

Effect of Algae *Spirulina* Extraction in treatment of intestinal tissues of mice infected with *Cryptosporidium* spp.

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Abstract:

Cryptosporidiosis caused by *Cryptosporidium* spp. It is a zoonotic disease that is the most prevalent, so the study was conducted to evaluate the anti-cryptosporidiosis efficacy of alcoholic and aqueous spirulina algae extracts in comparison with Azithromycin in the intestines of infected mice. Fecal samples were collected from patients at Al-Kout Hospital suffering from diarrhea in the period November 2021 to February 2022, and 124 microscopic samples of both sexes were examined using a modified Ziehl-Nelson stain to detect eggs infected with the parasite. Isolation and flotation purification with shifter sugar solution and preservation in potassium dichromate for the purpose of infection in mice. The Experimental study was on groups of 57 mice by dealing with oral parasite oral oocysts within 104 oocyst/ ml except for the negative group addressed by a fishery saline solution. To strict injury, the detailed mice has been examined with microscopic parasites using Ziehl-Nelson Stain, and molecular screening was performed using Multiplex PCR technology. 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 *Spirulina* at different concentrations 50, 100, 150 mg/ml on the respectively, while the second group which included 21 mice on three groups A, B, and C was treated with aqueous extract of *Spirulina* 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. The results of histological examination showed that treatment with alcoholic and aqueous *Spirulina* algae extracts led to remarkable repair and regeneration with

restructuring in all sections of the small intestine infected with *Cryptosporidium* spp. parasite to varying degrees according to the concentrations used. Whereas the groups treated with *Spirulina* algae extracts showed epithelial layers renewed with the formation of non-enlarged cells and less edema, in addition less infiltration of inflammatory cells was observed in the submucosal layer. There was a slight inflammation in the tissues of the duodenum, jejunum and ileum with a clear decrease in inflation and the degree of inflammation as well as less severe lesions in the intestinal tissues treated with alcoholic and aqueous extract of *Spirulina* algae in the highest concentrations with complete disappearance of parasite oocysts.

Key words: Intestine, *Cryptosporidium* spp., *Spirulina*.

1. Introduction

Cryptosporidiosis is a diarrheal disease caused by protozoa of the genus *Cryptosporidium* spp. Most human infections are caused by 2 species: *C. hominis*, which is reportedly restricted to humans, and *C. parvum*, which can also be found in a wide range of animals [1]. In which *Cryptosporidium* oocysts have ubiquitous presence in the environment, *Cryptosporidium* oocysts transmission can occur following direct or indirect contact with an infected host usually via the fecal–oral route, Person-to-person contact, zoonosis, and the consumption of contaminated food or water are well known mechanisms for fecal–oral transmission [2, 3]. Infection results from consumption of contaminated water or food including raw milk [4]. cryptosporidiosis is a short-term sickness in the form of diarrhea and weight

loss in immunocompetent children and adults. However, in immunocompromised individuals, infection could be prolonged and life-threatening [5]. The incubation period ranges from 1 to 12 days on average, about 7 days, and symptoms in immunocompetent patients can last up to 4 weeks. The most common symptom of cryptosporidiosis is watery diarrhea, but abdominal pain, fever, nausea and dehydration can also occur.[6]. Effective treatment for cryptosporidiosis has not already been demonstrated, although more than 200 chemotherapeutic agents have evaluated their anti-*Cryptosporidium* spp. effects, the only FDA-approved drug therapy is nitazoxanide, and clinical studies [7]. Those who suffer from diarrhea as a result of infection with cryptosporidiosis, there is a danger to their lives when they are treated with this medicine, and they need better treatment, especially those who suffer from other chronic diseases that lead to their

immune deficiency, As it was found that the toxicity and side effects of these treatments persist even after modification the recommended dose and duration of treatment as (MNZ) has been shown to be a toxic substance that has an effect on living organisms [8].

