A novel impact of *Boswellia serrata* on *Blastocystis* spp. infected mice

Original Article

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ABSTRACT

Background: The susceptibility of *Blastocystis* spp.to standard antimicrobials is not clear. The development of resistant strains against the recommended drugs has evoked the importance of using an alternative medicine. Metronidazole constitutes a mainstay and is considered the first line for treatment, yet, it is complicated with many drawbacks. The demand for finding alternatives introduced nitazoxanide (NTZ) and natural products to provide successful new regimens for treatment and to avoid resistant infections.

Objective: The present study was conducted to evaluate the anti-*Blastocystis* effects of *Boswellia serrata* compared to NTZ on experimentally infected mice.

Material and Methods: Three groups of BALB-c mice were used: untreated control group (G1); infected mice treated with NTZ (G2); infected mice treated with *B. serrata* (G3). Histopathological examination of colonic epithelium and immunohistochemical assessment was done for detection of TNF-α in the two groups of mice treated with *B. serrata* in comparison to those treated with NTZ.

Results: The test appraised the effect of *B. serrata* which succeeded in maintaining the intact surface epithelium and goblet cells. The mononuclear infiltrations were markedly decreased in the lamina propria and appeared as small aggregates at the base of the crypts. The submucosa showed marked reduction of inflammatory cells. Occasional intraepithelial lymphocytes were detected in-between epithelial cells. The negative Periodic Acid-Schiff (PAS) reaction detected in the intestinal crypts was comparable to NTZ treatment. Surpassing NTZ, *B. serrata*-treated group showed an apparently less positive reaction to TNF-α in the cells of the submucosa and lamina propria, while NTZ effect was restricted only to the submucosa.

Conclusion: This natural product can offer an alternative therapy for use instead of or concurrently with the conventional anti-*Blastocystis* treatment.

Keywords: *Blastocystis* spp., *Boswellia serrata*, histopathology, immunohistochemstry, *in vivo*, nitazoxanide, TNF-α.

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INTRODUCTION

Blastocystis spp. have been considered among the most common protists detected in human fecal samples globally[1]. Prevalence rates are higher in developing countries (63–100%)[2], than developed countries (0.5–24%)[3]. Its prevalence in Egypt reached 33%[4]. The risk of acquiring infection was associated with intra familial transmission, lack of piped water supply, poor maternal education and zoonotic transmission[2]. Blastocystosis can be asymptomatic or cause varying gastrointestinal symptoms[5], and the parasite may act as an opportunistic pathogen in immunocompromised patients[6]. *Blastocystis* spp. constitute an important cause of irritable bowel disease (IBD)[7,8]; and was reported in association with urticaria[9]. Transmission is by the feco-oral route[10]. An extensive genetic diversity of 17 subtypes of *Blastocystis* has been identified[11].

Blastocystosis damages the intestinal epithelium resulting in increased permeability by inducing apoptosis[12], and degrading the tight junction proteins (TJP)[13]. It modulates the immune response in intestinal epithelial cells[14], and has the ability to induce an *in vivo* pro-inflammatory response with production of IL-8 and GM-CSF by human colonic epithelial cells[15], up regulation of IFNγ, IL-12 and TNF-α mRNA[16], with the presence of inflammatory infiltrates in the sub-mucosa[17]. Lysates of subtype 7 resulted in an *in vivo* up

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regulation of IL1β, TNF-α and IL6 in intestinal explants and macrophages[18].

The diagnosis is currently based on microscopic detection in direct smears or on molecular identification[19]. It is generally acceptable that treatment is needed when debilitating symptoms are present with presence of several cysts in stool specimens and with exclusion of other clear causes[20]. Metronidazole (MTZ) was found to be an effective therapy yet, not in all situations[21]. It showed many side effects; nausea, abdominal pain, and diarrhea[22]. Serious neurotoxicity, optic and peripheral neuropathy, and encephalopathy have also been reported. Researchers proposed that the union of MTZ and its metabolites to RNA provokes the inhibition of protein synthesis and axonal degeneration of nerve fibers[23]. Cerebellar dysfunction, visual impairment, vestibule and cochlea toxicity, ataxic gait, dysarthria, and seizures also have been documented[24]. Rossignol *et al*. suggested that *Blastocystis* can be treated effectively with NTZ, which was equally effective in both children and adults[25]; moreover, the dose and duration of NTZ were much lower than with MTZ. Thus, still the susceptibility to standard antimicrobials is not clear[26]. Also, the development of resistant strains against the recommended drugs has evoked the importance of using an alternative medicine[27].

