Original Article

Metabolomics Based Study of the Antileishmanial Activity of Xanthium strumarium Leaf Extract on Promastigotes Phases of Leishmania major by Proton NMR Spectroscopy

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Abstract

Background: Xanthium strumarium L. is extensively used as a traditional herb to treat many diseases and is also known as a source of phytochemicals. It has been used traditionally to treat trypanosomiasis, malaria fever, eczema, cancer, ulcer, fever, herpes headache, and skin lesion such as leishmaniasis. In this preliminary study, nuclear magnetic resonance (NMR)-metabolomics approaches was used to evaluate the inhibitory effects and metabolic alterations caused by leaf extract of X. strumarium on the stationary phases of promastigotes in Leishmania major.

Methods: The promastigotes were cultured in Biochemistry Laboratory at Pasteur Institute of Iran in 2017, stationary phases were obtained from 5 to 6 day-old cultures and treated with different concentrations of the plant’s extract. Antileishmanial activity was assayed by MTT method and cell metabolites were extracted. 1H NMR spectroscopy was applied, and outliers were separated using multivariate statistical analysis.

Results: The most affected metabolic pathways in the experimental groups were limited to amino sugar and nucleotide sugar metabolism, cyanoamino acid metabolism, starch and sucrose metabolism, butanoate metabolism, and galactose metabolism.

Conclusion: The ethanolic leaf extract of X. strumarium is a potent growth inhibitor of Leishmania major and can affect vital metabolic pathways of Leishmania promastigotes. The assay provided new perspectives on the development of novel treatment strategies for leishmanial activity derived from natural products.
Introduction

Leishmaniasis, the infection caused by several species of intracellular protozoa of the genus *Leishmania*, is identified as a neglected health problem by WHO, especially in tropical and subtropical regions. These infections exist in two clinical manifestations, visceral and cutaneous leishmaniasis (1). Cutaneous leishmaniasis (CL) is the most prevalent clinical form worldwide with 0.7 to 1.3 million new cases each year and annually causes 20,000 infection cases in Iran alone (1, 2). Since 1923, the chemotherapy based on pentavalent antimonials (SbV) has been the first-line treatment for leishmaniasis, which is far from satisfactory. Alternative drugs such as amphotericin B (amphoB), pentamidine, miltefosine, and drug combinations have also been recommended (3). The first indications of drug resistance were reported in the early 80s first in India and then in Nepal, in patients who had relapsed (4). The toxicity and the high cost of the available drugs due to drug resistance, teratogenicity and the need for the hospitalization of patients have resulted in the loss of drug effectiveness (5).

Therefore, due to the mentioned reasons, there is an urgent need to develop safer and more effective antileishmanial drugs with high efficacy. Folk medicine based on natural plants is used by approximately 80% of the world population. It contains about 25% of modern medicine, widely applied in the treatment of anti-parasitic diseases (6).

*Xanthium strumarium* L. (Cocklebur) is an annual weed, including 25 species belonging to the Asteraceae family, which grows in Iran between August and September with local common name, Tough or Zardineh. *X. strumarium* is traditionally used to treat trypanosomiasis, malaria fever, eczema, cancer, ulcer, fever, herpes, headache and skin sores such as leishmaniasis (7). The species contain a class of plant terpenoids called Sesquiterpene lactones (STLs) which are classified based on their carboxylic skeletons, into xanthanolides and guaianolides. STLs are believed to be the active metabolites of *X. strumarium* and have caused considerable interest because of the broad range of their biological activities. Artemisinin for the treatment of malaria, xanthatin and xanthinosin for antitumor activity, parthenolide for treating migraine and thapsigargin for curing prostate cancer all belong to terpenoids classes (8).

