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Metabolomics-based studies in the field of *Leishmania*/leishmaniasis

Nasrin Amiri Dash Atan [a], Mehdi Koushki [b], Nayeb Ali Ahmadi [a,*], Mostafa Rezaei-Tavirani [a]

[a] Proteomics Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[b] Department of Biochemistry, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Leishmania is a neglected protozoan parasite which creates some problems for public health with different clinical infections in different countries around. Due to the lack of an effective drug without side effects and the emergence drug resistance, there is an urgent need to introduce the novel drug targets and new drugs and vaccines to control leishmaniasis during recent years, metabolomics and other "Omics" platforms has become an important approach to comprehensive knowledge of the *Leishmania* parasites biology. The study of metabolite profiles can open the insights for discovering novel therapeutic targets in this infection in both of the parasites and human host. In addition, specifying the metabolomics profile changes among promastigotes, amastigotes and during metacyclogenesis can pay the way for achieving parasite survival parameters and the host-parasite interaction. The previous studies in this field have been extracted from the databases, literature and their detailed major concepts. The present review highlights the role of metabolomics approach in the field of *Leishmania* research. Also, several important metabolite signatures introduced in various aspect of *leishmania* parasite such as drug resistance and parasite biology which would be useful in the field of biomarker and drug discovery process. Finally, metabolomics plays a potential role in introducing metabolic pathways related to *Leishmania* parasite and its treatment design.

Contents

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* Corresponding author at: Proteomics Research Center, and Department of Medical Laboratory Technology, Faculty of Paramedical Sciences, Shahid Beheshti University of Medical Sciences, Darband Street, Tajrish, 1971653313Tehran, Iran.
E-mail address: nayebalia@sbmu.ac.ir (N.A. Ahmadi).

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1. Introduction

Personal health is considered as one of the principles of health care in the future. The system biology plays a significant role in understanding the interactions of biological system at different levels such as genomes, proteins, and metabolites. Recently, "Omics" approaches including genomics, proteomics, and metabolomics analysis and also bioinformatics and computational prediction are from the modern technologies which have gained much attention in recent years to find potential markers for different diseases using various biological samples such as tissues, biological fluids and cell cultures.[1,2] As one of the components of the system biology, metabolomics is also rapidly developing, which has attracted a lot of attention in various biomedical studies. In addition, the metabolomics research has a great potential for coming into the medical arena.[3–5] Metabolomics analyzes the whole metabolites which are present within an organism in a specific condition. Through detecting and quantifying all metabolites in a specific sample, metabolomics provides a "snapshot" during the metabolic changes related to the disease.[6] In fact, metabolomics studies reflect changes in the upstream stages of system biology research (genome and proteome). Due to more dynamical status of metabolomics rather than both genomics and proteomics analysis, this approach can detect metabolic changes associated with different physiological states within a shorter time frame.[3,7] A large body of research emphasized the importance of metabolites as invaluable biomarkers for diagnosing and detecting disease. However, metabolomics can detect and introduce biomarkers in a broad range of samples such as whole blood, serum, plasma, urine, saliva, and the like in various disease conditions[8]

2. Leishmania and leishmaniasis

Protozoan *Leishmania* causes a wide spectrum of clinical disease among humans, which is globally regarded as a major public health problem in the Middle-East.[9] Leishmaniasis is a vector-borne disease which is transmitted by sandflies and caused by obligate intracellular protozoan of the genus *Leishmania*. Human infection appears by about 20 species of parasite which are transformed to human through biting by sand-fly vector.[10] Different species are morphologically the same although they are different by isoenzyme analysis. These species include the *L. donovani* complex with two main species such as *L. donovani*, *L. infantum*, the *L. mexicana*, *L. tropica*, *L. major* and *L. aethiopica* and the subgenus *L. viannia* with four main species. It is estimated that over 350 million people are at risk for leishmaniasis, among whom 12 million were infected and 1.5–2 million new cases are globally reported each year.[9,11] There are several different forms of leishmaniasis in the human based on species-dependent disease, ranging from self-healing skin ulcers in cutaneous leishmaniasis to the most severe pathologies associated with visceral leishmaniasis by *L. donovani*, *L. infantum* and *L. chagasi*, which is fatal if it is not treated appropriately.[12] Cutaneous leishmaniasis is the most common form of leishmaniasis and *L. major* and *L. tropica* are the main cause for this form of leishmaniasis making skin lesions.[13] The *Leishmania* parasite has a dimorphic life cycle including an infective promastigote form inside the gut of the sand-fly, which are transmitted with saliva into a human host during blood feeding. Within the mammalian host, promastigotes are rapidly engulfed by phagocytic cells at the infection site and undergo cytodifferentiation to the amastigote stage within the phagolysosome of the host macrophage with acidic environment Fig. 1. The differentiation of the promastigote form into the amastigotes is determined by several morphological and biochemical changes which are essential for surviving and propagating parasite during accommodation in phagolysosome vacuoles. Interestingly, promastigote forms can be easily cultured *in vitro* and the amastigotes of some *Leishmania* species can be proliferated in cell free medium[14,15] Thus, most studies were conducted on the promastigote form and axenic amastigote and in the meantime, metabolomics studies also are no exception.

