ISSN:1994-7801
المجلة العراقية لدراسات الصحراء
Iraqi. J. Des. Stud. 9 (2): 1-7, 2019
2019 :(2) 9

VULGARIS ACTIVITY AGAINST OF *Leishmania tropica* PROMASTIGOTES; *IN VITRO*.

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ABSTRACT

Cutaneous leishmaniasis (CL) is a major public health problem and an endemic disease in Iraqi population, *Leishmania tropica* is one of the causes of leishmaniasis in Baghdad. 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 therapy with fewer side effects. Therefore, our research group conducted studies on new therapeutic agents. This study is intended to investigate the effect ethanol extract of *Chara vulgaris* at concentrations (15.6-500) µg/mL in the growth rate and viability of *leishmania tropica* isolates and compared with pentostam (3.12-100) µg/mL *In-vitro*. (15.6, 31.25, 62.5, 125, 250, 500 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. Three incubation periods (24, 48, 72 hr.).

Keywords: Cutaneous leishmaniasis, *Chara vulgaris*, Ethanol extract.

دراسة تأثير فعالية مستخلص الإيثانول لطحلب *Chara vulgaris* ضد الطور أمامي السوط لطفيلي اللشمانيا الجلدية *Leishmania tropica* في المختبر.

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المستخلص

داء اللشمانيا الجلدي هو مشكلة صحية عامة كبيرة ومرض مستوطن في السكان العراقيين، *Leishmania tropica* هو أحد أسباب داء اللشمانيا في بغداد. وبالنظر لعدم كفاءة الأدوية الحالية وحقيقة أن بعض أصناف الليشمانيا تقاوم هذه العلاجات، يتم البحث عن أدوية جديدة من أجل إيجاد علاج أكثر انتقائية وفعالية مع آثار جانبية أقل، لذا أجرت مجموعة الأبحاث لدينا دراسات حول عوامل علاجية جديدة. وقد هدفت هذه الدراسة إلى دراسة تأثير مستخلص الإيثانول من مستخلص *Chara vulgaris* بتركيز (15.6 و 31.25 و 62.5 و 125 و 250 و 500 ميكروغرام/مل) في معدل وحيوية طفيلي اللشمانيا ومقارنتها مع نتائج علاج البنتوستام وبتركيز (3.12-100) ميكروغرام/مل في المختبر بواسطة MTT assay [3-(4.5-dimethylthiazol-2-yl)- 2.5-diphenyl tetrazolium bromide)] لدراسة تأثيره على تكاثر السوطي خلال ثلاث فترات من الحضانة (24 ، 48 ، 72 ساعة).

الكلمات المفتاحية: اللشمانيا الجلدية، طحلب الكارا، مستخلص الإيثانول.

البحث مستل من رسالة الباحث الاول.

INTRODUCTION:

The leishmaniases are a spectrum of different diseases caused by more than 20 species and subspecies of parasites belonging to the genus *Leishmania*. Approximately 350 million people in 88 countries are exposed to these parasites which cause an estimated 12 million infections world-wide (Nasereddin, 2010). Cutaneous leishmaniasis is classified into Old World- and New World- disease. Also, there is mucocutaneous and visceral leishmaniasis, also known as Kala-Azar (Handler *et al.,* 2015). CL disease transmission by the bite of sand flies from the genus *Phlebotomus* in the Old world and *Lutzomyia* in New World separately (Figueira *et al.,* 2017).

Treatment options against *Leishmania* infections are limited for a few drugs with inconsistent efficacy and many side effects: pentavalent antimonials (sodium stibogluconate, meglumine antimoniate), second-line pentamidine, amphotericine B (also formulated as liposome), allupurinol and ketoconazole. In addition, oral miltefosine with fewer side effects has recently been introduced, which appears to be efficient against visceral and cutaneous leishmaniasis (Murray *et al.*, 2005; Soto and Toledo, 2007). 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 therapy with fewer side effects. Therefore, our research group conducted studies on new therapeutic agents (Charret *et al.*, 2009; Charret *et al.*, 2013; Marra *et al.*, 2012). The literature has reported several studies about biological activities of extracts from marine algae (Shalaby, 2011). These also have exhibited appreciable anticoagulant, anti-inflammatory, antitumoral, antiparasitic, antibacterial, and antiviral activities (Mayer *et al.*, 2009).

