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Effect of *Vigna radiata, Tamarix ramosissima* and *Carthamus lanatus* extracts on *Leishmania major* and *Leishmania tropica*: An *in vitro* study

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ABSTRACT

Objective: Current therapy strategies of leishmaniasis have some problems such as high cost, toxicity and side effects. Plant extracts can be a source of drugs to control leishmaniasis. In this study, the effect of hydroalcoholic and chloroformic extracts of *Vigna radiata, Tamarix ramosissima*, and *Carthamus lanatus* on *Leishmania major* and *L. tropica* was studied.

Methods: The plant samples were collected from west of Iran and their extracts were prepared. Anti-promastigote activity assay of all extracts was done using tetrazolium-dye assay.

Results: Only high concentrations of *V. radiata* and *C. lanatus* were able to inhibit *Leishmania*, while both high and low concentrations of *T. ramosissima* had antileishmanial effect. No difference was observed between hydroalcoholic with chloroformic extract of each plant.

Conclusion: Altogether, the results revealed the antileishmanial activity of *T. ramosissima* extracts against *L. major* and *L. tropica*, indicating its potential as an antileishmanial agent.

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1. Introduction

Cutaneous leishmaniasis (CL) is a disease caused by several *Leishmania* species. Annually, CL affects numerous populations around the world mainly in underdeveloped and developing countries. Incidence of CL is around 1.5 million cases per year (World Health, 2010). CL in Middle East is mainly caused by *Leishmania major* and *Leishmania tropica* (Akya & Hamzavi, 2017; Rostamian & Niknam, 2019).

In Asia, Africa, and Europe, the first line therapy for CL is pentavalent antimonials, *i.e.*, meglumine antimoniate and sodium stibogluconate (Markle & Makhoul, 2004). However, several severe side effects have been reported for antimonials such as myalgia, cardiac arrhythmia, pancreatitis, hepatitis, and drug accumulation in liver and spleen. Therefore, there is an urgent need for novel effective and nontoxic treatments of leishmaniasis (Brooker et al., 2004; Markle & Makhoul, 2004).

Applying natural products and medicinal plants are increasing in health-related industries (Sharifi-Rad et al., 2018; Yuan, Ma, Ye & Piao, 2016). Since last decades, the most attractive source for new antileishmanial drug development has been natural products and their compounds (Rocha, Almeida, Macedo & Barbosa-Filho, 2005; Sharifi-Rad et al., 2018; Sharifi-Rad, Salehi, Sharifi-Rad, Setzer & Iriti, 2018).

Vigna radiata (Linn.) Wilczek (mung bean) is a plant that belongs to the Fabaceae family. It is known that *V. radiata* possess dietary and medicinal properties. Up to now, numerous medicinal effects of *V. radiata* have been reported including antioxidant, antimicrobial, anti-inflammatory, antidiabetic, antihypertensive, antitumor, and antisepsis effects (Tang, Dong, Ren, Li & He, 2014). Traditionally, in the border of Iran and Iraq, people put chewing *V. radiata* seeds on the CL lesions to accelerate healing. However, according to a deep survey of the literature, the effect of *V. radiata* extract on *Leishmania* species remains unexplored experimentally.

Tamarix ramosissima Lcdcb belongs to the family Tamaricaceae and is one of the oldest herbal medicines, and has been used for the treatment of several diseases. The antioxidant and antimicrobial effects of *T. ramosissima* have been reported previously (Akya, Mojarrab, Farshchian & Ahmadi, 2015; Ren et al., 2019; Sultanova et al., 2001). It is also traditionally used for healing of wounds in the west of Iran. However, there is no report on *T. ramosissima* effects for *Leishmania* species.

