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## Evaluation of Leishmanicidal Effect of *Euphorbia bungei* Boiss Extract by *in vitro* Leishmanicidal Assay Using Promastigotes of *Leishmania major*

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**Abstract:** In this study, the leishmanicidal effect of different extract of *Euphorbia bungei* Boiss aerial part was evaluated on promastigotes of *Leishmania major in vitro*. Therefore, dried and ground aerial part of the plant was extracted using either maceration in 80% ethanol or Soxhlet in methanol. Then, 5 different concentrations of each extract, one positive control, one negative control and one solvent control were prepared and were placed in a 24-well plate containing 40,000 parasites/well. The extract concentrations were 0.06, 0.12, 0.25, 0.5 and 1 mg mL<sup>-1</sup>. Amphotricin B (0.5 mg mL<sup>-1</sup>) was used as positive control while negative control contained only culture medium. The plate was incubated at 25°C for six days and the amount of parasites in each well was determined on days 2, 4 and 6 of experiment microscopically using Neubar chamber. It was observed that amphotricin B and both macerated and soxhlet extracts at concentration of 1 mg mL<sup>-1</sup> killed all parasites. Lower doses exhibited a dose-dependent leishmanicidal activity. The EC<sub>50</sub> for macerated and soxhlet extracts in DMSO was between 0.5 and 0.25 mg mL<sup>-1</sup>. The control solvents had no significant effect on the *L. major* promastigotes. These results indicated that both macerated and soxhlet extracts of *E. bungei* Boiss have favorable leishmanicidal activity.

**Key words:** *Euphorbia bungei* Boiss, leishmanicidal activity, *Leishmania major*, promastigotes

### INTRODUCTION

Leishmaniasis is a protozoal disease of man that occurs in most parts of the world and according to the World Health Organization (WHO) while 12 million people are infected by the parasites, 350 million people are living at risk of infection with *Leishmania* parasite (Ashford *et al.*, 1992). The annual incidence of new cases is about 2 million from which 1.5 million have cutaneous leishmaniasis (CL) (Moddaber, 1993). Cutaneous leishmaniasis (CL), which caused by the different species of *Leishmania*, produces a skin ulcer that heals spontaneously in most cases, leaving an unsightly scar. Treatment of leishmaniasis is based on antimony compounds which have unpleasant side-effects and sometimes is not effective (UNDP/WB/WHO, 1989). The effects of available topical leishmanicidal products are minimal. Therefore, there is a great and urgent need for the development of new, effective and safe drugs for the treatment of leishmaniasis (McGregor, 1998). One strategy to discover new drug leads is to investigate natural products from medicinally used plants. Most people in areas where leishmaniasis is endemic depend largely on

traditional medicine. *Euphorbia* species have been successfully used for the treatment of CL by local people in Mashhad suburb, Iran where growing number of cases are reported recently. To provide a scientific reason for ethnomedicinal use of *E. bungei* Boiss, in this study the leishmanicidal effect of ethanolic macerated and methanolic soxhlet extracts of *Euphorbia bungei* Boiss was evaluated on promastigotes of *L. major in vitro*.

### MATERIALS AND METHODS

**Plant material:** *Euphorbia bungei* Boiss was collected from near Mashhad (Khorasan Province, Iran). The aerial part of the plant was dried in shade and powdered. It was identified in the Herbarium of Ferdowsi University and voucher samples were preserved for reference at the Herbarium of the Mashhad School of Pharmacy with reference number of (189).

#### Preparation of extract

**Soxhlet methanolic extract:** The plant powder (50 g) was extracted with methanol (200 mL) for 12 h using Soxhlet apparatus. The methanol was removed under

reduced pressure and dried. The extract was kept in refrigerator until use.

**Macerated ethanolic extract:** The powdered plant (100 g) was extracted with ethanol (1000 mL, 80%, v/v) by maceration. The extract was collected every 24 h for three days. The combined extracts were dried under reduced pressure. The dried extract was kept in refrigerator for further testing.

**Leishmania parasites:** *Leishmania major* strain MRHO/IR/75/ER was maintained with passage in BALB/c female mice. The amastigotes were isolated from the lesions of infected BALB/c mice and transformed to promastigotes on NNN medium then subcultured in RPMI 1640 (Sigma) containing 10% v/v heat inactivated FCS, 2 mM glutamine, 100 U mL<sup>-1</sup> of penicillin and 100 mg mL<sup>-1</sup> of streptomycin sulfate (RPMI-FCS) at 25°C. Leishmanicidal assays were conducted using stationary-phase promastigotes.

