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## Evaluation of Amygdalin (B17) and *Cucurbita pepo* (Pumpkin seed) Activity Against *Blastocystis* from Diarrheic Patients in Baghdad, Iraq: *in Vitro* Study

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### Abstract:

*Blastocystis* is a ubiquitous human and animal protozoa that inhabit the gastrointestinal tract. Metronidazole is considered the standard drug for the treatment of *Blastocystis* infection; however, there is growing evidence of treatment failure, hazardous side effects, and appearance of strains resistant to metronidazole. In the last era, many studies have been implicated in the quest for new treatments for *Blastocystis* infection, especially natural products. Attention has been focused on the effect of Amygdalin (B17) and pumpkin seed on eradicating parasitic infections. The current work was built up to explore the *in vitro* efficacy of two natural compounds, Amygdalin (B17) and pumpkin seeds against *Blastocystis* isolated from symptomatic patients. *In vitro* incubation of the parasite with B17 (200, 400  $\mu\text{g mL}^{-1}$ ), pumpkin seed (200, 400  $\mu\text{g mL}^{-1}$ ) and metronidazole (100, 150  $\mu\text{g mL}^{-1}$ ) was counted at different periods (one, two, twenty-four and forty-eight hours) and morphological changes were evaluated using Light Microscope. *Blastocystis* detected from patients with symptoms was subtype 1. The B 17 and pumpkin seed demonstrated statistically significant ( $p < 0.05$ ) growth reduction of *Blastocystis* in culture. Such results showed the possible therapeutic effects of B 17 and pumpkin seed against blastocystosis as effective safe natural alternatives.

**Keywords:** Amygdalin (B17), *Blastocystis*, *In vitro* cultivation, Metronidazole, Pumpkin seed.

### Introduction:

*Blastocystis* is one of the world's most widely distributed intestinal protists that infect humans and animals<sup>1</sup>. The prevalence of *Blastocystis* in industrialized and developing countries varies between 0.5-30% and 30-76%, respectively<sup>2</sup>. The *Blastocystis* pathogenicity remains a controversial issue. Originally, it was thought to be a commensal protozoan, but recent studies have supported its pathogenicity<sup>3</sup>. *Blastocystis* causes enteritis, which results in diarrhea, bloating, abdominal discomfort, and/or vomiting<sup>4</sup>. Clinical studies also connect the parasite with other inflammatory conditions of intestine and skin, like irritable bowel syndrome and urticarial disorders. Immunocompromised people (patients of HIV/acquired immunodeficiency syndrome or cancer) are especially susceptible to infection, indicating that *Blastocystis* can work as an opportunistic pathogen<sup>5</sup>. The morphological forms recorded from stool samples and/or culture media

are vacuolated, granular, trophozoite, and cyst forms<sup>4</sup>. The vacuolated form is the most commonly observed in feces and is responsible for the fecal-oral transmission of infection.

*Blastocystis* sp. is diagnosed by microscopic detection in direct smears performed before or after fecal sample cultivation or molecular parasite DNA identification<sup>6</sup>. Small-subunit (SSU) rRNA gene molecular studies categorized *Blastocystis* into 17 subtypes (ST1-ST17). From these, the subtypes 1–9 were reported in humans, and ST1 to ST4 being the prevalent subtypes in humans found in >90% of examinations<sup>2</sup>. In a recent study from South America, subtype 12 has also been reported found in humans<sup>7</sup>. Due to the uncertainty surrounding the potential pathogenicity of *Blastocystis* and the self-limiting nature of the symptoms, this disease is treated equivocally<sup>8</sup>. While the standard treatment for infection with *Blastocystis* is metronidazole, recent documents

have referred to treat insufficiency indicating the development of isolates not responding to therapy<sup>9</sup>. Also, metronidazole has many drawbacks and risks, like migraine, dizziness, queasiness a metallic taste in the mouth, reversible neutropenia with rare major adverse reactions including pancreatitis, peripheral neuropathy and CNS toxicity consisting of seizures, encephalopathy, cerebellar dysfunction, paresthesia, mental confusion, and depression. These neurologic reactions generally occur only with high, prolonged, cumulative doses. As a result, a new therapy for *Blastocystis* especially the analysis of natural active agents has been requested<sup>10</sup>.

In the developing countries, medicinal plants have been widely used for centuries due to their abundance, inexpensiveness, and conventional uses<sup>10</sup>. Amygdalin is a natural plant-derived from pebbles of rosaceous fruit such as apricots, almonds, cherries, peaches, and plums<sup>11</sup>. It is made up of two glucose molecules, a benzaldehyde molecule and a hydrocyanide molecule, it has been shown that benzaldehyde of amygdalin is capable of inducing an analgesic effect and hydrocyanide of amygdalin is capable of inducing an anticancer effect<sup>12</sup>. It is also known as vitamin (B17), it possesses several benefits and used to treat many disorders such as asthma, nausea, leprosy, bronchitis, and leukoderma<sup>13</sup>. 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<sup>14</sup>.

