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Investigation the effect of dietary *Spirulina* supplement on the viability and proliferation of *Leishmania donovani* parasite *in vitro*

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Abstract :

The aim of this study is to evaluate the *in vitro* antileishmanial activities of dietary *Spirulina* supplement and to determine its effectiveness in comparsion with a pentostam drug against *Leishmania donovani*. The effect of *Spirulina* on the visceral leishmaniasis viability was investigated using the MTT test after processed by different concentrations of 100%, 75%, 50%, 25% and compared to the effect of pentostam treatment at a concentration of 0.041 mg / mL at different times of 24 and 48 hours. Parasite after 24 hours for percentages (100%, 75%, 50%, 25%) µg/ml were (33.3%, 42.8%, 60%, and 75%) respectively, while after 48 hours they were (22.2%, 28.5%, 45.4% and 64%) respectively, compared to pentostam treatment, where the parasite viability after 24 and 48 were 50%, 20% respectively, also observed that the *Spirulina* effect on the parasite growth, initially the parasite was grown at a rate of 1×10^4 cells / mL in three groups (*Spirulina*, pentostam, control) and the parasites grow at low rate of division compared with control groups, then the numbers of parasites began to gradually decrease in *Spirulina* and pentostam groups with the time until it became zero in 6 days with no notable growth has occurred

Keywords: *Leishmania donovani*, *Spirulina*, visceral leishmaniasis, MTT assay, pentostam.

Note: The research is based on an M.A. thesis.

Introduction

Leishmania donovani parasites, which are obligate protozoan parasites, cause the vectorborne disease are spread by different species of sandflies of the genus *Phlebotomus* as extracellular flagellated promastigotes that develop into intracellular parasites (aflagellate amastigotes) in mononuclear cells of mammalian hosts, reticuloendothelial cells can be found in the spleen, liver, bone marrow, neutrophils, macrophages [1]. There are three main clinical types of the disease, which is brought on by several species of *Leishmania*. Visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and

Mucocutaneous leishmaniasis (MCL) are the most common clinical syndromes of leishmaniasis [2]. *Spirulina* algae is a type of microscopic blue-green algae that is simple and has one cell. Its scientific name is *Arthrospira platensis*.It looks like a long, thin, blue-green spiral thread when viewed under a microscope. It has a taste and smell similar to seaweed [3], also known as a cyanobacterium. *Spirulina* algae is present in a variety of freshwater habitats, such as ponds, lakes, and rivers. It has a high content of nutrients such as vitamins, protein, betacarotene food, minerals, and fats(especially, the essential omega-3 fatty acids and omega-6 fatty acids), with high concentrations of bioactive substances such as polysaccharides, phenols, phycocyanin pigment [4]. As a result, the dietary *Spirulina* supplement is a superfood and offers a wide range of advantages. It can regulate immunological processes and demonstrates anti-inflammatory characteristics by preventing mast cells from releasing histamine [3], Due to its high protein, polysaccharides, and lipid content it possesses multiple pharmacologic properties like antimicrobial (that include antibacterial and antiviral), immunostimulant, antioxidant, metalloprotective (prevention of heavy-metal poisoning against Cd, Pb, Fe, Hg), and anticancer effects [5]. As an anti-inflammatory, antibacterial, antimalarial pigment phacocyanine is one of the most researched pigments found in *Spirulina* [6]. Is well known to inhibit (NADPH) oxidase nicotinamide adenine dinucleotide phosphate, which contributes to the development of oxidative stress [7].

Materials And Methods

Preparation of *Spirulina* concentration

Spirulina tablets were obtained from the pharmacy, grinding and weighing an amount of 250 gm with a sensitive balance, then dissolved in the distilled water 100 mL to obtain a concentration of 2.5 mg/mL each 0.1mL containing 0.3 mg.

Parasites strain and culture

Leishmania donovani was obtained from the Department of Biology/ College of Science/ University of Babylon, it was serially passaged in NNN-media every 8 days and incubated at 27 C ◦to cultivate and maintain it.

Measured the effect of *Spirulina* on the *L.donovani* promastigotes viability *in vitro*.

