Prediction of potential drug targets for cutaneous leishmaniasis by *Leishmania major* and *Leishmania tropica:* a quantitative proteomics and bioinformatics approach

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***Leishmania* spp. cause life-threatening infectious diseases which affect universal health. Novel treatments for leishmaniasis are crucially needed since those available are limited by emerging drug-resistant species, low efficacy and side effects. In this study, we have employed a quantitative shotgun proteomics and bioinformatics method to identify differentially expressed proteins (DEPs) between *Leishmania major* and *Leishmania tropica* and to detect novel potential drug targets for cutaneous leishmaniasis, which may aid in the future drug discovery process. A total of 57 proteins were differentially expressed between the studied species. Based on KEGG pathway analysis, the more upregulated proteins in *L. major* are clearly related to proteasome and metabolic pathways. In *L. tropica*, most of the upregulated proteins are related to the metabolic pathway and carbon metabolism. According to gene ontology analysis based on biological process, the upregulated proteins mainly participated in translation and carbohydrate metabolism in *L. tropica* and *L. major* respectively. We have constructed a protein–protein interaction network that is common for the two species. We detected the top 10 potential targets for drug design by topology analysis of the protein network. Additional *in vivo* studies are needed to confirm these targets. We have identified several new DEPs between the species which would help in the understanding of pathogenesis mechanisms, and offer potential drug targets and vaccine candidates. Analysis of the predicted protein network provides a catalogue of key proteins, which can be considered in future studies to be validated as druggable targets against cutaneous leishmaniasis.**

Keywords: Cutaneous leishmaniasis, *Leishmania tropica*, *Leishmania major*, protein interaction network, quantitative proteomics.

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LEISHMANIA spp. cause leishmaniasis, a vector-borne disease. Clinical manifestation of the disease includes simple, self-limiting, cutaneous lesions, severe mucocutaneous and fatal visceral disease[1]. Leishmaniasis is classified as one of the neglected tropical diseases by the World Health Organization because 98 tropical and subtropical countries are known to be endemic to this disease, with an estimated risk to 350 million people, prevalence of 12 million infected subjects and 0.9–1.6 million new patients each year worldwide[2]. Iran is one of the endemic regions to cutaneous leishmaniasis (CL), which is mostly caused by *Leishmania major* and *Leishmania tropica*[3,4]. *Leishmania* is a digenetic organism which shifts between the flagellated, free-living promastigotes (procyclic and metacyclic) form and a non-motile and intracellular amastigote form. Given the technical difficulty in generating large quantities of amastigotes, we used logarithmic-phase procyclic promastigotes in this study. Due to lack of effective drugs and vaccines against leishmaniasis, it remains a major health issue worldwide. The present anti-leishmanial treatment relies on pentavalent antimony (including Pentostam and Glucantime) therapy. This classical treatment is largely unsuccessful due to toxicity, *Leishmania* species diversity, and differences in their susceptibility to drugs and varying sensitivity of host immune response. Severe side effects and the emergence of drug-resistant species are major problems in many endemic regions[5,6]. Thus, new and safe compounds are necessary. Over the past few years, proteomics study based on mass spectrometry (MS) has expanded its role in almost all diverse research fields of science. As drug discovery is an inherently complex and expensive process, new emerging technologies such as proteomics integrated with bioinformatics can accelerate this process. The drug discovery process has many stages in which proteomics plays a major role in target identification as the first step in the process. Extensive research has provided information on *Leishmania*

biology, human host–parasite interaction and identification of various protein targets for vaccine development[7]. 'Omics' techniques are popular in disease phenotype and parasite biology research[2,8]. Indeed, proteomics along with computational biology has provided a snapshot of the biological activity of the *Leishmania* parasite[9–11]. Several procedures used in proteomics study including label-free and labelled methods for detection of quantified proteins. In the label-free technique, proteins/peptides are quantified based on the precursor signal intensity or on spectral counting with mass analysers[12,13]. *Leishmania* proteome analysis is done by a combination of two-dimensional electrophoresis (2-DE) and MS. In general, label-free proteomics methods to study *Leishmania* are at early stage[14]. In this study, we used sequential window acquisition of all theoretical mass spectra (SWATH-MS) approach to compare the protein expression of *L. tropica* and *L. major*, to predict novel potential drug targets by documenting proteome differences of these causative agents of cutaneous leishmaniasis. The cells were collected from both species at the same phase of promastigotes (log phase) and subjected simultaneously to label-free SWATH-MS protein profiling. We looked for proteins that are differentially expressed in either *L. tropica* or *L. major* based on fold change to predict protein–protein interaction network. Protein network data were analysed to identify novel potential drug targets which are applicable to both species. This is the first step for experimental validation using *in vitro* and *in vivo* assays for novel therapies.

Materials and methods

Parasite sample

This study was performed on clinically suspected samples of CL referred to medical diagnostic laboratories for parasite diagnostic tests. Smears were prepared using the edges of skin lesions of suspected cases. Then, fixation and staining of smears were performed with methanol and Gimsa respectively. Search for leishman bodies in each smear was done using a light microscope. The positive samples for leishmaniasis primarily were cultured in Novy–Nicolle–Mc Neal (N.N.N.) medium at 24°C to obtain the procyclic promastigote forms (log phase). This study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran. All patients participating in the study gave written informed consent. The PCR–RFLP method was used to detect *Leishmania* species (*L. major* and *L. tropica*). In order to obtain a massive volume of parasites, they were cultured in RPMI1640 medium supplemented with %10 FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (all Gibco, Germany). The parasites were cultured for 3–5 days for collection of procyclic promastigotes phase. After bulk culturing of parasites and gaining 10^7 cells, they were collected by centrifugation at 3500 rpm for 20 min and then washed three times with sterile PBS (pH: 7.4) and stored in –70°C.