One of the well-known edible plants is the pumpkin. Since some special natural edible substances are present, where it has important medicinal properties. Many Phyto-constituents of alkaloids, flavonoids, palmitic acid, oleic and linoleic groups are present in the pumpkin. Thus, various essential medical characteristics are well known, in particular anti-diabetes, antioxidants, anti-carcinogens, anti-inflammatories, etc.<sup>15</sup>. Pumpkin seed has been used as a traditional medicine in various parts of the world treating gastrointestinal parasites with antihelmintic properties especially against tapeworm<sup>16</sup>.

## Materials and Methods:

**Collection of fecal samples:** 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 Sciences for women.

**Processing of fecal samples:** The microscopic examination of fecal samples were done immediately using direct wet smear in saline solution and colored with Lugol's iodine then the concentration technique was done using formalin-ethyl acetate, stool smears were stained using modified Ziehl-Neelsen acid-fast staining to eliminate the possibility of multiple parasitic infections and to detect *Blastocystis*<sup>10</sup>. Stool samples contain *Blastocystis* sp. were divided into two parts; part was used for culture on the same day and the other part for molecular assays and genotyping was stored at -20 °C.

***Blastocystis* culturing In Vitro:** 25µg/ml taken from fecal samples with *Blastocystis* were cultured in Jones' medium (3ml) not containing rice starch and enhanced with 10% horse serum<sup>17</sup>, and synergistic antibiotics were added 100 IU/ml penicillin, 100 µg/ml streptomycin and 1.25 µg/ml amphotericin B to prevent contamination<sup>18</sup>. The culture media were incubated with 5% CO<sub>2</sub> at 37°C for 72 h and light microscopic examination was done daily<sup>19</sup>. We studied to consider the culture media negative when there is no *Blastocystis* growth after 72h<sup>8,10</sup>. Cultures were used for drug testing when vacuolated forms of *Blastocystis* were more than 1×10<sup>4</sup> / µL.

***Blastocystis* molecular characterization:** sixteen stool samples were infected with *Blastocystis* and were used in this work.

**DNA extraction:** 1- Separated genomic DNA of the isolated samples was used by the maker's instructions (Geneaid biotech, Taiwan), using a Geneaid Kit (STLD004, STLD050, STLD100), as indicated. 2- DNA density and purity was measured by nanodrop, with a higher concentration of ethidium bromide (0.7 µg / ml) pre-colored in the buffer of TAE (40 mM tris-acetate, 2 mM EDTA (pH 8.3), and with a 1 kb of the ladder as molecular weight marker (Cat # D-1040, Bioneer, Daejeon, South Korea), the integrity of the DNA was assessed by electrophoresis in standard agarose gel at 0, 8% (w / v). 3- The PCR has been used as disengaged DNA.

**Polymerase chain reaction(PCR):** recommended that one PCR fragment was selected for amplification, which is supposed to partially cover 607 bp fragment indented to partially amplify the small subunit ribosomal RNA (SSU) *rRNA* locus within *Blastocystis* sp. genomic DNA sequences (Table 1).

**Table 1. The specific primers' pairs selected to amplify the SSU rRNA locus within *Blastocystis* sp. genomic DNA sequences. The bold letters refer to the start and end of the amplicon fragment.**

| Primer | Sequence (5'-3')      | Amplicon size | Annealing temperature | GenBank Accession Number | Corresponding species   |
|--------|-----------------------|---------------|-----------------------|--------------------------|-------------------------|
| RD5-F  | ATCTGGTTGATCCTGCCAGT  | 607 bp        | 59°C                  | MK719675.1 (1–20)        | <i>Blastocystis</i> sp. |
| Dr-R   | GAGCTTTTAACTGCAACAACG |               |                       | (586 – 607)              |                         |

The freeze-dried primers were purchased from (Bioneer, Daejeon, South Korea). 1-The PCR reaction was carried out utilizing the AccuPower PCR premix (Cat # K-2012, Bioneer, Daejeon, South Korea). 2- Every 20 µl of PCR premix contained 1 U of Top DNA polymerase, 250 µM of dNTP, 10 mM of Tris-HCl (pH 9.0), 30 mM of KCl and 15 mM of MgCl<sub>2</sub>. 3- The mixture of reactions was completed with 10 pmol of each primer and 50 ng of genomic DNA. 4- In PCR thermocycler (MyGenie™ 96/384 Thermal Block, Bioneer, Daejeon, South Korea), the accompanying program has been applied. 5- Amplification started with an initial denaturation at 94 ° C for 5 min, followed by 30 denaturation cycles at 94 ° C for 30 seconds, annealing at 59 ° C for 30 seconds and extension at 72 ° C for 30 seconds, and ending with a final extension at 72 ° C for 5 minutes. 6-The amplification was verified by electrophoresis on an ethidium bromide (0.5 mg/ml) 1.5% (w / v) pre-colored agarose gel in 1 × TBE buffer (2 mM EDTA, 90 mM Tris-Borate, pH 8.3), utilizing a Ladder of 100 bp (Cat # D-1010, Bioneer, Daejeon, South Korea) as a molecular weight marker. All PCR bands were assured to be explicit and consist of only one spotless and sharp band to be effectively submitted for sequencing.