On the other hand, *B. serrata* is an oleo-gum resin used as a traditional remedy for inflammatory diseases. Its antioxidant/anti-inflammatory properties have been studied for the pharmacological potential in arthritis, asthma, colitis and cancer[28,29]. The phytochemical content of *B. serrata* is dependent on its botanical origin including 30–60% triterpenes (such as α- and β-boswellic acids, lupeolic acid), 5–10% essential oils, and polysaccharides. The 11-keto-β-boswellic acid (KBA) and acetyl-11-KBA have been considered the main active derivatives[30]. Its effect on immune system was documented through decreased cytokines (interleukins and TNF-α) and diminished complement system and leukocyte elastase activities. Additionally, β-boswellic acids have been suggested as anti-inflammatory, acting through inhibition of serine protease cathepsin G and microsomal prostaglandin E synthase inhibition of 5-lipoxygenase (5-LO)[30], and reduction of ROS formation and P-selectin-mediated recruitment of inflammatory cells[31]. Furthermore, clinical studies suggested that *B. serrata* resin could be effective in IBD[32] as it preserves the intestinal epithelial barrier from oxidative and inflammatory damage[33]. Its acids have been confirmed to regulate inflammation and immune responses[30]. Likewise, it attenuated pulmonary and colonic fibrosis in rats[34], thus proving its value for the treatment of fibrosis associated with diverse clinical diseases. Its water-soluble acids significantly attenuated *S. japonicum* egg-induced granuloma and significantly improved the hepatic gross appearance[35]. Also, oral *B. serrata* extract provided a useful anti-cancer agent with significantly lower toxicity on normal liver tissue[36].

Consequently, the present study was conducted to evaluate the therapeutic effect of *B. serrata* compared to NTZ on experimentally infected mice with *Blastocystis* spp. *via* histopathological evidence and immunohistochemical assessment for TNF-α.

MATERIAL AND METHODS

This case control experimental study was carried out during the period from June 2018 to January 2019 in the Parasitology Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt.

Culture and isolation of *Blastocystis* spp.: Stool samples were collected from patients attending the Parasitological Research and Diagnostic Laboratory Unit, Faculty of Medicine, Ain Shams University, complaining of GIT manifestations. The samples were immediately examined for intestinal parasites by a wet smear stained with Lugol's iodine and followed by formalin ethyl acetate concentration technique. Positive stool samples for *Blastocystis* spp. were anaerobically cultured in a biphasic medium of inspissated whole egg slants overlaid with Locke's medium (LE) and 25% heat-inactivated horse serum (ATCC medium 1671) and incubated at 35°C[37].

Preparation of *B. serrata*: The dried oleo-gum-resin of *B. serrata* (family Burseraceae) was taxonomically authenticated and prepared[38]. Briefly, the resin was first dissolved in ether and evaporated to dryness under vacuum. A supplement of 1% of the *B. serrata* powdered resin was mixed with water and apple juice to improve the taste then given to mice.

Experimental animals: Eight-week-old male BALB/c mice weighing 20–25 g were obtained from the experimental house, Faculty of Medicine, Ain Shams University. The animals were freely fed by standard rodent chow and water.

Experimental design: The animals were divided into 3 groups, six mice each: infected untreated control group (G1); infected mice treated with NTZ (G2); and infected mice treated with *B. serrata* (G3). All the animals were infected with 500 µl LE medium containing 2×106 *Blastocystis* spp. To warrant that each mouse in the study was infected, fresh stool samples were collected from each mouse starting on 4th day post infection (PI). Infected mice in G2 were treated with 500 mg of NTZ[39], and G3 were administered *B. serrata*. The treatments were given twice daily for three consecutive days[40]. *B. serrata* and NTZ were administered to the mice intragastrically with a syringe fitted with a cannula needle to prevent tissue damage[27].

Cysts were counted in at least three fields with the average no./HPF documented. The cyst shedding ranged from 10 to 15/HPF. The bedding in the cages was cleaned daily to avoid re-infection[41].

Histological examination: All animals were sacrificed one day after the treatment regimen was completed. Segments of colon were freshly prepared, fixed in 10% neutral buffered formalin, and embedded in paraffin. The paraffin sections were cut and stained with hematoxylin and eosin[42].

