Ann. For. Res. 65(1): 5067-5084, 2022
ISSN: 18448135, 20652445

ANNALS OF FOREST RESEARCH
https://www.e-afr.org/

ASSESSMENT STUDY FOR THE ACTIVITY OF SPECIFIC ALGAL EXTRACTS IN THE TREATMENT OF CUTANEOUS LEISHMANIASIS IN MAYSAN GOVERNORATE

Weam. S. Turky[1], Prof. Dr. Sabeeh H. Al-Mayah[2] and Jassim Fathi. Ali[3]
[1]College of Science, University of Basra, Iraq
[2]College of Education for Pure, University of Basra, Iraq
[3]College of Education for Pure, University of Mosul, Iraq

Abstract:
The results of the current study recorded an assessment of the biological activity of the methanolic extract of two types of green algae: Enteromorpha intestinal and Cladophora crispata against the parasite L.major cutaneous leishmaniasis in vitro using BALB/c mice (males and females) and compared it with Pentostam. Mice were brought at an age ranging between 2-3 months and weighing between 30-35 grams obtained from the animal house at the Faculty of Science, University of Maysan. The results of the current study in vitro showed that the concentration of 2 g/ml methanolic extract of C. crispata was more efficient in reducing the number of parasites compared to Pentostam and the control group at a rate of (15.078, 10.720, and 18.679) parasites/ µm, after 72 hours of the experiment. As for the methanolic extract of E. intestinal, the percentage of decrease in the number of parasites was (19.316, 17.303, and 21.312) parasites/ µm for the same concentration and time. The results of the in vivo study showed that treatment with the methanolic extract of C. crispate was the best in treating cutaneous leishmaniasis lesions during the treatment periods, using several treatments compared with the control group, which recorded an arithmetic mean of 4.88 ± 0.36 cm. The group treated with Pentostam 120mg/kg recorded 3.1±0.29 cm compared to the control. Conversely, C.craspata recorded 10% more efficiency than Pentostam with an average lesion diameter of 2.85±0.28 cm, this efficiency was increased by increasing the concentration by 20% for the algae to record 1.85 ± 0.35 cm compared to the control treatments. The alga E.intestinals (10, and 20%) also recorded significant therapeutic efficacy to reduce the effects of ulceration caused by Leishmania infection, recording 3.76 ± 0.22 cm and 3.7 ± 0.27 cm, respectively. However, Vaseline did not record any significant differences compared to the control and according to the value of LSD. The cure was achieved when ulceration was treated with C.crispata extract 20%, as the highest cure rates were recorded in 9 out of 10 cases, with a partial improvement rate of 1 case. Followed by the group treated with C. crispata extract. crispata 10%, which was represented by 7 cases, and the partial improvement was 3 cases. The group treated with Pentostam recorded a cure rate of 5 cases and a partial improvement rate of 4 cases. As for the Vaseline group and the group treated with the algal extract E.intestinalis 10% and E.intestinalis 20%, the highest non-cure rates were recorded with (10, 7, and 5) cases, respectively. The cure rate, partial improvement, and no cure were recorded for those with cutaneous leishmaniasis, and those treated with C.crispata, E.intetinalis, and Vaseline, as it was shown that C.crispata 10% recorded a cure rate of 7%, followed by C.crispata 10 with a cure rate of 9%. Finally, the alga

E.intetinalis recorded 10% non-cure by 7%, and then 20% E.intetinalis, which recorded 5% non-cure, 3% partial improvement, and 2% cure, while Vaseline recorded no cure and by 10%.

Keywords: Algal, Cutaneous Leishmaniasis, neglected tropical disease

Introduction:

Leishmaniasis is widespread throughout the world, especially in tropical and subtropical regions, the Mediterranean basin, and the countries of the Middle East. It affects both humans and animals. This neglected tropical disease is a major public health problem in 98 endemic countries. The number of new infections annually is estimated to be approximately 2-4 million worldwide and about 70,000 deaths annually (Khraiwesh et al., 2016; Armeli Minicante et al., 2016). This disease is caused by infection with a primary parasite belonging to the class Trypanosomatidae and the genus Leishmania, which forcibly parasitizes the cells of the reticular endothelial system, the skin, in the mucous membranes, or the viscera. Therefore, leishmaniasis appears in multiple forms, it may affect the skin and is called cutaneous leishmaniasis. Leishmaniasis (CL) or internal organs is called visceral leishmaniasis (VL) (Steverding, 2017). Over the past 60 years, the first line of selected drugs for the treatment of cutaneous leishmaniasis have been pentavalent antagonists with two formulations: sodium stibogluconate (Pentostam) and meglumine antimoniate (glucantime) of equal efficacy. The emergence of resistance by the parasite to these treatments has been recorded, in addition to their toxic side effects, and their high costs. The drug Amphotericin B, which is a second line with excellent activity against resistant parasites, was used, but the long period of use and its harmful effects constituted major shortcomings that reduced its use. It is also too expensive and unaffordable for poor patients in many affected countries (Hamill, 2013; Tchokouaha Yamthe et al., 2017). Other drugs such as Pentamidine, Miltefosine, Paromomycin, Azoles, and Allopurinol have been used against Leishmania parasites (Meena et al., 2017; Soares et al., 2012). But its undesirable side effects, the emergence of parasite resistance to these drugs, relapse after treatment as well as the long course of treatment, and the high cost also prevented its widespread use. In practice, the new treatment must have more innovative mechanisms of action, higher effectiveness, cheapness, and ease to get by everyone, as well as less toxicity and shorter duration of use, and less likelihood of resistance by parasites (Khraiwesh et al., 2016; Armeli Minicante et al., 2016). Algae have medical importance in being able to produce a large number of biologically active compounds represented by a group of phenols, essential oils, alkaloids, triple terpenes, saponins, and tannins. These compounds, in turn, negatively or positively affect the biological activity of other living organisms, even if they are in low concentrations, especially microorganisms, as some algae extracts have been characterized by their anti-parasitic activity, including Leishmania (Al-Kaabi, 2018). Enteromorpha spp., a marine alga, is a valuable food source as it contains a high amount of carbohydrates, proteins, fats, vitamins, amino acids, and minerals, as well as bioactive compounds (Escobido et al., 2016). Regardless of nutritional value, they also contain phytochemicals that are widely used in pharmaceutical and other applications (Seenivasan et al., 2012). These include phytochemicals, alkaloids, anthraquinones, reducing sugars, tannins, phenols, saponins, flavonoids, steroids, and terpenoids (Tresina and Mohan,

