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Inhibitory effect of thymoquinone and capsaicin on *Blastocystis* grown *in vitro*

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Blastocystis is enteric parasites that live in both humans and animals gastrointestinal tracts. Metronidazole (MTZ) is generally preferred in the treatment of *Blastocystis* infection. However, it has been shown to have teratogenic and carcinogenic potential, causing various side effects. In this study, we have made an attempt to find an alternative drug with less toxic side effects in the treatment of *Blastocystis*. For this purpose, the anti-*Blastocystis* activities of thymoquinone (TQ) and capsaicin (CAP) were evaluated *in vitro*. *Blastocystis* isolate was inoculated in Jones medium in 1.5 mL eppendorf tubes supplemented with fetal calf serum (FCS) and incubated at 37°C. The anti-*Blastocystis* effect of TQ and CAP was evaluated by light microscopy (LM) and scanning electron microscopy (SEM). Both TQ and CAP had anti *Blastocystis* effects. It was observed that 500 ug/mL of TQ and 1000 ug/mL of CAP inhibited 100% of *Blastocystis* growth. In the LM and SEM images, it was observed that *Blastocystis* treated with TQ decreased in size and CAP had an effect on the cell surface when compared to the control group. It was concluded that TQ could be a more reliable anti-*Blastocystis* drug compared to MTZ and CAP, but more comprehensive studies should be performed.

Keywords: Capsaicin, Gut infection, Metronidazole, Thymoquinone

Blastocystis is a unicellular anaerobic opportunistic protozoan parasite that lives in the human intestinal tract as well as many animals[1]. Its prevalence is over 50% and 100% in developing countries and between 10% and 15% in developed countries[2]. Transmission of this parasite occurs by the fecal-oral route, especially in people who are frequently exposed to animals and contaminated food, and whose environment is poorly sanitized. As it can be seen asymptomatically, it can cause many symptoms, such as nausea, vomiting, abdominal cramps, fatigue, and diarrhea, especially in children and immuno-compromised patients. In addition, blastocystosis has been associated with irritable bowel syndrome, inflammatory bowel disease, and dermatological disorders such as urticaria and rash[3]. In a particular research investigation, it was determined that the *Blastocystis* antigen is effective in the growth of tumor cells occurring in the colon[4].

Although Metronidazole (MTZ) is the most recommended drug for the treatment of *Blastocystis*, it has been reported that this drug has undesirable side effects such as metallic taste, headache, dry mouth and nausea, glossitis, urticaria, itching, and dark urine[5].

In Türkiye, the leaves, stems, fruit, and seeds of many plant species have been used for years to treat diarrhea in alternative medicine. Because of the significant side effects and expensiveness of medicinal drugs, herbal compounds are widely used by the local population. Some of these herbal compounds may have therapeutic effects against different parasites and may turn into licensed drugs for these infections in the future[6].

Thymoquinone (TQ) is the bioactive constituent of the volatile oil of black seed; It exhibits outstanding antioxidant, anti-inflammatory, anticancer, and other important biological activities[7].

Capsaicin (CAP) is the active ingredient of chili peppers obtained from the plants of genus *Capsicum*, the most heavily consumed chili in the world. A class of chemicals with great potential through anti-inflammatory, anti-oxidant, and antitumoral effects is phytochemicals[8].

In this study, considering the need for alternative drugs with fewer side effects for the treatment of *Blastocystis*, we evaluated the efficacy of Thymoquinone (TQ) and Capsaicin (CAP) *in vitro*.

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Materials and Methods

Culture of *Blastocystis*

For the human-derived isolate of *Blastocystis*, a stool sample was obtained from a *Blastocystis* positive patient after obtaining informed consent. A small amount of the stool sample was taken (approximately the size of a grain of rice). They were cultured in Jones medium in 1.5 mL eppendorf tubes supplemented with fetal calf serum (FCS) and incubated at 37°C. After 72 h, when the vacuolar/granular forms of *Blastocystis* were observed, they were subcultured[9].

Preparation of the TQ, CAP, and MTZ solutions

TQ (Thymoquinone, Santa Cruz Biotechnology, USA), CAP (Capsaicin, Santa Cruz Biotechnology, USA), and MTZ (Nidazol®, İ.E. Ulagay Pharmaceutical Industry, Turkey) as a reference drug for anti-*Blastocystis* were used. These compounds and the drug were dissolved in dimethyl sulfoxide (DMSO), which was previously proven to have no effect on parasite reproduction[10]. The stock solution was prepared for different concentrations of the TQ, CAP, and MTZ.

