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RESEARCH ARTICLE

THE EFFECT OF PROBIOTICS, OREGANO OIL AND *QUILLAJA SAPONARIA* ON ISOLATED *B. HOMINIS*: AN EXPERIMENTAL VIEW

[1]Shaimaa Fouad, [2]Fadia M. Anwar, [1]Neimat M. Amer, [2]Nahed F. Farrag, [1,*]Zeinab H. Fahmy, [3]Mona Magdy, [4]Magda Azmy and [1]Soad Ismail

[1]Parasitology Department, TBRI
[2]Parasitology Department, Faculty of Medicine, Cairo University
[3]Pathology Department, TBRI
[4]Electron Microscopy Department TBRI

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ABSTRACT

Blastocystis is a protozoan parasite that inhabits the human intestinal tract. Various epidemiological surveys have recorded 50-60% prevalence in developing countries. Nitazoxanide is a commonly used drug in treatment of *Blastocystis* infection especially in metronidazole treatment failure. However, undesirable side effects and treatment failures were reported. To investigate the effect of probiotics, Oregano oil and *Quillaja saponaria* (*QS*)as natural compounds against isolated *Blastocystis* in comparison to nitazoxanide , fresh stools samples positive for *Blastocystis* were processed for in-vitro cultivation using locke serum media. Three criteria were used to test the drug's efficacy, Living cell count (LCC), Living cell rate (LCR) and ultra-structural changes as seen by transmission electron microscope (TEM). All the tested compounds used at higher concentrations showed a significant reduction in both LCC and LCR with p value< 0.001 and significant ultra-structural changes as seen by TEM. The tested compounds were arranged according to their LCR % on day one as follows: *QS* (1000 µg/ml) (47.1%), oregano oil (3200µg/ml) (49.5%), probiotics (500µg/ml) (57.0%) and nitazoxanide (0.776µg/ml) (62.5%). It was found that *Quillaja saponaria (QS)*, oregano oil and probiotics are promising new herbal therapeutic agents against *Blastocystis.*

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INTRODUCTION

Blastocystis species (spp.) are anaerobic intestinal parasite of humans and a wide range of animals (Tan, 2004). Numerous epidemiological surveys carried out in different countries identifying *Blastocystis spp*. as the most common eukaryotic parasite reported in human fecal samples (Tan, 2008). World Health Organization (WHO) (2000) has suggested that *Blastocystis spp*. are emerging parasite with a worldwide distribution. Prevalence varies widely from country to country and within communities of the same country (Alfellani *et al.,* 2013). Physicians usually have low awareness that *Blastocystis* is a cause of human disease. The number of infections appears to be high in most populations; however, the frequency is grossly underestimated (El-Shazly *et al.,* 2005). *Blastocystis spp*. isolates from humans and other animals have been reported to be morphologically indistinguishable. However, extensive genetic variation among numerous *Blastocystis spp*. isolates from both humans and animals was mainly observed by restriction fragment length polymorphism using polymerase chain reaction (RFLP-PCR) (Hoevers *et al.,* 2000) and PCR using sequenced-tagged site primers (Yoshikawa *et al.,* 2009). Treatment is usually considered if diarrhea is persistent and no other pathogen apart from *Blastocystis spp*. is identified in fecal specimens (Coyle *et al.,* 2012). A number of antimicrobial agents have been used to treat *Blastocystis* infection. This includes metronidazole, trimethoprim-sulfamethoxazole (TMP-SMX), nitazoxanide, paramomycin, emetine iodoquinol, ketoconazole, secnidazole and Tinidazole, however being a chemotherapeutic agent has multiple side effects especially when used for long time (Mirza *et al*., 2011). Many new natural products groups have revealed anti parasitic of surprising efficacy and selectivity. Following an initiative by WHO (2000). There is now opportunity to evaluate scientifically many more traditional medicines and other natural products in validated anti-parasite and toxicity screens.

Corresponding author: **Zeinab H. Fahmy,**
Parasitology Department, TBRI.

Aim of Work: To date a number of antimicrobial agents have been used to treat *Blastocystis* infection. Some of these drugs are effective for certain patients but does not provide complete eradication for others, particularly those with severe infection. Other drugs have severe side effects and drug resistance may occur. As a result, the necessity of finding alternative safe and highly effective agents against *Blastocystis* infection is required. There is an increasing awareness of the therapeutic potential of natural products and medicinal plants that are frequently considered to be less toxic and free from side effects than synthetic drugs .The present study was designed to evaluate the effect of probiotics, oregano oil and *Quillaja sapponaria (QS)*on isolated *Blastocystis*.

MATERIALS AND METHODS

Study Design: In order to assess the effect of probiotics, oregano oil, *QS* and nitazoxanide on isolated *Blastocystis spp*. thirty stools samples were collected from patients attending the outpatient clinic of Theodor Bilharz Research Institute (TBRI), Cairo, Egypt. The stool samples were collected from Patients complaining of gastrointestinal symptoms ± diarrhea. The study was conducted in the period from April to August, 2012. All stool samples were subjected to different parasitological examinations aiming to identify cases of *Blastocystis* infection. Only positive samples (three out of thirty) were subjected to culture to assess the efficacy of the tested drugs by counting living cell count (LCC), living cell rate (LCR) and TEM examination.

