

Isolation and Treatment of Blastocystis sp. with Natural Products

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ABSTRACT

Blastocystis sp. is a mysterious, polymorphic anaerobic single-celled protozoan that can reside in the intestinal tract of humans and various animals. Metronidazole is the most widely accepted treatment for Blastocystis; however, several cases of treatment failure and resistant strains have been reported. In recent years, numerous studies have been conducted to discover new treatments for Blastocystis infections, with a special focus on natural products. In vitro, incubation of the parasite with *Artemisia annua* (200, 400 µg/mL), Chitosan nanoparticles (20, 50 µg/mL), and metronidazole (100 µg/mL). Viable parasites were counted after 24, 48, and 72 hours of incubation. Chitosan nanoparticles loaded with *Artemisia annua* demonstrated a statistically significant ($p < 0.05$) reduction in the growth of Blastocystis sp. in culture, 98.3% after 72 hours of incubation. Thus, the present research represents the first in vitro evaluation of the potential of using chitosan nanoparticles in combination with *Artemisia annua* against Blastocystis sp, compared to single treatment. The results demonstrated that the synergistic action of these compounds has potential therapeutic effects against Blastocystis sp., suggesting they could serve as effective and safe natural alternatives.

Keywords: Blastocystis, Artemisia, in Vitro, Chitosan Nanoparticles, Metronidazole

INTRODUCTION

Blastocystis is a common parasite that inhabits the gastrointestinal tract of many different organisms. This disease is widespread worldwide, with a higher incidence in developing countries due to the lack of sanitation and hygienic facilities [1]. It is transmitted through the fecal-oral route and is associated with contaminated food, water, and exposure to infected animals [2]. Their morphological forms have been recognized: Granular, cyst, ameboid, vacuolar, and multivacuolar, the cyst serving as the infectious stage. After ingestion, the cyst undergoes excretion in the host's digestive tract, releasing vacuolated forms capable of binary fission. These forms later encyst in the intestinal lumen, producing new cysts excreted in the feces [3]. Seventeen distinct subtypes (STs) of the parasite have been identified using the molecular analysis of the small subunit rRNA (SSU-rRNA) gene [4]. However, the effect of *Blastocystis* also varies depending on the subtype causing the infection. Thus, the question of whether they are pathogenic or commensal involves extensive genetic diversity [5]. The pathogenic role of *Blastocystis* in causing disease in humans remains dialectical. Some scientists debate the extent of the parasite's harm to humans because of its association with asymptomatic carriers [6]. Others support the view that the parasite may play a potential pathogenic role in symptomatic cases [7]. *Blastocystis* infection; in addition, loss of appetite, weight loss, fatigue, fever, and depression are among the other symptoms of the disease, especially among infected children [8, 9]. Today, *Blastocystis* sp. is recognized as a potential etiological agent of chronic diarrhea, particularly in immunocompromised individuals [10], patients with colorectal cancer [11], and those with autoimmune diseases such as ulcerative colitis [12]. Recent research has demonstrated a link between Blastocystis sp. and symptoms of irritable bowel syndrome. A study by Ibrahim et al. [13] and Nahhas [14] reported a significantly higher prevalence of Blastocystis sp. in patients with irritable bowel syndrome. However, recent studies on animal models suggest that even asymptomatic Blastocystis sp. infections can induce histopathological changes in the intestine of hosts [15]. Patients infected with *Blastocystis* sp. who experience gastrointestinal symptoms are typically