2. Materials and Methods:

2.1 Samples collection

124 stool samples were collected from patients admitted to Al-Kut Hospital suffering from diarrhea for both sexes, for the period from November 2021 to February 2022.

2.2 Experimental injury

Specimens examination was carried out by modified Ziehl Nelson, part of the stool was taken and swab mixed drops of methyl alcohol 11% for 5 minutes and then concentrated red dye was added to 5 minutes, after that washed, then washed with acid and short alcohol, then washed with tap water, then stained with water with methylene blue dye for 2 minutes, then washed with double amount of water and left to dry. Then, samples were scanned at powers of 40× and 100×. Parasite oocysts in preserved fecal samples were isolated and purified by flotation using Sheather's glucose solution according to [9]. The number of cysts was

calculated according to the formula: The number of oocytes in 1 ml = (the number of oocytes counted) / (8) x 1000 [10]. The experimental group in the body (white mice), which included (57) male mice, aged (8-10) weeks and weighing (28-30) g, divided into (5) groups and treated with 104 oocysts / ml per mouse for experimental infection events.

2.3 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 Researcher. Co. Ltd, Iraq as following Table (1).

Table 1: Primer sequences used in this study.

Primers	Sequence 5'-3'		Product size
<i>C. parvum</i> hsp 70 gene	F	GCTGTTGCTTATGGTGCTGC	625 bp
	R	CCTTGATCTTCTTCTCAGCCTCA	
<i>C.hominis</i> hsp 70 gene	F	TCTGCGCTGATTACTTCCGT	310 bp
	R	CCACCAGCAGTTTCTAAACCG	

2.4 Preparation of an aqueous and ethanolic extracts of *Spirulina* and the Concentrations were used in the Study (50 %, 100 %, 150 % mg / L)

A sample of *Spirulina* in powder form was obtained from Kuching Company, Sarawak, Malaysia. Where 100 grams of dry *Spirulina* powder were weighed and placed in a glass beaker, then 1000 ml of distilled water was added. Then placed in an electric mixer for 15 minutes and then the mixture was left for 24 hours. Moreover, 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 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. Prepared in the same way as an aqueous extract that replaces distilled water

with ethanol. Prepared according to the method [11]. The different concentrations used in the study were prepared from the crude *Spirulina* extract, according to the method [12], three concentrations (50 %, 100 %, 150 % mg) mg / ml were prepared according to the following equation: concentration = (wt (mg)) / (v (ml)) × 100 Wt (mg) = Extract weight , V (ml) = distilled water or ethanol.

2.5 Executing the experiment.

After the groups of mice were divided into five groups, infection was confirmed in all groups except for the negative control group that was dosed with physiological saline. The first group treated 21 rats after they were divided into three subgroups A, B and C. Each group of seven mice was treated with alcoholic extract of *Spirulina* at concentrations of 50 %, 100 % and 150 %, while the second group was treated with

aqueous *Spirulina* extract at the same previous concentrations, and the third group was treated with azithromycin. While the fourth group remained infected with the parasite and was not treated as a positive control.

2.6 Azithromycin

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

2.7 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 [13].

3. Results

Detection of *C. Parvum* and *C. hominis* in Experimental animals by Using Multiplex PCR Techniquil. 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 1: Agarose gel electrophoresis image that showed the Multiplex PCR product analysis of hsp70 gene in *C. parvum* and *C. hominis* from 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) stool samples taken from mice inoculated with the parasite. The result using Multiplex PCR technology in table 2 showed

that the infection rate was 38.4 % with a distribution rate of (20) samples.

Table 2: Confirmation percentage of infection with *Cryptosporidium Spp.* according to Multiplex PCR Technique.

Multiplex PCR for <i>Cryptosporidium Spp.</i>	Results	
	No	%
Specimen Positive for <i>Cryptosporidium Spp.</i>	20	38.4 %
Specimen Negative for <i>Cryptosporidium Spp.</i>	32	61.5 %
Total	52	100 %

The results of the extracted DNA are shown in table 3 from 20 positive stool samples, 75% were recorded for *C. parvum* in 15 samples, and 15% were for *C. hominis* in 3 samples, and 10% in 2 samples were infected with *C. parvum*, and *C. hominis*.

