Effects of combination of Amphotericin B and *Fumaria parviflora* ethanolic extract against *Leishmania major* and expression of miR146a-5p and miR499 levels

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Abstract

Cutaneous leishmaniasis is a common zoonotic parasitic disease in the Middle East caused by *Leishmania major* and *L. tropica*. The aim of this study was to investigate Amphotericin B and *Fumaria parviflora* (*F. parviflora*) ethanolic extract of antiparasitic effects against *L. major* and expression of miRNA (miR)146a-5p and miR499 in culture cells. Amphotericin B (50 and 100µg/mL) and *F. parviflora* ethanolic extract (10-100%v/v) were prepared and subjected to ER/75/IR/M L. major strain promastigotes in the RPMI medium wells and BALB/c mice (n=60). Promastigotes suspensions (2×10⁶) were cultured in wells in triplicate. After incubation at 25°C for 24hrs, the number of parasites into macrophages were counted using Gimsa dye in each group. The mice cutaneous infection was performed for a week and the effect of Amphotericin B (100µg/mL) singly and combined with 50% v/v of the *F. parviflora* ethanolic extract was evaluated for 10 days. The expression of miR146a-5p and miR499 into macrophages in each group was evaluated using real-time PCR.

The 50% lethal dose (LC50) of *F. parviflora* ethanolic extract included 100% v/v while the combination of 50% extract with 100 µg/mL of Amphotericin B killed 90% (LC90) of promastigotes during 48h. The in vivo study exhibited improvement of lesions sizes significantly in combination therapy compared to the control group (p<0.05). The expression of miR146a-5p and miRA499 was not significantly affected in treatment to the Amphotericin B and *F. parviflora* ethanolic extract. Combination of Amphotericin B (50 and 100µg/mL) and *F. parviflora* ethanolic extract can significantly inhibit the promastigotes growth during 72hrs. Moreover, this combination was promising towards in vivo treatment of skin Leishmania lesions.

Keywords: Amphotericin B, *Fumaria parviflora* ethanolic extract, *Leishmania major*, miR146a-5p, miR499.

Introduction

Cutaneous leishmaniasis is a common zoonotic parasitic disease in the Middle East caused by *Leishmania major* and *L. tropica*. Leishmaniasis is divided into three groups of cutaneous, skin-mucosal and visceral leishmaniasis in terms of clinical signs and outcomes in patients.²⁷¹⁰ Skin lesions or cutaneous leishmaniasis is one of the most common endemic zoonotic diseases in most parts of the world. The main reservoir of the *L. major* has been identified as domestic animals and wildlife, while the carriers of the disease in the old and new world respectively include female mosquitoes of phlebotomus and lutzemia.²² These flies carry promastigote form of Leishmania. When entered into the human cells, they transform into amastigote form. Moreover, at culture conditions at 22°C, the promastigote form will be developed.

South American species *L. braziliensis* (27.84), *L. major* and *L. tropica* also cause the disease in Europe and Asia. The number of people affected by Leishmaniasis currently reach more than 21 million individuals in 88 countries and it is estimated that 351 million people are at risk of infection and 1 to 2 million new cases occur annually²⁻⁷ while treatment of the disease is still based on old therapies such as pentavalent antimonials with potential of systemic toxicity. Glucantime is the most common drug for local use and to improve pain and recover appetite but is not recommended in patients liver and kidney impairment conditions.

*Fumaria parviflora* (*F. parviflora*) is a traditional herbal medicine consumed for various ailments such as increase fertility, anti-inflammatory, antispastic and pain, anti-nausea and for treatment of burn lesions. This herb grows in humid and semi-humid areas. It contains various alkaloids such as fumarin, protopin, cryptocavin, scolerin and tetrahydrocupicin. Minerals such as potassium and various bioactive compounds such as fumaric acid, cinamic acid, fumaramidin parfomin, bicoculin and flavonoids are also considerable. Noticeably, flavonoids, resins, alkaloids, resins and tannens in various herbs have conferred anti-parasitic effects.⁸¹²¹⁸

A previous study has demonstrated that N.octacosan- 7β-α bioactive compound from *F. parviflora* exhibited...
antimicrobial, antifungal and anti-leishmanial (anti-promastigote) effects. This herb has also exhibited antiplasmodium and anti-trypanosome properties. The aim of this study was investigation of Amphotericin B and F. parviflora ethanolic extract antiparasitic effects against L. major and expression of miRNA (miR)146a-5p and miRA499 in macrophage culture.

Material and Methods
Preparation of F. parviflora ethanolic extract: The herb was prepared from humid area and confirmed by traditional drug keeper in the laboratory university. A 25mg weight of herb powder was dissolved into 25mL of ethanol and the extract was obtained using the maceration method.

