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Journal of Chemical and Pharmaceutical Research, 2015, 7(6):972-978

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

In vitro leishmanicidal of *Euphorbia microsciadia* Bioss against promastigotes of Leishmania major

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ABSTRACT

There is a great tendency toward the consumption of traditional plants in the cases of leishmaniases. The aim of this study was to evaluate the leishmanicidal effects of different extracts of Euphorbia microsciadia Bioss on promastigotes of Leishmania major in vitro. Arterial parts of the plant were dried and extracted with methanol or macerated in ethanol. Five different concentrations of each extract (1, 0.5, 0.25, 0.125, 0.0625 mg.mL^{-1}), amphotericin B (0.5 mg.mL^{-1}) (positive control) and culture medium (negative control) were placed in 24-well plates containing 40,000 parasites/well. The plates were incubated for six consecutive days at 25˚C and the number of the parasites in each well was determined microscopically on the days 2, 4 and 6 of the experimental procedure. Amphotericin B at the specified concentration killed all the parasites. A concentration-dependent lethal toxicity was observed for different doses of the extracts. Exposure of promastigotes to 1 mg.mL^{-1} of both extracts exhibited the most leishmanicidal activity. The EC_{50} values for the methanol (Soxhlet) and ethanol (macerated) extracts were determined (0.078 - 0.331 mg.mL^{-1}) four days after incubation. Our results indicate that both extracts of E. microsciadia Bioss exhibit favorable lethal toxicity against promastigote of L. major and may be suitable candidates for further research in future leishmanicidal studies.

Keywords: *Euphorbia microsciadia*, leishmaniases, lethal toxicity, Soxhlet extract

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INTRODUCTION

Leishmaniases are the prevalent tropical diseases arising from the infectious of *Leishmania* parasites. More than two million new cases of leishmaniases occur annually worldwide which lead to a wide spectrum of morbidity and mortality. It has been reported that this disease is endemic in 98 countries throughout the world [1]. Visceral, mucocutaneous and cutaneous leishmaniases are three main clinical forms of the disease [2]. Amongst these, cutaneous manifestations are the most prevalent form of leishmaniasis, whereas, the sever form that parasites migrate to the vital organs, is considered to be the visceral leishmaniasis. More than 90% of reported cases from these two forms of disease occur in 11 countries including Bangladesh, Nepal, Sudan, India, Syria, Iraq, Algeria, Afghanistan, Brazil, Saudi Arabia and Iran [1].

The genus *Leishmania* (Kinetoplastida: Trypanosomatidae) which is responsible for the disease is a protozoan parasite. Two distinct developmental stages have been identified for the parasite life cycle. The form that develops within sand flies (*Phleobotomus* spp.) vector is a known extracellular promastigote and transmitted by the fly during biting. In the vertebrate host the promastigotes are internalized by phagocytes and develop into intracellular amastigotes [3].

Pentavalent antimonial compounds have been used since the 1940s as the drugs of choice for treatment of leishmaniasis [4, 5]. The treatment can be also conducted by using pentamidine, amphotericin B, paramomycin and miltefosine as second-choice drugs depending on the species clinical form of *Leishmania* [6]. Consumption of these medications leads to serious side effects including cardiac and renal failure as well as pancreatic abnormalities. Other disadvantages of these drugs are a requirement for long treatment time, resistance of the parasites to the drugs and inconsistence of the medication efficacy [6, 7]. In view of the present clinical scenario the investigation for developing new drugs that are safe, efficacious and more accessible to patients are necessitated. Traditional medicines, as a source of chemotherapeutic compounds, were found to be effective against a wide spectrum of diseases such as leishmaniasis.

Euphorbia is the largest genus of the family Euphorbiaceae with over 2000 species worldwide [8]. This genus of plant has been used as a folk medicine in treatment of gout and back pain in Iran [9]. Besides, pharmacological studies have shown that the members of this genus are effective against intestinal parasites, bacterial infections, gonorrhea and asthma [10, 11]. *E. microsciadia* is one of the species of *Euphorbia* genus which grows in Iran and is used in folk medicine for different purposes. Salmasi et al (2011) has shown that *E. microsciadia* extracts have favorable antiviral activities [12]. In another study, the flavonoids which isolated from aerial parts of *Euphorbia microsciadia* have been shown to have immune-modulatory activities. This effect was manifested by lymphocyte suppression activity of the flavonoids [13].

