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IN VITRO RESPONSE OF *BLASTOCYSTIS* SPP. TO AQUEOUS EXTRACTS OF SOME EGYPTIAN HERBS

By
ASMAA KAMAL ABD ELLAH
Department of Medical Parasitology, Faculty of Medicine, Sohag University, Egypt.
(Correspondence: Asmaakamal@med.sohag.edu.eg)

Abstract

Blastocystis spp. is a protozoan parasite with controversial clinical significance and consensus for treatment. Metronidazole (MTZ) was considered the main line of treatment, but drug resistance and many side effects motivated the search for effective safe therapy.

This study evaluated the in vitro therapeutic effects of aqueous extracts of nine Egyptian herbs on *Blastocystis* spp. compared to MTZ as standard control drug.

Blastocystis-positive stool samples were obtained from patients with gastrointestinal symptoms by direct smears and FECT. Aqueous extract of herbal plants (*A. sativum*, *N. sativa*, *P. nigrum, C. cyminum*, *Z. officinale, T. vulgaris, O. majorana*, *M. chamomilla* & *A. Judaica*) were prepared. Positive samples were incubated with 0.1mg/ml concentration of tested herbal extracts on Jones medium at 37ºC versus 0.1mg/ml MTZ as a control. Parasite count was assessed after 24, 48, & 72hr post incubation.

The *A. sativum, M. chamomilla, A. Judaica* & *N. sativa* extracts showed highly significant growth inhibition rate of *Blastocystis* species compared to MTZ followed by *O. majorana, C. cyminum* & *T. vulgaris* extract. But, *P. nigrum* & *Z. officinale* extracts showed insignificant growth rate.

Keywords: *Blastocystis* spp., Herbal extracts, MTZ, In-vitro culture, Egypt.

Introduction

Blastocystis spp. is an intestinal protozoan parasite primarily found in human stools and in many animals (Yoshikawa *et al,* 2016). Its' prevalence ranged from 7% to 50% in developed countries to 55% to 100% in developing (Darwish *et al*, 2023). Infection is by consumption of contaminated raw vegetables or water, and contact with infected animals (Lee *et al*, 2019). Blastocystosis may be asymptomatic or with gastrointestinal symptoms mainly diarrhea with inflammatory bowel mediated disorders, gut dysbiosis, and colon cancer (Al-Khalaf *et al*, 2023). The variability of human clinical pictures is due morphological genetic diverse (Rudzińska and Sikorska, 2023).

Metronidazole (MTZ) is the drug used to treat blastocystosis. However, MTZ showed side effects as well as parasite resistance (Stensvold *et al*, 2010). Also, this drug causes changes in gut microbiota with side effects, including embryotoxic, carcinogenic and teratogenic (Lepcz yńska *et al*, 2017). So, a more effective drug, with few or without side effects and low cost was indicated (Abdel- Hafeez *et al*, 2015).

Aromatic herbs and spices showed antimicrobial efficacy in folk medicine and food preservation (Christaki *et al*, 2012). They act on microorganisms, and parasites (Moon *et al*, 2006). All parts of these plants contain odor, volatile, hydrophobic and essential oils (Negi, 2012), consist of secondary metabolites low-boiling-point phenylpropenes and terpenes (Greathead, 2003). Families contain essential oils are Liliaceae as *Allium sativ um*, Ranunculacea as *Nigella sativa*, Piperaceae as *Piper nigrum*, Apiaceae as *Cuminum cyminum*, Zingiberaceae *as Zingiber officinale*, Myrtaceae as *Thymus vulgaris*, Lapmiaceae as *Origanum majorana* and Asteraceae or Compositae as *Artemisia judaica* and *Matricaria chamomilla* (Raut and Karuppayil, 2014). The antimicrobial properties are mainly attributed to essential oils (Giannenas and Kyriazakis**,** 2009) hydrophobicity accumulates in cell membrane lipid bilayer rendering them more permeable (Solorzano-Santos and Miranda-Novales, 2012). Also, some essential oils inhibited growth and death of antimicrobials and parasites by homeostasis (Devi *et al*, 2010).

This study aimed to evaluate the *in-vitro*

effects of nine Egyptian Medicinal Herbs (*A. sativum*, *N. sativa*, *P. nigrum, C. cyminum*, *Z. officinale, T. vulgaris*, *O. majorana*, *M. chamomilla* and *Artemisia Judaica*) versus Metronidazole® (MTZ) as a standard drug control against *Blastocystis* spp. isolated from symptomatic patients.