Carthamus lanatus L. is a species of thistle and belongs to the family Asteraceae. The anti-inflammatory, antibacterial, antifungal,

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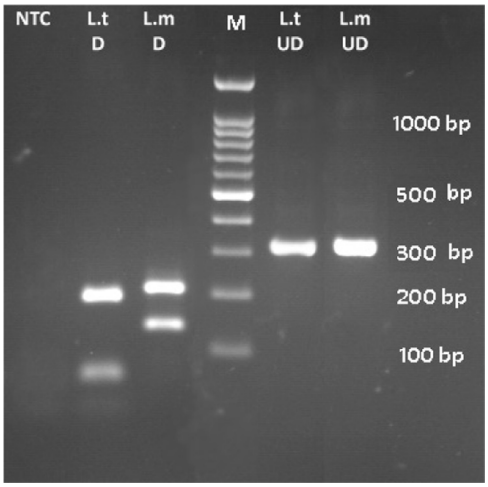

Fig. 1. Species identification of parasites by RFLP. *Note:* Amplification of ITS-1 gene of both isolates resulted in a single distinct band of about 300 bp (L.m UD and L.t UD). HaeIII-digestion of the PCR product of *L. major* and *L. tropica* isolates showed a 220 bp–150 bp (L.m D) and 200 bp–100 bp (L.t D) pattern that were concordant to *L. major* and *L. tropica*, respectively. M, DNA marker; D, digested ITS1 PCR product; UD, un-digested ITS1 PCR product; NTC: non-template control.

and analgesic effects of *C. lanatus* extracts have been previously reported (Bocheva, Mikhova, Taskova, Mitova & Duddeck, 2003; Jalil et al., 2003; Taskova, Mitova, Najdenski, Tzvetkova & Duddeck, 2002). Further, in the west of Iran, the ashes of this plant are used for treatments of CL lesions. However, there is no report on *C. lanatus* effect on *Leishmania* in the literature.

Therefore, in the present study we aimed to evaluate the *in vitro* antileishmanial potentials of *V. radiata, T. ramosissima*, and *C. lanatus* extracts against *L. major* and *L. tropica*.

2. Materials and methods

2.1. Parasites and their identification

In the present study, two *Leishmania* species were used: *L. major* (Fredline strain) and *L. tropica* (IPAS4 strain). Both parasites, which have been previously isolated from Iranian patients, were gifts from Dr. Hamid Mahmoudzadeh-Niknam (Immunology Department, Pasteur Institute of Iran, Tehran, Iran).

The frozen parasites were thawed and cultured in Novye-MacNeale-Nicolle (NNN) medium for 3 d at 24–26 °C. Then, the parasites were transferred to liquid RPMI 1640 medium supplemented by 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 1 mg/mL streptomycin. The number of parasites was determined by counting using Neubauer chamber.

The parasite identification was done as reported previously (Mahmoudzadeh-Niknam et al., 2011; Rostamian, Akya & Niknam, 2019). Briefly, genomic DNA of the parasites was extracted by QIAamp DNA Mini Kit (Qiagen, USA). Using specific primers (Forward: 5′-CTGGATCATTTTCCGATG-3′, Reverse: 5′-AAGTGCGATAAGTGGTA-3′), the ITS1 region of *Leishmania* DNA was amplified and further analyzed by restriction fragment length polymorphism (RFLP), using HaeIII enzyme.

The whole study was approved by the Ethics Committee of Kermanshah University of Medical Sciences on 14 Aug, 2018 (license number IR.KUMS.REC.1397.371).

2.2. Plant collection and authentication

The *V. radiata* seeds, *T. ramosissima* barks, and *C. lanatus* flowers were collected from Kermanshah Province, west of Iran from May to September in 2018. The identities of the plants were confirmed by botanic experts in Department of Pharmacognosy & Biotechnology, Kermanshah University of Medical Sciences, Kermanshah, Iran.

2.3. Preparation of extracts

To prepare the extracts, the plant samples (*V. radiata* seeds, *T. ramosissima* barks, and *C. lanatus* flowers) were chopped into small pieces, dried in a shaded place, and ground mechanically by a blender. Two types of extraction (hydroalcoholic and chloroformic) were used in the present study. To prepare hydroalcoholic or chloroformic extracts, 100 g of each dry powder was added to 500 mL of pure ethanol or chloroform respectively and mixed gently for 4 h by a magnetic stirrer.