**DNA Sequencing of PCR products:** The settled PCR amplicons were commercially sequenced from termini, forward, and reverse, as indicated by guidance manuals of the sequencing organization (Macrogen Inc. Geumchen, Seoul, South Korea). Additionally, Specific chromatographic information obtained from the ABI sequence documentation was analyzed to ensure that the analysis and classes did not result from PCR or artifact sequence. By looking at the DNA sequences observed for live samples with the DNA sequences obtained from *Blastocystis* sp., the virtual locations, and other details of the recovered PCR pieces were differentiated.

**Interpretation of sequencing data:** The consequences of sequencing the PCR results of various samples were different and adjusted, then examined along with the individual arrangements of the reference database utilizing Bio Edit Sequence Alignment Editor Version 7.1 (DNASTAR, Madison, WI, USA). The varieties observed in each sequenced test were numbered in PCR amplicons as

well as in their relative position within the alluding genome.

**Drugs: Metronidazole:** It has been used as an antiprotozoal reference drug, supplied by Ajanta pharma limited<sup>21</sup>. To set up the stock solution of 1 mg mL<sup>-1</sup>, the tablet was granulated and disintegrated in sterile refined water, and at that point stored in a dim container at 4 ° C. The last levels of Metronidazole have been standardized to 100 and 150 µg mL<sup>-1</sup>.

**Pumpkin seed powder:** About 650 mg per capsule manufactured and provided by Earth Natural Supplements, Florida, and USA. The capsule's powder was broken down in sterile refined water to achieve a 1 mg mL<sup>-1</sup> final stock solution. Final concentrations of 200 and 400 µg mL<sup>-1</sup> were adjusted.

**Amygdalin (B17) Powder:** About 500 mg per capsule manufactured in the USA for Zilek Nutrition, New York. The capsule's powder was broken down in sterile refined water to achieve a 1 mg mL<sup>-1</sup> final stock solution. Final concentrations of 200 and 400 µg mL<sup>-1</sup> were adjusted.

**The activity of Metronidazole, Pumpkinseed, and Amygdalin B17 as anti-*Blastocystis* in vitro**

**In vitro experiment:** Inoculums size of 8×10<sup>4</sup> parasites taken from cultures were brought into each group of culture tubes which have Jones medium at the various concentrations of Metronidazole, Pumpkinseed, and Amygdalin B17 to detect the growth of the parasite. Untreated cultures of positive control (only parasites) and negative control (only culture media) were exposed to a similar condition as those utilized for the rest of the studied cultures. Triplicate tubes containing culture media were utilized for each concentration of Metronidazole, Pumpkinseed, Amygdalin B17, and untreated cultures. *Blastocystis* was tested with a graduated concentration of Metronidazole (100 and 150 µg mL<sup>-1</sup>), Pumpkinseed (200 and 400 µg mL<sup>-1</sup>), and Amygdalin B17 (200 and 400 µg mL<sup>-1</sup>). After 1, 2, 24, and 48 h, the cultures tested were incubated at 37 ° C, then the culture was examination. In previous standardized studies, the treatment time of at least 1 h was utilized and indicated that this timeframe is important to stimulate the cytotoxic reaction in these parasites<sup>22</sup>.

### Assessment of in vitro anti-*Blastocystis* activity of Metronidazole, Pumpkinseed, Amygdalin B17:

The concentration of parasite cells/ml of culture was estimated at different periods (1, 2, 24, 48 hours) after incubation with different concentrations of drugs.

**Light microscopic examination of the in vitro experiment:** the effect of the used drugs against the parasites was assessed by enumerating the number of viable cells after 1, 2, 24, and 48 h using Lugol's iodine solution which stained viable cells. Counting was performed twice and the intact organisms were only enumerated utilizing a light microscope cell counting chamber. According to Eq 1, the percentage of reduction of *Blastocystis* proliferation in cultures that were treated was measured in comparison to non-treated culture.

$$\text{Growth inhibition (\%)} = \frac{a-b}{a} \times 100 \dots 1$$

Where, in control cultures, "a" is the mean number of organisms and "b" is the mean number of organisms in treated cultures (10).

