IJMS
Vol 46, No 1, January 2021

Original Article

A Nuclear Magnetic Resonance-Based Metabolomic Study to Identify Metabolite Differences between Iranian Isolates of *Leishmania major* and *Leishmania tropica*

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Received: 18 June 2019
Revised: 10 August 2019
Accepted: 18 August 2019

What's Known

- *Leishmania tropica* and *Leishmania major* cause cutaneous leishmaniasis in Iran and many other countries.
- Metabolomics is a powerful tool to identify the metabolite profile and differentially expressed metabolites and can help gain further information on the metabolic pathways between species, the presentation of potential drug targets, and host-pathogen interactions.
- No data exist on comparative metabolomics between the Iranian clinical isolates of *Leishmania tropica* and *Leishmania major*.

What's New

- Our data showed markedly different metabolic patterns and their biochemical pathways for *Leishmania tropica* and *Leishmania major*.
- Betaine metabolism, ammonia recycling, and the metabolism of methionine, asparagine, and aspartate were the most important metabolomic pathways between the two species.
- Significant metabolites confer greater insights into novel potential drug targets and how pathogens adapt to their hosts.

Abstract

Background: Cutaneous leishmaniasis caused by *Leishmania* species (*L. spp*) is one of the most important parasitic diseases in humans. To gain information on the metabolite variations and biochemical pathways between *L. spp*, we used the comparative metabolome of metacyclic promastigotes in the Iranian isolates of *L. major* and *L. tropica* by proton nuclear magnetic resonance (^{1}H-NMR).

Methods: *L. tropica* and *L. major* were collected from three areas of Iran, namely Gonbad, Mashhad, and Bam, between 2017 and 2018, and were cultured. The metacyclic promastigote of each species was separated, and cell metabolites were extracted. ^{1}H-NMR spectroscopy was applied, and the data were processed using ProMatab in MATLAB (version 7.8.0.347). Multivariate statistical analyses, including the principal component analysis and the orthogonal projections to latent structures discriminant analysis, were performed to identify the discriminative metabolites between the two *L. spp*. Metabolites with variable influences in projection values of more than one and a P value of less than 0.05 were marked as significant differences.

Results: A set of metabolites were detected, and 24 significantly differentially expressed metabolites were found between the metacyclic forms of *L. major* and *L. tropica* isolates. The top differential metabolites were methionine, aspartate, betaine, and acetylcarnitine, which were increased more in *L. tropica* than *L. major* ($P<0.005$), whereas asparagine, 3-hydroxybutyrate, L-proline, and kynurenine were increased significantly in *L. major* ($P<0.01$). The significantly altered metabolites were involved in eight metabolic pathways.

Conclusion: Metabolomics, as an invaluable technique, yielded significant metabolites, and their biochemical pathways related to the metacyclic promastigotes of *L. major* and *L. tropica*. The findings offer greater insights into parasite biology and how pathogens adapt to their hosts.

Please cite this article as: Tabrizi F, Seyyed Tabaei SJ, Ahmadi NA, Arefi Oskouie A. A Nuclear Magnetic Resonance-Based Metabolomic Study to Identify Metabolite Differences between Iranian Isolates of *Leishmania major* and *Leishmania tropica*. Iran J Med Sci. 2021;46(1):43-51. doi: 10.30476/ijms.2019.82120.0.

Keywords ● Leishmaniasis ● *Leishmania tropica* ● *Leishmania major* ● Metabolomics ● Proton magnetic resonance spectroscopy

Introduction

Leishmaniasis is a parasitic and vector-borne disease created by various *Leishmania* species (*L. spp*). Cutaneous leishmaniasis (CL)

is regarded as a major public health concern in the Middle East, including Iran.[1] The Leishmania parasite has different two stages including infective metacyclic promastigote forms inside the sand fly midgut, which is transmitted during the blood feeding, by the bite of phlebotomine sand flies into a vertebrate (mammalian) host. In the vertebrate hosts, metacyclic promastigotes are rapidly swallowed by macrophages and converted to amastigotes.[2, 3] CL is the most common form of leishmaniasis; it is endemic in more than half of the provinces of Iran.[1, 4] *Leishmania tropica (L. tropica)* and *Leishmania major* (*L. major*) cause CL in Iran; they represent the two main forms of CL: anthroponotic (dry) CL and zoonotic (wet) CL, respectively.[1] The recent years have witnessed a rise in the number of CL cases in different parts of Iran.[1]