Quantitative LC-MS/MS and data analysis

The cells (1×10^7 *Leishmania* cells/ml) were collected and dissolved using lysis buffer with 8M urea, dithiothreitol (DTT), Tris-HCl, glycerol, Tween-20 and 1X protease inhibitor cocktail, and then incubated for 2 h at room temperature. The cell lysate was centrifuged at 15,000 *g* for 15 min at 4°C to remove cell debris. Supernatant was isolated and centrifuged at 15,000 *g* for 15 min. The 2D Quant (Cytiva (ex GE Healthcare)) kit was used to measure protein concentration of samples. The soluble proteins were precipitated as single-use aliquots and maintained at –70°C until further use. Quantitative shotgun proteomics analysis (SWATH-LC-MS/MS) was performed at PhenoSwitch Bioscience, Canada (using ABSciex Triple TOF 5600 instrument). The precipitated protein samples were resuspended in a buffer containing 4M urea and 25 mM Tris (pH 8.0). Digestion and acidification of proteins were performed with 1 µg of trypsin/LysC (lysine) overnight at 37°C and 2% formic acid. For the SWATH mode, 5.5 kV at 225°C was used. Separation step was performed on a reverse-phase HALO C18-ES column 0.3 mm i.d., 2.7 µm particles, 150 mm long (Advance Materials Technology, Wilmington, DE, USA) that was preserved at 60°C. Ion library for sample analysis was obtained using ProteinPilot software running on the 12 IDA Wiff files with the mixed proteins from *L. major* taken from the UniProt database. The quantification of proteins in each studied sample was done using the obtained ion library in the Peakview software (ABSciex), utilizing three transition/peptide and six peptide/protein maximum. A score higher than 1.5 and false discovery rate (FDR) < 1%, calculated using Peakview software, were considered as sufficient measure of the peptide. Student's *t* test was used to analyse quantitative variables, and $P < 0.05$ and fold change (FC) > 2 were considered significant.

Real-time PCR validation

From the significantly altered proteins, increased levels pyruvate kinase (PK) and glutathione peroxidase (TDPX) in *L. tropica* and increased levels of phosphoglycerate kinase (PGK) and tryparedoxin peroxidase (TRYP1) in *L. major* were validated by real-time PCR (RT-PCR). The experiment was performed using the manufacturer's instructions as follows: Procyclic promastigotes of samples (10^7) were used to extract total RNA employing the RiboEx kit (GeneAll Biotech, Korea). RNA quantification was done with the Nano Drop device (ND-1000, Thermo Fisher Scientific, USA). For the synthesis of

complementary DNA (cDNA), RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific), was used with 3 µg of total RNA. Primers of target genes in this study were designed using Gene Runner software, Version 6.5.50 (www.generunner.net). The sequences of primers used in real-time PCR are as follows: PK (F: CTAACGCGCACACGATCTCT, R: AAGATCATGTCCACGCCCTG), TDPX (F: TTCATGGTACTGGCGTTCCC, R: ACCCTTGCACGTGTTCTTCA), PGK (F: GAGATGCCGTGCTACTCGAA, R: CATGCGACATGTTCTTCGCC), TRYP1 (F: AGTCGCTTCAACGAGCT, R: CTTGTCGGCTAGCATTG) and GAPDH (F: GAAGTACACGGTGGAGGCTG, R: CGCTGATCACGACCTTCTTC). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was chosen as internal gene control. RT-PCR was carried out in 20 µl reactions (including 1 µl cDNA target, 100 nM forward and reverse primers and 1X SYBR Green RealQ Plus Master Mix (Ampliqon, DK-5230 Odense M, Denmark)). Each sample was performed in triplicate, and repeated three times with similar results using the StepOne TM RealTime PCR System (Applied Biosystems, Life Technologies, USA). The PCR programme was as follows: activation at 95°C for 10 min, amplification at 95°C for 15 sec and 60°C for 1 min for 40 cycles. The expression level of each gene was calculated based on the threshold cycle (CT) value of the studied genes, normalized to that of control gene (GAPDH) using the 2-ΔΔct procedure; the level of significance acceptable was 95% (P-value < 0.05).

GO enrichment and KEGG pathway analysis

The proteins that were considerably altered between *L. major* and *L. tropica* promastigotes (log phase) were analysed in TriTrypDB based on their biological processes. The COG database (http://www.ncbi.nlm.nih.gov/COG/) was used to categorize these differentially expressed proteins. Both *L. tropica* and *L. major* promastigote upregulated proteins were analysed using STRING database with links to KEGG to determine the pathways involved.

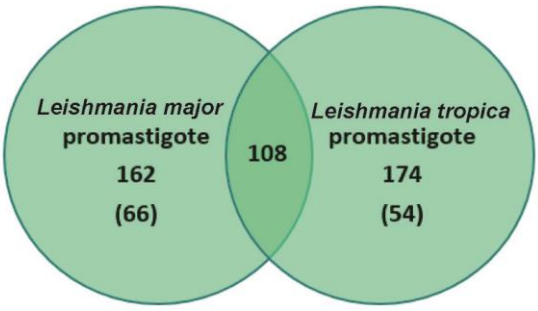

Figure 1. The number of proteins and common proteins identified for *Leishmania tropica* and *Leishmania major* promastigotes. The number of proteins identified only in *L. tropica* or *L. major* is given within brackets.