Table 3: Percentages of samples infected with both *C. parvum*, and *C. hominis* according to Multiplex PCR Technique.

Multiplex PCR for <i>Cryptosporidium Spp.</i>	Results	
	No.	%
Specimen Positive for <i>Cryptosporidium Parvum</i>	15	75 %
Specimen Positive for <i>Cryptosporidium Hominis</i>	3	15 %
Specimen Positive for <i>Cryptosporidium Parvum and hominis</i>	2	10 %
Total	20	100 %

*Chi-Square at $p \leq 0.05$ P Value 0. 252.

The results also showed in table 4 display the difference in infection percentage in groups of infected mice that will be treated

with alcoholic and aqueous *Spirulina* extract and azithromycin by using the Multix PCR technique, where it recorded 33.3% in the

alcoholic extract group with 7 samples, while in the aqueous extract group it was recorded 38% with 8 , while the azithromycin group

recorded 60% with 3 samples, and the control group was 40% with 2 samples.

Table 4: Distribution of infection percentages for groups of mice by using Multiplex PCR Technique

Specimen Positive for <i>Cryptosporidium</i> spp.	Multiplex PCR for <i>Cryptosporidium</i> Spp.							
	Alcoholic		Aqueous		Azithromycin		Control	
	No.	%	No.	%	No.	%	No.	%
	7	33.3	8	38	3	60	2	40
Total	21		21		5		5	

*Chi-Square at $p \leq 0.05$ P Value 0.03

3.1 Histopathological changes

The results of the histological examination of the infected group showed pathological changes that represented significant damage to the tissues of the small intestine in all its parts, while the small intestine tissues in the negative control group were in a normal condition. In the duodenum of the infected and untreated control group, a clear atrophy and detachment was observed in the upper ends of the villi with sloughing of the basal layer clearly with a large

enlargement of the epithelium. In the jejunum part of the infected and untreated control showed the oocyst of *Cryptosporidium* spp. with dangerous infiltration of inflammatory cells in the lamina propria. Histological examination of the ileum part in the infected and untreated control group showed that there was a clear enlargement of the villi with *Cryptosporidium* spp. oocytes, in addition to a marked enlargement of the epithelium and a significant infiltration of inflammatory cells present in the lamina propria in as shown in figure 2.