"Omics" approaches, including proteomics and metabolomics, have gained much attention in the recent years.[3, 5] Metabolomics is an instance of such modern technologies and is capable of detecting and quantifying metabolites in specific samples. Thus, it provides a global picture in time of the metabolic changes that occur during the course of a disease.[3] Metabolomics enjoys better dynamism than both genomics and proteomics, which enables to detect metabolic changes associated with different physiological sates in a shorter time frame.[6] There are multiple changes in biochemical and metabolite pathways in the different stages of *L. spp*,[3] including *L. tropica* and *L. major*, which are responsible for the creation of the two different clinical forms of cutaneous ulcer. A thorough understanding of these differences in the life cycle of the parasite and the identification of the metabolites related to these biochemical dissimilarities that play an important role not only in the prevention and treatment of leishmaniasis but also in the prediction of new potential drugs against it.

For all the metabolomics-based studies performed on *L. spp* to assess their drug resistance mechanisms, metabolomics, and stage-specific metabolic profiling,[3, 7] there is a dearth of data on comparative metabolomics between the Iranian clinical field isolates of the parasites. We, accordingly, performed the present study to identify differentially expressed metabolites between the Iranian isolates of *L. major* and *L. tropica* via the analytical platform of proton nuclear magnetic resonance (^{1}H-NMR).

Materials and Methods

Patients and Parasite Species Identification

Iranian clinical field isolates were obtained from the cutaneous lesions of seven patients infected with *L. major* and nine patients infected with *L. tropica* who referred to laboratories in Gonbad, Golestan Province, and Bam, Kerman Province, for parasitological diagnoses between 2017 and 2018. All the subjects were new cases, and had not been on medication. The study protocol was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences (Code No: IR.SBMU.REC.1398.086), and written informed consent was obtained from all the participants.

Samples (smears) were prepared through sampling the border of the skin lesions of each case with clinically suspected CL. The samples were then stained with Giemsa stain and examined for the amastigote stage by light microscope (ZEISS, Germany). Positive skin scrapings were aseptically inoculated into tubes containing the Novy-MacNeal-Nicolle medium (NNN). The isolates were identified via the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) technique, in which the internal transcribed-spacer-1 (ITS1) region of the parasites' ribosomal-RNA gene was amplified, followed by the resulting amplicons digested by the *Hae*III enzyme (Fermentas, Leon-Rot, Germany). PCR was conducted through the use of a forward primer (5'-CTGGATCATTTTCCGATG-3'), and a reverse primer (5'-TGATACCACTTATCGCACTT-3'). After the use of the restriction enzyme, the banding patterns of the isolates were obtained in comparison with the molecular profiles of the World Health Organization (WHO) reference strains of *L. tropica* (MHOM/IR/02/MHOM/R), and *L. major* (MRHO/IR/75/ER).

Preparation of Metacyclic Promastigotes

The promastigotes were initially grown on the NNN medium and for mass culture. The parasites were prepared in the RPMI 1640 medium (Gibco, Germany), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco, Germany), and Pen-Strep (100 U/mL of penicillin and 100 µg/mL of streptomycin; Gibco, Germany) at 24 to 25 °C. The promastigotes were cultured with repeated medium refreshment every three to five days until the number of parasites reached approximately 2×10^7 cells/mL by Neubauer chamber counting (Marienfeld, Germany). The culture was incubated without adding the fresh medium for 10 (range=8–12) days, and then the metacyclic promastigotes (the stationary phase) were evaluated using both the morphometric analysis and the agglutination assay by lectin.[8, 9] The cell body size and flagellum lengths for 300 parasites in each day were measured, and the percentage of metacyclic forms was

