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Original paper

A zymographic study of metalloproteinase activities in whole cell extracts and extracellular secretions of *Leishmania* (*L.*) *tropica*, *L. major* and *L. infantum* from Iran

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ABSTRACT. Leishmaniosis encompasses a group of diseases that is transmitted by sand flies and caused by different species of *Leishmania*. The skin is the initial organ to be infected by the *Leishmania* in cutaneous, mucocutaneous and visceral forms of leishmaniosis. The matrix metalloproteinases (MMPs) are capable of degrading all kinds of extracellular matrix (ECM) proteins. The aim of this study was to investigate the protease activity through zymography in cell extracts and extracellular secretions of *L. major*, *L. tropica* and *L. infantum* as three prevalent *Leishmania* spp. in Iran. The three *Leishmania* spp. were cultured in RPMI-1640 medium supplemented with fetal calf serum. Promastigotes and axenic amastigotes were harvested and lysed at various phases, and extracellular secretions and cell extracts were collected. *Leishmania* spp. were proved by targeting kDNA gene. Enzymes were characterized according to gelatin zymography and sensitivity to distinct proteinase inhibitors. We observed proteinase bands with molecular weights (MWs) between 66 to 180 kDa in cellular extracts of axenic amastigotes of *L. infantum*, *L. tropica*, and *L. major*, and from 66 to 92 kDa in extracellular secretions of *L. infantum*. No proteinase activities were observed in extracellular secretions of axenic amastigotes and in cellular extracts of promastigotes in logarithmic and stationary phases of *L. major* and *L. tropica*. Using specific inhibitors, we determined that these proteolytic activities are due to metalloproteases. Our study demonstrated that amastigotes of all three *Leishmania* spp. have distinct amounts of proteinase activities and therefore can cause various types of lesions and outcomes of the disease.

Keywords: metalloproteinase activity, zymography, *Leishmania tropica*, *Leishmania major*, *Leishmania infantum*, Iran

Introduction

Leishmaniosis is a global vector-borne disease, caused by various species of *Leishmania* with a broad spectrum of clinical presentations, including cutaneous (CL), mucosal (ML), diffuse cutaneous (DCL), and fatal visceral leishmaniosis (VL) [1]. It is transmitted by the bite of infected female phlebotomine sand flies [2,3]. Depending on the species of *Leishmania* involved, humans and an extensive range of mammals can function as reservoirs [4,5]. CL is the most frequent form of the disease with 0.7 to 1.3 million new cases per year being reported worldwide [6]. CL is characterized by the presence of a papule at the bite site, which subsequently becomes an ulcerated lesion. ML is characterized by destructive lesions in the oral, nasal, and pharyngeal mucosa [7,8]. VL has an average annual incidence of 0.5 million cases, and is the most severe form of disease which is manifested with anemia, hepatosplenomegaly, and pancytopenia and is potentially fatal if untreated [9,10]. Four

epidemiological forms of leishmaniosis are present in Iran, anthroponotic (ACL), zoonotic (ZCL), ML and VL and are caused by *L. tropica*, *L. major*, and *L. infantum*, respectively [7,8,11,12]. However, *L. tropica* and *L. major* may also cause infrequently VL with *L. tropica* being more usual than *L. major* [9].

Following *Leishmania* infection, both macrophages and dendritic cells phagocytose the parasite. These cells produce cytokines and chemokines, thereby initiating events that help to cause the arbitration of the host immune response and establishment of infection [13,14]. In this context, the expression of protein phosphatases is of special interest. Protein phosphatases are involved in parasite biology and virulence due to the modulation of host cytokine production and impairment of the microbicide potential of macrophages [15,16]. Recently, it has been reported that *Leishmania* enter other cell types such as mesenchymal stem cells, neutrophils, and fibroblasts [17–19].

Furthermore, during *Leishmania* sp. infection, T and B lymphocytes, mononuclear and plasma cells are activated, Toll-like receptors 2 and 4 are overexpressed, and TNF-α, TGF-β, IL-10 and IFN-γ are produced in high levels in CL patients [20–22]. TGF-β and TNF-α partakes in the inflammatory process through the induction of nitric oxide, necrosis, tissue damage, cytotoxicity and expression of matrix metalloproteinases (MMPs) [23]. MMPs or matrixins are calcium-dependent, zinc-containing enzymes that degrade extracellular matrix (ECM) molecules (collagen, laminin, and fibronectin) by releasing concealed epitopes from the ECM [24,25]. However, these enzymes have a broad range of biological functions and also operate on several biomolecules, including cytokines, hormones and chemokines, therewith regulating immune responses [24,26].

MMPs can be categorized according to their ECM substrates, primary structure and cellular location as collagenases (MMP-1, MMP-8 and MMP-13), stromelysins (MMP-3, MMP-10, MMP-11 and MMP-12), matrilysins (MMP-7 and MMP-26), membrane type MMPs (MT1-MMP to MT6-MMP) and gelatinases (MMP-2 and MMP-9) [24,26]. Leishmanolysin GP63 by *Leishmania* [27], falcilysin by *Plasmodium falciparum* [28], and toxolysin 4 secreted from *Toxoplasma gondii* micronemes [29] are the main MPs of parasites. Among the MMPs, MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) are well known for their participation in the pathogenesis of a wide spectrum of parasitic infections [23,30–34]. MMP-2 and MMP-9 are endopeptidases that specialize in cleaving gelatin and type IV collagen, the major constituent of skin basement membrane. Gelatinases can also degrade collagen type V, VII, and X, elastin, and aggrecan. While MMPs are essential for prosperous annihilation of infection by arousing the migration of effector cells to the inflammatory area, high production of these molecules may induce pathology [25]. An imbalanced production of MMPs and its natural regulator, tissue inhibitor of metalloproteinases (TIMPs) happens in a variety of diseases where tissue damage occurs [35]. *Leishmania*-infected macrophages secrete MMP-9, and its production is increased in patients with CL [23]. Moreover, MMP-9 secreted by macrophages infected with *L. chagasi* may contribute to the liver injury observed in VL [36]. It is hypothesized that the skin lesion formation in CL is due to breakdown dysregulation of the basement membrane caused by imbalance in levels of MMP-9 and TIMP-1, cooperatively with cell recruitment and edema [23].

In this study, the protease activities of *L. infantum*, *L. major* and *L. tropica* were characterized and compared using axenic amastigote forms and whole-promastigote extracts and extracellular secretions. To the best of our knowledge, this is the first study executed on metalloproteinase activity of these *Leishmania* spp. in Iran.

Materials and Methods

In vitro culture

Standard strains of *L. major* (MRHO/IR/75/ER), *L. tropica* (MHOM/AF/88/K27), and *L. infantum* (MCAN/IR/07/MOHEB) were cultured in the modified NNN medium and incubated at 25°C. Modified NNN medium consists of two phases, horse blood agar base and an overlay Locke's solution. After 72 h, the liquid phase of each culture tube was examined under inverted microscope in order to observe motile promastigotes. Cultures were mass cultivated in RPMI-1640 medium (Gibco, Frankfurt, Germany) buffered with 25 mM HEPES and 2 mM $NaHCO_3$ (pH 7.2) and supplemented with 20% heat-inactivated FCS (Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco), and maintained at 25±1°C. From a starting inoculum of 5×10^5 promastigotes/ml,

cell densities in the range of 2×10^7 to 7×10^7/ml were obtained on day seven. The pH of culture medium was changed to 5.0 by adding sterile 10 mM succinic acid (Merck, Darmstadt, Germany). Transformation of promastigotes to fully differentiated amastigotes in liquid medium were completed within five days with pH 5.0 at 33°C. Axenically grown amastigote forms of *L. tropica* and *L. major* were preserved at 32±1°C, and *L. infantum* was maintained at 36±1°C with 5% CO_2 in 25-cm^2 flasks [37].

