

**Effect of Algae *Chlorella* extracts for treatment of intestinal tissues  
of mice infected with *Cryptosporidium* spp.**

**Russl Jalil Kazem El-Heluo, May Naji Al-Khanaq, Wassan Hamza Mazal**

**Department of Life Sciences, College of Science, Wasit University, Al-Kut Iraq**

**Abstract**

*Cryptosporidium* is a protozoan parasite of medical importance that causes gastroenteritis in a variety of vertebrate hosts, so the study was conducted to evaluate the anti-cryptosporidiosis efficacy of alcoholic and aqueous *Chlorella* algae extracts in comparison with Azithromycin in the intestines of infected mice. Fecal samples were collected from patients at Al-Kut Hospital suffering from diarrhea in the period December 2021 to end of March 2022, and 90 microscopic samples of both sexes were examined using a modified Ziehl-Nelson stain to detect oocysts infected with the parasite. Isolation and flotation purification with Sheather's 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 *Chlorella* 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 *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. The results of histological examination showed that treatment with alcoholic and aqueous *Chlorella* 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 *Chlorella* 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 inflammation and the degree of inflammation as well as less severe lesions in the intestinal tissues treated with alcoholic and aqueous extract of *Chlorella* algae in the highest concentrations with complete disappearance of parasite oocysts.

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

## **1. Introduction**

Cryptosporidiosis is a disease caused by a microscopic parasite, *Cryptosporidium* species [1]. an intracellular obligatory protozoan that infects microvilli epithelial cells in the digestive tract, is the disease-causing agent [2,3]. In 2016, diarrhea was one of the major causes of death, accounting for more than 1 to 6 million fatalities globally, One of the three causes of death in children under the age of five is cryptosporidium [4]. and it will be more severe if it affects kids who are HIV-positive [5]. Transmission is effective and only a few dozen oocysts are needed to produce sickness in healthy people, though it can get serious in immunocompromised people [6,7]. Currently, cryptosporidiosis is the main cause of chronic diarrhea in HIV-positive individuals and a substantial source of morbidity and mortality worldwide [8,9].

Infection occurs through oral ingestion of the oocyst stage of the parasite from contaminated feces, food, drink, and pasture (for grazing animals), and following ingestion, the sporozoites are released from the oocysts and invade and undergo asexual development in the epithelial cells of the gastrointestinal tract of the host. This is followed by a sexual phase of development resulting in the production of potentially genetically diverse oocysts that are shed fully infective in the feces. Oocysts may also 'hatch' before they are shed from the host, causing re-infection and exponential increases in parasite burden, leading to chronic infection, particularly in immunocompromised hosts [10]. *Cryptosporidium* parasites have a remarkable capacity to reproduce in the host. The rapid multiplication of the parasite in the gut cells causes tissue damage and destruction of the

intestinal epithelial cells with stunting of the villi, reducing the absorptive surface of the gut, leading to malnutrition, dehydration, and diarrhea [11]. In immunocompetent patients' cryptosporidiosis is a self-limiting condition; nevertheless, in immunocompromised patients, it can be fatal. Mild to severe diarrhea is caused by invasive *Cryptosporidium* infection of the small intestine, which also destroys the intestinal epithelium and impairs absorption and barrier function [12]. To lower comorbidity and mortality, the main goals of treatment are to shorten the length of diarrhea, avoid complications, and remove the organism from the host. Research demonstrates that if there is a *Cryptosporidium* infection, therapy for diarrhea in PLHIV is insufficient [13]. Diarrhea that becomes profuse is usually followed by significant weight loss, anorexia, malabsorption syndrome, and fever, and accompanied by abdominal pain [14].