Ethical Consideration: The protocol of this study was approved by scientific research ethics committee of TBRI. Patients included in the study, were informed verbally about the purpose of the study, and the collection of stool samples was performed after obtaining their consent.

Parasitological Examination

- Sample collection.
- Sample processing.
- Stool analysis:
- Macroscopic examination.
- Microscopic examination:
  - Direct smear (Melvin and Brooke, 1974).
  - Formol ether concentration technique (Moody, 1997).
  - Permanent staining using Giemsa stain (Van Gool *et al*., 1990).
  - Culture technique using Locke serum media (Craig, 1926).

Drug Preparation

- **Probiotics** (Rameda Pharmaceutical Industries & Diagnostic Reagents. 6th of October City, Egypt). Lacteol fort *LAB* sachets, each sachet Corresponding to *LAB delbruekii* and *LAB fermentum*10 billion. Three different concentrations were done 150µg/ml, 300µg/ml and 500 µg/ml (Perez *et al.,* 2001).
- **Oregano oil** (Sigma Aldrich, St. Louis, Mo, Unites States) one bottle containing 100 g with a density 0.939 g/mL at 25 °C, with a solubility 1ml/2ml of 70% Ethanol. Oregano oil of different four concentrations (200µg/ml, 800µg/ml, 1600 µg/ml, and 3200µg/ml)was tested in comparison to ethanol (solvent) as control (Machado *et al.,* 2010).
- ***Quillaja* bark saponin** (Sigma Aldrich, St. Louis, Mo, United States) containing 10% saponin was adjusted and added to culture media so as to yield four different concentrations (50µg/ml,100µg/ml,500µg/ml and 1000µg/ml) (Yang *et al.,* 1996).
- **Nitazoxanide** (Medizen pharmaceutical industries for utopia pharmaceuticals).Containing 100mg/5ml, two concentrations were used 0.017 and 0.776 µg/ml respectively (Cedillo-Rivera *et al.,* 2002).

Determination of *Blastocystis* LCR: (Yang *et al.,* 1996).

Transmission Electron Microscopy (TEM): (Robinson *et al.,* 1987; Zierdt and Swan, 1981).

Statistical analysis: Data were analyzed using Minitab (statistical software 13.1/ Minitab Inc., PA, United States) for each duplicate tube logarithm (log) 10 transformation was conducted to calculate mean and standard deviation (± SD) values. Comparison between different groups was analyzed using paired student (t -test). Significance was established at P value ≤0.05 (Elgayar and Soliman, 2011).

RESULTS

To assess the effect of probiotics, oregano oil, *QS* and nitazoxanide on *Blastocystis spp.* in vitro, 30 stools specimen were collected from patients with (gastrointestinal symptoms ± diarrhea) attending the outpatient clinic of TBRI. All the samples were subjected to different parasitological examinations (macroscopic examination, direct smear unstained and stained with iodine, formol ether concentration technique and permanent staining using Giemsa stain) (Figures 1, 2 and 3).

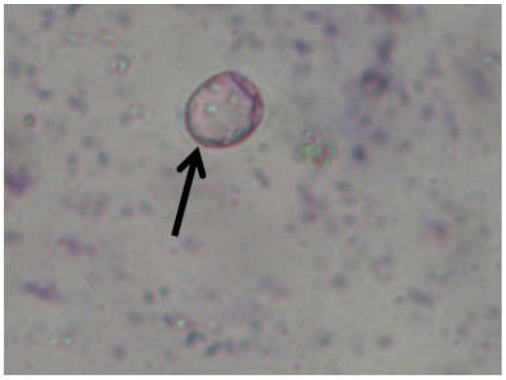

Fig. 1. Vacuolar form of *Blastocystis spp*. stained with Giemsa stain (x 1000)

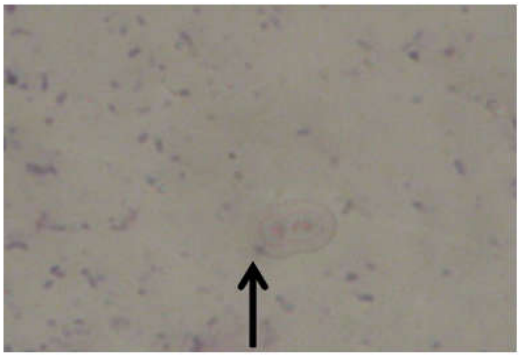

Fig. 2. *Blastocystis spp*. (binary fission stained with Giemsa stain (x 1000)