3. Metabolomics

The metabolomics is a part of "Omics" science in system biology which has been developing for understanding the alteration of molecular level in disease conditions. Metabolomics, along with other "Omics" platforms such as genomics, transcriptomics and proteomics, can complete the systemic view of biology in different research areas.[16] Metabolomics is defined as an informative analytical tool for metabolites measurement in both quantitative and qualitative conditions.[17] Regarding "Omics", metabolomics is

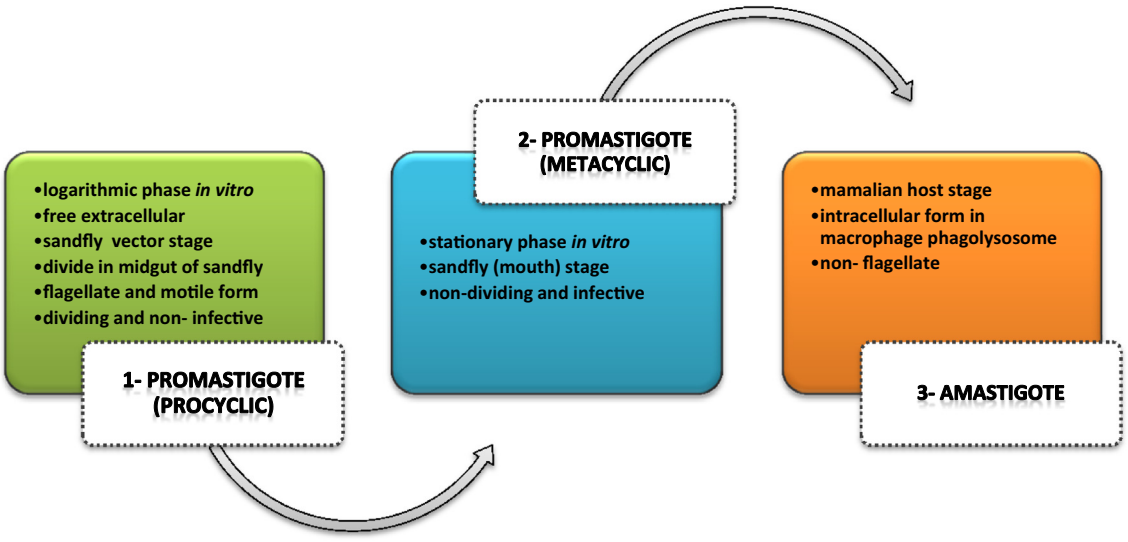

Fig. 1. Different forms of *Leishmania* parasites during their infectious cycle. Procyclic promastigotes exist in the gut of sand-fly vector and divide quickly. Then this form develops into the metacyclic promastigotes forms. Transformation of procyclic to metacyclic is named metacyclogenesis process. In mammalian hosts metacyclic transforms in to intracellular amastigote forms in macrophage cells.

