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EFFECT OF GREEN ALGAE CHLORELLA EXTRACTION IN TREATMENT OF CRYPTOSPORIDIOSISI INFECTION IN EXPERIMENTAL INFECTED MICE

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Abstract

The objective of this study was to conduct in vivo to detect the effect of alcoholic and aqueous Chlorella algea extracts in comparison with azithromycin on Cryptosporidium spp. and to examine its in male laboratory mice (Mus musculus) type Albino mice of c-Balb strain. Stool samples were collected from patients attending in Al-Kut Hospital suffering from diarrhea from December 2021 to end of March 2022, and 90 samples were examined for both sexes microscopic by using the modified Ziehl-Nelson Stain to detect infected the parasite's oocysts , Isolation and purification by flotation with Scheither's sugar solution and preserved in potassium dichromate for the purpose of using it in causing infection in laboratory mice. The experimental study was conducted on groups of 57 mice by oral administration of parasite oocysts at 104 oocyst / ml; while, the negative control group that drenched a physiological saline. The feces of mice inoculated with oocysts of parasites were microscopically examined using the modified Ziehl-Nelson Stain, as well as the molecular examination was conducted using Multiplex PCR technique. After the mice were divided into five groups with the uninfected and untreated group kept as a healthy negative control. The first group which included 21 mice was treated after it was divided into three subgroups A, B, C for each secondary group 7 mice they were treated with alcoholic extract of Chlorella at different concentrations 50, 100, and 150 mg/ml, respectively; while, the second group which included 21 mice on three groups A, B, and C was treated with aqueous extract of Chlorella at the previous concentrations for three consecutive days for each concentration. The third group was treated with azithromycin at a concentration of 500 ml, and the positive control group remained infected with the parasite and was not treated. After treatment a microscopic examination was performed by evaluating the excretion average of parasite oocysts using a hematocytometer slide.

There was a decrease in the shedding of fecal oocysts in the groups treated with alcoholic extract at different concentrations, reaching down as 250 with the highest concentrations at the end of the treatment period. The average of shedding of fecal parasite oocysts of groups of mice at concentrations 50, 100, and 150 mg/ml reached 1.875, 625, and 250 oocyst / ml, respectively. The groups treated with the aqueous extract also showed a clear decrease in the shedding of fecal oocysts, as the average reached the three concentrations 50, 100, 150 mg/ml to 2.750, 1000, 700 oocyst/ml respectively, while the average of shedding of oocysts in the group treated with azithromycin reached 0 oocyst/ml in comparison with the infected positive control group in which the number of oocysts continued to increase to 14.863 oocyst/ml at the end of the treatment period, the results showed statistically significant differences between groups with different concentrations at $P \leq 0.05$. The results of the therapeutic efficacy of Chlorella alga extracts showed

percentages of 87%, 95%, and 98% for the alcoholic extract at concentrations 50, 100, and 150 mg/ml, respectively; while the rates were recorded 81%, 93%, and 95% for aqueous extract at concentrations 50, 100, and 150 mg/ml. However, the therapeutic efficacy of azithromycin reached 100% at the end of the treatment period.

Key words: Cryptosporidium spp., Chlorella Extract, Azithromycin

INTRODUCTION

Cryptosporidium is a protozoan parasite of medical importance that causes gastroenteritis in a variety of vertebrate hosts (Gerace et al., 2019). Cryptosporidium spp. is a single-celled, obligate intracellular (extracytoplasmic apicomplexan) parasite that infects the epithelial cells of the digestive tract of humans and animals, causing diarrhea (cryptosporidiosis) in immunodeficiency individuals (Utami et al., 2020).More than 20 species are perceived, however most of human cases are brought about by C. parvum or C. hominis (Wilke et al., 2018). Profuse watery diarrhea can last up to 3 weeks in immunocompetent patients and can lead to life-threatening malnutrition and wasting in immunocompromised patients. Fecal-oral transmission can occur by ingestion of contaminated, drinking water, food, or through contact with infected persons or animals (Ahlinder et al., 2022). The entire development of Cryptosporidium comprises asexual reproduction, gametogenic, and formation of oocyst containing four sporozoites that occur within the samehost (Cruz-Bustos et al., 2021). In the immunocompromised patient , Cryptosporidium infection is distinguished by gastrointestinal signs that include profuse , sudden- onset, watery diarrhea which may be accompanied by vomiting, abdominal pain or cramps, and weight loss and more non-specific symptoms include fatigue, malaise, nausea, muscle weakness and fever (Carter et al., 2019; Carter et al., 2020). Cryptosporidium has a complex life cycle consisting of both an asexual phase (merogony) and a sexual phase (gametogony) that culminates in oocyst formation (Wilke et al., 2018), it is includes six evolutionary stages, which are excystation stage, merogony stage, gametogony stage, fertilization, oocysts stage and sporogony stage (Dhal et al., 2022).

Feces are the most commonly examined sample while sometimes small bowel aspirates, biopsies or tissue samples may be available, Cryptosporidium can be diagnosed by a number of techniques including microscopic examination either by the wet mount preparation or staining the smears with modified acid-fast stain or by fluorescent stains ,Immunological methods detecting both antigen and antibody are available, Histological examination of the biopsy and various molecular methods for detection of DNA are also available (Pumipuntu and Piratae, 2018).

Due to the pathological importance caused by the parasite and its danger, especially in children and immunosuppressed, and the lack of treatments used to treat this parasite, plant and algal extracts were approved for its treatment (Besednova et al., 2021), one of the most important algae is Chlorella, as a result of the importance of this parasite, Chlorella has been used the Chlorella contain abundant amounts of antioxidants and phytonutrients, as well as several alkaloids known for their powerful anti-inflammatory properties Antioxidant, which makes them very useful in supporting the immune system to fight carcinogens and algae are known for their powerful anti-inflammatory properties that cause chronic diseases, In addition to their role in delaying aging

(Mushtaq et al., 2018). For this reason, cryptosporidiosis is considered one of the riskiest opportunistic infections for patients with acquired immune deficiency syndrome (Innes et al., 2020).

MATERIALS AND METHODS

Samples collection

90 stool samples were collected from patients admitted to Al-Kut Hospital and Al- Zahra Teaching Hospital in the Kut city suffering from diarrhea for both sexes, for the period from December 2021 to the end of March 2022, and kept in potassium dichromate solution for the purpose of conducting examinations.

