Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 8408 - 8420
Received 25 April 2021; Accepted 08 May 2021.

The Efficiency of Alcoholic Extract of Green Algae *Chlorella Sp.*Onleishmania *Major*infection (In Vivo)

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ABSTRACT

The current study revealed the effectiveness of extract of chlorella alga on *Leishmania major* infection,extract of chlorella with different concentrations (25%, 50%, 75%, 100) and compared it with the drug Pentostam, the duration of the study lasted three weeks in vivo (white mice). Alcoholic extract of chlorella alga compared with Pentostam and positive control group, As was the efficiency of the therapeutic extract alcohol concentration of 100% is the highest was recorded (96%). The results of the statistical analysis showed significant differences clear at the moral level of 0.05 when compared with drug SSG reaching (39%) as well as the control group, as for the alcoholic extract with a concentration of 75%, it showed clear therapeutic efficacy, but less than 100% concentration, reaching (88%), while the therapeutic efficiency of 25% concentration was recorded (74%), which is an approach to the therapeutic efficacy of 50% concentration, which was recorded (83%). We conclude from the study that the effect of the alcoholic extract of *chlorella sp.* depends on its concentration during the three weeks of the study, the higher concentration of the treatment, have the greater effect and the rate of healing for infected mice with cutaneous leishmaniasis. Therefore, this treatment is one of the most promising treatments in the future for treatment of cutaneous leishmaniasis.

Keywords
Cutaneous leishmaniasis, Chlorella algae, Leishmaniasis, Alcoholic extract

Introduction

Leishmaniasis is considered a dangerous parasitic disease and is caused by Leishmania spp., And this disease spreads in the form of epidemic waves in various countries of the world (Webb *et al.*, 1998). Leishmaniasis is characterized by the diversity of the clinical features of the disease, where the degrees of infection vary from skin lesions. In Cutaneous Leishmaniasis to lesions and erosion of the nose and the nasopharyngeal cavity in Mucocutaneous Leishmaniasis up to visceral leishmaniasis affecting the liver and spleen (de Souza *et al.*, 2011).

Among all cases, Cutaneous Leishmaniasis recorded the highest rate of infection (70-50%) of infections, which appears in two types: the first is: Zoonotic Cutaneous Leishmaniasis and is caused by *L. major* parasite, and it appears in rural areas and is caused by this type. Rural sore, and Anthroponotic Cutaneous Leishmaniasis, caused by the parasite *L. tropica*. This is the most common form in urban areas. This type causes a

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dry sore (AlSamarai & AlObaidi, 2009). In Iraq, the two types of leishmaniasis were founded. Dry and wet CL is one of the endemic diseases in Iraq (Postigo, 2010).

Leishmaniasis is transmitted by the bite of a female sand fly carrying the Leishmania parasite, where the Promastigote is inside the intestine of the insect, and Amastigote lives within the phagocytes of the vertebrate host, where they are considered reservoir hosts such as wild animals such as rodents and pets such as goats, dogs, camels and cats (Devault & Bañuls, 2008).

Cutaneous leishmaniasis has different names according to the type of lesion or according to the region in which the infection was recorded, such as the Baghdad seed, the eastern blister, the Aleppo kiss, the Delhi pill, the Lahore ulcer and the Kandahar ulcer (Akhoundi *et al.*, 2017; Alvar *et al.*, 2012). Finally described as Gulf War Syndrome (WHO, 2003).

Cutaneous leishmaniasis takes a long time to heal itself leaving a trace or a scar, as the disease begins in the form of a small papule and reddish color at the site of the sand fly bite and then begins with ulceration and takes a gradual increase in size, after which the ulceration spreads gradually and its shape is peripheral. Ulcers are single or multiple (Arthur & Burns, 2004).

