Potent lethal effect of *Syzygium aromaticum* essential oil on *Blastocystis* spp.: An *in vitro* study

Original Article

Hayam M Ezz Eldin

Medical Parasitology Department, Faculty of Medicine, Ain-Shams University, Cairo, Egypt

ABSTRACT

Background: *Blastocystis* spp. are protozoan parasites that cause a wide range of gastrointestinal manifestations and is incriminated of being a possible element in the development of irritable bowel diseases as well as colorectal carcinoma. Metronidazole (MTZ) is commonly prescribed for treatment of blastocystosis. However, the reported increase in MTZ resistant parasites and undesirable side effects make the search for an alternative a priority. *Syzygium aromaticum*-eugenol rich essential oil has been widely investigated for its medicinal properties.

Objective: The present study was carried out to investigate the *in vitro* effects of *S. aromaticum*-eugeonl rich essential oil on *Blastocystis* spp. *in vitro*.

Material and Methods: Stool samples were collected from patients complaining of diarrhea, referred for stool examination to the Research and Diagnostic Laboratory Unit of the Parasitology Department, Faculty of Medicine, Ain Shams University. Microscopically, positive stool samples for *Blastocystis* spp. were cultured. Compared with MTZ, the effects of different concentrations of *S. aromaticum* essential oil on the viability of *Blastocystis* spp., tested by trypan blue dye, at different time points were evaluated. The diameter of *Blastocystis* treated with *S. aromaticum* essential oil, MTZ and untreated parasite control was measured.

Results: The minimal lethal concentrations for *S. aromaticum* were 300 µg/ml at 24 h, 200 µg/ml at 48 h, 100 µg/ml at 72 h and 50 µg/ml at 96 h, as compared to MTZ 1 mg/ml that did not induce complete inhibition till the end of the studied intervals. Notable shrinkage in the size of *Blastocystis*-treated with *S. aromaticum*, was significantly smaller than that of parasite control.

Conclusion: These results highly suggest that *S. aromaticum* essential oil may be a promising and safe agent for treatment of blastocystosis.

Keywords: *Blastocystis*, eugenol, essential oil, *Syzygium aromaticum*.

Received: 15 March, 2019**, Accepted:** 8 April, 2019.

Corresponding Author: Tel.: 00201005281590, **E-mail:** hayamezz@hotmail.com

Print ISSN: 1687-7942, **Online ISSN:** 2090-2646**, Vol. 12, No. 1, April, 2019.**

INTRODUCTION

Blastocystis spp. are protozoan inhabiting the gastrointestinal tract, with an incidence usually reaching 5% in developed countries, exceeding 40% in individuals with chronic gastrointestinal illness, and can reach up 76% in developing countries[1,2]. Infection with *Blastocystis* spp. can be detected in stool samples of asymptomatic individuals or patients complaining of diarrhea[3]. However, as the data depend on methods used for diagnosis, blastocystosis is likely underestimated[4]. Currently, *Blastocystis* spp. are included in WHO water sanitation programs[5]. It has been linked to the increased risk of allergic skin manifestations[6], and inflammatory bowel disease[1]; recently, a relation between blastocystosis and development of irritable bowel syndrome was reported[7]. Colorectal carcinoma was also linked to blastocystosis[8], as solubilized antigen of *Blastocystis* spp. was reported to promote the growth of human colorectal cancer cells[9]. Thus, therapy of blastocystosis is required to avoid or reduce the development of its possible complications. Lately, contradictory findings about the response of blastocystosis to MTZ treatment emerged, with efficacy rates ranging from 0% to 100%[10-12].

In this context, there is a need for new therapeutic agents against blastocystosis with high effectiveness and low toxicity. In recent times, the study of plants used by traditional medicine is a mean of alternative treatment, and several anti-parasitic properties of natural products have been identified[13]. Of those natural products is *Syzygium aromaticum* eugenol rich essential oil. Commonly known as clove, it is native to tropical America and Australia[14]. The oil extract has been widely investigated due to its availability and high essential-oil contents, and has long been considered to have medicinal properties[15], including anti-bacterial[16], anti-trypanosomal[17], and anti-malarial actions[18]. Islamuddin *et al.*,[19] reported that *S. aromaticum* essential oil induced apoptosis in *L. donovani* promastigotes. Additionally, it was found to have anti-*Giardia* activity[20]. *S. aromaticum* essential oil medicinal actions were attributed to the major component 'eugenol', that constitutes 89% of the oil[21].