The aim of this study was to assess the leishmanicidal effects of ethanol and methanol soxhlet extracts of *E. microsciadia* Bioss on promastigotes of *L. major*.

EXPERIMENTAL SECTION

E. microsciadia Bioss was collected from Mashhad suburbs (Khorasan Razavi Province, Iran), identified by in the Herbarium of Ferdowsi University (Mashhad, Iran) and a voucher sample was preserved as reference in the Herbarium of Mashhad Pharmacy School (Iran) with reference number 108-0513-3. Aerial parts of plant were cleaned and dried in the dark at 25-30°C. The dried parts were then powdered by a mechanical grinder.

Preparation of *E. microsciadia* Bioss extract

Soxhlet methanol extract

The plant powder (50 g) was subjected to extraction in methanol (200 ml) for 8 h using Soxhlet apparatus. The methanol was subsequently removed through heating in *vacuo* and dried. The extract was then kept in 4˚C until use.

Macerated ethanol extract

Powdered plant (100 g) was macerated in 1000 mL ethanol (80%, v/v) for three consecutive days, then filtered and concentrated under *vacuo* at 40˚C to remove the ethanol content. The extract was then kept at 4˚C until use.

Lieshmania parasites

BALB/c female mice were used to maintain the *Lieshmania major* strain MRHO/IR/75/ER. The amastigotes were separated from the infected lesions of the mice and transformed to promastigotes by culturing in NNN and then RPMI 1640 medium containing 2 mM glutamine, 10% (v/v) heat inactivated FCS, 100 mg mL^{-1} streptomycin sulfate and 100 U mL^{-1} of penicillin at 25˚C.

Leishmanicidal activity assay

Leishmanicidal activity of the extract was performed according the method of Rahman A-U *et al*. (2001) [14]. Briefly, *L. major* promastigotes in stationary phase were seeded at 40,000 parasites/400 µL/well in a 24-well plate containing RPMI-FCS. The plant extract was dissolved either in DMSO or in ethanol to give final concentrations of 1 $mg.mL^{-1}$. A serial dilution of the extracts (0.5, 0.25, 0.125, 0.0625 $mg.mL^{-1}$) was also prepared. The promastigotes were incubated for six consecutive days at 25˚C with different concentrations of extracts and the numbers of the

parasites in each well were counted on the days two, four and six of the experimental procedure using Neubauer chamber under a microscope. Amphotericin B (0.5 mg. mL $^{-1}$) was used as positive control for leishmanicidal activity, DMSO and ethanol were used as solvent controls. The medium (RPMI-FCS) was also employed as negative control.

Statistical analysis

The experimental results are presented as mean ± standard error (SEM). Following the assurance of normal distribution of data, One-Way analysis of variance (ANOVA) with the Tukey-Kalmer *post hoc* test was done in SPSS (17.0). The EC_{50} was determined by Litchfield and Wilcoxon method.