located at the downstream of others which make the system-level rich by metabolites, their concentration assessment, and flux through metabolic pathways. The development of metabolomics and conducting a large body of research in this area are regarded as some evidence of the progression and applicable presence of metabolomics in many areas.[18] Identification of the changes in metabolites level can help to diagnose disease in early stages. Metabolites are small endogenous molecules and the end products of biochemical pathways with low molecular weight between 1500 Da and less such as lipids, peptides, organic acids, nucleic acids, amino acids, vitamins and carbohydrates which metabolomics allow for profiling the metabolites globally in various biological samples such as blood, tissue, urine and the like.[19] The metabolites are invaluable small molecules playing an important role in biological systems and representing useful data to understand disease phenotypes.[20] In addition, profiling metabolite markers and their amounts is regarded as an important method for diagnosing the disease in early stages. Comparative metabolomics by metabolite profiling from different conditions (e.g. disease vs. control) resulted in detecting metabolic changes and supporting the inference of different aspects of the disease such as molecular mechanisms, biochemical pathways, disease prediction or progression, and even screening markers of early diagnosis.[21] Metabolites variation levels are applied as the prediction of disease before clinical symptoms of disease. Regarding the metabolomics field, targeted and non-targeted approaches are available as two main categories. Based on targeted approach, specific and known groups of metabolite are associated with certain metabolic pathways while non-targeted approach focuses more on all possible metabolite analyses in any sample in certain condition. Thus, the second approach is preferred.[22] Biomarker discovery for various diseases and integrative system biology are considered as the most common areas for metabolomics analysis. Biomarker discovery by using metabolomics tool is a promising approach for disease diagnosis in the early stage before appearing the related symptoms. A comprehensive profile of metabolites in biological specimen is defined as metabolomics.[21] In addition, a large number of techniques are used for profiling metabolites, among which we can refer to one dimensional proton nuclear magnetic resonance (^{1}H NMR) and mass spectrometry (MS) as the most commonly used techniques. The development of metabolomics-based studies usually relies on the analytical techniques such as chromatography separation methods, integrated with MS.[23] Based on MS-based metabolomics experiments, the quantitative analysis of metabolites is performed with high sensitivity while using sample complexity reduction prior to detection can simplify the identification of metabolites based on LC-MS approach. The type of biological sample is very important in choosing the technique in metabolomics. Recently, the detection of metabolites and their metabolic pathways is made possible by advanced techniques-based MS such as GC-MS, HPLC-MS, UPLC-MS and CE-MS. Each of these applied analytical platforms for detecting metabolites has its own advantages and disadvantages. Thus, these platforms cannot individually detect all metabolites in biological samples. There are metabolome analyses of many types of organisms conducted for obtaining information about metabolic pathways. Therefore, an appropriate metabolomics analysis is required to obtain full understanding of *Leishmania* metabolic pathways in different aspects of this parasite. The quality of sample preparation is very important for achieving high quality results. This step should be highlighted in different resources of biological samples in *Leishmania* and an individual protocol is necessary for assessing the metabolome profile of cultured parasite ($\sim 10^8$ cells) by NMR or MS-based methods Fig. 2.

3.1. Analytical approach in metabolomics

3.1.1. NMR (Nuclear Magnetic Resonance)

NMR is considered as one of the most commonly used analytical platforms in metabolomics. Single proton ^{1}H NMR involves the use of a large and powerful magnet to align the protons which are available in a sample. In this method, each metabolite appears with a distinct peak in the NMR spectrum and the area under the peak represents each metabolite concentration. The advantages and disadvantages of this method are summarized in Fig. 3. One of the most important advantages of NMR for detecting metabolites in this method is that the sample is not degraded and it is even used in subsequent studies.[3] Low sensitivity and detection of limited mass range are regarded as the limitation of NMR. The general process of NMR-based metabolomics includes the following steps[16]

- Experimental design
- Sample preparation
- NMR raw data

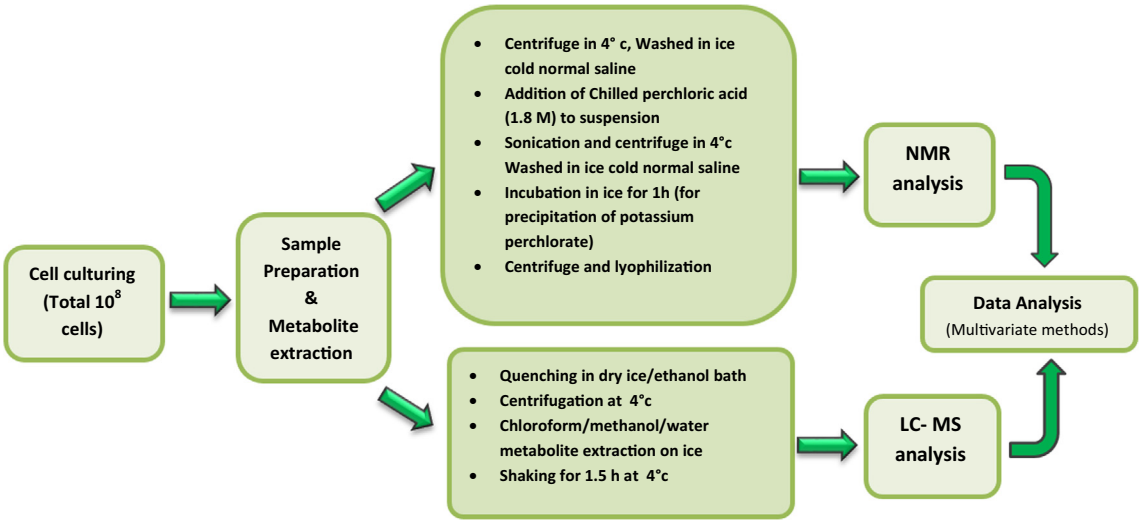

Fig. 2. The metabolomics-based study of *Leishmania* parasite process for NMR and LC-MS analysis. Sample preparation step of cells for NMR and MS-base analysis is different from each other which applying appropriate protocol could be obtain better results. The study of the metabolome of *Leishmania*-infected cells using metabolomics, can lead to the identification of metabolite targets for chemotherapeutic treatment and introducing of novel molecular markers that can be used for the diagnosis of different diseases.