 DOI: 10.21608/puj.2019.10650.1035

Based on the reported anti-protozoal activities of *S. aromaticum* essential oil, we were encouraged to assess its *in vitro* effect on *Blastocystis* spp. As far as we know, this is the first report demonstrating the potential anti-*Blastocystis* spp. activity of *S. aromaticum* essential oil.

MATERIAL AND METHODS

This descriptive analytical study was conducted at the Research and Diagnostic Laboratory Unit of the Parasitology Department, Faculty of Medicine, Ain Shams University, during the period from September, 2018 to January, 2019.

Stool collection and examination: *Blastocystis* was obtained from fecal samples of patients complaining of diarrhea, referred for stool examination to the Research and Diagnostic Laboratory Unit of the Parasitology Department, Faculty of Medicine, Ain Shams University. Stool specimens were examined for intestinal parasites immediately after collection by direct saline and lugol's iodine wet mount.

***In vitro* cultivation:** Positive specimens for *Blastocystis* spp. were isolated *in vitro* by inoculation of ~50 mg of positive stool samples into NIH modification of Boeck Drbohlav biphasic medium[22]. The cultures were incubated at 37°C and examined every 24 h by inverted microscope under ×10 and ×40 magnifications for *Blastocystis* growth. Subsequently, positive parasites culture was maintained in the laboratory by sub-culturing every 48 h when organisms were in the log phase of growth.

Materials: *S. aromaticum*-eugenol rich essential oil was purchased from Starwest Botanicals, Inc., Canada SKU-442060-01, and diluted in dimethylsulfoxide 0.2% (DMSO; Sigma-Aldrich). MTZ was supplied as 250-mg tablets (Flagyl, Sanofi-Aventis, Egypt). Tablets were dissolved in 250 ml of sterile distilled water, and stored in a dark bottle at 4°C till use[23].

Determination of MLCs: The anti-*Blastocystis* activity of the tested plant material was compared with MTZ as a reference antiprotozoal drug[24]. *Blastocystis* inoculum of $2x10^5$ parasites/ml from cultures in the log phase was introduced into each set of biphasic culture media tubes and incubated for 24 h prior to introduction of the different concentrations of *S. aromaticum* essential oil and MTZ to study parasite growth and viability. Untreated parasite control, solvent control and negative control were included in the experiment. Parasites were challenged with graded concentrations of *S. aromaticum* essential oil (300, 200, 100, 50, 25 µg/ml), and MTZ (500 µg/ml and 1 mg/ml). The tested cultures were examined at 24, 48, 72 and 96 h. The minimal lethal concentrations (MLCs) was considered as the lowest drug concentration needed to reach the maximum reduction of growth and multiplication of the parasite *in vitro* up to no growth[25]. MLCs of *S. aromaticum* essential oil (300, 200, 100, 50, 25 µg/ml) and MTZ were determined by parasite counting. The experiment was performed twice in triplicate for each group, cultures containing non-viable cysts were re-inoculated in fresh culture tube, incubated at 37°C for additional 96 h and examined to detect any viable parasite.

Parasite counting: Ten µl of 0.4% Trypan blue dye were added to the same amount of cells and incubated for 5 min at 30°C. Unstained (viable) parasites, were counted after 24, 48, 72 and 96 h using a Neubauer cell counting chamber (Hausser Scientific, Horsham, PA, USA)[26]. Mean parasitic counts were calculated and the distribution percentage of different morphological forms vacuolar, granular, cyst and dividing or amoeboid forms were determined in each group. An estimate of 100 cells were examined along the different time intervals for each group and the average of percentages at all intervals were calculated for all studied groups[27].