calculated. (The promastigotes whose flagellum/body length ratio was ≥2 were considered metacyclic forms, as described by da Silva and colleagues.[8]) To ensure the collection of purified metacyclic promastigotes, we also performed the agglutination assay with peanut agglutinin (PNA) (Sigma, CA, USA).[9] Briefly, we added PNA to phosphate-buffered saline (PBS)-washed metacyclic promastigotes to a final concentration of 30 µg/mL for 2×10^7 cells/mL. After 30 minutes at room temperature, the separation of PNA^- cells (metacyclic promastigotes) from PNA^+ cells (procyclic promastigotes) was performed by centrifugation at 200 *g* for five minutes. The non-agglutinated metacyclic promastigotes were then harvested from the supernatants. Stationary phase *L. major* and *L. tropica* promastigotes were washed at least three successive times in sterile PBS (pH 7.4) to remove the culture medium compounds, and collected by centrifugation at 3500 *g* at 4 °C for 20 minutes. The practical conditions and processes/steps for both parasite species examined were thoroughly identical.

Cell Extraction and Proton Nuclear Magnetic Resonance (^{1}H-NMR) Spectroscopy

Cell disruption and metabolite extraction were performed according to Gupta and others,[10] with slight modifications. Child 1.8 M perchloric acid, Merck, Germany, (800 µL) was added to the cell suspension, and then each sample was vortexed and sonicated for five minutes at 4 °C. The lysed cells were centrifuged at 12000g for 10 minutes at 4 °C, and the pH of each supernatant was adjusted to 6.8 with potassium hydroxide (Merck, Germany). The supernatant was kept on ice for one hour to allow the precipitation of potassium perchlorate and centrifuged again as above. The lysate supernatant was transferred into new Eppendorf tubes (Sigma Aldrich, Germany). For ^{1}H-NMR spectroscopy, D_2O (20%) with trimethylsilyl propionate (TSP, 0.25 mM) (Sigma, CA, USA), as the NMR chemical shift reference (as an internal standard), was added to each sample, and mixed by vortexing. The samples were spun down at 12000*g* for 15 minutes at 4 °C, and then 550 µL of the supernatant was transferred into 5-mm NMR tubes (Sigma Aldrich, Germany). All the experiments were performed using a Bruker AVANCE 400 MHz (Bruker, Germany), equipped with a 5-mm probe at 298 K. The Carr-Purcell Meiboom-Gill (CPMG) spin-echo pulse sequencing was used to remove macromolecule signals. The water impact on the baseline was minimized by irradiating the water frequency during the relaxation delay of two seconds and a mixing time of 0.1 seconds. ^{1}H-NMR spectra were acquired in 150 scans per sample, at 298 K, with an acquisition time of 3.0 seconds and a spectral width of 8389.26 Hz.

Data Processing and Statistical Analysis

The ^{1}H-NMR spectra were processed using ProMatab in the MATLAB (version 7.8.0.347) environment, and necessary corrections were made on the spectra. The spectra were referenced to the TSP standard at 0.0 ppm. Chemical shifts between 0.2 and 10 ppm were normalized and subjected to spectral binning in 0.01 ppm. The spectral region affected by the water-related signal was removed in the range of 4.54 to 5.0 ppm. The 2D data matrix obtained from ^{1}H-NMR was 16 at 813, comprising the number of the studied samples (nine samples of *L. tropica* and seven samples of *L. major*) and bins, respectively.

Multivariate statistical analyses, including the unsupervised principal component analysis (PCA) and the supervised orthogonal projections to latent structures (OPLS) discriminant analysis, were applied to identify the most significant relevant metabolites between *L. tropica* and *L. major*. PCA was applied to detect similarities or trends in different samples, as well as to reveal the outliers and relationships that existed between the observations. The OPLS discriminant analysis was employed to maximize the covariance between the measured data and to compare the two classes of samples. The PCA and OPLS diagrams were plotted in the R software (Version 3.3.2). In each comparison, the variable influence in projection (VIP) was used to find the most significant discriminating metabolites. Metabolites with a VIP of greater than 1 and a P value of less than 0.05 were marked as significant variables.

Metabolite Identification and Pathway Analysis

The metabolites were identified using metabolite validated databases such as the Biological Magnetic Resonance Data Bank (BMRB)[11] and the Human Metabolome Database (HMDB).[12] Finally, the significantly changed metabolites were used to determine the most important pathway differences between the two *L. spp.* The pathway enrichment analysis was performed using the MetaboAnalyst server.[13] MetaboAnalyst (www.metaboanalyst.ca) is a web server for metabolomic data analysis and interpretation.