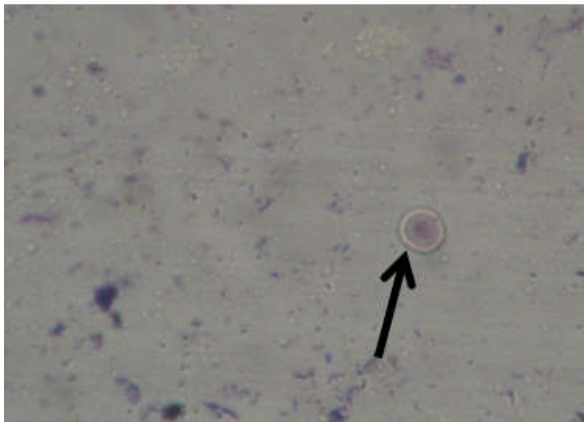

Fig. 3. Cyst form of *Blastocystis spp.* stained with Giemsa stain (x 1000)

Only positive samples (three out of thirty) as confirmed by direct smear, formol ether concentration and Giemsa stain were subjected to culture to assess the efficacy of the tested drugs. Criteria of effectiveness were based on (LCC, LCR and EM changes).

The effect of *QS* on *Blastocystis spp*

Based on counting only, intact vacuolar forms of *Blastocystis spp*., there was significant reduction in LCC by using different concentrations of *QS* with p value <0.001 compared to non-treated control group. On first day LCC decline folds increased(2.68, 3.04, 3.28 and 5.35) by using four ascending different concentrations of *QS*(50µg/ml, 100µg/ml, 500µg/ml and1000µg/ml) in order, while on day three and day six LCC decline folds were (2.55, 2.70, 2.99 and 4.98) and (1.70, 1.93,2.38 and 5.42) with the same concentrations in order. The previous results showed that decline folds were high on day one for all concentrations of *QS* in comparison to day three and six except for *QS*(1000µg/ml) on day six as decline fold was 5.42 compared to decline folds on day one and three(5.35 and 4.98)respectively (Table 1). Based on counting viable (living) *Blastocystis* cells which were stained greenish tinge with EC stain with intact membrane (Figure 4)while dead forms were red in color with non-intact membrane (Figure 5),it was observed that *QS* (50µg/ml) showed non-significant reduction with p value 1 and median LCR 83%,while the other three concentrations(100µg/ml ,500µg/ml and 1000µg/ml) showed significant LCR reduction (70%, 65% and 47.1%) in order with p value < 0.001 (Figure 6).*QS* showed to be significantly effective against *Blastocystis spp.* in all concentrations used except 50µg/ml. This effect was increased by increasing the concentration.

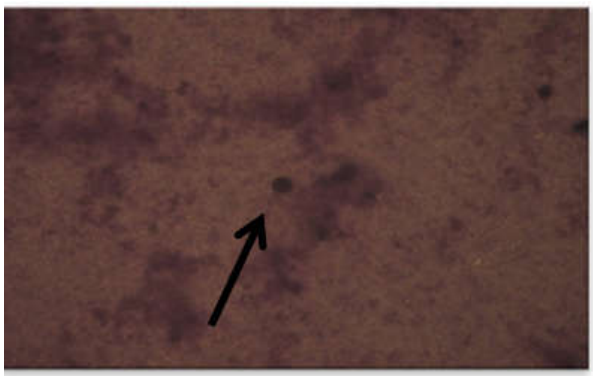

Fig. 4. Viable *Blastocystis spp.* (intact membrane) Stained greenish tinge with EC blue stain (x400).

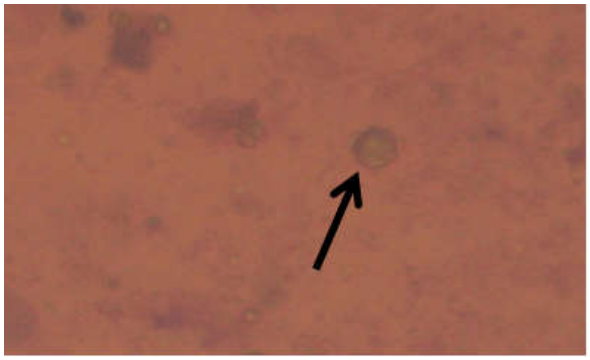

Fig. 5. Non-viable *Blastocystis spp.* stained red with EC blue stain (x400)

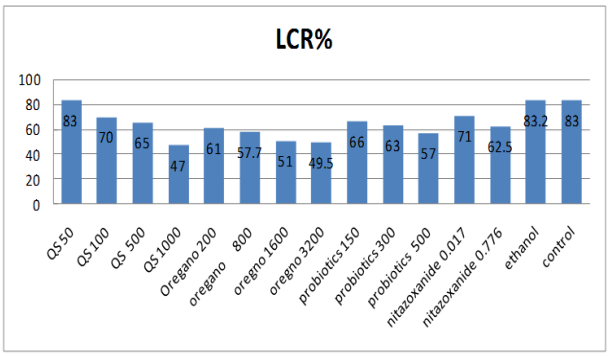

Fig. 6. Median LCR % for day one *Blastocystisspp.* exposed to different concentrations of *QS*, oregano oil, probiotics and nitazoxanide compared to non-treated control and ethanol treated control (for oregano oil only).