## **2. Materials and Methods**

### **2.1 Samples collection**

90 stool samples were collected from patients admitted to Al-Kut Hospital suffering from diarrhea for both sexes, for the period from December 2021 to end of March 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 [15]. 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 [16]. 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 (U11761.1 and EF591787.1) and primer 3 plus design. These primers were provided by Scientific Research. 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	615 bp
	R	CCTTGATCTTCTTCTCAGCCTCA	
<i>C.hominis</i> hsp 70 gene	F	TCTGCGCTGATTACTTCCGT	310 bp
	R	CCACCAGCAGTTTCTAAACCG	

#### **2.4 Preparation of Aqueous and Ethanolic Extracts of Chlorella and The Concentrations Used in The Study (50, 100, 150 mg/L)**

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, 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 medicine. 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. Prepared in the same way as an aqueous extract that replaces distilled water with ethanol. Prepared according to the method [17]. The different concentrations used in the study were prepared from the crude Chlorella extract, according to the method [18], three concentrations (50, 100, 150) mg/l.

#### **2.5 Executing the experiment.**

After the groups of mice were divided into 5 groups, infection was confirmed in all

groups except for the negative control group that was dosed with physiological saline. The first group treated 21 mice after they were divided into three subgroups A, B and C. Each group of 7 mice was treated with alcoholic extract of *Chlorella* at concentrations of 50, 100 and 150, while the second group was treated with aqueous *Chlorella* 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**

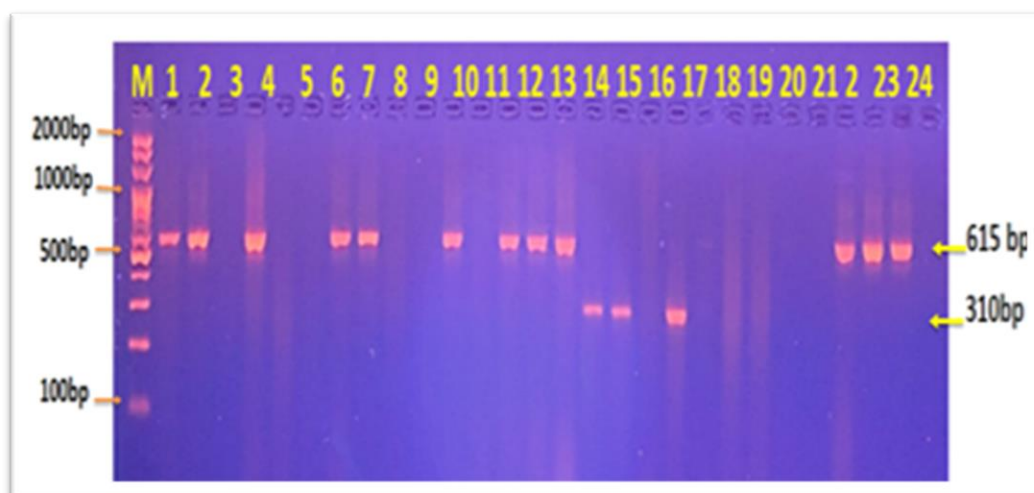
Statistical significance was determined by entering the obtained data into a computer

database, the Statistical Package for Sciences SPSS-25 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 [19].

## **3. Results**

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

The PCR multiplex results are shown in figure 1, 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.

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

Multiplex PCR for <i>Cryptosporidium</i> Spp.	Results	
	No	%
Specimen Positive for <i>C. Parvum</i>	24	46.15%
Specimen Positive for <i>C. hominis</i>	3	5.76%
Specimens Negative	25	48.07%
Total	52	

\*Chi-Square at  $p \leq 0.05$  P Value 0.024.

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) 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 51.9% with a distribution rate of (27) samples. The results of the extracted DNA are shown in table (3) from 27 positive sample, 46.1 % were recorded for *C. parvum* in 24 samples, and 5.7% were for *C. hominis* in 3 samples

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

Multiplex PCR for <i>Cryptosporidium</i> Spp.	Results	
	No.	%
Specimen Positive for <i>Cryptosporidium</i> Spp.	27	51.91 %
Specimen Negative for <i>Cryptosporidium</i> Spp.	25	48.07 %
Total	52	100 %

\*Chi-Square at  $p \leq 0.05$  P Value 0.355

The results also showed in table (4) the difference in infection percentage in groups of infected mice that will be treated with alcoholic and aqueous *Chlorella* extract and azithromycin by using the Multix PCR technique, where it recorded 57.1% in the alcoholic extract group with 12 samples,

while in the aqueous extract group it was recorded 47.6% with 10, while the azithromycin group recorded 60% with 3 samples, and the control group was 40% with two samples.