Results

Diagnosis of Parasite Species

The PCR products of both positive specimens

and standard strains with approximately 350 bp were digested by the *HaeIII* enzyme. The reference strain on agarose gel exhibited two bands (135 bp and 215 bp) that corresponded to *L. major* and two bands (57bp and 185bp) corresponded to *L. tropica* (figure 1).

Discrimination between L. major and L. tropica Using Multivariate Analysis

Multivariate analyses were performed on the result matrix to find metabolites, that mostly discriminated *L. major* from *L. tropica*. The values of the first two components in the PCA with the highest role in separating the two groups (*L. major* and *L. tropica*) in *in vitro* conditions were 92.9% (PC1) and 4% (PC2) (figure 2). The score plot based on the PCA analysis showed that the two groups were well-separated based on the first two dimensions of the analysis. Additionally, all the data were within the 95% confidence interval, and no outlier data were observed. The OPLS discriminant analysis is a supervised method for finding a combination based on existing variables with the most variance between the groups. The OPLS discriminant analysis separated *L. major* and *L. tropica* clusters, which is visualized in score plots in figure 3.

The OPLS model is validated by R^2 (coefficient of determination) and Q^2 (cross-validated R^2), which was performed in the present study via the seven-fold *cross-validation* measure. All the models had Q^2 values of greater than 0.4 with the intercept of less than zero, which demonstrated that the models were validated. The R^2X, R^2Y, Q^2Y, and RMSE values of the OPLS model were 0.983, 0.595, 0.566, and 0.355, respectively (figure 3), which demonstrated the model's good predictive ability.

Metabolite Identification of Two Leishmania Species and Pathway Analysis

The differentially expressed metabolites were determined among the metacyclic

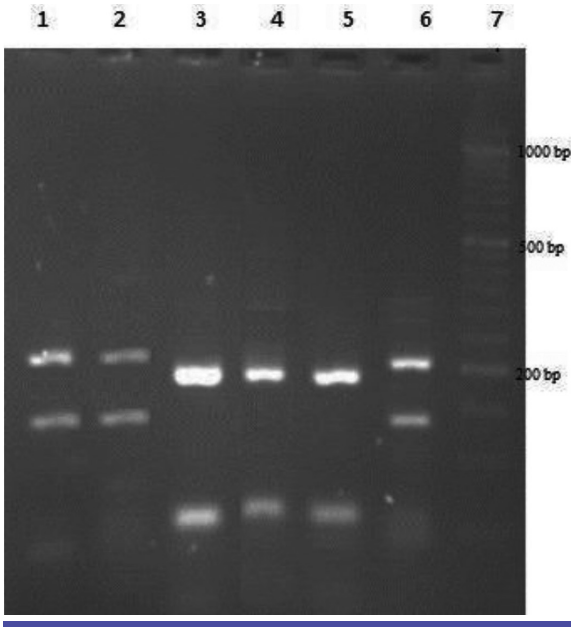

Figure 1: Results of the restriction fragment length polymorphism patterns of ITS1-rDNA amplicons digestion with the *HaeIII* (*BsuRI*) enzyme for the *Leishmania* isolates are illustrated. Lane 1 and 2: *Leishmania tropica* sample; Lane 3 and 4: *Leishmania major* sample; Lane 5: standard strain of *Leishmania major* (MHOM/IR/75/ER); Lane 6: standard strain of *Leishmania tropica* (MHOM/IR/02/MHOM/R); Lane 7: marker 50 bp