The obtained solution was left for 24 h at room temperature. Then the solvent was removed from the solution by evaporation in

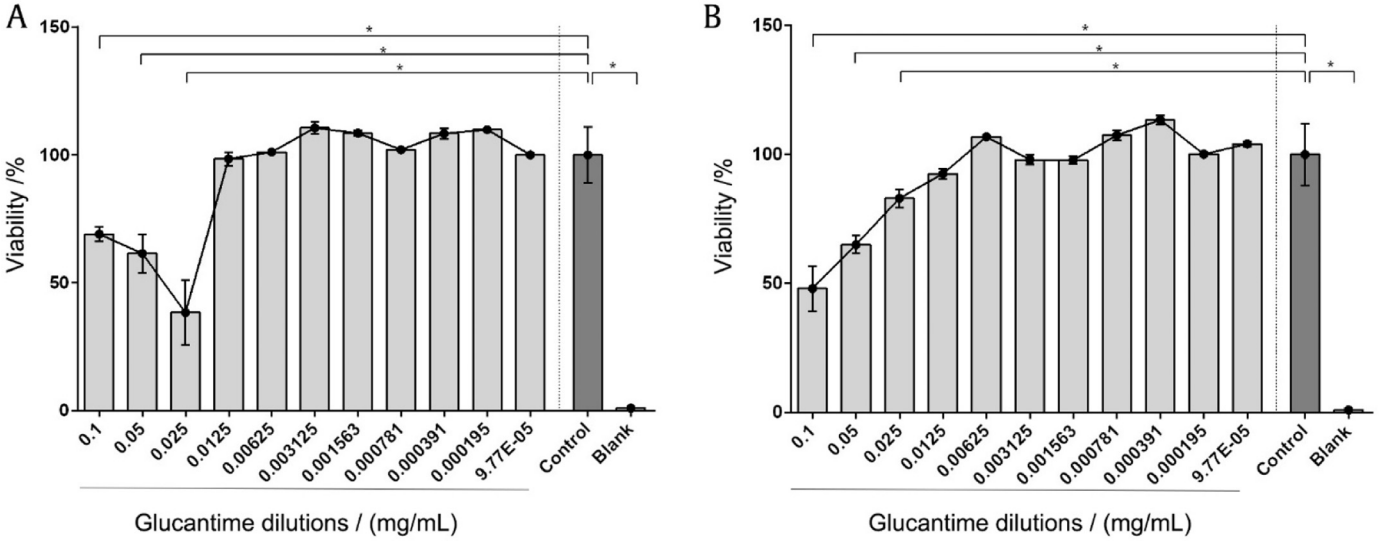

Fig. 2. Effect of Glucantime on *Leishmania* parasites. *Note:* Glucantime inhibited growth of parasites at 0.1, 0.05, and 0.025 mg/mL, and the parasite viabilities were significantly lower than control group (shown by "*"). Blank column shows the viability of the medium alone (without parasite) group.