Experimental design

The study comprised four groups. In the first group, five different concentrations of MTZ, at final amounts of 3.125, 6.25, 12.5, 25 and 50 µg/mL were used in the culture[11]. In the second and third groups, TQ and CAP in six different concentrations, with final amounts of 31.25, 62.5, 125, 250, 500, and 1000 µg/mL were used in the culture. The negative control for the fourth group consisted of Jones medium only.

Cultures containing approximately 2×10^5 parasites/mL of *Blastocystis* were obtained by subculturing. MTZ, TQ, and CAP (50 µL of each mixed with DMSO) were added separately to tubes containing *Blastocystis* and Jones culture. Culture tubes were incubated at 37°C for 24, 48 and 72 h. Each sample was tested in triplicate[9].

Counting viable cells under a light microscope

The viable cells in all of the culture tubes were counted at 24, 48 and 72 h of inoculation. After the culture tubes were turned upside down, 10 µL of the culture was taken and mixed with 10 µL of 0.1% eosin solution. The preparation prepared with a Neubauer hemocytometer was examined under a light microscope with a 40X objective lens and the living forms of *Blastocystis* were counted. Parasites that stained orange were considered dead, while parasites that did not stain were considered viable[9].

Each culture tube was counted in triplicate and the mean parasitic counts were calculated. Accordingly, the minimum inhibition concentration (MIC) of the TQ, CAP and MTZ achieved in the lowest concentrations were calculated. The parasite growth inhibition percentage was calculated according to the equation (A – B/A) × 100, where A is the mean number of viable *Blastocystis* in the control cultures and B is the mean number of viable *Blastocystis* in the treated cultures. MIC90 is the value at which 90% of *Blastocystis* growth is inhibited. This value was obtained from the curve of growth inhibition percentage versus different concentrations of tested compound[9].

Scanning electron microscopic study

The effects of the TQ and CAP on *Blastocystis* were examined under scanning electron microscopy. Preparations were prepared by taking samples from cultures containing 1000 µg/mL in the TQ and CAP groups, and the control group culture at 72 h for examination. Cell culture samples were washed thrice using phosphate buffered saline (1M PBS, pH 7.4) and then centrifuged for five min at $2000\times g$[12]. The resulting pellet was fixed with methanol and spread on a slide for SEM images.

Statistical analysis

The study used means, standard deviations, counts and proportions as descriptive statistics. SPSS (Ver. 26) was used for calculations.

Results

Forms of *Blastocystis* isolated from stool samples showed an increase in cell culture after 72 h (Fig. 1).

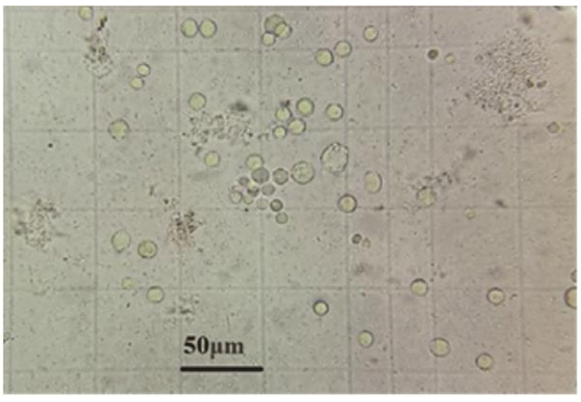

Fig. 1 — *Blastocystis* forms grown in culture

It was determined that both TQ and CAP had anti-*Blastocystis* effects (Table 1). It was observed that TQ at 500 µg/mL and CAP at 1000 µg/mL inhibited 100% of *Blastocystis* growth.

The MIC90 values of the MTZ, TQ and CAP are given in Fig. 2. According to the inhibition data, the MIC90 value was calculated at a 14.67 times higher concentration at 24 h of incubation, 10.89 times higher at 48 h of incubation, and 4.24 times higher at 72 h of incubation for the TQ when compared to the MTZ. Similarly, the MIC90 value was calculated at a concentration of 25.35 times higher at 24 h of incubation, 28.42 times higher at 48 h of incubation, and 46.98 times higher at 72 h of incubation for the CAP when compared to the MTZ (Fig. 2).