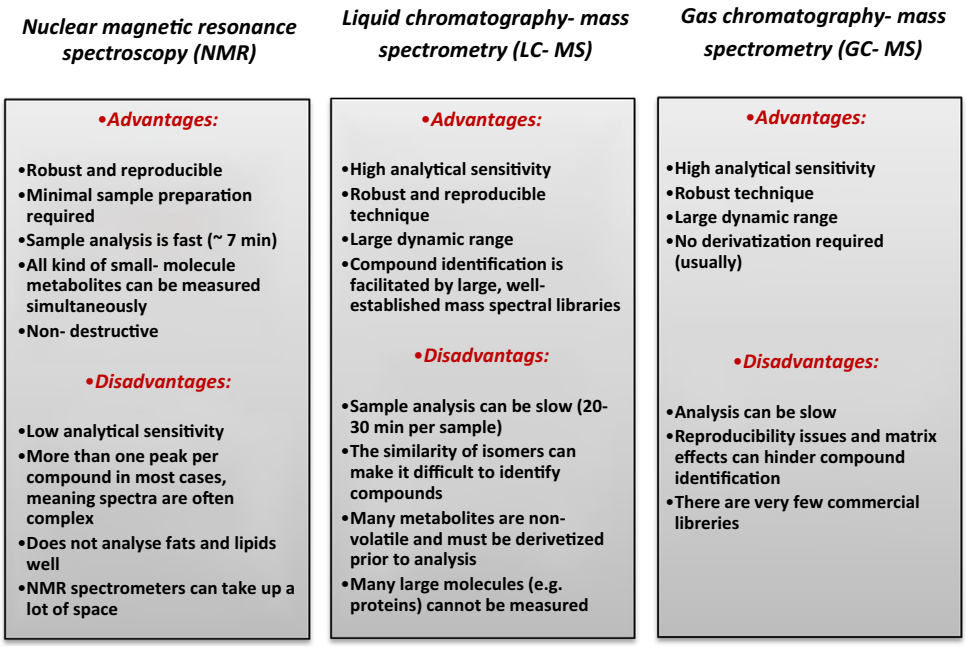

Fig. 3. A summary of the most common analytical techniques (with advantages and disadvantages) used in metabolomics.

- Spectral processing
- Data preprocessing
- Multivariate data analysis (PCA, PLS-DA, OPLS-DA)
- Metabolic pathway identification (potential biomarkers)

3.1.2. MS-based techniques

Metabolite spectrum data are considered as mass-to-charge (m/z) ratios by MS.[24] MS is primarily coupled with liquid chromatography or gas chromatography in metabolomics studies.[3] It is worth noting that the use of LC-MS in metabolomics studies results in detecting a wide range of metabolites.[25] Regarding some idiosyncratic properties of LC-MS method, we can refer to the simultaneous assessment of aqueous and lipid metabolites, more separation, and detection of different types of metabolites.[26] This method covers a greater mass range and no derivation of samples is required. However, the variability of LC-MS instruments and absence of standard metabolic library are considered as the disadvantages of this approach Fig. 3.[27] In order to detect metabolites by LC-MS, some steps should be taken such as separating by chromatography, ionizing the sample, determining the compound mass by mass analyzer, and detecting the metabolites by detector. TOF and quadrupole are regarded as the most common mass analyzers coupled with LC. During recent years, some advance techniques such as FT-ICR with high mass accuracy and consecutive MS (tandem MS) ability has attracted a lot of attention. However, it is less implemented due to the high cost of FT-ICR. GC is another separation method which is coupled with MS. GC-MS is used for separating and detecting volatile metabolites at high temperature with high sensitivity. The efficiency of this method is related to the high sensitivity and the detection of volatile metabolites capability. However, regarding the major challenges of GC-MS, we can refer to the derivation of readily volatile samples and limited mass range that incomplete derivation can lead to loss of metabolite and intricate explanation of results.[26]

