	Percentage %
Diarrhea	5	50
Abdominal pain	4	40
Flatus	3	30
Vomiting	1	15
Nausea	2	10
Anorexia	2	10
Weight loss	1	5

Table 2: *B. hominis* \pm SD (10^5) in culture tubes after exposure to various concentrations of *N. sativa* and Metronidazole among groups.

Group	Day 1	Day 3	Day 6
I: - ve Control	8.1 \pm 3.56	25.3 \pm 5.03	45 \pm 32.32
II: <i>N. sativa</i> =10 μ g/ml	4.6 \pm 0.4	6.4 \pm 0.76 ^{a,c}	7.7 \pm 1.73 ^a
III: <i>N. sativa</i> =100 μ g/ml	2.4 \pm 0.53 ^a	0.12 \pm 0.01 ^{a,c}	0.4 \pm 0.2 ^a
IV: <i>N. sativa</i> =500 μ g/ml	1.8 \pm 0.61 ^a	0.3 \pm 0.21 ^{a,c}	0.00 ^a
II: MTZ=10 μ g/ml	6.5 \pm 3.04	3.1 \pm 1.01 ^a	0.00 ^a
III: MTZ=100 μ g/ml	1.3 \pm 0.75 ^a	0.01 \pm 0.01 ^{a,c}	0.00 ^a
IV: MTZ 500 μ g/ml	0.00 ^{a,b}	0.00 ^{a,c}	0.00 ^a
P value	0.0008*	0.00001*	0.0037*

*P value = significant, ^aP=significant vs. control, ^bP = significant vs. MTZ=10 μ g/ml, ^cP = significant vs. to *N. sativa*=10 μ g/ml

Table 3: Aqueous extracts of *N. sativa* on *B. hominis* infection in mice versus MTZ.

Drug	Dose (mg/kg/ day)	No. of mice cured/treated
Infected untreated GA	-	0/3 (0%)
MTZ GBI [†]	62	3/4 (75%)
GBII [†]	125	4/4 (100%)
<i>N. sativa</i> GCI	250	2/4 (50%)
GCI [†]	500	4/4 (100%)
P value		0.028*

* P value = significant, [†]P = significant vs. control.

Histopathological data: *In vivo* response was evaluated by microscopic examination of iliocecal tissues from the infected MTZ and *N. sativa* treated mice compared to infected untreated ones. Intestinal mucosa of unaffected controls revealed thin, folded mucosa of the cecum with short crypts of Lieberkühn and a lamina propria forms deep cavities. The epithelium contains absorptive enterocytes with apical microvilli and many oval, mucous goblet cells (Fig. 3; Anc). Cecal mucosa of *B. hominis* untreated infected mice (GA) showed moderate to severe sloughing of the intestinal villi with destruction of both muscularis interna and the muscularis externa with pronounced inflammatory cells infiltration (Fig. 3; Apc). The low dose metronidazole (62mg/kg/d) treated mice (GBI) showed mild inflammation with less inflammatory cells infiltration and dam-

age of the epithelial mucosa (Fig. 3; BI) in compared to MTZ high dose (125mg/kg/d) treated mice (GBII) which showed normal cecal mucosa more or less healthy, but associated with few inflammatory cells infiltration (Fig. 3; BII). On the other hand, cecal mucosa from low dose *N. sativa* (250 mg/kg/d) treated mice (CI) showed mild damage of cecal mucosa with mild infiltration with inflammatory cells (Fig. 3; C1) but high dose *N. sativa* (500 mg/kg/d) treated mice (GCI) exhibited a normal histological structures of the cecal mucosa with mild pathological alteration (Fig. 3; CII). Interpretation revealed that *B. hominis* caused destruction of cecal epithelium with sloughing of intestinal villi. Mild to moderate infection was recorded by evaluating the number of pathological alteration and numbers of the infected cells. In high magnification parasite

showed inflammatory reactions in all groups, significant decrease in *N. sativa* treated mice.

