The potential therapeutic effect of Amygdalin (B17), Cobalamin (B12), and Cucurbitapepo (pumpkin seed) extract on Blastocystis in the murine model.

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ABSTRACT:

Background: The pathological role of Blastocystis sp. is very controversial which has led to debates on the treatment of this organism, apart from the absence of established diagnostic criteria. Metronidazole is the recommended drug for the treatment of Blastocystis sp. infection but drug-resistant strains appeared and many complications were met by this treatment. Therefore, many studies have been involved in the research of new Blastocystis sp. therapies. Natural medicine has many positive aspects and provides a very successful new diet for humans.

Aim: The current research has been designed to investigate how amygdalin (B17), cobalamin (B12), pumpkin seeds, and metronidazole are effective against Blastocystis sp. versus the nowadays drug of choice Metronidazole.

Material and methods: 80 Swiss albino mice were divided into two main groups. The first group (I) comprising of 20 immunocompetent mice and subdivided into 2 sub-groups: subgroup Ia; noninfected non treated (control negative) and subgroup Ib; infected non treated. The second group (II) comprising of 60 mice immunosuppressed by Dexamethasone and subdivided into 6 sub-groups: subgroup IIa; noninfected non treated, subgroup IIb; infected non treated, subgroup IIc; infected and treated with B17 (100 mg/kg), subgroup IId; infected and treated with B17 (50 mg/kg) + B12 (50 µg/mg/kg), subgroup IIf; infected and treated with pumpkin seeds (120 mg/kg) and subgroup III; infected and treated with metronidazole (100 mg/kg) Blastocystis sp. shedding (cysts) in feces, Liver function tests in serum and glutathione (GSH) and malondialdehyde (MDA) levels were assessed for the study groups.

Results: Treatment of infected mice reduced the shedding of cysts significantly compared to the infected untreated group. Metronidazole showed a statistically significant reduction (p < 0.05) about 85%. While B17, B17 + B12, and pumpkin seeds decreased shedding of cysts by 25, 31, and 57% respectively. Serum Liver enzymes, aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) levels were significantly (p<0.05) increased in Blastocystis sp. infected groups but the levels decreased in groups treated with B17, B17 + B12 and pumpkin seeds especially when treatment with vitamins B17 + B12 compared with control mice. Our study indicates that Blastocystis sp. infestation induces biochemical alterations in mice in the form of increased MDA level and
decreased the GSH level (p<0.05). Furthermore, the altered MDA and GSH values tend to be standardized by using B17, B17 + B12, pumpkin seeds, and metronidazole compared to that of the uninfected controls.

**Conclusion:** Metronidazole is the best treatment for *Blastocystis* sp. while B17, B17 + B12, and pumpkin seeds are promising natural therapeutic agents against *Blastocystis* sp.

**Keywords:** *Blastocystis*, Amygdalin (B17), Cobalamin (B12), pumpkin seed, glutathione

**Introduction:**
Parasitic diseases affecting the intestine are one of the main public health problems of the world [1]. *Blastocystis* sp. is one of the most prevalent human intestinal protozoa in the world [2]. Its prevalence varies from country to country depending on the used diagnostic and sanitary conditions. In developing countries, the prevalence exceeds 50% [3] and it reached around 20% in developed countries [4].

*Blastocystis* sp. was shown to be involved in various intestinal disorders. Several studies have identified its possible pathogenic factors and the presence of human symptoms was linked to *Blastocystis* sp. infection [5].

The controversy remains about *Blastocystis* sp.’s pathogenic function. Different variables that may connect the parasite itself, including the parasite load, with its subtype, or with the host such as host immune and dysbiosis, may influence the onset of the disease. [6,7].

The transmission of this organism could be directly through contaminated hands or indirectly through polluted food or water with human/animal feces [8,9].

A variety of hepatic disorders have investigated the therapeutic importance of serum aspartate aminotransferase ratios (AST) to alanine aminotransferase (ALT) (10). In a study done by [11], no substantial association exists between certain parasites such as *Entamoeba histolytica*, *Entamoeba dispar*, *Giardia lamblia*, *Blastocystis hominis* or *Cryptosporidium parvum* in Egyptian children with chronic liver disorders and elevated liver function tests (AST and ALT). Lipid peroxidation which causes systemic biological damage from infections with parasites is one of the best indicators of reactive oxygen (ROS) levels [12]. In many studies, free radicals are imbalanced with antioxidants and lipid peroxidation where Malondialdehyde (MDA) can be used to indicate the final product [13].