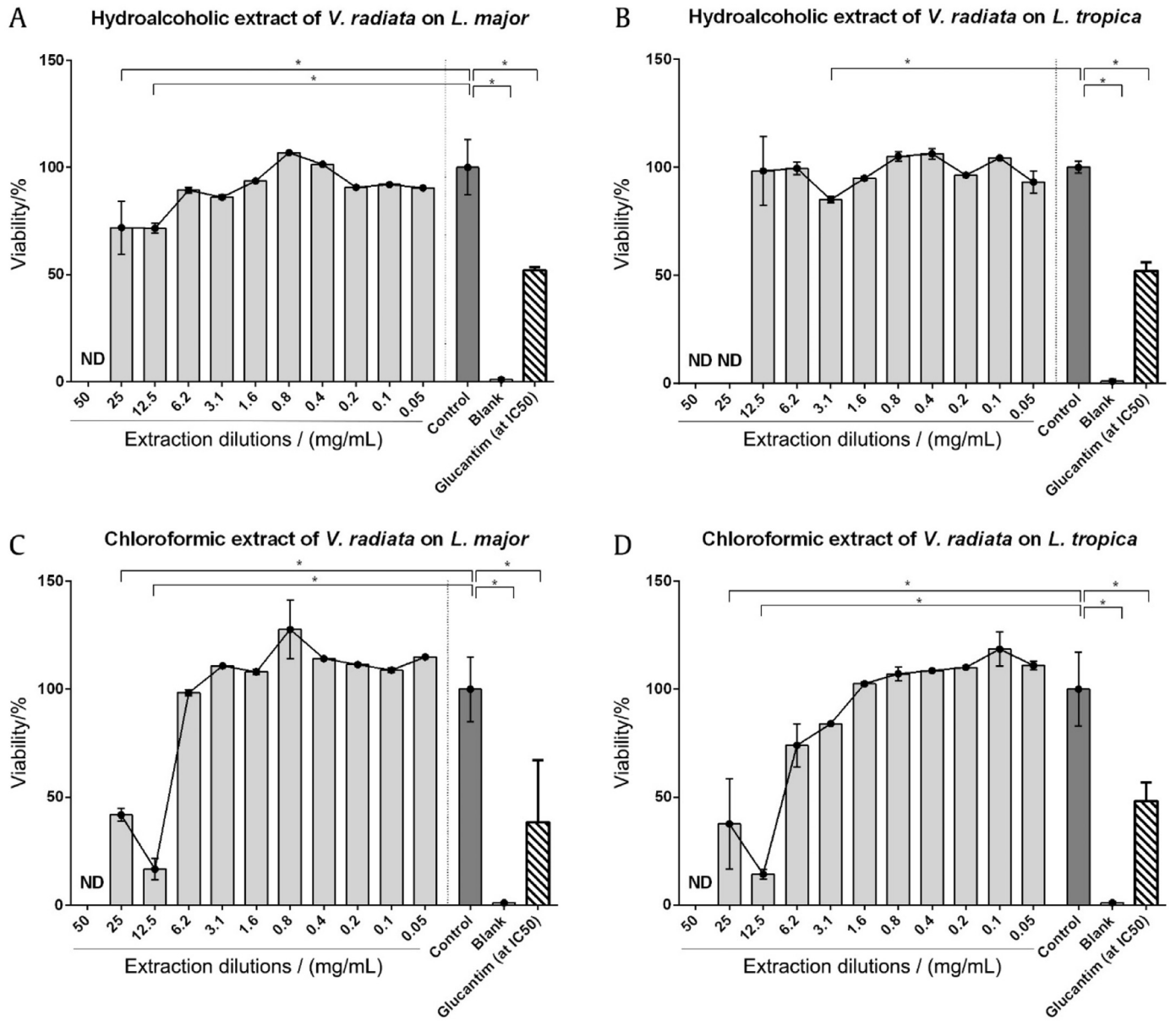

Fig. 3. Effect of *V. radiata* extracts on *Leishmania* isolates. A) Effect of hydroalcoholic extract of *V. radiata* on *L. major* promastigotes. B) Effect of hydroalcoholic extract of *V. radiata* on *L. tropica* promastigotes. C) Effect of chloroformic extract of *V. radiata* on *L. major* promastigotes. D) Effect of chloroformic extract of *V. radiata* on *L. tropica* promastigotes. Control and blank columns show the parasites without extract and medium alone groups, respectively. ND (not determined) shows extract dilutions in which ELISA reader could not read OD due to high turbidity.

a rotating evaporator at 45 °C and the remained semisolid material was kept in the 4 °C until use.

2.4. Anti-promastigote activity assay

L. major and *L. tropica* promastigotes were cultured in RPMI medium as above for 5 d to reach the logarithmic phase. The plant extracts were dissolved in DMSO and were added to a 96-well microplate at pre-determined serial dilutions from 50 to 0.05 mg/mL. Then, 2.5×10^6 promastigotes/mL of each parasite was prepared and added to each well (100 µL/well). The plate was incubated for 72 h at 24–26 °C.

The viability of promastigotes was evaluated by 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT) dye colorimetric assay as previously reported (Mosmann, 1983). Briefly, the MTT solution (at final concentration of 0.5 mg/mL) was added to the plate and incubated at 37 °C for 3–4 h. Afterwards, 100 µL/well of DMSO was added to the plate to dissolve the MTT formazan and the optical density (OD) was recorded by an ELISA plate reader at 570 nm. The negative control wells contained DMSO without extracts.

Glucantime (SANOFI, France) was used at its half maximal inhibitory concentration (IC_{50}) as the positive control. To find the IC_{50} of the Glucantime on each *Leishmania* species, two folds serial dilutions of it from 10^{-1} to 10^{-4} mg/mL were used in a separate experiment.

