Original Article

Anti-*Blastocystis in vitro* activity and *in vivo* efficacy of the ethanol extracts of *Origanum syriacum* L. and *Ceratonia siliqua*

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ABSTRACT

Background: Blastocystosis is significantly implicated in irritable bowel disease, and its current available drugs do not provide a complete cure and have several side effects.
Objective: In order to search for a safe natural alternative treatment for blastocystosis, the study investigated both *in vitro* activity and *in vivo* effect of two natural medicinal plants extracts, *Origanum syriacum* L. (Marjoram) and *Ceratonia siliqua* (Carob) on *Blastocystis* spp. isolated from symptomatic patients.
Material and Methods: The ethanolic extracts of the two plants, *O. syriacum* L. and *C. siliqua* were prepared in 3 concentrations (0.5, 2.5, 5.0 mg/ml), and screened phytochemically. *Blastocystis*-positive stool samples from symptomatic patients were cultured in Jones medium. Cultured *Blastocystis* cysts were used to screen the *in vitro* anti-*Blastocystis* activity of both plant extracts. Lab-bred mice were experimentally infected with cultured *Blastocystis* and treated with different concentrations of the extract of both plants to assess their *in vivo* effect. Metronidazole (MTZ) was used as a control drug in both *in vitro* and *in vivo* studies.
Results: Ethanolic extract of both plants showed dose and time dependent anti-*Blastocystis* effects both *in vitro* and *in vivo*. *C. siliqua* at 5.0 mg/ml showed the highest anti-*Blastocystis* efficacy and had a maximum significant growth inhibition rate compared to MTZ.
Conclusion: The ethanolic extract of *C. siliqua* is a promising, effective, safe, natural, environmentally-friendly, anti-*Blastocystis* alternative therapy. *O. syriacum* L. had a fair anti-*Blastocystis* effect and could be used as a supportive additive.

Keywords: *Blastocystis*; *Ceratonia siliqua* (Carob); *Origanum syriacum* L. (Marjoram); plant extracts; Syria.

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INTRODUCTION

Blastocystis spp. is one of the most common enteric, worldwide, single-celled protozoan parasites colonizing the large intestine of both animals and humans. It has a variable prevalence in humans, reaching 23% in industrialized countries and 100% in developing countries[1,2]. Blastocystosis may be asymptomatic or symptomatic with a wide range of unclear pathogenicity and several outcomes. It may lead to gastrointestinal symptoms, mainly diarrhea, or be associated with inflammatory bowel mediated disorders, gut dysbiosis, and colon cancer. It also causes inflammation in immunocompromised individuals[3,4].

Metronidazole (5-nitromidazole), the standard therapeutic drug for blastocystosis, is also the drug of choice to treat other parasitic infections including trichomoniasis, amoebiasis, and giardiasis, as well as infections caused by several anaerobic microorganisms[5,6]. Resistance of intestinal pathogens to MTZ was reported and attributed to the over prescription and empirical use of MTZ in abdominal disorders[7].

Natural plants and their products were used in traditional herbal medicine to treat different disorders and diseases. *O. syriacum* L., also known as Marjoram, Zaatar or green thyme, and *C. siliqua* known as Carob, are known medicinal plants common in the Mediterranean basin[8,9]. While *O. syriacum* L. belongs to the plant family Lamiaceae and its dry leaves are used as spices[10,11], *C. siliqua* belongs to family Fabiaceae[13]. Both plants are used as nutritional food, and medicinally to treat various abdominal disorders, especially diarrhea and abdominal pain, as well as promising eco-friendly green insecticides[9-14]. The pods and fruits of *C. siliqua* are also used as an anti-parasitic treatment for enteric parasites[9].

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In order to search for a safe alternative natural treatment for blastocystosis, we investigated both the *in vitro* activity and the *in vivo* effect of the ethanolic extract of these two natural medicinal plants against *Blastocystis* spp. isolated from symptomatic patients.

MATERIAL AND METHODS

This experimental study was conducted at the Parasitology Department, Faculty of Medicine, Aleppo University during the period from April to June 2021.

Study design: Stool samples were collected from patients attending the Internal Medicine Department in Aleppo University Hospital, and examined immediately for intestinal parasites. *Blastocystis* positive samples were cultured on Jones media. Isolated *Blastocystis* cysts were challenged *in vitro* and *in vivo* with the ethanolic extract of leaves of *O. syriacum* and dried pulp of *C. siliqua* at different concentrations. We used parasite burden as parasitological parameter to evaluate plant extract efficacy.