*Leishmania* parasites undergo fluctuating environmental conditions in their life cycle; promastigotes proliferate in the mildly alkaline and glucose-rich environment of the midgut of sandfly and amastigotes grow in the acidic and glucose-poor environment of the phagolysosome of macrophages. There were significant changes in parasite metabolism during these developmental stages (9). The altered metabolic fluxes undoubtedly reflect adaptation to differing challenges that *Leishmania* naturally confronts in its two hosts. Glycolysis rather than gluconeogenesis is more important in promastigotes than in amastigotes. Differentiation of procyclic promastigotes to infective metacyclic promastigotes involves changes in membrane fluidity in lipophosphoglycan (LPG) and glycerolipids structures (10).

Antimonials have a complex multifactorial mode of action such as an inhibitory effect on trypanothione reductase of the parasite, and sodium stibogluconate [Sb(V)] inhibits fatty acid oxidation, glycolysis, and energy metabolism (11).

Metabonomics is one of the omics technologies, which is regarded as one of the functional responses of biological systems to pathophysiological stimuli or genetic modifications and is relevant to drug resistance studies (12). It is defined as a promising approach to study metabolic alterations within living samples after drug treatment (13). In fact, this approach directly links the metabolic profile of an organism to its corresponding genomic

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profile, which is widely used to find new pharmaceutical compounds (12).

Due to the lack of effective chemotherapy, the emergence of drug resistance and the growing interest in plant compounds for the treatment of parasitic diseases, this preliminary study aimed to explore the inhibitory effects of X. strumarium leaf extract and compare the metabolome fingerprint alterations on stationary phases of promastigotes in L. major treated with this substance.

Materials and Methods

Plant material
Leaves of X. strumarium were collected from August-September 2014 from around Kermanshah City, Kermanshah Province, Iran. Voucher specimens were authenticated by Central Herbarium of Tehran University (TUH) under voucher specimen number 48241.

Preparation of crude plant extracts
The dried and powdered leaves of X. strumarium (40 g) were exhaustively macerated with an alcohol-water solution (80%) at room temperature for 72 hours. This mixture was filtrated and concentrated under reduced pressure to obtain a light-green extract. The resulting extract was mixed with active charcoal, centrifuged to isolate plant pigments and stored at -20°C until required for bioassay.

Determination of total polyphenol content
Total phenolic content was determined using the Folin-Ciocalteau reaction (2014). The calibration curve of gallic acid standard solutions was applied to calculate the number of total polyphenols. Measurements were done in tetraplicate (14).

Leishmania parasites
Amastigote forms of L. major (strain MRHO/IR/75/ER) were originally isolated from infected Balb/c mice followed by transformation into promastigote forms in RPMI 1640 medium supplemented with 10-20% heat-inactivated fetal bovine serum (FBS), 100 µg/ml streptomycin, and 100 U/ml penicillin G at 23-26°C (Sigma Aldrich, St. Louis, MO, USA). To perform leishmanicidal assays, Stationary-phase promastigotes were obtained from 5 to 6 day-old cultures and centrifuged at 3000 rpm for 10 min at 4°C (15).

Assay of leishmanicidal activity
The leishmanicidal assay was carried out following a protocol (16). The promastigote forms, in stationary phase, were seeded at 27°C for 24 h at 2×10^5 promastigotes/well in a 96-well microplate. The stock solutions of each of the test samples (150 µg/mL) were added and serial dilution was carried out with RPMI medium. After 48 hours, promastigotes viability was calculated by tetrazolium-dye (MTT) colorimetric method and absorbance was measured at 545 nm in the presence of reference length wave 630 and the percentage of the viability of the promastigotes was measured according to IC_{50}. The results were expressed according to the amount of the extract concentration (15-0.000015 µg/mL) required to reduce the absorbance to half (IC_{50}) that of the negative control wells.

Amphotericin B (0.5 mg/ml) and promastigotes with no drugs were used as positive and negative controls, respectively.