Also the increases in decline folds were more prominent on day one than day three and day six in most of the used concentrations.

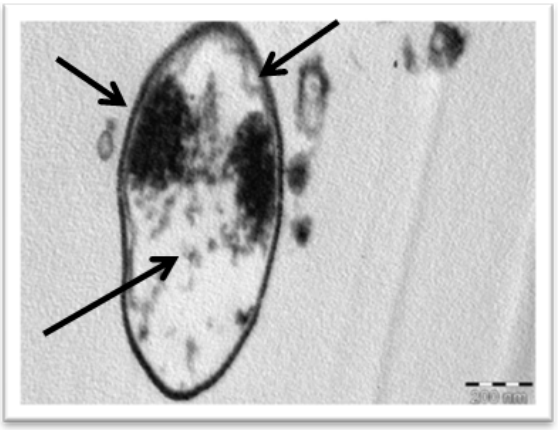

Fig. 7. TEM of *Blastocystis spp*. exposed to *QS* 100 µg/ml showing programmed cell death with apoptotic features (x 35,000)

TEM showed that *Blastocystis spp.* treated with *QS* 100 µg/ml showed morphological changes suggestive of programmed cell death with apoptotic changes includes (cell shrunk , nuclear chromatin produced distinct clumps along the periphery and large cytoplasmic vacuoles appears empty) (Figure 7).

The effect of oregano oil on *Blastocystis spp:* It was observed that using ascending concentrations of oregano oil (200 µg/ml, 800µg/ml, 1600µg/ml and 3200µg/ml)against *Blastocystis spp*. has significant reduction in LCC with p value <0.001.

Table 1. LCC (p value and decline folds) for *Blastocystis spp.* exposed to different concentrations of *QS*, oregano oil, probiotics and nitazoxanide compared to non-treated control

Control versus different drug concentration	Living cell count								
	Day 1			Day 3			Day 6		
	N x 10^3	Decline fold	P value	N x 10^3	Decline fold	P value	N x 10^3	Decline fold	P value
Control	4700			3300			2100		
QS 50µg/ml	1770	2.68	<0.001	1300	2.55	<0.001	1250	1.70	<0.001
QS 100µg/ml	1550	3.04	<0.001	1220	2.70	<0.001	1100	1.93	<0.001
QS 500µg/ml	1440	3.28	<0.001	1100	2.99	<0.001	900	2.38	<0.001
QS 1000µg/ml	880	5.35	<0.001	660	4.98	<0.001	400	5.42	<0.001
oregano 200µg/ml	1150	4.14	<0.001	700	4.82	<0.001	350	6.32	<0.001
oregano 800µg/ml	900	5.35	<0.001	550	6.15	<0.001	250	8.97	<0.001
oregano 1600µg/ml	880	5.45	<0.001	300	11.41	<0.001	100	20.94	<0.001
oregano 3200µg/ml	700	6.87	<0.001	200	17.23	<0.001	60	28.86	<0.001
probiotic 150 µg/ml	2880	1.65	<0.001	2400	1.37	<0.001	2000	1.07	<0.001
probiotic 300 µg/ml	2600	1.82	<0.001	1900	1.74	<0.001	1800	1.19	<0.001
probiotic 500 µg/ml	2200	2.14	<0.001	1800	1.83	<0.001	1120	1.88	<0.001
nitazoxanide 0.017 µg/ml	1220	3.91	<0.001	1150	2.89	<0.001	1100	1.91	<0.001
nitazoxanide 0.77µg/ml	800	5.86	<0.001	650	5.06	<0.001	500	4.19	<0.001

On first day LCC decline folds with the same concentrations were (4.14, 5.35, 5.45 and 6.87) in order, while on day three LCC decline folds with the same concentrations were(4.82, 6.15, 11.41 and 17.23) in order. On day six with lower concentrations(200µg/ml and 800 µg/ml) decline folds were (6.32 and 8.97)in order, while with higher concentrations of oregano oil (1600 µg/ml and 3200µg/ml) decline folds were very high (20.94 and 28.86) respectively (Table 1). LCR of *Blastocystis spp.* exposed to four different concentrations of oregano oil showed significant reduction with p value <0.001. Median LCR with ascending concentrations of oregano oil (200 µg/ml, 800µg/ml, 1600µg/ml and 3200µg/ml) were (61%, 57.7 %, 51% and 49.5%) in order (Figure 6). From these results it is clear that oregano oil is significantly effective against *Blastocystis* especially in higher concentrations (1600µg/ml and 3200µg/ml).