2.5. Statistical analysis

The statistical differences were analyzed by one-way ANOVA followed by Tukey multiple comparison test. $P \leq 0.05$ was considered statistically significant.

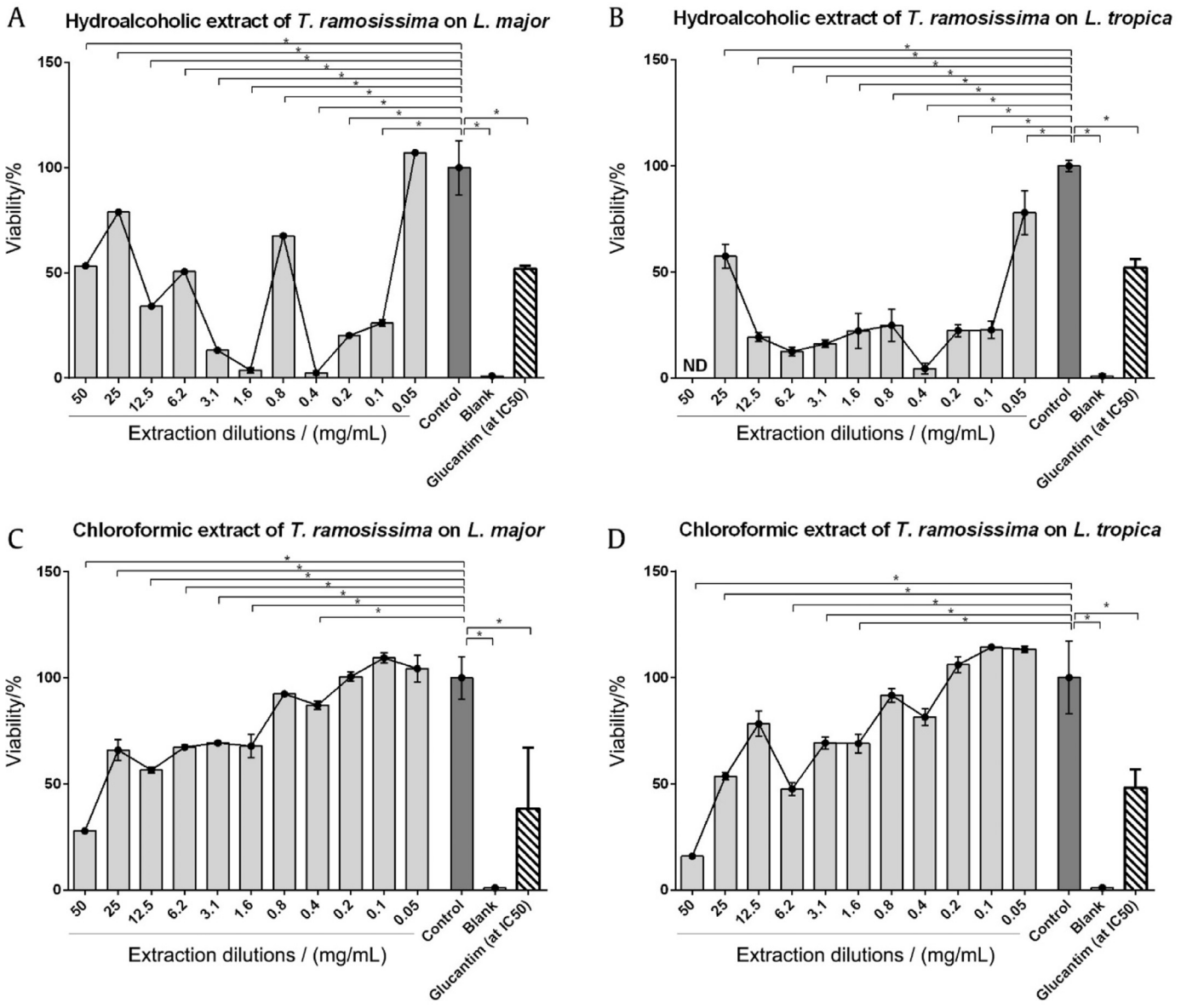

Fig. 4. Effect of *T. ramosissima* extracts on *Leishmania* isolates. (A) Effect of hydroalcoholic extract of *T. ramosissima* on *L. major* promastigotes. (B) Effect of hydroalcoholic extract of *T. ramosissima* on *L. tropica* promastigotes. (C) Effect of chloroformic extract of *T. ramosissima* on *L. major* promastigotes. (D) Effect of chloroformic extract of *T. ramosissima* on *L. tropica* promastigotes. Control and blank columns show parasites without extract and medium alone groups, respectively. ND (not determined) shows extract dilutions in which ELISA reader could not read OD due to high turbidity.