Cell extraction for metabolite analysis
Stationary promastigotes (initial concentration 1×10^9 parasites/vial) were treated with IC_{50} concentrations of leaf extract (0.003 mg/ml) leaf extract/2×10^9 parasites). After

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incubation at 25 °C for 72 hours, the cells were centrifuged at 4000 rpm for 10 min at 4 °C. Then pellets were harvested for the extraction process of metabolites for 1HNMR spectroscopy. In brief, pellets (1×10⁹ promastigotes/vial) were resuspended in RPMI 1640 without FBS, centrifuged at 15000 rpm for 15 min at 4°C and washed twice with PBS. 1.0 ml of chilled perchloric acid (1.8 M) was added to the cell suspension, vortexed and sonicated for 5 min at 4°C followed by centrifugation at 12000 rpm at 4°C for 10 min. The pH of the supernatant was adjusted to 6.8 and it was kept on ice for one hour to allow the precipitation of potassium perchlorate and then was centrifuged again as above. The supernatant was lyophilized (17).

1HNMR Spectroscopy
Before NMR acquisition, lyophilized powder samples (n=10) were resuspended in D₂O containing trimethylsilyl propionate (1mM) as a chemical shift reference (δ=0 p.p.m.) and imidazole (2 mM) as pH indicator (δ=5.50 to 8.80 p.p.m.). Samples were centrifuged (18000 rpm for 30 min at 4 °C). 500 µl of the extracted samples were transferred to a NMR tube, analyzed on a Bruker AV-500 NMR spectrometer with field gradient operating at 500.13 MHz for proton observation at 298K. One-dimensional 1HNMR spectra were recorded using a 10-µs pulse, 0.1 s mixing time, 3.0 s relaxation delay, 6009.6 Hz spectra width, and 3000 transients with standard 1D NOESY pulse sequence to suppress the residual water peak (18).

Data analysis
NMR spectra were preprocessed using custom written ProMatab function in MATLAB (v.7.8.0.347) environment. Following the standard processing steps, each 1D spectra were aligned and binned in 0.005 ppm and selected signals arising from water at 4.7 ppm were removed (19). Partial Least Square - Discriminant Analysis (PLS-DA), which is a supervised technique to visualize the class-specific segregation and to obtain the significant bins contributing to the variation across the classes, was used. This, in turn, led to the identification of the metabolites corresponding to the spectral bins using Human Metabolome Database (HMDB) and LeishCyc database, which is the pathway/genome database for L. major. Metabolic pathways were also investigated by the help of Metaboanalyst (v3.0) online software package.

Results

Total polyphenol content
The total phenolic content (TPC) of leaf extract of X. strumarium was equated to 0.150 µg/ml.

Antileishmanial assay
X. strumarium stock extract (150 µg/mL) showed the highest anti-promastigote activity, while a weaker inhibitory activity against this parasite was observed in other concentrations. In comparison, amphotericin B presented an inhibitory activity almost similar to the stock fraction. Concerning the antileishmanial activity, we observed that only one concentration could be considered as having promising activity with an IC₅₀ value of 0.15 µg/ml treating culture of stationary promastigotes (Table 1). There was a dose-dependent decrease in the percentage of viability.

Effect of leaf extract of X. strumarium on metabolome profile
1D NMR assay showed that many metabolic pathways were influenced by leaf extract of X.strumarium. In the current study, the class-specific segregation can be refined using PLS-DA. This aided in the visualization of the data as scores plot (Fig. 1). Outliers were also separated by the use of loading plot (Fig. 2). Important features were identified by PLS-DA as variable importance of projection (VIP) (Fig.3).
Table 1: In vitro viability of *L. major* freidlin promastigotes against *Xanthium strumarium* extract activity

| Concentration (µg/ml) | Xanthium Strumarium | Amphotericin B (Positive control) |
|-----------------------|---------------------|----------------------------------|
| Negative Control      | 85                  | 80                               |
| 150(Stock)            | 9                   | 3                                |
| 15                    | 23                  | 20                               |
| 1.5                   | 36                  | 28                               |
| 0.15                  | 56                  | 49                               |
| 0.015                 | 62                  | 58                               |
| 0.0015                | 76                  | 76                               |
| 0.00015               | 83                  | 80                               |
| 0.000015              | 82                  | 79                               |