DNA extraction

Total genomic DNA was extracted using the AccuPrep® Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea), according to manufacturer's instructions. The quantity and quality of the extracted DNA was assessed using a Nano spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The DNA was stored at –20°C until used.

kDNA conventional PCR

All cultured samples were pre-identified to *Leishmania* species level before they were subjected to gelatin zymography. The conserved area of the minicircle kDNA of all the three *Leishmania* samples was amplified by using the primers LINR4 (forward) (5'-GGG GTT GGT GTA AAA TAG GG-3'), and LIN17 (reverse) (5'-TTT GAA CGG GAT TTC TG-3') for species identification.

The PCR reactions were performed in a final volume of 25 µl consisting of 12.5 µl of 2× Master Mix Red (Ampliqon, Odense, Denmark), 1.25 U *Taq* DNA polymerase, 200 µM of dNTPs, 1.5 mM $MgCl_2$, 10 pM of each primer and 2 µl of template DNA. The PCR temperature profiles were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 10 min in an Eppendorf Mastercycler Gradient (Hamburg, Germany). Amplicons were analyzed on 1.5% agarose gels by electrophoresis at 90 V in 1× TAE buffer and visualized by UV light after being stained with GelRed® (Biotium, Hayward, CA, USA) [8].

Protein extraction

Proteins and soluble enzymes extracts were prepared separately for each *Leishmania* spp. Promastigotes and axenic amastigote forms were harvested by centrifuging at 3000 rpm at 4°C, following washing three times in cold PBS. Afterwards, whole cells were resuspended in enzyme stabilizer solution (ε-aminocaproic acid, DTT and EDTA), and following were immersed in liquid nitrogen and thawed at 37°C. After freezing-thawing procedure, they were sonicated and the disrupted parasites were centrifuged at 12000 × *g* for 15 min at 4°C, and aliquots of the supernatants were separated and kept frozen at –20°C until further analysis [37].

Secreted proteins are often masked by high amounts of protein supplements in the culture medium. We have therefore established a serum-deprived efficient method for the enrichment and analysis of the secretome of promastigotes and axenic amastigote forms. After three washes with 10 ml of PBS, the whole cells were deprived of serum. This technique has been previously shown to induce secretion of proteins. Serum deprivation periods did not affect cellular viability from 48 h and inward. Whole cell's supernatants were collected and centrifuged at 300 × *g* at 4°C for 10 min and kept at −20°C until use [37].

In order to standardize the procured results, the samples were examined for total protein concentration with bicinchoninic acid (BCA) using a BCA Protein Assay Kit (Boster Bio, Pleasanton, CA, USA).

Gelatin zymography

The gelatinolytic activity of these samples was assessed by gelatin zymography. Proteins (from supernatants of the cultures or from each *Leishmania* spp. amastigotes lysate) were subjected to electrophoresis under non-reducing conditions in a 10% SDS-PAGE gel copolymerized with 1% gelatin (G8150, Sigma-Aldrich, St. Louis, MO, USA). The samples were prepared by mixing 30 µg of the sample protein with equal amount of non-reducing Laemmli sample buffer (125 mM Tris–HCl, pH 6.8; 20% glycerol, 4% SDS, and 0.2% bromophenol blue) for 5 min, and were then subjected to electrophoresis at 4°C for 3 h. Subsequently, the gels were incubated with quiet stirring (30 min, 25°C) in enzyme renaturing buffer (50 mM Tris, 200 mM NaCl, 5 mM $CaCl_2$, 2.5% Triton X-100, 0.2% Brij-35, pH 7.5), refreshing with new buffer for a total of four times. The gels were then incubated for 20 h at 37°C in the enzymatic activation buffer (50 mM Tris, 200 mM NaCl, 5 mM $CaCl_2$, 0.2% Brij-35, pH 7.5) with gentle shaking. Following incubation, the buffer was replaced with 0.5% Coomassie brilliant blue (R-250; Bio-Rad), 45% methanol and 10% glacial

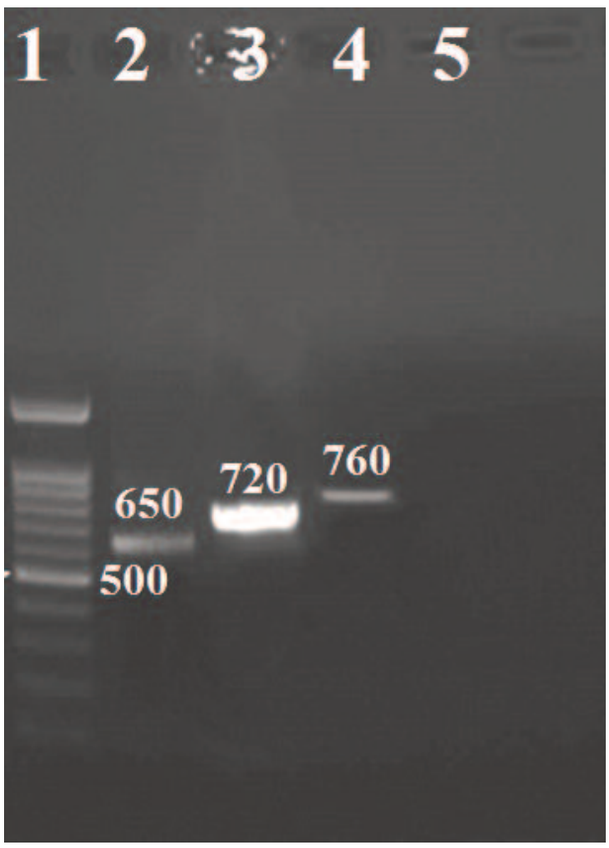


Figure 1. Electrophoresis of PCR products of DNA extracted from *Leishmania* spp. cultures. The five lanes contained the products from *L. major* (lane 2), *L. infantum* (lane 3), *L. tropica* (lane 4), negative control (lane 5), and a molecular marker (lane 1)

acetic acid for 1 h. The gels were destained in the same solution without the dye for 45 min. The molecular mass of proteinases was calculated by comparison with the mobility of a commercial molecular mass standard. Clear gelatinolytic bands on a uniform dark blue background were measured by densitometry with the image analyzer GS-800 Calibrated Imaging Densitometer (Bio-Rad). The gels were digitalized and the integrated densities of the bands, expressed as arbitrary units (A.U.), were calculated by summing the pixel values within the digested area of the band and subtracting the background density (gelatinase activity = number of pixels × surface area), using Quantity One® software (Bio-Rad). As a positive control, human recombinant MMP-2 (PF037; Calbiochem, San Diego, CA, USA) and MMP-9 (PF038; Calbiochem) were used [38–40].

Inhibition assays

Stock solutions of 1,10-phenanthroline (200 mM, inhibitor of metalloproteinases) and pepstatin A (1 mg/ml, inhibitor of aspartic proteinases) were solubilized in ethanol. Trans-epoxysuccinyl-L-leucylamide-(4-guanidino) butane (E-64, 100 μM, inhibitor of cysteine proteinases), was dissolved in deionized water. Phenyl-methyl-sulfonyl fluoride (PMSF, 250 mM, inhibitor of serine proteinases) was diluted in isopropanol.

Inhibition assays were performed with 10 μM E-64, 10 mM 1,10-phenanthroline, 500 μM PMSF, and 5 μM pepstatin A.

Statistical analysis

The correlation between the levels of the enzymes was measured by linear regression analysis. A value of $P<0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad Prism (GraphPad, La Jolla, CA, USA).