Percentage of inhibition of *Blastocystis* multiplication was calculated according to the equation[28]: $[(a-b)/a]\times100$, where a = mean number of organism in control tubes and b = mean number of organism in treated culture tubes. MLCs were determined by transfer of a drop of sediment from all tubes showing no viable parasite into fresh medication-free medium to assess viability. MLC was confirmed by absence of viable parasites after the tubes were examined for 4 d to determine whether the parasite growth resumed[29].

Diameter estimation: The diameter of *Blastocystis* treated with *S. aromaticum* essential oil, MTZ and untreated parasite control was measured by means of eyepiece graticule and stage micrometer; the average size of parasites/4 fields was determined for each group[30].

Statistical analysis: Data were analyzed using SPSS version 19 for Windows (SPSS Inc., Chicago, IL, USA). Data were presented as the mean±SD of triplicates percent of growth inhibition. The mean numbers were compared at the same time interval using student's t test, the difference was considered significant when the *P* value was <0.05.

Ethical consideration: An informed consent was obtained from all patients after explaining the aim of the study to them. The study was approved by the Research Ethics Committee, Faculty of Medicine, Ain Shams University. Patients with positive results were referred to the physician with laboratory reports to start treatment.

RESULTS

S. aromaticum treated *Blastocystis* cultures showed 100% inhibition of the parasitic growth with concentration of 300 µg/ml after 24 h. incubation, and 96.2% inhibition with concentration of 200 µg/ml after 24 h, progressing to complete inhibition of growth after 48 h. With concentration of 100 µg/ml, 95.1 % inhibition was observed after 24 h, progressing to 100% inhibition at 72 h, 89.75% and 89.1% inhibition at concentrations of 50 µg/ml and 25 µg/ml respectively at 24 h; and reaching complete and 96% inhibitions at concentrations of 50 µg/ml and 25 µg/ml, respectively after 96 h (Figure 1). All tested *S. aromaticum* essential oil concentrations gave lower means of parasite count that showed significant differences as compared to MTZ, and parasite control (Table 1). Minimal lethal concentrations of *S. aromaticum* essential oil were 300 µg/ml, 200 µg/ml, 100 µg/ml and 50 µg/ml at 24, 48, 72 and 96 h, respectively (Table 1). Viability was assessed by Trypan blue exclusion dye stain where non-viable parasites stained blue and viable parasites remained unstained (Figure 2 a, b and c).

Different morphological forms of *Blastocystis* were encountered including vacuolar, granular, amoeboid as well as dividing forms (Figure 2 a-f). The vacuolar form was the most abundant among all forms in the studied groups (Figure 3). The mean diameters of *Blastocystis* treated by *S. aromaticum* essential oil showed highly significant difference as compared to the mean diameter of parasite control along the studied intervals with all tested concentrations. One way ANOVA revealed no significant differences within the group along the duration of the study (Table 2 and Figure 4).

