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Effect of *Eucalyptus Camaldulensis* Extracts on *Leishmania major In Vitro* and *In Vivo*

Hind Mahdi Jarallah[1*], Abdul Hussein Hubash Awad[2], Abdul-Mohsin Hamed Jassim[3]

[1]*Marine Science Center, University of Basrah, Basrah, Iraq*
[2]*College of Education, University of Basrah, Basrah, Iraq*
[3]*College of Medicine, University of Basrah, Basrah, Iraq*

ABSTRACT

The leaves extract of Eucalyptus camaldulensis (aqueous, alcoholic and oils) were studied as antileishmanial inhibition on Leishmania major promastigotes form. Oils were more active against promastigotes than aqueous and alcoholic extract, thus, the LD_{50} for oils, alcoholic and aqueous extract was (0.602, 3.630 and 4.786) mg/ml respectively. Oils and alcoholic extract were used by topical application for treated BALB/c mice experimentally infected with L. major, on the footpad thickness and mice tail base ulcers.

Keywords: *Leishmania major, Cutaneous Leishmaniasis, Eucalyptus camaldulensis, Plant extracts, BALA/c mice*

INTRODUCTION

Leishmania is a genus of protozoan flagellated parasites [1,2]. Leismaniasis is a major public health problem, disseminated in different regions of world especially in tropical and subtropical regions with between two and three million humans affected by the diseases annually [3,4]. It has several disease forms, Cutaneous, mucucutaneous and visceral leishmaniasis. In Iraq cutaneous leishmaniasis (CL) caused by *Leismaniasis major* and *Leismaniasis tropica* [5], disseminated in different regions, especially in marshlands villages, southren Iraq [4,6]. *Leishmania* parasites are resistance for pentavalent antimony drug that indicated the need of new treatment [7]. The development of tradition herbal therapy has been practiced to some degree in all cultures. Plants extracts can be classifieds according chemical compounds such as tannins, terpenoids, alkaloids and flavonoids, which have been found to have antimicrobial properties. The medical plants have been active materials against protein synthesis or DNA synthesis and others [8].

Eucalyptus belongs to family myrtaceae, and to genus Eucalyptus. It is a large genus, embracing about 500 species. *E. Camaldulensiss*, is a large tree, leaves narrowly lanceolate, acuminate, 10-15 cm or more [9]. It is composed of kino-tannic acid (47%) and small quantities of catechin, pyrocatechin, kinoin and in some specimens, an essential oil. Essential oils or Eucalyptus oil is widely used in medicine. The gum is sometimes used in diarrhoea, for relaxing throats and as an astringent in dentistry, cuts, astringent in dysentery. Pharyngitis and laryngitis in the form of lozenges [9,10]. Aqueous extract (0.05 g/0.1 ml) of Eucalyptus showed a good therapeutic effect against *Trichomonus vanginalis* after 24 h [11]. On the other hand, the oil of Eucalyptus leaves have been used for treatment of patients infected with skin fungi as a topical therapy (external ointment) [12]. The aqueous extracts of Eucalyptus have a highest activity against protoscolices of human *Echinococcus granulosus* that decreased the viability to 0% in 72 [13].

MATERIALS AND METHODS

Source of parasite

Leishmania major strain was isolated from female patient aged 35 years by specialized clinic of dermatology. Diphasic (NNN) medium was used in this study, it's made of 2 phases a solid [14] and liquid [15]. The promastigotes were cultivated at (26-28ºC) then harvested on the 6th day for animal's infection and sub-culturing. The number of promastigotes was determined by counting with aid of the hemocytometer (chamber slide) and adjusted to 1×10^7/0.1 ml for experimental animal infection [16]. BALB/c mice, 8-10 weeks old were used in this study.

Animal inoculation

Experimental animals were divided in to two groups each mouse in both groups was inoculated subcutaneously with a dose 1×10^7/ 0.1 ml of promastigotes in hind footpad for group (I) and in a shaved area for group (II).

Post infection

After 4 weeks post infection mice in group (I) used for treated (4 mm) footpad thickness, while, mice in group (II) used after 8 weeks post infection for treated ulcerative lesions.

Plant extraction

The procedures of plant extractions were according [17-19].