2014). C. crispate also has antiparasitic medicinal importance, as some studies have shown that the alga contains many bioactive compounds, including alkaloids and ethyl acetate compounds, which have high lethal activity against protoscoleces of Echinococcus granulosus (Athbi et al., 2014; Kini et al., 2020). It also has an anti-growth inhibiting role of a hydatid cyst parasite (Bianco et al., 2013; Divyashree et al., 2019). Moreover, (Athbi et al., 2013) in Basra indicated that alkaline and ethyl acetate extracts of Cladophora crispata have a positive effect on the protoscoleces of hydatid cyst in comparison with the activity of albendazole, which is associated with a reduction in the number and weight of the hydatid cyst, as well as blockage of the germ layer and is responsible for the proliferation of protoscoleces. As well as a study by (Al-Mousawi et al., 2021) in Basra on the effectiveness of using E. intestinalis and C. crispata extracts for effective control of sarcoptic mange. Given the lack of available studies on the treatments for leishmaniasis and the side effects of the used treatment (Pentostam), the difficulty of its availability in hospitals, and the high cost of it. In particular, this study attempted to find an alternative natural medicine from the extracts of Enteromorpha intestinalis, and Cladophora crispate and evaluate its therapeutic efficacy in vitro and in vivo. Besides, comparing it experimentally in mice with the drugs already used such as the treatment of Pantostam after converting these extracts into an Ointment used instead of injections, as it is more receptive to the infected.

Materials and methods of work

Culture Media

1- NSemi-Solid Medium

This medium consists of the components mentioned by (Limoncu et al., 1998) as shown in Table (1), it was used for the initial isolation and growth of promastigote parasites after the initial withdrawal of skin lesions and the recovery of parasites from infected animal tissues. The semi-solid medium was prepared in the following manner: -

It attended as follows:-

1. All components except rabbit blood defibrinated and antibiotic were dissolved in a quantity of distilled water in a volumetric 1-liter vial. After making sure that the substances dissolved well, the volume was completed with distilled water up to the mark, the pH was adjusted to 7.4 and the solution was sterilized with an autoclave at 121 °C for 20 minutes.
2. The solution was cooled and add fibrin-free human blood to it by placing it in a sterile glass bottle covered with a tight-fitting lid containing glass balls and shaking it for three minutes to remove the fibrin (Evans, 1989).
3. Then the antibiotic was added to the solution.
4. The nutrient medium was divided into sterile 25-ml bottles covered with a tight-fitting lid, 5 ml in each bottle, and incubated in an incubator at 37°C for 24 hours to verify that the medium was free of contamination.
5. After making sure that the medium is free of contamination, the bottles are kept in the refrigerator until later use.

Table (1) Components of a semi-solid medium in 1 liter of distilled water

NaCl	6.91 g
$CaCl_2.2H_2O$	0.22 g
$NaHCO_3$	0.10 g
KCl	0.29 g
D-glucose	0.77 g
Agar	0.40 g
Pepton	1.00 g
Beef extract	0.30 g
Distilled water	800 ml
Defibrinated rabbit blood	100 ml
Gentamicin	1.5 ml

2- NacNeal-Nicolle (NNN) Media

The NNN media consists of two phases: a solid phase and a liquid phase (Chouihi et al., 2009). This media was used to obtain parasite sub-cultures, antigen preparation and treatment experiments. The solid blood agar phase consists of the materials shown in Table (2).

Table (2) Components of the solid phase in 1 liter of distilled water

Agar	20 .00 g
Brian heart infusion	37.00 g
D-Glucose	10.00 g
D.W	800 ml
Gentamycin	500 mg
Rabbit defibrinated blood	200 ml
Distilled Water	

This Media was prepared as follows:-

1. All components except the blood and the antibiotic were dissolved in an amount of distilled water in a volumetric bottle of 1 liter and after the process of dissolution of the substances is completed the volume was completed with distilled water to the mark. The pH was adjusted to 7.4 and the solution was sterilized with an autoclave at 121 °C for 20 min.
2. After cooling, fibrin-free human blood was added (as described above) and mixed well.
3. The media was divided under sterilization conditions into 10 ml bottles with a tight lid by 5 ml in each bottle and was placed in the incubator tilted to obtain a large surface area for growth at a temperature of 37 ° C.

As for the liquid phase, it was prepared according to the method of (Dawson et al., 1978) and included Locke's solution components shown in Table (3).

Table (3) The components of Locke's solution in 1 liter of distilled water

NaCl	9.00 g
$CaCl_2.2H_2O$	0.32 g
KCl	0.42 g
$NaHCO_3$	0.20 g
D-glucose	2.00 g
Gentamicin	1 ml
Distilled water	

All materials were successively dissolved in distilled water, the pH was adjusted to 7.4, the medium was sterilized, cooled, and antibiotics were added to it in the same manner mentioned previously. 2 milliliters of this solution were added to each vial containing the solid phase, which was previously prepared under sterilization conditions. The bottles were then incubated at 37°C for 24 hours to ensure sterilization and then kept in the refrigerator until use.