Fig. 1: PLS-DA scores plot of stationary phases of *L. major* ¹HNMR. One-color dots represent the sample available in a group. Red triangle = *L. major* – untreated and green cross = *L. major* - treated. The explained variance are shown in brackets

Fig. 2: PLS-DA loading plot of stationary phases of *L. major*. This plot shows the relative contribution of bins/spectral variables to the clustering of experimental and control groups. Each point in the figure represents a bin. The loading [2] axis represents the correlation of the bin towards the predictive variation shown in Figure 1. The loading [1] axis represents the magnitude of the spectral bins
**Pathway analysis**

Human Metabolome Database (HMDB) and LeishCyc were used to identify metabolites corresponding to the spectral bins. Also, metabolic pathways relating to these separated outliers were determined by generic databases, such as KEGG pathway database and Metaboanalyst online pathway analysis tools for metabolomics (Table 2 and Fig. 4). Five pathways with p value less than 0.5 were used in our discussion and other pathways were kept aside.

![Fig. 3: A VIP variable score is the measurement of the variable importance in PLS-DA model. The red color indicates the increase, and the green color stands for the decrease in variable concentrations](image)

![Fig. 4: Topology map of altered biochemical pathways in experimental and control group according to the degree of centrality. The geometric position of each node in the topology map is presented by -Log(p)](image)

1- Aminosugar and nucleotide sugar metabolism;  
2-Cyanoamino acid metabolism;  
3-Starch and Sucrose metabolism;  
4-Butanoate metabolism;  
5-Galactose metabolism;  
6-Phenylalanine Tyrosine and tryptophan biosynthes;  
7-Pantothenate and CoA biosynthesis;  
8-Synthesis and degradation of ketone bodies;  
9-Glycerolipid;  
10-Cysteine and methionine metabolism;  
11-Alanine, aspartate and glutamate metabolism;  
12-Pentose and glucuronate interconversions ;  
13-Valine, leucine and isoleucine biosynthesis;  
14-Aminoacyl-tRNA biosynthesis;  
15-Pyrimidine metabolism ;  
16-beta-Alanine metabolism;  
17-Inositol phosphate metabolism;  
18- Fructose and mannose metabolism;  
19-Glycine, serine and threonine metabolism;  
20- Sphingolipid metabolism;  
21-Arginine and proline metabolism;  
22- Glutathion metabolism;  
23- Purine metabolism;  
24- Glycerophospholipid metabolism;  
25-Valine, leucine and isoleucine degradation

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Table 2: Metabolome pathway analysis results

| Metabolic pathway                                                                 | Metabolites                                                                 | Total | Hits | Raw p |
|-----------------------------------------------------------------------------------|-----------------------------------------------------------------------------|-------|------|-------|
| Amino sugar and nucleotide sugar metabolism                                       | D-Fructose, Glucosamine6-phosphate, uridine diphosphate galactose, Fructose 6-phosphate, N-Acetyl-D-Glucosamine6-phosphate | 21    | 6    | 0.349 |
| Cyanoamino acid metabolism                                                        | L-Aspartic acid, L-Asparagine                                                | 6     | 2    | 0.419 |
| Starch and Sucrose metabolism                                                     | Glucose1-phosphate, D-Fructose                                               | 6     | 2    | 0.419 |
| Butanoate metabolism                                                              | 3-Hydroxybutyric acid, Butanol 1-Butanol                                     | 11    | 3    | 0.483 |
| Galactose metabolism                                                              | Glucose1-phosphate, Uridine diphosphate galactose                            | 7     | 2    | 0.506 |
| Phenylalanine, tyrosine and tryptophan biosynthesis                               | L-phenylalanine                                                              | 4     | 1    | 0.650 |
| Pantothenate and CoA biosynthesis                                                 | Pantothenic acid L-valine                                                    | 10    | 2    | 0.711 |
| Synthesis and degradation of ketone bodies                                         | 3-Hydroxy butyric acid                                                       | 5     | 1    | 0.731 |
| Glycerolipid                                                                      | D-glyceraldehyde                                                             | 11    | 2    | 0.762 |
| Cysteine and methionine metabolism                                                | Cysteic acid S-Adenosyl homocysteine L-Serine L-Cystathionine                 | 22    | 4    | 0.786 |
| Alanine, aspartate and glutamate metabolism                                       | Aspartic acid L-Asparagine glucosamine 6-phosphate                           | 17    | 3    | 0.791 |
| Pentose and glucuronate interconversions                                          | glucose-1-phosphate L-Valine                                                 | 6     | 1    | 0.794 |
| Valine, leucine and isoleucine biosynthesis                                       | L-Valine                                                                     | 6     | 1    | 0.794 |
| Aminoacyl-tRNA biosynthesis                                                       | L-Asparagine L-Phenylalanine L-Arginine L-Aspartic acid L-serine L-valine L-Lysine | 46    | 8    | 0.873 |