Results

PCR assay

The conserved area of the minicircle kDNA from the *Leishmania* sp. was amplified by PCR. A 760-bp fragment was amplified for *L. tropica*, whereas a 720-bp and 650-bp fragments were amplified for *L. infantum* and *L. major*, respectively (Fig. 1).

Culture

Promastigotes of *L. tropica*, *L. major*, and *L. infantum* were transformed to wholly axenic amastigotes in acidified RPMI-1640 medium within five days and maintained at 33°C and 37°C, respectively. The cellular population of axenically grown amastigotes were seemed uniform, circular to ovoid, aflagellated, and immotile when examined under an invert microscope.

Protease activities

In zymographic studies, cellular extracts and extracellular secretions of promastigotes and axenic amastigote-like forms of *L. major*, *L. tropica* and *L. infantum* were investigated. Using gelatin–SDS–PAGE analysis, protease bands were seen as clear bands against a stained dark-blue background. In cellular extracts of axenic amastigote-like forms, zymographic assays disclosed gelatinolytic activity connected to enzymes with molecular weights (MWs) of 180, 120, 92 (proMMP-9), 72 (proMMP-2), and 66 kDa (active MMP-2) for *L. infantum*; 92, 86 (active MMP-9), and 72 kDa for *L. tropica*; and

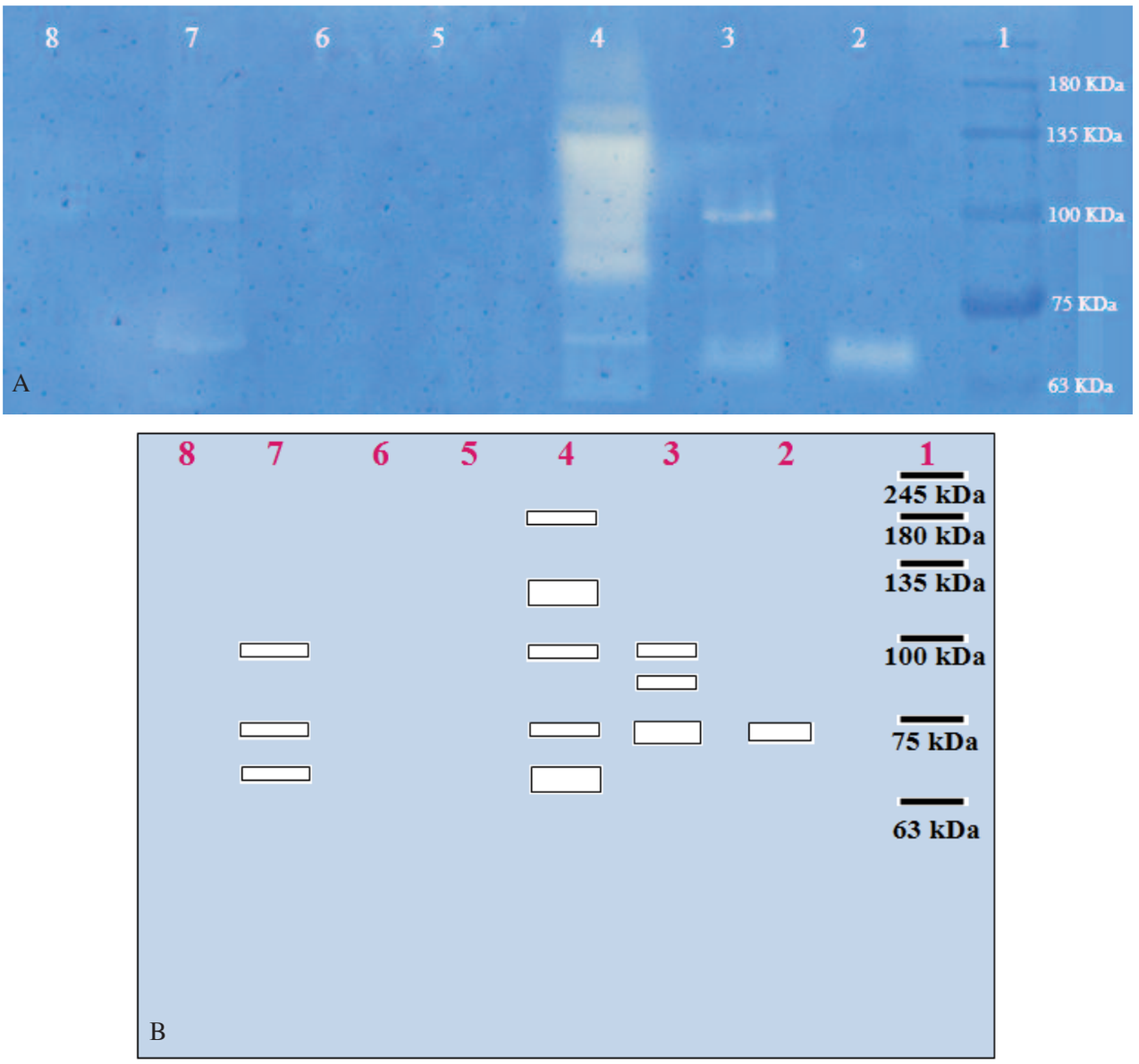
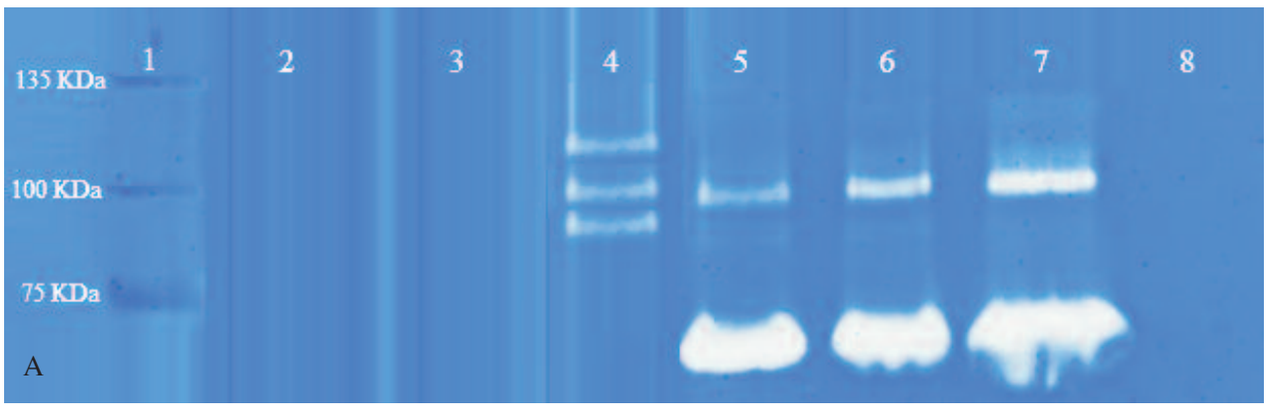