treated with antibiotics, such as metronidazole (MTZ). There have been accumulating reports of treatment failure. Furthermore, MTZ has many side effects, including headache, vertigo, nausea, and a metallic taste in the mouth. In addition, pancreatitis, central nervous system toxicity at high doses, and transient reversible neutropenia have been attributed to MTZ [16]. The discovery of new, safe, effective, and affordable medicines is essential. Herbal medicine has benefits, including being safer, healthier, and less expensive. *Artemisia* is one of the largest genera within the Asteraceae family. It comprises approximately 500 species distributed worldwide, and its plants are especially abundant in tropical and subtropical regions known for their aromatic characteristics [17]. The species within this genus are well recognized for their bitter taste, attributed to the terpenoids and sesquiterpene lactones they contain, which possess antibacterial, antifungal, antitumor, and insecticidal properties [18], as well as anti-parasitic properties [19]. Several terpenoid compounds, polymethoxylated flavonoids, coumarins, acetylenes, and sterols have been isolated from *Artemisia* species [20]. *Artemisia annua*, commonly known as sweet wormwood, has been widely studied for its broad-spectrum antiparasitic properties. Recent studies have highlighted its inhibitory effects on *Blastocystis* sp., an anaerobic intestinal parasite associated with gastrointestinal disturbances [21]. Nanoparticles are a promising drug delivery method that has proven to be a new hopeful therapeutic agent with a crucial role in the treatment of many parasitic diseases [22]. For the past few decades, chitosan from exoskeletons of crustaceans, such as shrimps, crabs, and squids, has been the material of choice for the preparation of NPs in various applications in medical and health fields due to its biodegradable and nontoxic properties, reducing side effects, improving bioavailability, and potentially overcoming drug resistance [23]. Where chitosan nanoparticles (CS NPs) are used for oral drug delivery, anti-parasitic agents are employed [24]. Many studies have been completed on chitosan nanoparticles in the treatment of cryptosporidiosis [25], amoebiasis [26]. Noo and Hanaa reported that CS NPs are effective in treating giardiasis [27]. Some researchers

suggest that antibiotics used to treat *Blastocystis* sp. may be effective, but adding certain natural products to the diet can also help to eliminate the parasite. Therefore, the study aimed to analyze the effect of two natural products, chitosan nanoparticles and *Artemisia annua* (*A. annua*), at different concentrations on the viability of *Blastocystis* sp. cells.

MATERIAL AND METHODS

Sample Stool Collection

Ten fresh stool samples were collected from both sexes of children aged 1- 12 years old suffering from various colonic manifestations like diarrhea and abdominal pain, recruited at AL-Kadhimiya Hospital for Children in Baghdad. Fresh samples were collected in clean, dry, labeled containers and transferred to the Parasitology Laboratory. There was no history of antibiotic administration in the patients. Stool specimens were examined for intestinal parasites immediately after collection by wet smear preparation in saline and staining with Lugol iodine solution. All samples were then processed using the formalin/ethyl acetate concentration technique. Specimens positive for *Blastocystis* sp. and negative for other parasites were selected for the *in vitro* cultivation.

Drugs

Metronidazole Preparation

Sanofi Ventes in France manufactures and purchases metronidazole (Flagyl), an antiprotozoal drug used. The stock solution was at a concentration of 1 mg/ml and stored in a dark bottle at 4°C. Finally, metronidazole was prepared at a concentration of 100 µg /mL [28].

Preparing Chitosan Nanoparticles

The synthesis was performed according to the method of Ohya et al. [27], using the Sol-Gel chemical method with some modifications. The size of these particles is 40 to 60 nm.

Preparation of Artemisia Powder

Artemisia annua (sweet wormwood) aerial parts: 650 mg per capsule, manufactured by Jiva Botanicals - USA. The capsule powder was dissolved in sterile distilled water to obtain a final solution of 1 mg/ml. Final concentrations were adjusted to 200 and 400 µg/ml.

Cultivation of *Blastocystis* sp. In Vitro

25µg/ml taken from fecal samples with *Blastocystis* cultured using the modified Jones' medium culture method without starch, and horse serum was replaced with sheep serum. After planting, 100

I.U./mL penicillin and 100 µg/mL streptomycin were added to prevent microbial contamination, and the mixture was incubated at 37°C for 48 – 72 hours. The culture results were then read after 48 hours using Lugol's iodine solution stain and examined with a light microscope at 400x [28]. Cultures were considered suitable for drug evaluation when the number of *Blastocystis* /ml exceeded 1×10^4 vacuolar forms.