3- Preparation of Phosphate Buffer Saline (PBS):

It was prepared according to the method of (Collee et al., 1996), pH 7.2, ingredients per liter as listed below

1000 ml	Distilled water	
0.2 g	Potassium Choride	KCl
8 g	Sodium Chloride	NaCl
2.88 g	Sodium hydrogen phosphate	Na_2HPO_4

4- Preparation of Stock Geimsa Stain:

50 milliliters of glycerin were mixed, then the mixture was put in a sealed bottle of dark color and put it in a water bath at a temperature of 60 ° C for two hours with stirring every half an hour. The mixture was left to cool and then 50 milliliters of methyl alcohol was added to it with a concentration of (95%) with continuous stirring, the dye was then filtered by a Whatman No. 1. Then, it was kept in a dark place at room temperature until it is used for dyeing after shaking it as the dye solution was prepared with one milliliter of the base dye, one milliliter of sodium bicarbonate solution, 1.25 milliliters of methyl alcohol (95%) and 40 milliliters of distilled water (Garcia et al., 1979).

5- Experimental animals

93 white Swiss mice (BALB/c) (males and females) with ages ranging between 2-3 months and weights between 30-35 grams were obtained from the animal house at the Faculty of Science, Maysan University. Plastic cages of dimensions (30 * 10 * 10 cubic centimeters) furnished with

sawdust were used to raise them and put them in an air-conditioned room with a temperature of 22 ° C and a cycle of light and darkness 10 / 14 hours/day. The mice were fed a standard diet consisting of the materials mentioned in Table (4) and clean drinking water, the cage litter is replaced weekly, and mice are checked periodically by a veterinarian.

Table (4) Components of the concentrated mice diet used during the study period

Feed Ingredients	%	For each 10 kg
Full Cream Milk Powder	20	2 kg
Wheat groats	17	1.7 kg
Wheat flour	17	1.7 kg
Barley groats	20	2 kg
Corn groats	25.5	2.5 kg
Salt	1	0.1 kg

A group of vitamins, amino acids, and mineral salts available in (Cholivet-A-M.) were added at a dose of (10) g per (10) kg of ration (Ward, 1970).

6- Experimental infection with Leishmania parasite\

6-1 Leishmania Sample

A sample of L.major parasite cultured on prepared culture media was obtained from RPMI 1640 in 10ml glass bottles from Kut University/College of Science/Department of Biology, these samples were transferred to the laboratories of the College of Basic Education, University of Maysan. The bottles were incubated in an incubator at 26°C, with daily monitoring of parasite movement and observation of shape, number, and movement by light microscopy.

6.2 Preparation of Parasites Dosage

Parasite samples were purified from the RPMI1640 culture medium by withdrawing 5 ml of culture medium and placing it in a test tube and centrifuging at 5000 rpm for 5 minutes. Then the filtrate was poured and the sediment containing the parasites was withdrawn and diluted with physiological solution (sodium chloride) the experimental mice to be infected were injected at the soles of the feet and the intercostal cavity at a dose of 1*106 ml (Green et al., 2017).

7- Vital tests for the cutaneous leishmaniasis parasite:

Parasite vitality was tested using 0.4% of Erythrocin-B stain, according to the method of (Hodgkinson et al., 1980).

8- Counting the Parasite

The parasite numbers were calculated according to the method of (Amoa-Bosompem et al., 2016). 20 µl of a dilute solution (phosphate buffer) containing promastigotes was taken and placed in a 1.5 ml Eppendorf tube containing 20% formalin and mixed well. 10 µl of the solution containing the inhibiting parasite was taken and placed on a Neubauer hemocytometer slide and left at room temperature for 5 minutes until the parasite settled on the slide. Then, the number of parasites was calculated in the counting squares and multiplied the resulting number by 150. Thus, the resulting

number represents the number of the parasite in 1 milliliter of the culture medium, this means, it was calculated in five squares of the median square designated for counting red blood cells. Meaning that the sum of squares = 5 x 16 = 80 squares, and since the volume of each square is 1/4000 mm^3, then the volume in 80 squares = 1/4000 x 80 = 1/50 mm^3. The appropriate volume of injection was prepared using the dilution law C1V1=C2V2.

Therapeutic study

9- The experiments of the study

The current study included the main aspect, which is the evaluation of the effectiveness of the algal extracts Enteromorpha intestinalis and Cladophora. crispata against L.major parasite in vitro and in vivo and its comparison with the treatment Pentostam.

10- Dry algae powder

Dry algae powder was obtained from the algae laboratory in the Department of Life Sciences - College of Education for Pure Sciences - the University of Basra after collecting and identifying algae by a professor. Ghazwan Talib Nouri.

11- Preparation of the methanolic extract of algae

The extraction process was carried out according to the method of (Rois et al., 1987). 50 grams of dry algae powder were taken and placed in a conical flask with a capacity of 500 milliliters, then 250 milliliters of absolute methanol were added to it. The mixture was mixed using a magnetic stirrer for 24 hours, then the mixture was filtered using filter paper, the filtrate was transferred to a glass Petri dish and left at laboratory temperature until the alcohol evaporated and the dry extract was obtained, and placed in glass bottles until use.

12- Injection of laboratory animals

7 groups of white mice (Bulb/c) were injected into the intraperitoneal area, foot, and the beginning of the tail with 10 mice per group to study the pathological, immunological, and therapeutic changes of the parasite.