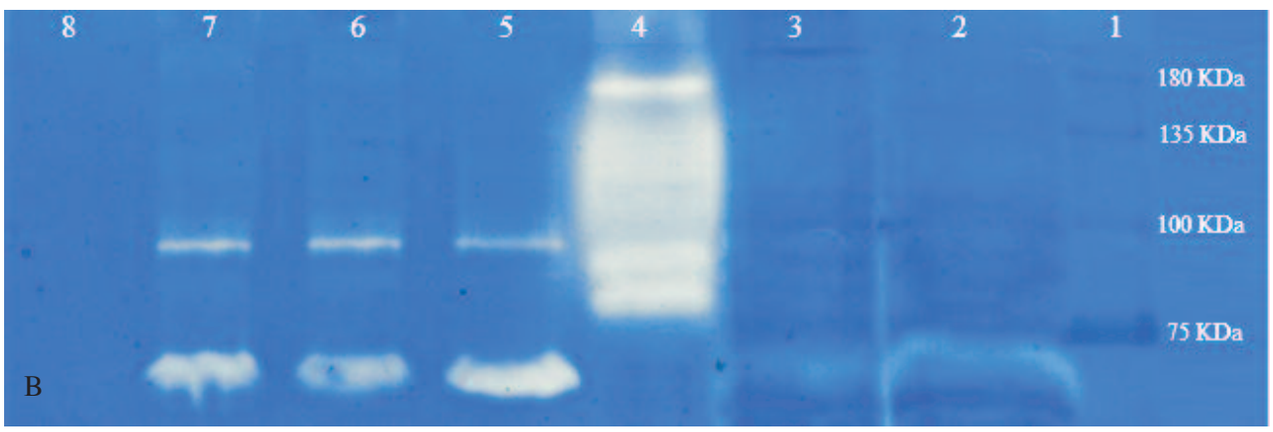
Figure 2. Representative (A) and schematic (B) gelatin-SDS–PAGE showing the protease profiles of *L. major*, *L. tropica* and *L. infantum* cellular extracts and extracellular secretions of axenic amastigote-like forms. Protease bands can be seen as clear bands against blue background. Lane 1: Protein ladder; lanes 2, 3 represent cellular extracts and lanes 5, 6 represent extracellular secretions of *L. major* and *L. tropica* in axenic amastigote-like forms; lanes 4, 7: represent cellular extracts and extracellular secretions of *L. infantum* in axenic amastigote-like forms; lane 8: negative control

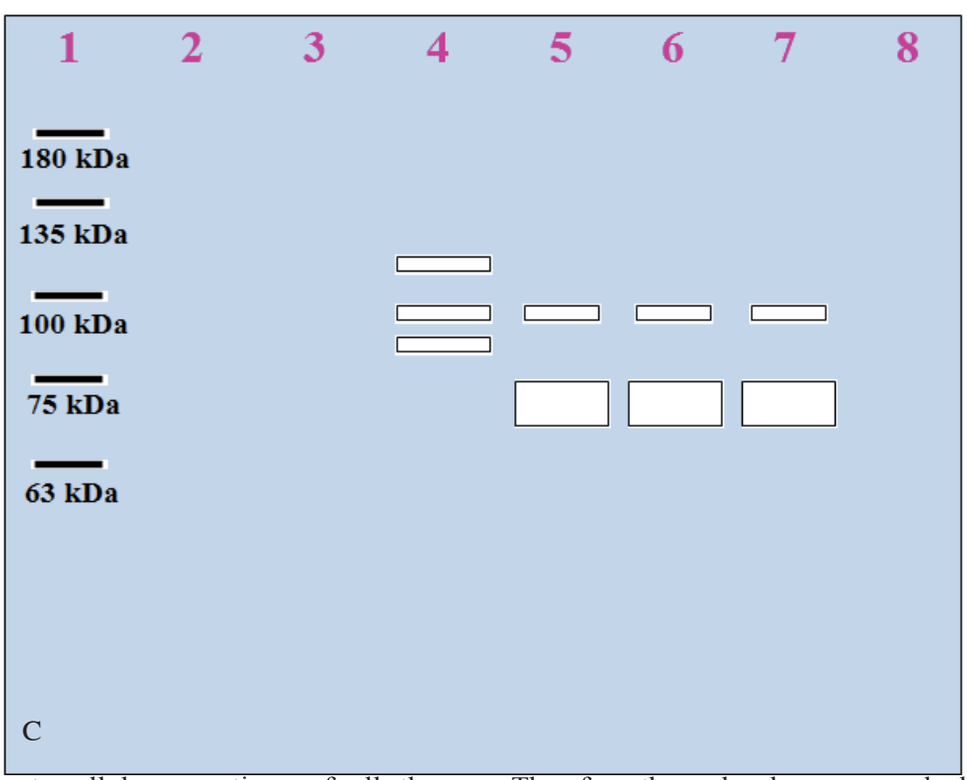
72 kDa for *L. major*. In *L. major* and *L. tropica* extracellular secretions of axenic amastigote-like forms, no gelatinase activities were observed and both parasites have lacked protease activities. In *L. infantum* extracellular secretions of axenic amastigote-like form, three protease bands with 92, 72 and 66 kDa were observed (Fig. 2A, B).

In the stationary phase, no gelatinase activities were observed in *L. major* and *L. tropica* promastigote extracts. While in cellular extracts of *L. infantum* promastigotes, three protease bands with 120, 92, and 86 kDa were discerned. In extracellular secretions of all three *Leishmania* spp. at this stage, similar protease bands with 92 and 72 kDa were remarked. No gelatinase activity was observed in the negative control sample (Fig. 3A, C).

In the logarithmic phase, no gelatinase activities were seen in the cellular extracts of both *L. major* and *L. tropica*, while in *L. infantum*, four protease bands with 180, 120, 92 and 86 kDa were observed. Furthermore, exactly like in the stationary phase, similar protease bands with 92 and 72 kDa were







noticed in extracellular secretions of all three *Leishmania* spp. No gelatinase activity was observed in the negative control sample (Fig. 3B, D). It is worth noting that in zymographic analysis the proteins are not entirely denatured and reduced. Therefore, the molecular masses calculated here for proteolytic activities are estimates.

The values of gelatinolytic activity intensity proclaimed as arbitrary units (A.U.). In *L. infantum* cellular extracts of axenic amastigotes, gelatinolytic

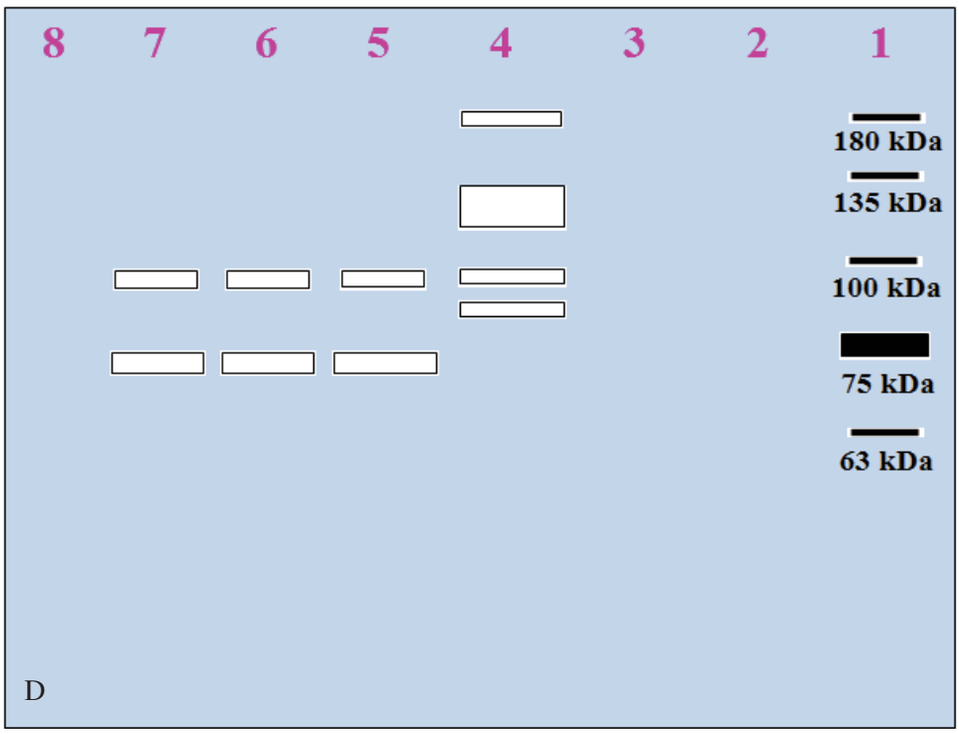


Figure 3. Representative (A, B) and schematic (C, D) gelatin-SDS–PAGE showing the protease profiles of cellular extracts and extracellular secretions of *L. major*, *L. tropica* and *L. infantum* promastigotes in the stationary and logarithmic phase. Lane 1: Protein ladder; lanes 2, 3 represent cellular extracts of *L. major* and *L. tropica*; lane 4 represent cellular extracts of *L. infantum*; lanes 5, 6, 7 represent extracellular secretions of *L. infantum*, *L. tropica* and *L. major*; lane 8: negative control

