The activity of extracts *chara vulgaris* against promastigotes of *leishmania tropica.*

فعالية مستخلصات طحلب الكارا ضد الطور المسوط للشمانيا المدارية الجلدية

Ban Hussein Ali **Thaer A. Saleh*** **Mohammad M.F Al-Halbosiy****

Department of Biology, Education College for women, Al- Anbar University, Iraq

Department of Biology, College of Science, AL-Anbar University, Iraq*

Biotechnology Research Center, AL-Nahrain University, Baghdad, Iraq**

بان حسين علي **ثائر عبدالقادر صالح*** **محمد محمود فرحان****

جامعة الأنبار/ كلية التربية للبنات

* جامعة الأنبار/ كلية العلوم

** جامعة النهرين، مركز بحوث التقنيات الأحيائية

E-mail: ma8jed@yahoo.com

Abstract:

Leishmaniasis is a widespread parasitic disease caused by *Leishmania* parasite, this disease considers as a major health problem worldwide. The available therapy is unsatisfactory expensive with a cytotoxic side effects. Studies of marine algae as a source of pharmacological active compounds have increased worldwide. This study was aimed to investigate the effect of a type of green algae (*Chara vulgaris*) on promastigotes of *L. tropica,* by using. various concentrations (500, 250, 125, 62.5, 31.25, 15.6 *µg/mL)* in vitro by MTT assay [3-(4.5-dimethylthiazol-2-yl)- 2.5-diphenyl tetrazolium bromide)], to investigate its effect on the proliferation of promastigotes, by three incubation periods (24, 48, 72 hr.) The results showed a significant ($p< 0.05$) decrease in survived of promastigotes in treatment groups with concentrations that ranged between 15 to 500 µg/ ml. This study revealed a major growth inhibition effect of the organic extract of *C. vulgaris* against *L. tropica* promastigotes, and the extract of ethyl acetate showed potential activity is better than the aqueous extract.

Keywords: Cutaneous leishmaniasis, *Chara vulgaris*, Promastigot.

الملخص :

داء الليشمانيا مرض طفيلي واسع الإنتشار يسببه طفيلي الليشمانيا، ويعتبر هذا المرض مشكلة صحية رئيسية بين جميع أنحاء العالم. العلاج المتاح غير مُرضٍ وباهظ الثمن مع تأثير جانبي سام للخلايا. مؤخراً إزدادت الدراسات حول الطحالب البحرية كمصدر للمركبات النشطة دوائياً في جميع أنحاء العالم. هدفت هذه الدراسة إلى التحقق من تأثير نوع من الطحالب الخضراء (Chara vulgaris) على الطور المسوط من اللشمانيا تركيزات مختلفة (500 ، 250 ، 125 ، 62.5 ، 31.25 ، 15.6 ميكروغرام/ مل) في المختبر بواسطة فحص MTT لدراسة تأثيره على إنتشار بروماستيجوتس على ثلاث فترات من الحضانة (24 ، 48 ، 72 ساعة). أظهرت النتائج انخفاضاً ملحوظاً ($p< 0.05$) في النجاة من الطور المسوط في المجموعات المعالجة ذات تراكيز تراوحت بين 15 و 500 ميكروغرام / مل . هذه الدراسة كشفت عن تأثير كبير للمستخلص العضوي C. الشائع ضد الطور المسوط للشمانيا ، حيث أظهرت خلاصة النتائج ان لمستخلص خلات الأثيل نشاط محتمل أفضل من المستخلص المائي.

الكلمات الدالة: حبة بغداد، طحلب الكارا، فحص MTT

Introduction

Leishmaniasis is a parasitic disease, clinically divided into three forms: cutaneous, mucocutaneous and visceral. Geographically, the disease is divided into: / Old World leishmaniasis found in Africa, Asia, the Middle East, the Mediterranean, and India and New World leishmaniasis found in Central and South America [1]. The disease is vector-borne disease caused by protozoa of the genus *Leishmania*, are transmitted via sand flies of the genus *Phlebotomus* in the old world and *Lutzomyia* the new world [2]. There are over 14 species of Leishmania which may cause up to three different clinical syndrome [3, 4].