Protein–protein interaction network analysis

A protein–protein interaction network (PPIN) was constructed for shared proteins in *L. tropica* and *L. major* protein profiles using the STRING server (http://string-db.org). The resulting networks were displayed and analysed using CytoHubba plugin in Cytoscape software, version 3.6.1 based on degree method to identify hub proteins.

Results

Protein expression changes in L. tropica and L. major isolates

Using proteomics experiment (LC-MS/MS), 162 and 174 proteins were detected in *L. major* and *L. tropica* procyclic promastigotes (log phase) respectively (Figure 1, Supplementary Table 1). Comparative analysis of proteins identified from *L. major* and *L. tropica* indicates that the two species share 108 proteins (Supplementary Table 1). Among the proteins, 66 were observed uniquely in *L. major* and 54 in *L. tropica*. Among the shared proteins, 57 were expressed differentially (FC > 2 and P-value < 0.05) while 51 proteins were unchanged between the two species. According to the results, in differentially expressed proteins, upregulation of 30 and 27 proteins was observed in *L. tropica* and *L. major* respectively (Tables 1 and 2).

Gene ontology enrichment analysis

To gain insights into the biological changes in the procyclic forms of *L. major* compared to those of *L. tropica*, the altered proteins (including those upregulated) were categorized according to the biological process. For the 57 differentially expressed proteins, most of the *L. tropica* upregulated proteins were associated with translation and amino acid metabolic process (Figure 2 *a*), while most of the *L. major* upregulated proteins were involved in carbohydrate metabolic process, oxidation–reduction process and RNA processing (Figure 2 *b*). Further database mining indicated that both the *L. major* and *L. tropica* upregulated proteins could be categorized into 13 groups according to the cluster of orthologous groups of proteins (COG) function classification (Figure 2 *c*).

PPIN analysis and identifying potential drug targets

The total number of shared proteins identified in *L. tropica* and *L. major* promastigotes (log phase) was analysed using the protein–protein interaction network to identify potential drug targets in cutaneous leishmaniasis by the two species (Figure 3). The top 10 hub (key) proteins

Table 1. Proteins quantified (up-regulated) in *Leishmania tropica* promastigotes

Protein	Protein ID	Gene	Gene ID	Chromosome no.	FC
Probable eukaryotic initiation factor 4A	O62591	LmjF.01.0770	12983087	1	2.5
40S ribosomal protein SA	Q4Q0Q0	LmjF.36.5010	5655803	36	2.4
Putative methylmalonyl-coenzyme a mutase	E9AD07	LmjF.27.0300	12982958	27	5.6
Putative paraflagellar rod protein 1D	E9AE37	LmjF.29.1760	12981038	29	4.2
Pyruvate kinase	E9AEI0	LmjF.35.0030	12982187	35	2.6
Putative NADH-dependent fumarate reductase	E9AEU1	LmjF.35.1180	12982345	35	2.2
Arginase	E9AEX1	LmjF.35.1480	12982375	35	3.7
Putative 60S ribosomal protein L2	Q4FWX5	LmjF.32.3900	5656514	32	3.4
Adenosylhomocysteinase	Q4Q124	LmjF.36.3910	5655685	36	2.9
Cysteine synthase	Q4Q159	LmjF.36.3590	5655650	36	7.3
Clathrin heavy chain	Q4Q1R2	LmjF.36.1630	5655418	36	3.3
Putative 2,4-dienoyl-coa reductase *fadh1*	Q4Q4A9	LmjF.33.0830	5654493	33	3.6
Glutamate dehydrogenase	Q4Q7X1	LmjF.28.2910	5653506	28	4.0
40S ribosomal protein S14	Q4Q8H1	LmjF.28.0960	5653305	28	2.4
40S ribosomal protein S26	Q4Q8L6	LmjF.28.0540	5653260	28	2.7
Glutathione peroxidase	Q4Q9B4	LmjF.26.0800	5652808	26	2.8
GTP-binding nuclear protein	Q4Q9V1	LmjF.25.1420	5652553	25	7.2
Putative cytochrome c oxidase VII	Q4Q9Y0	LmjF.25.1130	5652524	25	3.8
Putative calpain-like cysteine peptidase	Q4QCS9	LmjF.20.1180	5651470	20	2.6
Glycosomal malate dehydrogenase	Q4QDF0	LmjF.19.0710	5651170	19	3.6
Putative fucose kinase	Q4QEX6	LmjF.16.0440	5650589	16	2.3
Putative glutaminyl-tRNA synthetase	Q4QF36	LmjF.15.1440	5650529	15	2.6
NAD-specific glutamate dehydrogenase	Q4QF83	LmjF.15.1010	5650482	15	3.9
Putative 60S ribosomal protein L13a	Q4QFG2	LmjF.15.0200	5650376	15	7.9
40S ribosomal protein S12	Q4QG97	LmjF.13.0570	5650086	13	5.8
Glucose-6-phosphate isomerase	Q4QGN9	LmjF.12.0530	5649944	12	3.8
Putative 40S ribosomal protein S15A	Q4QGW3	LmjF.11.1190	5649870	11	5.7
Putative 40S ribosomal protein S9	Q4QIM3	LmjF.07.0680	5649227	7	2.1
ATPase alpha subunit	Q4QJF1	LmjF.05.0500	5648912	5	2.6
Nucleoside diphosphate kinase	Q9U1E1	LmjF.32.2950	5656416	32	8.1

The list includes upregulated proteins in *L. tropica* procyclic promastigotes compared to *L. major* procyclic promastigotes in Iranian isolates using shotgun proteomics (SWATH-MS) analysis. Upregulated proteins are those with FC > 2 and *P*-value < 0.05. FC, Fold change.