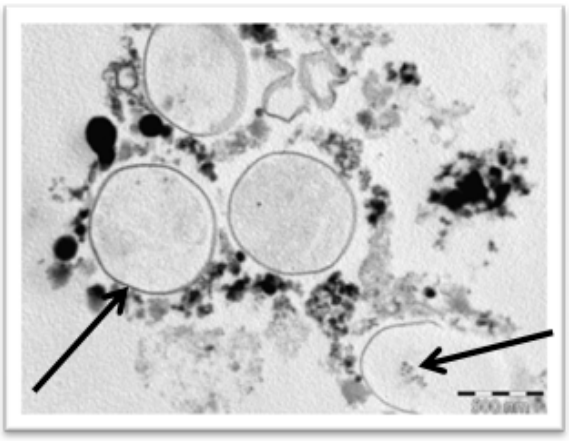

Fig. 8. TEM of *Blastocystis spp.* exposed to oregano oil 200µg/ml showing complete autolysis with cell membrane rupture(x 30,000)

The higher concentrations of oregano oil are more effective than the higher concentrations of *QS*. In contrast to *QS* the decline folds for oregano oil were higher on day three and six rather than day one. TEM showed that *Blastocystis spp.* Exposed to oregano oil 200 µg/ml showed complete autolysis with cell membrane rupture (Figure 8).

The effect of probiotics on *Blastocystis spp:* LCC of *Blastocystis spp*. exposed to ascending concentrations of probiotic (150µg/ml, 300µg/ml and 500µg/ml) has significant reductions in all days with p value < 0.001. On day one LCC decline folds with the same concentrations in order were (1.65, 1.82 and 2.14) while on day three decline folds were (1.37, 1.74 and 1.83) and day six were (1.07, 1.19 and 1.88) (Table1). LCR of *Blastocystis spp*. exposed to ascending concentrations of probiotics showed significant reduction with p value <0.001, median LCR with the same concentrations (150µg/ml, 300µg/ml and 500 µg/ml) in order were (66 %, 63 % and 57%) (Figure 6).These results showed that probiotics has significant effect on *Blastocystis spp.* as regards LCC and LCR. Also, it was found that the effect is more in higher concentrations and the decline folds were higher on day one than on day three and six. TEM showed that *Blastocystis spp*. exposed to probiotic (150µg/ml) has significant ultra-structural changes including cell wall shrunk and programmed cell death (Figure 9).

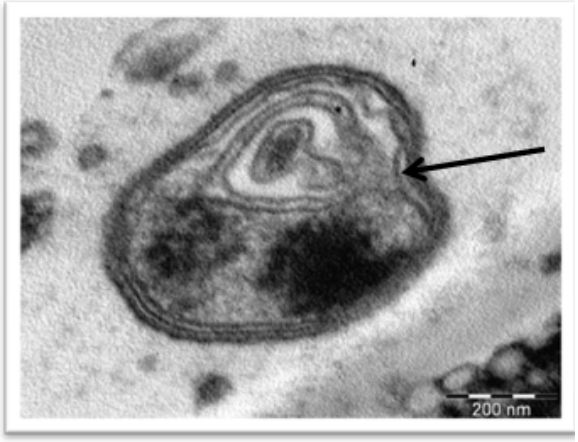

Fig. 9. TEM of *Blastocystis spp*. exposed to probiotic 150 µg/ml showing shrinkage of cell membrane with programmed cell death (x 38,000)

The effect of nitazoxanide on *Blastocystis spp:* *Blastocystis spp*. exposed to two different concentrations of nitazoxanide (0.017 µg/ml and0.77µg/ml) showed significant reduction in LCC with p value <0.001. By using ascending concentrations of nitazoxanide (0.017µg/ml and0.77µg/ml) decline folds were (3.91 and 5.86) on day one, while on day three (2.89 and 5.06) and day six (1.91 and 4.19) in order. It was observed from the results mentioned above that LCC decline folds were higher on day one than day three and day six (Table 1).

LCR of *Blastocystis spp*. treated with two different concentrations of nitazoxanide (0.017 µg/ml and0.77µg/ml) showed significant reduction with p value <0.001 and median LCR were (71 %and 62.5%) in order (Figure 6). These results showed that nitazoxanide is significantly effective against *Blastocystis spp.* and by increasing the concentrations, the effect is increased as shown by LCC and LCR. Similar to *QS* and probiotic the increase in decline folds were more on day one than day three and six. TEM for *Blastocystis spp*. treated with nitazoxanide 0.017µg/ml showed morphological changes suggestive of cell swelling and distorted cell shape, a redistribution of vacuoles, plasma membrane damage, and the formation of extensive empty areas in the cytoplasm (Figure 10).

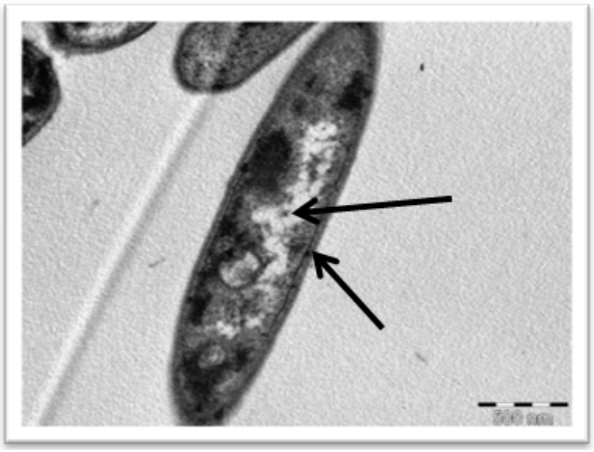

Fig. 10.TEM of *Blastocystis spp.* exposed to nitazoxanide 0.017 µg/ml showing cell swelling and distorted cell shape, a redistribution of vacuoles, plasma membrane damage, and the formation of extensive empty areas in the cytoplasm (x 35,000).