activity of protease band with 66 kDa (110.32) was higher than protease bands with 120 (70.99), 180 (10.12), 86 (9.58) and 92 kDa (5.58), respectively (Fig. 4A). Moreover, the amount of gelatinase activity of enzyme with 66 kDa (57.81) in *L. infantum* cellular extracts of axenic amastigotes was higher than similar stage of *L. major* (14.28) and *L. tropica* (7.56), respectively (Fig. 4B). In *L. tropica* cellular extracts of axenic amastigotes, the gelatinase activity of 92 kDa (64.34) band was higher than bands with 72 (45.03) and 86 kDa (10.97), respectively (Fig. 4C).

In the stationary phase, the amount of gelatinase activity of 72 kDa band in *L. infantum* (21.52) extracellular secretions was higher than *L. tropica* (18.07) and *L. major* (16.98), respectively. Besides, in cellular extract of *L. infantum*, gelatinolytic activity of 120 kDa (60.24) protease band was higher than 86 (15.60) and 92 kDa (3.89) protease bands, respectively (Fig. 5A, B).

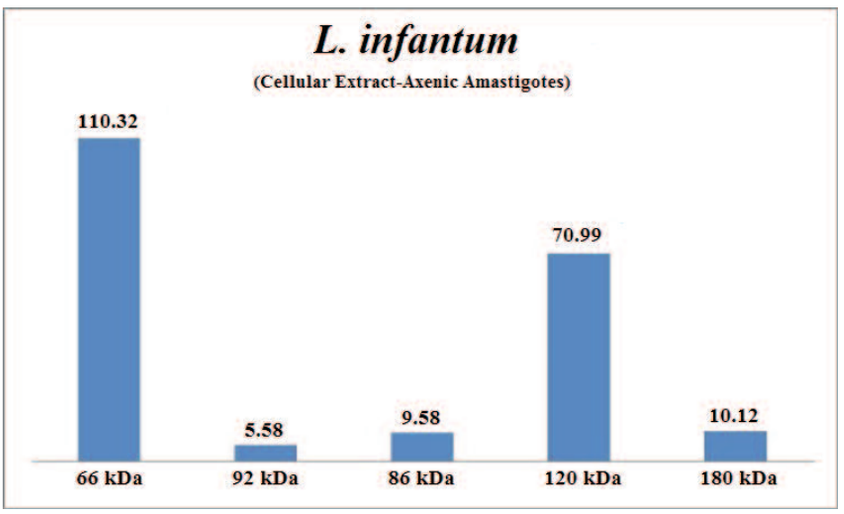
In the logarithmic stage, in cellular extracts of *L. infantum*, gelatinolytic activity of 120 kDa (53.35) protease band was higher than 180 (9.58), 86 (5.90) and 92 kDa (4.40) protease bands, respectively (Fig. 6A). Additionally, in *L. infantum* extracellular secretions, the gelatinase activity amount of 92 (83.11) and 72 kDa (35.94) bands in logarithmic phase were higher than stationary phase (54.31 and 30.50, respectively) (Fig. 6B). Meanwhile, the gelatinase activity amount of 72 kDa band in extracellular secretions of *L. infantum* (20.49) was higher than *L. tropica* (10.69) and *L. major* (9.96), respectively (Fig. 6C, D).

Enzymatic inhibition assays

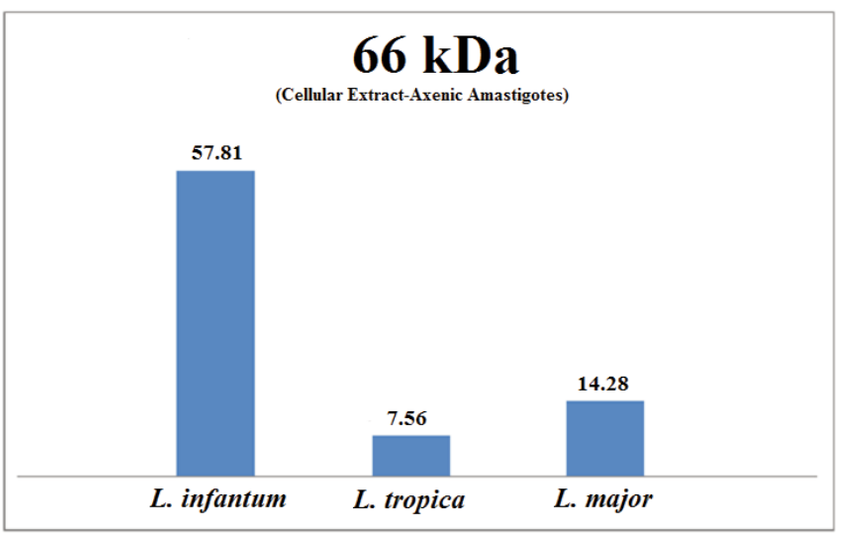
The effects of proteinase inhibitors on proteolytic activities were investigated. The proteinase activities of *L. major*, *L. tropica*, and *L. infantum* extracts were not affected by 10 μM E-64, 500 μM PMSF, or 5 μM pepstatin A. In contrast, 10 mM 1,10-phenanthroline completely annulled the proteinase activities from three species, indicating they belong to the metalloproteinase class.

Discussion

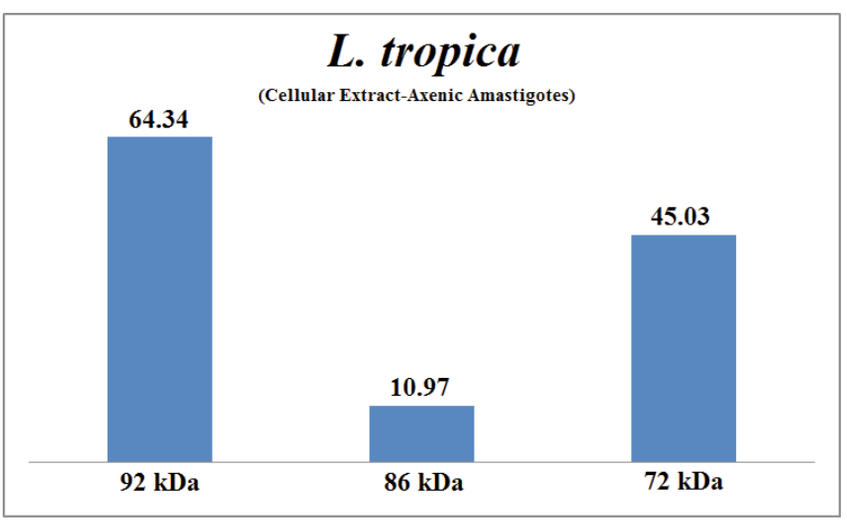
Leishmanioses are one of the most significant forsaken vector-borne tropical diseases. In humans, the disease occurs in at least four major forms: cutaneous, diffuse cutaneous, mucocutaneous and