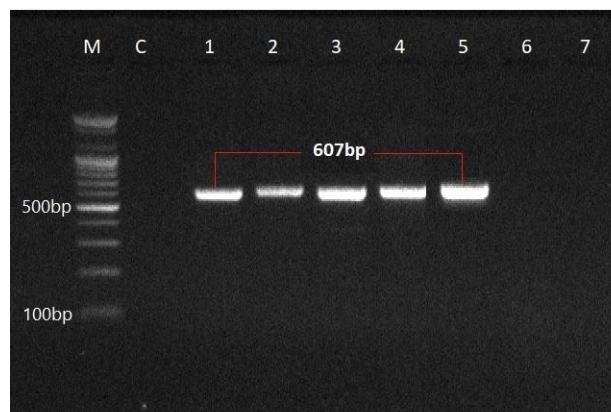
**Statistical analysis:** Data as Mean counts± Standard deviation were recorded. Statistical analyzes were conducted using computerized SPSS statistical program version 15 the independent sample t-test was used for assessing the statistical significance of the mean difference between the two study groups. Statistical value determination as  $p < 0.05$ .

**Ethics Committee approval:** The proposal of this study was endorsed by the research ethics committee at the College of Sciences for women. The patients involved in this study verbally on the motivation behind the study and the collection of fecal samples were carried out after obtaining their verbal approval.

### Results: Amplification and genotyping of *Blastocystis* isolates:

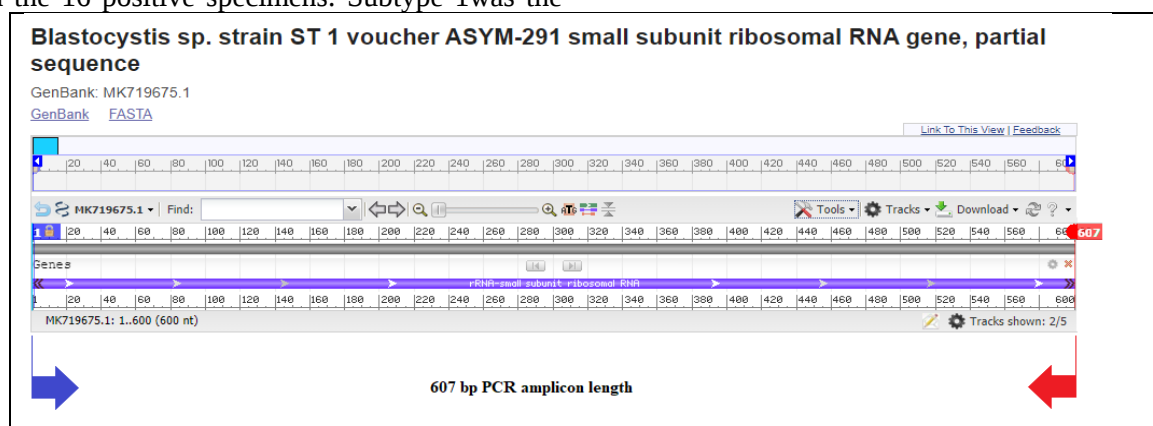
Five samples were positive for *Blastocystis* by PCR from the 16 positive specimens. Subtype 1 was the

genotype that was detected using genotypic assessment (Fig. 1).



**Figure 1. DNA analysis of *Blastocystis* samples:** Electrophoresis of *Blastocystis* gene amplification products (607bp) on 1.5% agarose gel and 70 volts/cm for 1:00 h using DNA Ladder (100bp-1000bp). In lane M and starts from 100bp, C: negative control, lanes1, 2, 3, 4, 5 positive samples, lanes 6 and 7 negative.

**Sequencing results:** About the investigated samples of S1 – S5, the sequencing reactions indicated the exact positions after performing the National Center of Biotechnology Information (NCBI) BLASTn for these PCR amplicons. NCBI BLASTn engine has shown about 99% sequences of similarities between the sequenced samples and this target. NCBI BLASTn engine has indicated the presence of remarkable homology with the expected target that covered a portion of the SSU *rRNA* within *Blastocystis* sp. genomic DNA sequences. By looking at the DNA sequences observed from these nearby samples with the DNA sequences recovered (GenBank acc.) MK719675.1, the specific positions and different subtleties of the recovered PCR fragment were recognized (Fig. 2).



**Figure 2. The exact position of the retrieved 607 bp amplicon that covered a portion of the SSU *rRNA* locus within the *Blastocystis* sp. DNA genomic sequences (acc. no. MK719675.1). the red arrow refers to the starting point of this amplicon while the cyan arrow refers to its endpoint.**

After positioning the 607 bp amplicons' sequences of its sequences were highlighted (Table 2).  
within the SSU rRNA DNA sequences, the details