Microscopic Examination of Stool

For the detection of cryptosporidium spp. oocysts, each sample was examined by smears stained with a by modified Ziehl- Nelson stain according to the method (AL-Ezzy and Kadhim, 2021) in which a portion of feces the size of the tip of a match was taken and mixed on a clean glass slide and mixed with a drop of distilled water, then distribute it over the entire area of the slide and leave it in the open air to dry , For 10 minutes without using a flame, the swab was fixed by adding drops of 11% methyl alcohol for 5 minutes and left to dry at room temperature, after which carbol red concentrated fuchsin dye was added to the fixed swab and left for 3-5 minutes and passed over a quiet flame. The stain was washed off with a weak stream of tap water and left to air dry then shortened swab with acidified alcohol for 30 seconds, washed with tap water and left to dry. Then the swab was dyed with methylene blue dye for two minutes, washed with a weak stream of water, and dried. The stained specimens were examined with a light microscope under the objective lens of X40 and then the oil lens of X100 for the examination of Cryptosporidium spp. oocysts and to confirm its presence in the stool samples.

Isolation and purification of Cryptosporidum spp. oocysts

Parasite oocysts were isolated from stool samples preserved in potassium dichromate solution in the first stage by flotation using Scheither's sugar solution according to (Al-Dahhan and Zghair, 2020) where stool samples were washed three times using phosphate-buffered saline (PBS) centrifugation at IO rpm for 5 minutes, Each time the filtrate was poured out and the precipitate was shaken until the yellow color of the potassium dichromate solution was removed. Then add 10ml of precipitated sugar sheather solution and mix well and then centrifuge at 700 rpm for 20 minutes, this process is known as flotation because the cysts float in a highly concentrated sugar solution. Then collect the floating portion containing the oval sacs using a Pasteur pipette and dilute it with distilled water in a volume ratio of 1:10 to prevent the sugar solution from affecting the oocysts. Then the diluted solution was precipitated in a centrifuge at 700 rpm for 15 minutes, then the liquid was poured out, the precipitate was re-washed three times with distilled water at the same speed and time. Precipitates containing oocysts were collected in conical tubes and a solution of l% sodium hypochlorite was added to it in a volume equal to the volume of the precipitate, then distilled water was gradually added to the wall of the conical tube. The Qasr product was washed several times with distilled water by rapid disposal (700 rpm for 15 min) and

isolation steps were carried out at 4°C to prevent oocysts from breaking. After each separation process, a drop of sediment is taken on the glass slide; the sliding cover is placed on it and examined under the microscope to ensure the presence of the oocysts of the parasite. Then the oocysts were counted for each millimeter of the suspension using the counting slide scale. Finally, it was used in experimental injury.

Cryptospordium spp. oocysts counting

The number of cysts of the parasite that were used in animal doses was calculated using a hemocytometer slide and based on the method (Kawan, 2018) where a drop of iodine solution was placed as a dilution agent to suspend the pure cysts to stain them and make them more visible under the microscope. The counting slide was washed with distilled water and covered with a cover slide, then put a drop stuck in the counting chamber to spread under the cover with the diffusion characteristic and put it under the microscope and adjust the power of the lenses to get a clear view, as the bags were counted in the eight corners on both sides of the slide according to the equation:

Number of oocysts in 1 ml

$$= \frac{\textbf{calculated oocysts number}}{\textbf{8}} \textbf{x}\ 1000$$(Kadhim and Al-Zubaidi, 2018).

Experimental infection animals

The experimental study was conducted in vivo (white mice), which included (57) male mice aged (8-10) weeks and weighing (28-30) gm, divided into (5) groups and dosed with 104 oocyst/ml for each mice by the oral dosing syringe (Yuddhakaran and Veeraseatakul, 2002) except for the negative control group, which was dosed with physiological saline only, The stool was examined daily using M.Z.N stain to ensure that the parasite oocysts shed, as infection was confirmed on the seventh day by 100%.

Microscopic examination of feces

Stools of mice inoculated with parasite oocysts were examined microscopically with MZN stain on a daily basis for parasite detection and experimental infection was achieved (Khan et al., 2018) in which a portion of the stool sample was placed on a clean glass slide and mixed with a drop of distilled water and distributed over the entire area of the slide Leave in the air for 10 minutes to dry, taking into account the numbering of stool. A few drops of 95% methanol were then placed for 1 minute for the purpose of fixation. Then Carbol Fusion red dye was added to the fixed slide and left for 15 minutes. The sample was then washed with distilled water and left to dry. Acid alcohol was added for the purpose of default and washed with tap water. Then the slide was stained with methylene blue for two minutes. The slide was washed with light water and left to dry. Examination was performed under optical microscopy of 40X and 100X.

DNA extraction multiplex PCR

Fecal samples DNA was extracted using the Presto™ Stool DNA Extraction Kit and performed according to the company's instructions, all samples were treated with heat shock for 5 cycles and boiled in a water bath each for 5 min, then incubated at 56°C for 10 min, extended for 1 h. at 95°C. DNA was extracted and amplified by multiplex PCR targeting the heat shock protein 70 (hsp70)

gene. The Multiplex PCR primers for detection Cryptosporidium parvum, Cryptosporidium hominis based on heat shock protein 70 (hsp70) gene were designed in this study using NCBI-Genbank (KM116517.1 and EF591787.1) and primer 3 plus design. These primers were provided from Scientific Resercher. Co. Ltd, Iraq as following Table (1).

Table 1.Primer sequences used in this study

Primers	Sequence 5'-3'		Product size
C. parvum hsp 70 gene	F	GCTGTTGCTTATGGTGCTGC	625 bp
	R	CCTTGATCTTCTTCTCAGCCTCA	
C. homini *s*hsp 70 gene	F	TCTGCGCTGATTACTTCCGT	310 bp
	R	CCACCAGCAGTTTCTAAACCG	

Preparation of aqueous and ethanolic extracts of Chlorella

A sample of Chlorella in powder form was obtained from Kuching Company, Sarawak, Malaysia.Where 100 grams of dry Chlorella powder were weighed and placed in a glass beaker, then 1000 ml of distilled water was added to it, then placed in an electric mixer for 15 minutes and then the mixture was left for 24 hours. The next day, the mixture was filtered using several layers of medical gauze and placed in a centrifuge at 300 rpm for 10 min. Then the solution was placed in clean and sterile metal dishes and dried in the oven at a temperature of 40°C, and the dried product was placed in sterile opaque vials and kept at a temperature of 4°C until use, and the ethanolic extract was placed and prepared in the same way as an aqueous extract that replaces distilled water with ethanol. Preparation was carried out according to the method El-Hamed et al. (2021).