13- In vitro study

To evaluate the effectiveness of the algal extracts and compare it with the effectiveness of Pentostam treatment currently used to treat cutaneous leishmaniasis in Iraq, three concentrations of E. intestinalis extract and C. crispata were prepared using 10% glycerin with a weight (0.5, 1, 1.5 and 2 g) of each algal extract, respectively, one milliliter of 10% glycerin was added to each of these weights, thus the concentrations (0.5, 1, 1.5 and 2 ml/g) for each algal extract. They were preserved in small glass bottles, and 0.5 ml of each concentration of algal extracts was added to each tube of culture tubes of L.major parasite, which is in the logarithmic state (cell 1 mL 106) with 3 replicates for each concentration. Meanwhile, 0.5 mL of 10% Glycerin was added to three other parasite culture tubes, which were considered as a control group. As well as Pentostam (SbV) (100 mg/ml) was added to three parasite culture tubes, which were considered a positive control

group. The average number of live parasites in each tube during 72 hours was calculated by using a Haemocytometer.

14- In Vivo study

- Ointment preparation

Concentrations (10% and 20%) of the methanolic extract were prepared for each type of algae studied by adding a weight of the algae extract to a weight of Vaseline to prepare the ointment used in the treatment. The concentrations were kept individually in glass bottles until use (Tabassam et al., 2008).

15- Treatment of infected animals

Laboratory mice experimentally injected with 0.1 ml of L.major promastigotes parasites were divided into 7 groups by 10 mice in each group. After three weeks of injection, the clinical signs of cutaneous leishmaniasis infection appeared on the body of mice and they were treated with ointment for 6 weeks as follows:

1. A negative control group. Mice infected with the cutaneous leishmaniasis parasite L.major were left untreated.
2. Positive control group: Mice infected with the parasite were injected with Pentostam (SbV) treatment (100 mg/ml) for 5 days for 6 weeks, and the diameter of the lesion was measured with a micrometer ruler.
3. The Control group of parasite-infected mice was treated with Vaseline only daily (morning and evening) for 6 weeks.
4. Groups of mice infected with the parasite and treated with algae extracts were divided into four groups:

- Group treated with C. crispate extract at a concentration of 20%.
- Group treated with C. crispate extract at a concentration of 10%.
- Group treated with E.intestinalis extract at a concentration of 20%.
- Group treated with E.intestinalis extract at a concentration of 10%.

In all of these treatment groups, the diameter of the lesions formed as a result of infection with the Leishmania parasite was measured to know the differences between them and to take notes and photograph them before and after treatment.

Statistical Analysis

All study results were subjected to statistical analysis to find out the significant differences between the rates of infection with cutaneous leishmaniasis according to the IBM-SPSS version 24 program. The significant differences were determined at the 5% probability level by using the T-test of the difference between two paired samples before and after injury Paired sample t-test. With the use of (Pearson correlation coefficient) to measure the correlation between two variables (SPSS, 1998).

Results:

Therapeutic study

1- Effect of algae extracts on L.major in vitro

The results of the current study showed that the extracts of E. intestinalis and C. crispata had an effect on the vitality of the cutaneous leishmaniasis parasite compared with Pentostam (SbV) 100 mg/ml. Besides, glycerin 10% at three concentrations of each of them (0.5 mg/ml, 1 mg/ml, 2 mg/ml) with three treatments for each concentration as shown in Table (5). The first, second, and third treatments with E.intestinalis extract recorded a decrease in the number of parasites, and the best effect was at the concentration of 2 mg/ml. It was recorded (19.316, 17.303, and 21.312) compared to the positive control group (32, 33, and 34) and the glycerin 10% group. C.crispata recorded more efficiency in reducing the number of parasites at the concentration of 2 mg/ml compared to the other treatment groups (15.078, 10.720, and 18.679). The statistical analysis results showed that there were significant differences ($P<0.05$).

Table (5) Effect of the methanolic extract of algae on L. major after 72 hours (in vitro)

Treatment type	Concentration mg/ml	The first treatment	The second treatment	The third treatment	Mean±SD
		Promastigote concentration 1 X 106			
Control	0	32	33	34	a 33±1
E.intestinalis	0.5	24.132	22.166	26.176	b 24.16±2.01
	1	21.361	18.372	24.362	b 21.37±2.995
	2	19.316	17.303	21.312	bc 19.3±2.005
C.crispata .	0.5	23.754	17.733	24.920	b 22.1±3.8
	1	18.580	14.490	21.590	c 18.2±3.6
	2	15.078	10.720	18.679	c 14.8±3.99
Pentostam (SbV) (100mg/ml)	0.5	24.166	19.273	24.178	b 22.54±2.83
	1	17.244	18.388	23.372	bc 19.67±3.26

	2	18.305	16.323	20.325	bc 18.32±2.0
L.S.D . value	4.604				

In vivo study

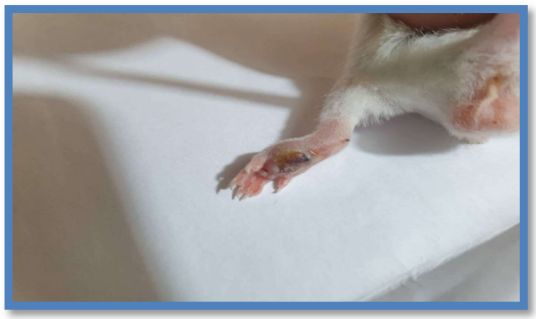
16- Determination of the response rate of infected animals to methanolic extracts of algae

A laboratory experiment was conducted to determine the efficacy of methanolic extracts on the L.major parasite in vivo. 93 mice were infected with the parasite and the infection rate was 100%. Mice were left for four weeks until signs of infection appeared on them, and 70 mice were selected for treatment. They were divided into seven groups, with 10 mice for each group. The mice were treated with algae extracts, Pentostam (SbV) (100mg/ml) and Vaseline for six weeks. The average diameter of the lesions in infected mice was calculated, and the results were recorded and compared with the control group. The lesions of the control group recorded an average diameter of 4.88 ± 0.36 cm, while the average lesion diameter of the group of mice treated with Pentostam (SbV) (100mg/ml) was 3.1±0.29 cm. There was a preference for C.crispata extract at 20% and 10%, respectively, as they reduced the lesion diameter compared to the rest of the treatments and the differences were significant according to the value of LSD, while Vaseline did not record any significant differences compared to the control.