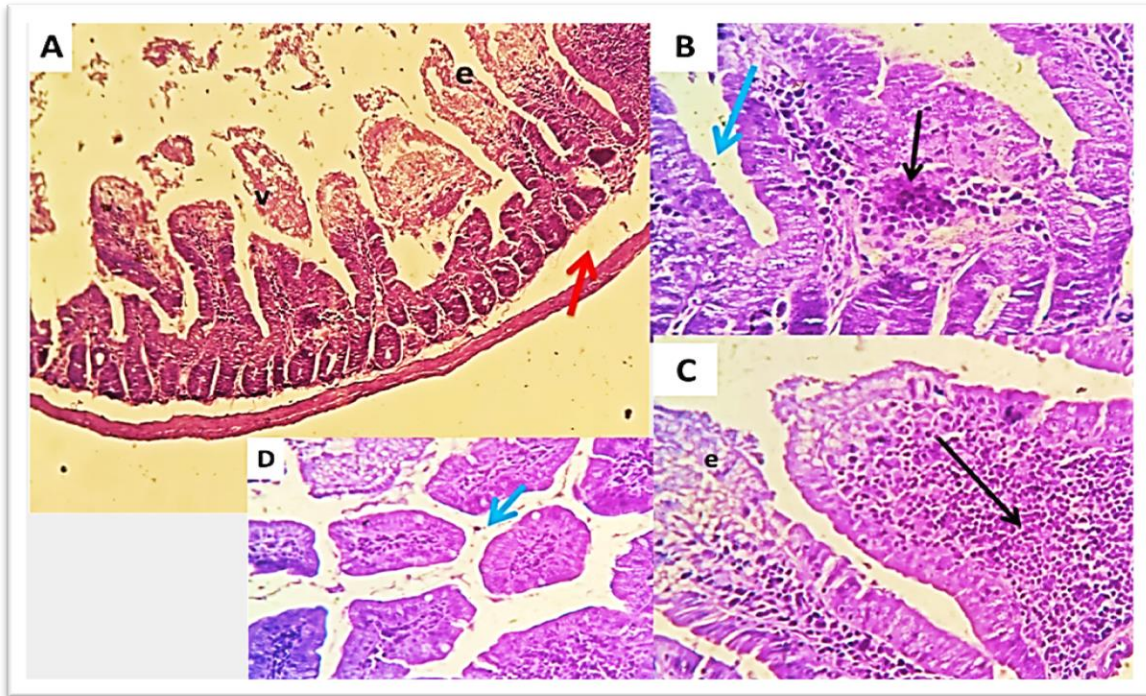


Figure 2: Histopathological of control (infection and not treated) small intestine duodenum(A) : villi (v), basal layer (red arrow) . jejunum (B) ileum (C and D): Cryptosporidium spp. (blue arrow), lamina propria (black arrow). H and E stain (A:X100, B, C and D: X400).

The negative control (uninfected and untreated) which showed a normal histological structure in the duodenum part. The jejunum showed a normal tissue

structure. The histological structure was normal in the ileal part with normal villi (vi) and normal lining epithelial cells (e) as in figure (3).