L. major standard strain and Amphotericin B: L. major standard strain was provided by Pasteur Institute with strain number ER/75/IR/M. Amphotericin B was purchased from Sigma (USA).

Culture conditions and anti-promastigote effects: After first culture of L. major into the RPMI1640 complete medium (Biosera, France), the promastigotes were taken at the stationary phase (one week), they were diluted and passaged. Moreover, the macrophage cell line, RAW264.7 cells were cultured into the DMEM medium and passaged. A macrophage suspension equal to 1×10⁶ cells/mL in duplicate and L. major and various concentrations of ethanolic extract (5, 10, 25, 50 and 100%) and amphotericin B (50 and 100µg/mL) were added into a 6-well plate of DMEM medium and incubated for 24h. Two other same culture conditions using each extract and amphotericin B were also incubated.

A culture without treatment was considered as a control. The dilutions of treatment were prepared and non-adherent promastigotes were washed. The infected and non-infected plus treated macrophages were kept at -80°C for RNA extraction. Another culture was used for enumeration of amastigotes into the macrophages using methanol fixation and Gimsa dye.

Detection of LC50 and LC90: The culture of promastigotes and same treatments as aforementioned were performed in the DMEM medium and the LC50 and LC90 of ethanolic extract and amphotericin B various dilutions and their combination were assessed at 24, 48 and 72h. The test was implemented in triplicate.

BALB/c mice: BALB/c mice were infected with 10⁶ cells/mL (100µL) of promastigotes subcutaneously each using an insulin syringe and a scar was formed on the mice back after a week. The parasites were sampled and observed using dye under the light microscopy. An ointment was prepared for each group (100µg/mL Amphotericin B singly and combined with 50% v/v of extract, 50% of extract and control, n=60).

Expression of miRNAs: Of 4 × 10⁷ cells from the groups, the total RNA was extracted using TriZOL-chloroform method (Ambion, Thermo scientific, USA). Expression of miR146a-5p and miRA499 levels was evaluated using the real-time PCR and the fold change was calculated using 2⁻ΔΔCT method. The β-glubin was used as the internal control.

Analysis of data: Using the SPSS version 21 and Graph Pad Prism, the t-test and ANOVA were used for data analysis and comparisons at p<0.05.

Results
Cell cytotoxicity: The cytotoxicity of various concentrations of F. parviflora ethanolic extract on HaCaT human normal skin cells has been exhibited in figure 1. Accordingly, no cytotoxic effects were observed at 24-72h of exposure in concentrations 5-100% v/v.

Figure 1: The cytotoxicity of various concentrations of F. parviflora ethanolic extract
Parasites counts and LC50 and LC90 values: The 50% lethal dose (LC50) of *F. parviflora* ethanolic extract included 100% (v/v) while the combination of 50% of extract with 100 µg/mL of amphotericin B killed 90% (LC90) of promastigotes during 48h. The LC90 of amphotericin B was >100µg/mL. The effect was time and concentration-dependent (figure 2). Moreover, the combination of *F. parviflora* ethanolic extract and amphotericin B exhibited a synergy effect.

**In vivo results:** The *in vivo* study exhibited improvement of lesions sizes significantly in combination therapy compared to the control group (p<0.05). The lesions size changes have been exhibited in table 1 and figure 3. Accordingly, the size of lesions in the treatment groups after a week was not significantly smaller than that of the control. However, a significant difference was found during a month. The combination of amphotericin B and extract significantly reduced the size of lesion compared to other treatment groups (p<0.0001).

![Figure 2: The inhibitory levels of *F. parviflora* ethanolic extract singly and in combination to the Amphotericin B at various time spans, Amp: Amphotericin B](image)

![Figure 3: The size of lesions in the control (A) and treated (B: Amphotericin B50, C: Amphotericin B+extract and D: extract at 50%) groups after a month](image)
Gene expression: The expression of miR146a-5p (0.3 fold decrease) and miRA499 (0.11 fold decrease) was not significantly affected in treatment to the amphotericin B (50 and 100µg/mL) and F. parviflora ethanolic extract.

Discussion
Owing to the multifaceted aspects of leishmaniasis spread in environment, perspectives for eradication of infection have not been successful.\(^5\,\text{5}\) Side effects, unavailability, resistance and costs of chemical drugs are examples of uncertainty with this regard.\(^5\,\text{5}\) In this study, no cytotoxic effects were observed at 24-72h of exposure in concentrations of 5-100% v/v of F. parviflora ethanolic extract. The 50% lethal dose (LC50) of F. parviflora ethanolic extract included 100% (v/v), while the combination of 50% of extract with 100 µg/mL of amphotericin B killed 90% (LC90) of promastigotes during 48h. The LC90 of amphotericin B was >100µg/mL.