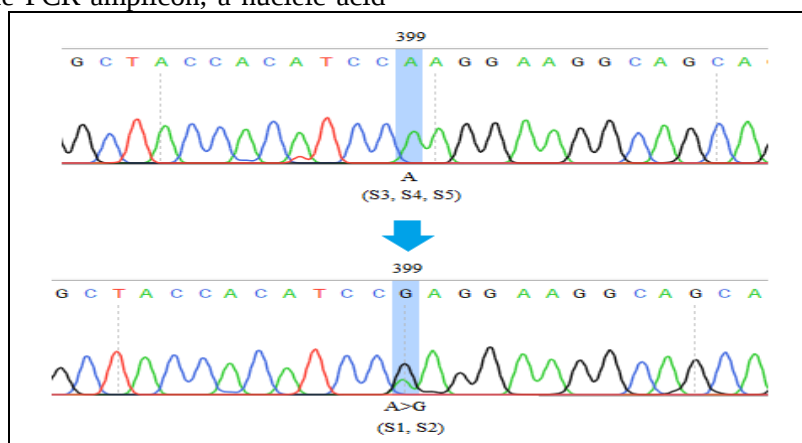
**Table 2. The site and length of the 607 bp PCR amplicons utilized for amplifying a portion of the SSU rRNA fragment. The amplified sequences were extended from 1 to 607 of the NCBI reference DNA sequence (GenBank acc. no. MK719675.1).**

| Amplicon                     | Referring to locus sequences (5' - 3')                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | length |
|------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------|
| SSU rRNA<br>DNA<br>sequences | * <sup>1</sup> TTTGGTTGATCCTGCCAGTAGTCATACGCTCGTCTCAAAGATTAAGCCATGCATGTGTA<br>AGTGTAATATCAAAGTTTGGAACGCGAATGGCTCATTATATCAGTTATAGTTTATTTGG<br>TGAAGTGTACTACTTGGATAACCGTAGTAATTCTAGGGCTAATACATGAGAAAGTCCTCT<br>GGTGAGGTGTGTTTATTAGAATGAAAACCATATGCTTCGGCATGATAGTGAGTAATAGTA<br>ACCTATCGTATCGCATGCTTAATGTAGCGATGAGTCTTTCAAGTTTCTGCCCTATCAGCTT<br>TCGATGGTAGTATATGGGCCTACCATGGCAGTAACGGGTAACGAAGAATTGGGTTTCGAT<br>TTCGGAGAGGGAGCCTGAGAGATGGCTACCACATCCAAGGAAGGCAGCAGGCGCGTAAA<br>TTACCCAATCCTGACACAGGGAGGTAGTGACAATAAATCACAATGCGGGACTATCAGTCT<br>TGCAATTGGATTGAGAACAATGTACAACCTTATCGATAAGCCATTGGAGGGCAAGTCTG<br>GTGCCAGCAGCCGCGTAATTCCAGCTCCAATAGCGTATATTAACGTTGTTGCAGTTAAA<br>AAGCTC | 607 bp |

\*<sup>1</sup> **Notice;** the reverse primer was placed in a reverse complement mode.

The sequencing chromatogram of DNA sequences in addition to its particular interpretation was recorded and these variants pattern within the amplified sequences are shown in (Fig.3). In the position 399<sup>th</sup> of the PCR amplicon, a nucleic acid

substitution of Adenine (A) to Thymine (T) was detected in both S1 and S2 samples, while S3 – S5 samples kept an entire homology with the referring sequences.



**Figure 3. The chromatogram profile of the observed genetic variants of the SSU rRNA gene within *Blastocystis* sp. local isolates. Each substitution mutations featured by their positions in the PCR amplicon. The symbol “>” alludes to “substitution” mutation. The symbol “S” refers to the sample code.**

To summarize all the results obtained from the sequenced 607 bp fragments, the specific

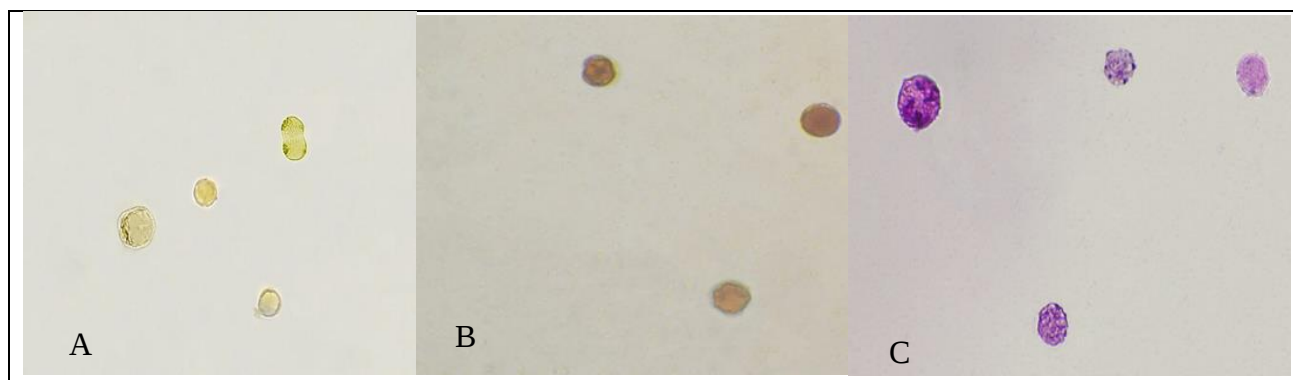
situation of the watched mutation was described in (Table 3).