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

Specimen Positive for <i>Cryptosporidium</i> <i>spp.</i>	Multiplex PCR for <i>Cryptosporidium Spp.</i>							
	Alcoholic		Aqueous		Azithromycin		Control	
	No.	%	No.	%	No.	%	No.	%
	12	57.1 %	10	47.6 %	3	60 %	2	40 %
Total	21		21		5		5	

\*Chi-Square at  $p \leq 0.05$  P Value 0.126.

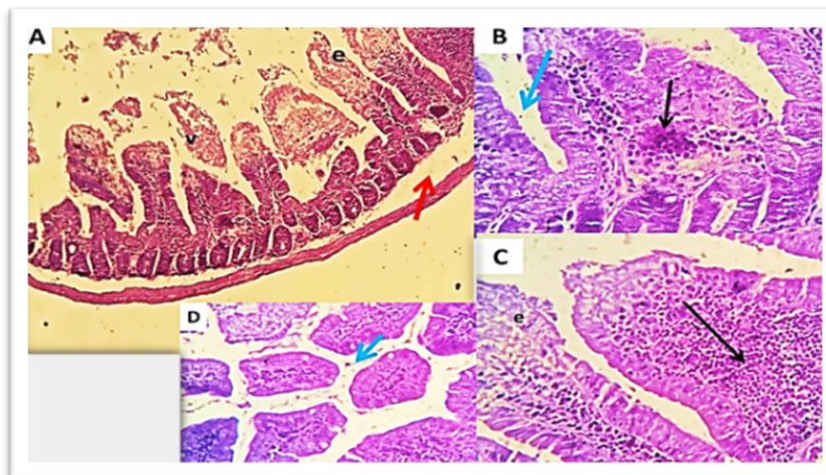
### 3.2 Histopathological changes

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

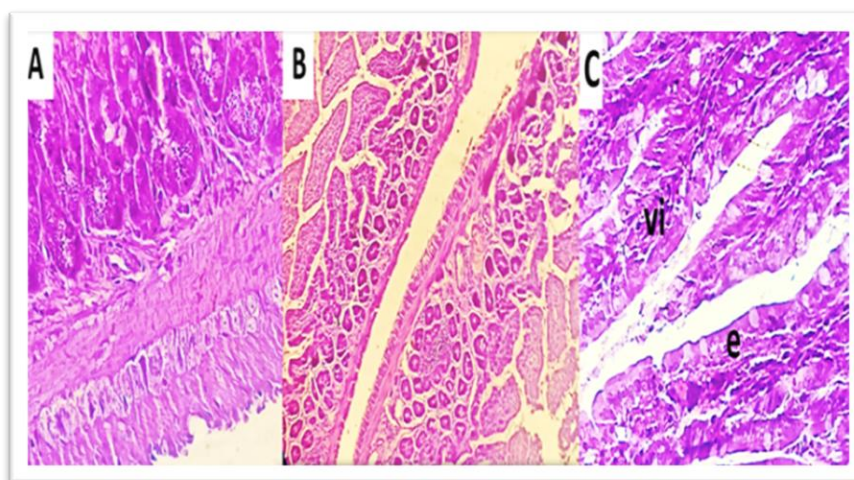
a significant infiltration of inflammatory cells present in the lamina propria in as shown in figure (2).



**Figure 2:** Histopathological of control (infection and not treated) small intestine (A) duodenum villi, basal layer (red arrow). (B) Jejunum, C and D ileum: *Cryptosporidium* spp. (blue arrow), lamina propria (black arrow) . H and E stain (A:  $\times 100$ , B, C, and D:  $\times 400$ ).

The negative control (uninfected and untreated) tissues were normal in the uninfected control group, we noticed normal

Elongated villi and normal intestinal glands in the parts duodenum, jejunum, and ileum. as in figure (3).