Therefore, at concentrations of antiparasitic effects, the herb did not confer any toxicity to human HaCaT normal cells. This is crucial for application of its ointment for skin healing due to scars. The effect was time and concentration-dependent (figure 2). Moreover, the combination of F. parviflora ethanolic extract and amphotericin B exhibited a synergy effect. Synergistic antiparasitic effects have been exhibited against hydatid cyst in previous studies using albendazole and some herbal extracts and bioactive compounds.\(^1,\text{1}5,\text{16,21}\)

In addition, the in vivo study exhibited improvement of lesions sizes significantly in combination therapy compared to the control group (p<0.05). Accordingly, the size of lesions in the treatment groups after a week was not significantly smaller than that of the control. However, significant differences were observed in extract and amphotericin B+extract compared to the control during a month. The combination of amphotericin B and extract significantly reduced the size of lesion compared to other treatment groups (p<0.0001).

<table>
<thead>
<tr>
<th>Group</th>
<th>A week</th>
<th>A month</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amp</td>
<td>2.25±3.2</td>
<td>2.12±1.6</td>
</tr>
<tr>
<td>Extract</td>
<td>2.5±1.6</td>
<td>3.9±06</td>
</tr>
<tr>
<td>Amp+extract</td>
<td>2.1±11</td>
<td>1.06±49</td>
</tr>
<tr>
<td>Control</td>
<td>2.96±66</td>
<td>4.64±16</td>
</tr>
<tr>
<td>(p) value</td>
<td>0.378</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Table 1: The lesions size in various groups after a week and a month

Similar to our study, Lawsonia inermis methanolic extract had exerted no cytotoxic effects on host cells.\(^6\) The extract of Peganum harmala combined to the potassium antimony Tartrate has exerted anti-promastigotes effects in vitro. Methanolic extracts of Artemisia aucheri, Ferula assa-foetida and Gossypium hirsutum have also exhibited inhibitory effects against L. major promastigotes.

We observed that the expression of macrophage miR146a-5p (0.3 fold decrease) and miRA499 (0.11 fold decrease) was not significantly affected in treatment to the amphotericin B (50 and 100µg/mL) and F. parviflora ethanolic extract. It has been exhibited that mutations within the miR146a affect the skin susceptibility in exposure to L. guyanensis.\(^4\) Moreover, miR146a has been association with several diseases.

On the other hand, miRA499 rs3746444 has been related to development of cutaneous leishmaniasis.\(^7\) These variations have impaired the neutrophil migration into the site of the sand fly bite. A recent study also demonstrated that miR146a-5p has a role in the macrophage polarization and immune regulation which result in the immune therapy of visceral leishmaniasis.\(^3\) Limitations of our study included lack of sequencing for miR146a and miR449 to find a relation between them and development of L. major infection.

Conclusion
Combination of amphotericin B (50 and 100µg/mL) and F. parviflora ethanolic extract can significantly inhibit the promastigotes growth during 72hrs. Moreover, this combination was promising towards in vivo treatment of skin Leishmania lesions after a month. The expression of miR146-a and miR499 was not affected by treatments.

References
3. Das S., Mukherjee S. and Ali N., Super enhancer-mediated transcription of miR146a-5p drives M2 polarization during parthenium, Maesa balansa, Peschiera australis and Piper aduncum have been demonstrated.
4. Similar to our study, Lawsonia inermis methanolic extract had exerted no cytotoxic effects on host cells. The extract of Peganum harmala combined to the potassium antimony Tartrate has exerted anti-promastigotes effects in vitro. Methanolic extracts of Artemisia aucheri, Ferula assa-foetida and Gossypium hirsutum have also exhibited inhibitory effects against L. major promastigotes.
5. We observed that the expression of macrophage miR146a-5p (0.3 fold decrease) and miRA499 (0.11 fold decrease) was not significantly affected in treatment to the amphotericin B (50 and 100µg/mL) and F. parviflora ethanolic extract. It has been exhibited that mutations within the miR146a affect the skin susceptibility in exposure to L. guyanensis. Moreover, miR146a has been association with several diseases.
6. On the other hand, miRA499 rs3746444 has been related to development of cutaneous leishmaniasis. These variations have impaired the neutrophil migration into the site of the sand fly bite. A recent study also demonstrated that miR146a-5p has a role in the macrophage polarization and immune regulation which result in the immune therapy of visceral leishmaniasis. Limitations of our study included lack of sequencing for miR146a and miR449 to find a relation between them and development of L. major infection.


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