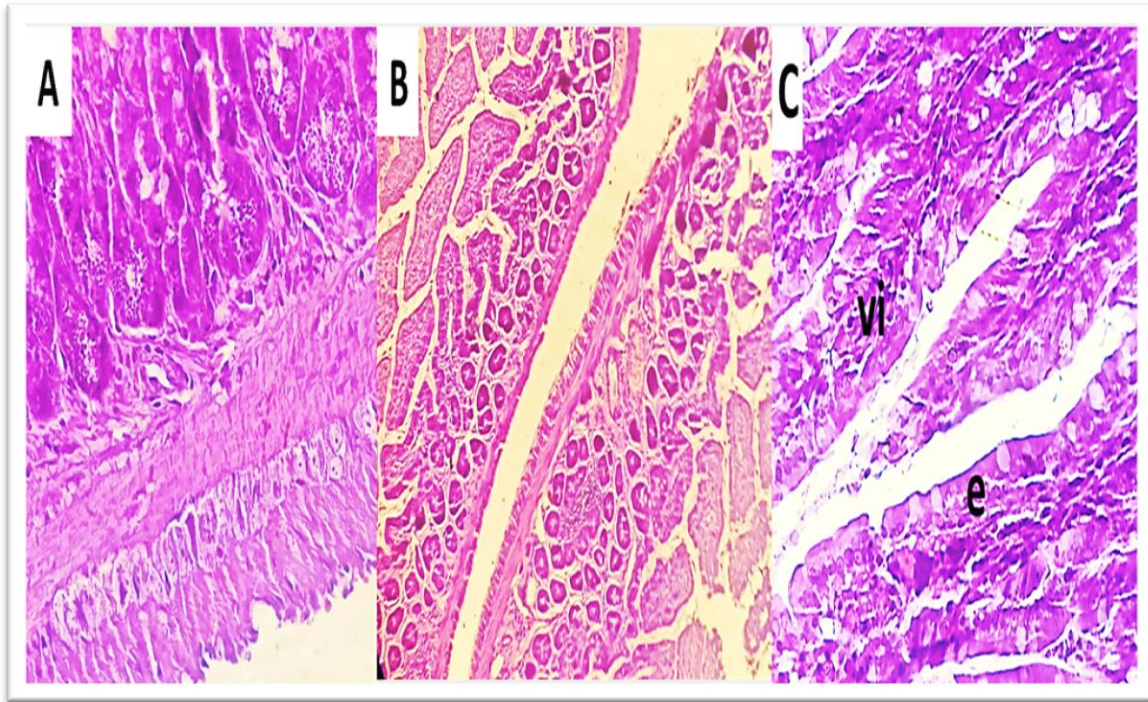


Figure 3: Histopathological of small intestine duodenum (A), jejunum (B), ileum (c) control (no treated and not infection) epithelium (e) , villi (vi) , H and E stains (A and B:X200. D:X400).
Effect of Treatment with Alcoholic and Aqueous Spirulina Extract at Concentrations (50, 100, 150 mg/ml) on Small Intestins Tissues of Mice Infected with *Cryptosporidium* spp.

3.2 The effect of treatment with extract at concentration of 50 mg/ml

The duodenum part in the infected and treated group showed a slight improvement in subepithelial edema with a decrease in epithelial hypertrophy, in addition to mild muscle edema and the examination showed the presence of lymphocyte infiltration and no infiltration of inflammatory cells in this part. The jejunum fraction in the treated group showed

desquamation repair in the villous limbs and a healthy undamaged vault with intermuscular edema with observation of parasite oocyst and slight infiltrating lymphocytes. The ileum part of the treated group showed intact villi free of hyperplasia with a slight enlargement of the epithelium, in addition to observing the parasite oocyst in this section with a slight infiltration of inflammatory cells as in figure 4.