**Assay for Leishmanicidal activity:** The assay was performed according to Atta-ur Rahaman *et al.* (2001). Briefly, *L. major* promastigotes in stationary phase were seeded at 40,000 parasite/400 µL/well in 24-well plate in RPMI-FCS. The extracts were dissolved in DMSO and added further 400 µL/well to give final concentrations of 1 mg mL<sup>-1</sup> and serial two-fold dilutions thereof. Promastigotes were incubated over a period of 6 days at 25°C and the amount of the parasites in each well determined on days 2, 4 and 6 of experiment using Neubauer chamber under a microscope. Amphotericin B (0.5 mg mL<sup>-1</sup>) was used as positive control, culture media was used as negative control and DMSO alone was used as solvent control.

**Statistical analysis:** Statistical analysis was carried out using one-way ANOVA and multiple comparison Tukey-Kramer test was used to compare the means of different treatment groups. The EC<sub>50</sub> was determined by Litchfield and Wilcoxon method.

**RESULTS**

**Leishmanicidal activity of macerated ethanolic extract of *Euphorbia bungei* Boiss in DMSO:** Amphotericin B (0.5 mg mL<sup>-1</sup>) and macerated ethanolic extract of *Euphorbia bungei* Boiss (1 mg mL<sup>-1</sup>) in DMSO killed all of the *L. major* promastigotes (Fig. 1-3) and lower doses of macerated ethanolic extract of *Euphorbia bungei* Boiss in DMSO killed *L. major* promastigotes dose-dependently

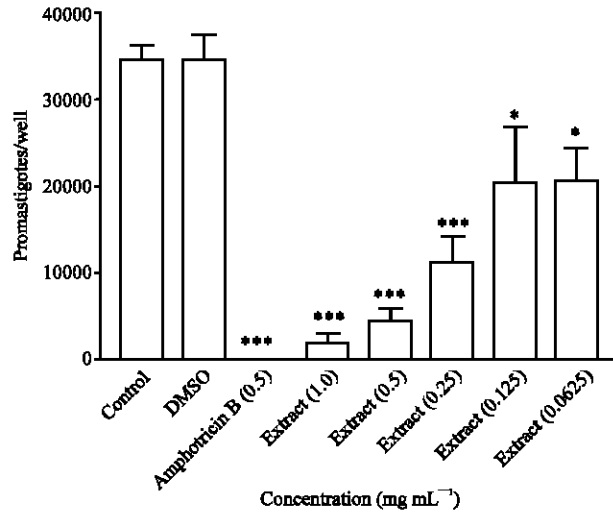


Fig. 1: Effect of different concentration of *E. bungei* Boiss macerated extract against *L. major* promastigotes after 2 days of incubation. Each bar represents the mean±SEM of the number of promastigotes in 12 wells. \*p<0.005, \*\*\*p<0.001, Turkey-Kramer test

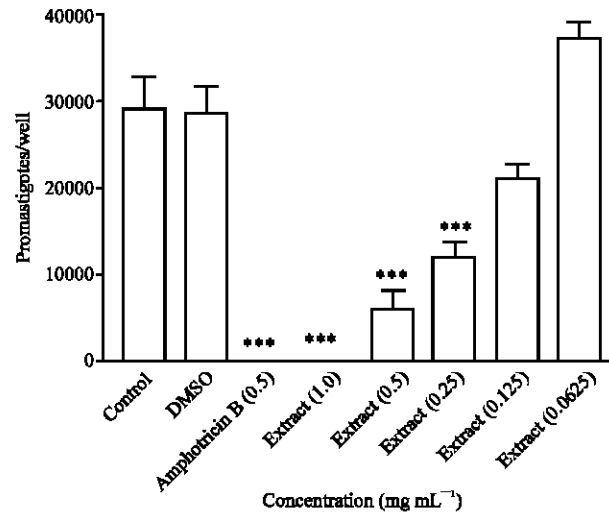


Fig. 2: Effect of different concentration of *E. bungei* Boiss macerated extract against *L. major* promastigotes after 4 days of incubation. Each bar represents the mean±SEM of the number of promastigotes in 12 wells. \*\*\*p<0.001, Turkey-Kramer test

while DMSO did not have any effect on the *L. major* promastigotes. The EC<sub>50</sub> for macerated ethanolic extract of *Euphorbia bungei* Boiss in DMSO was 0.112 mg mL<sup>-1</sup> after 2 days of incubation. The leishmanicidal activity was lower after 6 days of incubation although it was not significantly different from days 2 and 4.