Metronidazole is also the recommended medicine for *Blastocystis* infection. However, insufficient treatment indicates the development of isolates that do not respond to treatment. However, insufficiency in treatment indicates the development of isolates not responding to therapy [14]. Many disadvantages and hazards are met with Metronidazole such as migraine, dizziness, queasiness, and metallic tastes in the mouth, pancreatitis, increased central nervous system dose toxicity, and reversible neutropenia.
In nature, many sources contain Amygdalin such as the fruit of Prunus Pipes as apricot, water, feather, bitter almond like coarse nuts, clover, lima beans, and the sorge[15]. These sources have obtained a great interest in cancer therapy[16].

Amygdalin and a modulated version named laetrile both have been utilized as an alternative therapy for tumors nearly 60 years ago[17]. Also, it was used to treat many disorders such as asthma, nausea, leprosy, bronchitis, and leukoderma[18]. It also benefits the digestive system, where it has a calming and defensive effect; as well as the urinary system, where human renal fibroblast apoptosis is encouraged and kidney function improved[19]. The amygdalin community also significantly down-regulates the levels of ALT and AST. These enzymes can be perceived in the liver as inflammatory indexes[20].

Vitamin B12 [cobalamin, Cbl, B12] is an essential micronutrient water-soluble that is used to mitochondrial methylmalonyl-CoA mutase (MCM) and cytosolic methionine synthase (MS) coenzyme, deficiency of B12 leads to neurological decline and anemia and may lead to death if left untreated[21].

Nutrition with Vitamin B12 has two unique features that make human nutrition pose problems: One is pernicious anemia (PA), unique human autoimmune disease, and the other is the virtual restriction of B12 to foods from animal origin[22].

Pumpkin, a common palatable plant, has major medicinal characteristics because of the presence of essential substances to the body involving specific Phyto-constituents with alkaloid classifications and different biologically active amino acids[23]. Pumpkin seed is used in various parts of the world as a traditional medicine to treat gastrointestinal parasites with anthelmintic properties, especially against tapeworms[24].

Materials and methods

1. Animals:

A. Animal source and handling:
For the research, 80 Swiss albino mice, (weighing 20-25 gram and of 10-12 weeks’ ages) obtained from the Iraqi center for Cancer Research, Baghdad, Iraq were used. The animals were kept in well-aerated plastic cages with a normal diet of 24% protein, 4% fat, and around 4 to 5% fiber. Water ad libitum was given during the study. Mice were housed at 25°C ±2°C, with the humidity maintained at 55%, wood chip bedding, on a 12:12 hour dark/light cycle, twice a week, the cages had been washed to ensure good safety before they had to be used. The mice were allowed 1 week before the experiment to adjust to the laboratory environment and their feces were tested daily microscopically to eliminate the existence of parasites.

B. Animal groups:
80 Swiss albino mice were divided into two main groups. The first group (I) comprising of 20 immunocompetent mice and subdivided into 2 sub-groups of 10 mice each: subgroup Ia; noninfected non-treated (control negative) and subgroup IIa; infected non-treated. The second group (II) comprising of 60 mice immunosuppressed by Dexamethasone and subdivided into 6 sub-groups of 10 mice each: subgroup IIb;...
IIa: noninfected non treated, subgroup IIb: infected non treated, subgroup IIC: infected and treated with B17, subgroup IID: infected and treated with B17 + B12, subgroup IIE: infected and treated with pumpkin seeds and subgroup IIIF: infected and treated with metronidazole.

C. Immunosuppression:

Immunosuppression was performed by giving the animals synthetic corticosteroids (dexamethasone sodium phosphate) (Dex), produced and supplied by (DEXOJECT, Turkey), intramuscularly at a dose of 100µL/mice twice in the first week then the mice were continues taken 4mg L⁻¹Dex.added water daily for another two weeks before oral inoculation with Blastocystis cysts[25].

D. Infection:

Fresh fecal samples were taken from 16 patients complaining of gastrointestinal symptoms like diarrhea, abdominal pain, and distention from the Central Pediatric Teaching Hospital in Baghdad, Iraq. The stool samples were collected in sterile clean stool cups and transferred to Parasitology Laboratory at the college of life Sciences for women. Fecal samples were microscopically tested using direct wet smear immediately in saline and stained with Lugol's iodine then the concentration technique was done using formalin-ethyl acetate[26]. fecal samples with Blastocystis sp.were cultured in Jones’ medium (3ml) not contain rice starch and enhanced with 10% horse serum [27], 100 IU/µL penicillin, and 100 μg/ µL streptomycin. The culture media was incubated with 5% CO2 at 37°C for 72 h and light microscopic examination was done daily [28].