Leishmaniasis treatment includes four main options, pentavalent antimony, polyene amphotericin B, the alkylphosphocholine miltefosine and aminoglycoside paromomycin [5]. Despite advances, safety, resistance and cost issues necessitate the continued effort to identify an improved anti-leishmanial drug. Studies have also been undertaken to test the drug susceptibility of clinical isolates [6]. New drugs are being researched in order to find a more selective and effective therapy with fewer side effects [7,8]. The literature has reported several studies about biological activities of extracts from marine algae [9], are also have exhibited appreciable anticoagulant, anti-inflammatory, antitumoral, antiparasitic, antibacterial, and antiviral [10]. Moreover, the ability of marine algae to grow through mariculture and their short generation time make them sustainable sources of active ingredients, are considered an environment-friendly strategic approach that overcomes problems associated with the overexploitation of marine resources and the use of destructive collection methods [11].

Materials and Methods

1. Chemicals used.

MTT powder, fetal calf serum (FCS) and RPMI-1640 medium with L-glutamine were purchased from Capricorn Scientific. All other chemicals and solvents were of analytical grade.

2. Green algae *Chara vulgaris* isolates.

The algae *C. vulgaris* was collected from North of Iraq (AL- Sulaymaniyah Governorate) in April 2016 and diagnosed by Dr.Khaled Faiq Al Balani, University of Garmian. The algae was brought to the laboratory in plastic bags containing water to prevent evaporation. Algae was then cleaned from epiphytes and rock debris and given a quick fresh water rinse to remove surface salts.

a. Preparation of water extract.

C. vulgaris powder 50g was added to 500 ml of distilled water (50 – 60 °C) in a glass flask and operated with a magnetic stirrer for 1 hour, then leave for 30 minutes, used a soft cloth to filter the solution and were separated by a centrifugal at 3000 rpm for 10 minutes. The solution was filtered again with type 1 paper Whatman, No.1; and the leachate was collected by rotary evaporator at a temperature of 45 °C and leaved to dry at room temperature, weighed dry matter and preserved in glass jars 20° C until used [12].

b. Preparation of Ethyl acetate extract.

According to [13] method preparation of the extracts were carried out [13].The dried plant materials (50g) were ground and extracted by Soxhlet extractor device in room temperature. Solvent was removed in a rotary evaporator and extracts were concentrated to dryness and stored at -20 °C, until testing.

3. Culture of *Leishmania tropica*.

Promastigotes were cultured in RPMI 1640 media (pH 7.2, 10% Fetal Calf Serum (FCS) with antibiotics (Penicillin/Streptomycin, Euroclone®) at 26˚C and sub-cultured at cell densities of 2×107 to 2.5×107 cells/ml. Promastigotes were seeded in 96-well culture plates at a density of 1×105 cells/ml and treated in triplicate with extracts in final concentrations ranging from 500-15.2 μg/mL. The plates were incubated at 26˚C for 24,48 and 72 hr. before MTT assay.

4. Measurements of cell viability by MTT colorimetric assay.

MTT is a water soluble tetrazolium salt yielding a yellowish solution. Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzymes [14]. This water insoluble formazan can be solubilized using Dimethyl sulfoxide (DMSO), and the dissolved material is measured spectrophoto-metrically yielding absorbance as a function of concentration of converted dye [15]. Relative numbers of live cells were determined based on the optical absorbance of the treated and untreated samples. *L. tropica* promatigotes was prepared in 96-well plates in a final volume of 100µl/well and incubated at 25˚C for three days. Ten µl of MTT solution was added per well and then the plate was incubated for 4 hr. at 25˚C. The media was removed and 100µl of DMSO solution was added in order to solubilize the formazan

crystals. The plate was stirring gently then, left for 15 minutes. Absorbance was recorded at 490 nm by micro-plate reader and viability determined using the formula:

Percentage of viability = Plate-absorption reading of each test triplicate/Mean of plate reading of control triplicate X 100 [16].