Table (6) Average diameters of lesions in infected mice after treatment (6 weeks)

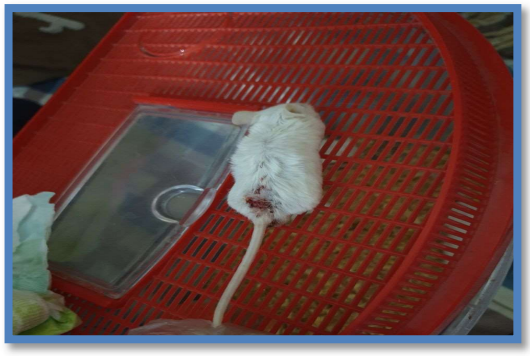
Treatment method (groups)	The number of mice used in the experiment	Average lesion diameter measurement Main±SD
Control	10	a 4.88±0.36
Pentostam (SbV) (100mg/ml)		3.1±0.29 c
C.craispata 10%		2.85±0.28 c
C.craispati 20%		1.85±0.35 d
E.intestinalis 10%		3.76±0.22 b
E.intestinalis 20%		3.7±0.27 b
Vaseline		4.9±0.45 a
LSD value		0.29

Picture (1) An uninfected or treated control mice



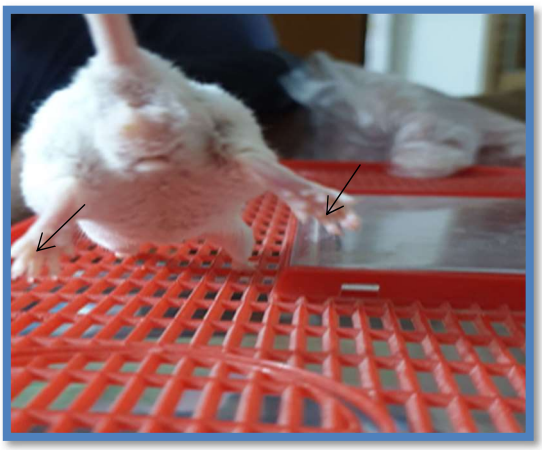
Picture (2) A- A mouse infected with cutaneous leishmaniasis (CL) before treatment

B- Cutaneous leishmaniasis (CL) after treatment with Pentostam for six weeks



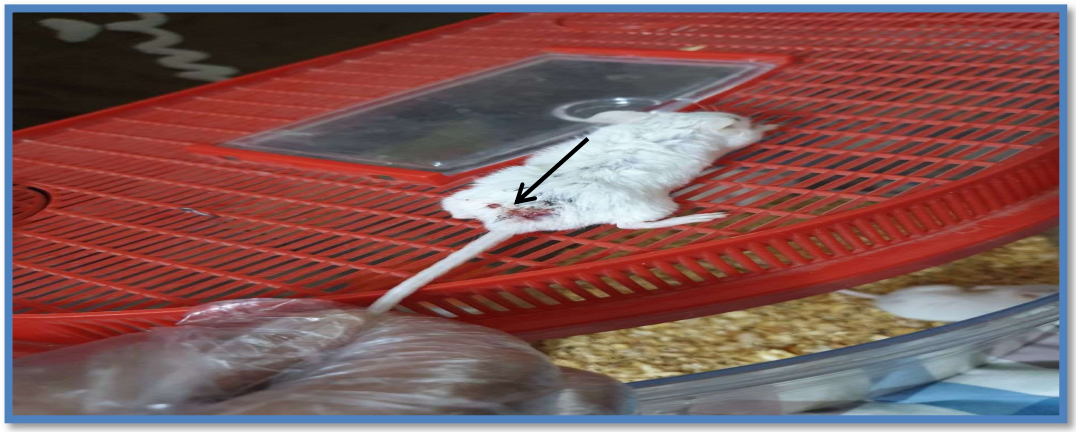
Picture (3) A- A mouse infected with cutaneous leishmaniasis (CL) before treatment.

b- Cutaneous leishmaniasis (CL) after treatment with C.craispati alga extract



Picture (4) A- A mouse infected with cutaneous leishmaniasis (CL) before treatment

B- Cutaneous leishmaniasis (CL) after treatment with E.intestinalis extract for six weeks.



Picture (5) of cutaneous leishmaniasis (CL) after treatment with Vaseline for six weeks.