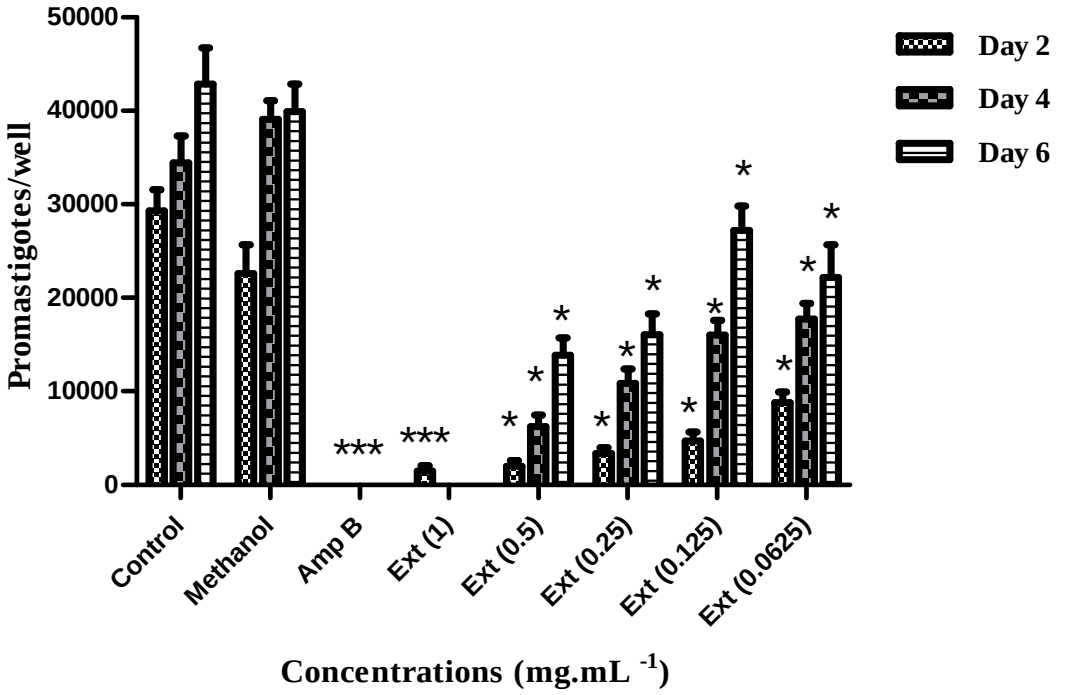

Fig. 1: The effect of different concentrations of *E. microsciadia* Bioss Soxhlet extract in methanol against *L. major* promastigotes after 2, 4 and 6 days incubation

*Each bar represents the mean±SEM of the numbers of promastigotes in 24 wells. Amp B: Aamphotericin B, Ext: Extract. * p<0.001, Tukey-kramer test*

RESULTS AND DISCUSSION

Cutaneous leishmaniasis is the second most prevalent vector-borne infection after malaria in Iran [15]. The occurrence of this infectious disease has been gradually increasing over the past decade and about 30,000 leishmaniasis cases have been reported in 2011 from Iran [16]. According to the fact that injection therapy is painful and somewhat ineffective, there is a great tendency toward the consumption of traditional plants for treatment of wounds. Therefore, several plants have been studied as leishmanicidal agents [17-19]. Local people from different parts of Iran have successfully used *Euphorbia* genus for treatment of cutaneous leishmaniasis. Some studies were also done to evaluate the efficacy of *Euphorbia* against leishmania infection [20, 21]. Jaafari *et al*. (2006) showed that *Euphorbia bungei* extracts had favorable leishmanicidal activity [22]. The focus of this study was to verify the *in vitro* leishmanicidal effects of ethanol macerated and methanol Soxhlet extracts of *E. microsciadia* Bioss on promastigote of *L. major*.

Our results showed that the final yield of the soxhlet methanol extract of *E. microsciadia* was 16% (w/w), whereas, macerated ethanol extract yielded 12% (w/w).

Leishmanicidal activity of the extract of *E. microsciadia* in methanol was evaluated two, four and six days after incubation (Fig. 1). The results indicated that methanol as a solvent control did not kill *L. major* promastigotes compared to negative control ($P>0.05$). However, a dramatic lethal toxicity was occurred due to the methanol extract on the test days ($P<0.001$). Table 1 indicates the LC_{50} of the extract in methanol four days after incubation.

Table 1: leishmanicidal activity of *E. microsciadia* Bioss extracts [LC_{50} (mg.mL^{-1})] against *L. major* promastigotes after 4 days incubation)

Extracts	LC50 (mg.mL-1)
Soxhlet methanol extract in DMSO	0.175
Soxhlet methanol extract in methanol	0.078
Ethanol extract in DMSO	0.331
Ethanol extract in methanol	0.215

As shown in Fig. 2, parasites were completely killed by amphotericin B (0.5 mg.mL^{-1}) during the test days whereas, DMSO treatment did not have lethal effects on *L. major* promastigotes ($P>0.05$). A concentrate-dependent lethal toxicity on parasites was occurred as the result of 1, 0.5 and 0.25 mg mL^{-1} of methanol extract ($P<0.001$), while no significant larvicidal activity was observed when the 0.125 and 0.0625 mg mL^{-1} concentrations of extract were used (on the second day of incubation). Likewise, the leishmanicidal activity of extract was significantly increased on the days 4 and 6 after incubation in comparison with the second day ($P<0.05$). The LC_{50} of the extract in DMSO was 0.175 mg mL^{-1} after 4 days incubation (Table 1).