Recent studies indicated that infection with the parasite is more frequent in areas that coincide with the presence of HIV and the presence of the parasite (WHO, 1998). The size of the ulcer until it reaches a few centimeters and contains inside it live and dead cells and the remains of dead parasites (Markle & Makhoul, 2004).There are many chemical compounds used in the treatment of leishmaniasis, the results of which vary in treatment depending on the type of parasite causing the disease, the duration of the disease, the number, size and location of the lesion, and the presence of secondary infection (AlSamarai & AlObaidi, 2009),Among the most important drugswhich used in the treatment of cutaneous leishmaniasis is Pentostam as well as Glucantime. These drugs have side effects such as muscle pain, arthritis, white blood cell deficiency, pancreatitis, and heart muscle enlargement, and the length of treatment can cause the drug to accumulate in body tissues, especially In the liver and spleen(Desjeux*et al.*,2013; SCHOLAR, 2007). Pentostam is considered the best treatment for patients with leishmaniasis, despite its toxic effects, and as a result, many studies have been made to find types of therapeutic drugs that are less toxic than pentostam (Alamri, 2020).

Green algae contain abundant amounts of antioxidants and phytonutrients, as well as many alkaloids known for their strong antioxidant properties, making them very useful for preventing cancer and supporting the immune system to fight cancer cells and algae are known for their strong anti-inflammatory properties that cause chronic diseases, as well as their role in Delayed aging(Zainul-Azlan*et al.*,2019; Torres *et al.*, 2014), because of green algae importance as anantioxidant ,this study aimed to know the Therapeutic efficacy of *Chlorella sp.* against Cutaneous leishmaniasis.

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Methods

Preparation of*Chlorella sp.* extract concentrations

Firstly, to prepare*Chlorella sp*.extract a 25 g. from dry powder of*Chlorella sp*.was weighted and put in a thamble bowl, and then this container was put in a Soxhlet, 99% ethyl alcohol used as an organic solvent, the sample was left for two hours to saturate the solute with the solvent, the extraction process was done according to (Weggler *et al.*, 2020).

Four concentrations (25% , 50% , 75% and 100%)are used in the study were prepared from the crude extract of *Chlorella sp*, according to the following equation (Ucko, 2012),and then they kept at 4°C until the laboratory experiments were carried out.

$$concentration = \frac{wt.(mg)}{V\ (ml)} \times 100$$

Wt = weight of extract (mg)

V = volume of distilled water (ml)

Parasite culture

Isolation of *Leishmania major*was cultivated by using RPMI-1640 medium. Promastigote was grown in the lab as follows:

1- The culture room was sterilized (hood) in order to avoid contamination in the laboratory environment, which may lead to contamination of the lab culture with fungi or other microorganisms.

2- 10 ml of RPMI 1640 medium was added in test tubes, after that1.0 ml from fetalbovine serum (FBS) was added to each tube so that the medium was ready for culturing the parasite.

3- A 0.1 ml of Leishmania isolation was added to all culture tubes except one which leave as a control group,all tubes incubated at 27°C.

4- The growth of parasite in the tubes was monitored daily, in thefourth day the color of the medium was changed from pink to a pale-yellow color as an indicator ofparasite growth clearly in the tubes.

5- To prepare parasite for injection, culture tubes of the parasite were placed in a centrifuge (5000 rpm) where the parasites were deposited at the bottom of the tube, after that the clear liquid was discarded and then added 0.5ml from nutritional supplement solution per tube for preservation until used to injection into mice for experimental infection.

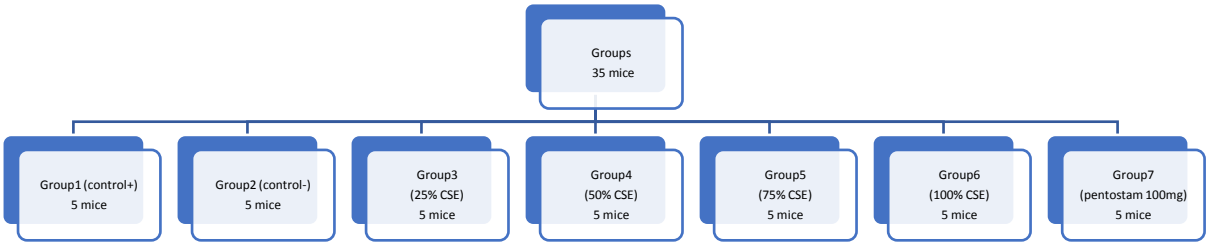
Injection of Laboratory whitemice:

Mice were injected in the tail area and the bad foot with (0.1) ml promastigote of L. major at parasitic density of (1×10^6) after sterilizing the injection area with 70% ethyl alcohol (Wangoo *et al.*, 1989).