MATERIALS AND METHODS:

Chemicals used

MTT powder, Dimethyl sulfoxide (DMSO) fetal calf serum (FCS) and RPMI-1640 medium with L-glutamine were purchased from Capricorn Scientific.

The algae *C. vulgaris* was collected from North of Iraq (Al- Sulaymaniyah Governorate) in April 2016 and diagnosis 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.

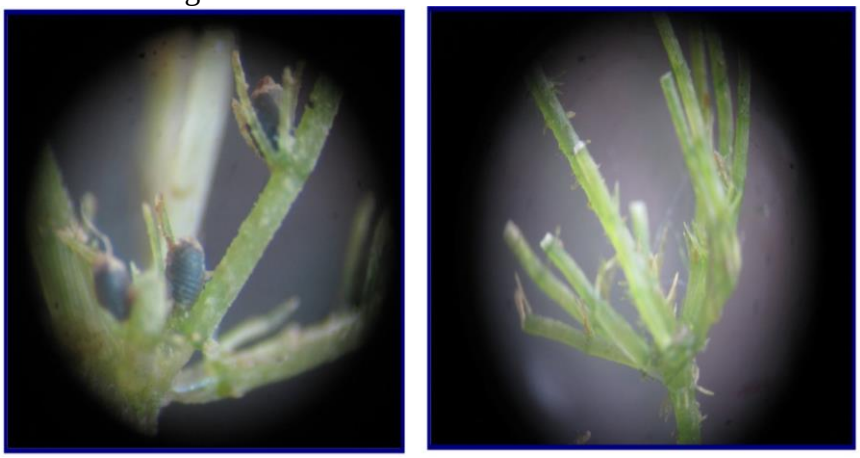

Figure 1. *Chara vulgaris* spp. (A) The virtual shape of the naked eye (B) Microscopic shape under 16x (Shaker *et al.*, 2010).

According to Ladd *et al.*, (1978) method preparation of the extracts, 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.

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 (Terry *et al.*, 2004). 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 (Mosmann, 1983). 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 (Ali, 2014).

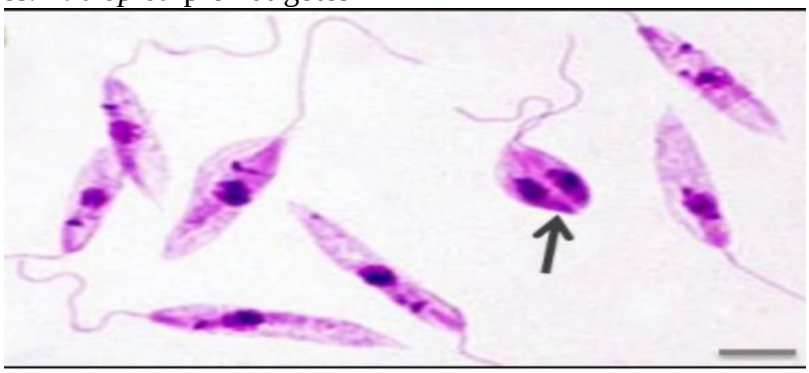

Figure 2. Extracellular promastigote of *Leishmania* parasite (Oryan, 2015).

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 (Quinn and Keough, 2002).

RESULTS AND DISCUSSION:

Leishmaniasis has been considered a neglected disease, despite its high rates of mortality and morbidity (Alvar *et al.*, 2012; WHO 2014). The drugs used in leishmaniasis treatment have serious side effects (Ameen, 2010), and the search for anti-leishmanial activity among natural products such as algae may be useful in the development of new drugs .Recently, marine algae have been highlighted as resources that contain a variety of biologically active compounds, with antibacterial, antitumor, and immunostimulating activities (Harada *et al.*, 1997; Sims *et al.*, 1975).

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 softwear 2010 (Abe *et al.*, 2012).