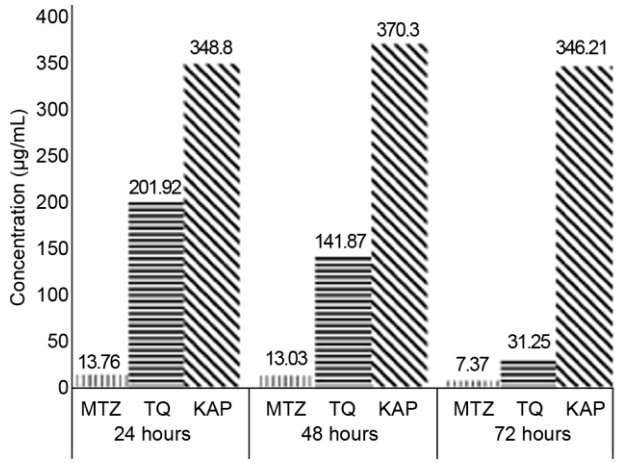

Fig. 2 — MIC90 values of the Metronidazole (MTZ), Thymoquinone (TQ), and Capsaicin (CAP) after incubation in the cultures of *Blastocystis* for 24, 48 and 72 h

While vacuolar, granular, and cystic forms of *Blastocystis* in the control group were detected, in the TQ and CAP groups, only the granular form was detected. LM and SEM images showed that the *Blastocystis* forms affected by TQ decreased in size when compared to the *Blastocystis* forms in the control group. It was observed in the LM and SEM images that CAP destroyed the cell surface (Figs 3 and 4).

Discussion

Although many *Blastocystis* infections are asymptomatic, studies have suggested that the infection causes gastrointestinal symptoms in children and adults. Therefore, researchers insist on the necessity of treatment in *Blastocystis* infections[13]. Various drugs have been used against *Blastocystis*. MTZ is most commonly used in the treatment. Other treatment options include nitazoxanide, trimethoprim-sulfamethoxazole (TMP-SMX), paramomycin, ornidazole, fluconazole, secnidazole, furazolidone and albendazole. Although the infection can be treated with these drugs, it has been reported that *Blastocystis* develops resistance to these drugs, as in some other protozoa[14].

Table 1 — Growth inhibition rates of the MTZ, TQ, and CAP after incubation with cultures of *Blastocystis* for 24, 48 and 72 h (2×10^5 cells/mL in Jones's culture)

	Concentration (µg/mL)	24 h		48 h		72 h	
		Mean±SD*	% Growth inhibition	Mean±SD*	% Growth inhibition	Mean±SD*	% Growth inhibition
Control	-	4.63±0.14	0	3.44±0.06	0	2.52±0.04	0
MTZ	50.00	0	100	0	100	0	100
	25.00	0.27±0.03	94.1	0.13±0.03	96.2	0	100
	12.50	0.41±0.03	91.1	0.29±0.03	91.5	0.18±0.03	92.8
	6.25	0.67±0.04	85.5	0.39±0.04	88.6	0.21±0.03	91.6
	3.12	1.14±0.04	75.3	0.97±0.05	71.8	0.36±0.03	85.7
TQ	1000.00	0	100	0	100	0	100
	500.00	0	100	0	100	0	100
	250.00	0.38±0.06	91.7	0.21±0.03	93.8	0.12±0.03	95.2
	125.00	0.53±0.05	88.5	0.33±0.03	90.4	0.14±0.03	94.4
	62.50	0.68±0.03	85.3	0.4±0.03	88.3	0.17±0.04	93.2
	31.25	0.78±0.03	83.1	0.51±0.03	85.1	0.23±0.04	90.8
CAP	1000.00	0	100	0	100	0	100
	500.00	0.34±0.02	92.6	0.19±0.03	94.4	0.09±0.03	96.4
	250.00	0.48±0.04	89.6	0.29±0.05	91.5	0.12±0.03	95.2
	125.00	0.82±0.03	82.2	0.9±0.2	73.8	0.63±0.03	75.0
	62.50	1.43±0.06	69.1	1.21±0.02	64.8	1.02±0.03	59.5
	31.25	1.91±0.03	58.7	1.59±0.04	53.7	1.17±0.03	53.5

[MTZ, Metronidazole; TQ, Thymoquinone; and CAP, Capsaicin]