Discussion

There are controversies about *B. hominis* pathogenesis (Taamasri *et al*, 2002). It is asymptomatic in immune-competent persons but more pathogenic in immune-suppressed patients as AIDs and renal failure (Thathaisong *et al*, 2003). Prevalence of *B. hominis* in present study agreed with Zaki *et al*. (1991) who found that its prevalence in Saudi Arabia was 17.5%. The present study found that diarrhea was the commonest symptom in patients that agreed with Hegazy *et al*. (2008). There are trends for using plants in therapy as a result of side effects and complications of drugs. *N. sativa* aqueous are folk medicinal plants used for centuries to treat various illnesses. Regarding *N. sativa* therapeutic use, Holy Mohammad (pbuh) said "There exist, in the black seed grains, health and cure of all the diseases except death". Biological activities of *N. sativa* included antifungal, anti-bacterial, antiviral, anti-inflammatory, antioxidant, antihelminthic (Salem *et al*, 2000, Morsi, 2000; El Dakhakhny *et al*, 2000; Daoud *et al*, 2004) and preventive role against the toxic effects of Oxytetracycline (Abdel-Daim and Ghazy, 2015). Noor *et al*. (2015) stated that Multiple sclerosis (MS) is a major, immune-mediated and reported that *N. sativa* suppressed inflammation observed in experimental autoimmune encephalomyelitis (EAE)-induced rats. In addition, *N. sativa* enhanced remyelination in the cerebellum. Moreover, *N. sativa* reduced the expression of transforming growth factor beta 1 (TGF β 1). They concluded that *N. sativa* seeds could provide a promising agent effective in both the protection and treatment of EAE.

On the other hand, in experimental toxoplasmosis Mady *et al*. (2016) reported that although *N. sativa* oil, if administered alone, has significant immunostimulant and antioxidant properties, but it failed to decrease tachyzoite counts and that its combination of

NSO with pyrimethamine had synergistic effect in treatment of toxoplasmosis. The present study showed that *N. sativa* significantly inhibited *B. hominis* growth on 6th day with 500 μ g/ml. MTZ higher activity at certain concentration may be due to the fact that *N. sativa* was a crude extract compared to raise activity of purified MTZ. This agreed with El Wakil (2007) showed that *N. sativa* at 500 μ l/ml conc. had significant inhibitory effect against *in vitro* growth of two different *B. hominis* isolates. A part from *N. sativa*, Yang *et al*. (1996) of 20 crude extracts of traditional Chinese medicine on *B. hominis*, found that extracts of *Coptis chinensis* and *Brucea javanica* inhibit growth on 1st day as rapid growth occurred at first day. The same result was proved by Sawagjaroen and Sawangjaroen (2005). The present study showed therapeutic effect of *N. sativa* in treatment of *B. hominis* in experimentally infected mice as high dose produced complete remission of infection with 100% cure rate, which might be due to *N. sativa* inhibitory effect on *B. hominis* growth (El-Wakil, 2007). Besides, *N. sativa* proved effective against *Trichinella spiralis* infected rats infected with and increased the production of antibodies generated during life cycle of the parasite (Abu El-Ezz, 2005), *Echinococcus granulosus* (Kasim *et al*, 2012) and *Fasciola gigantica* (Shalaby *et al*, 2012). *N. sativa* seeds alcoholic extract gave antileishmanial activity (Bafghi *et al*, 2011). Tonkal (2009) showed a promising effect using wheat germ agglutinin and *N. sativa* aqueous extract in treating *Trichomonas vaginalis*, compared to MTZ (50 μ g/ml) after 24 hours whereas, lower doses of wheat germ agglutinin did not inhibit *T. vaginalis* growth. Both medications remarkably inhibited trophozoites motility. In the present study, cure rate of MTZ was 75% with lower dose (60mg/kg). El-Masry *et al*. (1993) found that Furazol gave a satisfactory eradication of *B. hominis* (cure rates 75%). In contrary to the present result, MTZ had no beneficial effect on parasites (Nigro *et al*, 2003).

Conclusion

No doubt, *Nigella sativa* possesses anticestode and antinematode actions and have effect on *B. hominis* in experimentally infected mice with high dose but in human, still need a prove by double blind controlled clinical trials to assess *N. sativa* as treatment of human *B. hominis*. *N. sativa* is of popular use as safe low cost spice and its use for *B. hominis* treatment is recommended

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Explanation of figures

Fig.1: Mean count in culture tubes post-exposure to various concentrations of *N. sativa* and Metronidazole in groups versus control.

Fig. 2a: *B. hominis* (vacuolar form) iodine stained smear (x400), Fig. 2b, c: from positive culture (x400)

Fig. 3: Effect of *N. sativa* aqueous extract on *B. hominis* vs. MTZ in experimentally infected mice.

A nc: Cecal mucosa showed normal histological characteristics of unaffected controls. A pc: Infected cecal mucosa showed moderate to severe sloughing of intestinal villi with destruction of musculatures (red arrow) infiltrated with inflammatory cells. B I: Normal histological structures of cecal mucosa with mild pathological alteration (black arrow). B II: Cured cecal mucosa. C I: Mice treated with low dose of *N. sativa* showed mild cecal mucosa damage with mild infiltration of inflammatory cells. C II: Mice treated with high dose of *N. sativa* showed normal cecal mucosa tended to be healthy, but associated with few inflammatory cells infiltration.