Effect of *E. Camaldulensis* plant extracts on *L. major* promastigote

Various concentrations of *E. Camaldulensis* extracts after prepared and test were sterilized by filtration. The screw-capped vials were prepared containing 5 ml solid phase medium and 0.9 ml liquid phase medium (Lock's solution) which contain various concentrations of *E. Camaldulensis* extracts were added to screw-capped vials. Each concentration was done in triplicate. Other vials of diphasic medium were kept as the vehicle control without drug. It was done also in triplicate. Promastigotes were adjusted to 1×10^6 cell/0.1 ml at logarithmic phase. Then, added to each vial. The vials were then incubated at 25-26ºC.

The parasites were counted once daily with the aid of the haemacytometer and preparation of a 1:20 dilution in saline together with the 0.4% trypan blue dye. The viable parasites exclude the dye while the dead forms are permeable to it [20,21].

Percentage of Growth Index (GI%) would be depended in detecting the *in vitro* effect of tested plant extract on parasite forms calculated according El-On, & Messer [22] as follows:

GI% =No. of treated parasite ÷ No. of untreated parasites (control) × 100

The LD_{50} after 3 days from extract addition was calculated according Healy [23].

***In vivo* effect of *E. Camaldulensis* (alcoholic and oils) extracts Preparation**

10 g from powder alcoholic extract of Eucalyptus was mixed with 90 g of Vaseline and used as applied locally ointment.

Oils of Eucalyptus: This oil used directly as local application.

A-Mice foot pad thickness

Oils and alcoholic extract were applied locally on footpad thickness (started at the 4^{th} weeks post infection and continued for 4 weeks). Measure of increasing in thickness was monitored every week aid of the Caliper.

B-On the mice tail base ulcer

The treatment of ulcer was by Daily topical applied of Oils and alcoholic extract for 30 days and their effects on ulcerative lesions was assessed by the followings:

1. Clinically: depended on clinical improvement signs by reduction the erythema and size of ulcer.

2. Parasitology: the density of parasite in stained smear was counted under microscope and recorded by scan 50 fields.

Statistical analysis

Each value was expressed as mean ± standard deviation (SD). The LD_{50} was calculated according Healy [23]. The differences were recorded as significant whenever probability (p) was less than 0.05 SPSS [24].

RESULTS

The effects of leaves extract of *Eucalyptus camaldulensis* on *L. major* promastigote (aqueous, alcoholic and total oils):

The results show high effect of aqueous, alcoholic and oils of Eucalyptus extracts on *L. major* promastigote activity. Tables 1-3 show the density of promastigote of *L. major* in control compared with the untreated groups during 4 days of experiment for each type of leaves extracts aqueous, alcoholic and oils respectively.

Table 1: The effects of various concentration of aqueous extract of *Eucalyptus Camaldulensis* on the *L. major* promastigote *in vitro*

Total No. of parasite cells/ml (× 10^6) plant extract concentrations (mg/ml)					
Days after plant extract exposure	**0 Control**	**8**	**6**	**4**	**2**
1	4.2 ± 0.282	1.3 ± 0.424	2.2 ± 0.282	3.2 ± 0.848	3.9 ± 0.424
2	7.2 ± 1.414	1.6 ± 0.282	3 ± 0.565	5.2 ± 0.282	6.2 ± 0.565
3	12.6 ± 0.848	2.6 ± 1.131	4.8 ± 0.848	8.9 ± 1.272	10.2 ± 0.282
4	22 ± 2.82	2.2 ± 0.565	6 ± 0.565	14.3 ± 0.707	16.5 ± 0.424

Data are represented as (mean ± SD) from 3 experiments

Table 2: The effects of various concentrations of alcoholic extractof *Eucalyptus Camaldulensis* on the *L. major* promastigote *in vitro*

Total No. of parasite cells/ml (x 10^6) plant extract concentrations (mg/ml)					
Days after plant extract exposure	**0 Control**	**4**	**3**	**2**	**1**
1	2.5 ± 0.141	1.8 ± 0.282	2.1 ± 0.424	2.2 ± 0	2.4 ± 0.565
2	6.5 ± 0.707	4.2 ± 1.131	5.2 ± 1.414	5.4 ± 0.848	6.2 ± 0.848
3	12 ± 1.414	7.2 ± 1.697	8.8 ± 0.282	9 ± 0.565	11.1 ± 1.272
4	18 ± 0.131	7.3 ± 0.989	12.5 ± 0.141	12.6 ± 1.131	16.2 ± 2.262