3.2. Statistical analysis of metabolomics-based data

Multi-dimensional dataset includes the signal from a large number of metabolites generated in metabolomics studies. Thus, developing a coherent hypothesis explaining any changes in metabolite profile by intuition alone is difficult due to a large number of measurements. Therefore, employing a multi-dimensional statistical analysis is essential for reducing the number of variables in a dataset. The most common methods used for reducing data are related to unsupervised methods such as PCA and supervised methods including PLS, PLS-DA.[28] PCA method is utilized for outlier identification within the dataset. Then, PLS-DA and OPLS-DA as unsupervised multivariate data analytical methods are performed for ^{1}H NMR spectral data analysis. PLS-DA is considered as a pattern recognition approach used to identify potential disease-specific biomarkers. After identifying the metabolites, their interpretation into biological process and pathways is an important step in analyzing the results of metabolomics study.[29] The existence of valid database including invaluable information about metabolites and the involved pathways play a significant role in mapping metabolites on biological system. There are a number of public information databases about metabolites and their reactions, as well as some of open source tools such as MetaCore which provide some helpful ways to experimentally map the observed changes of metabolites into metabolic pathways.[3,30] Finally, the data are imported into the Metscape plugin by Cytoscape software for more analysis of differentiated metabolites in the groups under study. Metscape plugin use to map of the pathway of metabolomics data. Finally, this application demonstrates seed metabolites, metabolic reactions, enzymes, and genes.[3,31]

4. Metabolomics and *Leishmania*/leishmaniasis

Recently, a number of metabolomics-based studies have been published with respect to *Leishmania*/leishmaniasis. Metabolomics as a new field in post-genomic era represents an emerging discipline related to a comprehensive analysis of small molecule metabolites, which provides a powerful approach to discover biomarkers in biological systems. *L. major* Fredlin strain is the first protozoan model which metabolic network was reconstructed by Chavali et al.[32] In this regard, NMR and MS spectrometry are considered as the most useful methods.

4.1. ^{1}H NMR-based metabolomics in the leishmania/leishmaniasis research

Most of the studies in the field of *Leishmania* by 1HNMR-based metabolomics have been conducted on life cycle stages in metabolic changes of parasite in different species. However, metabolomics-based studies about the drugs affected metabolomics map have been less emphasized and only one study was reported on blood serum of cutaneous leishmaniasis. Table 1 indicates all of the previous studies on *Leishmania* by ^{1}H NMR-based metabolomics. Arjmand et al.[33] focused on metabolomics by using ^{1}H NMR in order to compare two stages of promastigote forms of parasite including procyclic and metacyclic in *L. major* species. The main altered metabolites in logarithmic and stationary phases included citraconic acid, isopropylmalic acid, L-leucine, ornithine, caprylic acid, capric acid, and acetic acid. Amino acids play an important role in energy production in *Leishmania* parasite.[34] The major alterations in amino acids in metacyclogenesis process include citraconic acid, isopropylmalic acid, L-leucine related to valine, leucine and isoleucine biosynthesis pathways, respectively. In the present study, caprylic and capric acids in fatty acid biosynthesis pathway indicated some alterations between the two phases. Based on the results of the previous studies, the oxidation rate in fatty acids is faster in stationary phase rather than the logarithmic phase, which is consistent with the results of previous studies.[35] The final significant change in metabolites is related to acetic acid in pyruvate and sulfur metabolic pathways. In the mentioned study, the detection of metabolite alterations could be helpful for better understanding of metacyclogenesis process and reopening new horizons in control of disease. In another study, Boroujeni et al.,[36] performed NMR-based approach for metabolic profiling of *L. major* in the control group (without artemisinin reception in culture media) and the case group (with artemisinin reception in culture media). Based on the results, the most significant changes were related to galactose metabolic cycle. Other significant metabolites belong to sphingolipid, valine, leucine and isoleucine biosynthesis pathways. The results showed that galactose metabolism is the most important pathway in cutaneous leishmaniasis pathogenesis and can be considered as an important drug target in parasite extermination. According to sphingolipid role in apoptosis, the results of this study on the changes of this metabolic pathway indicate that artemisinin is effective for parasite clearance. Drug resistance represents a major challenge for controlling leishmanial infectious. In addition, based on the results, antimonial (sodium stibogluconate; SSG) resistance was closely related to metacyclogenesis process in *L. donovani* as SSG-resistant strains could increase the capacity for generating metacyclic-infective forms. Also, by using ^{1}H NMR, Lamour et al.,[37] characterized the cell growth medium, parasite extract and intra and extracellular metabolome of activated and non-activated macrophages in the presence and absence of *L. major*. The results demonstrated some metabolic differences between infection state and macrophage activation. Succinate, acetate, and alanine metabolites placed at higher level in the samples exposed to parasite, compared to uninfected macrophages. Furthermore, Gupta et al.,[38] characterized map/profile of *in vitro* cultured axenic amastigote metabolites, compared to intracellular amastigote and promastigote forms employing proton NMR spectroscopy. In this study, two new metabolites including betaine and β-hydroxybutyrate were reported. It is worth noting that betaine was presented in all of parasite forms while β-hydroxybutyrate was identified only in the promastigote and axenic amastigote stages. Among other metabolites, acetoacetate could only be detected in axenic and intracellular amastigote stages. All the remaining metabolites were present at all three stages of parasite which were considered in the present plan with different levels. Tafazzoli et al.,[39] in another study, utilized a non-targeted study by using gas NMR in order to compare the metabolite profiling of

Table 1
Summary of ^{1}H NMR-based metabolomics studies conducted in the field of *leishmania* research.