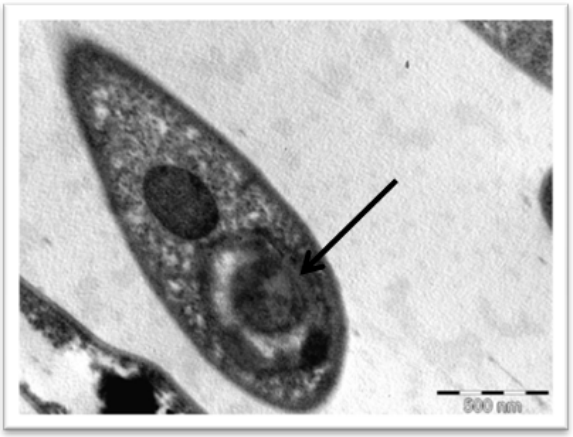

Fig. 11. TEM of *Blastocystis spp.* in Drug free culture media showing characteristic nuclear morphology (x 35,000)

In conclusion, from the previously mentioned data the four substances used (*QS*, oregano oil, probiotics and nitazoxanide) were significantly effective against *Blastocystis spp.*, however *QS* and oregano oil were superior as they were more effective in achieving a higher decline folds in LCC than probiotics and nitazoxanide at their highest concentrations on the sixth day (5.42 and 28.86) compared to (1.88 and 4.19) respectively. Similarly *QS* and oregano oil achieved lesser values for median LCR at their highest concentrations on the first day than probiotics and nitazoxanide (47.1 % and 49.5%) compared to (57% and 62.5%) respectively.

Drug free culture tubes (non-treated control)of *Blastocystis spp:* Two groups of culture tubes were maintained one free from any drugs (culture containing tubes) and the other culture tubes containing ethanol (only for oregano oil as it is used as solvent). Non -significant reduction in LCR was observed in both control groups (drug free culture tubes and culture tubes containing ethanol 70%) with p value 1.0 and high median LCR (83.2% and 83%) in order. TEM of *Blastocystis* from culture containing tubes showed non-significant ultra-structural changes with characteristic nuclear morphology (Figure 11).

DISCUSSION

Blastocystis is an enteric protozoan parasite of humans and many animals (Tan, 2004). It has a worldwide distribution and is often the most commonly isolated parasite in parasitological surveys (Rayan *et al.,* 2007). Many genotypes exist in nature and recent observations indicate that humans are in reality host to numerous zoonotic genotypes, such genetic diversity has led to a suggestion that previously conflicting observations on its pathogenesis are due to pathogenic and non pathogenic genotypes (Moosavi et *al.,* 2012). The need to treat *Blastocystis* is equivocal, considering the controversial pathogenesis of the organism and the apparent self-limiting nature of the symptoms (Zaki *et al.,* 1991). Although nitazoxanide is considered the drug of choice in metronidazole-treatment failures (Rossignol *et al.,* 2005), being a chemo therapeutic agent, nitazoxanide has many side effects which may limit its use for long time (Parashar and Arya, 2005). Natural products are not only the basis for traditional or ethnic medicine, but also screening natural plant products provides highly successful new regimens for human welfare. Many new natural products have revealed anti parasitic of surprising efficacy and selectivity (Hussein *et al.,* 2008).

The present study was carried out to evaluate the in vitro effect of different concentrations of *Qs*, oregano oil, probiotics and nitazoxanide against *Blastocystis spp.* in vitro. In the present study LCC for *Blastocystis spp.* treated with *QS* showed significant reduction with all concentrations used (50 µg/ml, 100 µg/ml , 500 µg/ml, 1000 µg/ml) and p value <0.001. It was also observed that LCC decline folds for *Blastocystis spp.* treated with different concentrations of *QS* were higher on day one than day three and six. This is in agreement with Zierdt and Swan (1981) who conducted a study on the generation time of *Blastocystis* over six days, they found that the most rapid growth of *Blastocytstis* occurred during the first 24 –h period therefore, evaluation of the drug efficacy against *Blastocystis spp.* could be based on its effect on the first day. We observed that *QS* (1000 µg/ml) declined the LCC by 5.35 folds on day one followed by 4.98 folds on day three leaving only resistant *Blastocystis* forms. On day six by using drug free culture tubes (although the drug effect was removed) the LCC declined to 5.42 folds this could be explained by autolysis of the remaining persistent parasites from day three to day six. As regards, the LCR % for *Blastocystis spp.* treated with ascending concentrations of *QS* (50 µg/ml, 100 µg/ml , 500 µg/ml, 1000 µg/ml) showed significant reduction with p value < 0.001 except for *QS* (50 µg/ml)which showed p value 1 and median LCR 83%. The present study showed that the p value for LCC of *Blastocystis* treated with *QS* (50 µg/ml) is < 0.001 while the p value for LCR of *Blastocystis* treated with the same concentration of *QS* is 1.