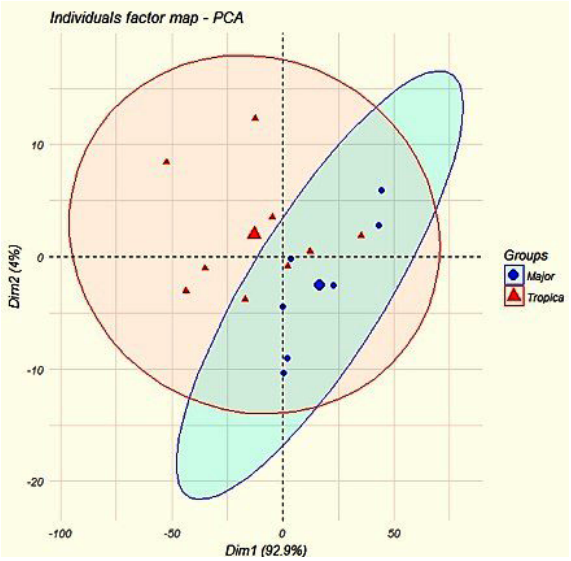

Figure 2: Scatter score plot of the principal component analysis displays the discrimination between the Iranian isolates of *Leishmania major* (○) and *Leishmania tropica* (Δ). Principal component 1 (Dim1) and principal component 2 (Dim2) explain 92.9% and 4.0% of the total variance, respectively.

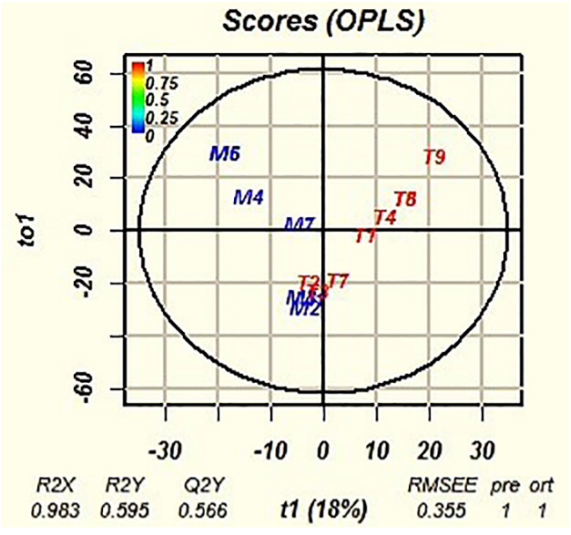

Figure 3: Score plot of the orthogonal projections to latent structures discriminant analysis illustrates a clear discrimination between the Iranian isolates of *Leishmania major* and *Leishmania tropica*. M: *Leishmania major*; T: *Leishmania tropica*

Table 1: Significant discriminating metabolites between the metacyclic promastigote extracts of *Leishmania major* and *Leishmania tropica*

Metabolite*	HMDB ID	KEGG ID	bin	VIP	P value	L.t/ L.m
3-hydroxybutyrate	HMDB0000357	C01089	4.175	2.09	0.005	0.79
Phosphoserine	HMDB0000272	C01005	4.165	2.05	0.005	0.822
L-proline	HMDB0000162	C00148	4.125	2.03	0.006	0.801
kynurenine	HMDB0000684	C00328	4.145	1.99	0.005	0.813
Lactate	HMDB0000190	C00186	4.105	1.86	0.005	0.838
Choline	HMDB0000097	C00114	4.055	1.85	0.005	0.89
Creatinine	HMDB0000562	C00791	4.045	1.67	0.004	1.35
Galactose	HMDB0000143	C00124	4.085	1.65	0.004	1.515
Tryptophan	HMDB0000929	C00078	4.035	1.59	0.004	1.7
Histidine	HMDB0000177	C00135	3.985	1.55	0.004	0.88
Asparagine	HMDB0000168	C00152	3.995	1.55	0.005	0.75
Phenylalanine	HMDB0000159	C00079	3.975	1.52	0.003	0.91
Tyrosine	HMDB0000158	C00082	3.935	1.50	0.002	0.88
L-serine	HMDB0000187	C00065	3.955	1.50	0.004	1.5
Hippurate	HMDB0000714	C01586	3.945	1.49	0.003	1.965
Pantothenate	HMDB0000210	C00864	3.965	1.45	0.004	1.45
Betaine	HMDB0000043	C00719	3.895	1.45	0.002	2.87
Creatine	HMDB0000064	C00300	3.915	1.36	0.001	2.35
Mannose	HMDB0000169	C00159	3.925	1.30	0.001	2. 216
Aspartate	HMDB0000191	C00049	3.885	1.30	0.002	3.48
Methionine	HMDB0000696	C00073	3.855	1.22	0.002	3.45
Homocitrulline	HMDB0000679	C02427	3.725	1.20	0.015	1.3
Leucine	HMDB0000687	C00123	3.715	1.18	0.010	0.8
Acetylcarnitine	HMDB0000201	C02571	3.845	1.01	0.002	2.85