Data are presented as (mean ± SD) from 3 experiments

Table 3: The effects of various concentrations of total oils of *Eucalyptus camaldulensis* on the *L. major* promastigote *in vitro*

Total No. of parasite cells/ml (× 10^6) plant extract concentrations (mg/ml)					
Days after plant extract exposure	**0 Control**	**1**	**0.8**	**0.6**	**0.4**
1	4.4 ± 0.282	2.8 ± 0.565	3.2 ± 0.565	3.4 ± 0.5656	3.6 ± 0
2	8.9 ± 0.707	5.5 ± 0.424	6.2 ± 0.282	6.5 ± 0.707	6.6 ± 0.565
3	16.7 ± 0.989	6.5 ± 0.707	7 ± 0	9 ± 0.282	10 ± 0.565
4	28 ± 0.23	6 ± 0.848	10 ± 0	14 ± 1.131	16 ± 1.414

Data are presented as (mean ± SD) from 3 experiments

Different extract of Eucalyptus show high effect on *L. major* promastigote. Growth Figures (1), (2), (3) show the percentage of growth index at different time and different concentrations on day 4 in the treated groups which contain the highest concentration of each type of extract methods aqueous, alcoholic and oils respectively. On day 4 of experiment, it was noticed that the total oils of leaf extract have a higher efficiency with LD_{50} (0.602) mg/ml in comparison with alcoholic extract LD_{50} (3.630) mg/ml. The aqueous extract shows the lowest with LD_{50} (4.786) mg/ml. Statistical analysis data of growth index (GI)% on day 4 of experiment revealed highly significant differences ($P<0.05$) among different plant concentrations.

8 mg/ml > 6 mg/ml > 4 mg/ml > 2 mg/ml	For the aqueous extract.
4 mg/ml > 3 mg/ml > 2 mg/ml > 1 mg/ml	For the alcoholic extract.
1 mg/ml > 0.8 mg/ml > 0.6 mg/ml ≥ 0.4 mg/ml	For the total oils extract.

Among various concentrations for 3 type extracts

In vivo effects of alcoholic and oils Eucalyptus Camaldulensis

A- On footpad thickness:

The effect of alcoholic and oils of Eucalyptus on footpad thickness during one month is illustrated in Table 4. Statistically, there are significant differences ($P<0.05$) between 2 groups (infected treated and infected untreated control).

Table 4: The effects of plant extracts on mice footpad thickness

Weeks after infection	***Eucalyptus Camaldulensis* (alcoholic)**	***Eucalyptus Camaldulensis* (oils)**	**Control**
1	3.575 ± 0.17	3.475 ± 0.095	4.175 ± 0.095
2	3.35 ± 0.129	3.125 ± 0.125	4.3 ± 0.115
3	2.975 ± 0.125	2.875 ± 0.262	4.575 ± 0.17
4	2.675 ± 0.17	2.125 ± 0.095	4.75 ± 0.129

Data are presented as (mean ± SD)

B- on the mice tail base ulcer:

Clinically

Treated groups have improvement signs for lesions after 30 days post treatment with plant extracts as topical applications, while in control group the ulceration was worsening Table (5). The clinical signs of treated mice with alcoholic and oils of Eucalyptus extract show in Figure 4.

Parasitology

Microscopical examination of stained smear show positive results in 2 groups animals (infected treated and infected untreated control). Table (6), illustrates the density of parasites in 2 groups before and after treatment with Eucalyptus extracts.

Table 5: Ulcer's diameter in control and infected treated groups before and after treatment for 30 days with *Eucalyptus Camaldulensis*

	Ulcer diameter (mm)			
	Infected treated group		Control group	
	Two months post-infection	One month post-treatment	Two months post-infection	One month without treatment
E. camaldulensis (alcoholic)	5 x 11	3.5 x 9	5 x 11	11 x 15
E. camaldulensis (oils)	7 x 10	4 x 5	7 x 10	15 x 15

Table 6: The density of *L. major* amastigotes in stained smears from the margin of ulcers before and after treatment with *E. camaldulensis*

	Before treatment (20 fields were scanned)	After treatment (50 fields were scanned)
Eucalyptus camaldulensis (alcoholic)	100-200/ field	4
Eucalyptus camaldulensis (oils)	100-200/ field	1