Preparation of different concentrations (50%, 100%, 150% mg/L) of raw Chlorella extract

The different concentrations used in the study were prepared from the crude Chlorella extract, according to recent method (Okechukwu et al., 2019), three concentrations (50%, 100%, and 150% mg) mg/ml were prepared according to the following equation:

$$\text{concentration} = \frac{\boldsymbol{wt\ (mg)}}{\boldsymbol{v\ (ml)}} \times 100$$

Wt (mg) = Extract weight

V (ml) = distilled water or ethanol.

Azithromycin

Azithromycin (500 mg/5 ml syrup, Iraq. Al Kut) was purchased and used as the control standard drug.

Evaluation of therapeutic efficacy

The therapeutic efficacy of the aqueous and ethanolic extract of Chlorella and azithromycin was calculated according to (Ganai et al., 2021) according to the following equation:

$$\text{Therapeutic efficacy} = \frac{\text{The average number of oocysts in the control group} - \text{The average number of oocysts in the treatment group}}{\text{The average number of in the c oocysts ontrol group}} \times 100$$

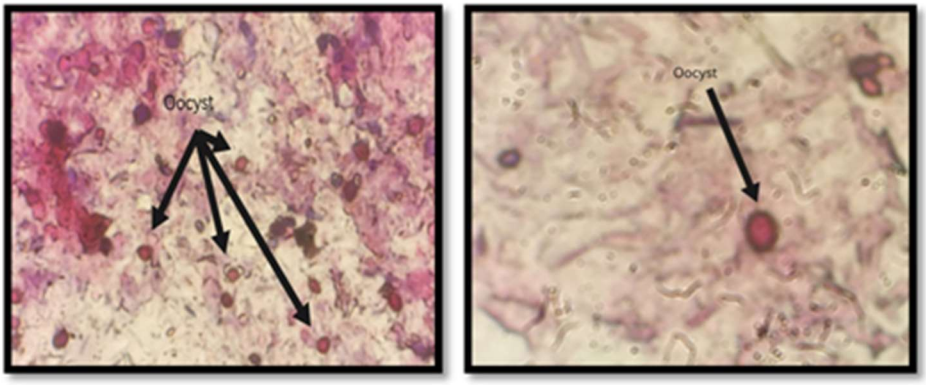
Statistical Analysis

Statistical significance was determined by entering the obtained data into a computer database, the Statistical Package for Social Sciences (SPSS) program was used for statistical analysis, data were recorded in numbers and percentages, numbers were compared using chi-square test, and $P \leq 0.05$ was considered significant (Gharban, 2022).

RESULTS

Detection of Cryptospordium spp. by Using Modified Ziehl-Nelson Stain

As expected, the results of microscopic examination of 124 stool samples isolated from the patients showed that Cryptosporidium spp. oocysts were spherical in shape, red in color with a blue background, containing four spores , using an oil lens after staining it with a modified Ziehl Nelson stain as shown in Figure (1)



A **B**

Figure 1: Oocyst of Cryptosporidium stained by M.Z.N stain Figure A Parasite oocyst with magnification 100X, Figure B Parasite oocyst with magnification 40X.

The result in Table (2) showed that the percentage of those infected with the disease amounted to 57.7% as the number of positive samples reached 52 samples.

Table 2: Ppercentage of positive and negative using MZN

Modified ziehl- neelsen stain	Results	
	No.	%
Specimen Positive for *Cryptosporidium* spp.	52	57.7

Specimen Negative for *Cryptosporidium* spp.	38	42.2
Total	90	99.9

Isolation and purification of Cryptosporidium spp. oocyst by using floatation method

The distinctive morphological shape of Cryptosporidium spp. oocysts was observed, as the ovules appeared in a circular to oval shape of green color surrounded by a thin membrane and containing uncharacteristic spores as in Figure (2).

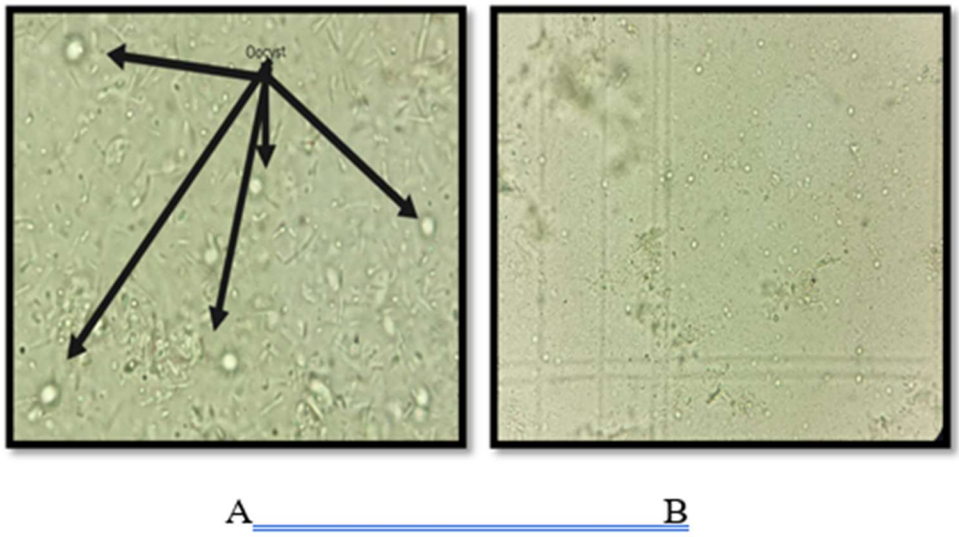


Figure 2: Oocysts Cryptosporidium spp. flotation with Sheather's solution, Figure A Parasite oocyst with magnification 100X, Figure B Parasite oocyst with magnification 40 X.

The results in Table (3) showed the percentage of isolated samples amounted to 57.6% and the average distribution 30 out of 52 positive samples.

Table 3: Percentage Isolated samples by flotation method with Schether's sugar solution

Flotation method with Schether's solution	Results		
	No	Total	%
	30	52	57.6

Experimental Infection

After using the oral dosing method to induce experimental infection in groups of mice, the current study showed that a dose of 104 oocyst/ml of the parasite severely infected mice within 7 days.