While the results of the viability of ethanol extract figure (3) show non-significant ($p \leq 0.05$) differences between all times (24, 48 and 72 hr.) but was showed significant ($p < 0.05$) differences between the values of extract concentrations. Except the lowest concentration 15.6 µg/mL which have the highest values of mean ± SD of percentages of viable cells (90.67±3.75) after 24, 48 and 72 hours of follow-up showed significant ($p \leq 0.05$) differences with the highest concentrations (125, 250 and 500 µg/mL), which have high impact and the lowest values of mean ± SD (71.98±6.32, 66.02±1.96 and 60.24±0.93) respectively. While other concentrations 31.2, 62.5 µg/mL, recorded non-significant ($p \leq 0.05$) differences mean ± SD of a percentage of viability which is (87.72±1.32, 84.25±1.93) respectively, as shown in table -1.

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

Extract Concentrations	Percentages of promastigotes viability after exposed to ethanol extract			mean ± SD	LSD P ≤ 0.05
	24 hr.	48 hr.	72 hr.		
15.6	86.64	94.06	91.33	90.67±3.75	7.418
31.2	86.31	87.90	88.95	87.72±1.32	
62.5	85.43	85.31	82.02	84.25±1.93	
125	67.21	79.15	69.58	71.98±6.32	
250	64.34	65.55	68.18	66.02±1.96	
500	59.38	61.23	60.12	60.24±0.93	
mean ± SD	74.88±12.87	78.86±12.98	76.69±12.57		
LSD P ≤ 0.05	16.920				

According to the results of MTT assay the IC_{50} is calculated to determine the most effective concentrations on the viability of *L. tropica* promastigotes. The IC_{50} of ethanol extract after 24, 48 and 72 hr. are 969.03, 974.73 and 942.123 µg/ml respectively. There is a significant ($p \leq 0.05$) difference between them.

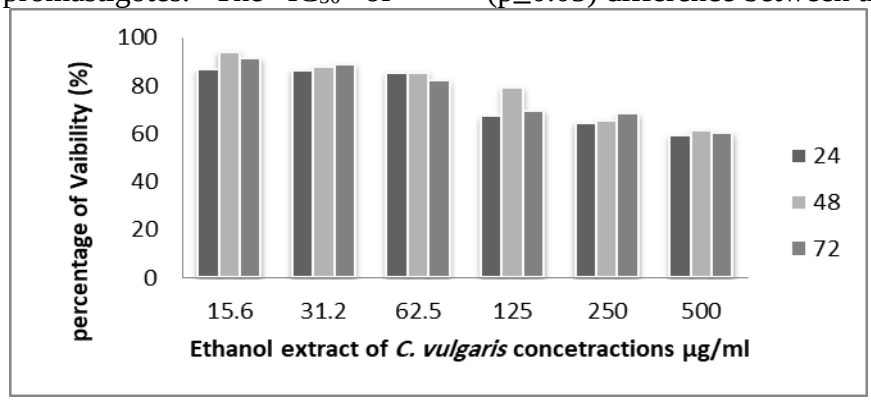

Figure 3. Cell viability of *L. tropica* Promastigote treated with Ethanol extract of *C. vulgaris*, after (24, 48, 72) hours incubation.

The literature has reported about the diagnosis of compounds that the phenolic extract contains (Phytol) compound, *Plazaa et al.*, (2010) explain the phytol compound extracted from the *hematalia elonyata* and *Synechocystis* sp. has antimicrobial and toxin efficiency. Al-Dosari, (2010) has explained the efficiency

of phenols derived from plant *Quercus aegilops* and *Nigella sativa* in *leishmania* parasite viability. The effect is that phenols act to inhibit protein and carbohydrate metabolism by interfering in a parasitic chain of interactions leading to a lack of proteins that are important for the survival of organism. Phenolic compounds may be associated with proteins, forming complexes that are difficult to digest by the parasite (Al-Mansour, 1995). Al-Rubaie, (2014) has pointed to the ability of the (phytol) compound to inhibit the growth of the fungus *Alternaria solani*. As showed Al-Akabi, (2014) the ability of Phytol to kill *Giardia lamblia* parasites and *Entamoeba histolytica* parasites, Al-Maliki, (2008) has pointed to the ability of phenolic compounds isolated from the plant Coriandrum sativum to kill the *E. granulosus* parasite *Ex-vivo*. The reason for this is that the presence of phenolic compounds may lead to disruption of breathing processes in mitochondria and thus induce inhibition of metabolism of carbohydrates, fats and proteins leading to parasite death.