In this experiment, there were six groups, each group contained six tubes with one mL of SHE- medium where the steps were prepared according to

the source [8] and inoculated with *L.donovani* promastigotes (1×10^3 cells/mL), then added to each group's tube from this material listed below:
• Group 1: added 1 mL (0.041 mg/mL) of pentostam.
• Group (2,3,4, and 5): added 1mL of *Spirulina* in different concentration (100%, 75%, 50%, 25%) respectively.
• Group 6: without any addition and regarded as a control group.
For 24 and 48 hours all groups were incubated at 26C°, after each incubation period, put 100µL from each tube in the microplates well.MTT solution 50 µL was added to the plates according to the manufacturer's and incubated the plate for 3 hours at 37°C. 150µL of MTT solvent was added in each well after incubation to dissolve the MTT formazan which was produced and incubated for 30 min at room temperature. Photometrically, the quantity of formazan generated by live cells per well was determined at 630 nm. From the OD values, the percentage of viability was determined using the following formula [9].

$$\textbf{Viable cells} = \frac{\textbf{absorbance of treated cells}}{\textbf{absorbance of control cells}} \times 100$$

Measured the effect of *Spirulina* on the *L.donovani* promastigotes growth *in vitro*.
The effect of *Spirulina* on the *L.donovani* promastigotes growth was tested. Included three groups each group containing six vials of NNN-media with 4 mL of lock solution then cultured with 1×10^3cells / mL of *L.dondvani* promastigote added each group as follows:
- **Group 1**: added 0.1mL of *spirulina* (0.3mg)
-**Group 2:** added 0.1mLdaily of Pentostam(0.041 mg).
-**Group 3**: without any addition and regarded as a control group.
After (2,4,6,8,) days, the numbers of the parasite were calculated by using the hemocytometer.

Results and Discussion

The effect of *Spirulina* on the *L.donovani* promastigotes viability

The effects of four concentrations of 100%, 75%, 50%, and 25% of *Spirulina* tablets on the viability of promastigotes *in vitro* were evaluated after 24 and 48 hours and compared with pentostam. The results showed that the percentage of viability will decrease with time and concentration. The percentage of viability of promastigote after 24hr was 33.3%,42.8%,60%, and 75%, respectively, while in pentostam group was 50% as shown in Figure (1)