Experimental Infection Detection of Cryptosporidium spp. oocysts in Experimental Animals by **Using Modified Ziehl-Nelson Stain**

The results of the examination of 52 stool samples isolated from mice after conducting the experimental infection by MZN stain showed that Cryptosporidium spp. oocysts are spherical in shape with red to pink color with a blue background.

The results in Table (4) showed that the infection percentage was 100% in the groups of mice inoculated with the parasite, and the average distribution was 52% out of (52) samples.

Table 4: A table showing the percentage of microscopic infection of the parasite in the experimental group

Microscope for *Cryptosporidium* spp.	Results			P value	df
	Number	No.	%		
Alcoholic	21	21	100	0.042	2
Aqueous	21	21	100		
Positive control	5	5	100		
Azithromycin group	5	5	100		
Total	52				

Detection of C. Parvum and C. hominis in Experimental animals by Using Multiplex PCR Technique

The PCR multiplex results are shown in Figure 4, where amplification of the (hsp70) gene yielded a clear (2000–100) bp range, confirming the presence of C. parvum and C. hominis in stool samples from lab mice infected with the parasite Cryptosporidium spp.

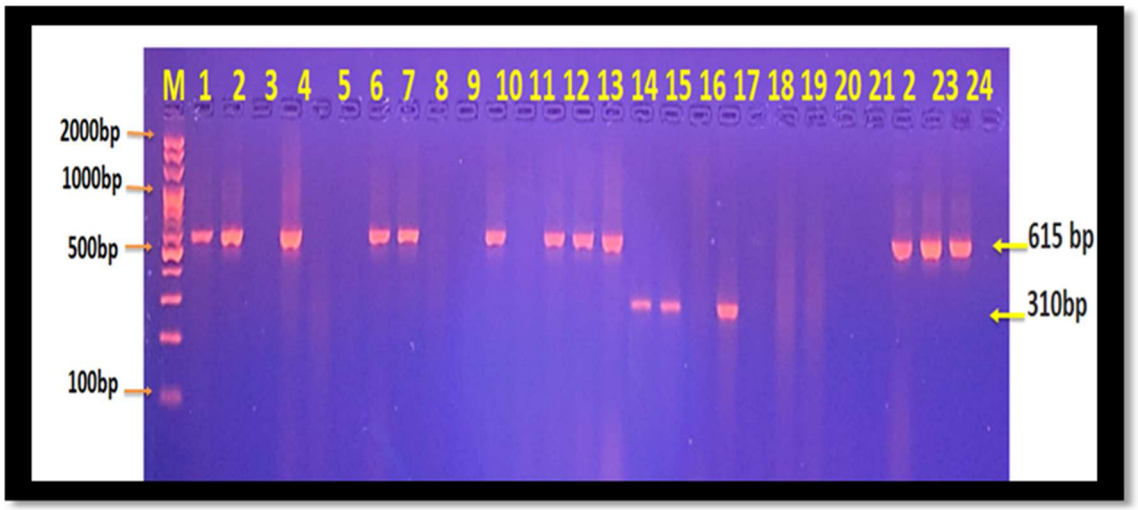


Figure 4: Agarose gel electrophoresis image that showed the Multiplex PCR product analysis of hsp70 gene in C. parvum and C. hominisfrom Rats feces samples. Where, the Lane (M): DNA marker ladder (2000-100 bp) and the Lane (1-24) were showed some positive hsp70 gene in C. parvum at 615 bp and C. hominis at 310 bp PCR product size.

This study included the examination of 52 samples were examined using Multiplex PCR, where the total positive samples showed 51.91% (27/52)while negative samples appeared 48.07% (25/52). As shown in the table (5)

Table 5: The total percentage of infection with the parasite

Multiplex PCR for *Cryptosporidium* spp.	Results		P value	df
	No.	%		
Specimen Positive for *Cryptosporidium* spp.	27	51.91%	0.024	1
Specimen Negative for *Cryptosporidium* spp.	25	48.07%		
Total	52			

Evaluation of the Effect of ChlorellaExtracts Compared with Azithromycin on Cryptosporidium in Vivo (white mice)

The results of the therapeutic study on the third day of treatment with Chlorella alcoholic extract showed a decrease in the number of oocysts released when compared to the number of oocysts immediately after infection, as the mean of oocysts in the group treated with a concentration of 50 mg/ml reached 10.000 oocyst/ml, while the group treated with a concentration of 100 mg/ml reached 9.375 oocyst / ml. While the rate of oocytes released in the group treated with the concentration reached 150 mg/ml to 8.625 oocyst/ml compared with the group treated with azithromycin 500 ml at 10.776 oocyst/ml, the positive control group showed a slight increase in the shedding mean of oocysts in the feces arrived to 11.624 oocyst/ml compared with the negative control group as shown in Table (6). The decrease in the mean of released oocyst continued during the tenth day of treatment in the group treated with concentration 50 mg/ml, reaching 8.250 oocyst/ml, while the highest mean was recorded at concentrations 100 and 150 mg/ml, reaching 5.125 and 4.125 oocyst/ml, respectively. The group treated with azithromycin 5.183 oocyst/ml, compared to the negative control group the positive control group recorded a increase with an average of 13,263 oocyst /ml. On the seventeenth day very large reduction oocysts were recorded as the rate of oocyst shedding in the 50 mg/ml group treated 3.625 oocyst/ml, while the 100 mg/ml group treated with a mean of 1.750 oocyst/ml, and the lowest mean at 150 mg/ml reached 1000 oocyst/ml compared to azithromycin which recorded 301 oocyst/ml, And in comparison with the negative control group recorded a increase in the shed at a mean of 14.697 oocyst/ml. The results of the treatment on the twentieth day recorded a sharp decrease that reached 250 at the highest concentration of 150 mg/ml at a mean of 0 oocyst/ml, while the concentrations of 50 and 100 mg/ml recorded a very low mean of 1.875, 625 oocyst/ml respectively, compared to the group treated with azithromycin with a decrease that reached to stop at a mean of 0 oocyst/ml, the positive control group continued to shed oocysts in the last day at a very high mean of 14,863 oocyst/ml compared to negative control group.