Study groups

To determine anti-Leishmania activity of the *Chlorella sp.* extract concentration,five experimental groups were designed as follows:

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Experience design

35 mice (BALB/ c), which used with different in ages and sexes, were injected with *L. major* (0.1ml), and after 96 hours of the appearance infection, they were treated with *Chlorella sp.* extractsand withPentostam as follows:

1- Treatment groups:

Five groups included (5 mice) for each group, four of them were treated with extract of *chlorella sp.*, as four concentrations of the extract were selected, which are **(25%, 50%, 75%, 100%)**and the fifth group treated with Pentostam.the treatment was done by subcutaneous injection in the affected areas after 96 hours from the onset of infection and it was up to one dose every week for three weeks.

2- Control group:

It consists of two groups (5 mice for each group), and the first group represent the positive control which injected with parasite without treatment by any drug, and the second group,representthe negative control, which wasn't infected with parasite or treated with any drug.

Histopathologicalstudy:

Both of the treated and normal parts of all groups were cut offand preservative in 10% formalin to prepare them to histological sectioning which done according to(Luna, 1968) , and then the slides were examined and photographed.

Statistical Analysis:

The statistical analysis of the results was done by using SPSS version 16 program, where the one and two ANOVA test was applied with LSD and the chi-square test was used to find the relationship among groups, as the confidence interval equal to 95% was adopted and the value of the probability level less than 0.05 ($P \leq 0.05$).

Results and Discussions

Study of the efficacy of Chlorella extract and Pentostam in treatment of infected mice:

The results showed that the average number of parasites, as shown in Table (1), in infected mice which treated with *Chlorella sp.* extract compared to Pentostam, as the average number of parasites in infected mice decreased with increasing of chlorella extractconcentration compared to the control group.

In the first week, when treatment groupscompared with Pentostamgroup and control group, it revealed that the number of infected mice decreased significantly, as the average

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of healing percentage was 38.75% at concentration25% of chlorella extract, and it were 48.75%63.75,%,88.75%at concentration 50%, 75% and 100% respectively, as shown in Figure (1).

While in the second week, and through the results that appeared in the statistical analysis shown in Table (1), which states that there are a significant differences among concentrations in their effect, which showed that all concentrations used in the study have a significant differences when comparing among themselves , as well as when comparing them with Pentostam and with control group, as the three concentrations (100%, 75% and 50%) recorded the highest healing rate which was (98.94%), and the affected mice gained full recovery, While the concentration (25%) recorded lower healing rates (83.98%), and Pentostam recorded (43.74%), which was the lowest of healing rate at second week(as shown in Figure 1).

Finally in the third week, the results of the statistical analysis showed that there are differences among concentrations. These effects were shown that the concentration of 25% has asignificant difference when compared to Pentostam and control group, as it recorded a healing rate (100.00%). All mice infected and treated with this concentration gained full recovery, while Pentostam and control group recorded (43.74%, 28.75%) respectively, as in Table (1) and Figure (1).

Table (1) Effect of different concentrations of *Chlorella sp.* algae extract in the treatment of cutaneous leishmaniasis in white mice compared to Pentostam.

Drugs	The healing percentage rate for mice			Total Percentage
	1st Week Mean ± SD	2nd Week Mean ± SD	3rd Week Mean ± SD	
25%	11.30±38.75	83.980.49 ±	100.000.46 ±	%74
50%	48.75 ± 11.30	98.940.4 ±6	100.000.46 ±	%83
75%	63.75 ± 11.30	98.940.4 ±6	100.000.46 ±	%88
100%	88.75 ± 11.30	98.940.4 ±6	100.000.46 ±	%96
Pentostam	38.75 ± 11.30	43.740.6 ±1	43.740.61 ±	%39
Control	0.00 ± 0.00	0.00 ± 0.00	28.75± 13.60	
X^2	43.768	43.768	30.00	