Discussion:

Therapeutic study

Algae contain secondary metabolites that are an important source of bioactive chemical compounds (TÜney et al., 2006). C. crispate is a rich source of bioactive compounds such as polysaccharides, tannins, terpenes, alkaloids, fatty and amino acids, proteins, flavonoids, phenols, sterols, aromatic organic acids, and aldehydes (Krish and Das, 2014; Kalid et al, 2012). Green algae E. intestinalis also contains significant amounts of alkaloids, polysaccharides, fats, terpenes, proteins, phenols, sterols, amino acids, and saponins and is a rich source of these compounds. (Benjama and Masniyom, 2011; Al-Jaber, 2016). Previous studies indicated the high biological

activity of phenols, saponins, sterols, terpenes, and alkaloids as secondary metabolites. These compounds were isolated from several types of algae and were considered of medical importance (Bernhoft, 2010). WHO (2018) indicated that 80% of the world's population depends mainly on traditional medicine for primary health care. Cyanophyta and Chlorophyta are rich source of bioactive metabolites of medical importance. Methanol alcohol can dissolve and extract large amounts of chemical compounds compared to other solvents (Goud et al., 2007), so it was used in the extraction of compounds in the current study.

Effect of algae extracts on L.major in vitro

The results of the methanolic extract test of E. intestinalis and C. crispate on the vitality of L. major in vitro showed that the extract concentrations of both algae showed a clear effect on the parasite's vitality with varying percentages. Besides a probability for the methanolic extract of C. crispate compared with the treatment group with Pentostom and the positive control group. The two algae extracts had an upward effect by increasing the concentration, which indicates the toxicity of the methanolic extracts of both algae against L. major, as the methanolic extract of algae C. crispate and E. intestinalis with a concentration of 2 mg/ml reduced parasite numbers in the treatment group, and the parasite numbers were 14.8, 19.3 compared to the parasite numbers in culture tubes treated with Pentostam and in the same concentration, in which the average number of parasites was 18.3 with a significant difference. Previous studies were conducted in Iraq to evaluate the biological activity of the methanolic extract of the same algae against other parasites, and the results were promising. (AL-Mayah et al., 2011) were used three concentrations of the methanolic extract of C. crispate to evaluate its effectiveness against protoscoleces of Echinococcus granulosus compared with Albendazole, and its effectiveness was close to that of the algal extracts. (Al-Miusawi et al., 2021) also conducted a study to evaluate the effectiveness of the methanolic extract of C. crispate, E. intestinalis against the scabies parasite in vitro and in vivo. The study findings recorded the superiority of the algal extracts compared to Ivermectin in killing the parasite and healing the infected rabbits. They indicated that the toxicity of the extracts against the scabies parasite depends on time and concentration, that is, whenever the concentration increased, the recovery period decreased. The high efficiency of the alkaloids present in the ethanolic extract of C. crispate was previously observed in killing Escherichia coli (Athbi et al., 2011). In addition to the study conducted by (Khalaf, 2012), which noted a high efficiency of the same alga extracts against hydatid cyst disease in vitro and in vivo. As for the study of extracts of the alga E. intestinalis, (Bhakuni and Rawat 2005) indicated the efficiency of the extracts of this alga against the parasite E. histolytica. The efficiency of the methanolic and aqueous extract of this alga in killing the parasite Plasodium falciparum was also recorded (Rovikumar et al., 2011). The mechanism by which biologically active compounds act on how to kill and exterminate parasites is not known. Some hypotheses have been suggested that explain the possible mechanisms of killing, as (Sepulveda - Bozaaad Cassels, 1996) believed that the active compounds kill parasites either through their effect on the parasite's respiratory chain. Otherwise, through their effect on the defenses of parasite cells against respiratory stress, sometimes the active compounds bind with the parasite DNA strands. It may also affect the enzyme Dihydrorotate dehydrogenase (DHOD),

which is the fourth enzyme in the vital pathway for the manufacture of pyrimidine, which is necessary for the parasite, leading to its inactivation and death of the parasite (Nara et al., 2005). Tannins may bind to proteins inside the parasite's body, causing them to not be degraded and disrupting the metabolism related to nitrogen and essential amino acids in maintaining its vitality. Or it may be attributed to the ability of the active compounds to destroy the cell membrane of the parasite by affecting the proteins and lipids in it, or it may penetrate the cell membrane and block the active sites of some enzymes necessary for its reproduction and growth (Inabo and Fathnddin, 2011; Al-Maliki, 2008). (Khan et al., 2013) pointed out that the Naphthaguinine compound impedes the oxygen metabolism of the parasite cells and thus prevents its respiration, which leads to the formation of free radicals and nitric oxide, and then the parasite dies due to lack of oxygen and toxicity. This activity may also be explained by the presence of terpenes that can interact with groups of Iron (Fe I, Fe II) leads to the release of free radicals that cause damage to the parasite and then its death. (Spavieri et al., 2010) studied the natural sources of antiparasitic compounds, raw extracts of four green marine algae (Cladophora rupestris, Codium fragile ssp. tomentosoides, Ulva intestinalis and Ulva lactuca) collected from Dorset region in England. These compounds tested on Trypanosoma brucei rhodesiense, T. cruzi, L. donovani, and Mycobacterium tuberculosis, where it was found that all algae extracts are active against T. brucei rhodesirupestris, and C. rupestris was the most effective. However. only C. rupestris and U. lactuca had moderate activity against T. cruzi leishmanicidal activity, as none of the extracts showed cytotoxicity towards cells. A study by (Spavieri et al., 2013) demonstrated the role of the antimalarial algal extract P. falciparum, which was extracted from the brown seaweed Cystoseira tamariscifolia, C. baccata and the green seaweed Ulva. lactuca, in inhibiting parasite growth without any apparent effect on the viability of human hepatoma cells (Huh7). The highest efficacy was demonstrated by an extract of U. lactuca, red seaweed Ceramium virgatum and Halopitys incurvus. The active extracts are an inhibitor of one or more major enzymes of the malaria type II fatty acid synthesis pathway (FAS-II), a specific drug target for LS, as all LS active extracts showed dual activity against both intracellular malaria parasites.