HMDB: Human Metabolome Database; KEEG: Kyoto Encyclopedia of Genes and Genomes; VIP: Variable importance in projection; *L.m: Leishmania major*; L.t: *Leishmania tropica;* *Independent t-test were applied and Metabolites with VIP values of more than 1 and P values of less than 0.05 were considered significant

promastigotes of the Iranian isolates of *L. major* and *L. tropica*, and the spectral bins of the highest importance according to VIP values were selected. Twenty-four significantly differentially expressed metabolites were identified among the metacyclic promastigotes of the *L. major* and *L. tropica* samples (P≤0.0152). The details of the metabolite alterations that reached statistical significance are indicated in table 1.

The metabolite set enrichment analysis of biochemical pathways was performed using the MetaboAnalyst database. In this analysis, the significantly differentially expressed metabolites between *L. tropica* and *L. major* were measured relative to the predefined biochemical pathway sets in the database, which were involved in specific biological pathways, as is shown in figure 4. According to the result, the significantly altered biochemical pathways with a P value of less than 0.05 were eight metabolic pathways. Most of the altered metabolic pathways in the metacyclic promastigotes of the Iranian isolates of *L. major* and *L. tropica* are presented in table 2.

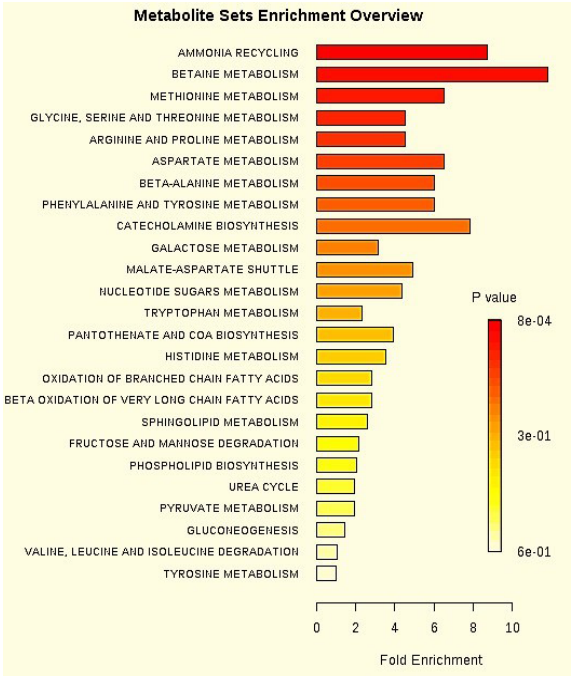

Figure 4: Metabolite set enrichment analysis results of differentially expressed metabolites are shown between the Iranian isolates of *Leishmania tropica* and *Leishmania major*.

Discussion

In this study, the metabolites detected by the ^{1}H-NMR spectroscopy of the Iranian clinical isolates

Table 2: Significant metabolic pathways in the metacyclic promastigotes of the Iranian isolates of *Leishmania major* and *Leishmania tropica*

Metabolic Pathway	Metabolite	Total	Hits	Raw P	FDR
Ammonia recycling	L-asparagine, L-histidine, L-serine, L-aspartic acid	16	4	<0.001	0.0603
Betaine metabolism	betaine, choline, L-methionine	10	3	0.001	0.0606
Methionine metabolism	betaine, choline, L-serine, L-methionine	24	4	0.002	0.0631
Glycine, serine, and threonine metabolism	betaine, creatine, L-serine, phosphoserine	26	3	0.025	0.403
Arginine and proline metabolism	creatine, L-proline, L-aspartic acid	26	3	0.025	0.403
Aspartate metabolism	L-asparagine, L-aspartic acid	12	2	0.035	0.403
Beta-alanine metabolism	L-aspartic acid, pantothenic acid	13	2	0.040	0.403
Phenylalanine and tyrosine metabolism	L-tyrosine, L-phenylalanine	13	2	0.040	0.403