Sample (Method)	Study Target	Metabolites/Metabolic Pathways	Ref.
L. major (^{1}H NMR)	*Examination of the metabolites changes in the metacyclogenesis process (procyclic VS metacyclic)*	***Valine, leucine, and isoleucine biosynthesis**: Citraconic acid, isopropylmalic acid, L-leucine* ***Pyruvate metabolism:** Acetic acid, isopropanol* ***Glutathione metabolism:** Ascorbic acid, ornithine* ***Fatty acid biosynthesis:** Caprylic acid, capric acid* ***Sulfur metabolism:** Acetic acid*	33
L. major (^{1}H NMR)	*Assessment of Artemisinin drug on metabolites profile (control VS case)*	***Galactose metabolism:** α-D-glucose 6-phosphate, melibiose, raffinose, lactose staciose* ***starch and sucrose metabolism:** Amylase, α-D-glucose 6-phosphate* ***sphingolipid metabolism:** Sphingosine, Sphingaine valine, leucine, **isoleucine biosynthesis:** Threonine biotin metabolism : pimelate, 6-carboxyhexanol coA*	36
L. major (^{1}H NMR)	*Metabolic Characterization of L. major Infection in Activated and Nonactivated Macrophages*	***Discriminating Metabolites Associated with L. major Infection:*** ***Cell culture supernatants:** Acetate, Alanine, Citrate, Fumarate, Glucose, Lactate, Ornithine, Pyruvate, Succinate* ***Cell Extracts:** Acetate, β-Alanine, Citrate, Creatine, Creatine Phosphate, Glycerophosphocholine, Glycine, Lactate, Phosphocholine, Taurine* ***Discriminating Metabolites Associated with Macrophage Activation:*** ***Cell culture supernatants:** Acetate, Alanine, Citrate, Glucose, Glycine, Lactate, Ornithine, Pyruvate, Succinate* ***Cell Extracts:** Acetate, β-Alanine, Citrate, Creatine, Creatine Phosphate, Glucose, Glycerophosphocholine, Glycine, Lactate, Phosphocholine, Taurine*	37
L. donovani (^{1}H NMR)	*Comparing of axenic amastigotes metabolites with those of promastigotes and intracellular amastigotes*	***Common metabolites in three stages:** succinate, lactate, α-glycerophosphoryl choline (α-GPC), betaine, arginine, alanine, valine* ***Promastigote and axenic amastigte metabolites:** βhydroxybutyrate* ***Axenic and intracellular amastigote metabolites:** acetoacetate*	38
Blood serum (^{1}H NMR) (atomic absorption)	*Metabonomic Study on Samples of Cutaneous Leishmaniasis and its Correlation with the Selenium Level in the Blood Serum*	*Threonine, Lipid, Lactate, Alanine*	39

^{1}H NMR; one dimensional proton nuclear magnetic resonance.

20 leishmaniasis patients sera and 44 healthy individuals. In addition, they measured the concentrations of selenium in all 64 serum samples by using an atomic absorption device. In this study, PCA and PLS methods were applied for distinguishing the outliers and classifying the patients based on selenium concentration in blood serum. The most important metabolites were related to threonine, lipids, lactate and alanine. The findings of this study indicated a significant relationship between selenium concentration and the related variables. In case of drug resistance mechanism evaluation in *leishmania*, Rainey and Mackenzie, 1991 performed metabolomics study in order to clarify the drug resistance mechanisms in *Leishmania* species which is regarded as a problem in treatment output.[40]