Table (6): Effect of alcoholic extract of Chlorella compared to azithromycin on infection with parasite C. parvum, C. hominis

Groups		**Average number of oocysts /ml during the treatment period after confirming the injury**

	Concentration used mg /kg days	Immediately after confirming injury	3	6	10	13	17	20	P value	x2
Treatment with alcoholic *Chlorell*a extract	50 %	10.375	10.000	9.125	8.250	5.875	3.625	1.875	0.221	7
	Azithromycin drug-treated mg/kg	11.539	10.776	8.645	5.183	2.034	301	0		
	positive control	11.223	11.624	12.996	13.263	13.923	14.697	14.863	0.949	0.266
	100 %	10.625	9.375	7000	5.125	3.375	1.750	625	0.870	1.846
	Azithromycin drug-treated mg/kg	11.539	10.776	8.645	5.183	2.034	301	0		
	positive control	11.223	11.624	12.96	13.63	13.923	14.697	14.863	0.407	5.077
	150 %	11.875	8.625	6.125	4.125	2.125	1000	250		
	Azithromycin drug-treated mg/kg	11.539	10.776	8.645	5.183	2.034	301	0	0.345	5.615
	positive control	11.223	11.624	12.96	13.63	13.923	14.697	14.863	0.194	7.385
Azithromycin drug-treated mg/kg	11.539		10.776	8.645	5.183	2.034	301	0	0.321	5.846
positive control	11.223		11.624	12.996	13.263	13.923	14.697	14.863		
Negative control	0		0	0	0	0	0	0	df	5

The third day of treatment With Chlorella aqueous extract showed a slight decrease in the number of oocytes shedding in the groups treated with concentrations 50, 100 and 150 mg/ml, which recorded 11.500, 11.125, and 10.250 oocyst/ml respectively compared to the group treated with azithromycin at mean of 10 .776 oocyst/ml, while there was an increase slight drop in the mean of

oocysts shedding in the positive control group was 11.624 oocyst/ml compared to the negative control group as shown in Table (7). The therapeutic study recorded on the sixth day in the group treated with a concentration of 50 mg/ml the highest mean of oocytes shedding reached 10.625 oocyst/ml while the group treated with a concentration of 100 mg/ml amounted to 9.375 and the lowest shedding mean at the concentration of 150 mg/ml reached 8.750 oocyst/ml compared with the group treated with azithromycin 8.645 oocyst/ml, while the positive control group continued to increase the shedding of oocysts at a mean of 12,996 oocyst/ml. The results of the thirteenth day of treatment showed a decrease in the number of oocysts in the group treated with a concentration of 150 mg/ml, reaching an average of 3.125 oocyst/ml while the mean of egg shedding was recorded 4.500 and 7.125 oocyst/ml for both concentrations 100 and 50 mg/ml respectively compared to the group treated with azithromycin, which recorded 2.034 oocyst/ml, and compared to the negative control the increase persisted in the positive control group at a mean of 13.923 oocyst/ml. The results of the treatment on the twentieth day and last day of the treated groups showed a decrease in the shedding of oocysts in the treated group at a concentration of 150 and 100 mg/ml, which amounted to 700 and 1000 oocyst/ml, respectively and the highest mean at concentration 50 reached 2.750 oocyst/ml compared to the group treated with azithromycin in which oocytes shedding stopped it reached a mean of 0 oocyst/ml, while the positive control group reached its peak in shedding oocysts at a mean of 14 .863 oocyst/ml.

Table (7): Effect of aqueous extract of Chlorella compared with azithromycin on infection with C. parvum C. hominis

Groups	Concentration Used mg/kg days	Average number of oocysts /ml during the treatment period after confirming the injury								
		Immediately after confirming injury	3	6	10	13	17	20	P Value	X^2
Treatment with aqueous *Chlorella* extract	50 %	12.125	11.500	10.625	9.500	7.125	5.375	2.750	0.277	6.308
	Azithromycin drug-treated mg/kg	11.539	10.776	8.645	5.183	2.034	301	0		
	positive control	11.223	11.624	12.996	13.263	13.923	14.697	14.863	1	0.179
	100 %	12.375	11.125	9.375	7.375	4.500	2.625	1000	0.942	1.231
	Azithromycin drug-treated mg/kg	11.539	10.776	8.645	5.183	2.034	301	0		
	positive control	11.223	11.624	12.996	13.263	13.923	14.697	14.863	0.465	4.615

	150 %	11.750	10.250	8.750	6.250	3.125	1.125	700	0.942	1.231
	Azithromycin drug-treated mg/kg	11.539	10.776	8.645	5.183	2.034	301	0		
	P. control	11.223	11.624	12.996	13.263	13.923	14.697	14.863	0.465	4.615
Azithromycin drug-treated mg/kg		11.539	10.776	8.645	5.183	2.034	301	0	0.321	5.846
positive control		11.223	11.624	12.996	13.263	13.923	14.697	14.863		
Negative control		0	0	0	0	0	0	0	df	5

Therapeutic Efficacy of Alcoholic and Aqueous Chlorella Extract and Azithromycin against Cryptosporidiosis

After applying the therapeutic efficacy equation on the third day of treatment, the highest percentages of therapeutic efficacy were recorded for the alcoholic extract with the highest concentration of it. Where the efficiency of the alcoholic extract concentrations reached 50, 100 and 150, azithromycin mg/ml different percentages reached 13%, 19%, 25% and 7%, respectively as shown in Table (8). The therapeutic efficacy on the tenth day showed that the alcoholic extract recorded a slightly superior Percentage of efficacy over azithromycin, Where the concentrations reached 50, 100, and 150, azithromycin percentages of 37%, 61%, 68%, and 60%, respectively. On the seventeenth day of treatment the therapeutic efficacy of azithromycin was slightly higher than that of the alcoholic extract with the highest concentration , Where it scored 75%, 86%, 93% and 97% for each of the concentrations 50, 100, and 150, azithromycin respectively. On the last day of treatment the highest therapeutic efficacy Percentages were recorded for concentrations 50, 100, and 150 and azithromycin, reaching 87%, 95 %, 98% and 100%, respectively.