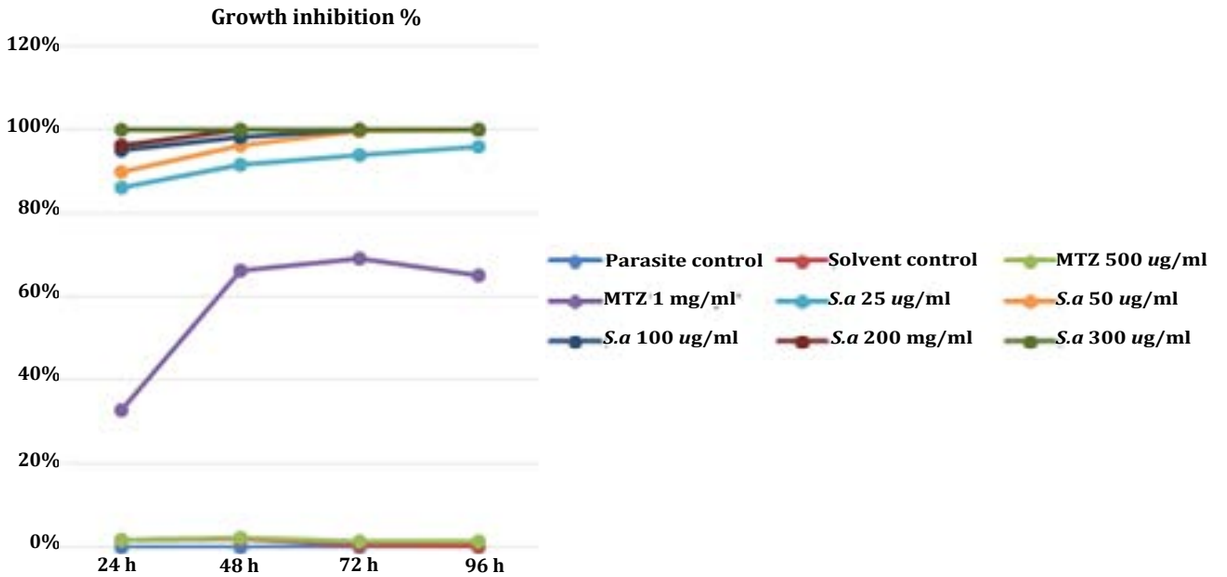

Fig. 1. Percentage of growth inhibition of *Blastocystis* spp. in culture medium after exposure to different concentrations of *S. aromaticum* (*S.a*) essential oil, and MTZ at 24, 48, 72 and 96 h.

Table 1. Effect of *S. aromaticum* essential oil on the *in vitro* growth of *Blastocystis* spp. ($2x10^5$) at different incubation periods.

	Time interval			
	24 h (Mean±SD)	**48 h (Mean±SD)**	**72 h (Mean±SD)**	**96 h (Mean±SD)**
Control				
Parasite	86.3 ± 3.05	74.0 ± 4.4	66.0 ± 7.9	52.5 ± 9.8
Solvent	86.0 ± 1.0 *, @	72.6 ± 2.5 *	64.3 ± 5.0 *	52.4 ± 11.7 *
MTZ				
500 µg/ml	85.0 ± 1.0 *	72.3 ± 0.57 *	65.0 ± 2.0 *	51.8 ± 10 *
1 mg/ml	57.9 ± 8.7 **	24.9 ± 5.4 **	20.3 ± 16.0 **	18.3 ± 4.2 **
***S. aromaticum*-eugenol rich essential oil**				
300 µg/ml	0.00 ± 0.00 **, @	0.00 ± 0.00 **, @	0.00 ± 0.00 **, @	0.00 ± 0.00 **, @
200 µg/ml	3.40 ± 0.60 **, @	0.00 ± 0.00 **, @	0.00 ± 0.00 **, @	0.00 ± 0.00 **, @
100 µg/ml	4.16 ± 0.87 **, @	1.30 ± 0.50 **, @	0.00 ± 0.00 **, @	0.00 ± 0.00 **, @
50 µg/ml	8.58 ± 2.10 **, @	2.70 ± 1.25 **, @	0.30 ± 0.38 **, @	0.00 ± 0.00 **, @
250 µg/ml	12.0 ± 4.30 **, @	6.16 ± 0.90 **, @	3.75 ± 0.66 **, @	1.90 ± 0.14 **, @

SD: Standard of deviation, **MTZ:** Metronidazole.
*No significant difference, **Significant difference as compared to parasite control, **@** Significant difference as compared to MTZ (1 mg/ml).