**Table 3. The pattern of the observed mutation in the 607 bp SSU rRNA amplicons in comparison with the NCBI referring sequences (GenBank acc no. MK719675.1). The symbol “S” refers to the “sample” code.**

| Sample No. | Native | Allele | Position in the PCR fragment | Position in the reference genome | Variant summary      |
|------------|--------|--------|------------------------------|----------------------------------|----------------------|
| S1, S2     | G      | A      | 399                          | 399                              | MK719675.1:g.399 G>A |

**In vitro experiment:** activity of various concentrations of Metronidazole, Pumpkinseed, and Amygdalin (B17) on *Blastocystis* subtype 1 development after 1, 2, 24, and 48h is illustrated in

(Table 4). Culture sample of *Blastocystis* was stained with iodine after 2 h without treatment (Fig.4).



**Figure 4.** A- *Blastocystis* in culture stained with iodine after 2h untreated(x40), B-*Blastocystis* in culture stained with iodine after 2h treated with Pumpkin seed (x40), C- *Blastocystis* in culture stained with Ziehl –Neelsen acid –Fast stain after 2h treated with Amygdalin (B17) (x40).

Impact of specific Metronidazole, on *Blastocystis* subtype 1 development in culture media after several incubation times (Table 4).

**Table4.** Effect of different concentrations of Metronidazole, Pumpkinseed, and Amygdalin B17 on *Blastocystis* subtype 1 development in culture media after various incubation times.

| Drugs/concentration ( $\mu\text{g mL}^{-1}$ ) | Cells $\text{mL}^{-1}$ (Mean $\pm$ SD) $\times 10^4$ (1h) | Cells $\text{mL}^{-1}$ (Mean $\pm$ SD) $\times 10^4$ (2h) | Cells $\text{mL}^{-1}$ (Mean $\pm$ SD) $\times 10^4$ (24h) | Cells $\text{mL}^{-1}$ (Mean $\pm$ SD) $\times 10^4$ (48h) |
|-----------------------------------------------|-----------------------------------------------------------|-----------------------------------------------------------|------------------------------------------------------------|------------------------------------------------------------|
| Control                                       | 8.33 $\pm$ 0.91                                           | 8.35 $\pm$ 0.92                                           | 8.37 $\pm$ 0.93                                            | 8.43 $\pm$ 0.94                                            |
| metronidazole 100 (%)                         | 7.10 $\pm$ 1.10<br>14.76                                  | 2.35 $\pm$ 0.57*<br>71.85                                 | 0.50 $\pm$ 0.11* 94.02                                     | 0.09 $\pm$ 0.07*<br>98.93                                  |
| Metronidazole150 (%)                          | 5.25 $\pm$ 0.77<br>36.97                                  | 1.97 $\pm$ 0.19* 76.40                                    | 0.40 $\pm$ 0.49* 95.22                                     | 0*<br>100                                                  |
| Pumpkin seed 200 (%)                          | 4.00 $\pm$ 0.26*<br>51.98                                 | 1.49 $\pm$ 0.26*<br>82.15                                 | 0.38 $\pm$ 0.19* 95.45                                     | 0.25 $\pm$ 0.13* 97.03                                     |
| Pumpkin seed 400 (%)                          | 1.93 $\pm$ 0.32* 76.83                                    | 1.13 $\pm$ 0.11*<br>86.46                                 | 0.36 $\pm$ 0.14 * 95.69                                    | 0.10 $\pm$ 0.02* 98.81                                     |
| B17 200 (%)                                   | 0.80 $\pm$ 0.61*<br>90.39                                 | 0.31 $\pm$ 0.22*<br>96.28                                 | 0.12 $\pm$ 0.07* 98.56                                     | 0.04 $\pm$ 0.03* 99.52                                     |
| B17 400 (%)                                   | 0.24 $\pm$ 0.13* 97.1199                                  | 0.09 $\pm$ 0.01*<br>98.92                                 | 0.03 $\pm$ 0.02* 99.64                                     | 0*<br>100                                                  |

Values are shown as Mean $\pm$ SD and percentage of reduction (%) from control, \*Statistically significant in comparison with the infected control group ( $p < 0.05$ ), Pumpkinseed, and B17.

## Discussion:

Inhuman, *Blastocystis* is the most common enteric protozoan. Though Metronidazole is the treatment of choice, doctors are still skeptical about an antibiotic prescription for *Blastocystis* as there is still some controversy about its pathogenicity and frequent reports of failure to respond to chemotherapy<sup>23</sup>. Variation from strain to strain in *Blastocystis* of sensitivity to metronidazole, over-prescription as well as abuse of antimicrobial drugs might be some explanations for the failure of the treatment<sup>24</sup>.

Considering the side effects and resistance of many antiparasitic medications, attention has been focused on plant extracts and herbal compounds used in traditional medicine that have therapeutic potential as sources for new treatments<sup>25</sup>. Also, WHO has also recommended the

start of studies to identify and characterize new herbal preparations from traditionally known plants and the development of new effective therapeutic agents, particularly in areas where many individuals lack access to needed preventive and treatment care. Natural sources were considered the best option in this regard to isolating new and novel anti-microbial components<sup>26</sup>.