Immunohistochemical studies: Immunohistochemical staining was performed on 4 µm, formalin-fixed, paraffin-embedded intestinal sections. Followed by sections incubation with primary antibodies (Anti-TNF -rabbit polyclonal IgG, 100 µg/ml, 1:50 dilution, cat. no. sc-130220; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4°C. The primary antibody was identified by avidin-biotin peroxidase detection solution (Dakocytomation labelled streptavidin biotin reagent; Dakocytomation, Glostrop, Denmark and System-horse radish peroxidase; Dako, Glostrup, Denmark) and the signal was visualized using diaminobenzidine (Dakocytomation) and Substrate Chromogen-System (Dako). Slides were counterstained with Harris's haematoxylin, dehydrated, cleared and mounted. Positive and negative control sections were used for each assay[43].

Ethical consideration: Written permissions were taken from patients to use their stool samples. All experimental procedures were conducted according to the ethical standards approved by the Institutional Animal Ethics Committee guidelines for animal care and use, Ain Shams University, Cairo, Egypt under registration number FWA 00006644.

RESULTS

Appraisal of the effect of *B. serrata* (G3) showed that it succeeded in maintaining the intestinal surface epithelium and goblet cells intact (Fig. 2-B). The mononuclear infiltrations were markedly decreased in the lamina propria and appeared as small aggregates at the base of the crypts. The submucosa showed marked reduction of inflammatory cells. Occasional intraepithelial lymphocytes were detected in-between epithelial cells. The negative (PAS) reaction detected in the intestinal crypts of G3 was comparable to NTZ treatment. Nitazoxanide-treated group (G2) shows a decrease in the positive reaction to TNF-α in the cells of the submucosa and increase in the lamina propria. Surpassing, NTZ, *B. serrata* treated group shows an apparently less positive reaction to TNF-α in the cells of the submucosa and lamina propria. (Fig.4-B,C).

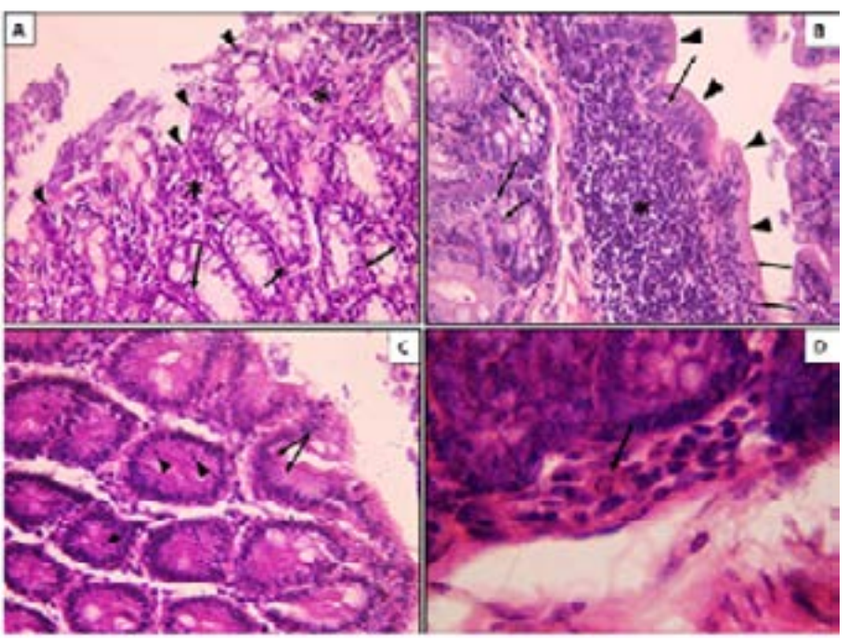

Fig 1. A photomicrograph of colon section from infected control (G1):
(A): Sloughing of surface intestinal epithelium (▲), infiltration of the lamina propria in between the crypts by mononuclear inflammatory cells. Intraepithelial lymphocytes are seen in between epithelial cells. Notice the presence of a giant macrophage.
(B): Loss of crypts on one side of an intestinal fold. The crypts are replaced by heavy mononuclear inflammatory cells covered by intact epithelium (▲). The surface epithelium on this side of the fold shows intraepithelial lymphocytic infiltration, but is lacking goblet cells. On the other side of the fold, transverse sections of bases of crypts are showing intraepithelial lymphocytic infiltration in between the cells.
(C): Transverse sections of crypts illustrating a remarkable decrease in goblet cells and intraepithelial infiltration by lymphocytes (▲). Notice the presence of two *Blastocystis* vacuoles invading the surface epithelium). A, B and C: H&E x400.
(D): *Blastocystis* invading the lamina propria of the colon just beneath bases of crypts (H&E x1000).