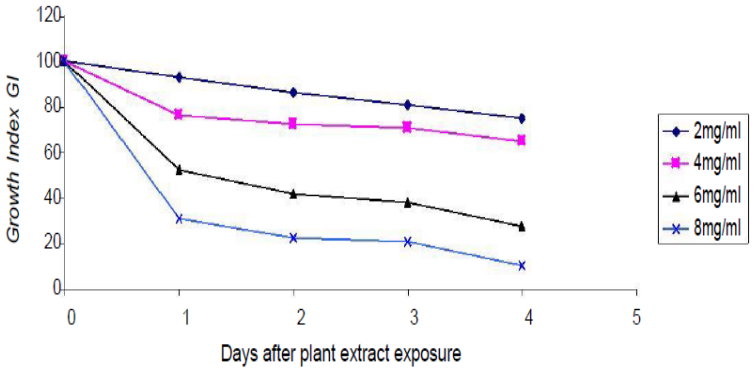

Figure 1: The effect of aqueous extractof *Eucalyptus Camaldulensis* on the growth of *L. major* promastigote

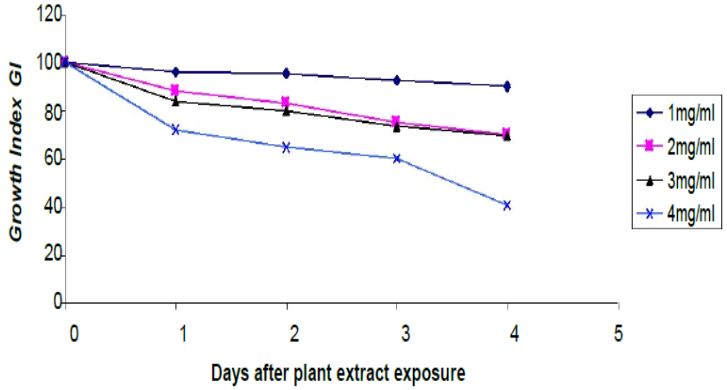

Figure 2: The effect of alcoholic extract of *Eucalyptus camaldulensis* on the growth of *L. major* promastigote

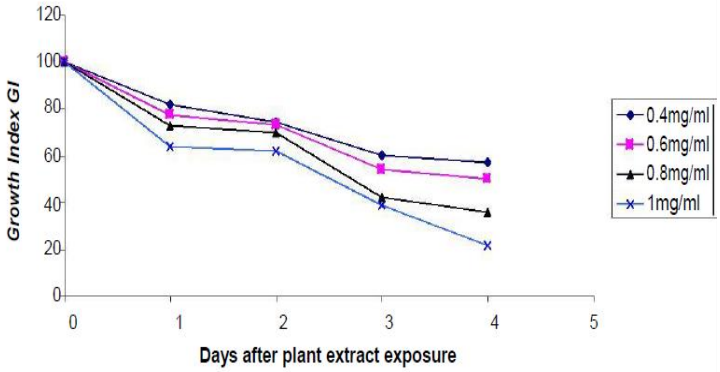

Figure 3: The effect of total oils of *Eucalyptus camaldulensis* on the growth of *L. major* promatigote

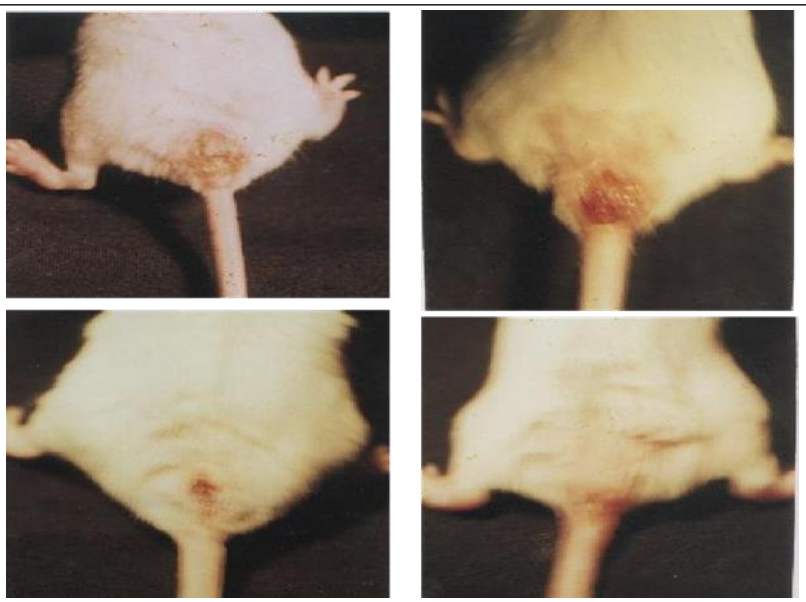

Figure 4: Mouse with CL lesion before treatment (above) and after 30 days of treatment with ointment of *Eucalyptus Camaldulensis*, alcoholic extract (below) and total oils (below)