In this study, genotypic evaluation of the isolated positive samples of *Blastocystis* showed infection with subtype I. This finding is following many epidemiological studies worldwide and, also in Iraq that reported STI as the mostly detected subtype<sup>27,28</sup>. In agreement with our results, several studies stated that ST1 is the prevalent subtype that showed an important role in pathogenic consequences in humans<sup>29,30</sup>.

In the present study, Amygdalin (B17), pumpkin seed, and metronidazole demonstrated a statistically valuable ( $p < 0.05$ ) reduction of *Blastocystis* development according to the used concentration. Amygdalin B17 showed the highest reduction in *Blastocystis* numbers for all incubation times with statistical valuable change ( $p < 0.05$ ) in comparison with the control untreated group. Amygdalin B17 at concentrations of 200  $\mu\text{g}/\text{mL}$  showed significant inhibition ( $p < 0.05$ ) in *Blastocystis* numbers for all incubation times (1, 2, 24, 48 h). It is shown that the highest concentration of Amygdalin B17 (400  $\mu\text{g}/\text{mL}$ ) caused the parasite to die quickly after 48 h and triggered considerably more grounded impacts with mean growth inhibition of 100% similarity with 150  $\mu\text{g mL}^{-1}$  MTZ after 48h.

During the late 1970s, amygdalin got one of the most mainstream natural compounds utilized by tumor patients. Notwithstanding alerts of the potential danger of cyanide harmfulness, amygdalin has encountered a solid renaissance as another alternative for treating cancer<sup>31</sup>. Still, controlled clinical investigations have not been completed and inquiries concerning amygdalin's effectiveness have not yet been agreeably replied<sup>32</sup>.

In a study done by<sup>33</sup>, amygdalin possessed a significant protective effect for chronic liver injury in rats. Furthermore, amygdalin intake abolished the majority of the pathological alterations of the LPS-induced liver damage of rats, the useful effects of amygdalin can be attributed to its action against inflammation and enhancement of liver impairment by inhibition of PI3K/AK JAK2/STAT3 and NF- $\kappa$ B signaling pathways. In another study done by<sup>34</sup> when rats were treated orally with 500 mg/kg of pepsin hydrolysate of almond water-solution on carbon tetrachloride, the action of pepsin was inhibited by the benzaldehyde resulted from the disintegration of amygdalin. Additionally, the level of AST and ALT decreased, hydroxyproline content raised and the extension of euglobulin lysis time was lowered. Also, it could diminish the proliferation of connective tissue of rat liver. Moreover, amygdalin was found to be an effective medication for chronic gastritis and atrophic gastritis in rats<sup>34</sup>.

Pumpkin seed at concentrations of 200  $\mu\text{g}/\text{mL}$  showed significant inhibition ( $p < 0.05$ ) in *Blastocystis* numbers for all incubation times (1, 2, 24, 48 h). On the other hand, the most elevated concentration of pumpkin seed which produced the highest reduction of parasite counts was 400  $\mu\text{g}/\text{mL}$  after 48 h.

There are different varieties of pumpkin, the most noticeable species being *Cucurbita*

*Maxima*, *Cucurbita pepo*, and *Cucurbita moschata*, which are discovered around the world<sup>35</sup>. Pumpkin is one of the most notable palatable plants and has significant medical properties due to the presence of important natural consumable substances; it includes a few Phyto-constituents having a place among the classifications of alkaloids, fatty acid and different amino acid which are biologically active specific nurturance<sup>35</sup>.

Separating different broad spectrum of anti-microbial compounds from pumpkins. In a study done by<sup>36</sup>, the extracted oil of Pumpkin caused growth slight inhibition of *Acinetobacter baumannii*, *Aeromonas veronii* bio group *sobria*, *Enterococcus faecalis*, *Candida albicans*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica* subsp, *enterica* serotype *Typhimurium*, *Serratia marcescens* and *Staphylococcus aureus* at 2•0% (v/v) concentration. Malaria is brought about by Plasmodium species, a specific microorganism. In zones where malaria is endemic, influenced individuals depend on homegrown drugs for treatment<sup>37</sup>. In 1991<sup>38</sup> stated that the crude ethanolic extract of *C. maxima* is viewed as valuable *in vivo* inhibition and prevention of the development of parasitemia *in vivo*. The utilization of *C. maxima* unrefined ethanolic extract and pyrimethamine expanded invulnerability against malaria. Leaf concentrates of *C. maxima* display larvicidal and ovicidal properties and can be utilized as a defensive boundary against mosquito chomp<sup>39</sup>.