Stool sample collection: Stool samples were collected from 27 patients of both sexes, aged 18 years and older, complaining of GIT symptoms and attending the Internal Medicine Department at Aleppo University Hospital. Stool samples were examined immediately for intestinal parasites using wet mount before and after concentration[15]. Five samples were *Blastocystis* positive and were cultured on Jones media.

Plant extract preparation: Leaves of *O. syriacum* L. and dried pulp of *C. siliqua*, were collected from the local market of Aleppo city. The leaves were washed and dried at room temperature. Both dry plant materials were ground with a mortar and pestle into fine powder. Ethanol extraction of ground plants was performed as previously reported[15,16]. Briefly, in a glass flask 100 ml of 70% Ethanol, as extractor solution, was added to 40 gm of each dried plant. The solution was soaked for seven days in the dark, at room temperature with frequent shaking. The solution was then filtered to remove undissolved plant materials, and the extracted filtrate was evaporated at 45°C/90 rpm in a rotary evaporator to remove the solvent. The extracts were finally suspended in distilled water and kept until used[15-16].

Chemicals and supplies: Unless specified, all chemicals used in the present study were purchased from Merck (Germany).

Phytochemical analysis of plant ethanolic extracts: The ethanolic extracts of each plant filtrate were phytochemically screened for the presence of alkaloids, flavonoid, saponins, and tannins as follows:

1. **Alkaloid:** In water bath 2 ml of 1% HCl was added to 5 ml extract, followed by 2–4 drops of Dragendoff's reagent after a few min. The alkaloid was detected by the appearance of an orange reddish color[17].
2. **Flavonoid:** One ml of 2% NaOH solution was added to 1 ml extract in a test tube. The solution turned into an intense yellow color which became colorless on addition of a few drops of dilute acid indicating the presence of flavonoids[18].
3. **Saponins:** Ten ml of hot distilled water were added to 1 gm extract, then shaken vigorously for 15 min. Formation of stable persistent foam indicated the presence of saponins[19].
4. **Tannin:** Two drops of 1% $FeCl_3$ added to 2 ml of extract solution produced a dark blue color in the presence of tannins[17].

Drug control: Metronidazole, the reference anti-*Blastocystis* drug was used as drug control. It was purchased from a local pharmacy as 'Flagyl'. Metronidazole tablet (500 mg) was dissolved in 500 ml double distilled water to produce a stock solution of 1 mg/ml, kept in a dark bottle. Final concentrations of MTZ added to culture were adjusted to 100 µg/ml[20] and used in three replicates.

Isolation of *Blastocystis* parasite: Stool samples from symptomatic patients were cultured in fresh Jones' medium enriched with 10% horse serum (Liofilchem, Italy) in an incubator at 37°C[21-22]. Cultures containing more than 10^6/ml vacuolar forms of *Blastocystis* were used to assess the biological activities of the plant extract.

***In vitro* study:** Tubes of 500 µl of Jones' medium containing 3.8×10^6/ml *Blastocystis* spp. vacuolar forms were prepared. Both plant extracts were added to obtain a final concentration of 0.5, 2.5 and 5.0 mg/ml for each plant, in three replicates for each concentration. In addition to the three MTZ drug control replicates, three culture tubes were left unchallenged as negative control. All experiment culture tubes were incubated at 37°C[20]. After incubation for 72 h at 37^0C, the challenged and unchallenged cultured *Blastocystis* cysts were assessed for their viability using Trypan blue solution (0.4%). The number of viable cysts in each culture tube was counted and the percentage of inhibition in their growth was calculated using a Neubauer's hemocytometer[3].

***In vivo* study:** Four weeks old BALB/c mice (n=63), lab bred in the animal house of Faculty of Sciences, Aleppo University, were used for the *in vivo* experiment. Each mouse was infected orally with 4.8×10^6 *Blastocystis* vacuolar form/ml in 0.9% saline. The infection was confirmed by direct examination of mice stool samples six days post inoculation. Then, the animals were divided into 5 groups as follows:

- Group I (7 mice): Non-infected, untreated group (negative control).
- Group II (7 mice): *Blastocystis*-infected, untreated group (positive control).

- Group III (21 mice): *Blastocystis*-infected and treated orally with three concentrations (0.5, 2.5, and 5.0 mg/ml) of Marjoram extract.
- Group IV (21 mice): *Blastocystis*-infected and treated orally with three concentrations (0.5, 2.5, and 5.0 mg/ml) of Carob extract.
- Group V (7 mice): *Blastocystis*-infected and treated orally with 100 mg/ml MTZ (drug positive control).

After 24 h post treatment, the number of excreted cysts in stool samples were counted daily for each one of the experimentally infected mice for 6 d using Neubauer's Hemocytometer.