4.2. MS-based metabolomics in the leishmania/leishmaniasis research

Metabolomics studies based on mass spectrometry are proving of great utility in the analysis of different aspects of protozoan parasites such as *leishmania* genus. Metabolomics has also given insight into the biology, pathogenesis, mechanisms of drug against parasites and also drug resistance in *leishmania*. The list of LC-MS-based metabolomics studies along with details of the studies are summarized in Table 2. In a metabolomics investigation, Akpunarlieva et al.,[41] integrated proteomics and metabolomics approaches to identify proteins and metabolites, which represent altered abundance in mutant *L. Mexicana* (*ΔLmGT*) promastigotes. In this study, the molecular variations of wild-type and mutant model of *Leishmania* were investigated in proteome and metabolome levels. The results indicated some variations such as deficiency in glucose transport and several phenotypic changes in mutant model, as well as seeking molecular interpretation of these phenotypes. In addition, the results demonstrated a series of changes in metabolic pathways of glycoconjugate production and redox homeostasis involved in glucose metabolism in response to oxidative stress, which may represent the compatibility to the loss of sugar uptake capacity and elucidate the low virulence of this mutant among the hosts. In other study, Westrop et al.,[42] compared the metabolome profile of promastigotes of *L. donovani*, *L. major* and *L. mexicana* by LC-MS technique. The analysis revealed 64 metabolites with confirmed identity differing 3-fold or more between the cell extracts of species and 43 metabolites of confirmed identity in culture media of species, respectively. Furthermore, the recognized metabolites were detected in cell extracts of three species and their culture media included 161 and 87 metabolites. Significant differences in metabolic pathways were observed in amino acids, especially Trp, Asp, Arg and Pro, compared to the global metabolome of the studied *Leishmania* spp. This study failed to play a role in detecting inter-species differences about metabolic adaptation of parasites in their hosts. In this regard, Vincent et al.,[43] implemented an untargeted LC-MS metabolomics approach to clarify the metabolic profile changes involved in miltefosine action at time intervals of 0, 1.25, 2.5, 3.75 and 5 h of drug treatment in *L. infantum* species. Totally, 876 metabolites were detected, among which alkane fragment and sugar release related to metabolites significantly altered after 3.75 h. Fragment release was concurrent with ROS production, while no changes has occurred in membrane

Table2
Summary of MS-based metabolomics studies conducted in the field of *leishmania* research.

Leishmania spp. (Method)	Study target	Metabolites	Ref.
L. Mexicana (*LC-MS) (**GC-MS)	*Elucidation of metabolic adaptation in mutant model of Leishmania*	*Glycerol, Nicotinate, Phosphoethanol-amine, Glutamate 5-semialdehyde, Ovothiol A disulfide, Cytosine, Phosphocholine, Betaine, 2-Oxoglutaramate, Dihydroorotate, Deoxyribose, L-Glutamate, L-Aspartate, L-Alanine, Glutathione, Succinate*	41
L. donovani ***L. mexicana*** ***L. major*** (LC-MS)	*Comparative analysis of the metabolomes of promastigotes of L. donovani, L. major and L. mexicana*	***Differentiated confirmed metabolites between the cell extracts of three species:*** *64 metabolites* ***Differentiated confirmed metabolites between the cell extracts of three species****: 43 metabolites* ***Number of confirmed and putative metabolites:*** *355 metabolites* ***Large differences metabolites :*** *tryptophan, aspartate, arginine and proline*	42
L. infantum (LC-MS)	*Elucidation of the metabolic changes involved in miltefosine action on JPCM5 promastigote cell line*	*Over 800 metabolites detected, 10% of which were significantly altered after 3.75 h(many of the changes related to an increase in alkane fragment and sugar release)*	43
L. donovani (LC-MS)	*Identification of metabolic differences on three different levels: (i) during in vitro promastigote growth; (ii) between strains with a different drug susceptibility; and (iii) when a resistant strain is maintained under drug pressure*	***Differential Significant decreased metabolites between BPK275_SbIII and BPK275:*** ***LOG:*** *butyryl-, capryloyl-, valerylglycine, 2 fatty acyls, 2-oxoarginine and acetyllysine* ***STAT:*** *Xanthosine 5′-phosphate, trypanothione, low unsaturated GPL Differential metabolites change in resistant VS. sensitive strains growth stages:* ***Up LOG R/LOG S:*** *GPL building blocks* ***Up STAT R/STAT S:*** *Unsaturated GPLs, Amino acids, Purine nucleosides, Sugar phosphates* ***Down LOG R/LOG S:*** *Nucleobases and nucleosides* ***Down STAT R/STAT S:*** *Saturated GPLs*	44
L. donovani (LC-MS)	*Untargeted metabolite profiling and comparison their levels between L. donovani Promastigotes during in vitro culturing from logarithmic to stationary phase*	*Alchols, amines, amino acids, carbohydrates, fatty acyls, glycerolipids, glycerophospholipids, heterocyclic molecules, ketones and aldehydes, nucleosides, organic acids, SL and sphingoid bases, sterol and prenol lipids, vitamins and cofactors*	45
L. donovani (LC-MS)	*Characterization of the global metabolic differences between antimonial-sensitive and antimonial-resistant L. donovani isolates*	*Glycerophospholipids, sphingolipids and bases, glycerolipids, sterol and prenol lipids, amino acids, polypeptides and conjugates, purines/ pyrimidines and conjugates, miscellaneous*	46