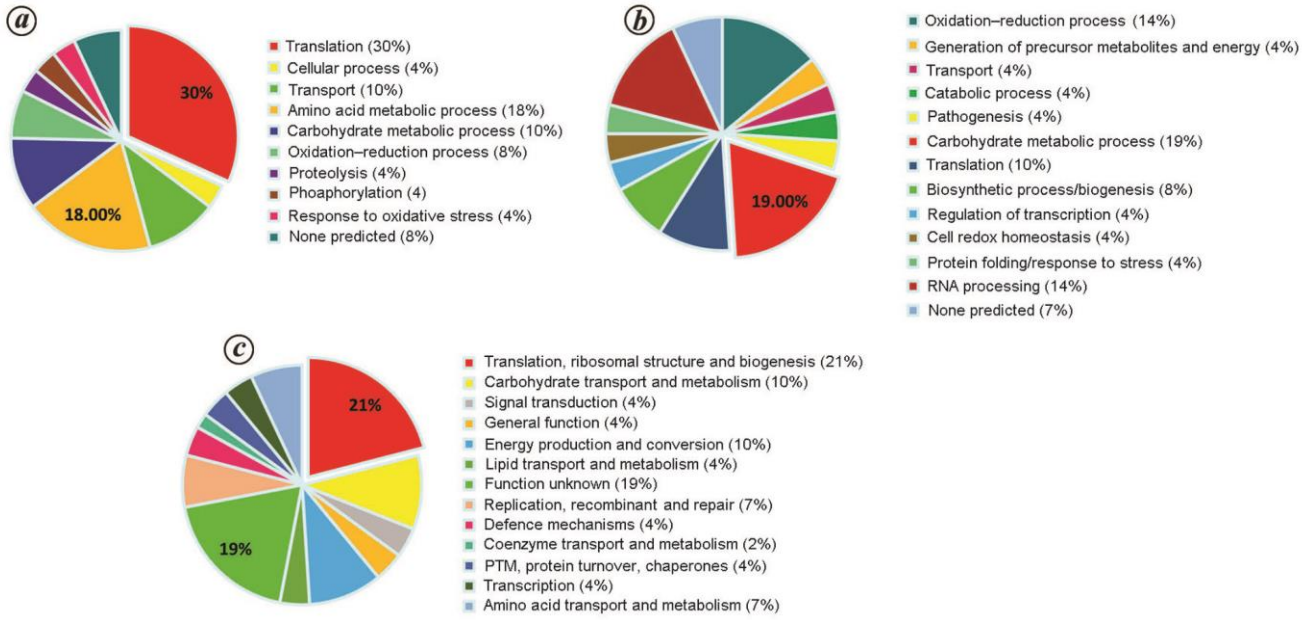

Figure 2. Clustering of gene ontology enrichment based on biological process (GOBP) for upregulated proteins observed in (***a***) *L. tropica* and (***b***) *L. major* promastigote stage, and (***c***) cluster of orthologous groups (COG) function classification. The proteins that were considerably upregulated in promastigotes stage of *L. tropica* and *L. major* were analysed on TriTrypDB based on the biological processes they are involved in. PTM, Post-transcriptional modification.