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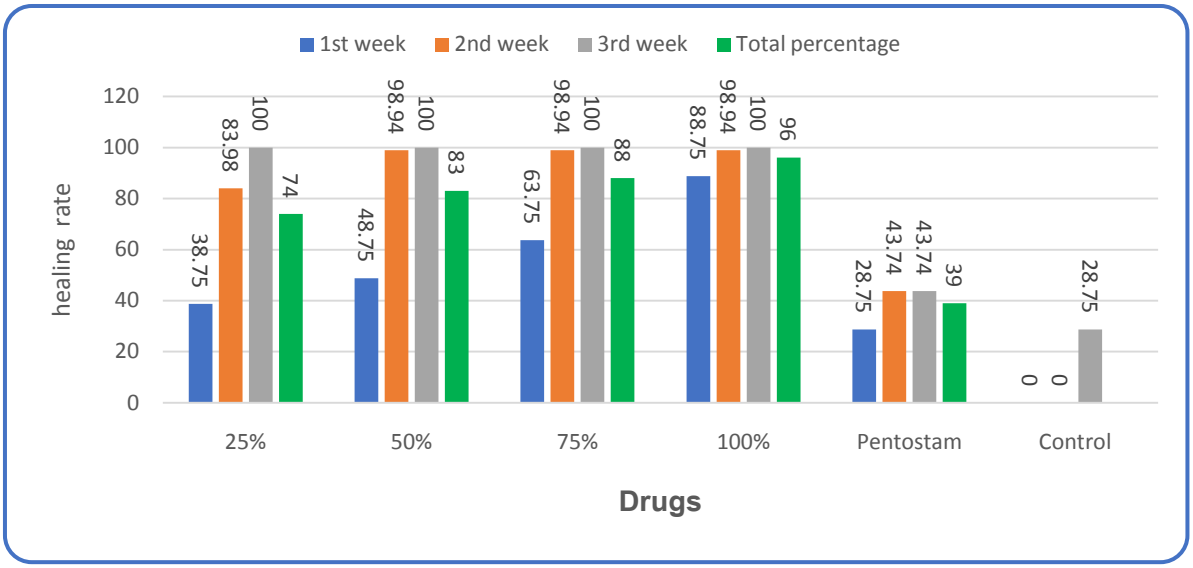


Figure (1) The percentage of healing rates of infected mice with cutaneous leishmaniasis which treat with different concentrations of *Chlorella sp.* extract and Pentostam in different growth periods.

After injection of mice with isolateof *L. major*, the injected groups were characterized by the emergence of slight swelling not accompanied by an ulcer after 4-5 days, and the results showed the difference in the severity of pathogenicity in laboratory mice injected with different conditions of cutaneous leishmaniasis. The infected laboratory mice were treated with*Chlorella sp.*extract in different concentrations (25%, 50%, 75%, 100%).

Treated skin lesions showed a good response to the extract with different concentrations and signs of ulcer healing were noticed after 14 days of treatment, but at different levels according to the concentration of extract, as the swelling and redness decreased at the end of the experiment and led to the disappearance of the treated ulcers, while the skin ulcers of untreated control group were more disintegration and increased in size.

The results of the study showed that the extract of chlorella, with its different concentrations, led to a clear decrease in the size of the ulcers with an increase in the concentration, and this could be explained by the chemical compounds of algae extracts, which can be attributed to this effect due to their high production of long-chain unsaturated fatty acids and phenolic compounds, volatile compounds, sterols, proteins, amino acids, peptides, vitamins and polysaccharides.

The presence of long-chain polyunsaturated fatty acids produced by these algae as nutritional supplements, from which they manufacture the omega-6 family, which includes linolenic acid and arachidonic acid, and the omega-3 family, which includes eicosapentaenoic acid and docosahexaenoic acid (Golmakani *et al.*, 2012; Jubie *et al.*, 2012). As well as containing phenol compounds, which are considered one of the most important categories of natural antioxidants, and the phenolic compounds produced by these microalgae include caffeic acid, ferulic acid and coumaric acid(Cabrita *et al.*, 2010; La Barre *et al.*, 2010).

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This alga contains volatile compounds, and due to its biological activities (Anggadiredja & Andyani, 1997; Brown & Miller, 1992), which have been identified as carbonyls, alkines, saturated and unsaturated aliphatic alcohols, aldehydes, quinones, esters, thiosters, and terpene sulfides, And fatty acids, Brominated Hydroquinones and Phycotin, VOCs provide anti-bacterial, fungal, virus and cancer-fighting activities(Borowitzka, 1997; Mathew *et al.,* 1995; Morimoto *et al.,* 1995).