Table (8) Therapeutic efficacy of alcoholic Chlorella extract compared with azithromycin against cryptosporidiosis

Treatment period	The therapeutic efficacy ratios of alcoholic *Chlorella* extract					
	50% mg\ml	Azithromycin 500 mg\ml	100% mg\ml	Azithromycin 500 mg\ml	150% mg\ml	Azithromycin 500 mg\ml
3	13%	7%	19%	7%	25%	7%
6	29%	33%	46%	33%	52%	33%
10	37%	60%	61%	60%	68%	60%
13	57%	83%	75%	83%	84%	83%
17	75%	97%	86%	97%	93%	97%
20	87%	100%	95%	100%	98%	100%
P value	0.232		0.189		0.189	
chi square x^2	6.846		7.462		7.462	
Df	5		5		5	

The results after applying the therapeutic efficacy equation on the third day showed little change of the aqueous extract reverse the drug azithromycin, where varying percentages were recorded for each of 50, 100, 150, azithromycin mg/ml amounted to 1%, 4%, 11%, 7%, respectively as in the Table (9). On the sixth day of treatment, azithromycin had a higher therapeutic efficacy than the aqueous extract, as the percentages reached 18%, 27 %, 32 %, and 33 % for each of the concentrations 50, 100 and 150 azithromycin respectively. On the thirteenth day of treatment, the superiority of azithromycin continued with a difference from the highest concentrations of the aqueous extract, with percentages of 48%, 67%, 77%, 83% for each of the concentrations 50, 100 and 150 and azithromycin respectively. On the twentieth day of treatment, the study recorded increase in the therapeutic efficacy Percentages for all concentrations with a marked superiority of azithromycin and the highest concentration of the aqueous extract at percentages of 81%, 93%, 95% and 100% for each of the concentrations 50, 100, 150 and azithromycin respectively.

Table (9): Therapeutic efficacy ratios of aqueous Chlorella extract compared with azithromycin against cryptosporidiosis

Treatment period	The therapeutic efficacy ratios of aqueous *Chlorella* extract					
	50% mg/ml	Azithromycin 500 mg/ml	100% mg/ml	Azithromycin 500 mg/ml	150% mg/ml	Azithromycin 500 mg/ml
3	1%	7%	4%	7%	11%	7%
6	18%	33%	27%	33%	32%	33%
10	28%	60%	44%	60%	52%	60%
13	48%	83%	67%	83%	77%	83%
17	63%	97%	82%	97%	92%	97%
20	81%	100%	93%	100%	95%	100%
P value	0.161		0.179		0.189	
chi square x^2	7.615		7.923		7.462	
df	5		5		5	

DISCUSSION

Regarding the detection of Cryptosporidium spp. by MZN in laboratory mice the current study agree with Wang et al.,(2021) this was done by infecting all groups of experimental mice with the cryptosporidium parasite after dose, It also agrees with the results of the study Khater et al. (2017) Infect all experimental mice with the parasite suspension. The results of the current study disagree with Gaber et al. (2022) when laboratory mice were drenched with cryptosporidium, not all groups were infected, which means that the infection was partial.

As for the detection of C. Parvum and C. hominis in experimental animals by using multiplex PCR technique the current study agree with Ježková et al. (2021) in wild brown mice, when conducting the polymerase chain reaction (PCR), 55% of samples positive for the parasite appeared. Also, the current study agrees with Zhao et al.,(2019) this was done by collecting the feces of wild mice infected with Cryptosporidium and diagnosing them using PCR, where the percentage appeared (75/150) 50%.

The results of this study disagree with the results of Ayinmode et al. (2017) It was studied in Nigeria on laboratory mice, 134 fecal samples were obtained and examined for the presence of Cryptosporidium oocyst using PCR where only 2 samples were positive 1.4%, and disagree with the study Wei et al. (2019) 2.1% (9/435) were seen in laboratory mice using the PCR test.

The results of the current study showed through the use of aqueous and alcoholic extract of Chlorella algae, which belongs to green algae. The effectiveness of this algae against Cryptosporidium parasite during the current study, water and ethanol alcohol were used as solvents due to the fact that water is the most widely used solvent at the global level in extraction (Mostafa et al., 2018). As for ethanol alcohol, it is the safest solvent in terms of health for the preparation of extracts (Gunathilaka et al., 2019). The results showed significant effects against the parasite Cryptosporidium through the inhibitory effect of the aqueous and alcoholic extracts of this alga at different concentrations and with varying rates on the appearance of parasite cysts in the stool, which is attributed to the effect of the extracts at the vegetative phases of the parasite, compared with the positive control group, whose infection continued to increase throughout the trial period. As for the difference in the effectiveness of the extracts against the parasite, it may be due to the nature of the compounds extracted from each solvent (Ayal, 2021).

The inhibitory mechanism of the extract may be attributed to the ability to weaken the enzymatic system of the parasite, including the enzymes involved in energy production, as well as its effect on the integrity and structure of the parasite membrane (Abdelmohsen et al., 2017).

The presence of alkaloids in the aqueous extract has its inhibitory ability on microorganisms through its interference in the chain of metabolic reactions of proteins necessary for the continuation of its vitality and its ability to destroy the cell wall and its contents of proteins and fats, and then destroy it (Alghazeer et al., 2022). As for the phenolic compounds, they led to the parasite's cell membrane losing its selective permeability, and thus the unregulated entry and exit of substances from the parasite, and then the parasite's death (Antwi et al., 2019). There are studies that indicated that phenolic compounds in algal extracts have the ability to bind with lipids in the cell membrane and the membranes of cell organelles and change their functional structure and thus the death of the living cell (Khadija et al., 2020). The effect may be due to the presence of other active substances, such as flavonoids which have the ability to denature of proteins and to stop the action of enzymes accompanying the glycolysis process, the most important of which is Hexokinase, thus losing the microorganism's ability to continue to live (Fernandes et al., 2017). Or the effect may be due to the impact of metabolic processes related to nitrogen and amino acids that are essential in building cell membranes, nuclei, and the Golgi body, which is important in the continuation of the vitality of microorganisms, or because of its ability to unite with proteins, which leads to changes in the chemical properties of the cell wall or changes the shape of the entire cell, and leads to her death (Chiaradonna et al., 2018).

As for the alcoholic extract of Chlorella, the current study confirmed that the extract of Chlorella algae contains proteins, amino acids and peptides, which consist of a high percentage of protein more than 50% dry weight , and therefore it can be used as a source of essential amino acids including isoleucine, leucine , lysine, valine and tryptophan and the study showed that the alcoholic

extract of Chlorella algae with its concentrations The different types of cysts led to a clear decrease in the rate of the number of oocyst presented with the increase in concentration, and this can be explained by the chemical content of the alcoholic extract of algae, to which this effect can be attributed due to Its high content of long -chain unsaturated fatty acids, and these results are consistent with what was mentioned (Andrade et al., 2018).