5. Statistical Analysis.

To determine the significant differences between means of control and test values for each concentration after time (24, 48, and 72 hr), using t-test and different between means have analyzed at ($p \leq 0.05$) and expressed as Mean ± SD [17].

Results and Discussion

In order to determine the cytotoxicity of *C. vulgaris* extracts *in vitro* and *ex-vivo* infection and its effect on the viability of *Leishmania*. The compound cytotoxicity has been screened against *L. tropica* Iraqi strain on culture of promastigotes. Colorimetric MTT assay had been used to examine the cell viability and it was determined by the ability of cells for transforming yellow tetrazolium crystal to insoluble blue formazan. Thus, the quantities of formazan produced were rate as a measure of cell viability. The results were plotted and compared with control group for all *C. vulgaris* extracts concentrations. Cytotoxicity was assessed by data of the microtiter-plate reader and calculated as mean ± standard deviation (SD).

Also, IC50 was estimated, the concentration that inhibited 50% of cell growth, which was calculated by SPSS software 2010[18].

Considering the inefficiency of current drugs and the fact that some varieties of *Leishmania* are resistant to these treatments, new drugs are being researched in order to find a more selective and effective therapies with fewer side effects. *In vitro* promastigotes of *L. tropica* were screened with *C. vulgaris* extracts following three times of follow up (24, 48, 72 hr.) Promastigotes were cultured in the RPMI-1640 medium at 26ºC [7, 8]. Therefore, our research conducted studies on new therapeutic agents.

1. Effect of different concentrations of *C. vulgaris* (Aqueous Extract) on *L. tropica* Promastigote, after (24, 48, 72) hours incubation.

Figure (1) revealed to *L. tropica* promastigotes viability after 24, 48 and 72 hr of exposure to aqueous extracts of *C. vulgaris*, showed significant differences ($p< 0.05$) between (24, 72 hr.) and non-significant ($p< 0.05$) among other times and between different concentrations of aqueous extract of *C. vulgaris*, except the lowest concentration (15.6 µg/mL), which have the highest values of mean ± SD of percentages of viable cells (83.29±2.83) after 24, 48 and 72 hours of follow-up showed significant ($p< 0.05$) differences with the highest concentrations (250, 500 µg/mL), which has high impact and the lowest values of mean ± SD (62.02±10.26, 59.73±9.47) respectively. While other concentrations of 31.2, 62.5 and 125 µg/mL was non-significant ($p< 0.05$), mean ± SD of percentage of viability were (74.67±5.77), (67.05±10.65) and (65.27±9.13) respectively, as shown in (table 1).

Table (1) : The percentage of viable cells of *L. tropica* promastigotes treated with aqueous extract of *Chara vulgaris* after 24, 48, 72 hours of incubation

Extract concentrations	Percentages of promastigotes viability after exposed to water extract			mean ± SD	LSD P ≤ 0.05
	24 hr.	48 hr.	72 hr.		
15.6	80.02	85.09	84.77	83.29±2.83	
31.2	68.10	76.99	78.93	74.67±5.77	
62.5	56.62	66.63	77.92	67.05±10.65	
125	55.62	66.41	73.79	65.27±9.13	19.271
250	53.64	58.96	73.48	62.02±10.26	
500	49.77	60.79	68.64	59.73±9.47	
mean ± SD	60.62±11.31	69.14±10.03	76.25±5.55	------	
LSD P ≤ 0.05		11.961			