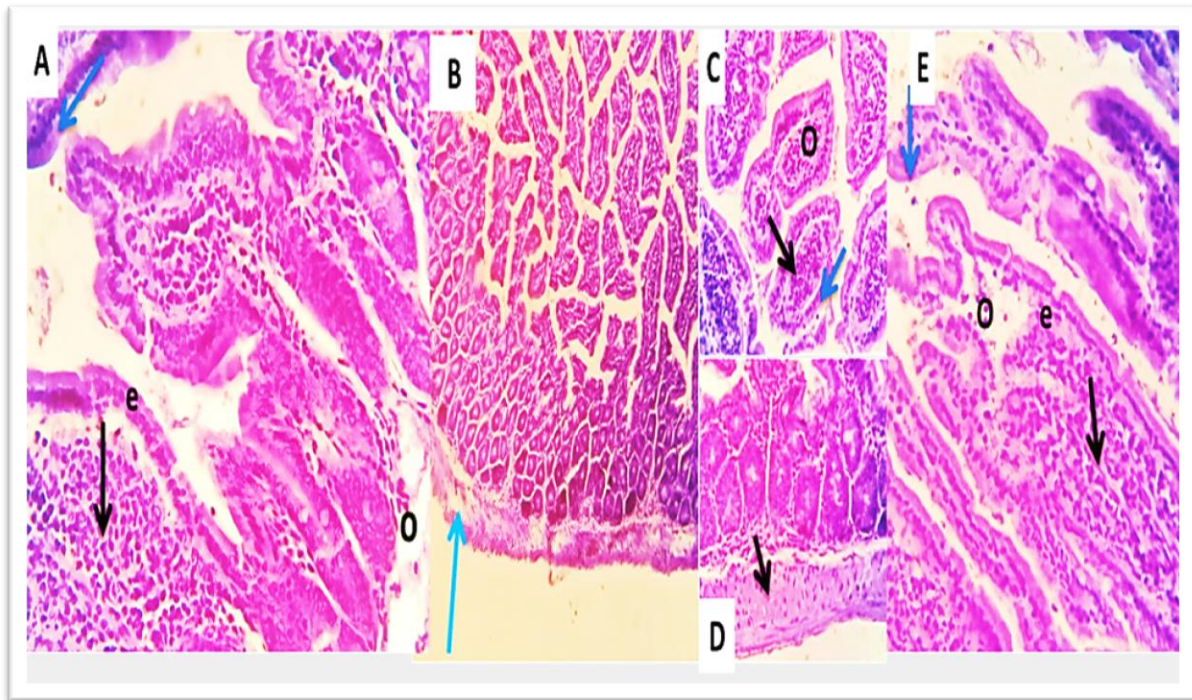


Figure 4: Histopathological of small intestine Duodenum (A): lymphocytes (black arrow), epithelium (e), edema Subepithelial (o). jejunum (B): Cryptosporidium spp. (blue arrow). Ileum (E): epithelium (e), lamina propria (black arrow A), Cryptosporidium spp. (blue arrow). of mice treated with 50 % Spirulina algae extract (H and E stain) (B: X100: A, D, E and C: X400), C and D magnified for Fig. B.

3.3 The effect of treatment with extract at concentration of 100 mg/ml

Histological examination of the duodenum in the treated group showed regeneration of the cells lining the villi with a slight elongation. The jejunum part of the

treated group showed regeneration in the epithelium and the absence of any cell infiltration with a regression in the brush border. The ileum portion in the treated group did not observe any villi enlargement as in figure 5.

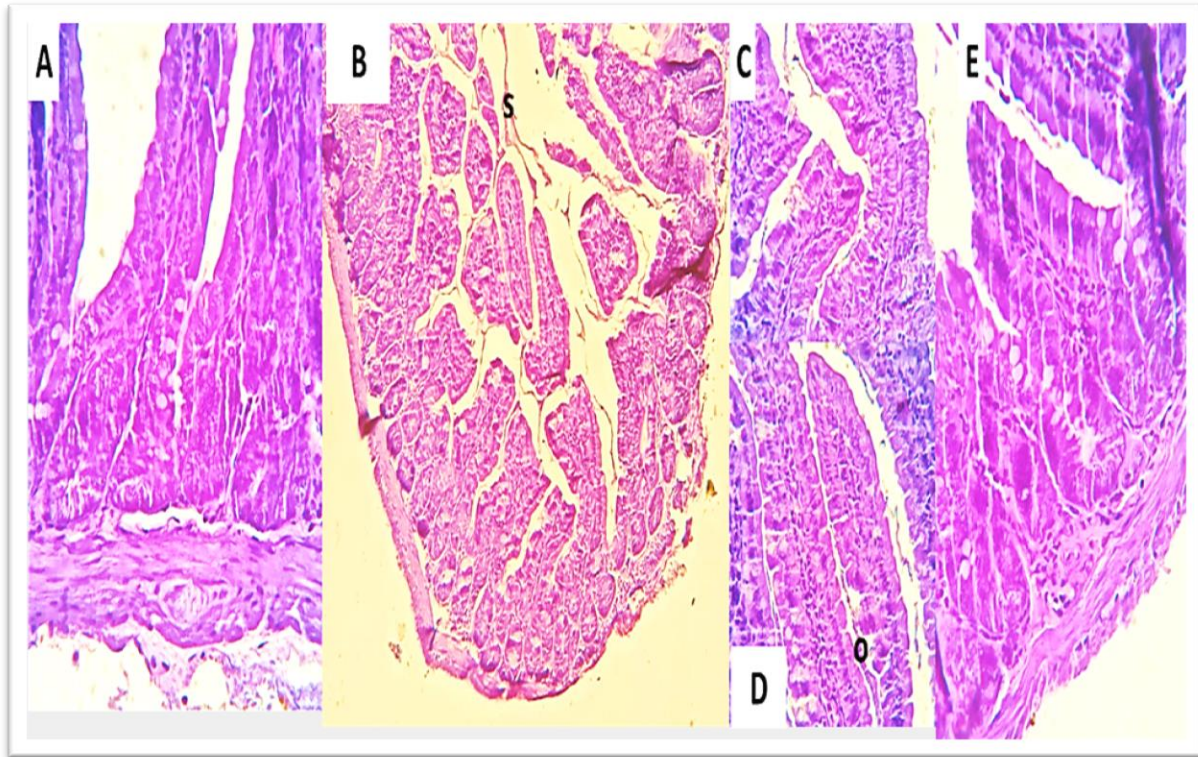


Figure 5: Histopathological of small intestine: duodenum (A), jejunum (B): brush strokes (s), ileum (E) of mice treated with 100% Spirulina algae extract (H and E stain) (B:X100: A and C: X200: C and D: X400), C and D magnified Fig. B.