DISCUSSION

In the present study Eucalyptus leaves extract was shown to have highly antileishmanial activity against promastigote forms of *L. major in vitro*. The percentage growth index (GI)% is depended on the increasing the applied concentrations. Previous studies in Basrah indicated that aqueous extract of *E. Camaldulensis* leaves have an antiprotozoal effect against urogenital flagellate *Trichomonas vaginals* [11] and anti-primary scolex of *Echinococcus granulosis* [13]. A high rate growth inhibition of *L. major* promastigote was obtained by oils of Eucalyptus with different concentration which is more than alcoholic and aqueous extracts. Moreover, alcoholic extract was more active than aqueous extract, thus, the LD_{50} for oils, alcoholic and aqueous extract was (0.602, 3.630 and 4.786) mg/ml respectively. The antileishmanial effect may be due to inhibition of glucose phosphate isomerase enzyme (GPI). Al-Dulaimi, 2002 [25] demonstrated that the leaves extract of *Mytrus comminus* has great effects on (GPI) enzyme of promastigote *L. tropica*, *L. major* and *L. donovani*.

The *in vitro* study has shown that alcoholic extract of Eucalyptus was more active than aqueous extract against dermatophytes [26]. The alcoholic extract of *Trigonellafoenum-graecum* seeds shows high activity towards *L. major* promastigotes than aqueous extract at different concentrations compared to the control [27]. The high activity of alcoholic extract than aqueous extract in inhibition of (GI)% of *L. major* promastigote may be related to the nature of the active compounds (volatile oils) and also the solvents used in the extraction. Oils are arise non-polar compound, not easy to be dissolved in water but it is dissolved in nonpolar organic solvent such as ethanol [28]. Rai et al., [29]. 1999 reported a moderate inhibitory effects of essential oils of six species of the genus Eucalyptus which is markedly inhibited fungal growth. *In vivo* study, topical application of alcoholic and oils extracts of Eucalyptus prevented lesion development in mice footpads infected with *L. major*. Slight footpad thickness was observed in treated mice, compared with control mice.

Statistically, there are significant differences ($P<0.05$) noticed in the mean footpad thickness between alcoholic and oils extracts. Similarity results were noticed in other study with used Licochalcone (flavonoids isolated from the root of Chinese Licorice) on *L. major* [30]. The clinical examination of the infected treated mice showed high improvement and reduction in size of ulcer after treated with each of alcoholic and oils Eucalyptus extract. In Iran, 86 patients suffering from cutaneous leishmaniasis were treated with the topical herbal extracts (named Z-HE) as a black paste, showing complete healing 6-weeks post treatment [31].

Highly differences were found between the density of *Leishmania* amastigote in cutaneous smear in infected control and infected treated mice. In other experimental studies, it was found that oils of Eucalyptus were effective against 22 bacterial strains, and fungi among 10 essential oils of 10 plants [32]. Other study by Shahi, et al., [33] demonstrated the activity of essential oils of Eucalyptus leaves by topical ointment against skin fungal disease.

The mechanism of action for Eucalyptus plant extracts against *L. major* is not known. The plant Licochalcone A, effect on growth and activity of *L. major, L. donovani* promastigotes and amastigotes *in vitro*. The mode of action for this plant was on parasite mitochondria [34]. Phenolic substances and other compounds such as cineole, eucalyptol and, β pinene showed high activity against growth of microorganisms [28]. It was found that phenolic substances have an enzymatic effects, especially on actyl-choline esterase [35] which is important in physiological actions forming organism. The change in flexibility of cell membrane may be attributed to the effects of this enzyme [36,37] thus the parasite loses its ability in allowing movement of different material in and out of the cell causing cellular death.

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