**Figure 3:** Histopathological of small intestine (A) duodenum, (B) jejunum, (C) ileum control (no treated and not infection), epithelium (e), villi (vi), H and E stains (A and B:  $\times 200$ . D:  $\times 400$ ).

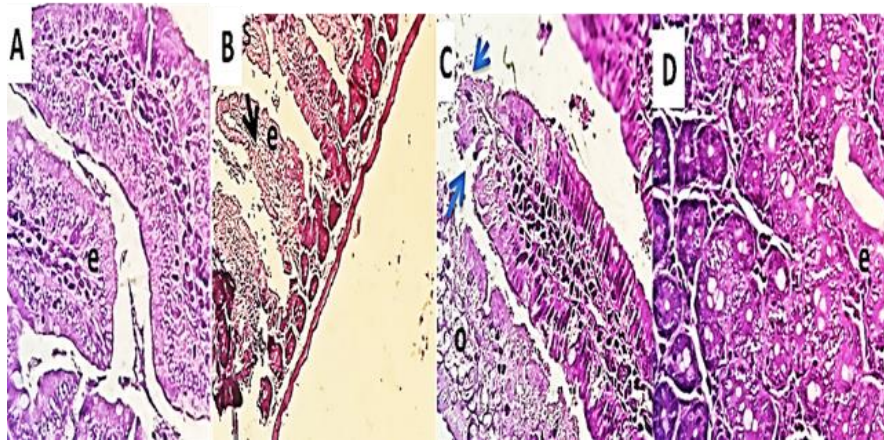


### **3.2.1 Effect of Treatment with Alcoholic Chlorella Extract at Concentrations (50, 100, 150 mg/L) on Small Intestines Tissues of Mice Infected with *Cryptosporidium* spp.**

#### **1- Effect of Treatment with Extract at a Concentration of 50 mg/L**

Through the microscopic examination of the infected mice tissues treated with a

concentration of 50 mg/L, it was found that the duodenum had enlarged and destroyed the epithelium in the form as shown in Figure A .In figure B; C, the jejunum shows an enlargement of the epithelium with the appearance of edema in the sub epithelial cells with the presence of the parasite at the brush borders of the villous epithelial cells. As for the ileum part, enlargement of the epithelium layer. figure D shows the tissue sections.as in figure 4.

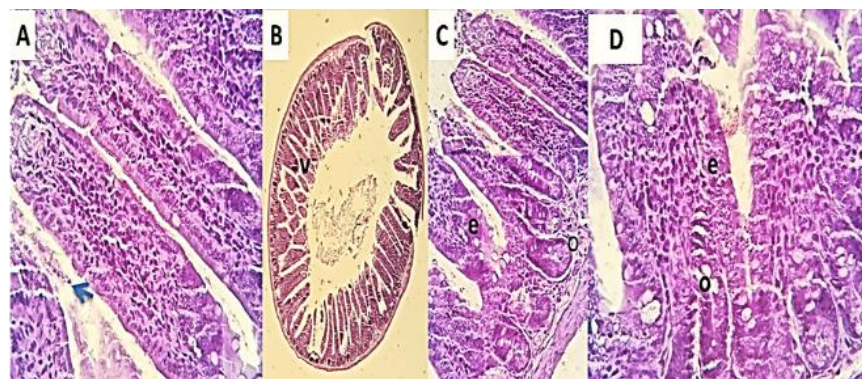


**Figure 4:** Histopathologica of small intestine (A) duodenum, (B) and (C) jejunum, (D) ileum of mice treated with 50 Chlorella algae alcoholic extract. (H and E stain) (A:  $\times 100$ ; B  $\times 400$ .C magnified for Fig. B.

#### **2- Effect of Treatment with Extract at a Concentration of 100 mg/l**

A and C shows the presence of *Cryptosporidium* in duodenum at brush borders of the villous epithelial cell with goblet cell enlargement and the appearance of edema around *Cryptosporidium*, as for the

jejunum part, the appearance of thickened and flattening of villus, degenerations, some atrophy and the removal of some villi from the upper extremities. as shown in Figure B. In the ileum part, edema around *Cryptosporidium* and goblet cell hyperplasia are indicated.as in figure 5.