Mice were orally infected with 1×10⁴±10 Blastocystishominis vacuolar forms via gavage using esophageal tube per mouse[29]. Three weeks after infection of the mice [21st day post-infection (PI)], fecal pellets were collected from infected groups and subjected to parasitological examination to verify the existence of the parasitic infection.

2. Drugs:

A. Metronidazole:

In tablet 500 mg form Ajanta pharma limited, India. To set up the stock solution of 1 mg mL⁻¹, the tablet was granulated and disintegrated in sterile refined water, at that point stored in a dim container at 4 °C. It was administered orally for 10 successive days in a dose of 100 mg/ kg/day as the dose often used for relevant mouse reports.

B. Pumpkin seed powder

About 650 mg per capsule manufactured and provided by Earth Natural Supplements, Florida, USA. The capsule's powder was broken down in sterile refined water. It was administered orally for 10 successive days in a dose of 120 mg/ kg/day.

C. Amygdalin (B17) Powder:

About 500 mg per capsule manufactured in the USA for Zildek Nutrition, New York. The capsule's powder was broken down in sterile refined water. It was administered orally for 10 successive days in a dose of 100 mg/ kg/day.

D. Cobalamin (B12):
About 500µg per capsule manufactured in Eisai, TOKYO, JAPAN. The capsule’s powder was broken down in sterile. It was administered orally for 10 successive days in a dose of 50µg / kg/day with 50µg / kg/day of Amygdalin (B17) refined water. The doses were computed by extrapolating human therapeutic doses to animal doses by table [30]. After the establishment of infection (three weeks after infection of the mice) the drugs used were given for ten consecutive days

3. Drug assessment:

   A. Parasitological examination:
   fecal pellets were collected from infected mice and were microscopically examined to detect cysts shedding. It was done after 10 daily doses of drug administration followed by the sacrifice of the tested mice. The quantitative assessment of the infection intensity of *Blastocystis* sp. in the fecal samples in mice was done to assess the burden of the parasitic infection. In each experiment, the mean ± Standard deviation of the mean number of cysts/gram feces was determined.

   B. Liver function tests in serum:
   By the end of the experiment, mice were sacrificed. Blood was collected from each mouse in non-heparinized glass tubes before being left for thirty minutes to clot at room temperature before being subject to a 3000-rpm centrifugal for ten minutes. Sera were separated in Eppendorf tube and stored in aliquots at -20°C until required. Serum AST and ALT levels were detected using Kit (RANDOX GTIN:05055273200447, 05055273200188 Cat.No. AS147, AL146-United Kingdom) by Colorimetric method (31) for determination of serum. According to the method used by the manufacturer.

   C. Biochemical determinations:
   The liver was removed, cleaned in cold saline, and 0.5 g of it was used for comet assay to measure toxicity in liver tissues of all studied groups. This was done by the measurement of Lipid peroxidation (Malondialdehyde) and Glutathione (GSH) content using the colorimetric method by using Kit [BIODIAGNOSTIC.CAT.No.MD 25 29, GR 25 11, Egypt]. About MDA, the method based on reacts of thiobarbituric acid (TBA) with Malondialdehyde (MDA) in the acidic medium at a temperature of 95 °C for 30 min to form thiobarbituric acid reactive product the absorbance of the resultant pink product can be measured at 534 nm. As for GSH, the method based on reduction of 5, 5dithiobis(2-nitrobenzoic acid)(DTNB) with glutathione(GSH) to produced chromogen a yellow compound. The reduced chromogen directly proportional to GSH concentration and its absorbance can be measured at 405 nm.

4. Statistical analysis of data:
   Data as Mean ± Standard deviation of the mean were recorded. Statistical analysis was conducted using SPSS version 15. The independent sample t-test was used to assess the statistical significance of the mean difference between the two study groups. The percentage of reduction in number of *Blastocystis* cysts/g according to the following equation:

   \[
   \text{Reduction of parasitic cysts(%) = } \frac{a - b}{a} \times 100
   \]

   where:
   - a: number of *Blastocystis* cysts/g in control group
   - b: number of *Blastocystis* cysts/g in experimental group
Where, in immunosuppressed infected non treated, "a" is the mean number of cysts and "b" is the mean number of cysts in immunosuppressed infected with treated groups.

The degree of significance (p-value) was obtained from corresponding tables. The degree of significance was expressed as p<0.05.

5. Ethics Committee approval:

The proposal of this study was endorsed by the research ethics committee at the College of Sciences for women.