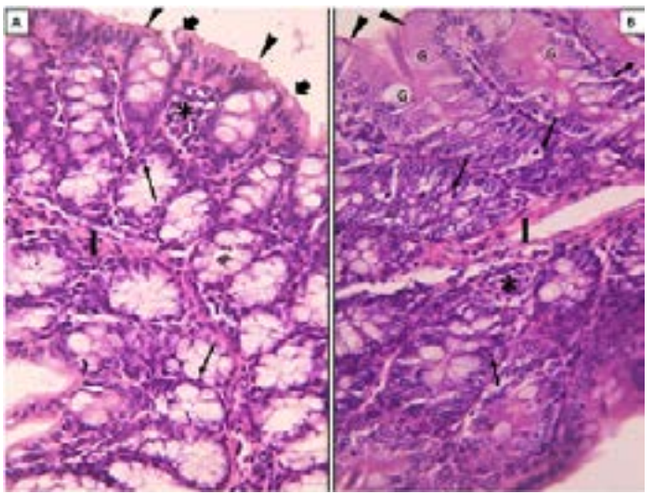

Fig 2. A photomicrograph of colon section from:
(A) NTZ-treated group (G2) showing intact surface epithelium (▲) with few goblet cells (thick arrow). Mononuclear infiltrations are apparently decreased in the lamina propria and are confined to the subepithelial areas. The submucosa in the core of the intestinal fold shows moderate mononuclear infiltration (l). Few intraepithelial lymphocytes are still detected.
(B) *B. serrata*-treated group (G3) showing intact surface epithelium (▲) with goblet cells (G). Mononuclear infiltrations are markedly decreased in the lamina propria and appear as small aggregates at the base of the crypts. The submucosa shows marked reduction of inflammatory cells (l). Occasional intraepithelial lymphocytes are detected in between epithelial cells (H&E x400).

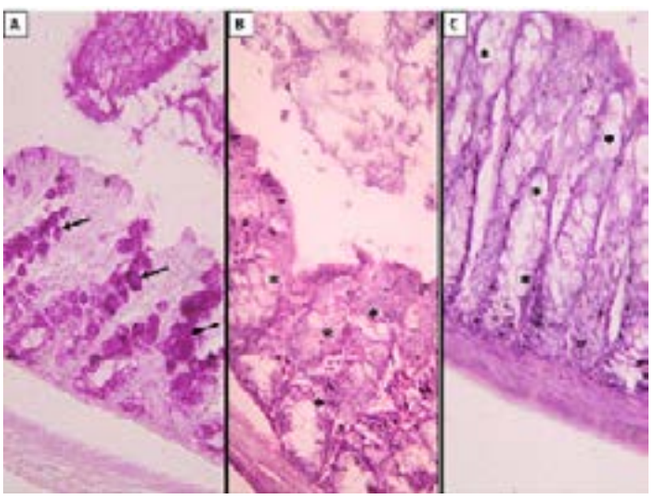

Fig. 3. A photomicrograph of a colon section of **(A)** Infected control (G1) showing numerous PAS stained mucin in goblet cells lining intestinal crypts. **(B) and (C)**; NTZ and *Boswellia* (G2, G3)-treated groups respectively showing negative PAS reaction in the intestinal crypts (□) (PAS x400).

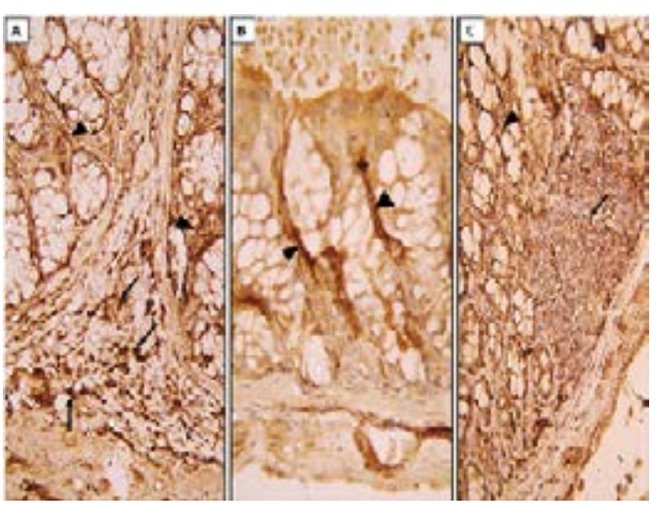

Fig. 4. A photomicrograph of a colon section of reflecting reaction to TNF-α. **(A):** Infected control (G1) showing numerous inflammatory cells demonstrating positive reaction in the submucosa of an intestinal fold (□). Also, in the lamina propria there is an increase in the expression of the immune stain (▲). **(B):** NTZ-treated group (G2) showing an apparent decrease in the positive reaction in the cells of the submucosa. However, the lamina propria shows an increase in the positive reaction (▲). **(C):** *Boswillia*-treated group (G3) showing apparently less positive reaction in the cells of the submucosa (□) and lamina propria (▲) (Immunohistochemical stain x400).