3.4 The effect of treatment with extract at concentration of 150 mg/ml

Histological examination of the duodenum in the treated group showed significant repair, represented by the absence of subepithelial or submucosal edema and no cell infiltration with histological repair of any enlargement. The jejunum part of the infected

and treated group showed stimulation of the tissue structure in the villi elongated and branched with intact epithelium. The ileum part in the infected and treated group showed that the villi were elongated and had a normal end with clear cells lining them and intestinal glands, and no parasitic oocyst were observed in this part as in figure 6.

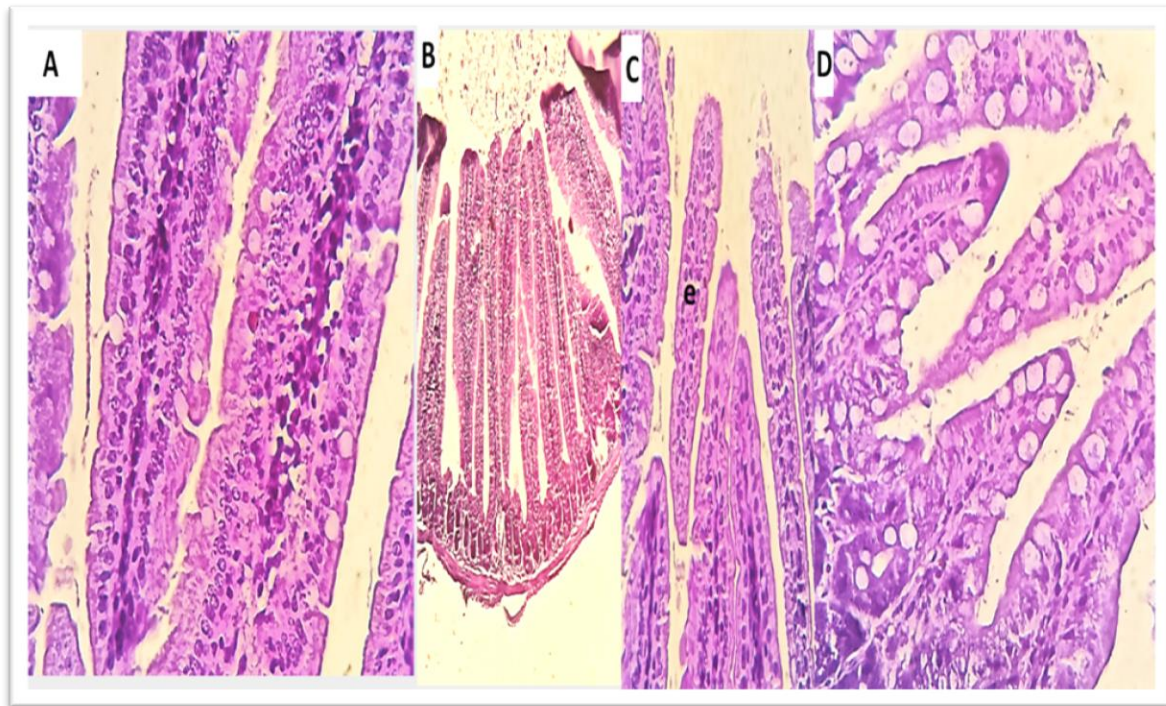


Figure 6: Histopathological of small intestine (duodenum (A), jejunum (B), ileum (D): epithelium (e) of mice treated with 150 % Spirulina algae H and E stains (A, C, D: X400, B: X100).