Since, many pathways were tested at the same time; the statistical p values from enrichment analysis are further adjusted for multiple testing. Total is the total number of compounds in the pathway; Hits, the actually matched number from the user upload data;
Raw p, the original P value calculated from the enrichment analysis by using Metaboanalyst (v.3) online software.

**Discussion**

The toxicity profile of antimony justified the search for safer drugs in treating leishmaniasis. Antimony drugs inhibit energy metabolism and macromolecular biosynthesis via inhibition of glycolysis and fatty acid beta-oxidation (20). ¹H NMR spectroscopy comparison of metabolome profile of *L. major* promastigotes treated with leaf extract of *X. strumarium* showed that there were important variations in some levels of individual metabolites and their corresponding pathways. The most significantly altered pathways between the two groups were amino sugar and nucleotide sugar metabolism, cyanoamino acid metabolism, starch and sucrose metabolism, butanoate metabolism, and galactose metabolism.

*Leishmania* parasites contain a variety of nucleotide sugars (21). One of the most distinctive roles of these nucleotide sugars is the biosynthesis of glycoconjugates, forming the cell surface coat (22). This dense glycocalyx is composed of glycosylphosphatidylinositol-like structures including lipophosphoglycans (LPGs), GPI-anchored glycoproteins, proteophosphoglycans (PPGs) and, free GPI glycolipids which are essential for parasite survival in the sandfly vector and the mammalian host (23).

The distinct role of sugar nucleotides in *Leishmania* molecular adjustment has been reported (22). *Leishmania* requires uridine diphosphate glucose (UDP-GLC) in the nucleus to synthesize an unusual DNA base called base J (β-D-glucosyl-hydroxymethyluracil) which plays an important role in regulating the onset and end of transcription (22). In the present study, treatment of *Leishmania* promastigotes with leaf extract of *X. strumarium* was associated with changes in N-acetylglucosamine 6-phosphate, glucose-1 phosphate, glucosamine-6 phosphate and glucose and fructose uridine diphosphate metabolites in amino sugar and nucleotides sugar metabolic pathways.

Collectively, it is implied that the plant extract, by affecting these metabolites, causes complex glycocalyx destruction, which in turn causes protective surface coat formation and mediates essential host-parasite interactions.

In the present study, the major alterations of amino acids in the cyanoamino metabolic pathway were confined to two metabolites, namely aspartic acid and asparagine. In another investigation, to find a novel drug, there were 97 unique metabolic reactions in *L. donovani*, which cyanoamino acid was one of them (24). Asparagine amino acid had an inhibitory effect on the autophagosome pathways of *Leishmania* (25). Increasing the amount of asparagine and decreasing the amount of aspartic acid by inhibiting asparaginase enzyme, could enhance the inhibitory effect of phagolysosome fusion and consequently inhibit promastigotes infection in the host body (26). In addition, the biological activity and the role of asparagine synthase enzyme are more emphasized today, due to the role of asparagine. Asp and Asn amino acids can be synthesized from oxaloacetate by mitochondrial enzymes of aspartate aminotransferase and asparagine synthase which were present in *L. major* (27).