L; Leishmania, LC-MS; liquid chromatography, GC-MS; gas chromatography, LOG; logarithmic growth stage, STAT; stationary growth stage, up; significant increase, down; significant decrease.
* Untargeted metabolomics platform.
** Targeted metabolomics platform.

phospholipids. In addition, the levels of some thiols and polyamines changed indicating DNA damage in the treated cells. Due to cell membrane damage, the evacuated levels of most metabolites occurred after 5 h of miltefosine treatment. In miltefosine resistant cells, no change was found in the internal metabolites levels. The results are important for understanding miltefosine and can be regarded as fundamental information in designing treatment program. In this respect, Berg et al.,[44] implemented an untargeted LC-MS platform for evaluating the metabolic differences of *L. donovani* during promastigote stages in the absence or presence of drug pressure. The study presented new approaches in the context of antimonial resistance and differentiation. The results of metabolites changes demonstrated that the SSG-resistant parasites can play a significant role in encountering with oxidative stress process. Further, Silva et al.,[45] applied an untargeted metabolomics approach for evaluating the metabolic changes during promastigote by developing *in vitro* culturing between 3 and 6 days. Based on the results, the metabolome profile of promastigotes differed significantly from each other during culture. The most striking metabolic differences were related to the structural variations in glycerophospholipids and an increase in the amount of sphingolipids and glycerolipids as cells progressed from procyclic to metacyclic stages. Furthermore, Kindt et al.,[46] implemented an untargeted LC-MS metabolomics approach to global comparative metabolic profiling of antimonial-sensitive and antimonial-resistant *L. donovani* isolates. They determined the antimonial-sensitive and antimonial-resistant differences by using metabolomics study and measured the relative abundance of 340 metabolites and a large number of lipids. Based on the results, distinct metabolic profiles were observed in drug-sensitive and drug-resistant parasites and significant differences were shown between two phenotypes among100 metabolites. The most significant difference in metabolites was related to the pathways of lipid metabolism. However, the results failed to involve resistance mechanisms for antimonial drugs. Also, in the field of comparative metabolomics of wild type and mutant parasite model, De Souza et al.,[47] described a GC-MS peak clustering approach for discriminating the wild-type of *L. Mexicana* from two mutant parasite lines based on their metabolic profile.

5. Conclusion

Leishmania as a protozoan parasite is considered as an etiological agent of wide range of clinical manifestations known as ‘‘leishmaniasis”. The disease represents a major public health problem and is endemic in 98 countries across five continents. However, no effective vaccine and decisive treatment has been introduced yet. Over the past few years, ‘‘Omics” technologies have played a pivotal role in recognizing parasite biology in its different life cycle stages, identifying the parasite-host interaction, and introducing novel drug target. Metabolomics approach based on a large-scale study of metabolite profiles both in parasite and the host can accelerate the discovery of novel metabolite markers and reveal their related biochemical pathways to understand the underlying pathology of leishmaniasis. In addition, metabolomics approach has attracted a lot of attention among researchers, due to the regulation of gene expression of trypanosomatidae in post-transcriptional level. This review provides a reference including all studies in the field of *Leishmania* metabolomics. In most of the conducted studies, metabolomics-based study of *L. donovani*, *L. major* and *L. mexicana* spp. was investigated by LC-MS and NMR methods while other species such as *L. tropica*, *L. infantun*, etc. were neglected. The most common objectives in these studies are related to a comparative metabolomics of the biology of *Leishmania* parasite in different stages such as procyclic, metacyclic and amastigote. However, a few studies were conducted by using biological samples of mammalians hosts for example blood, urine and tissue samples in response to infection. In this respect, the results of the study on blood serum of cutaneous leishmaniasis showed that the most important metabolites include threonine, lipid, lactate and alanine. Further, the limitation of metabolomics study of parasite isolates has great potential to increase our understanding of metabolism in the clinically relevant stage. The study of drug resistance and drug action by metabolomics obtain some important information about designing integrated therapies, understanding parasite metabolism, and highlighting some new targets for chemotherapy important. In metabolomics-based study, the study of metabolic pathways in natural plant-derived products is very critical in confronting the disease in a natural program with the least amount of side effects. The present review will provide a reference to conduct new studies for better understanding of the pathways playing a role in the biology of *Leishmania* and its control. Additionally, the integration of metabolomics with other platforms of system biology such as genomics, transcriptomics, proteomics and lipidomics will provide further and better insights into evaluating different pathophysiology aspects and treating the disease.

Conflict of interest

The authors declare that there is no conflict of interest.

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