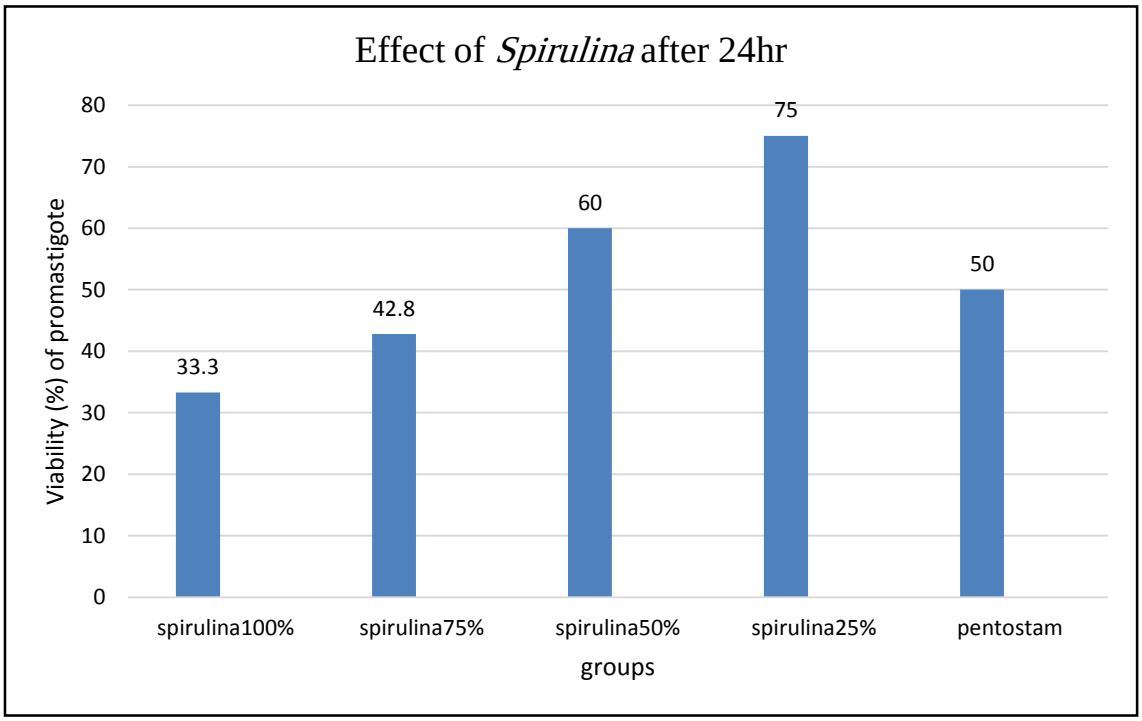

Figure (1): The viability percentage of *Leishmania donovani* promastigotes after exposure to different concentrations of *Spirulina* and a pentostam drug by MTT assay after 24 hours.

After 48 hours the percentage of viability decreased and became 22.2%, 28.5%, 45.4% 64%, respectively, compared with pentostam was 20% as shown in figure(2)

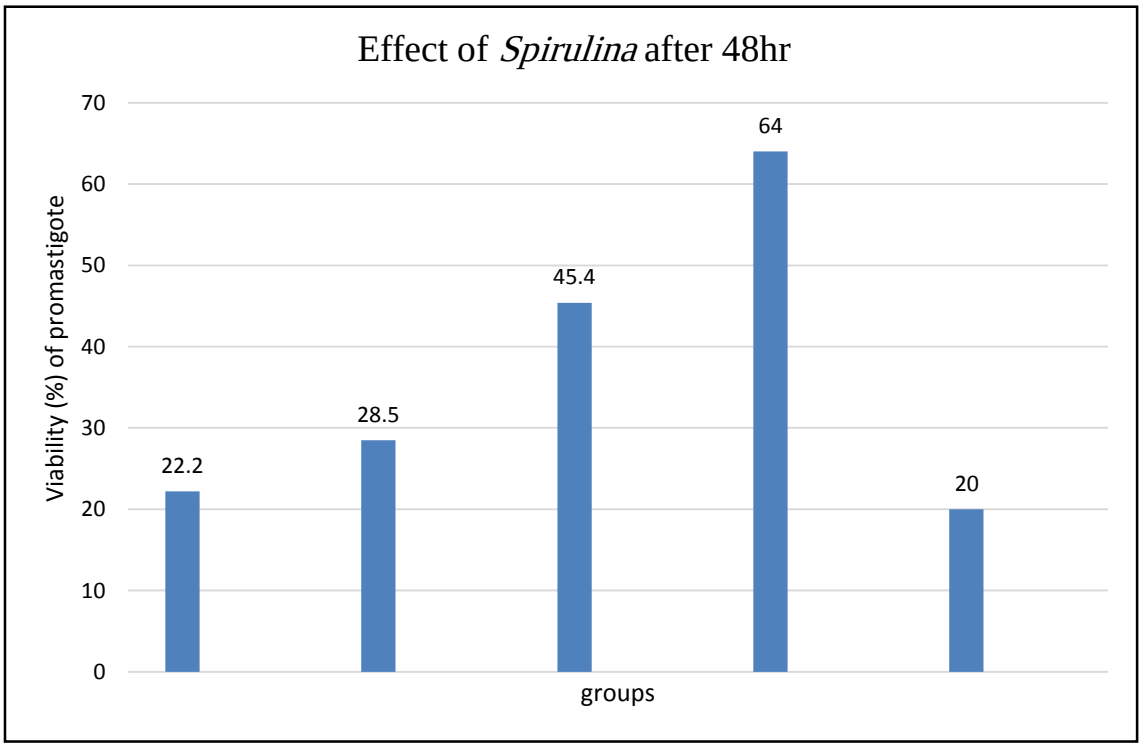

Figure (2): The viability percentage of *Leishmania donovani* promastigotes after exposure to different concentrations of *Spirulina* and a pentostam drug by MTT assay after 48 hours.

It is clear from the results, that the rate of viability for promastigotes decreases with the increase in the *spirulina* concentrations. Selenium, magnesium, copper, calcium, sodium, nickel, and other minerals are abundant in *Spirulina* [10]. A trace element that is important to human health is selenium (Se). It has been used in many medical treatments such as cancer prevention including esophagus, prostate, lung, and gastric-cardiac cancers, as well as antioxidant and antiviral effects [11].