Because of algae contains sterols which including phytosterol, brassicasterol, ergostenol, poriferasterol, and ionasterol, which play a fundamental role in microalgal physiology, especially with regard to membrane integrity, phytosterol is structurally similar to cholesterol is one of the most promising sterols. Healthy diets, especially those aimed at reducing coronary heart disease (Luo *et al.,* 2015).

Its constituents include proteins, amino acids and peptides which are high in protein (more than 50% dry weight) and thus can be used as a source of essential amino acids including lysine, leucine, isoleucine, tryptophan and valine(Mišurcová *et al.,* 2014), Vitamins are essential for maintaining human health. Chlorella produces vitamin A (beta-carotene), vitamin C, vitamin E, thiamine (B1), riboflavin (B2), niacin (B3), pantothenic acid (B5), pyridoxine (B6), folic acid (B9) and cobalamin (B12)(Gomes, 2013). This alga produces many polysaccharides that are highly complex immurella, which has an effective anticancer effect, which has been explored by pharmaceutical companies(Chaiklahan *et al.,* 2013; Spolaore *et al.,* 2006).

The results of this study showed that the use of Pentostam in the treatment of skin ulcers in affected laboratory mice gave a good result in healing during the study period, and this result was similar to their study (Sharquie *et al.,* 2017), who found good results from this treatment, and these results are attributed to the fact that Pentostam is a traditional treatment for cutaneous leishmaniasis consisting of pentavalent antimony (sodium stibgluconate and meglumine antimoniate) and given for 42 days at a dose (20 mg / kg). This drug, despite its therapeutic efficacy, was limited to its use with visceral leishmaniasis due to its strong interaction with it and this reaction it leads to pancreatitis and cardiac toxicity, as well as with cutaneous leishmaniasis, because it causes sensitivity, irritation and redness of the treated area, relapse of treatment with this drug is common, and anemia resistance fluctuates around 50% (Aït-Oudhia *et al.,* 2011).

Histopathological study

The results of present study showed that the tissue sections of the skin of laboratory mice of the positive control group (infected) presence of heavy infiltration of inflammatory cells (mixed inflammatory cells) with necrosis in the animal skin, and after 21 days of infection, several layers of vaculated cells that are filled with parasites Leishmaniasis around it and many macrophages filled with the non-flagellar phase in the necrotic area with neutrophils in the dermis and subcutaneous tissues, and the tissue changes showed necrosis in the epidermal layer and infections reached the fatty tissues as in Figure (3) and Figure (4). In the negative control group, the results showed that the skin layer is normal and intact, as in Figure (5) and Figure (6).

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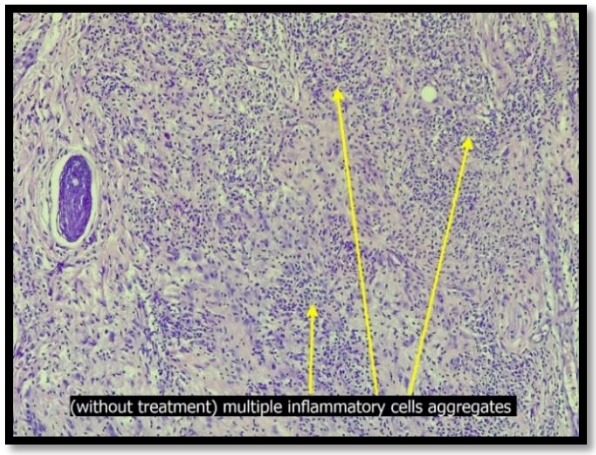


Figure (3) section in the skin tissue of the affected trial group in the third week after infection. It is noticed that there is heavy infiltration in the inflammatory cells and the appearance of many macrophages filled with Leishmania parasite in the necrotic area aggregated with neutrophils (H&E stain 10X)