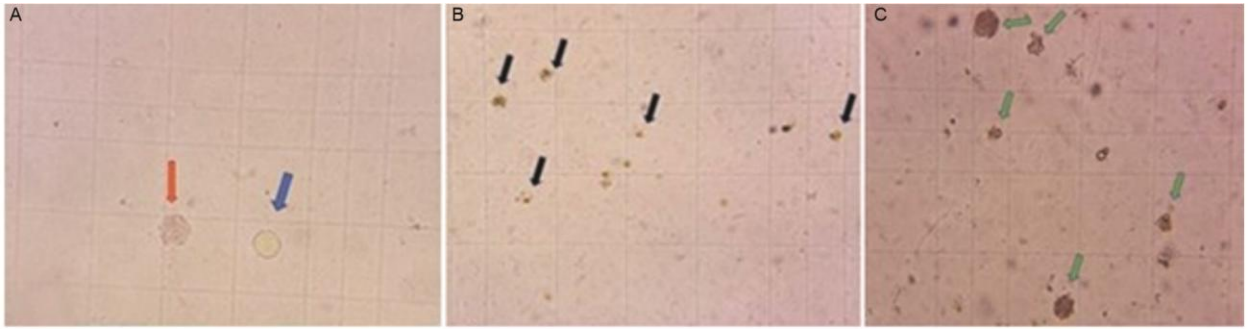

Fig. 3 — LM appearance of *Blastocystis* (A) Control live (blue arrow) and dead (red arrow) blastocystis; (B) Thymoquinone (TQ) dead (black arrow) *Blastocystis*; and (C) Capsaicin (CAP) dead (green arrow) *Blastocystis*

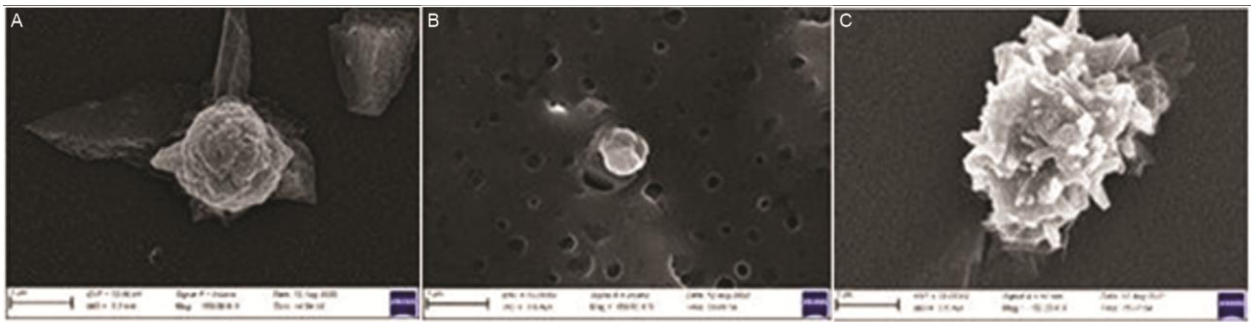

Fig. 4 — SEM appearance of *Blastocystis* (A) Control; (B) Thymoquinone (TQ); and (C) Capsaicin (CAP)

Blastocystis may gain resistance to drugs due to reasons such as emergence in different geographical regions, pathogenicity, the immune system of the host, human intestinal microbiota and the subtype. This may result in treatment failure. In this status, it may be beneficial to use natural herbs, vegetables, or spices as an alternative in the treatment of blastocystosis. In addition, such treatments reduce the side effects of drugs and the cost of treatment[15]. Natural extracts from plants are sources of valuable bioactive compounds that have traditionally been used to treat diseases from simple headaches to parasitic infections[16]. Numerous *in vivo* and *in vitro* studies have been conducted against *Blastocystis* using different plant extracts and have been successful[13]. It is known that the aqueous extracts of *Origanum majorana* and *Foeniculum vulgare* inhibited *Blastocystis* proliferation[16], the use of *Artemisia judaica* with dichloromethane (DCM), ethyl acetate (EtOAc) and n-hexane is effective against *Blastocystis* subtypes ST1 and ST3[11]. In addition, the aqueous solution of *Allium tuncelianum* is effective against symptomatic diarrhea caused by the subtype of *Blastocystis* ST3[9]. Another study reports that *Syzygium aromaticum* has a strong lethal effect on *Blastocystis* compared to MTZ, and a significant reduction in the cell diameter was observed in *Blastocystis* treated with this plant compared to the control group[17]. Similar to our study, extracts of *Eurycoma longifolia*[18], *Ceratonia siliqua*[3], *Boesenbergia rotunda* and *Ganoderma lucidum*[19] have shown potential medicinal benefits in inhibiting the growth of *Blastocystis*.