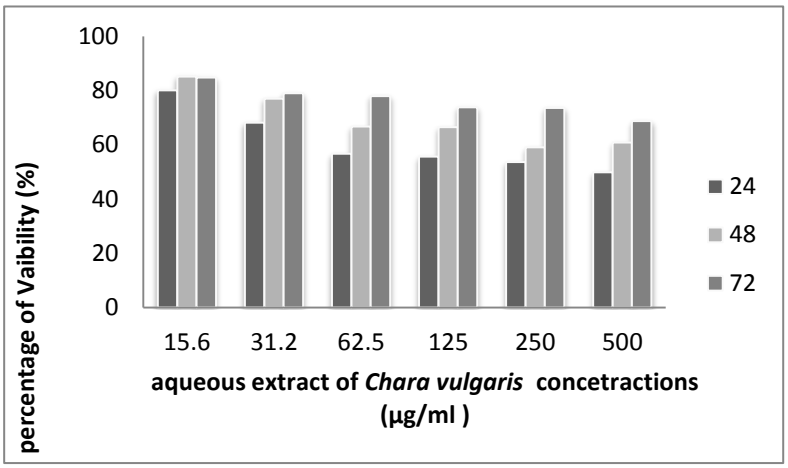

Figure (1): Cell viability of *Leishmania tropica* Promastigote treated with aqueous extract of *Chara vulgaris* after (24, 48, 72) hours incubation.

According to the results of MTT assay the IC50 was calculated to determine the most effective concentrations on the viability of *L. tropica* promastigotes. The IC50s of water extract after 24, 48 and 72 hr. were 318.94, 1006.27 and 1764.76 µg/ml respectively, there was a significant ($p< 0.05$) difference between them.

This is consistent with a previous study [19], where shown water extract of *H. opuntia* chlorophyta algae showed activity on promastigotes and intracellular amastigotes of *L. amazonensis.*

As showed the chemical test for the general constituents of the aqueous extract of *C. vulgaris* that's contained flavonoids, sapoins, terpenoids and alkaloids an absence of phenols and flavonoids in the water extract [20,21], the absence of phenols and flavonoids is justified the low efficiency of the water extract on the viability of *leishmania* parasite.

3.2. Effect the different concentrations of organic extract (ethyl acetate) from *C. vulgaris* on *L. tropica* Promastigote, after (24, 48, 72) hours incubation.

Ethyl acetate it extracts all medium-polar compounds such as terpenes, phenols, free radicals, salts, and some nitrogen oxides, the last solvent petroleum ether is non-polar, so it extraction non-polar compounds such as fats, terpenes and alkaloids free [22, 23]. As that elucidate the successful isolation of bioactive compounds depends primarily on two factors: extraction pattern and the type of solvent used [24].

Where it revealed to *L. tropica* promastigotes viability after 24, 48 and 72 hr of exposure to ethyl acetate extracts of *C. vulgaris*, showed non-significant ($p> 0.05$) differences between different times and different concentrations, also showed non-significant ($p< 0.05$) differences, except the lowest concentration (15.6 µg/mL), which have the highest values of viable cells 59.15±4.37 after 24, 48 and 72 hr. of follow-up showed significant ($p<0.05$) differences with the highest concentrations (250, 500 µg/mL), which have high impact and the lowest values (46.95±5.11; 41.29±6.44) respectively. While other concentrations 31.2, 62.5 and 125µg/mL was non-significant ($p> 0.05$) viability which were 57.61±3.57, 54.8±2.76 and 48.38±5.77 respectively, as shown in (table 2).

Table (2): The percentage of viable cells of *L. tropica* promastigotes treated with ethyl acetate extract of *Chara vulgaris* after 24, 48, 72 hours of incubation.

Extract concentrations	Percentages of promastigotes viability after exposed to ethyl acetate extract			mean ± SD	LSD P ≤ 0.05
	24 hr.	48 hr.	72 hr.		
15.6	54.30	60.36	62.80	59.15±4.37	
31.2	53.53	60.15	59.17	57.61±3.57	
62.5	53.09	57.99	53.32	54.8±2.76	
125	41.72	51.83	51.61	48.38±5.77	10.972
250	41.05	50.21	49.59	46.95±5.11	
500	36.86	38.33	48.68	41.29±6.44	
mean ± SD	46.75±7.73	53.14±8.41	54.19±54.19		
LSD P ≤ 0.05		9.458			

and also indicate that the most effective compounds were semi-polar compounds, and since these solvents (organic) gave a good killing rate, than water extracted compounds, (figure,2).