Experimental Design

To study parasite growth, the vacuolar form of *Blastocystis* cells, 1×10^4 /ml from cultures, was introduced into each set of culture tubes containing modified Jones medium at different concentrations of *A. annua*, CS NPs, (*A. annua* + CS NPs), MTZ, and non-treated control (NTC) and negative control (culture media only).

Parasites were challenged with a graded concentration of *A. annua* (200, 400µg/mL), CS NPs (20,50 µg/mL), *A. annua* + CS NPs (200 µg/mL and 20 µg/mL; 50 µg/mL and 400 µg/mL), and MTZ 100 µg /mL. The tested cultures were incubated at 37°C.

Drug Susceptibility Assays

To assess the biological activities of natural products, the vacuolate form of *Blastocystis* multiplication was harvested, then counted at 24, 48, and 72 hours using a Neubauer cell counting chamber and screened using Lugol's iodine solution, which stained viable cells, by light microscopy to determine the minimal inhibitory concentrations of *A. annua*, CS NPs, (*A. annua* + CS NPs), and MTZ compared to a non-treated control [28].

The percentage of parasite reduction was calculated according to the growth inhibition equation:

$$\text{Growth inhibition \%} = \frac{\text{Infected non-treated} - \text{Infected treated}}{\text{Infected non-treated}} \times 100$$

Analytical Statistics

In this study, Data were expressed as mean values $\pm$ SD by the statistical software SPSS (version 19). A t-test was used to assess the statistical significance of means. A significant difference was $P < 0.05$, and $p < 0.001$ was considered a highly significant difference.

Ethical consideration

Ethical clearance for this study was secured from the Ministry of Health in Baghdad. Before including young children in this study, their consent was obtained.

Results

Details of these results are illustrated in Table 1 and Figures 1 and figure 2.

Table 1: *Blastocystis* sp. Mean Counts ($\times 10^4$) and Inhibition% Incubated with Graded Concentrations of Natural Products

Dosage of Treatment	24h Mean $\pm$ SD	24h % Growth Inhibition	48h Mean $\pm$ SD	48h % Growth Inhibition	72h Mean $\pm$ SD	72h % Growth Inhibition
NTC	37.3 $\pm$ 3.7	0.0	33.6 $\pm$ 2.8	0.0	30.0 $\pm$ 1.2	0.0
MTZ (100 µg/mL)	15.5 $\pm$ 1.8	65.5	11.6 $\pm$ 0.8	69.8	8.2 $\pm$ 1.0	74.0
<i>A. annua</i> (200 µg/mL)	9.8 $\pm$ 1.2	72.7	8.7 $\pm$ 0.6	75.5	8.3 $\pm$ 0.2	79.0
<i>A. annua</i> (400 µg/mL)	7.5 $\pm$ 0.3	83.6	5.9 $\pm$ 0.8	85.4	4.1 $\pm$ 0.7	89.2
CS NPs (20 µg/mL)	14.3 $\pm$ 1.4	66.7	12.4 $\pm$ 1.2	67.0	11.9 $\pm$ 0.3	70.1
CS NPs (50 µg/mL)	4.7 $\pm$ 0.1	91.2	4.2 $\pm$ 0.8	92.8	3.1 $\pm$ 0.8	93.0
<i>A. annua</i> + CS NPs (200, 20 µg/mL)	10.5 $\pm$ 1.7	78.1	7.9 $\pm$ 1.4	79.2	6.6 $\pm$ 1.2	79.8
<i>A. annua</i> + CS NPs (400, 50 µg/mL)	2.9 $\pm$ 0.3	95.0	2.5 $\pm$ 0.1	96.7	1.8 $\pm$ 1.4	98.3

Data are Expressed as Mean $\pm$ SD. Statistically Significant =* $P < 0.05$, and $P < 0.001$ =Highly Statistically Significant Difference in Comparison with NTC.