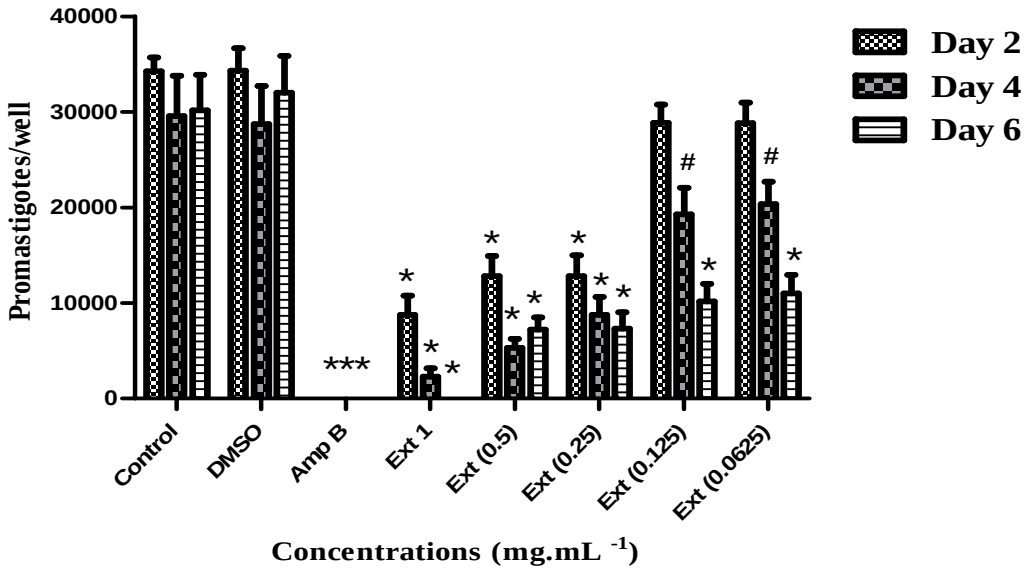

Fig. 2: The effect of different concentrations of *E. microsciadia* Bioss Soxhlet extract in DMSO against *L. major* promastigotes after 2, 4 and 6 days incubation

*Each bar represents the mean±SEM of the numbers of promastigotes in 24 wells. Amp B: Aamphotericin B, Ext: Extract. # p<0.05, * p<0.001, Tukey-kramer test*

The data obtained form leishmanicidal evaluation of *E. microsciadia* ethanol extract in methanol revealed that the extract (1, 0.5 and 0.25 mg.mL^{-1}) significantly killed the promastigotes during the test days (Figure 3). The LC_{50} of this extract was 0.215 mg mL^{-1} after 4 days exposure (Table 1).

Changes in survival rate of parasites exposed to *E. microsciadia* ethanol extract revealed that the concentrate-dependent lethal toxicity was occurred at the second day after incubation with extract (Figure 4). The concentrations of 0.125 and 0.0625 mg.mL^{-1} of the extract did not show any lethal toxicity against the promastigotes on the days 4 and 6 of incubation ($P>0.05$). As indicated in Table 1, the calculated LC_{50} of the ethanol extract at the fourth day was 0.331 mg. mL^{-1}.

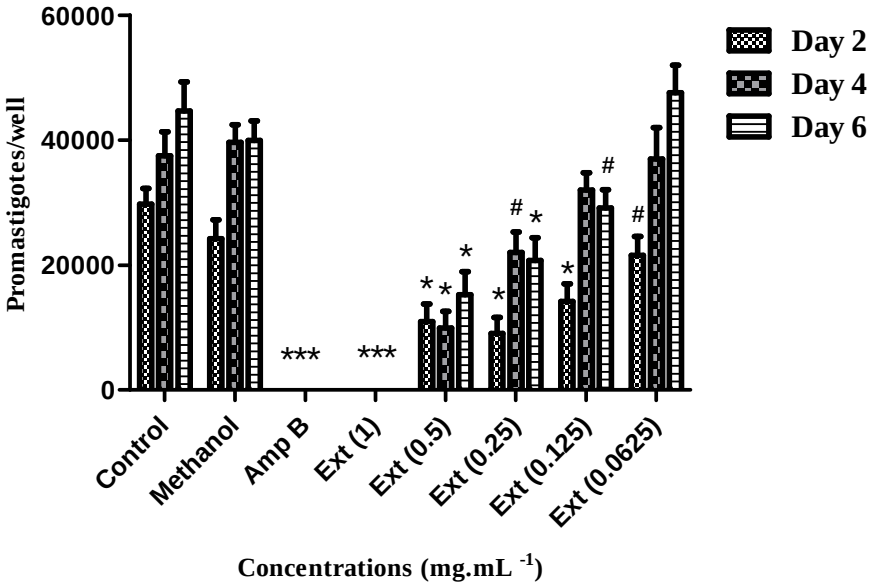

Fig. 3: The effect of different concentrations of *E. microsciadia* Bioss macerated extract in methanol against *L. major* promastigotes after 2, 4 and 6 days incubation