Results:

A. Shedding of cysts:

Treatment of the infected mice by the used drugs significantly reduced Blastocystis sp. cysts shedding compared to immunosuppressed infected non treated group. The study revealed that the number of Blastocystis sp. cysts/gram feces in amygdalin (B17), compound (B17 + B12), pumpkin seeds, and metronidazole treated groups was 49340, 45090, 28160 and 10003 respectively compared to immunosuppressed infected non treated group (65770). The best response between the test groups with marked reduction of the mean cyst count in the feces was shown in treated group metronidazole with 85% percentage of reduction while pumpkin seeds treated group showed improvement more than amygdalin (B17) and compound (B17 + B12) treated groups (Table 1).

Table 1: The mean number and the percentage of reduction in the number of Blastocystis cysts/g feces ten days’ post-treatment with Amygdalin (B17), (B17) +(B12), Pumpkinseed, and Metronidazole comparison with immunosuppressed infected group.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Cysts g⁻¹ feces (mean±SD) ×10⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>IIb; immunosuppressed infected non treated</td>
<td>6.577±3.472</td>
</tr>
<tr>
<td>Iic; immunosuppressed infected and treated with B17 (%)</td>
<td>4.934±4.124</td>
</tr>
<tr>
<td>IIId; immunosuppressed infected and treated with B17 + B12 (%)</td>
<td>4.509±2.605</td>
</tr>
<tr>
<td>Ile; immunosuppressed infected and treated with pumpkin seeds (%)</td>
<td>2.816±2.426*</td>
</tr>
<tr>
<td>IIIf; immunosuppressed infected and treated with metronidazole (%)</td>
<td>1.003±0.384*</td>
</tr>
</tbody>
</table>

Data are shown as mean ±SD. p<0.05* =significant.
B. Liver function tests in serum:

Serum AST and ALT levels were significantly (p < 0.05) increased in the infected untreated - immune-suppressed groups compared to the control of negative mice (Fig. 1). Conversely, when the infected untreated - immune-suppressed groups were treated with amygdalin (B17), (B17) + (B12), Pumpkinseed, and Metronidazole. AST and ALT were significantly (p < 0.05) highly decreased in infected immune-suppressed treated with B17+ B12 compared to the other groups.

C. Biochemical determinations

Mice Infected with *Blastocystis* sp. exhibited decreased levels of GSH (4.10±0.88, 8.36±0.81) and increase MDA production (7.38±0.29, 6.86±0.53) compared to that of uninfected controls. Other effects of B17, B17 + B12, pumpkin seeds, and metronidazole on the levels of glutathione (GSH) and malondialdehyde (MDA) shown in Table (2) indicate that there was a significant increase (p<0.05) in the levels of glutathione (GSH) but decrease in the levels of malondialdehyde (MDA) compared to the Infected group. The highest significant effect was the compound B17 + B12 increase in the levels of glutathione (GSH) (7.09±0.57, 3.16±0.38) and a decrease in the levels of malondialdehyde (MDA) (6.71±0.32, 7.70±0.39) compared to the immunsupp, infected non treated group. While both effect B17 and pumpkin seeds were close to the result of increased GSH (6.59±0.57, 6.12±1.17) respectively and decreased MDA (6.52±0.83,
Table 2: Comparison of GSH and MDA levels in liver all studied groups.

<table>
<thead>
<tr>
<th>Sample groups</th>
<th>GSH (mg /g. tissue)</th>
<th>MDA (mg /g. tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ia; noninfected non treated (control negative)</td>
<td>8.36±0.81</td>
<td>6.86±0.53</td>
</tr>
<tr>
<td>Ib; infected non treated</td>
<td>4.10±0.88</td>
<td>7.38±0.29</td>
</tr>
<tr>
<td>IIa; immunsupp. noninfected non treated</td>
<td>5.92±0.90</td>
<td>6.98±0.52</td>
</tr>
<tr>
<td>IIb; immunsupp. infected non treated</td>
<td>3.16±0.38</td>
<td>7.70±0.39</td>
</tr>
<tr>
<td>IIc; immunsupp. infected and treated with B17</td>
<td>6.59±0.57*</td>
<td>6.52±0.83</td>
</tr>
<tr>
<td>IIId; immunsupp. infected and treated with B17 + B12</td>
<td>7.09 ±0.41*</td>
<td>6.71±0.32</td>
</tr>
<tr>
<td>IIe; immunsupp. infected and treated with pumpkin seeds</td>
<td>6.12±1.17*</td>
<td>6.22±0.39*</td>
</tr>
<tr>
<td>IIf; immunsupp. infected and treated with metronidazole</td>
<td>5.28±0.52*</td>
<td>6.07±0.27*</td>
</tr>
</tbody>
</table>

(Data are given mean ±SD. Degree of significance *P<0.05 compared with control by Student’s independent t-test (SPSS Version 15.0).