Anti-leishmanial activities of selenium nanoparticles and selenium dioxide (chemical form of selenium) were determined by adding six dilutions of 2.5, 5, 10, 25, 50, and 100 µg/mL of selenium nanoparticles on *L. infantum in vitro*, The colorimetric MTT assay was used to investigate the cytotoxicity of promastigote, Results showed that SeO_2 and Selenium NPs have a growth-inhibitory effect on promastigotes[9]. In the study of the Leishmanicidal effect of selenium nanoparticles (Se NPs) on the form of promastigote of both glucantime-resistant and sensibility of *Leishmania tropica*, using colorimetric cell viability MTT assay to evaluate the viability of

promastigote, found that the growth rate of promastigotes of both strains was significantly inhibited by various concentrations of Se NPs in a dose-dependent manner, Se NPs were toxic to promastigotes and it might cause some morphological and biochemical changes such as DNA fragmentation during apoptotic cell death in the parasite [12].investigation of SeNPs for their anti-leishmanial activity against 10^6 live of promastigote forms of *Leishmania major* at concentrations of 1.25, 2.5, 5, 10, 25, 50, and 100 µg/mL in exposure periods of 24, 48, and 72 hours, Trypan blue was used to calculate the number of live parasites, the results of the investigation showed that SeNPs have anti-leishmanial activity at all concentrations, But after 72 hours of exposure, 100 µg/mL of SeNPs had the highest anti-leishmanial effect 100%[13].

Anti-leishmanial activity of CuSALwas performed with different concentrations of Salisylaldoxime complex of Copper (CuSAL), in this experiment 3.0×10^6 cells/mL of *L.donovani* promastigotes were incubated with15 µM, 30 µM and 45 µM of CuSAL for different times, The trypan blue exclusion method was used to count the numbers of viable promastigotes, growth was inhibited by 30 µM of CuSAL at a rate of 97% after 8 h, and 100% after 12 h, CuSAL selectively inhibits the catalytic activity of *L. donovani* Topoisomerase I (LdTOPILS) leading to apoptosis [14].

The effect of *Spirulina* on the *L.donovani* promastigotes growth.

Spirulina tablets were tested for antileishmanial activity, and the results showed a strong antileishmanial activity and their antiproliferative effects were comparable to that of the pentostam as a potent antileishmanial agent. The results showed that the *Leishmania donovani* parasites grown in the *Spirulina* group was 5×10^4 cells / mL compared with control group was 9.80×10^4 cells / mL while the pentostam was the lowest group in the number of *Leishmania donovani* parasites was 4.20×10^4cells / mL after one day of cultivation, then the numbers of parasites began to gradually decreased with the time until it became in 6^{th} day the numbers of *Leishmania donovani* parasite in *Spirulina* and pentostam groups was zero cells / mL, while in the control group, the division of parasites continue increased till 8 days the numbers of parasites reached 46.20×10^4. the results showed there was a significant difference between all groups ($p \leq 0.05$) as shown in table(1).

Table (1): Mean numbers of *L.donovani* promastigotes ×10^4cell\mL±SD during different periods of growth in study groups.*ANOVA

Days	Control M±SD×10^4	Pentostam M±SD×10^4	*Spirulina* M±SD×10^4	P value
1	9.80 ± 1.10	4.20 ± 0.84	5.00 ± 0.71	0.05
2	13.40 ± 1.14	3.60 ± 0.55	7.20 ± 0.84	0.05
3	16.80 ± 0.84	2.60 ± 0.55	4.80 ± 0.84	0.05
4	19.60 ± 1.14	1.80 ± 0.45	2.60 ± 0.55	0.001
5	25.20 ± 0.84	0.80 ± 0.45	1.60 ± 0.55	0.001
6	29.60 ± 0.89	0.00 ± 0.00	0.80 ± 0.45	0.001
7	33.40 ± 1.52	0.00 ± 0.00	0.00 ± 0.00	Non
8	46.20 ± 1.64	0.00 ± 0.00	0.00 ± 0.00	Non
P value	0.001	0.05	0.05	
There is a significant difference between all groups that were calculated at alpha 0.001*= p ≤0.05***= p ≤0.05***				

In this study, the results showed that *Spirulina* tablets effect on the *L.donovani* promastigotes growth.