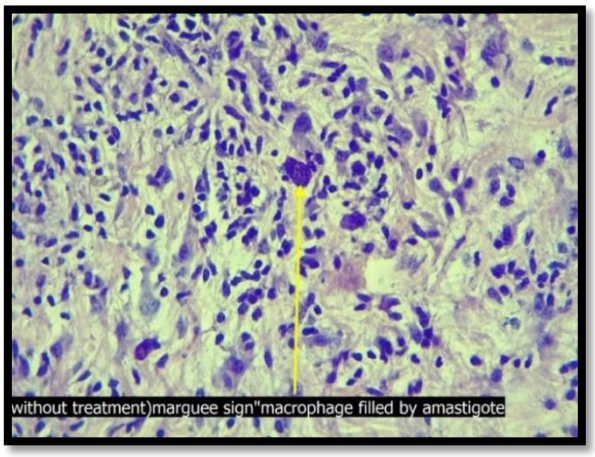


Figure (4) section in the skin tissue of the affected trial group in the third week after infection, and it is noticed that there is heavy infiltration in the inflammatory cells and the appearance of many macrophages filled with Leishmania parasite in the necrotic area, clustered with neutrophils (H&E stain 40X)



Figure (5) section in the skin tissue of the negative experiment group. In the third week, a normal and intact skin layer is observed (H&E stain 10X)

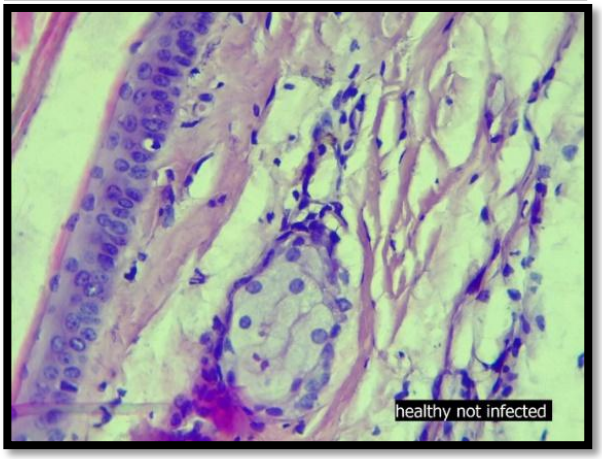


Figure (6) section of the skin tissue of the negative experiment group. In the third week, a normal and intact skin layer is observed (H&E stain 40X)

The group of infected and treated mice with extract of chlorella algae extractat concentrations of 100% and 75% in 21 days of treatment, upon histological sectioning , showed healing completely , return of tissue to normal condition, final disappearance of infections, and removal of the lesion from the epidermis and dermis layer as in Figures (7, 8), However, when treated with extract at 50% during the same period, the epidermal layer showed healing and the disappearance of the lesion, with a few infections in the dermis layer and some minor infiltration in the inflammatory cells as in Figure (9), while the histological section of the mice treated at 25% showed Macrophages and

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lymphocytes, disappearance of necrosis, and healing lesion , in the same time period as in Figure (10).

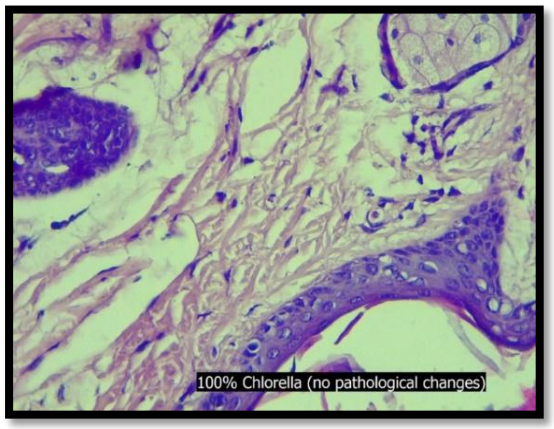


Figure (7) section in the skin tissue of an infected experiment group treated with 100% extract of *Chlorella sp*. During the third week of treatment, a normal and intact skin layer is noticed
(H&E stain 40X).

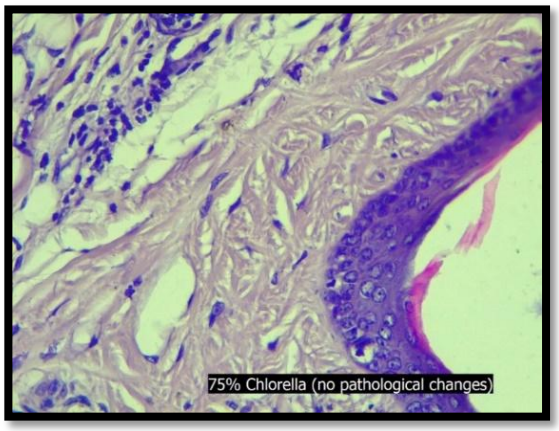


Figure (8) section in the skin tissue of the infected trial group which treated with a concentration at 75% of the extract of *Chlorella sp*. In the third week of treatment, a normal and intact skin layer is observed (H&E stain 40X)