Materials and methods

Study design: This study was conducted at Sohag University, Medical Parasitology Department from January to March 2024.

Stools collection: Morning stool samples were obtained from symptomatic patients attended Parasitology Laboratory, Sohag University Hospitals. Those received or on antiparasitic treatment were excluded. Samples were collected in labeled disposable boxes and examined immediately by direct saline and iodine wet smears and formalin ethyl acetate concentration technique (FECT) for *Blastocystis* spp.

Blastocystis isolation: Positive *Blastocystis* spp stools (50mg formed or 0.5ml diarrheic) were cultured in Jones media tubes with 10% horse serum, 100UI/ml penicillin and 100µg/ml streptomycin (Sigma-Aldrich) and incubated at 37°C for 2-3 day and then subcultured for 2-3 days in fresh medium. Cultures were examined microscopically every 12 hr for *Blastocystis* spp. growth (Abdel-Hafeez *et al*, 2015).

Herbal and drug extracts**:** A total of nine medicinal herbs were purchased from insecticide-free source. Aqueous extracts were measured, grounded, and soaking in distilled water for 4 days at 4°C, and then filtered to have 100mg/ml solution of each, with pH 7.0 neutralized infusions and stored at-20°C. Garlic was used fresh. Each herbal extract was added to media to give a final concentration of 0.1mg/ml (Ismail *et al*, 2012).

MTZ tablets 500mg (reference drug) were purchased, crushed, and dissolved in 500ml bi-distilled water to produce a stock solution of 1mg/ml and final concentration of 0.1 mg/ml.

Latin, common name and plant extracts used.

Latin name	Common name	Part used
Allium sativum	Garlic	fresh bulbs
Nigella sativa	Black seed	dried seeds
Piper nigrum	Black pepper	dried seeds
Cuminum cyminum	Cumin	dried seeds
Zingiber officinale	Ginger	dried rhizome
Thymus vulgaris	Thymus	dried leaves
Origanum majorana	Marjoram	dried leaves
Matricaria chamomilla	Chamomile	dried flowers
Artemisia Judaica	Shih Baladi	dried aerial parts

Drug susceptibility assays: All experiments were done using an inoculum of $2x10^5$/ml from *Blastocystis* isolate. In each tube, herbal extract or MTZ was added to media to 0.1 mg/ml. This was side by side with negative control. The culture tubes were incubated at 37ºC and examined after 24, 48, & 72hr. Efficacy were assessed by removing supernatant of each tube, the 0.4% Trypan blue stain sediment was microscopy examined and viable cysts were counted by a hemocytometer. Mean of two counting was compared with control. Growth inhibition (GI) percent was calculated as follows: a-b/a ×100, where; a = viable cysts in control cultures, b = viable cysts in treated cultures.

Ethical considerations: The study was approved by Medical Research Ethical Committee (MREC) Faculty of Medicine, Sohag University, with IRB registration No (Sohmed-23-12-02PD), which went with Helsinki Declaration (2008). Written informed consent was obtained from patients after explaining the study aim and that their data are only for study purpose.

Statistical analysis: Data were collected, tabulated and analyzed by using IBM SPSS Statistics for Windows version 25.0. Quantitative data were expressed as Mean ±SD. Significance of variations between data was determined by using ANOVA test. P value < 0.05 was significant difference.

Results

The results were shown in table (1) and figures (1, & 2).

Table 2: In vitro effects of herbal extracts GI versus MTZ on *Blastocystis* for different incubation periods.