There are two different A and B forms of asparagine synthase enzyme. Form A is strongly dependent on ammonium and form B uses glutamine. Form A of this enzyme is not observed in humans while it is considered a potential drug target in trypanosomes (28). Form A of this enzyme is necessary for the survival and infectivity of *L. donovani*. Therefore, it is considered a drug target (28). Previous studies revealed the important role of these two metabolites. Therefore, it is likely that *X. strumarium* leaf extract inhibits the growth of promastigotes by cyanoamino acid metabolic pathway alteration through increasing asparagine and reducing aspartic acid.
In addition, the present study indicates the alteration of two metabolites, fructose, and glucose 1 phosphate, in the pathway of starch and sucrose metabolism. Thus, sucrose is an intermediate metabolite in this metabolic pathway. The presence of a sucrose enzyme, namely fructofuranose, α-glucosidase, α-amylase, and cellulose in *L. major* was proven while it was absent in a few trypanosomes species (29).

*L. major* promastigotes experience glucose-rich conditions in the digestive tract of their sandfly vector, as the sugar meals consumed from the plant sap were digested by amylase, kinase and sucrose enzymes (30).

In addition, the plant starch is hydrolyzed by α-amylase enzyme and converted to maltose, and maltose is often converted to glucose by α-glucosidase enzyme. Therefore, given that the main fuel of promastigotes is ATP production through glucose glycolysis, and since an important quantity of parasite glucose is provided through the pathway of starch and sucrose metabolism, it can be concluded that any change in the metabolites of this pathway can inhibit parasite growth by interfering with the energy of the promastigotes, which is consistent with our results.

In the current study, butanoate profile alterations are confined to three metabolites, 3-hydroxy-butanoic acid and butanal and 1-butanol. No significant study was observed on the role and probable importance of butanol and butanal in the maintenance and survival of promastigotes. The previous metabolomics-based investigation, confirmed 3-hydroxybutyric alteration in mice treated with new amphoterocin B drug compound (31). This drug compound had less toxic effects on patients with Leishmaniasis (32). Therefore, it can be postulated that butanol and butanal metabolites, especially 3-hydroxybutyric acid, play an important role in the parasite metabolism and the leaf extract of *X. stumarium* inhibits the growth of *Leishmania* promastigotes by disrupting this metabolic pathway and inhibiting ATP supply. Our results are supported by previous finding (31).

UDP-Gal can be interconverted to UDP-galactofuranose (UDP-Galf) by the flavor dependent enzyme UDP-galactopyranose mutase (33). The LPGs were the most abundant glycoconjugates on the surface of *Leishmania* promastigotes, about 6×10^7 copies per cell (34), which play an important role in protection against the hydrolytic enzymes of blood-fed phlebotomines (22). In *L. major*, The LPG galactose side chains are progressively capped with b1,2-linked D-arabinose during the transition from procyclic to metacytic promastigotes. These changes lead to an increase in the thickness of the surface glycolyx, which results in additional resistance to complement-mediated lysis (32). The defective galactose metabolism present in altered glycolyx was associated with parasite attenuation (35).

Therefore, the importance of enzymes involved in the biosynthesis of Galf might provide new targets for the development of effective drugs to combat Leishmaniasis (21). Deletion of the UGM gene in *L. major* resulted in the complete depletion of β-Galactofuranose (β-Galf) in the structure of repeated units of phosphoglycans in GPIs and LPGs. Furthermore, mice infection by this mutant of *L. major* was significantly attenuated (36). Moreover, galactose metabolism acts as a key factor in the occurrence of African sleepiness caused by *Trypanosome brucei* (37). In the present study, two key metabolites of glucose 1-phosphate and uridine diphosphate galactose in the galactose metabolic pathway changed in promastigotes treatment with promastigotes with leaf extract of *X. strumarium* caused disruption or change in the glycoprotein cover of the parasite, by disrupting or changing the galactose metabolism. Therefore, increased susceptibility to host complement and oxidative stress, caused promastigotes attenuation.
Conclusion

Ethanolic leaf extract of X. strumarium exhibits antileishmanial activity even in low doses and affects vital metabolic pathways of Leishmania promastigotes. Further research is underway to find out the potential fractions of the leaf extract of this plant.