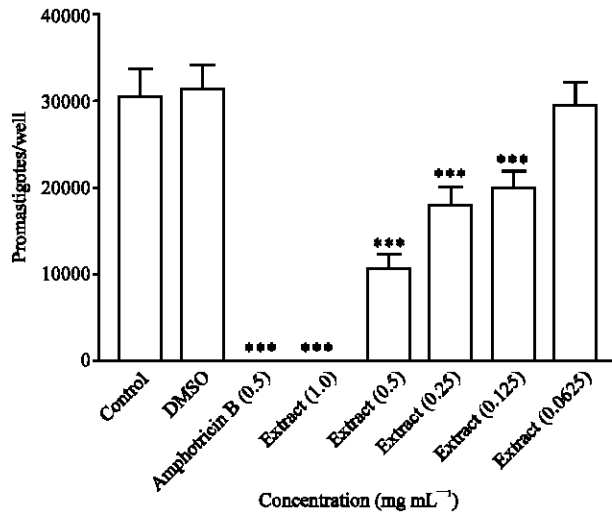


Fig. 3: Effect of different concentration of *E. bungei* Boiss macerated extract against *L. major* promastigotes after 6 days of incubation. Each bar represents the mean±SEM of the number of promastigotes in 12 wells. \*\*\*p<0.001, Turkey-Kramer test

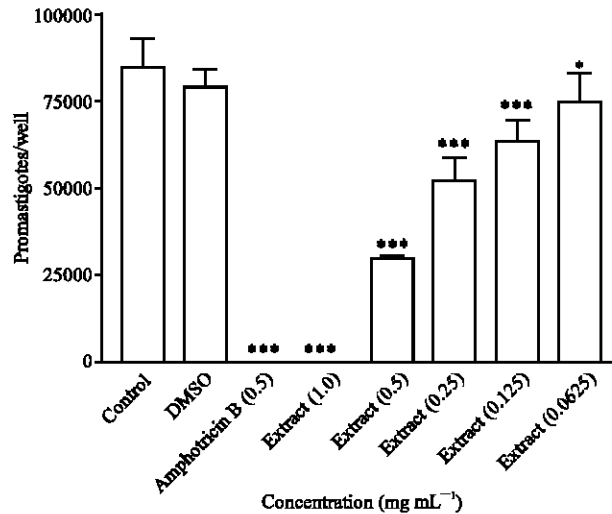


Fig. 4: Effect of different concentration of *E. bungei* Boiss soxhlet extract against *L. major* promastigotes after 2 days of incubation. Each bar represents the mean±SEM of the number of promastigotes in 12 wells. \*p<0.05, \*\*\*p<0.001, Turkey-Kramer test

**Antileishmanial activity of soxhlet methanolic extract of *Euphorbia bungei* Boiss in DMSO:** Different concentrations of soxhlet methanolic extract of *Euphorbia bungei* Boiss in DMSO killed parasites dose-dependently (Fig. 4-6). The EC<sub>50</sub> for soxhlet methanolic extract of *Euphorbia bungei* Boiss in DMSO was 0.137 mg mL<sup>-1</sup> after 2 days of incubation. DMSO did not have any effect on the *L. major* promastigotes.

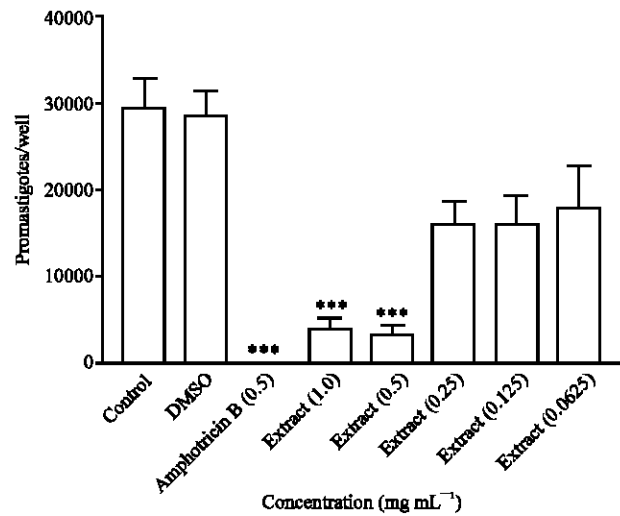


Fig. 5: Effect of different concentration of *E. bungei* Boiss soxhlet extract against *L. major* promastigotes after 4 days of incubation. Each bar represents the mean±SEM of the number of promastigotes in 12 wells. \*\*\*p<0.001, Turkey-Kramer test