A



B



C

Figure 4. Data in the histograms represent the area for comparing gelatinase activity (corresponding to the clear areas on the gel) in cellular extracts of *L. infantum* (A), *L. major* and *L. tropica* (B, C). The values, expressed as arbitrary units (A.U.), represent the integrated density of the gelatinolytic bands. The values were obtained by densitometry analysis of the gel

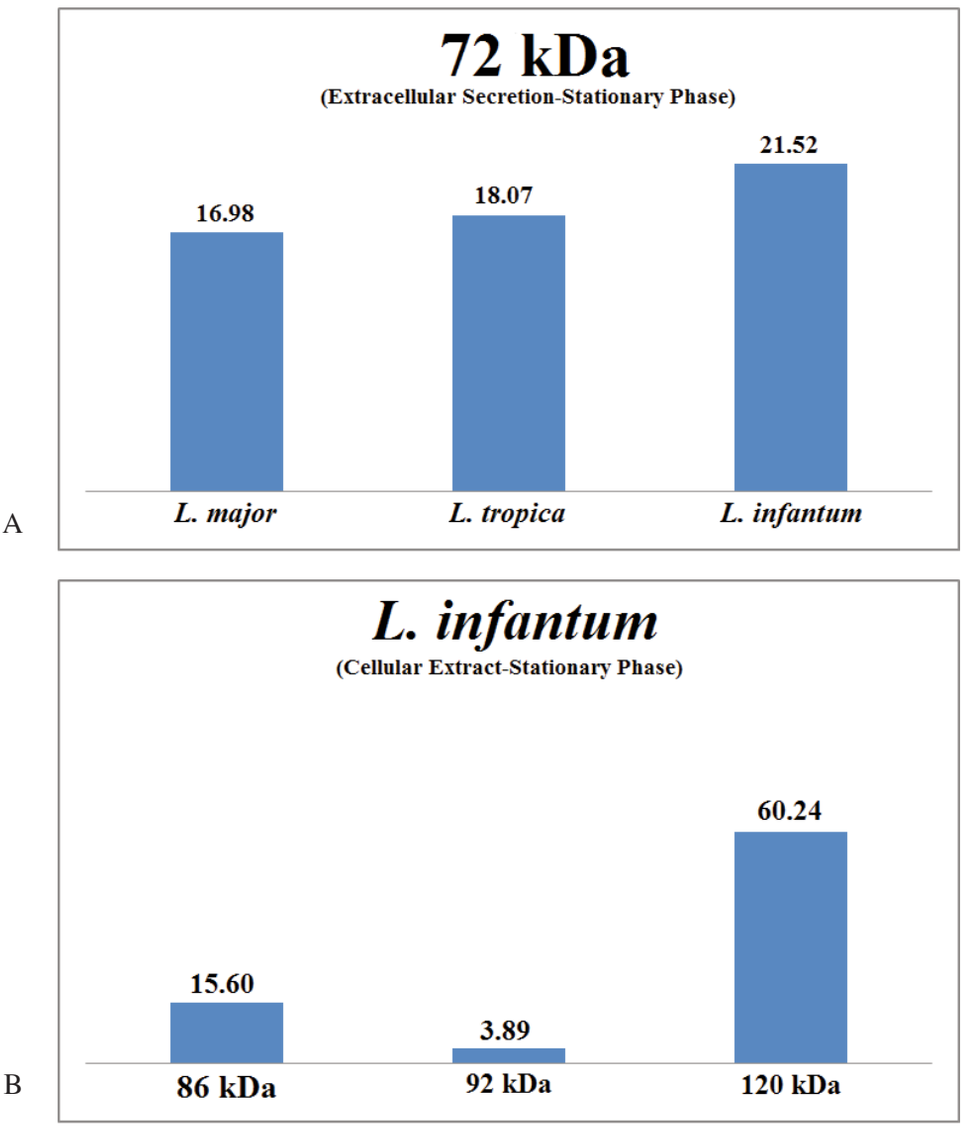


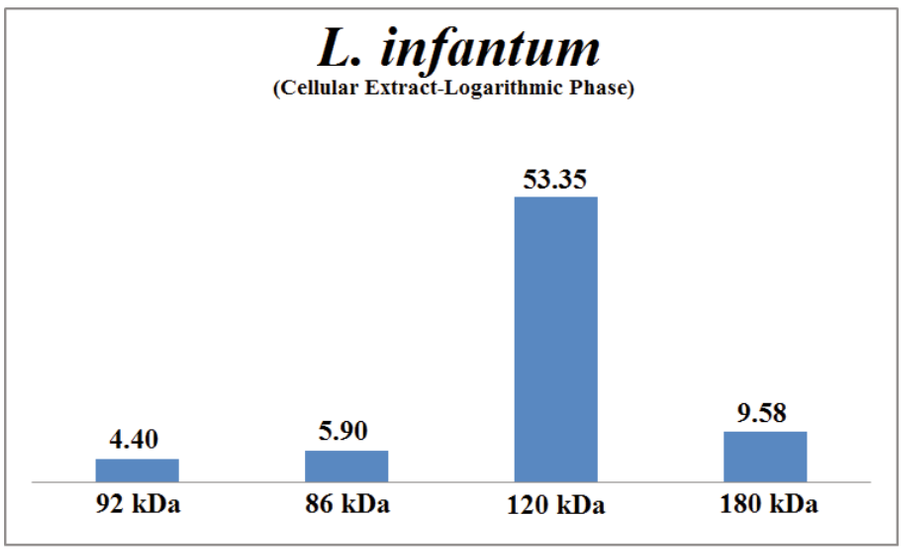
Figure 5. Histograms for comparing the amount of gelatinase activity in extracellular secretions of *L. infantum*, *L. major*, and *L. tropica* (A) and cellular extract of *L. infantum* (B) in the stationary phase

visceral [1]. The wide clinical range of the leishmanioses makes the diagnosis difficult. Parasitological detection abides the gold standard in leishmaniosis diagnosis because of its high specificity. Culture in conjunction with multilocus enzyme electrophoresis (MLEE) permits the parasite species identification and characterization [41]. Several serological tests are usually used in VL diagnosis, but these methods are rarely used in CL diagnosis [42]. PCR-based methods presently comprise the principal molecular diagnostic approach for leishmaniosis diagnosis [43].

The sand fly vector inoculates promastigotes into the human host, where they differentiate into amastigotes. Amastigotes are liable for all clinical outcomes in the vertebrate host. The *in vitro* cultivation system of amastigotes can be an excellent and unlimited source of viable organisms that are free from contaminating host-derived constituents and can be efficiently used in proteomics and genomics studies [44,45]. The first successful establishment of axenic amastigotes of *Leishmania* spp. were achieved in culture media only at elevated temperatures and/or in conjunction with diminished pH and/or in the presence of 5% CO_2. Axenic amastigotes are similar in ultrastructure, infectivity, stage-specific antigens and biochemical properties, when compared with intracellular amastigotes [37,44,45].