In addition, it contains phenolic compounds, which are among the most important classes Natural antioxidants, including the phenolic compounds produced by these micro-algae on caffeic acid, ferulic acid and bucomaric acid (Salehi et al., 2019).

This algae contains volatile compounds, and due to its biological activity (Caporgno and Mathys, 2018). These are identified as carbonyls, alkenes, and aliphatic alcohols saturated and unsaturated, aldehydes, quinones, sulfur esters, terpene sulfides, acids Steroids, Advance volatile organic compounds offer anti-bacterial, anti-fungal, anti-viral and anti-cancer efficacy (Zuo, 2019).

Conclusions

The alcoholic and aqueous extract of Chlorella algae showed an effective and safe therapeutic effect for cryptosporidiosis, and the alcoholic extract had a greater effect than the aqueous extract. Increasing the therapeutic efficacy of the alcoholic and aqueous extract of Chlorella algae by increasing the concentration, as high concentrations showed greater therapeutic efficacy than low concentrations of both extracts. The extracts of Chlorella algae have an important effect on the parasite, and when compared with the drug azithromycin it was found that the alcoholic extract of Chlorella algae with the highest concentration it has a therapeutic efficacy to that of azithromycin, while the efficiency of the aqueous extract of Chlorella algae less effective than efficacy of azithromycin. Alcoholic and aqueous Chlorella algae extracts are important not only in eliminating the parasite, but also in restoring and reconstructing the damaged parts of the intestine.

References

Abdelmohsen, U. R., Balasubramanian, S., Oelschlaeger, T. A., Grkovic, T., Pham, N. B., Quinn, R. J., and Hentschel, U. (2017). Potential of marine natural products against drug-resistant fungal, viral, and parasitic infections. The Lancet Infectious Diseases, 17(2): e30-e41.

Ahlinder, J., Svedberg, A. L., Nystedt, A., Dryselius, R., Jacobsson, K., Hägglund, M.,and Troell, K. (2022). Use of metagenomic microbial source tracking to investigate the source of a foodborne outbreak of cryptosporidiosis. Food and waterborne parasitology, 26: e00142.

Al-Dahhan, I. N., and Zghair, Z. R. (2020). Isolation of Cryptosporidium spp. from rabbits in Baghdad city, Iraq. Plant Arch, 20(Suppl 2), 2911-4.

AL-Ezzy, A. I. A., and Kadhim, A. T. (2021). Clinical Evaluation for the wet mount preparations versus Ziehl–Neelsen staining modifications for Diagnosis and severity scoring of cryptosporidium parvum in children under 5 years. Diyala Journal For Veterinary Sciences, 1(2): 126-138.

Alghazeer, R., Azwai, S., Garbaj, A. M., Amr, A., Elghmasi, S., Sidati, M., and Alansari, W. S. (2022).Alkaloids Rich Extracts from Brown Algae Against Multidrug-Resistant Bacteria by Distinctive Mode of Action. Arabian Journal for Science and Engineering, 47(1): 179-188.

Andrade, L. M., Andrade, C. J., Dias, M., Nascimento, C., and Mendes, M. A.(2018).Chlorella and spirulinamicroalgae as sources of functional foods.Nutraceuticals, and Food Supplements, 6(1): 45–58.

Antwi, C. A., Amisigo, C. M., Adjimani, J. P., and Gwira, T. M. (2019). In vitro activity and mode of action of phenolic compounds on Leishmaniadonovani. PLoS neglected tropical diseases, 13(2): e0007206.

Ayal, A. W. R. (2021). Effect of Methanolic Extract of Green Algal EnteromorphaIntestanalis on the Bioactivity of Giardia lamblia Parasite. Indian Journal of Forensic Medicine and Toxicology, 15(3): 1847-1852.

Ayinmode, A. B., Ogbonna, N. F., and Widmer, G. (2017). Detection and molecular identification of Cryptosporidium species in laboratory rats (Rattusnorvegicus) in Ibadan, Nigeria. Annals of Parasitology, 63(2).

Besednova, N. N., Zaporozhets, T. S., Andryukov, B. G., Kryzhanovsky, S. P., Ermakova, S. P., Kuznetsova, T. A.,and Shchelkanov, M. Y. (2021). Antiparasitic Effects of Sulfated Polysaccharides from Marine Hydrobionts. Marine Drugs, 19(11): 637.

Caporgno, M. P., and Mathys, A. (2018). Trends in microalgae incorporation into innovative food products with potential health benefits. Frontiers in nutrition, 5: 58.

Carter, B. L., Chalmers, R. M., and Davies, A. P. (2020). Health sequelae of human cryptosporidiosis in industrialised countries: a systematic review. Parasites and vectors, 13(1): 1-14.

Carter, B., Stiff, R., Elwin, K., Hutchings, H., Mason, B., Davies, A., and Chalmers. (2019). Health sequelae of human Cryptosporidiosis—a12-month prospective follow-up study. European Journal of ClinicalMicrobiology and Infectious Diseases, 38(9): 1709-1717.

Chiaradonna, F., Ricciardiello, F., and Palorini, R. (2018).The nutrient -sensing hexosamine biosynthetic pathway as the hub of cancer metabolic rewiring. Cells, 7(6): 53.

Cruz-Bustos, T., Feix, A. S., Ruttkowski, B., and Joachim, A. (2021). Sexual development in non-human parasitic apicomplexa: just biology or targets for control?. Animals, 11(10): 2891.

Dhal, A. K., Panda, C., Yun, S. I., and Mahapatra, R. K. (2022).An update on Cryptosporidium biology and therapeutic avenues. Journal of Parasitic Diseases, 1-17.

El-Hamed, A., Fayez, W., Yousef, N. S., Mazrou, Y. S., Elkholy, W. A., El-Refaiy, A. I., Albadrani,M., El-Tokhy,A, I., and Abdelaal, K. (2021). Anti Cryptosporidiumefficacy of Oleaeuropaea and Ficuscarica Leaves Extract in Immunocompromised Mice Associated with Biochemical Characters and AntioxidativeSystem.Cells, 10(9) : 2419.

Fernandes, I., Pérez-Gregorio, R., Soares, S., Mateus, N., and De Freitas, V. (2017). Wine flavonoids in health and disease prevention. Molecules, 22(2): 292.