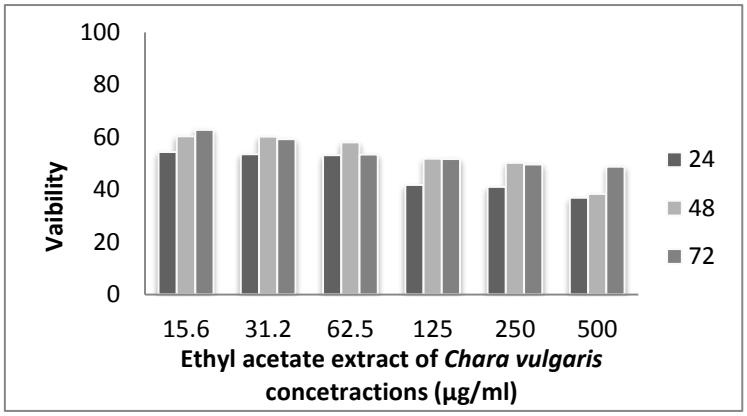

Figure (2): Cell viability of *Leishmania tropica* promastigote treated with ethyl acetate extract of *Chara vulgaris*, after (24, 48, 72) hours incubation.

The IC50s of ethyl acetate extract after 24, 48 and 72 hr. were 49.41, 289.97 and 241.7912 µg/ml respectively, there was a significant ($p< 0.05$) difference between them.

The activity of *C. vulgaris* extracts is due to the presence of very important compounds, terpenes which have biological activity for viruses, bacteria, fungi and protozoa and phenols which is one of oxidizing compounds toxic to microorganisms as well as tannins, which works to stop bleeding and secretions and inhibit enzymes and proteins tanker in the cell membrane [25].

Conclusion.

In conclusion, results suggest that *Chara vulgaris* extracts proved a potential anti-Leishmania activity, in vitro, and it is recommended for further in vivo studies to examine the effect of *Chara vulgaris* on amastigotes.

References.

1. Hailu, A., Dagne, D.A. and Boelaert, M. (2016). Leishmaniasis. In *Neglected Tropical Diseases-Sub-Saharan Africa* (pp. 87-112). Springer, Cham.
2. Dostálová, A. and Volf, P. (2012). Leishmania development in sand flies: parasite-vector interactions overview. *Parasites & vectors*, *5*(1), p.276.
3. Herwaldt, B.L. (1999). Leishmaniasis.Lancet., 354, pp: 1191–1199.
4. Barratt, J.L.N., Harkness, J., Marriott, D., Ellis, J.T. and Stark, D. (2010). Importance of nonenteric protozoan infections in immunocompromised people. *Clinical microbiology reviews*, *23*(4), pp.795-836.
5. Alvar, J., Croft, S. and Olliaro, P. (2006). Chemotherapy in the treatment and control of leishmaniasis. Advances in parasitology, 61, pp.223-274.
6. Kumar, D., Kulshrestha, A., Singh, R. and Salotra, P. (2009). In vitro susceptibility of field isolates of Leishmania donovani to Miltefosine and amphotericin B: correlation with sodium antimony gluconate susceptibility and implications for treatment in areas of endemicity. *Antimicrobial agents and chemotherapy*, *53*(2), pp.835-838.
7. Charret, K.S., Rodrigues, R.F., Bernardino, A.M., Gomes, A.O., Carvalho, A.V., Canto-Cavalheiro, M.M., Leon, L. and Amaral, V.F. (2009). Effect of oral treatment with pyrazole carbohydrazide derivatives against murine infection by Leishmania amazonensis. *The American journal of tropical medicine and hygiene*, *80*(4), pp.568-573.
8. Marra, R.K., Bernardino, A.M., Proux, T.A., Charret, K.S., Lira, M.L.F., Castro, H.C., Souza, A.M., Oliveira, C.D., Borges, J.C., Rodrigues, C.R. and Canto-Cavalheiro, M.M. (2012). 4-(1H-Pyrazol-1-yl) benzenesulfonamide derivatives: Identifying new active antileishmanial structures for use against a neglected disease. *Molecules*, *17*(11), pp.12961-12973.
9. Shalaby, E. (2011). Algae as promising organisms for environment and health. *Plant signaling & behavior*, *6*(9), pp.1338-1350.
10. Mayer, A.M., Rodríguez, A.D., Berlinck, R.G. and Hamann, M.T. (2009). Marine pharmacology in 2005–6: Marine compounds with anthelmintic, antibacterial, anticoagulant, antifungal, anti-inflammatory, antimalarial, antiprotozoal, antituberculosis, and antiviral activities; affecting the cardiovascular, immune and nervous systems, and other miscellaneous mechanisms of action. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1790*(5), pp.283-308.
11. Lauritano, C., Andersen, J.H., Hansen, E., Albrigtsen, M., Escalera, L., Esposito, F., Helland, K., Hanssen, K.Ø., Romano, G. and Ianora, A. (2016). Bioactivity screening of microalgae for antioxidant, anti-inflammatory, anticancer, anti-diabetes, and antibacterial activities. *Frontiers in Marine Science*, *3*, p.68.
12. المنصور، ناصر عبد علي (1995). تأثير مستخلصات مختلفة من نبات قرن الغزال Iatea *Ibicella* في الأداء الحياتي للذبابة البيضاء *Bemissia tabacis* . أطروحة دكتوراه، كلية العلوم، جامعة البصرة. 124 صفحة.
13. Ladd Jr, T.L., Jacobson, M. and Buriff, C.R. (1978). Japanese beetles: extracts from neem tree seeds as feeding deterrents. *Journal of economic entomology*, *71*(5), pp.810-813.
14. Terry, L. R., Richard, A. M., Andrew, L, Helene, A. B., Tracy, J. W., Lisa, M., Douglas, S. and Yvonne, R. (2004). Cell viability assay, In: Assay Guidance Manual, Sittampalam, G. S., (eds.), National Library of Medicine, USA.
15. Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, 65, pp: 55- 63.