One of the aims of the present study was to establish a simple procedure to yield tremendous amounts of three *Leishmania* spp. amastigote forms in axenic cultures. A high percentage of *L. tropica* and *L. major* axenic amastigotes were observed in

A



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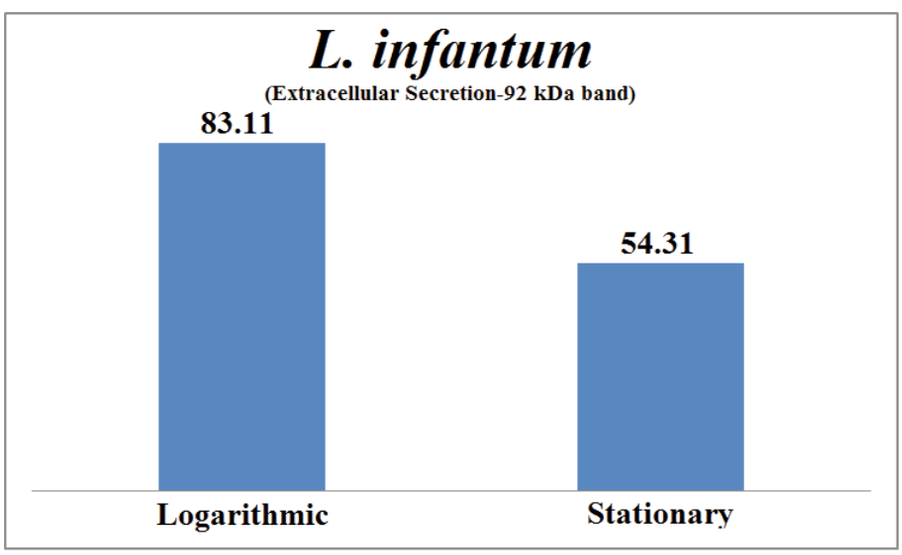

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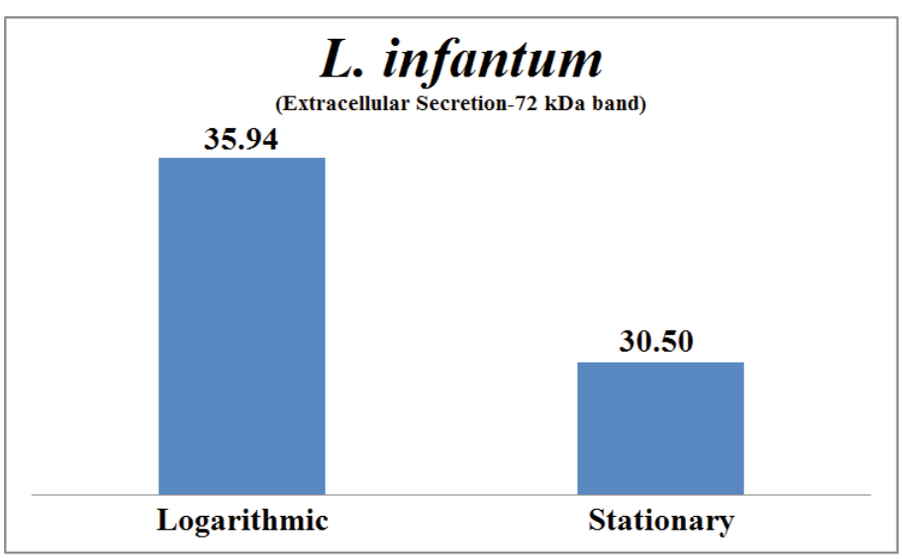

RPMI-1640 medium supplemented with 20% FCS, pH 5.0, and incubated at 33°C with 5% CO_2. Moreover, the optimal condition for differentiation of *L. infantum* promastigotes to axenic amastigotes was RPMI-1640 medium containing 20% FCS at pH 5.0, and incubated at 37°C in the presence of 5%

D

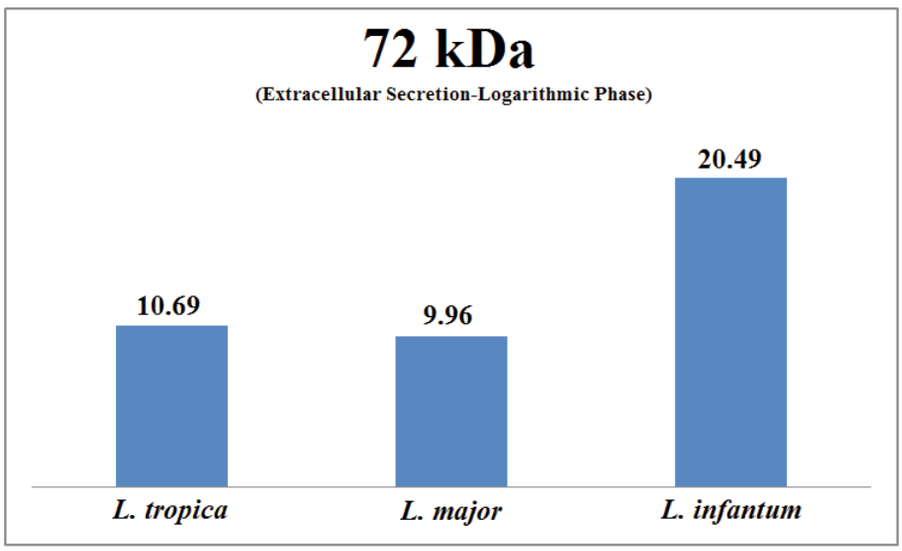

Figure 6. Data in the histograms represent the amount of gelatinase activity in cellular extracts of *L. infantum* (A), extracellular secretions of *L. infantum* between logarithmic and stationary phase (B, C), extracellular secretions of *L. infantum* compared to *L. tropica* and *L. major* in the logarithmic phase (D)

CO_2. This is in conformity with previous studies [37,46].

The other aim of the present study was the assessment of protease activity in axenic amastigotes and whole-promastigote extracts of three *Leishmania* spp. in Fars Province, southern Iran. Metallo-proteinases represent a large group of Ca^{2+}– dependent and Zn^{2+}– containing endopeptidases, involved in degradation of the ECM molecules and play a major role in cell behaviors such as cell proliferation, migration (adhesion/dispersion), differentiation, angiogenesis, apoptosis, and host defense [24]. In addition, these molecules are concerned with the cleavage of cell surface receptors, the release of apoptotic ligands (such as the FAS ligand), and chemokine/cytokine inactivation [14,24]. Due to the sensitivity of gel zymography, being able to detect protease activity from as little as picograms of protein, zymograms are best interpreted as qualitative data, and densitometry analysis of bands exhibited by zymograms may not correlate with protein expression as determined by Western immunoblotting. Although MMPs exist in two forms, as a 'latent' zymogen and an 'activated' form following cleavage of the propeptide, zymography is able to detect activity for both of these forms, and so multiple bands for a single enzyme are to be anticipated [39,47].

Proteases of *Leishmania* spp. play major roles in the establishment of infection, inhibition of host immune responses, survival and pathogenicity. The major surface protease of 63 kDa (gp63) has been implied in the resistance to complement-mediated lysis, binding of promastigotes to macrophages *via* receptor-mediated endocytosis, protection against degradation within macrophage phagolysosome, and migration through the ECM [27,48,49]. Various studies have appraised the existence and amount of protease activities in different *Leishmania* spp. The cysteine-, metallo-, serine-, and aspartic-proteinase activities of *L. amazonensis* promastigotes differentiation into amastigotes have been well explored and described. Results disclose that cysteine-proteinase activity heightened but metallo, serine-, and aspartic-proteinase activities were downregulated during the promastigote transformation into amastigote-like forms [50]. Furthermore, differential expression of protease profiles in virulent and avirulent promastigotes of *L. amazonensis* has been depicted. The surface protein profiles exhibited two great polypeptides of 65–60 and 50–47 kDa that were expressed in both virulent and avirulent promastigote forms, but polypeptides of 115, 52, 45, 32, and 25 kDa were expressed in the virulent forms [51]. Moreover, proteolytic activities of 5 strains of *L. braziliensis* secluded from Brazilian and Colombian patients, were characterized and compared using whole-promastigote extracts and extracellular secretions. Zymographic assays regarding whole-cell extracts and supernatants resulted in the discovery of high molecular weight bands, ranging from 50 to 125 kDa [52]. In addition, the proteolytic profile of *L. peruviana* and *L. braziliensis* isolates from Peru were investigated and compared by zymographic analysis in SDS-PAGE copolymerized with gelatin.