Statistical analysis: The software SPSS V.21 was used to statistically analyze the recorded data presented as mean and standard deviation (SD). Means were calculated for each of the three independent replicates. Means were compared and variances were analyzed. *P* values of <0.05 were considered statistically significance.

Ethical considerations: The research was approved by the ethical committee of the Faculty of Science at Aleppo University. All patients were informed verbally about the research purpose, and the collection of stool specimens was completed after obtaining their consent. The animal experiments were performed in accordance with the Aleppo ethical committee for laboratory animals research guidelines and with the ethics guidelines of the Helsinki Declaration.

RESULTS

Qualitative phytochemical screening of the ethanolic extracts of the plants showed that they contained secondary metabolic substances that included alkaloids, flavonoid, saponins, and tannins. While flavonoid and saponins were detected in *O. syriacum* L. leaves, dried pulp of *C. siliqua* contained all the screened phenols. The two tested plant extracts showed a dose dependent inhibition of growth of *Blastocystis* cysts after 72 h incubation at 37°C (Table 1). Ethanolic extract of *C. siliqua* (5.0 mg/ml) had the highest activity on growth of *Blastocystis* cysts with a statistically significant difference of 82.53% ($P<0.05$) inhibition rate compared to MTZ (76.44%).

Anti-*Blastocystis* efficacy of ethanolic extracts of both *O. syriacum* L. and *C. siliqua* in treating infected mice compared to MTZ is shown in table (2). Ethanolic extract of *C. siliqua* at 5.0 mg/ml showed the highest anti-*Blastocystis* efficacy with 92.51% reduction in excretion of *Blastocystis* cysts by the seventh day post treatment and statistically significant difference ($P<0.05$) compared to MTZ (82.05%).

Table 1. Anti-*Blastocystis* activity of *O. syriacum* L. and *C. siliqua* extracts compared to metronidazole.

	Conc.	No.	GR	IR
Negative control		3.72×10^6	100%	0%
C. siliqua	0.5	2.35×10^6	63.17%	36.83%
	2.5	1.71×10^6	45.96%	54.04%
	5.0	6.50×10^6	**17.47%***	**82.53%***
***O. syriacum* L.**	0.5	1.72×10^6	45.26%	54.74%
	2.5	1.52×10^6	40.00%	60.00%
	5.0	1.43×10^6	37.63%	62.37%
Metronidazole	100	9.46×10^6	25.26%	74.44%

Conc.: Concentration (mg/ml); **No.:** Number of vacuolar forms in cultures; **GR:** Growth rate; **IR:** Inhibition rate. ***:** Significant ($P<0.05$).

Table 2. Anti-*Blastocystis* efficacy of *O. syriacum* L. and *C. siliqua* extracts compared to metronidazole in infected mice.

	Conc.	Inhibition rate %					
		Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Negative control		0	0	0	0	0	0
C. siliqua	0.5	12.97	25.38	31.64	38.81	45.73	53.71
	2.5	15.40	30.12	37.54	46.06	54.27	63.74
	5.0	22.35	43.72	54.49	66.86	78.77	**92.51***
O. syriacum **L.**	0.5	15.19	29.70	37.03	45.43	53.53	53.53
	2.5	16.80	32.86	32.49	50.26	59.22	69.55
	5.0	18.08	35.37	44.09	54.10	63.74	74.85
Metronidazole	100	18.82	38.77	48.33	59.30	69.86	82.05

Conc.: Concentration (mg/ml); ***:** Significant ($P<0.05$).

Figure (1) shows that the ethanolic extract of *C. siliqua* at 5.0 mg/ml had the highest anti-*Blastocystis* efficacy both *in vitro* and *in vivo* (82.53% and 92.51% respectively) than MTZ (74.44% and 82.05% respectively) and ethanolic extract of *O. syriacum* L. (62.37% and 74.85% respectively), with statistical significance (P>0.001).