Dissection:

*Blastocystis* sp. single-cell intestinal protozoan parasites, are still enigmatic, clinically, and epidemiologically unknown organisms. [32]. Various studies have been shown that subtypes can be linked to pathogenicity[33] although others find it not pathogenic [34]. Some antimicrobial agents have been active against *Blastocystis* sp. *in vivo*, the most common medication in *Blastocystis* sp. is metronidazole, but some reports of failure and resistance to treatment have been documented. Puromycin and trimethoprim-sulfamethoxazole have also been proposed as other treatment options [35].

Some herbal compounds may have a major therapeutic impact and therefore, it has become more important to use natural medicine. In the future, some of these herbal compounds could become approved for the therapy of Blastocystosis[36].

In many earlier studies, a safe and efficient natural alternative therapy for *Blastocystis* sp has been identified *in vitro* and *in vivo* as garlic and ginger, oil sheet extract, and bee pollen agents. Infectiousness [29,37].

In this study, an *in vivo* assessment of some natural compounds[ amygdalin (B17), compound (B17 + B12), pumpkin seeds] compared to metronidazole had been done as an alternative treatment for that organism. Our results showed that Pumpkinseed treatments had significantly lowered the shedding of cysts less to that of the metronidazole treatment as shown in(Table 1). Metronidazole showed the highest percentage of reduction in the number of oocyst /gram feces of infected treated mice followed by Pumpkinseed with a statistically significant difference (p<0.05) in comparison with the control group. The mechanism by which Amygdalin (B17), (B17),(B17+B12), and Pumpkinseed treatments significantly lowered the shedding of cysts is still unclear.
However, these results could be explained on the basis that in 1991 [38] stated that the extracted crude ethanolic from Cucurbita maxima is viewed as valuable in vivo inhibition and prevention of the development of parasitemia. Pomegranate and pumpkin seed extracts in vitro and in vivo are anthelmintic efficacy, including Asckardiagallias proved by[39].

Amygdalin(cyanogenic diglycoside) is also administered for cancertherapy throughtits ability to hydrolyze in hydrogen cyanide(HCN), glucose, and benzoaldehyde with other Conventional and Alternative Medicine (CAM) therapies including vitamins and fruit seeds, such as apricots and bitter almonds [40].

Vitamin B12 is found to has rare side effects and is an over-the-counter drug. Ciccarelli et al., in 2012 study the effect of vitamin B12 (or cyanocobalamin) in vitro and in vivo on various types of Trypanosomacruzi was investigated [41].

The present study aimed to distinguish the hepatic ameliorative role of the used drugs against Blastocystissp. infection-induced liver toxicity. Laboratory examination found that liver functions were disordered based on elevated transaminase serum (AST 104 U/L ALT 68 U/L in mice with Blastocystis sp. infection).

In this study, untreated infected immune-suppressed groups compared to control negative mice serum, AST, and ALT were significantly increased (p < 0.05). A rise in AST and ALT levels may be taken into account due to liver damage or improvements in membrane permeability [42], thereby indicating severe hepatocellular damage.

In B17 + B12, B17, Pumpkin seed, and Metronidazole, the AST and ALT were significantly (p < 0.05) decline compared to the other groups in infected immune-suppressed treatments. Similarity with a study by researchers [43] shows that vitamin B17 can be a reliable and novel therapy for breast cancer, this vitamin treatment of Ehrlich solid tumors (EST) (EST + B17) modulates the changes in liver and kidney functions. Contrary to what appeared in a study (11) when no significant correlation has been observed in chronic liver disease (CLD) patients between parasite infection and liver function tests (AST and ALT).

Mice with Blastocystis showed decreased GSH levels for the biochemical results in this study (4.10±0.88, 8.36±0.81) and increase MDA production (7.38±0.29, 6.86±0.53) compared to that of uninfected controls. This may be because GSH has a protective effect on protozoans similar to that of human cells. GSH is therefore integral to some of the protozoans’ survival because some Protozoans use their main antioxidant compound trypanothon (T(SH)2). In turn, for its production, T(SH)2 requires GSH. Therefore the drop in GSH levels (the effects of protozoan parasites may be adverse to a known inhibitor like bithioninesulfonoxime [BSO]) [44]. And this is identical to [45] who reported that Blastocystis sp. infestation prompts biochemical alterations, the observed elevation in MDA values of infected mice in this study is in concordance with the findings observed in Blastocystosis. One of the key reasons why the Blastocystissp. infected mice have high MDA levels could be increased defense system activity protecting tissues from free radical damage caused by Blastocystis-infected activated phagocytes [46].

References:


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