Spirulina is abundant in minerals such as sodium, zinc, selenium calcium, potassium, iron, nickel, chromium, magnesium, manganese, copper, and lead in addition to essential fatty acids and carotenoids, which are considered essential for a completely balanced diet [15]. Also, it contains large quantities of phenolic compounds [16]. The effect of phenolic compounds on the viability of *L.donovani* promastigotes was studied, 2×10^5 parasite \mL were incubated with different concentrations of phenolic compounds (0–50 µg/mL) and discovered that when cultured in the presence of IC_{50} concentration of phenolic compounds there was a50% decrease in the number of promastigotes. therefore *Spirulina* has anti-leishmania promastigote activity due to the activity of the phenolic compound, Where the phenolic compounds that elicited significant anti-leishmanial activity such as rosmarinic acid and Apigenin which affected parasites' growth for their ability to better scavenge

iron from the *leishmania donovani* parasite and hence the more pronounced growth inhibitory effect, where it has many functions as antioxidants by chelation of metal ions and removal of free radical [17].

Spirulina algae has high biological effects against some parasites such as malaria attributing this to the presence of many chemical compounds such as fatty acids and vitamins[18]. The effectiveness of crude alcoholic extract of *Spirulina* algae was investigated at concentrations 25%,50%,75% and100%, *L. major* promastigotes were cultured in RPMI- 1640, and the number of total parasite and living parasites was calculated, found that the percentage of growth of *L. major* promastigote decreased and reached to 10% at a growth rate 0,36 with an increase of *Spirulina* concentration 100%[19].

Conclusion

Spirulina is rich with mineral and phenolic compounds that affect on the viability of *Leishmania donovani* and can be considered as a new antileishmanial agent, it affects on growth of *L.donovani* promastigote, which has leishmanicidal activity and affects the *Leishmania donovani* proliferation *in vitro*.

Further Work

Investigating the effects of *Spirulina* on other parasites.

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دراسة تأثير مكمل السبيرولينا الغذائي على حيوية وتكاثر طفيلي الليشمانيا دونوفاني في المختبر

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مستخلص البحث:

الهدف من هذه الدراسة هو تقييم الأنشطة المضادة لليشمانيا في المختبر من مكملات سبيرولينا الغذائية وتحديد فعاليتها بالمقارنة مع عقار البنتوستام ضد الليشمانيا دونوفاني. تم دراسة تأثير السبيرولينا على حيوية داء الليشمانيات الحشوي باستخدام اختبارالمثيل ثايوزول رباعي الزوليوم بعد معاملتها بتراكيز مختلفة 100%، 75%، 50%، 25% ومقارنتها بتأثير معاملة البنتوستام بتركيز 0.041 ملجم/مل في اوقات مختلفة 24 و 48 ساعة. وكانت النسب المئوية للطفيلي بعد 24 ساعة للتراكيز (100%، 75%، 50%، 25%) ميكروجرام/مل (33.3%، 42.8%، 60%، 75%) على التوالي، بينما بعد 48 ساعة كانت (22.2%، 28.5 ، 45.4% و 64%) على التوالي، مقارنة بمعاملة البنتوستام، حيث بلغت حيوية الطفيلي بعد 24 و 48 50%، 20% على التوالي، كما لوحظ تأثير السبيرولينا على نمو الطفيلي، في البداية زرع الطفيلي بمعدل 1 × 10^4 خلية / مل في ثلاث مجموعات (سبيرولينا،بنتوستام ،سيطرة) ، لقد كان نمو الطفيلي بمعدل انقسام منخفض مقارنة بمجموعات السيطرة ثم بدأت أعداد طفيلي اللشمانيا دونوفاني تتناقص تدريجياً في مجموعة السبيرولينا والبنتوستام مع الوقت حتى أصبحت صفراً في اليوم السادس دون حدوث نمو ملحوظ .

ملاحظة : البحث مستل من رسالة ماجستير.

الكلمات المفتاحية: اللشمانيا دونوفاني، سبيرولينا، داء اللشمانيا الحشوي، اختبارالمثيل ثايوزول رباعي الزوليوم، البنتوستام