Extract (0.1mg)	Before drug	24hr After drug	48hr After drug	72hr After drug	P^
Negative control	20 ±1.6 (0%)	35 ± 2.1 (0%)	45±1.6 (0%)	70±1.6(0%)	
Positive control	20±1.6 (0%)	10 ± 1.4 (71.4%)	4 ±1.4 (91.1%)	0(100%)	0.001*
A. sativum	20 ±1.6 (0%)	6.5 ± 1.2 (81.4%)	3.5± 1.2 (92.2%)	0(100%)	0.001*
N. sativa	20 ±1.6(0%)	11.7 ± 2.1 (66.5%)	6 ±1.6 (86.6%)	0(100%)	0.001*
P. nigrum	20 ±1.6 (0%)	34.5 ± 3.6 (1.4%)	43.2 ± 1.8 (4%)	67.2 ± 1.8 (4%)	0.3
C. cyminum	20±1.6(0%)	7.5 ± 1.2(78.5%)	9.5 ± 2.3(78.8%)	14.7 ± 0.9(79%)	0.001*
Z. officinale	20±1.6 (0%)	34.5 ± 1.2 (1.4 %)	44.2 ± 2.6 (1.7 %)	68.7 ± 2.9 (1.8 %)	0.7
T. vulgaris	20 ±1.6 (0%)	12.5±1.2 (64.2%)	15 ± 1.6 (66.6%)	21.5 ± 1.2 (69.2%)	0.001*
O. majorana	20 ±1.6 (0%)	7.5 ± 1.2 (78.5%)	8.5± 1.7 (81.1 %)	11.2 ± 1.7 (84%)	0.001*
M. chamomilla	20 ±1.6 (0%)	6.7± 1.5 (80.8%)	3.7± 1.7 (91.7%)	0 (100%)	0.001*
A. Judaica	20±1.6 (0%)	7 ± 2.1 (80%)	4 ± 1.4 (91.1%)	0 (100%)	0.001*

All live *Blastocystis* x104, P^: Repeated ANOVA, P<0.05* = significant difference as compared to NTC.

Discussion

In the present study, 0.1 mg/ml MTZ dose was significantly (P <0.001) lowered the parasite number and the growth inhibition rate reduced from (71.4%) after 24 h to (91.1%) after 48 h to 100% after 72h. Aqueous extract of *A. sativum, M. chamomilla, A. judaica and Nigella sativa* significantly (P <0.001) lowered *Blastocystis* spp. count as compared to MTZ and growth inhibition rat es increased (81.4%, 80.8%, 80% & 66.5%) after 24hr to (92.2%, 91.7, 91.1 & 86.6%) after 48hr respectively to 100% in all extracts after 72hr. These data agreed with Yakoob *et al*. (2011), who reported that *Blastocystis* isolates sensitivity to garlic was proportional to MTZ in suppressing its growth and Abdel-Hafeez *et al*. (2015), who found that garlic extract reduction rate after 48hr was 92.44%. Garlic has strong antioxidants inhibiting nitric oxide (NO) production. Intestinal NO increases upon *Blastocystis* infection as a host defense mechanism of epithelial cells against parasites. However, it may lead to cellular damage and gut barrier failure, as well as was involved in the pathogenesis of many inflammatory and autoimmune diseases (Lepczyńska *et al*, 2017). The result agreed with Eida *et al*. (2016), who reported that *N. sativa* inhibited *Blastocystis* in vitro. Saleh *et al.* (2023), who found that extracts of *A. sativum, N. sativa* gave a potent lethal effect on blastocystosis as compared to Nitazoxanid. Also, it agreed with Mokhtar *et al.* (2019), who found a significant growth inhibition of *Blastocystis* after exposed to ethanolic extracts of *A. judaica* and Sabatke *et al.* (2022), who found that *M. chamomilla* act synergistically with NTZ, increased its effectiveness and decreased drug dose needed for giardiasis treatment. The anti-*Blastocystis* activity of *A. judaica* extract is related to its bioactive compounds in the aerial part. Such Lipophilic sesquiterpene lactones increased fluidity of the protozoan membrane, resulting in uncontrolled ions reflux and cell death (Loo *et al*, 2017).

In the present study, anti-*Blastocystis* efficacy of *O. majorana, C. cyminum*, & *T. vulgaris* aqueous extracts were less than those of *A. sativum, M. chamomilla, A. judaica* & *N. sativa* with significant (P <0.001) that lowered *Blastocystis* count and growth inhibition rates increased from (78.5%, 78.5% & 64.2%) after 24hr to (81.1 %, 78.8%, & 66.6%) after 48hr to (84 %, 79% & 69.2%) after 72hr respectively. This agreed with El-Sayed, (2009), who found that growth reduction rates by 0.5 mg of *T. vulgaris* ethanolic extract were 68, 73.8, & 80.4% after 24, 48, & 72hr incubation respectively, Yakoob *et al*. (2011), who found that *Blastocystis* isolates were not sensitive to cumin compared to garlic and MTZ. Besides, Meabed *et al.* (2018) who found *O. majorana* at 400µg/ml showed significant results equivalent to NTZ, but lower concentration showed low efficacy rates. Also, Saleh *et al*. (2023), who found that *O. majorana* was effective, but less than *A. sativum*, *N. sativa* and NTZ.