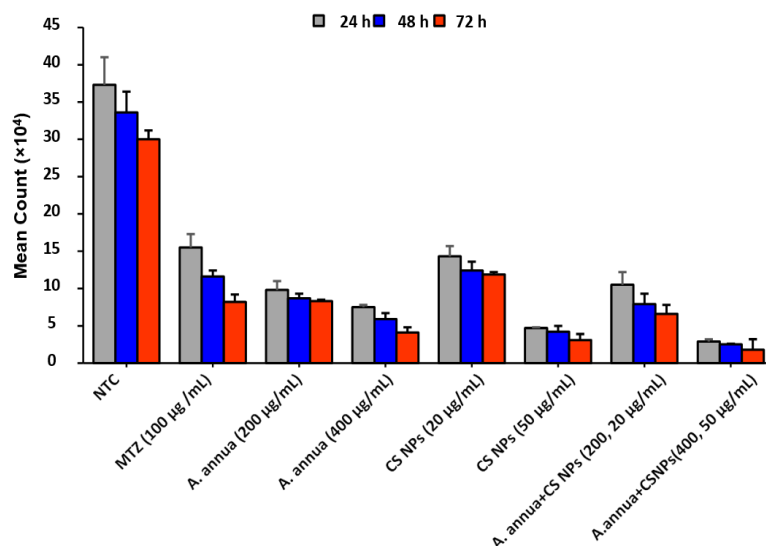


Figure 1: Mean Count of Blastocystis sp. ($\times 10^4$) in Cultures Challenged with Graded Concentrations of Natural Compounds & MTZ Compared to Non-Treated Culture (NTC)

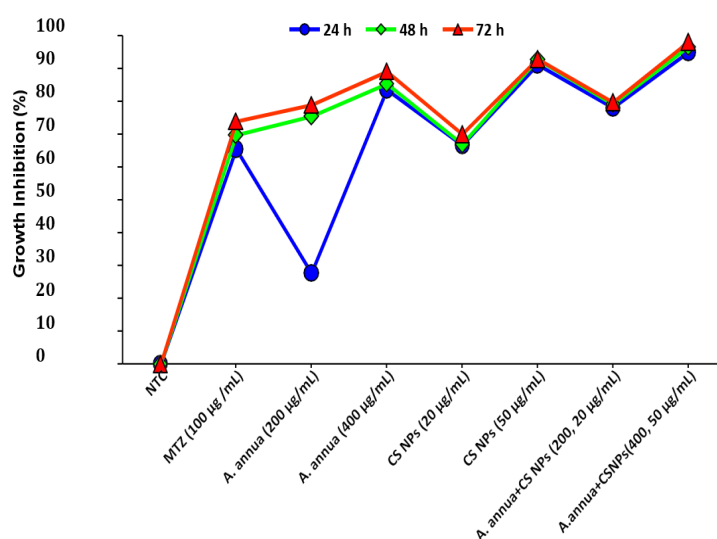


Figure 2: Growth Inhibition rate of Blastocystis sp. in the Presence of Different Concentrations of Natural Products

Discussion

The increasing incidence of *Blastocystis* resistance has become a problem, particularly to the most common anti-protozoal drugs, including metronidazole. Moreover, this drug can cause undesirable changes in the gut microbiota with various potential side effects, including embryotoxic, carcinogenic, and teratogenic effects [28]. Recent studies have explored the potential of natural compounds and medicinal plants as alternative therapies for *Blastocystis* sp. infections. Chitosan nanoparticles are a novel treatment currently under study. They have been investigated as antibacterial, antifungal, antiparasitic, and immune system stimulants, with promising outcomes [29]. To our knowledge, the *in vitro* activity of *Artemisia annua*-loaded CSNPs as an alternative method for treating *Blastocystis* has been researched for the first time. In the current study, the non-treated control group (NTC) exhibits a significant increase (37.3 ± 3.7 , 33.6 ± 2.8) after 24 and 48 hours. A slight decrease (30.0 ± 1.2) occurred after 72 hours, which might indicate a potential limit to growth or environmental changes affecting viability. The method for growing untreated cultures