Total: The total number of compounds in the pathway; Hits: The metabolite matches; Raw P: The original P value calculated from the enrichment analysis; FDR: False discovery rate

of *L. major* and *L. tropica* showed a considerable overlap; however, overall, 24 metabolites were significantly expressed in abundance at least with a P value of less than 0.05 between the cell extracts of the two species. The discriminating metabolites in *L. tropica* compared with *L. major* included the increment of creatinine, galactose, tryptophan, L-serine, hippurate, pantothenate, betaine, creatine, mannose, aspartate, methionine, homocitrulline, and acetylcarnitine along with the decline of 3-hydroxybutyrate, phosphoserine, L-proline, kynurenine, lactate, choline, histidine, asparagine, phenylalanine, tyrosine, and leucine. A comparative metabolomic analysis between the cell extracts of the promastigotes of *L. donovani*, *L. major*, and *L. Mexicana* by Westrop and others[14] revealed 64 different metabolites. They reported many interesting differences between the metabolites of the three *L. spp*, especially tryptophan, asparagine, arginine, proline, glycine, and serine.

We found many interesting differences between the metabolism of the two *L. spp*, particularly in the area of amino acid metabolism, which is somewhat in accordance with several other studies.[3, 14, 15]

Our comparative analysis revealed 13 out of 24 metabolites with significantly increased differential expressions in *L. tropica* and the remaining metabolites in *L. major*. Among them, the top four differential metabolites were methionine, aspartate, betaine, and acetylcarnitine, which were increased in *L. tropica* by 3.45-fold, 3.48-fold, 2.87-fold, and 2.85-fold compared with *L. major*, respectively. On the other hand, asparagine, 3-hydroxybutyrate, L-proline, leucine, kynurenine, and phosphoserine were increased remarkably in *L. major* in comparison with *L. tropica*. Consistent with these results, alterations in some metabolites have been previously recorded on the promastigotes and / or amastigotes of *L. donovani*, *L. major*, and *L. Mexicana*.[3, 10, 14] The results concerning amino acids also propound speculative hypotheses on how the species differ and could explain the distinct disease phenotypes resulting from the infections of humans with the two different species, if they occur also with the amastigote forms that are responsible for human diseases.[14] *Leishmania spp* are auxotrophic for L-leucine, and it can be converted through 4-methyl-2-oxopentanoate and hydroxymethylglutaryl coenzyme A into acetyl-CoA, and then metabolized through the tricarboxylic acid cycle.[16] Tryptophan Metabolism via the Kynurenine Pathway is leading to synthesis of nicotinamide adenine dinucleotide (NAD+); it also regulates the biological processes including immune cell response and inflammation.[17]

As indicated in table 2, we detected eight metabolic pathways with a P value of less than 0.05: ammonia recycling; betaine metabolism; methionine metabolism; glycine, serine, and threonine metabolism; arginine and proline metabolism; aspartate metabolism; beta-alanine metabolism; and phenylalanine and tyrosine metabolism. Based on the results of the previous studies, valine, leucine, and isoleucine biosynthesis, pyruvate metabolism, glutathione metabolism, fatty acid biosynthesis, and sulfur metabolism are the most important pathways changed between the procyclic and metacyclic phases of *L. major*.[3, 7]

In the present study, the ammonia recycling biochemical pathway analysis had the most important pathway involved in the biological pathways (P=0.007): L-aspartic acid, L-histidine, L-serine, and L-asparagine were the amino acids involved in this pathway. Amiri and others[5] and Arjmand and others[7] disclosed that the major alterations in metabolic pathways in the metacyclogenesis process of *L. major* were citraconic acid, isopropylmalic acid, L-leucine related to valine, leucine, and isoleucine biosynthesis pathways, respectively. One interesting hypothesis for these differences in metabolic pathways could be due to the fact that our findings pertain to comparisons between two species (*L. tropica* and *L. major*), while

their results are related to one species (only the standard strain of *L. major*). Asparagine/ glutamine related pathways in *Leishmania* play a key role in cellular homeostasis maintenance.[18] According to another study, aspartate uptake was essential in the tricarboxylic acid cycle in *L. Mexicana*.[19] Previous research supports the notion that the L-asparagine synthetase is a survival factor in *L. infantum* and can serve as a potential drug target in *Leishmania*.[20] As discussed above, similar roles may have been played by aspartate and asparagine in *L. tropica* and *L. major*.