In vivo study

Determination of the response rate of infected animals to methanolic extracts of algae

The results of the current study showed the efficiency of the methanolic extracts of E.inestinalis and C. crispate in the treatment of cutaneous leishmaniasis by treating skin lesions or ulcers, which changed significantly after treatment period with these extracts in the form of an ointment, which reduced the diameter of the lesions with a significant difference with the other treatment groups. The diameter of the ulcer in the control group was 4.88 ± 0.36 cm, and the average diameter was 3.1 ± 0.29 cm in the group treated with Pentostam. While the mean diameters of ulcers in the group treated with 10% C. crispate were 2.85 ± 0.28 and the mean diameters decreased when the concentration was increased to 20% to record 1.85 ± 0.35. The alga E.intestinals recorded 10%, and 20% significant therapeutic efficacy also in reducing the effects of ulcers caused by Leishmania infection, recording 3.76 ± 0.22 cm and 3.7 ± 0.27 cm, respectively. However, Vaseline did not record any reduction in ulcer diameter compared to the control. Sodium

stibogluconate Pentostam (SbV) (100mg/ml) is an organic derivative of antimony, and belongs to the class of drugs known as pentavalent antimonials, its mechanism is not clear but it may act by binding to thiol groups in the parasite and inhibiting the formation of high-energy phosphates (ATP) and guanosine triphosphate (GTP) used to treat leishmaniasis. Due to the side effects that this drug has on sufferers, in addition to its high price, it is necessary to use natural preparations. The use of natural products is considered an auxiliary therapeutic approach in medicine, as algae extracts are used to treat disorders and diseases such as wounds, fever, stomach pain and the prevention of irregular heartbeat. Recent trends in drug research from natural sources indicated that marine algae are a promising new source of bioactive compounds, especially with antiparasitic activity, since they have developed defensive strategies that have led to a large level of chemical structural diversity in different metabolic pathways. This inhibitory activity of the main materials synthesized by benthic marine algae is actively bioactive against Leishmania spp., Trypanosoma cruzi and T. brucei. T, Chagas disease, and African trypanosomiasis (Rais et al., 2020; Silva-Jardim et al., 2014). Current chemotherapy for leishmaniasis imposes difficult limitations such as cytotoxicity, and ineffectiveness in endemic areas, so glycosomal targets were reviewed for the discovery of antitrypanosomal drugs recently (Barros-Alvarez et al., 2014). Despite efforts to find new drugs against leishmaniasis, the treatment of leishmaniasis is still based on the use of pentavalent antibiotics (sodium stibogluconate and meglumine antimoniate), which have been developed for more than 60 years These antibiotics are known to have many noticeable side effects, including nausea, abdominal cramps, diarrhea, rash, hepatotoxicity and cardiotoxicity, as well as the development of pathogens, resistance to antimony has been a growing problem for nearly four decades (Sundar, 2019; Maltezou, 2001). Pentavalent antimony is also associated with a higher mortality rate, especially in Human Immunodeficiency Virus or HIV-infected patients (Balasegaram et al., 2012; Torres et al., 2014). The results of the current study agreed with what was found by (Fadhil & Marhoon, 2021) in studying the effectiveness of chlorella green alga extract on the parasite L. major at different concentrations (25, 50, 75, and 100%) and comparing it with Pentostam. The study lasted three weeks in vivo (in white mice), as it recorded the efficiency of the algae extract Chlorella compared with Pentostam (SbV) (100mg/ml). (Al-Mayah et al., 2011) confirmed that C. crispata contained bioactive compounds when it was used at three concentrations of the methanolic extract of the alga against protoscoleces of Hydatid cysts and after 5-6 days compared with albendazole.

Conclusions

The results showed the efficiency of the methanolic extracts of both algae C. crispata and E. intestinalis against the parasite L.major cutaneous leishmaniasis. The extracts showed an inhibitory activity that exceeded the inhibitory activity of Pentostam treatment both in vitro and in vivo.

Recommendations

1- Conducting in vitro studies on the possibility of using algal extract treatment for visceral leishmaniasis and other types.

2- Conducting a study to determine the active substance or substances in the algal extracts C. crispata and E. intestinalis, which had a role in killing the parasite that causes cutaneous leishmaniasis, as well as determining the killing mechanism used against the parasite.
3- Testing the biological activity of the algae extracts C. crispata and E. intestinalis against other types of parasites.
4- The possibility of using methanolic extracts of both algae as alternative and safe treatments against cutaneous leishmaniasis CL compared to traditional treatments.