has been reported by [30]. MNZ has been used as a standard anti-*Blastocystis* drug; The growth inhibition efficacy of 100 µg/ml was found to be statistically significant only after 72 hours, at 74% (Table 1; Fig. 1). This result is in agreement with the study conducted by [31]. This study suggested that *A. annua* has a significant effect on *Blastocystis* growth compared to MTZ. The inhibition of parasite multiplication was observed in proportion to the drug concentration and incubation duration (Fig. 2). The active concentration of *A. annua* that inhibited 89.2% of *Blastocystis* sp. growth was 400 µg/ml. Other protozoa have also been treated with *A. annua*, and its derivatives have been effective against *Cryptosporidium parvum*, *Giardia lamblia* [32], as well as *Leishmania* spp. [33]. Several studies have shown that both *in vitro* and *in vivo* assays have demonstrated that extracts of the aerial parts of *A. annua* can reduce the viability and growth of *Blastocystis*, suggesting a potential therapeutic role. The active components, sesquiterpene lactone artemisinin and its derivatives, are the primary bioactive compounds. These compounds exhibit potent antiparasitic activity by generating reactive oxygen species that damage proteins and membranes, and are widely recognized for their

proven efficacy against malaria [34]. *Artemisia annua* also contains other secondary metabolites, such as flavonoids, terpenoids, and essential oils, which contribute synergistically to its therapeutic effects [33]. In the present study, a significantly inhibitory effect of CS NPs on cultured *Blastocystis* parasites was observed at doses and exposure times. The high growth inhibition rate of 93.0% of chitosan nanoparticles at a concentration of 50 µg/ml for 72 hours outperforms MTZ by 74.0% over the same period. However, this is consistent with Sekar and Shanthi [30], who found that a significant reduction in the mean number of parasites occurred when 50 µg/ml of chitosan nanoparticles was used for 72 hours compared to MTZ for the same duration. Similarly, the study of Morua et al. [35] suggested that Cs NPs were more effective against *Blastocystis* than metronidazole. Additionally, a survey by [36] researchers found a relationship between the increase in the concentration of chitosan and chitosan nanoparticles and exposure time, as well as an increase in mortality rates. Chitosan nanoparticles possess several physicochemical characteristics that enhance their effectiveness against microbial agents. One of these is their mucoadhesive properties, which facilitate the opening of tight junctions, thereby improving permeability and cellular uptake [37]. In addition, Cs NPs exhibit antimetabolite activity, as they can inhibit membrane and nucleic acid synthesis due

to their ability to permeate the cell nucleus, bind to DNA, and subsequently inhibit mRNA synthesis and DNA transcription, ultimately leading to cell rupture and leakage of intracellular components [38]. In our recent work, the synergistic application of *A. annua* and Cs NPs (400, 50 µg/ml) has enhanced antiparasitic activity *in vitro*, with marked inhibition in parasitic viability (98.3%) observed following 72 hours of exposure to the combined treatment compared to single treatments.

In a similar study, Bargali et al. [39] researchers used *A. annua* L. essential oil-loaded chitosan nanoparticles against plant-parasitic nematodes. A Study by Husam et al. [40] documented that the biological activities of *A. judaica* extract, including antimicrobial and anticancer activities, were significantly enhanced when the extract was loaded with CS NPs.

Conclusion

These results indicate that the synergistic effect of combining *Artemisia annua* with CS NPs (400, 50 µg/ml) is active against *Blastocystis* sp. and has comparable effects to a single drug and MTZ, suggesting a potential therapeutic effect against the parasite, with the added advantage of being naturally safe compounds.

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