DISCUSSION

The association of *Blastocystis* spp. with human disease is still controversial[44]. While MTZ is the recommended chemotherapeutic drug for the treatment of human blastocystosis[21], a low dose of NTZ was reported to be more potent even for a shorter period of therapy[25]. However, development of drug resistance of some strains of *Blastocystis* has increased over the last few years. Thus, the use of natural agents has become more advantageous. Hence, our aim was to evaluate the anti- *Blastocystis* effect of *B. serrata in vivo*. In the infection control group (G1), results showed extensive sloughing with increased inflammation and decreased goblet cells reaction was revealed for TNF-α all over the colonic mucosa (Fig.1; Fig.3-A; Fig.4-A). Parallel studies of histological examination of the infection control group revealed only a slight increase in goblet cells in the cecal mucosa PI. Also, significant up-regulation of markers including TNF-α was demonstrated in the cecal mucosa 2 weeks PI. The induction of local host responses suggested that *Blastocystis* can elicit pro-inflammatory as well as protective responses in local tissues[16]. TNF-α expression by peripheral blood mononuclear cells and in rectal mucosa in diarrhea-predominant IBD with *Blastocystis* was similarly documented[45].

Likewise, previous histopathological studies in a mouse mode showed that *Blastocystis* localizes in the lumen or on the mucosal edge of the caecum and colon along with deposits of mucin[17]. In rats it resulted in chronic infections over several weeks[46,47], and remained luminal leading to an increase in neutral mucin containing goblet cells in the colon[16]. In naturally infected pigs, parasites were seen luminal and on the mucosal surface in association with fecal matter and mucus[48,49].

Our study reflected promising results for *B. serrata*, which succeeded in keeping the surface epithelium and goblet cells intact. In addition, the mononuclear infiltrations were markedly decreased in the lamina appearing as small aggregates at the base of the crypts, while the submucosa showed marked reduction of inflammatory cells. Occasional intraepithelial lymphocytes were detected in between epithelial cells. (Fig. 2-B). Negative PAS reaction in the intestinal crypts was detected which was comparable to NTZ (Fig 3- C). Surpassing NTZ, the *B. serrata*-treated group, showed less positive reaction to TNF-α in the cells of the submucosa and lamina propria while NTZ effect was restricted only to the submucosa, (Fig. 4-C).

It was proved that pro-inflammatory cytokines and reactive oxygen species (ROS) contribute to the initiation and/or propagation of damage within the mucosal intestinal barrier in IBD[50, 51]. Interestingly, and analogous to our study these alterations were significantly prevented by pre-treatment with *B. serrata*[52]. Furthermore, tight junction (TJ) proteins which were reportedly essential to maintain physiologic function of intestinal barrier[53], can be affected by various stimuli, including pathogens, oxidative stress, and pro-inflammatory cytokines[54, 55] including TNF-α in IBD[56–58]. *B. serrata* was reported to efficaciously prevent that damage[52]. Likewise, *B. serrata* was an effective agent against acute experimental ulcerative colitis experiments attributed to reduced lipid peroxidation and ROS[52, 59]. Herein, we showed that *B. serrata* prominently decreases the reactive TNF-α in the whole intestine unlike that in the infected non treated control or even NTZ treated control. Therefore, this study established the successful effect of *B. serrata* as anti-*Blastocystis* therapy, providing hope for the re-establishment of intestinal barrier function. Further investigations are needed to highlight its exact mechanism of action on the selective species.

Author's contribution: Hetta MH, prepared the drug and adjusted the doses; Sarhan RM, Saad GA and EzzEldin HM collected and cultured the stool samples and performed the *in vivo*, study, wrote and revised the manuscript. Baher W performed and evaluated the histopathology and the immunohistochemistry.

Conflict of interest: Authors confirm that there are no known conflicts of interest associated with this study.

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