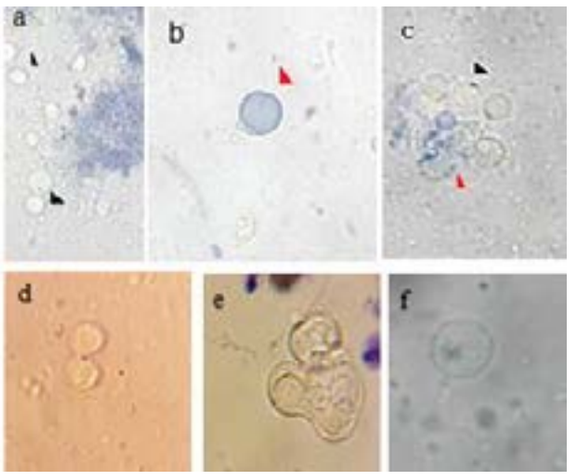

Fig. 2. Trypan blue 0.4% stained *Blastocystis* spp. **(a)** and **(c):** Unstained viable (black arrow head), **(b)** and **(c):** Stained non-viable (red arrow head); **(d)**: Dividing form; **(e):** Amoeboid form; **(f)**: Granular form. (x400).

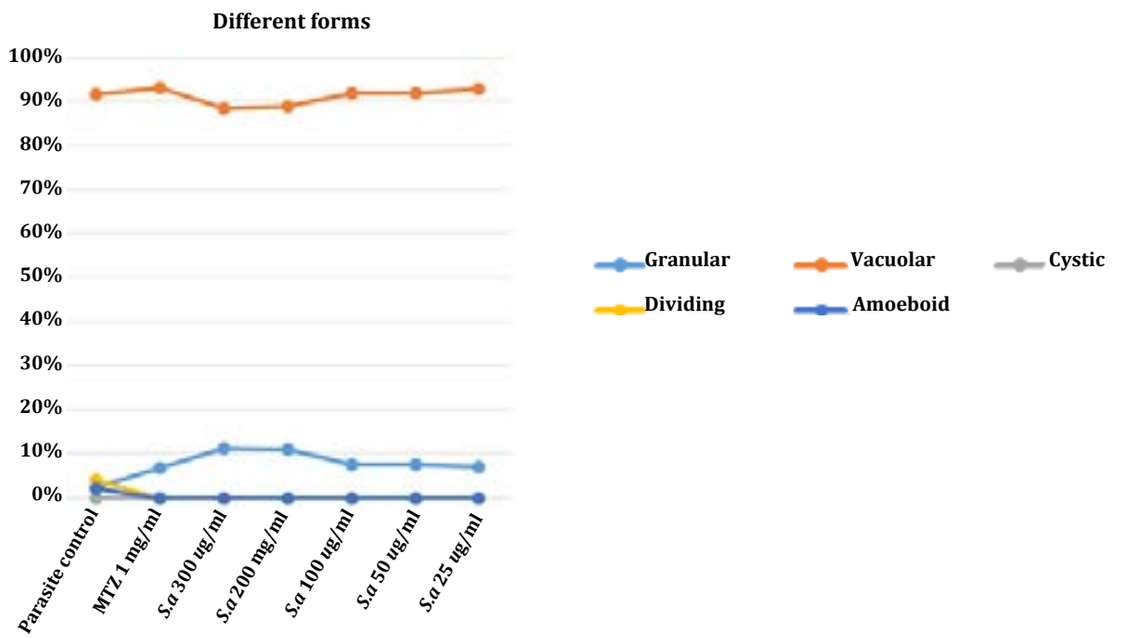

Fig. 3. Average percentage of *Blastocystis* spp. different forms distribution in different groups.

Table 2. Mean diameter (μm) of *S. aromaticum*-essential oil treated *Blastocystis* spp., MTZ treated and parasite control at different incubation periods.