3. Results

3.1. Species identification

Amplification of ITS1 region by PCR using specific primers resulted in a sharp single band of approximately 300 bp for both *L. major* and *L. tropica* isolates. The presence of this 300 bp band indicates that both isolates were *Leishmania* species.

HaeIII-digestion on ITS-1 PCR product of *L. major* isolate resulted in two bands of 220 bp and 150 bp, while for *L. tropica* the digestion resulted in two bands of 200 bp and 100 bp (Fig. 1). These patterns were the expected results for both isolates and confirmed their identities.

3.2. Finding IC_{50} of standard drug

In the present study, Glucantime was used as a standard drug. To find the half maximal inhibitory concentration (IC_{50}) of Glucantime, different dilutions was applied. IC_{50} was calculated as the minimum concentration of Glucantime that resulted in ≤50% viability of the parasites. As shown in Fig. 2, Glucantime inhibited the growth of both parasites at first three dilutions (0.1, 0.05, and 0.025 mg/mL). The measured IC_{50} of Glucantime for *L. major* and *L. tropica* was 0.025 and 0.1 mg/mL, respectively (Fig. 2). It showed that Glucantime had a more antileishmanial potency against *L. major* than *L. tropica*.

3.3. Antileishmanial effects

Antileishmanial effects of two types of extracts (hydroalcoholic and chloroformic) of *V. radiate, T. ramosissima, and C. lanatus* against promastigote forms of *L. major* and *L. tropica* were determined by MTT assay.

3.3.1. Antileishmanial effects of V. radiata

The hydroalcoholic extract of *V. radiata* were found to decrease cell viability of both *L. major* and *L. tropica* mainly at high concentrations (at 25 and 12.5 mg/mL for *L. major* and at 3.1 mg/mL for *L.*