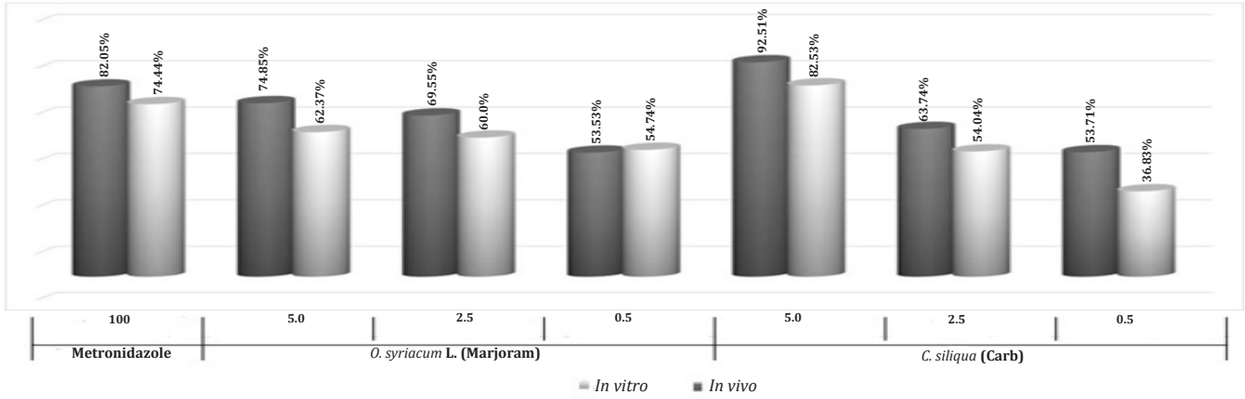

Fig. 1. Anti-*Blastocystis in vitro* activity and *in vivo* effect of *O. syriacum* (Marjoram) and *C. siliqua* (Carb) extracts compared to metronidazole.

DISCUSSION

To date, the emerging protozoal infection, blastocystosis, has no full cure. Due to its medicinal properties, being accessible, cost-effective, and naturally safe, herbal medicine gained particular interest as an alternative therapy for various human disorders and diseases. To find an alternative treatment for blastocystosis, the extract of two herbal medicinal plants were tested for their *in vitro* and *in vivo* effects[23-24]. Metronidazole was previously used in a small dose of 10 ug/ml as anti-*Blastocystis* treatment[25-26]. In the present study, a dose of 100 ug/ml of MTZ was used as a drug control.

The plants *O. syriacum* L. (thyme) and *C. siliqua* (Carob) and their products are safe and non-toxic, and are commonly used in food preparation and in many dishes in Mediterranean basin[8-9]. Buds and leaves of *C. siliqua* were used to treat abdominal disorders[8]. Lambs infected with nematodes helminth, *H. contortus* and *T. colubriformis*, were cured when fed *C. siliqua* pods. The study related the anti-helminthic effect of *C. siliqua* to its tannin contents[27]. The pods effectively treated lamb coccidiosis[28].

Additionally, *O. syriacum* L. showed antimicrobial properties in many studies[29]. Its alcoholic extract showed effective antiparasitic activity against the protozoan parasite, *A. castellanii*[29-30]; while its essential oil showed insecticidal effect against the mosquito *Culex*, and anti-parasitic effect against the nematode *A. simplex*[31].

Both plants have promising anti-parasitic activity, but their activity and efficacy as anti-*Blastocystis* require assessment. In the present study, we evaluated the *in vitro* activity and *in vivo* effects of their ethanolic extracts on the viability of the *Blastocystis* parasite for the first time. The ethanolic extracts of both plants showed promising anti-*Blastocystis* activity and efficacy that was dose- and time-dependent with all used concentrations compared to the MTZ drug control.

Our results also verified that in addition to tannins, flavonoids are a major constituent of the ethanolic extract of both plants. Several studies related these plants' anti-microbial activities to induction of cellular stress by reducing oxygen consumption by parasitic cells. Their antioxidant capacity is attributed to their high contents of flavonoids and other phenolic contents[32-33].

In conclusion, based on our findings using simple, cheap, reproducible, and eco-friendly techniques, we obtained effective, safe, natural, environmentally-friendly plants extracts. Particularly *C. siliqua*, proved effective as an anti-*Blastocystis* therapy, providing a new approach for designing promising novel alternative anti-protozoal and blastocystosis therapies. *O. syriacum* L. had fair anti-*Blastocystis* outcome and could be used as an additive to support the etiological treatment of blastocystosis. The antiparasitic effect of plant extracts may be attributed to the antioxidant capacity of their phenolic

components. More studies are required to purify and isolate the bioactive fractions of these extracts and determine their active ingredients.

In conclusion, the ethanolic extract of *C. siliqua* is a promising, effective, safe, natural, environmentally-friendly, anti-*Blastocystis* alternative therapy. *O. syriacum* L. (Marjoram) had a fair anti-*Blastocystis* effect and could be used as a supportive additive.

Author contribution: ALKhalaf E, Alabdurhman G and Assany Y proposed the study topic and performed the practical work. All authors planned the study design and equally contributed in data analysis, and writing of the original draft. Alabdurhman G and Assany Y supervised the work. All authors reviewed the final version.

Conflict of interest: The authors declare that there is no conflict of interest.

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