	Time interval				Statistical analysis One-way ANOVA	
	24 h Mean ±SD	48 h Mean ±SD	72 h Mean ±SD	96 h Mean ±SD	Σ squares	*P* value
Control						
Parasite	13.0 ± 2.62	13.0 ± 2.62	13.0 ± 2.62	12.6 ± 1.92	170.7169	0.9865 *
Solvent	13.1 ± 2.75 *	12.9 ± 2.36 *	12.8 ± 2.12 *	12.8 ± 2.12 *	150.2169	0.9957 *
MTZ (1 mg/ml)	10.0 ± 1.30 **	9.75 ± 1.16 **	9.75 ± 1.16 **	9.5 ± 9.26**	38	0.85 *
***S. aromaticum*-eugenol rich essential oil**						
300 µg/ml	4.89 ± 1.36 **	4.87 ± 1.39 **	4.67 ± 1.32 **	4.78 ± 1.20 **	57.6405	0.9819 *
200 µg/ml	4.78 ±0.97 **	4.56 ± 1.13 **	4.89 ± 1.05 **	4.89 ± 1.27 **	43.2211	0.9360 *
100 µg/ml	5.11 ± 0.78 **	4.89 ± 1.05 **	4.67 ± 1.00 **	5.10 ± 1.00 **	30.7495	0.7907 *
50 µg/ml	4.83 ± 1.05 **	5.11 ± 1.27 **	4.56 ± 1.01 **	4.67 ± 1.12 **	44.7484	0.7501 *
250 µg/ml	5.56 ± 1.13 **	5.00 ± 1.41 **	4.89 ± 1.27 **	5.11 ± 1.17 **	52.3065	0.6904 *

SD: Standard of deviation, **MTZ:** Metronidazole.
:** No significant difference, *** Significant difference as compared to parasite control.

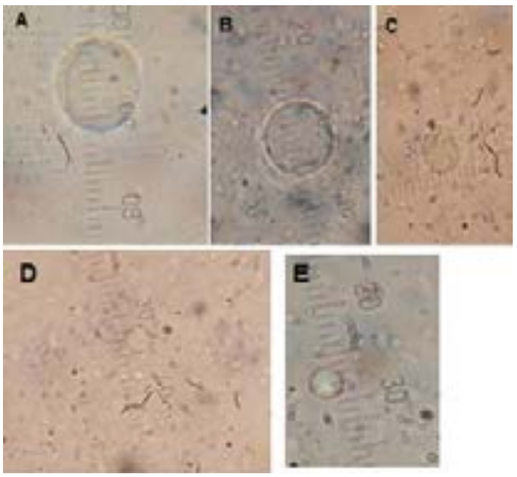

Fig. 4. Variation in diameter of vacular form *Blastocystis* spp., between untreated parasite control (A, B, and C) and *S. aromaticum* treated (D, and E), measured by eyepiece graticule callibrated with stage micrometer. (x1000).

DISCUSSION

The present study was carried out to evaluate the effects of *S. aromaticum*-eugenol rich essential oil against *Blastocystis* spp. Different concentrations were evaluated in comparison to parasite control and MTZ control. Our results demonstrated complete inhibition of *Blastocystis* viability in cultures treated with the concentration of 300 µg/ml after 24 h incubation. In correlation Machado *et al*.,[20] reported significant reduction of *G. lamblia* proliferation using *S. aromaticum* essential oil at a concentration higher than 200 µg/ml. Also in the present work, a significant 96.2% inhibition of the parasitic growth was observed with the concentration of 200 µg/ml after 24 h which progressed to complete inhibition of growth after 48 h. A significant inhibition of 95.1% was additionally observed with concentration of 100 µg/ml after 24 h, which also progressed to complete inhibition at 72h. In accordance, Ueda-Nakamura *et al*.,[31] reported 100% inhibition of L. amazonensis with the concentration of 100 µg/ml. We observed growth inhibition of 89.75% and 89.1% of *Blastocystis* spp. with the concentrations of 50 µg/ml and 25 µg/ml at 24 h, reaching up to 100% and 96% inhibition respectively at 96 h. That inhibition of viability was significantly higher than that of MTZ, which gave 32.8% inhibition at 24 h, and 65% inhibition at 96 h.

S. aromaticum essential oil inhibition of viability showed significant difference when compared to parasite control along the studied intervals. MLCs of *S. aromaticum* essential oil were 300 µg/ml, 200 µg/ml, 100 µg/ml and 50 µg/ml at 24, 48, 72 and 96 h respectively. Yakoob *et al*.,[32] tested other natural agents as garlic, ginger, black pepper, and white cumin for treatment of blastocystosis. In contrast to the viability results in our study, they reported that garlic and MTZ were equally effective, though the inhibitory effect reported in that study was 38% for MTZ and 44% for garlic. In addition, the tested *Blastocystis* isolates did not show any sensitivity to the other tested herbs.