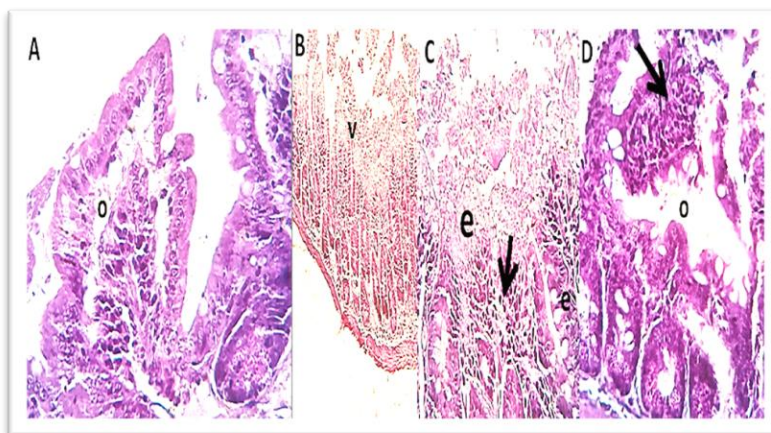


**Figure 5:** Histopathological of small intestine (A); (C) duodenum, (B) jejunum, (D) ileum of mice treated with 100% Chlorella algae alcoholic extract. (H and E stains) B:  $\times 100$ ; C:  $\times 200$ , A, D:  $\times 400$ , C magnified for Fig. A.

### 3- Effect of Treatment with Extract at a Concentration of 150 mg/L

At a concentration of 150 of the alcoholic extract, Clarify the duodenal section with the appearance of oedma in the sub-epithelial layer , the jejunum part shows an enlargement of the goblet cells and an enlargement of the epithelium with the

appearance of thickening and flattening of the villi, degeneration and some atrophy and detachment of the upper ends of some villi with the infiltration of inflammatory cells in the sub-epithelium, As for the ileum part, in the D figure shows the appearance of oedma in the sub-epithelium. As shown in figure 6.



**Figure 6:** Histopathological of small intestine (A) duodenum, (B); (C) jejunum, (D) ileum of mice treated with 150 Chlorella algae alcoholic extract. (H and E stains) B:  $\times 100$ ; A, C, D:  $\times 400$ , Fig. (C) magnified Fig. (B).

#### **4. DISCUSSION**

The current study agrees with [20] 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 [21] 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 [22] 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, Also, it disagrees with the study [23] 2.1 % (9/435) were seen in laboratory mice using the PCR test. The results of the histological study showed the positive control group in the small intestine Infection of intestinal changes, detachment of the upper edges of the villi, hyperplasia of the intestinal glands, with hyperplasia of epithelial cells due to this parasite. The current results agree with the study [24] In the normal control group, histopathological examination revealed normal villus/crypt ratio with a healthy mucosa. However, histopathological examination of intestinal tissues of immunosuppressed-infected mice

showed heavy parasitic colonization of the mucosa, the mucosa and submucosa showed severe inflammatory changes in the form of shortening and broadening of the villi in some areas and loss of brush border with villous atrophy in others, In addition, extensive necrosis associated with marked inflammatory cells infiltration of lamina propria, in the treated group, there was a partial reduction of mucosal parasitic burden with a moderate improvement of some pathological changes where the villi restored their normal architecture and the inflammatory infiltrates decreased. As for the histological changes of the groups infected with the parasite and treated with the aqueous and alcoholic extract of algae showed a relative improvement in the tissue of the small intestine when compared with the positive control group, which represented a slight infiltration in the inflammatory cells with repair and restoration of the villi with the presence of parasite cysts in the brush edges. These results are consistent with this study using ethanolic and aqueous extract of propolis [25].

#### **5. Conclusions**

Alcoholic *Chlorella* extracts showed a clear histological effect in restoring the damaged parts of the small intestine very

close to the normal state, and the efficiency of the extract appeared as the concentration increased.

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