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Conflict of interests

Authors declare that there is no conflict of interest.

References

1. WHO (2017). Fact sheet Leishmaniasis. https://www.who.int/news-room/fact-sheets/detail/leishmaniasis.
2. Mohebali M. Visceral leishmaniasis in Iran: Review of the epidemiological and clinical features. Iran J Parasitol. 2013;8:348-358.
3. Torres-Guerrero E, Quintanilla-Cedillo MR, Ruiz-Ensenjaud J, Arenas R. Leishmaniasis: A review. F1000Res. 2017;6:750.
4. Sundar S. Drug resistance in Indian visceral leishmaniasis. Trop Med Int Health. 2001;6:849-854.
5. Singh OP, Singh B, Chakravarty J, Sundar S. Current challenges in treatment options for visceral leishmaniasis in India: A public health perspective. Infect Dis Poverty. 2016;5:19.
6. Gachet MS, Lecaro JS, Kaiser M, et al. Assessment of anti-protozoal activity of plants traditionally used in ecuador in the treatment of leishmaniasis, J Ethnopharmacol. 2010; 128:184-197.
7. Lavault M, Landreau A, Larcher G, et al. Antileishmanial and antifungal activities of xanthanolides isolated from Xanthium macrocarpum. Fitoterapia. 2005;76:363-366.
8. Li Y, Chen F, Li Z, Li C, Zhang Y. Identification and functional characterization of sesquiterpene synthases from Xanthium strumarium, Plant Cell Physiol. 2016;57:630-641.
9. Subramanian A, Jhawar J, Sarkar RR. Dissecting Leishmania infantum energy metabolism - a systems perspective. PLoS One. 2015;10:e0137976.
10. Silva AM, Cordeiro-da-Silva A, Coombs GH. Metabolic variation during development in culture of Leishmania donovani promastigotes. PLoS Negl Trop Dis. 2011;5:e1451.
11. Berg M, Garcia-Hernandez R, Cuypers B, et al. Experimental resistance to drug combinations in Leishmania donovani: Metabolic and phenotypic adaptations. Antimicrob Agents Chemother. 2015;59:2242-2255.
12. Swanepoel CC, Loots du T. The use of functional genomics in conjunction with metabolomics for mycobacterium tuberculosis research. Dis Markers. 2014;2014:124218.
13. Atan NAD, Koushki M, Ahmadi NA, Rezaei-Tavirani M. Metabolomics-based studies in the field of Leishmania/leishmaniasis. Alexandria J Med. 2018
14. Scherer R, Godoy H. Effects of extraction methods of phenolic compounds from Xanthium strumarium l. And their antioxidant activity. Revista Brasileira de Plantas Medicinais. 2014;16:41-46.
15. Arjmand M, Madrakian A, Khalili G, Najafi Dastnace A, Zamani Z, Akbari Z. Metabolomics-based study of logarithmic and stationary phases of promastigotes in Leishmania major by 1H NMR spectroscopy. Iran Biomed J. 2016;20:77-83.
16. Wang X, Ge J, Wang K, Qian J, Zou Y. Evaluation of mt assay for measurement of emodin-induced cytotoxicity. Assay Drug Dev Technol. 2006;4:203-207.
17. Gupta N, Goyal N, Singha UK, Bhakuni V, Roy R, Rastogi AK. Characterization of intracellular metabolites of axenic amastigotes of Leishmania donovani by 1H NMR spectroscopy. Acta Trop. 1999;73:121-133.
18. Sheedy JR. Metabolite analysis of biological fluids and tissues by proton nuclear magnetic resonance spectroscopy. Methods Mol Biol. 2013;1055:81-97.
19. Viant MR. Improved methods for the acquisition and interpretation of NMR metabolomic data. Biochem Biophys Res Commun. 2003;310:943-948.