This might be explained by Yang *et al.* (1996) who studied the in vitro effect of traditional Chinese medicine against *Blastocystis* and concluded that LCR is considered more sensitive and accurate than LCC. This is because LCR basically calculates the percentage rate of *Blastocystis* viable cells (stained greenish with EC stain) while non-viable cells (stained red with EC stain). But LCC is unable to distinguish viable from non viable cells. It was observed in the present study that LCC decline folds for *Blastocystis* treated with *QS* **(**1000 µg/ml)on day one were 5.35.On the other hand Elgayar and Soliman (2011) studied the antiprotozoal effect of *QS* against two isolates of *Blastocystis* and found that by using the same concentration of *QS* (1000 µg/ml), the LCC decline folds were 70 and 527. The difference between the two decline folds in the previous study and the decline folds in the present study could be due to different virulence of different species of *Blastocystis* as stated by Hussein *et al.* (2008). Similarly in Elgayar and Soliman (2011) study the LCR % of the two isolates of *Blastocystis spp.* treated with *QS* (1000 µg/ml) were (60% and 45%) while in the present study LCR % was (47.1 %). In our study TEM showed that *Blastocystis spp.* treated with *QS* showed morphological changes suggestive of programmed cell death with apoptotic changes includes (cell shrunk , nuclear chromatin produced distinct clumps along the periphery and large cytoplasmic vacuoles appears empty). These changes were similar to Elgayar and Soliman (2011) study who reported ultra-structural changes of programmed cell death with apoptotic features. The ultra-structural changes of *Blastocystis* treated with *QS* appears to be related to altered permeability of the cyst wall by creating pores in the cell membrane as stated by Menin *et al.* (2001) who treated isolated cardio myocytes with saponin (50 µg/ml for 30 minutes or 600 µg/ml for 1 minute) to open the outer cellular membrane and release the metabolites from cytoplasm.

In the present study oregano oil appears to have significant effect on *Blastocystis* LCC with p value < 0.001 in all concentrations used (200 µg/ml, 800µg/ml, 1600µg/ml and 3200µg/ml). In contrary to other drugs used in the present study, it was observed that LCC decline folds of *Blastocystis* treated with oregano oil were higher on day six than day one and day three especially with higher concentrations (1600 µg/ml and 3200 µg/ml). Similar results were obtained by Ying *et al.* (2008) who studied the in vitro effect of traditional Chinese oregano oil on *Blastocystis*. The authors explained the higher decline folds after day three by spontaneous autolysis of remaining viable forms. As regards the LCR % for *Blastocystis* exposed to oregano oil showed significant reduction with ascending concentrations of oregano oil with median LCR % reaches up to 49.5 % with oregano 3200 µg/ml. While in Ying *et al.* (2008), study *Blastocystis* exposed to the same concentration of oregano oil (3200 µg/ml) has reduction rate of 98.6%. In the same context Grabensteiner *et al.* (2007) also studied the effect of essential oils against two isolates of *Blastocystis spp*. in comparison to other protozoa. The authors stated one hundred percent eradication of *Blastocystis* after 24-h of incubation. The differences in results between both Ying *et al.* (2008) study, Grabensteiner *et al.* (2007) study and the present study may be due to different dosage used or different compositions, quality and content of oregano oils which are influenced by diverse factors such as geographic and climatic changes as well as the conditions used for culture, drying and storage as stated by Escobar *et al.* (2010) who studied the chemical composition and antiprotozoal effects of essential oils**.**

It may be also due to different species of *Blastocystis* exists with different virulence (Hussein *et al.,* 2008). TEM showed that *Blastocystis* treated with oregano oil showed complete autolysis with cell membrane rupture. The same effect found by Lambert *et al.* (2001) who conducted a study on the minimum inhibitory concentration and the mode of action of oregano essential oil. The authors stated that the effect of oregano oil on protozoa may be due to altering the ion transport processes of the cell membrane and modify the activity of calcium channels which cause an increase in cell permeability and consequent release of vital intracellular constituents. Similarly Ueda-Nakamura *et al.* (2006) while studying *leishmanicidal* activity of *Origanum gratissimum* (*O. gratissimum*) essential oil found a considerable ultra-structural alterations including mitochondrial swelling, the inner mitochondrial membranes were altered with an increase in the number of cristae. Santoro *et al.* (2007) also studied the effect of *O. vulgare* on *Trypanosomacruzi*, TEM showed cytoplasmic swelling with occasional morphological alteration in the cytoplasmic membrane. As regards the in vitro effect of probiotics against *Blasocystis spp.* there was a significant reduction in LCC in all concentrations used **(**150 µg/ml **,** 300µg/ml and 500 µg/ml**)**with p value <0.001 , similar to *QS* decline folds were higher on day one than other days, this was explained before due to the fact that the most rapid growth of *Blastocytstis spp.* occurred during the first 24 –h period therefore, evaluation of the drug efficacy against *Blastocystis spp.* could be based on its effect on first day (Zierdt and Swan, 1981).