The TQ used in this study was the most abundant component in the *Nigella sativa* seed. *Nigella sativa* is known to have a strong lethal effect on *Blastocystis* isolates[20]. However, it is not known whether this effect is due to TQ or other components found in the *Nigella sativa* seed. TQ has been proven to be effective against *Encephalitozoon intestinalis*[21] *Leishmania donovani*[22], *Entamoeba histolytica*, *Giardia lamblia*[23], pyroplasma parasites[24] and *Fasciola gigantic*[25]. For the first time in this study, 100% *Blastocystis* inhibition was detected using TQ at a concentration of 0.5 mg/mL for 24 h.

CAP is a component found in chili pepper. In the literature review, no studies were found on the effect of this component on *Blastocystis*. However, it is known that CAP has a lethal effect on protozoa such as *Trypanosoma brucei*[26], *Trypanosoma cruzi*[27], and *Leishmania infantum*[28]. It was determined for the first time here in that CAP at a concentration of 1 mg/mL in 24 h resulted in 100% *Blastocystis* inhibition.

In this study, the MIC90 value was found to be effective at higher concentrations of both TQ and CAP when compared to MTZ. However, MTZ has various side effects such as microbiota disorder, dizziness, nausea, and headache. Therefore, it is important to know the toxic effects of TQ and CAP, which have anti-*Blastocystis* effects and can be a potential drug in this study. It was reported that oral TQ doses in the range of 10–100 mg/kg did not show any toxic or lethal effects in mice[29], the maximum tolerated dose in both male and female rats for oral ingestion was 250 mg/kg[30], when TQ was given orally to mice, the LD_{50} value was 870.9 mg/kg. It was emphasized that TQ is a very safe compound, especially when given orally to mice and rats[31].

The ingestion of CAP at low doses has been shown to have numerous benefits on the gut microbiota, primarily targeting metabolic and inflammatory diseases, including diverse and complex mechanisms[32]. It has been reported in many studies that the use of high doses of CAP has toxic effects. Santos et al (2023) showed that CAP concentrations of 100, 150, 175 and 200 µM for 24 h showed lower and different values than the negative control and therefore had a cytotoxic effect on hepatoma cells of *Rattus norvegicus*[33]. Other authors have also shown that Capsaicin reduces the viability and proliferation of pancreatic neuroendocrine tumor cells after 24 h of exposure with concentrations ranging from 10 to 200 µM, depending on the dose[34]. İn an *in vivo* study against *Toxoplasma gondii*, CAP exhibited the highest survival probability after pyrimethamine-sulfadiazine[35]. Other studies have shown that the use of CAP *in vivo* at low doses has shown high bioactivity, but the use of high doses has caused serious side effects[36]. The ingestion of large quantities of CAP can cause nausea, vomiting, abdominal pain, and burning diarrhea in humans[37].

Conclusion

This study has demonstrated that both thymoquinone (TQ) and capsaicin (CAP) have anti-*Blastocystis* effects. TQ at 500 µg/mL, CAP at 1000 µg/mL, and Metronidazole (MTZ) at 50 µg/mL inhibited *Blastocystis* growth by 100% after 24 h of treatment. *Blastocystis* cells treated with TQ, CAP and MTZ showed deformity in the form of withered shapes, when compared with the control. LM and SEM images showed that *Blastocystis* forms affected by TQ decreased in size compared to *Blastocystis* forms in the control group, and CAP also damaged the cell surface. According to these findings the effect of TQ and CAP on *Blastocystis* is promising. It was concluded that TQ may be a more reliable anti-*Blastocystis* drug when compared to MTZ and CAP, but more comprehensive studies are needed. In addition, it was concluded that the daily use of *Capsicum annuum* (hot pepper) and *Nigella sativa* (black cumin) containing these components may be a precaution against blastocystosis infections.

Ethical approval

The study protocol was approved by Van Yuzuncu Yıl University, Non-Interventional Clinical Research Ethics Committee (10.06.2022 / Decision no: 2022/06-12).

Conflict of interest

Authors declare no competing interests.

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