Table 2. Proteins quantified (upregulated) in *L. major* promastigotes

Protein	Protein ID	Gene	Gene ID	Chromosome no.	FC
Guanosine monophosphate reductase	Q4QEB3	LmjF.17.0725	5650809	17	2.3
Putative carboxylase	E9ABZ4	LmjF.01.0050	12983098	1	2.0
Putative fumarate hydratase	E9AE57	LmjF.29.1960	12981057	29	6.4
Putative ATP-dependent DEAD-box RNA helicase	E9AEL4	LmjF.35.0370	12982223	35	2.0
Putative threonyl-tRNA synthetase	E9AEW4	LmjF.35.1410	12982368	35	4.7
Putative ATP-dependent RNA helicase	E9AFD4	LmjF.35.3100	12980464	35	5.2
Polyadenylate-binding protein (PABP2)	E9AFP0	LmjF.35.4130	12980570	35	3.8
hypothetical protein	Q4Q079	LmjF.36.6760	5655991	36	2.4
hypothetical protein	Q4Q0H5	LmjF.36.5850	5655890	36	2.7
Putative universal minicircle sequence binding protein (UMSBP1)	Q4Q1R4	LmjF.36.1610	5655416	36	3.7
Putative heat shock protein	Q4Q3U8	LmjF.33.2390	5654663	33	7.4
Putative ATP-dependent RNA helicase	Q4Q5P5	LmjF.32.0400	5656149	32	6.4
Putative 26S proteasome regulatory subunit	Q4Q5P6	LmjF.32.0390	5656148	32	2.1
Putative RNA binding protein rbp16	Q4Q8I6	LmjF.28.0825	5653290	28	4.7
Succinate–CoA ligase [ADP-forming] subunit alpha, mitochondrial	Q4Q9M4	LmjF.25.2130	5652695	25	3.5
Ribosomal protein S25	Q4Q9X4	LmjF.25.1190	5652530	25	11.7
Putative cytochrome c oxidase subunit 10	Q4QBD7	LmjF.23.0370	5651997	23	2.9
Putative NADP-dependent alcohol dehydrogenase	Q4QBD8	LmjF.23.0360	5651996	23	2.3
Mannose-1-phosphate guanyltransferase	Q4QBG5	LmjF.23.0110	5651955	23	9.6
Phosphoglycerate kinase	Q4QD34	LmjF.20.0100	5651282	20	3.9
Inosine-5′-monophosphate dehydrogenase (IMP dehydrogenase)	Q4QD53	LmjF.19.1560	5651267	19	8.3
Putative 60S ribosomal protein L10a	Q4QDX9	LmjF.36.3760	5655670	36	6.9
Tryparedoxin peroxidase	Q4QF80	LmjF.15.1040	5650485	15	2.2
Cytochrome c oxidase subunit IV	Q4QGM6	LmjF.12.0670	5649957	12	2.6
Pyruvate, phosphate dikinase	Q4QGX9	LmjF.11.1000	564985	11	3.5
Histone H2B	Q4QHP1	LmjF.09.1340	5649574	9	7.2
6-phosphogluconate dehydrogenase (Fragment)	Q6Y9R4	–	–	–	4.7

The list includes upregulated proteins in *L. major* procyclic promastigotes compared to *L. tropica* procyclic promastigotes in Iranian isolates using shotgun proteomics (SWATH-MS) analysis. Upregulated proteins are those with FC > 2 and *P*-value < 0.05.

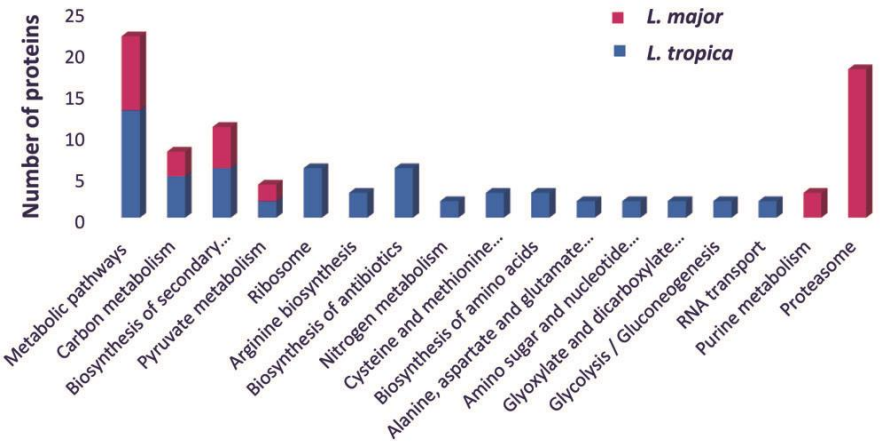

Figure 3. Pathway analysis on KEGG showing the most changed pathways in *L. tropica* and *L. major* promastigotes stage.

were selected by degree method (Table 3). EF2-1 (elongation factor 2-1) was the top score hub protein by the degree method and the other hubs included LmjF.36.5120 (40S ribosomal protein SA), LmjF.04.0950 (60s ribosomal protein L10), LmjF.32.3900 (60s ribosomal protein L2), LmjF.15.0200 (60s ribosomal protein L13a), RPL11 (60s ribosomal protein L11), RPL10a (60s ribosomal protein L10a), LmjF.18.0740 (elongation factor Tu), LmjF.29.1800 (40s ribosomal protein S15A) and LmjF.35.0030 (Pyruvate kinase). These hub proteins could be potential drug targets in leishmaniasis control (Figure 4).

Quantitative real-time PCR results

To verify the differentially expressed proteins (DEPs) generated by the proteomic experiment, the relative expressions of four target genes were evaluated by quantitative