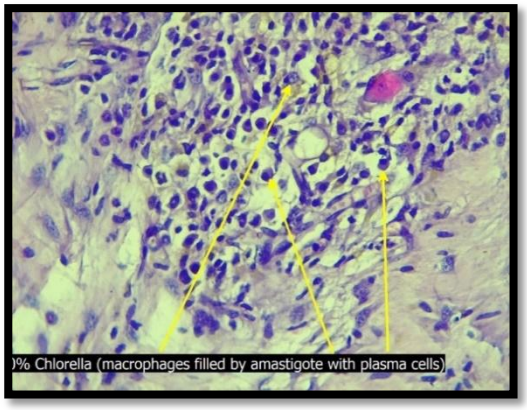


Figure (9): section of skin tissue of the infected experiment group treated with a concentration at 50% of the extract of Chlorella sp. In the third week of treatment, the lesion is observed with little inflammation in the mononuclear cells clustered in the dermis layer with the presence of plasma cells
(H&E stain 40X).

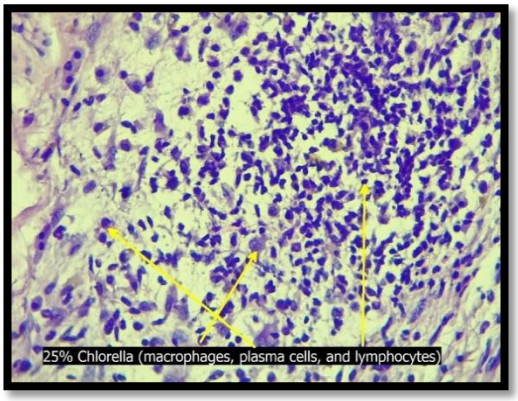


Figure (10): section of skin tissue of the infected experiment group treated with a concentration at 25% of the extract of *Chlorella sp*. In the third week of treatment, the lesion healing is observed with the presence of macrophages, plasma cells and lymphocytes (H&E stain 40X).

The histological study of the positive (infected) control group showed the presence of intense infiltration in the inflammatory cells, and these results were identical to the study (Peters *et al.*, 2008) which showed that neutrophils are ready to defend the body in the event of damage to the body tissues, such as the site of the sand fly bite The histological

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sections showed the presence of the parasite in the dermis and this is consistent with what was mentioned (Al-Difaie & Ghyda, 2014) that the leishmaniasis organisms do not exist inside the epidermis but are surrounded by a dense fibrous layer. Also, it was found that there are abscesses as a result of the destruction of the inflammatory cells, especially the neutrophil, because the period of Its life is short compared to other cells, especially in chronic cases of cutaneous leishmaniasis. Also, debris of neutrophil cells can be explained by the fact that these cells produce antibiotic-like substances called defenders that kill the pathogens due to the presence of strong oxidizing substances and in this type of ingestion the fate of the phagocyte cell. By day 21, wound healing, includingreconstruction of the epidermal layer and rebuilding of skin appendages, was progressing in mice treated with the alcohol extract as no sign of healing was observed in the lesions of control groups. It has already been shown that alcoholic extracts have an immune-regulatory effect on macrophages. Because of its ability to strengthen the immune system and this is in agreement with what was mentioned by(Mason, 2001) in his study, which proved that Chlorella are used in the balance of the body.

The algae used in the study contain EPA, which belongs to the Omega-3 family, and this is in agreement with what (Kim *et al.*, 2006) stated, that the topical application of eicosapentaenoic acid (EPA) to elderly human skin stimulates an extracellular matrix (ECM), which is an extracellular matrix (ECM). It consists of large molecules and minerals, such as collagen, enzymes, glycoproteins, and hydroxylapatite by increasing transforming growth factor-β (TGF-), which secretes the protein that controls cellular differentiation, and other functions in most cells and plays a role in immunity from diseases such as cancer. The topical application of EPA helps prevent and treat skin aging.

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