Available at: http://ijpa.tums.ac.ir
20. Croft SL, Sundar S, Fairlamb AH. Drug resistance in leishmaniasis. Clin Microbiol Rev. 2006;19:111-126.
21. Naderer T, Heng J, Saunders EC, et al. Intracellular survival of *Leishmania major* depends on uptake and degradation of extracellular matrix glycosaminoglycans by macrophages. PLoS Pathog. 2015;11:e1005136.
22. Damerow S, Hoppe C, Bandini G, Zarnovican P, Buettner FF, Ferguson MA, Routier FH. *Leishmania major* udp-sugar pyrophosphorylase salvages galactose for glycoconjugate biosynthesis. Int J Parasitol. 2015;45:783-790.
23. Zhang WW, Chan KF, Song Z, Matlaszewski G. Expression of a *Leishmania donovani* nucleotide sugar transporter in *Leishmania major* enhances survival in visceral organs. Exp Parasitol. 2011;129:337-345.
24. Meshram RJ, Jangle, SN. Comparative metabolomic investigation of *Leishmania major* and homo sapiens: An in-silico technique to discover and develop novel drug targets. Pharmacophore 2011;2 98-107.
25. Ren W, Rajendran R, Zhao Y, al. Amino Acids As Mediators of Metabolic Cross Talk between Host and Pathogen. Front Immunol. 2018;9:319
26. Singh J, Srivastava A, Jha P, Sinha KK, Kundu B. L-asparaginase as a new molecular target against leishmaniasis: Insights into the mechanism of action and structure-based inhibitor design. Mol Biosyst. 2015;11:1887-1896.
27. Oppendoes FR, Michels PA (2008) The metabolic repertoire of *Leishmania* and implications for drug discovery In: Myler PJ, Fasel N, editors. *Leishmania*: After The Genome Norfolk, U.K Caister: Academic Press; pp. 123–158
28. Faria J, Loureiro I, Santarem N, Macedo-Ribeiro S, Tavares J, Cordeiro-da-Silva A. *Leishmania infantum* asparagine synthetase a is dispensable for parasites survival and infectivity. Plos Negl Trop Dis. 2016;10:e0004365.
29. Jacobson RL, Schlein Y. Phlebotomus papatasi and *Leishmania major* parasites express alpha-amylase and alpha-glucosidase. Acta Trop. 2001;78:41-49.
30. Tielens AGM, van Hellemond JJ. Surprising variety in energy metabolism within trypanosomatidae. Trends Parasitol. 2009;25:482-490.
31. Santos DCM, Lima ML, Toledo JS, et al. Metabolomics as a tool to evaluate the toxicity of formulations containing amphotericin b, an antileishmanial drug. Toxicol Res (Camb). 2016;5:1720-1732.
32. McConville MJ, Turco SJ, Ferguson MA, Sacks DL. Developmental modification of lipophosphoglycan during the differentiation of *Leishmania major* promastigotes to an infectious stage. EMBO J. 1992;11:3593-3600.
33. Oppenheimer M, Valenciano AL, Sobrado P. Biosynthesis of galactofuranose in kinetoplastids: Novel therapeutic targets for treating leishmaniasis and chagas' disease. Enzyme Res. 2011;2011:415976.
34. Ilgoutz SC, McConville MJ. Function and assembly of the *Leishmania* surface coat. Int J Parasitol. 2001;31:899-908.
35. Damerow S, Lamerz A-C, Haselhorst T, Führing J, Zarnovican P, von Irzstein M, Routier FH. *Leishmania* udp-sugar pyrophosphorylase the missing link in galactose salvage? J Biol Chem. 2010;285:878-887.
36. Kleczka B, Lamerz AC, van Zandbergen G, et al. Targeted gene deletion of *Leishmania major* udp-galactopyranose mutase leads to attenuated virulence. J Biol Chem. 2007;282:10498-10505.
37. Roper JR, Guther MLS, Milhe KG, Ferguson MAJ. Galactose metabolism is essential for the african sleeping sickness parasite *Trypanosoma brucei*. Proc Natl Acad Sci USA. 2002;99:5884-5889.