L. peruviana isolates showed three proteolytic bands with molecular masses ranging from 55 to 80 kDa, whereas *L. braziliensis* isolates disclosed six proteolytic activities between 55 and 130 kDa [53]. In our study, we demonstrated the protease activities between 66 and 180 kDa in cellular extracts of axenic amastigotes, and between 66 and 72 in extracellular secretions of *L. infantum*, *L. tropica*, and *L. major*. It is important to emphasize that the nonexistence of other proteases is restricted to gelatin digestion, as the activities might be detectable using other substrates. Moreover, differences observed among the protease profiles of the three *Leishmania* spp. analyzed herein might signify geographical specialization of metalloprotease enzymes to distinct vectors involved in the transmission of the *Leishmania* spp. in Iran. In Iran, *L. infantum* is transmitted by *Phlebotomus major*, whereas *Ph. sergenti* and *Ph. papatasi* are the incriminated vectors for *L. tropica* and *L. major*.

In this study, we noted an individual variability in the levels of proMMP-2, MMP-2, proMMP-9 and MMP-9 in the cellular extracts and extracellular secretions of three *Leishmania* spp., according to the unequal patterns exhibited by the zymographic bands. This variability may be due to the different growth and development phases that each *Leishmania* spp. presented. There are a few examples of MMP-9 function in the pathogenesis of *Leishmania* sp. infection: in *L. chagasi*-infected mice, production of MMP-9 by macrophages was in agreement with liver damage in VL [36]; elevated levels of mRNA to MMP-2 were recorded in CL patient's ulcers and in macrophages from MCL patients; and upon infection with *L. braziliensis* human macrophages sharply increases the secretion of MMP-9 [54]. It was documented that TNF-α is a major regulator of MMP-9 production during *L. braziliensis* infection, and that tissue destruction may be associated with increasing in MMP-9 produced by $CD16^+$ monocytes and imbalance between MMP-9 and TIMP-1 [23,55]. MMP-9 performs a potential role in ulcer evolvement during CL, and excessive expression of MMP-9 was also correlated with therapeutic failure in CL [54,56]. In tissue samples from patients with MCL, mucosal lesion expansion is connected to excessive MMP-9 release from neutrophils [26]. In dermal tissue segments from CL patients under treatment, enlarged MMP-2 levels together with a high MMP-2 to TIMP-2 ratio was informed to be fundamental for successful cutaneous wound re-epithelialization and therapeutic success [56].

In serum samples from dogs naturally infected by *L. chagasi*, both proMMP-2 and proMMP-9 and also activated MMP-9 were upregulated, while MMP-9 and proMMP-9 were mutually related in infected dogs [57]. Exalted proMMP-2 and proMMP-9 expression levels were discovered in the brain from dogs infected with *L. chagasi* [58]. In the CSF of dogs with cerebral leishmaniosis, there was an enormous presence of active MMP-2, whereas solely the levels of both proMMP-2 and proMMP-9 were elevated in the serum [59]. These results recommend a role for MMP-2 in the neurological alterations of dogs with VL, particularly in the interruption of the cerebral barriers. Moreover, the interaction between proMMP-2 and GP63 could lead to activation of MMP-2 in dogs with VL [59]. In *L. chagasi* infected dogs, proMMP-9 is involved in the blood-brain barrier (BBB) disruption, since MMP-9 is implicated in the late phase of BBB failure. Hence, canine leishmaniosis is demonstrated by chronic immune stimulation and a raised high rate of T-lymphocyte influx into the CNS [57,58]. In addition, both the latent and activated forms of MMP-9 were discerned in the CSF of dogs infected with *L. chagasi* [38]. In the skin of dogs with VL, MMP-2 is released in larger concentrations in the beginning phase of the infection and MMP-9 becomes superior through chronic progression of the disease. Fibroblasts and cells of the dermal inflammatory infiltrate activated by the parasite, contributed towards higher expression of MMP-9 [60].

In addition to *Leishmania* spp., gelatinases are produced in other protozoan and helminth infections [32]. MMP-9 plays an important role in cerebral *Plasmodium falciparum* pathogenesis, increasing blood brain barrier permeability and infiltration of leukocytes. Especially, MMP-9 seems to be induced by the hemozoin at the endothelial cell level [61]. Besides, serum levels of TIMP-1 are connected with disease severity [62]. In the CSF of *Trypanosoma gambiense*–infected patients, MMP-2 and MMP-9 were considerably induced and were correlated with the presence of parasites and leukocytes in the CSF. This suggested that gelatinase activity participated in the infiltration into the CNS. Both MMP-2 and MMP-9 were assigned a critical role for leukocyte penetration of the outer parenchymal basement membrane into the brain parenchyma by the cleavage of the transmembrane anchor protein β-dystroglycan [26,63,64]. In Chagas disease, which is caused by *Trypanosoma cruzi*, high MMP-2 and

MMP-9 proteolytic activity raised inflammation, induced acute myocarditis and worsened the prognosis for the infected animals [31,65,66]. Moreover, plasma levels of MMP-9 are associated with the severity of Chagas' myocarditis [67]. Furthermore, MMP-2 and MMP-9 are involved in inflammation and cardiac remodeling in patients with the indeterminate and cardiac clinical forms of Chagas disease [65,67–70].

Toxoplasma gondii is an obligate intracellular parasite with a global distribution and a broad host range. Inflammation of the small intestine following oral infection with *T. gondii*-containing cysts has been investigated previously. IL-23 is basic for ileitis development and it forces the upregulation of MMP-2 and MMP-9 in the small intestine after *T. gondii* infection. Infection-induced MMP-2 is a key contributor in *T. gondii*-induced ileitis and may be an appropriate target for therapy [71]. Also, during *T. gondii*–induced immune response and inflammation, IL-1, IL-23, TNF-α, and cyclooxygenase-2 (COX-2) encouraged the manufacture of MMP in the brain [72].

Naegleria fowleri is a free-living and brain-eating amoeba discovered in freshwater lakes, ponds and pools and is the causative agent of primary amoebic meningoencephalitis (PAM). *N. fowleri* trophozoites secrete MMPs that play a role in motility, pathogenicity and invasion of the CNS. MMP-2 and MMP-9 are produced by *N. fowleri* trophozoites. MMP-2 is an inherent membrane protein in *N. fowleri* trophozoites, while MMP-9 is either a peripheral membrane protein or is contained in the cytosol. Accordingly, MMP-2 plays a more critical role in the invasive process for *N. fowleri* [33]. Moreover, *Acanthamoeba* spp. can pass into the human CNS and result in granulomatous amoebic encephalitis (GAE). A consequential upregulation of MMP-2 and MMP-9 levels in the cerebral cortex of *Acanthamoeba* spp. infected immunocompetent and immunosuppressed mice was demonstrated [34]. Intensified MMP-2 synthesis in the brains of mice infected with *Acanthamoeba* spp. may be allied with renewing processes that happen due to the beginning of CNS damage by this parasite [34].

In conclusion, this work is an initial study demonstrating the presence of a meaningful difference in protein profiles between three *Leishmania* spp. It is not known whether these differences are indicators of the different cutaneous and visceral pathologies of these parasites. It is important to assess the MMPs and their TIMPs in skin lesions and serum samples of patients infected with these three *Leishmania* spp. Further work requires to be performed to analyze molecular and genetic characterization of zymograms of these protozoa.

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