In the present study it was also noticed that using ascending concentrations of probiotics showed significant reduction in LCR % with p value <0.001. The present study up to the knowledge after long research may be the first study testing the effect of probiotics on *Blastocystis spp.* in vitro however, there is many clinical studies showing the efficacy of probiotics against protozoa. A clinical study by Dinleyici *et al.* (2011) has demonstrated the potential beneficial effects of probiotics against *Blastocystis* infection (symptoms, presence of parasites) in children treated with metronidazole or probiotics. Three groups were analyzed: group A, treated with probiotics (250 mg twice a day) for 10 days; group B, treated with metronidazole (30 mg/kg twice daily) for 10 days; and for group C no treatment was provided. On day 15, clinical cure was observed in 77.7% in group A; in 66.6% in group B; and 40% in group C. In contrary Goldman *et al.* (2006) disagree about probiotics clinical effectiveness as it could not demonstrate an adjuvant effect of the studied probiotic in the eradication of gastro-intestinal infections, indicating that other alternative therapeutic strategies should be studied. In the present study it was also observed that TEM of *Blastcystis spp.* exposed to probiotics showed ultrastructure changes including cell wall shrunk and programmed cell death. Similarly Amer *et al.* (2014) studied the in vitro effect of probiotics against *giardia* trophozoites. Ultra structural examination proved that probiotics showed marked changes in cellular architecture of the trophozoites with evident disorganization of the cell membrane, adhesive disc and cytoplasmic components. In the present study the in vitro effect of nitazoxanide against *Blastocystis* showed significant reduction in LCC with both concentrations (0.017µg/ml and 0.776 µg/ml) in all days with p value < 0.001. Similarly to *QS* and probiotics, nitazoxanide decline folds of LCC were higher on day one than day three and six. LCR% for *Blastocystis* was significantly reduced on day one (71% and 62.5 %) in order with p value <0.001.

Rossignol *et al.* (2005) had tested the in vivo effect of nitazoxanide in persistent diarrhea and enteritis associated with *Blastocystis* and proved to be effective as it caused complete remission of the treated patients. Another study by Elakkad *et al.* (2012) who tested the in vitro effect of nitazoxanide against *Blastocystis spp*. showed that by using neutral red stain for confirming viability as viable cells took the red stain while dead cells appear with corrupted morphology, loss of cell membrane continuity, pale and unstained. Nitazoxanide concentration of 1µg/ml, showed non considerable reduction of viability of *Blastocystis* while a concentration of 2µg/ml nitazoxanide was shown to induce 50% reduction after 48 hours in cultures and a concentration 10 µg/ml of nitazoxanide caused complete death of *Blastocystis* after 48 hours. While Elakkad *et al.*(2012) study showed non considerable reduction of viability of *Blastocystis* with a nitazoxanide concentration 1µg/ml, the present study showed considerable reduction of *Blastocystis* with a nitazoxanide concentration below 1µg/ml this difference could be due to different species of *Blastocystis* with different virulence (Hussein *et al.,*2008).

In the present study TEM of *Blastocystis spp.* treated with nitazoxanide 0.017 µg/ml showed cell swelling and distorted cell shape, a redistribution of vacuoles, plasma membrane damage and the formation of extensive areas in the cytoplasm. Similarly, in Elakkad *et al.* (2012) study, TEM showed drug induced cytoplasmic vacuoles with necrotic cells together with disruption of the normal morphology of *Blastocystis.* Cedillo-Rivera *et al.* (2002) upon studying the, TEM changes of protozoa exposed to nitazoxanide showed Ultra-structure changes in the form of cell swelling and distorted cell shape, and the formation of extensive empty areas in the cytoplasm. As shown in the present results *QS* (1000µg/ml) has the least LCR (47.1%) followed by oregano oil (3200µg/ml) as LCR is (49.5%) then probiotic (500µg/ml) showed LCR (57%) and at last nitazoxanide (0.776µg/ml) showed LCR (62.5%). In our study although all the drugs tested showed significant effect against *Blastocystis*, however some of the drugs showed more effect than others as measured by LCR%, LCC and TEM examination. Factors that determine efficacy could be due to intrinsic properties of the tested drugs and its pharmacokinetics (McFarland*,* 2010),the quality of these products from different sources may vary and many of the commercially available products may lack regulated quality control programs (Marcobal *et al.,* 2008), the stability of the products may significantly affect its potency over time (Graff *et al.,* 2008),the dose used in each drug tested (McFarland*,* 2010), different genotypes of *Blastocystis*, variation of drug susceptibility, mechanism of action and mode of resistance to the drug (Tan*,* 2008).

Conclusion

From the present study it could be concluded that natural products (Quillaja, Oregano Oil and Probiotics) might offer an alternative therapy to chemotherapeutic agents in treatment of *Blastocystosis* with less side effects.

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