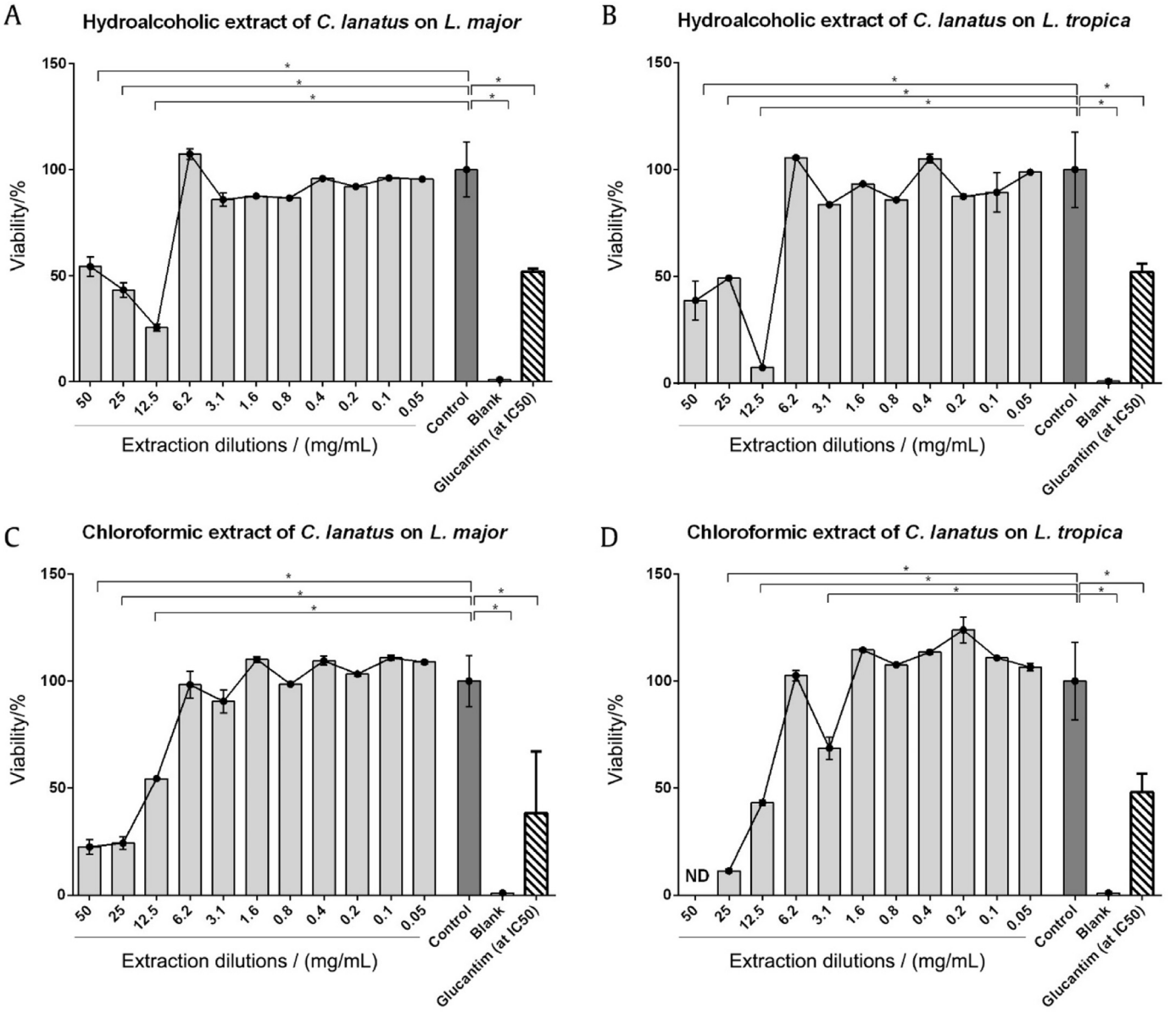

Fig. 5. Effect of *C. lanatus* extracts on *Leishmania* isolates. (A) Effect of hydroalcoholic extract of *C. lanatus* on *L. major* promastigotes. (B) Effect of hydroalcoholic extract of *C. lanatus* on *L. tropica* promastigotes. (C) Effect of chloroformic extract of *C. lanatus* on *L. major* promastigotes. (D) Effect of chloroformic extract of *C. lanatus* on *L. tropica* promastigotes. Control and blank columns show parasites without extract and medium alone groups, respectively. ND (not determined) shows extract dilutions in which the ELISA reader could not read OD due to high turbidity.

tropica) (Fig. 3A and B). However, none of these dilutions was able to decrease parasite viability to lower than 50%.

Similarly, the chloroformic extract of *V. radiata* reduced cell viability of both parasites at 25 and 12.5 mg/mL dilution of the extract (Fig. 3C and D). In contrast to hydroalcoholic extract, these dilutions of chloroformic extract were able to decrease the parasite viability to lower than 50%, indicating the more potency of chloroformic than hydroalcoholic extract of *V. radiata* in inhibition of *Leishmania* growth. The measured IC_{50} value for chloroformic extract of *V. radiata* was 12.5 mg/mL against promastigote forms of both parasites.

3.3.2. Antileishmanial effects of T. ramosissima

The hydroalcoholic extract of *T. ramosissima* were found to decrease cell viability of both *L. major* and *L. tropica* approximately in all concentrations used (Fig. 4A and B). The measured IC_{50} value for hydroalcoholic extract of *T. ramosissima* was 0.1 mg/mL against promastigote forms of both parasites.

The chloroformic extract of *T. ramosissima* reduced cell viability of *L. major* and *L. tropica* from its high concentration to 0.4 mg/mL and 1.6 mg/mL dilutions, respectively (Fig. 4C and D). The measured IC_{50} value for this extract was 50 mg/mL against both parasites.

3.3.3. Antileishmanial effects of C. lanatus

The hydroalcoholic extract of *C. lanatus* reduced cell viability of both parasites at its high concentrations (50, 25, and 12.5 mg/ml) (Fig. 5A and B). The measured IC_{50} value for this extract was 12.5 mg/mL against both parasites.