In the present study, extracts of *P. nigrum* and *Z. officinale* insignificantly lowered *Blastocystis* number (P =0.3 & P= 0.7, respectively) with low growth inhibition rates and slightly increased (1.4% & 1.4%) after 24h to (4% & 1.7%) after 48h to (4% & 1.8%) after 72hrs respectively. This agreed with Yakoob *et al*. (2011), who reported that *Blastocystis* isolates were neither sensitive to ginger nor black pepper compared to garlic and MTZ. But it disagreed with Abdel-Hafeez *et al.* (2015), who found that ginger reduction rate after 48h was 92.98%.

Herbs anti-*Blastocystis* activity is related to high phenolic and flavonoid compounds with antioxidant and free radical activities (Meabed *et al*, 2018). Isolation and purification of extracts' bioactive fractions reduced their effective dose (Al-Khalaf *et al*, 2023).

Conclusion

Blastocystis vary in degree of susceptibility to herbal extracts. Natural herbs showed an alternative blastocystosis treatment not only reduce drug resistance but also reduce its side effects and treatment costs.

Conflict of interest: Author neither has conflicts of interest nor received any funds.

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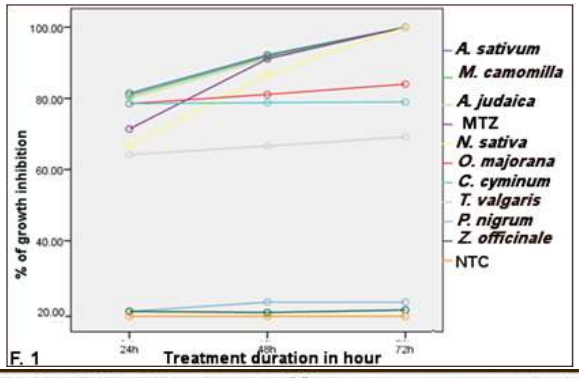
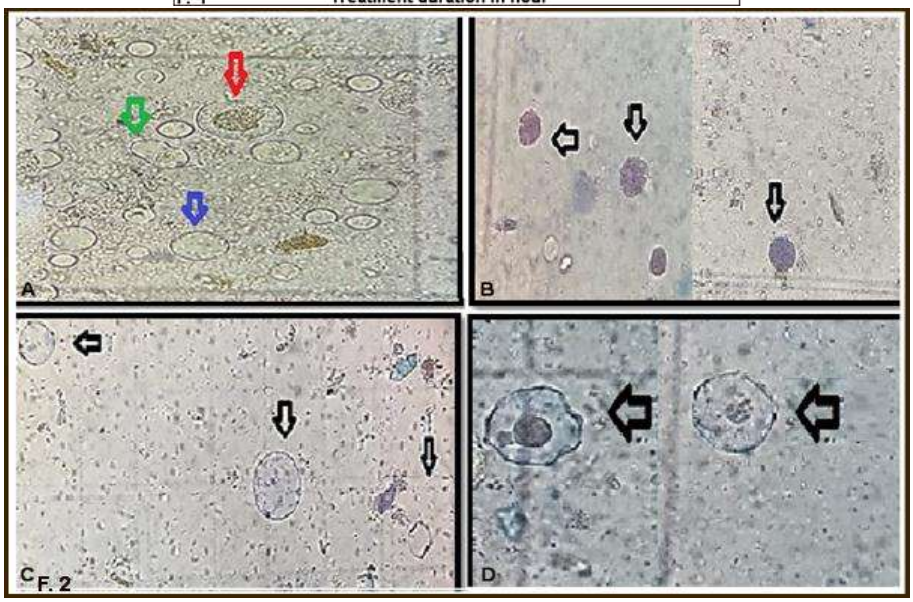
Explanation of figures

Fig. 1: *Blastocystis* spp growth inhibition% in culture medium after exposure to same concentration of herbal extracts and MTZ for different incubation periods. NTC showed 0% growth inhibition.

Fig. 2: A-Culture smear showed granular form (red arrow) and different vacuolar sizes (blue arrow) and binary fission (green arrow) of *Blastocystis* without exposure to extracts or MTZ (X1000). B, C &D- Trypan blue stained smears showed its death by herbal extracts (X1000).



F. 1



F. 2