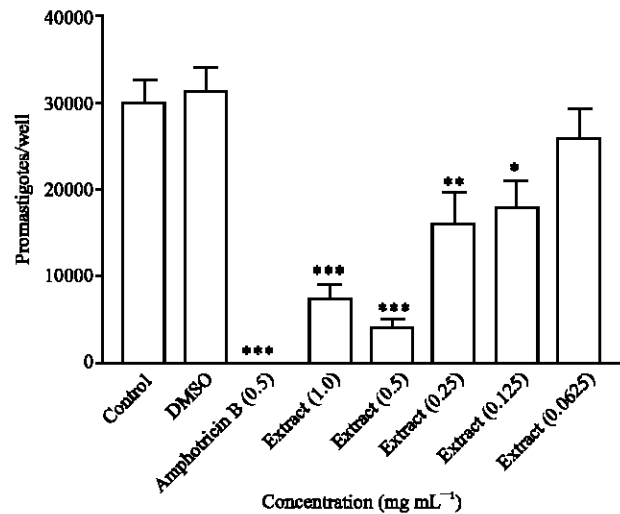


Fig. 6: Effect of different concentration of *E. bungei* Boiss soxhlet extract against *L. major* promastigotes after 6 days of incubation. Each bar represents the mean±SEM of the number of promastigotes in 12 wells. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, Turkey-Kramer test

## DISCUSSION

People customarily use the plant(s)/plant-derived preparations and consider them to be efficacious against cutaneous leishmaniasis without any scientific base to explain the action of such plants. Since cutaneous

leishmaniasis has become one of the major health issue in mashhad, a city located in north east of Iran and chemotherapy is somewhat ineffective and painful, people are using medicinal plants sold on the local market as a remedy to cure their wounds. The latex of *E. bungei* Boiss is one the remedies used topically to treat the cutaneous leishmaniasis. The current study was therefore carried out on promastigotes to evaluate its acclaimed efficacy using an *in vitro* assay based toxicity. Both macerated and Soxhlet extracts of the aerial part of *E. bungei* Boiss were prepared and tested against promastigotes of *Leishmania major*. All tested concentrations of both extracts exhibited leishmanicidal activity after 2, 4 and 6 days of incubation. Although the number of live promastigotes after 6 days of incubation was lower than those of 2 and 4 days of incubation, but no significant differences between these groups was observed. Since no significant difference between macerated and soxhlet extracts was observed, it could be suggested that the active constituents of the extracts were not thermally labile. The  $EC_{50}$  of both macerated and soxhlet extracts were in the range of 112-178  $\mu\text{g mL}^{-1}$  which could be considered as a moderate leishmanicidal activity. Although normally compounds with  $EC_{50}$  higher than 25  $\mu\text{M}$  is considered inactive and concentrations obtained in this study are not typically low, however the active leishmanicidal components may be present in very low concentration (Salehhen *et al.*, 2004) warranting the need for purification of active constituents of *E. bungei* Boiss. The phytochemical screening has shown the presence of alkaloids, saponins, tannins and flavonoids where flavonoids and tannins were the major constituents (Fazly Bazzaz *et al.*, 1997). Several reports have indicated alkaloids (Kam *et al.*, 1999; Mohanmad *et al.*, 2003), chalcones (Kayser and Kiderlen, 2001 and Liu *et al.*, 2003), di- and triterpenoids (Tan *et al.*, 2002), saponins (Ridoux *et al.*, 2001) and polyphenols (Kolodziej *et al.*, 2001) as the leishmanicidal constituents of some plants. With respect to preliminary phytochemical screening which indicates the presence of flavonoids, alkaloids and saponins (Fazly Bazzaz *et al.*, 1997), the leishmanicidal activity could be attributed to one these classes of compounds. Further fractionation of the *E. bungei* Boiss is required to pinpoint the leishmanicidal constituent(s).

### CONCLUSION

These results indicate that the ethanolic macerated and methanolic soxhlet extracts of *Euphorbia bungei*

Boiss have favorable leishmanicidal activity and kill the *L. major* promastigotes in a dose-dependent manner.

### ACKNOWLEDGMENT

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