16. Ali, H.Z. (2014). Cytotoxicity of myriocin against axenic culture of Leishmania mexicana. *Jornal of Biotechnology Research Center*, *8*(1), pp.36-40.
17. Quinn, G.P. and Keough, M.J.(2002). *Experimental design and data analysis for biologists*. Cambridge University Press.
18. Abe, Y., Sasaki, H., Osaki, T., Kamiya, K., Kawano, R., Miki, N. and Takeuchi, S. (2012). Rapid and accurate IC 50 determination using logarithmic concentration generator. In *Chemical and Biological Microsystems Society*.
19. García Parra, M., Monzote Fidalgo, L., Pasarón, C.O.C., Delgado, N.G. and Hernández, A.P., (2012). Antileishmanial activity of six extracts from marine organisms. *Revista cubana de medicina tropical*, *64*(1), pp.61-64.
20. Mohammed, I.H., Abed, A.K. and Nsaif, Q.H. (2018). The Cytotoxic Activity Chara elegans on Growth of same Cell Lines. *Diyala Journal of Medicine*, *14*(2), pp.9-16.
21. شاكر،هيا عبد .الظاهر وأريج ، حسن و حسن، وصال عودة (2010). فعالية بعض مستخلصات طحلب الكارا *Chara sp* على يرقات الطور الرابع لبعوض *Culex quinquefoseiatus* . مجلة ميسان للدراسات الأكاديمية. المجلد (9) العدد (17).
22. . Harborne, J. B. (1984). Photochemical methods. 2nd ed. Chapman and Hall, London, New York. 284 pp.
23. السلامي، وجيه مظهر(1998). تأثير مستخلصات نباتي *Convolvulus arvensis L.* و *Ipomoea cairica* في الأداء الحيوي لحشرة من الحنطة *Schizaphis grarainum*. رسالة دكتوراه ، كلية العلوم، جامعة بابل صفحة 111.
24. De Gives, P. M.; Arellano, M. E.; Hernandez, E. L. and Marcelino, L.A. (2012). Plant extracts: A potential tool for controlling animal parasitic nematodes, The bio sphere., pp119- 123.
25. Cowan, M. M. (1999). Plant products as antimicrobial agents. Clinical microbiology reviews, 12(4), 564–582.