4. Discussion

The current study agreed with the results obtained by [14] which recorded 37 % in feces of 100 mice using Multiplex PCR in Spain for molecular investigation of intestinal parasites, including *Cryptosporidium* spp. The current study disagree with the results ofthan [15] , which amounted to 22 % during the examination of 19 positive stool samples of 74 male and female mice of origin by multiplex pcr in the Republic of El Jik [16] which recorded 4.76 % by 84 stool samples of mice experimentally infected with *C. parvum* by

Multiplex PCR in Indonesia. Regarding histological changes in the parts of the intestine infected with the parasite that were not treated the present study agrees with [17] who found during their study of the therapeutic effect of phenyl vinyl sulfone and nitazoxanide on experimentally infected mice with cryptosporidiosis which took place in Egypt there is histological changes ranging from partial to complete villous atrophy and infiltration of inflammatory cells in sections of the small intestine infected with cryptosporidiosis. The current study is also in agreement with [18] who reported villous

shortening and villous atrophy with ulcers associated with cryptosporidiosis during their study Comparative efficacy of curcumin and paromomycin against *C. parvum* infection in a Balb/c model in Iran. The reason may be that the parasite has the ability to damage the mucous membranes of the intestine, which leads to the destruction of cells and the occurrence of significant histopathological changes in them [19]. It may be due to the production of inflammatory interleukins, which leads to the infiltration of inflammatory cells into the mucosal layer and thus leads to tissue damage and inflammation of the intestine, while the other leads to the stimulation of neighboring epithelial cells in different sections of the intestine [20]. With regard to the parts of the intestine treated for the alcoholic and aqueous extract of *Spirulina* the results of the current study agreed with [21] who found the ability of *Spirulina* to improve intestinal morphology, especially the duodenum and jejunum through the shape of the villi and increase the density of goblet cells during the study of microbial community modification in the intestines of mice. The current study is also in agreement with the results of the study of Abdul Daim [22] which showed a significant modification of the tissues of colitis caused by acetic acid after it suppressed the infiltration of

lymphocytes during the study of the anti-*Spirulina* effects in mice experimentally. the present study agreed with Ma H. [23] that reported the therapeutic effects of *Spirulina* represented by regeneration of damaged intestinal villi of the digestive tract affected by diphenoxylate induced constipation in mice due to the correcting activity of polysaccharides in *Spirulina* extract.

This protective activity of *Spirulina* extract on damaged tissues can be attributed to the flavonoids present in it, which have antidiarrheal effects that lie in its ability to improve the function of the tight junction barrier, the function of intestinal villi uptake, the permeability of the epithelial barrier, the suppression of muscle contraction with decreased intestinal motility and decreased fluid flavonoids intrainstestinal quercetin also has anti-inflammatory properties verified by reducing phagocytic infiltration into damaged intestinal tissues of mice [24]. Besides, polyphenol compounds and bioactive molecules present in *Spirulina* such as C-phycocyanin synergistically aid in erosive tissue healing by stimulating cell proliferation and tissue regeneration they also prevent cell death by removing free radicals and hyaluronic acid can be significantly richer generic polyphenols which have been shown to significantly cure local and

systemic infections as well as tissue damage [25]. This histological effect may be attributed to the chemical content of Spirulina extract from active compounds such as alkaloids that have biological activity against different pathogens and the mechanism of action of alkaloids, which have a great curative repair capacity [26]. Several compounds such as beta-carotene and zeaxanthin [27] as well as β -carotene, lycopene and may have both preventive and ameliorative effects in diseases [28]. The cell-renewing and anti-inflammatory effect of Spirulina may be due specifically to the presence of C-phycocyanin, whose high content in the aqueous extract of Spirulina [29].

5. Conclusion

Alcoholic and aqueous Spirulina extracts showed a clear histological effect in restoring the damaged parts of the small intestine very close to the normal state.

6. References

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