The other significant biochemical pathways in this study were betaine and methionine metabolism; betaine, choline, L-methionine, and L-serine were involved in these pathways (table 2 and figure 4). Betaine and methionine metabolites were increased by 2.87-fold and 3.45-fold, respectively, and choline was decreased more in *L. tropica* than *L. major*. A similar study on *L. donovani* life stages was performed by Gupta and others,[10] using the ^{1}H-NMR method. They detected significantly altered metabolites in the promastigote stage, including betaine and hydroxybutyrate; nonetheless, succinate and valine metabolites were present in the amastigote/ axenic amastigote stage. Choline and its derivatives are lipoprotein and membrane lipids components in eukaryote cells. Betaine serves as a substrate in the betaine-homocysteine–methionine synthesis pathway.[21]

We observed that methionine was markedly upregulated in *L. tropica* than *L. major*. This metabolite is also one of the amino acids involved in the betaine metabolism pathway, which is a sulfur-containing proteinogenic amino acid. L-methionine contributes to polyamine biosynthesis.[22]

As seen in figure 4 and table 2, the results of the pathway enrichment analysis showed that glycine, serine, and threonine metabolism, arginine and proline metabolism, beta-alanine metabolism, and phenylalanine and tyrosine metabolism constituted the other biochemical pathways with significant differences in metabolic pathways, particularly in amino acids. This observation is relatively consistent with reports from other studies.[3, 14] For example, Westrop and others[14] using the LC-MS method detected significant differences in metabolic pathways in amino acids, especially tryptophan, aspartate, arginine, proline, glycine, serine, and threonine compared with the global metabolome of the studied *L. spp*.

In the present study, phenylalanine and tyrosine were decreased more in *L. tropica* than *L. major*. These two amino acids were also involved in significant pathways. Lamour and others[15] found that these amino acids were differentially expressed in activated and non-activated macrophages affected by *L. major*.

In this study, proline was one of the other metabolites whose amount differed between the two species (*L. tropica*<*L. major*). *Leishmania* parasites in the insect stage of (promastigotes) use proline as an important carbon and energy source. A recent study by Westrop and others[14] showed that proline and asparagine were decreased more in *L. major* promastigotes than *L. infantum* promastigotes but were increased in comparison with *L. Mexicana* promastigotes. Tryptophan in *L. tropica* had a higher concentration than in *L. major* in this study. Furthermore, Westrop and others compared the metabolites of three *L. spp* via the LC-MS method and demonstrated that tryptophan had a considerably lower level in *L. major* than in *L. donovani* and *L. Mexicana*. Besides, its fundamental role in protein synthesis, tryptophan is an essential amino acid that is the precursor of many physiologically significant metabolites produced during the process of its degradation. It has been reported that 95% of the overall tryptophan degradation takes place in the kynurenine pathway.[23] According to this content, one of the methods against the pathogen by host cells is the evacuation of the parasite from tryptophan via the kynurenine pathway.[24]

A main limitation of the current investigation is that we evaluated the metabolic profile of promastigotes, while the metabolic profiling of amastigotes would have yielded more robust data on the species-specificity of survival and pathogenicity.

Conclusion

The metabolites of each species have a distinct metabolic profile and involve different numbers of amino acids and their catabolism. Significantly different metabolites may explain the distinct phenotypes and clinical patterns of CL resulting from the infection of humans with the two different *L. spp* (*L. major* and *L. tropica*), if they occur also with the amastigote forms, which are responsible for human diseases. Our findings could serve as a framework in future studies, aimed at exploring the host-parasite interaction and species-specific pathogenicity and can also help to present new potential drug targets.

Acknowledgement

This article was extracted from a thesis written by Fatemeh Tabrizi in the field of medical parasitology in the School of Medicine, Shahid

Beheshti University of Medical Sciences, (Registration No: 176), Tehran, Iran. The authors would like to thank the Medical Parasitology Department and the Proteomics Research Center in Shahid Beheshti University of Medical Sciences for their support (grant No: 20352).

Conflict of Interest: None declared.

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