All results showed that the extracts of *Chara vulgaris* have Alkaloids, Tannins, phenols, Flavonoids and saponins, while Glycosides, are absent. This results have agreed with many studies such as (Pelaez, 2002; Mayer *et al.*, 2007), they screened the most active compounds in macroalgae.

In this study sodium stibogluconate (3.12 - 100 µg/ml) are used as positive controls, where the results of the viability of Sb figure (4) show non-significant ($p \leq 0.05$) differences between the values of all times (24, 48 and 72 hr) and show significant ($p \leq 0.05$) differences between the values of drug concentrations, except the lowest concentration 3.12µg/ml which have the highest values of mean ± SD (56.75±2.43) is non-significant ($p < 0.05$) differences with concentration 6.26µg/mL which have values of mean ± SD (52.84±1.63) of percentages of viable cells for (Sb) after 24, 48 and 72 hours of follow up.

While the same concentration have significant ($p \leq 0.05$) differences with other concentrations like 12.5 and 25 µg/ml recorded mean ± SD of percentage of viability which are (46.1±2.94 and 40.89±4.83) respectively, but the effect is most apparent of the highest concentration 50 and 100 µg/mL, which have the lowest values of mean ± SD (36.91±5.2, 35.04±6.52) respectivel. as shown in table -2

Table 2. The percentage of viable cells of *L. tropica* promastigotes treated with Pentostam after 24, 48, 72 hours of incubation.

Extract concentrations	Percentages of promastigotes viability after exposed to Pentostam			mean ± SD	LSD P ≤ 0.05
	24 hr.	48 hr.	72 hr.		
3.12	55.40	55.29	59.57	56.75±2.43	9.719
6.26	54.63	52.48	51.41	52.84±1.63	
12.5	42.71	47.51	48.08	46.1±2.94	
25	35.54	44.92	42.23	40.89±4.83	
50	31.01	40.82	38.91	36.91±5.2	
100	27.59	39.74	37.80	35.04±6.52	
mean ± SD	41.14±11.87	46.79±6.22	46.33±8.35		
LSD P ≤ 0.05	11.733				

To date, the precise mechanism of action of antimonials remains an enigma and their antileishmanial action probably depend on the in-vivo reduction of SbV form to a more toxic SbIII form, due to

that only amastigotes are susceptible to the SbV (Berman *et al.*, 1998).

Currently, several limitations have decreased the use of antimonials: the variable efficacy against CL and VL, as well as the emergence of significant resistance, has been increased (Croft and Coombs,2003)

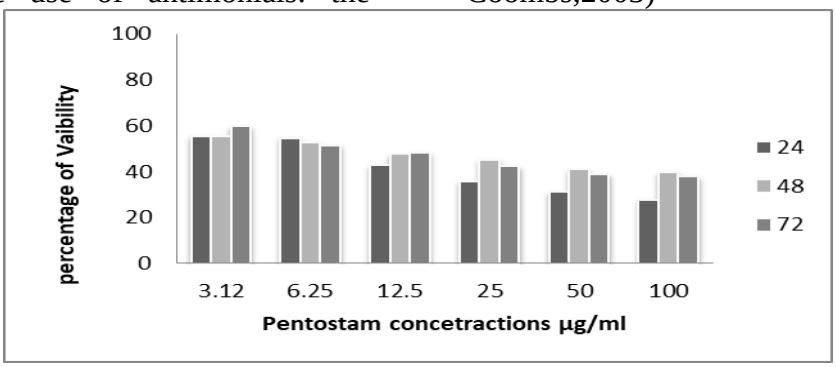

Figure 4. Cell viability of *L. tropica* Promastigote treated with Pentostam drug, after (24, 48, 72) hours incubation.

According to the results of MTT assay the IC_{50} is calculated to determine the most effective concentrations on the viability of *tropica* L.promastigotes. The IC_{50} of (Sb) after 24, 48 and 72 hr. were 32.38, 44.92 and 49.33 µg/ml respectively, there is a significant ($p \leq 0.05$) difference between them.

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