*Each bar represents the mean±SEM of the numbers of promastigotes in 24 wells. Amp B: Aamphotericin B, Ext: Extract. # p<0.05, * p<0.001, Tukey-kramer test.*

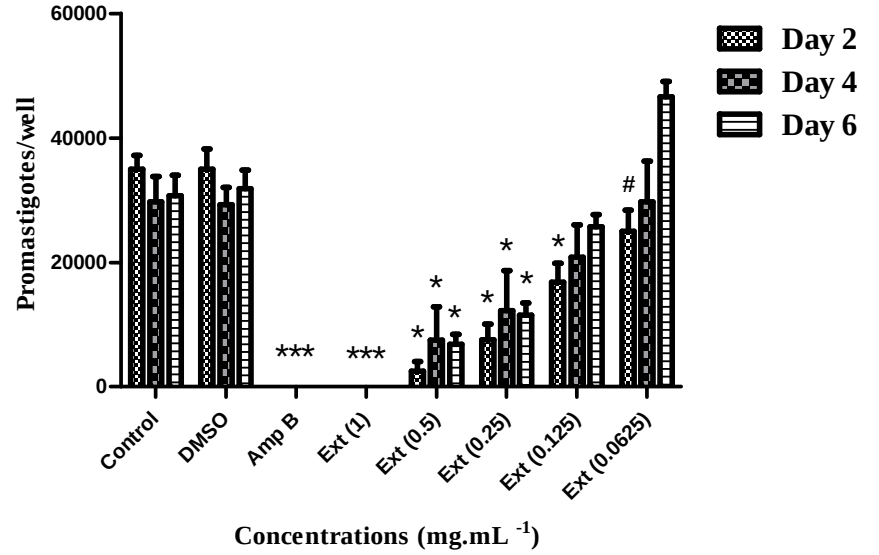

Fig. 4: The effect of different concentrations of *E. microsciadia* Bioss macerated extract in DMSO against *L. major* promastigotes after 2, 4 and 6 days incubation

*Each bar represents the mean±SEM of the numbers of promastigotes in 24 wells. Amp B: Aamphotericin B, Ext: Extract. # p<0.05, * p<0.001, Tukey-kramer test.*

Overall, our results indicated that all concentrations of methanol extract in methanol have lethal toxicity against the promastigotes, whereas the lower concentrations of the extract in DMSO did not have lethal effects against parasites on the 2nd day of experiment. The data indicate that the toxicity of extract in methanol against the parasites is higher than the extract in DMSO. Moreover, the comparison of EC_{50} value for the methanol and ethanol extract showed that stronger toxicity occurred by methanol extracts (Table 1). It seems that the Soxhlet extraction method is more efficient compared to the maceration technique for extraction of the plant active constituents which have

leishmanicidal properties. Furthermore, the calculated EC_{50} of both macerated and Soxhlet extracts were in the ranges of 0.078 - 0.331 mg mL^{-1} that could be considered as a moderate anti-leishmania activity.

The members of Euphorbiaceae family are famous for chemical diversity of their isoprenoid constituents [23].The chemical compounds of plants in this family include flavonoids, diterpenoids, sesquiterpenoids, triterpenes, phloracetophenones, glycerols, cerebrosides and steroids [24]. Although the chemical composition of *E. microsciadia* is not well studied, but some types of diterpenes and triterpenes were isolated from this species. Ayatollahi et al (2010) isolated pentacyclic triterpenes such as betulinic acid, oleanolic acid and ursolic acid from this plant [25]. Two diterpenoids which were structurally related to cyclomyrsinols also isolated from the *E. microsciadia* [26]. The leishmanicidal activity of diterpenoids and triterpenoid as the constituents of various plants has been shown by several investigations [27-29]. Therefore, with respect to previous studies, the leishmanicidal activity of the plant can be attributed to the above mentioned chemical constituents.

CONCLUSION

The results obtained indicate that both Soxhlet and macerated extracts of *E. microsciadia* Bioss exhibit a significant concentration-dependent lethal toxicity against promastigote of *L. major*. This lethal toxicity can be attributed to its constituent especially di- and triterpenoids.

Acknowledgements
The authors are thankful to the Vice Chancellor of Research, Mashhad University of Medical Sciences for financial support.

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