References

17- Bhakuni, D. and Rawat, D. (2005). Bioactive marine natural products Springer Science & Business Media, USA : 106pp.
18- Biological importance of Marine Algae- An overview A.Raja*, C. Vipin and A.Aiyappan Int.J.Curr.Microbiol.App.Sci (2013) 2(5): 222-227
19- Biotechnology, 25 (3), 73-95.
20- Blomster, J., Bäck, S., Fewer, D. P., Kiirikki, M., Lehvo, A., Maggs, C. A., & Stanhope, M. J. (2002). Novel morphology in Enteromorpha (Ulvophyceae) forming green tides. American Journal of Botany, 89(11), 1756-1763.
21- Bonde, C. S., Bornancin, L., Lu, Y., Simonsen, H. T., Martínez-Valladares, M., Peña-Espinoza, M., . . . Thamsborg, S. M. (2021). Bio-Guided Fractionation and Molecular Networking Reveal Fatty Acids to Be Principal Anti-Parasitic Compounds in Nordic Seaweeds. Frontiers in Pharmacology, 12(1155). doi: 10.3389/fphar.2021.674520
22- Bosch-Nicolau, P., Ubals, M., Salvador, F., Sánchez-Montalvá, A., Aparicio, G., Erra, A., . . . Molina, I. (2019). Leishmaniasis and tumor necrosis factor alpha antagonists in the Mediterranean basin. A switch in clinical expression. PLoS neglected tropical diseases, 13(8), e0007708.
23- Boussoffara, T., Boubaker, M. S., Ahmed, M. B., Mokni, M., Guizani, I., Salah, A. B., & Louzir, H. (2019). Histological and immunological differences between zoonotic cutaneous leishmaniasis due to Leishmania major and sporadic cutaneous leishmaniasis due to Leishmania infantum. Parasite, 26.
24- Brooks, F., Rindi, F., Suto, Y., Ohtani, S., & Green, M. (2015). The Trentepohliales (Ulvophyceae, Chlorophyta): an unusual algal order and its novel plant pathogen—Cephaleuros. Plant Disease, 99(6), 740-753.
25- Bruno de Sousa, C., Gangadhar, K. N., Macridachis, J., Pavão, M., Morais, T. R., Campino, L., . . . Lago, J. H. G. (2017). Cystoseira algae (Fucaceae): update on their chemical entities and biological activities. Tetrahedron: Asymmetry, 28(11), 1486-1505. doi: https://doi.org/10.1016/j.tetasy.2017.10.014.
26- Caldas, A., Favali, C., Aquino, D., Vinhas, V., Van Weyenbergh, J., Brodskyn, C., . . . Barral, A. (2005). Balance of IL-10 and interferon-γ plasma levels in human visceral leishmaniasis: implications in the pathogenesis. BMC infectious diseases, 5(1), 1-9.

27- Camacho, E., Rastrojo, A., Sanchiz, Á., González-De la Fuente, S., Aguado, B., & Requena, J. M. (2019). Leishmania mitochondrial genomes: maxicircle structure and heterogeneity of minicircles. Genes, 10(10), 758.
28- Carneiro, M. W., Fukutani, K. F., Andrade, B. B., Curvelo, R. P., Cristal, J. R., Carvalho, A. M., . . . de Oliveira, C. I. (2016). Gene expression profile of high IFN-γ producers stimulated with Leishmania braziliensis identifies genes associated with cutaneous leishmaniasis. PLoS neglected tropical diseases, 10(11), e0005116.
29- Centers for Disease Control and prevention. (2017). Leishmaniasis. 24/7:Saving ;Lives, Protecting People TM. https://www.cdc.gov/dpdx/leishmaniasis/index.html.
30- Chauhan, P., Nair, A., Patidar, A., Dandapat, J., Sarkar, A., & Saha, B. (2021). A primer on cytokines. Cytokine, 145, 155458.
31- Chelbi, I., Mathlouthi, O., Zhioua, S., Fares, W., Boujaama, A., Cherni, S., . . . Zhioua, E. (2021). The Impact of Illegal Waste Sites on the Transmission of Zoonotic Cutaneous Leishmaniasis in Central Tunisia. International journal of environmental research and public health, 18(1), 66.
32- Coelho, D. R., Miranda, E. S., Saint'Pierre, T. D., & Paumgartten, F. J. R. (2014). Tissue distribution of residual antimony in rats treated with multiple doses of meglumine antimoniate. Memórias do Instituto Oswaldo Cruz, 109, 420-427.
33- Compounds from the green alga Cladophoracrispata with their bioactivity test. MSc. Thesis, College of Education, University of Basrah
34- Cordi, B., Donkin, M., Peloquin, J., Price, D., & Depledge, M. (2001). The influence of UV-B radiation on the reproductive cells of the intertidal macroalga, Enteromorpha intestinalis. Aquatic toxicology, 56(1), 1-11.
35- Cox, F.E.G. (2002): History of Human Parasitology. Clin. Microbiol. Rev., 15 (4): 595-612
36- Cunningham, A. F., Flores-Langarica, A., Bobat, S., Dominguez Medina, C. C., Cook, C. N., Ross, E. A., Henderson, I. R. (2014). B1b cells recognize protective antigens after natural infection and vaccination. Frontiers in immunology, 5, 535.
37- Da Silva Santos, C., Attarha, S., Saini, R. K., Boaventura, V., Costa, J., Khouri, R., . . . Souchelnytskyi, S. (2015). Proteome Profiling of Human Cutaneous Leishmaniasis Lesion. Journal of Investigative Dermatology, 135(2), 400-410. doi: https://doi.org/10.1038/jid.2014.396
38- Dar, M. J., Din, F. U., & Khan, G. M. (2018). Sodium stibogluconate loaded nano-deformable liposomes for topical treatment of leishmaniasis: macrophage as a target cell. Drug delivery, 25(1), 1595-1606.
39- Dar, M. J., Khalid, S., Varikuti, S., Satoskar, A. R., & Khan, G. M. (2020). Nano-elastic liposomes as multidrug carrier of sodium stibogluconate and ketoconazole: a potential new approach for the topical treatment of cutaneous Leishmaniasis. European journal of pharmaceutical sciences, 145, 105256.

40- Diro, E., Lynen, L., Mohammed, R., Boelaert, M., Hailu, A., & van Griensven, J. (2014). High parasitological failure rate of visceral leishmaniasis to sodium stibogluconate among HIV co-infected adults in Ethiopia. PLoS neglected tropical diseases, 8(5), e2875.
41- Dolganyuk, V., Belova, D., Babich, O., Prosekov, A., Ivanova, S., Katserov, D., . . . Sukhikh, S. (2020). Microalgae: A promising source of valuable bioproducts. Biomolecules, 10(8), 1153.