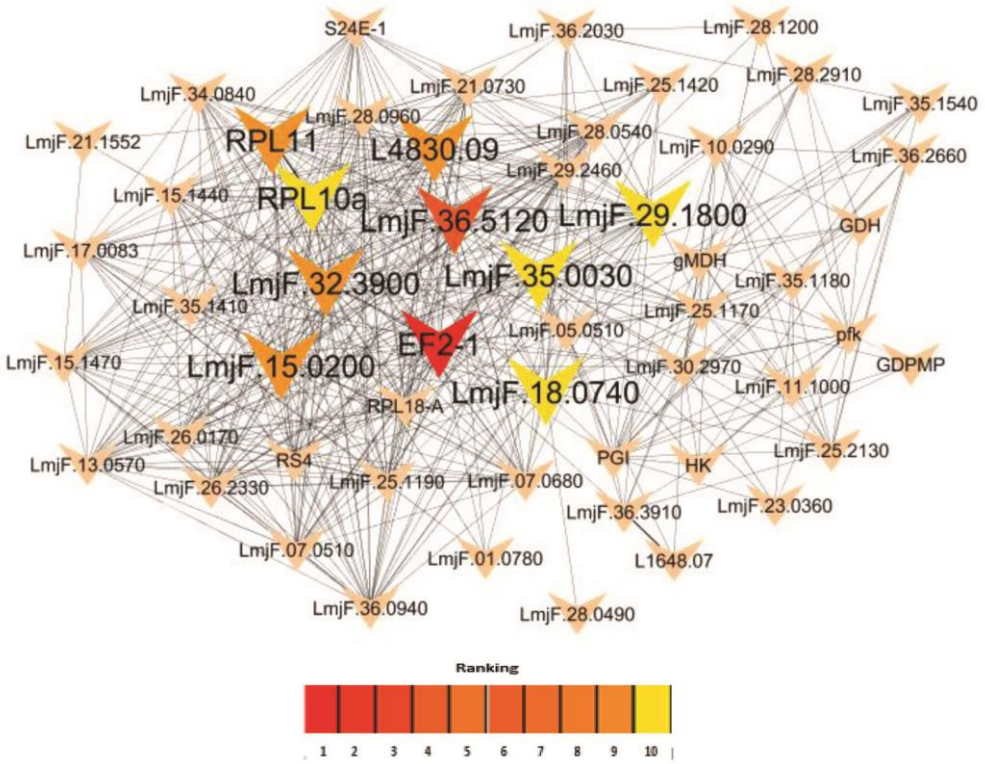

Figure 4. Protein–protein interaction network of *L. tropica* and *L. major*. The hub (high degree) proteins are shown bigger and dark coloured.

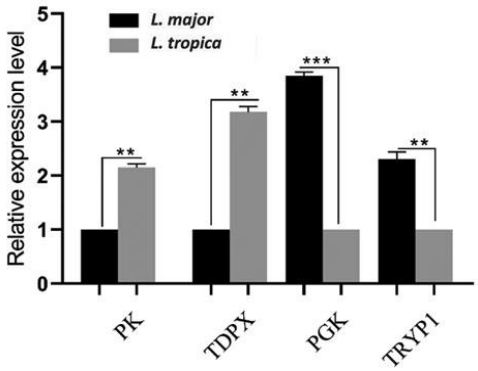

Figure 5. Relative gene expression of four selected genes between *L. major* and *L. tropica* by real-time PCR. The expression level of GAPDH gene was used as internal control. Values are mean ± SD of three independent analyses ($P < 0.05$). PYK, Pyruvate kinase; TRYP1, Tryparedoxin peroxidase; PGKC, Phosphoglycerate Kinase C; TDPX, Glutathione peroxidase; $^{**}P < 0.01$, $^{***}P < 0.001$: Significant alteration of gene expression in *L. major* compared to *L. tropica*.

Table 3. Top 10 hub proteins of *L. tropica* and *L. major* interaction network ranked by degree method

Hub gene ID	Hub protein	Score
EF2-1	Elongation factor 2-1	34
LmjF.36.5120	40s ribosomal protein SA	31
L4830.09 (LmjF.04.0950)	60s ribosomal protein L10	30
LmjF.32.3900	60s ribosomal protein L2	30
LmjF.15.0200	60s ribosomal protein L13a	30
RPL1 (LmjF.22.0030)	60s ribosomal protein L11	30
RPL10a	60s ribosomal protein L10a	29
LmjF.18.0740	Elongation factor Tu	29
LmjF.29.1800	40s ribosomal protein S15A	29
LmjF.35.0030	Pyruvate kinase	29

real-time PCR method. Sample collection conditions for proteomics and real-time methods were the same. The genes analysed using qPCR included pyruvate kinase (E9AEI0), glutathione peroxidase (Q4Q9B4), phosphoglycerate kinase C (Q4QD34) and tryparedoxin peroxidase (Q4QF80). The results confirmed that the expression levels of pyruvate kinase ($P < 0.01$) and glutathione peroxidase ($P < 0.01$) in *L. tropica* were higher than those in *L. major*, while the expression levels of phosphoglycerate kinase C ($P < 0.001$) and tryparedoxin peroxidase ($P < 0.01$) had decreased significantly in *L. tropica* compared to *L. major* (Figure 5).

Discussion

In this study, SWATH-MS proteomics analysis has been used to comprehensively outline the protein expression profiles of the procyclic (log phase) promastigotes of *L. tropica* compared to *L. major* in Iranian isolates, to uncover differences and species-specific *Leishmania* proteins of these two species. The results of this study can lead to identification of pathogenesis differences and the