A number of drugs have been used for treatment of blastocystosis, but they produced variable rates of cure. Although, MTZ is the most commonly utilized drug for blastocystosis, still, different *Blastocystis* isolates from different localities were reported to possess varying degrees of resistance to MTZ[23]. In the present study the concentration of 1 mg/ml of MTZ was used according to Haresh *et al*.,[23] who reported resistance to MTZ with concentrations up to 10 mg/ml. In our study initial evaluation of the concentration of 500 µg/ml MTZ did not show significant inhibition on the viability of tested isolate. That was also observed by El Deeb *et al*.,[33] who used MTZ at concentration of 500 µg/ml against Egyptian isolates, with incomplete inhibition of growth at lower concentrations. Bearing in mind the resistance to MTZ and its side effects, especially with higher doses, the control of blastocystosis constitutes a challenge. Investigation of the antiprotozoal activity of *S. aromaticum* essential oil against Leishmania by Coelho *et al*.,[34] showed that eugenol component of *S. aromaticum* inhibits leishmanial cysteine proteases, which are vital in several aspects of protozoa life cycle[35]. Furthermore, a surface-located cysteine protease was shown to be involved in the pro-survival role of *B. legumain* subtype 7[36]. Accordingly, the inhibitory effect of *S. aromaticum* essential oil observed in our study could be attributed to the ability of eugenol to inhibit cysteine protease's activity of *Blastocystis* spp. Another proposed mechanism of action of *S. aromaticum*-eugenol rich essential oil that could explain its influence on *Blastocystis* growth and viability is its ability to disrupt the cell wall, encourage cell leakage, increase cell permeability and cell shrinkage as observed with *Candida albicans*[37], *Proteus vulgaris* and *E. coli*[38]. This would explain the notable decrease in size of *S. aromaticum* treated *Blastocystis* in the current work to about 4.6-5.1 µm in comparison to 13 µm for untreated control. Size shrinkage was also reported with MTZ treated *Blastocystis*[26,39]. The observed reduction in cell diameter is one of the hallmarks of programed cell death[40], and as clarified by Lee *et al*.,[41] cell shrinkage is associated with inhibition of biomass

production, and is controlled by ion movement and ion channel regulation. Disruption of cells leads to shrinkage, inability of cells to maintain a volume balance causing outflow of intracellular contents and loss of cell volume. Additionally, modifications of the cell shape and inhibition of *G. lamblia* trophozoites adherence were also reported when treated by *S. aromaticum* essential oil[20].

Different morphological forms of *Blastocystis* spp. were encountered in the present study, though the vacuolar form was the most abundant among all studied groups, reaching to 92% in parasite control and 89% in *S. aromaticum*-300 µg/ml treated culture. The granular form, followed representing 11% in 300 µg/ml treated culture and 2% in parasite control. There was also a belief that the presence of granular form is the result of a triggering mechanism for apoptotic body deposition in *Blastocystis* central body, undergoing programmed cell death[42]. Stressful conditions have been reported to increase the number of granular forms[26]. As regard the cyst form it was not encountered in any of our studied groups in agreement with Yamada and Yoshikawa[43] who stated that the cyst form is mainly seen in fresh stool samples and rarely found in cultures. Concerning the safe use of *S. aromaticum* essential oil it was reported that it did not cause alteration of the viability of mammalian cells when compared to control cells[20]. It is generally recognized as a safe substance when consumed in concentrations lower than 1500 mg/kg[44]. Indeed, the World Health Organization established that the daily quantity acceptable of *S. aromaticum* essential oil per day in humans is about 2.5 mg/kg of weight[45].

It can therefore be concluded that the results of our study highlighted that *S. aromaticum* essential oil has a potent lethal effect on *Blastocystis* spp., as compared to MTZ, introducing an innovative mean for a new safe and effective therapeutic product.

Conflict of interest: There is no conflict of interest.

Financial support and sponsorship: Nil.

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