Similarly, the chloroformic extract of *C. lanatus* reduced cell viability of both parasites at its high concentrations (50, 25, and 12.5 mg/mL for *L. major* and 25, 12.5, and 3.1 mg/mL for *L. tropica*) (Fig. 5C and D). The measured IC_{50} value for this

extract was 25 and 12.5 mg/mL against *L. major* and *L. tropica*, respectively.

4. Discussion

Medicinal plant extracts have been claimed to be a potential treatment to control a wide range of diseases, including leishmaniases due to the less side effects, low cost and high availability (Rocha et al., 2005). In the present study, the antileisshmanial effect of *V. radiata* (Fabaceae), *T. ramosissima* (Tamaricaceae), and *C. lanatus* (Asteraceae) were studied. No studies were found in the literature about the antileishmanial effect of these plants. However, there are several reports on the effect of different species of Fabaceae, Tamaricaceae, and Asteraceae on *Leishmania* species.

Here, high concentrations of *V. radiata* reduced the growth of both *Leishmania* species. Moreover, it was showed that chloroformic extract of *V. radiata* has more antileishmanial potency than its hydroalcoholic extract. However, none of these extracts were able to decrease *Leishmania* viability at concentration as low as IC_{50} of Glucantime. The IC_{50} of Glucantime on *L. tropica* was 0.1 mg/mL, while the lowest concentration of *V. radiata* which surely inhibited *Leishmania* growth was about 12.5 mg/mL. These results showed that *V. radiata* extracts have no strong antileishmanial effects. Similar to our results, antileishmanial activity of another species of Fabaceae (*Campsiandra laurifolia* Benth.) was not detected in a previous study, although its seed extracts had satisfactory immunosuppressant potency (Chagas, Müller, Soares & Garcez, 2010). Inconsistently, there are some reports show the strong antileishmanial effects of Fabaceae family (Kheiri Manjili, Jafari, Ramazani & Davoudi, 2012; Rocha et al., 2005; Sartorelli, Andrade, Melhem, Prado & Tempone, 2007; Singh et al., 2005). This inconsistency may be referred to the different plant species used.

In the present study, both extracts of *T. ramosissima* showed strong antileishmanial effect and inhibited the growth of both *Leishmania* species even in low concentrations. There is only one old study that showed lectins extracted from a species of Tamaricaceae have an effect on the agglutination of *L. major* promastigotes (Jacobson & Schlein, 1999). It seems that our finding is the first data on *T. ramosissima* antileishmanial effect. It should be noted, that the effect of hydroalcoholic extract of *T. ramosissima* on *Leishmania* species (especially on *L. major*) was not dose-depended and the fluctuation was observed in the results of its different dilutions due to un-known reason. Although we dissolved the extract in different solvents and chose the best ones, it is hypothesized that the fluctuation in the results may be due to non-complete dissolving and remaining of debris in the solution.

Similar to the results of *V. radiata*, only high concentrations of *C. lanatus* were able to inhibit *Leishmania* growth. These concentrations are far higher than IC_{50} of Glucantim, suggesting that *C. lanatus* extracts do not have a strong antileishmanial effect. It is the first report about antileishmanial effects of *C. lanatus* and no more reports were found on this case in the literature. However, in contrast to our study, the antileishmanial effects of different species of Asteraceae family have been reported in several studies (Azizi et al., 2016; Fournet, Barrios & Munoz, 1994; Nikmehr, Ghaznavi, Rahbar, Sadr & Mehrzadi, 2014; Panda & Luyten, 2018). The inconsistency of our study with previous reports of Asteraceae, may refer to different plant species used.

5. Conclusion

Altogether, findings of the present study revealed the high antileishmanial activity of *T. ramosissima* extracts against *L. major* and *L. tropica*, indicating its potential as a natural source for the production of novel antileishmanial agents. Also, we found that *V. radiata* and *C. lanatus* have no antileishmanial effect in our experiment setting. However, further studies are needed to clarify these results specially by checking the extracts on amastigote forms of *Leishmania* species, their immunosuppressant effect, their effect on CL experimental models, and finally their effects on human volunteers.

Declaration of Competing Interest

The authors declare that there are no conflicts of interests.

Acknowledgement

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