presentation of common, novel and potential druggable proteins for urban and rural leishmaniasis caused by these species. Totally, 57 proteins were observed to be altered between *L. tropica* and *L. major*. Among the differentially expressed proteins identified, 30 were upregulated in *L. tropica* and 27 in *L. major* procyclic promastigotes. According to cluster of orthologue groups (COG) results, a cluster of proteins involved in translation, ribosomal structure and biogenesis displays significant differential expression. These were six and four upregulated proteins in *L. major* and *L. tropica* respectively. The cluster protein members play important roles in protein synthesis and assembly. Differences in the expression of the cluster indicate that protein expression may be distinct in *L. tropica* and *L. major*. Among this group, poly (A)-binding proteins (PABPs) have potential beneficial effects on post-transcriptional regulation of gene expression. Previous studies suggest that PABPs possibly interact with components of polyadenylation complex that facilitate PABP binding to newly synthesized poly(A) tails. Therefore, the conformation of mRNA closed-loop accelerates and eventually stimulates the protein translation and protecting versus PARN-mediated deadenylation[15]. The other largest clusters of identified proteins include energy production and conversion, and carbohydrate transport and metabolism groups. The energy production and conversion cluster consists of six differentially expressed proteins, including five upregulated proteins in *L. tropica* and one overexpressed protein in *L. major*. Overexpressed proteins in *L. tropica* in this cluster include putative fumarate hydratase, putative NADH-dependent fumarate reductase, putative 2,4-dienoyl-coa reductase fadh1, malate dehydrogenase and ATPase alpha subunit. Among these, fumarate hydratases are enzymes that catalyse the reversible hydration of fumarate to s-malate. In general, eukaryotic cells have two isoforms of fumarate hydratase enzyme: a cytosolic and a mitochondrial isoform[16,17]. There are several studies on human hydratases, but limited studies available on the role of fumarate hydratase in other organisms. For example, a RNA interference-based study showed that fumarate hydratase (FH) is vital for viability of procyclic form of *Trypanosome brucei*[18]. According to recent studies, the *Leishmania* genus contains two genes that encode different assumed FH enzymes. One of these genes is LmjF.29.1960 that encodes the putative LmFH-2 protein. Feliciano *et al.*[19] have confirmed FH activity in *Leishmania* genus. On the other hand, intracellular localization studies showed that LmFH-2 is located predominantly in the cytosol and possibly in the glycosome. Since different species of *Leishmania* have a high degree similarity in their genomic sequences, and also due to the important role of this enzyme in energy metabolism, FHs may be suggested as targets for anti-leishmanial drugs[19]. The other enzyme in this cluster is NADH-dependent fumarate reductase (FRD), which reduces fumarate to succinate. In most of the organisms, it is bound to the membrane and uses electron donors such as quinol. These enzymes can be classified into two groups: (i) those associated with respiratory chain complex, which transfer electron from quinol to fumarate, and (ii) soluble group and transferring electrons from NADH or $FADH_2$ cofactors to fumarate[20]. There is no information available on fumarate reductase in *Leishmania*. Recently, Coustou *et al.*[21] showed that *T. brucei* expresses a soluble FRD called glycosomes. This enzyme participates in the production of almost 70% of the end-product of glucose metabolism, namely succinate[21]. The high levels of this enzyme in *L. tropica* may be due to high levels of glucose consumption and energy production in this species. Glycosomal malate dehydrogenase is the other overexpressed enzyme in energy production and conversion cluster. According to Leroux *et al.*[22] the three MDHs (including cytosolic, glycosomal and mitochondrial malate dehydrogenase) are developmentally regulated. At the protein level, these isozymes are significantly more abundant in amastigote forms than in promastigote forms of *L. mexicana*. It has been reported that the three isoforms of MDH show little difference in biochemical characterization and subcellular localization in *Leishmania* spp. Probably the functional and biochemical aspects of these isozymes indicate the metabolic adaptation to various nutrient environments that these parasites are exposed to during their developmental stages[22]. Westrop *et al.*[23] validated that lysates of *Leishmania* showed malate dehydrogenase activity converting oxaloacetate to malate with NADH as the reducing agent and with special activities for *L. donovani*, *L. major* and *L. mexicana*. *L. tropica* and *L. major* in Iranian isolates show multiple differences in energy metabolism and metabolic pathways. Our results indicate six differentially identified proteins grouped in carbohydrate transport and metabolism that is similar to energy production cluster in terms of protein numbers. The two main proteins of this group are pyruvate kinase and glucose-6-phosphate isomerase. Pyruvate kinase catalyses the transfer of a phosphate group from phosphoenolpyruvate to adenosine diphosphate in the final step of the glycolysis process, yielding one molecule of pyruvate and ATP. Trypanosomatid genomes code for a large number of protein kinases. Study about active-site motif of protein kinase in predicted proteins indicate that there are roughly 199 protein kinases encoded by the *L. major* genomes. The predicted kinome constitutes 2% of genes encoding the predicted proteins. Therefore, the kinase gene family also shows a full family of potential protein targets to follow for anti-kinetoplastid agents[24]. Merritt *et al.*[25] provided data to show that kinases are druggable targets in trypanosomatid protozoan parasites[25]. Glucose-6-phosphate isomerase is another differentiated enzyme between *L. tropica* and *L. major* in the present study. It is a cytoplasmic enzyme that catalyses the reversible conversion of D-glucose 6-phosphate into D-fructose 6-phosphate. In addition,