Gaber, M., Galal, L. A. A., Farrag, H. M. M., Badary, D. M., Alkhalil, S. S., and Elossily, N. (2022).The Effects of Commercially Available Syzygiumaromaticum, Anethumgraveolens,

Lactobacillus acidophilus LB, and Zinc as Alternatives Therapy in Experimental Mice Challenged with Cryptosporidium parvum. Infection and Drug Resistance, 15: 171.

Ganai, A., Yadav, A., Katoch, R., and Chakraborty, D. (2021) .Therapeutic Efficacy of Ethanolic Extract of Curcuma longa and its Component, Curcumin against Experimental Cryptosporidiosis in Mice. Indian Journal of Animal Research, 1: 6.

Gerace, E., Presti, V. D. M. L., and Biondo, C. (2019). Cryptosporidium infection: epidemiology, pathogenesis, and differential diagnosis. European Journal of Microbiology and Immunology, 9(4) : 119-123.

Gharban, H. A. (2022). Clinical and Serological Diagnosis of Bovine Hypodermosis in Wasit Province. Revista Electronica de Veterinaria, 457-466

Gunathilaka, T. L., Samarakoon, K. W., Ranasinghe, P., and Peiris, L. D. C. (2019).In-vitro antioxidant, hypoglycemic activity, and identification of bioactive compounds in phenol-rich extract from the marine red algae Gracilariaedulis (Gmelin) Silva. Molecules, 24(20): 3708.

Innes, E. A., Chalmers, R. M., Wells, B., and Pawlowic, M. C. (2020). A one health approach to tackle cryptosporidiosis. Trends in parasitology, 36(3): 290-303.

Ježková, J., Prediger, J., Holubová, N., Sak, B., Konečný, R., Feng, Y.,and Kváč, M. (2021). Cryptosporidiumratti n. sp.(Apicomplexa: Cryptosporidiidae) and genetic diversity of Cryptosporidium spp. in brown rats (Rattusnorvegicus) in the Czech Republic. Parasitology, 148(1): 84-97.

Kadhim, A. K., and Al-Zubaidi, M. T. S. (2018). Isolation of Cryptosporidiumoocysts from slaughtered broiler chicken and Experimental infection in chicks. Basrah Journal of Veterinary Research, 17(3).

Kawan, M. H. (2018). Calculation of the Shedding Rate of CryptosporidiumOocysts from the Natural Infected Sheep. Iraqi Journal of Agricultural Sciences, 49(3).

Khadija, A. T., Abd-Wahab, R. A., and Fadhil, A. M. (2020). Effect of Ethanolic extract of Chlorella spp. On Entamoebahistolytica parasite in vivo. Plant Archives, 20(1): 1975-1978.

Khan, A., Shaik, J. S., and Grigg, M. E. (2018). Genomics and molecular epidemiology of Cryptosporidium species. ActaTropica, 184: 1-14.

Khater, M. M., El-Sayed, S. H., Yousof, H. A. S., Mahmoud, S. S., El -Dib, N., and El-Badry, A. A. (2017). Anti-Cryptosporidium efficacy of Oleaeuropaea and Actinidiadeliciosa in a neonatal mouse model. Kasr Al Ainy Medical Journal, 23(1), 32.

Mostafa, A. A., Al-Askar, A. A., Almaary, K. S., Dawoud, T. M., Sholkamy, E. N., and Bakri, M. M. (2018). Antimicrobial activity of some plant extracts against bacterial strains causing food poisoning diseases. Saudi journal of biological sciences, 25(2): 361-366.

Mushtaq, S., Abbasi, B. H., Uzair, B., and Abbasi, R. (2018). Natural products as reservoirs of novel therapeutic agents. EXCLI journal, 17: 420.

Nuchjangreed, C. (2018).An Investigation of Giardiasis and Cryptosporidiosis in Malawi and Cambodia. Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy : 241.

Okechukwu, P. N., Ekeuku, S. O., Sharma, M., Nee, C. P., Chan, H. K., Mohamed, N., and Froemming, G. R. A. (2019).In vivo and in vitro antidiabetic and antioxidant activity of spirulina. Pharmacognosy Magazine, 15(62): 17.

Pumipuntu, N., and Piratae, S. (2018).Cryptosporidiosis: A zoonotic disease concern. Veterinary world, 11(5): 681.

Salehi, B., Sharifi-Rad, J., Seca, A. M., Pinto, D. C., Michalak, I., Trincone, A.,and Martins, N. (2019). Current trends on seaweeds: Looking at chemical composition, phytopharmacology, and cosmetic applications. Molecules, 24(22): 4182.

Utami, W. S., Murhandarwati, E. H., Artama, W. T., and Kusnanto, H. (2020). Cryptosporidium infection increases the risk for chronic diarrhea among people living with HIV in Southeast Asia: a systematic review and meta-analysis. Asia Pacific Journal of Public Health, 32(1): 8-18.

Wang, L., Cao, L., Zheng, S., Chang, Y., Zhang, K., Zhang, S., and Zhang, L. (2021). Molecular identification and biological characterization of Cryptosporidiummuris from camels (Camelusbactrianus) in China. Parasites and Vectors, 14(1): 1-10.

Wei, Z., Liu, Q., Zhao, W., Jiang, X., Zhang, Y., Zhao, A., ... and Qi, M. (2019). Prevalence and diversity of Cryptosporidium spp. in bamboo rats (Rhizomyssinensis) in South Central China. International Journal for Parasitology: Parasites and Wildlife, 9: 312-316.

Wilke, G., Ravindran, S., Funkhouser-Jones, L., Barks, J., Wang, Q., VanDussen, K. L.,and Sibley, L. D. (2018). Monoclonal antibodies to intracellular stages of Cryptosporidium parvum define life cycle progression in vitro. Msphere, 3(3): e00124-18.

Yuddhakaran, Y., and Veeraseatakul, P. (2002). Experimental Study on Mixed Infections of Cryptosporidium muris and C. parvum in Severe Combined Immunodeficient (SCID) and BALB/c Mice. J. Trop. Med. Parasitol, 25 :1-5.

Zhao, W., Zhou, H., Huang, Y., Xu, L., Rao, L., Wang, S.,and Lu, G. (2019).Cryptosporidium spp. in wild rats (Rattus spp.) from the Hainan Province, China: Molecular detection, species/genotype identification and implications for public health. International Journal for Parasitology: Parasites and Wildlife, 9: 317-321.

Zuo, Z. (2019). Why algae release volatile organic compounds—the emission and roles. Frontiers in Microbiology, 10:491.