The antihelmintic effect of *C. moschata* was assessed *in vitro* from aquatic, methanolic, and dichloromethane seed separates against *Haemonchus contortus*, the parasitic nematode of little ruminants, and it was affirmed that *C. moschata* has larval growth reduction at all concentrations<sup>40</sup>. Extracts of pumpkin seeds and pomegranate peels against *Ascaridia galli* have been reported *In vitro* and *In vivo* anthelmintic activity by<sup>41</sup>. Pumpkin (*Cucurbita pepo* L) seeds are a valuable source of protein and bioactive compounds when mathematical models have been used to study the effect of pumpkin extracts on the weight gain of chicken<sup>42</sup>.

In this study, MTZ displayed inhibitory effects on *Blastocystis* with two concentrations (100 & 150  $\mu\text{g mL}^{-1}$ ). The 100  $\mu\text{g mL}^{-1}$  concentration produced highly significant inhibition in *Blastocystis* numbers within 24 h, whereas the 150  $\mu\text{g mL}^{-1}$  concentration produced no growth after (48 h).<sup>43</sup> reported that *Blastocystis* ST1 and ST3 were vulnerable to metronidazole at 50 and 100  $\mu\text{g mL}^{-1}$  concentrations with no growth within 24 hours, whereas the 10  $\mu\text{g mL}^{-1}$  concentration produced significant inhibition ( $p < 0.05$ ) in

*Blastocystis* numbers for all incubation times (24, 48, 72 h) with toxic effect within 72 hours.

So, in correlation with healthy controls at 0.01 and 0.1 mg mL<sup>-1</sup> concentrations, <sup>44</sup> recorded vulnerability of all *Blastocystis* isolates to metronidazole at 0.01 and 0.1 mg mL<sup>-1</sup> concentrations, these isolates were, for the most part, ST3 and coinfection of ST3 and ST1, yet a similar report indicated variable adequacy of metronidazole and was for the most part ST1. This dispute over the efficacy of metronidazole can be attributed to variations within subtype in the vulnerability mechanisms of the drugs, likely due to the presence of specific alleles in each subtype <sup>45</sup>

### Conclusion:

We conclude that Pumpkin seed and Amygdalin (B17) possessed potential beneficial activity and can be used as safe agents derived from nature for *Blastocystis* STI infections treatment.

### Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- The author has signed an animal welfare statement.
- Ethical Clearance: The project was approved by the local ethical committee in University of Baghdad.

### Authors' contributions statement:

N.A. Ardalan Designed the experiments, S.S. Salman, performed experiment, N.A. Ardalan and S.S. Salman, performed DNA sequencing analyses, S.S. Salman, performed parasitological effect of amygdalin (B17), Cucurbita pepo and metronidazole and analyzed it.

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تقييم نشاط Amygdalin (B17) وبذور اليقطين *Cucurbita pepo* ضد المتبرعمة الكيسية من مرضى المصابين  
بالإسهال في بغداد ، العراق: دراسة في المختبر

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الخلاصة:

تعد المتبرعمة الكيسية من الطفيليات الواسعة الانتشار في الجهاز الهضمي البشري والحيواني. يعتبر الميترونيدازول الدواء الاساسي لعلاج عدوى المتبرعمة الكيسية. ومع ذلك ، كان هناك دليل متزايد على فشل العلاج والاثار الجانبية الخطرة وحدوث سلالات مقاومة للميترونيدازول. شاركت العديد من الدراسات في الفترة الاخيرة في البحث عن علاجات بديلة لعدوى المتبرعمة الكيسية ، وخاصة دراسة العلاجات الطبية. تم توليد قدر كبير من الاهتمام حول دور أميغدالين B17 وبذور اليقطين في القضاء على الالتهابات الطفيلية. قُدمت هذه الدراسة للتحقق في فعالية اثنين من المركبات الطبيعية في المختبر أميغدالين B17 وبذور اليقطين ضد المتبرعمة الكيسية المعزولة من مرضى يعانون من أعراض مرضية ، في الحضانة المختبرية للطفيلي تم حساب B17 (200، 400 ميكروغرام/لتر)، بذور اليقطين (200، 400 ميكروغرام/لتر) و الميترونيدازول (100، 150 ميكروغرام/لتر) في فترات زمنية مختلفة (ساعة، ساعتان، اربع وعشرون ساعة، ثمان واربعون ساعة). تم تقييم التغيرات المورفولوجية باستخدام المجهر الضوئي. كانت المتبرعمة الكيسية المعزولة من المرضى الذين يعانون من أعراض مرضية من النمط الجيني الاول وظهر التأثير النوعي للأميغدالين B17 وبذور اليقطين عند  $p < 0.05$  في تثبيط نمو المتبرعمة الكيسية مختبرياً. أظهرت هذه النتائج الاثار العلاجية المحتملة للأميغدالين B17 وبذور اليقطين ضد داء المتبرعمة الكيسية كبديل طبيعى ، أمنة وفعالة.

الكلمات المفتاحية: المتبرعمة الكيسية، أميغدالين B17، بذور اليقطين، ميترونيدازول، الزراعة في المختبر.