glucose-6-phosphate isomerase is present in both cytosol and glycosome of *Leishmania* promastigotes, and represents a potential target in the drug design process[26]. The amino acid transport and metabolism cluster consisted of four differentially expressed proteins (including Arginase, Cysteine synthase, NAD-specific glutamate dehydrogenase, Glutamate dehydrogenase) all of which had higher expression in *L. tropica* compared with *L. major*. Hydrolysis of L-arginine to L-ornithine and urea is performed by arginase as a metalloenzyme. In addition, arginase activity has been observed in *Leishmania*. In both promastigote and amastigote forms, arginase is localized in the glycosome showing that arginine trafficking in the cell is used to supply the optimal concentration of substrate for arginase. Arginine uptake and arginase activity are crucial functions in establishing and maintaining *Leishmania* infection[27]. The first enzyme of the polyamine pathway in *Leishmania* is arginase[28]. Studies on *L. donovani* have indicated that both ornithine decarboxylase and spermidine synthase (enzymes of the polyamine biosynthetic pathway) are vital for promastigote cell proliferation and are needed for high infection in mice[29]. According to Boitz *et al.*[29] arginase is necessary for the survival of *L. donovani* promastigotes but not intracellular amastigote forms. In addition, *Leishmania* encodes its own arginase which is important to modulate its infectivity and pathogenesis. Muleme *et al.*[30] reported that *arg L. major* are impaired in their macrophage infectivity *in vitro* independent of host iNOS activities. The results of the present study show that arginase is expressed in high levels in *L. tropica*. This could indicate that arginase activity is low in *L. tropica*. Furthermore, high expression of arginase could be a compensatory method for further survival and infectivity of *L. tropica*. In proteins involved in amino acid metabolism, the expression of cysteine synthase was increased in *L. tropica* compared with *L. major* in the present study. Williams *et al.*[31] have demonstrated that cysteine is endogenously produced by promastigote cells of *Leishmania.* In the replication, recombination and repair cluster, three helicases were downregulated in *L. tropica* compared with *L. major*, including putative ATP-dependent DEAD-box RNA helicase and putative ATP-dependent RNA helicase. According to this study results, several protein synthesis and RNA processing-related proteins were differentially expressed between *L. tropica* and *L. major*. Among these, probable eukaryotic initiation factor 4A, putative ATP-dependent DEAD-box RNA helicase and putative ATP-dependent RNA helicase as RNA processing protein were differentially expressed between *L. tropica* and *L. major*. RNA helicases are enzymes that catalyse RNA unwinding and are important for various biochemical pathways such as mRNA splicing, ribosome assembly and translational initiation. Mojtahedi *et al.*[32] showed that RNA helicases were expressed abundantly in procyclic stage compared with metacyclic stage of *L. major*. Based on KEGG pathway analysis, more proteins upregulated in *L. major* have been related to proteasome and metabolic processes. In addition, most of *L. tropica* upregulated proteins have been associated with the metabolic pathway, generation of secondary metabolites, carbon metabolism and ribosomes. These differential biochemical pathways in each species can be a possible cause of pathological differences in the resulting cutaneous lesion caused by *L. major* or *L. tropica*. In the present study, the proteins related to Iranian isolates of *L. tropica* and *L. major* were also analysed using the PPIN constructed to identify hub proteins that could be predicted as useful drug targets. Using centrality indices, including node degree, the top-10 high-degree nodes were selected as important proteins in both *L. tropica*, and *L. major* (Table 3). The degree of a node is given by the number of links between the node and other nodes in the network. The nodes with high degree score (hub proteins) are three times more important than other proteins in maintaining network structure. Therefore, hub protein can be introduced as potential drug targets in diseases treatment[33]. As shown in Table 3, all the hubs, except pyruvate kinase belong to the protein synthesis machinery. Pyruvate kinase causes the conversion of phosphoenolpyruvate and ADP to pyruvate and ATP in the glycolysis process, and plays an important role in the regulation of cellular metabolism. In these organisms, the pyruvate kinase enzyme has a crucial regulatory role, and is special in response to fructose 2,6-bisphosphate as an allosteric activator[34]. Given the importance of the energy production cycle and the important role of pyruvate kinase during this cycle, this enzyme could be considered as a potentially important pharmaceutical target. Also, this enzyme is needed for parasite survival in the host environment and also it has differences with its homologous protein in the host[35]. Since procyclic (log phase) promastigote forms of *Leishmania* have rapid cell division and synthetic activity, components related to protein synthesis are important and manipulation of these proteins may lead to new strategies to block differentiation of leishmania *in vitro*. In addition, these key proteins can serve as vaccines and drug target candidates for the two species causing CL. They are important in terms of being able to jointly serve as a pharmaceutical and diagnostic candidate in both types of leishmaniasis derived from *L. tropica* and *L. major*. Further experimental studies are needed to validate the potential drug targets.

Conclusion

This study is an integrated comparative quantitative proteomics and bioinformatics analysis of the two main species causing CL in Iran. We have identified differentially expressed proteins between procyclic promastigotes of *L. tropica* and *L. major*. We observed that the proteins

involved in catalytic activity had the highest alterations among the *L. major* and *L. tropica*. These results indicate differences in metabolic function between the two species, which may also be involved in determining the features of cutaneous ulcers caused by each species. Also, two hypothetical proteins have been detected, which are downregulated in *L. tropica* procyclic promastigotes compared with *L. major* procyclic promastigotes. Our findings suggest new insight to study novel hypothetical proteins possibly playing a significant role in the metabolism of different *Leishmania* species. Moreover, the results may also provide beneficial data for the discovery of species-specific proteins/genes, biological markers and a deep understanding of *L. tropica* and *L. major* biology. We have also constructed a protein network using a computational method to predict essential (hub) proteins as potential drug targets. Among them, pyruvate kinase which belongs to the kinase family, has also been identified as a potential drug target in previous studies. It is necessary for parasite survival while having no homolog proteins in the encoded proteome by humans. Further *in vitro* and *in vivo* studies are needed for specific inhibitors. The results of this study will support future research on drug design for this neglected tropical disease.

Ethics approval and consent to participate. This study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences. All patients gave written informed consent. The study was approved by the Proteomics Research Center of Shahid Beheshti University of Medical Sciences.

Conflict of interest. The authors declare that there is no conflict of interest.

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