

Antileishmanial Activity of Date (*Phoenix dactylifera* L) Fruit and Pit Extracts In Vitro

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Abstract

Leishmaniasis is considered as a major public health problem worldwide. Current drugs in treatment of leishmaniasis have some limitations; thus, the current study was aimed to assess the methanolic extracts of pit and fruit of *Phoenix dactylifera* against *Leishmania major* promastigotes. *L. major* promastigotes were cultured in RPMI 1640 and incubated at 25°C ± 1°C for 24, 48, and 72 hours. For obtaining the IC₅₀ (half maximal inhibitory concentration) value, MTT assay was employed. Furthermore, promastigotes were examined in terms of morphology under light microscope. About 48 hours after treatment, IC₅₀s were estimated 23 µg/mL and 500 mg/mL for methanolic extracts of pit and fruit of *P. dactylifera*, respectively. Both extracts exhibited a dose and time-dependent antileishmanial activity against *L. major* parasites. Also, some visible morphological changes were seen. This finding revealed both date fruit and pit, are effective against *L. major* promastigotes. Further studies should be designed in future based on apoptosis induction in vitro and in vivo.

Keywords

Leishmania major, *Phoenix dactylifera*, in vitro

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Leishmaniasis is a vector-borne infection caused by an intracellular protozoa belonging to *Leishmania* genus, which according to clinical manifestation and causative agent, are categorized into 3 groups and ranged from self-curing cutaneous lesions to chronic and severe mucocutaneous and visceral forms. Leishmaniasis exists in 100 countries worldwide and 12 million people are infected currently; in addition 350 million individuals are at risk for acquiring the disease.^{1,2}

Pentavalent antimonial compounds currently are being used as first-line drugs for treatment of leishmaniasis. Since there is no efficient and suitable vaccine against leishmaniasis forms till now; in addition current common drugs for treatment of infection possess some limitations to use such as high cost, toxicity, numerous systemic side effects, parasite resistance, prolonged course of remedy, and painful injection; hence, there is an urgent for investigators to discover new drugs and compounds as candidate for leishmaniasis treatment.³ There is ample evidence suggesting that use of natural compounds like plant extract and plant-derived compounds could be useful against *Leishmania* spp. both in vitro and in vivo, and this has been confirmed in several studies.⁴⁻⁶ These plant compounds, in addition to being less in cost, have lower side effects, easy availability, and involve noninvasive administration. Based on World Health Organization report, more than 80% of world's population tends to use traditional medicine for their disease.⁷

Phoenix dactylifera L. (date palm) as a monocotyledonous woody perennial fruit belongs to the Arecaceae family and possesses nearly 200 genera and 3000 species. From ancient times, date pit and fruit have been consumed in different cultures and folks as herbal formulations. The pit of *P. dactylifera* is also called kernel, pip, seed, and stone.⁸ Numerous therapeutic properties for *P. dactylifera* have been mentioned, including anti-inflammation, antitumor, antioxidant, antimicrobial, anti-diabetic, nephroprotective, hepatoprotective, and sex hormone modulator.⁸

After World Health Organization's emphasis for finding new leishmanicidal drugs from natural products and plant compounds,⁷ extensive investigations have been performed worldwide during recent years on plants and plant-derived compounds

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Table 1. Value of IC₅₀ (Half Maximal Inhibitory Concentration) and IC₁₀₀ (Full Maximal Inhibitory Concentration) of Methanolic Extracts of Date Pit and Fruit.

Preparation	IC ₅₀			IC ₁₀₀		
	24 Hours	48 Hours	72 Hours	24 Hours	48 Hours	72 Hours
Pit (methanolic)	42 µg/mL	23 µg/mL	16 µg/mL	89 µg/mL	63 µg/ml	41 µg/mL
Fruit (methanolic)	1600 mg/mL	500 mg/mL	280 mg/mL	2900 mg/mL	800 mg/ml	650 mg/mL
Glucantime (control drug)	37 µg/mL	21.96 µg/mL	14.27 µg/mL	59 µg/mL	35 µg/ml	22 µg/mL

in order to introduce efficient candidate drugs against different forms of leishmaniasis. Lack of data about antileishmanial effect of *P. dactylifera* encouraged us to design the current study; hence, in the present investigation we screened the efficacy of methanolic extract of both pit and fruit of *P. dactylifera* against Iranian standard strain of *Leishmania major* (MRHO/IR/75/ER) promastigotes in vitro.

Materials and Methods

Materials

All materials were purchased as follows: RPMI 1640 (Roswell Park Memorial Institute), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), DMSO (dimethyl sulfoxide), MTT colorimetric assay kit (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), phosphate-buffered saline, and L-glutamine from Sigma Chemical Co (St Louis, MO, USA); antibiotics (streptomycin and penicillin) and fetal calf serum (FCS) from Gibco (Gibco, New York, NY, USA).

Preparation of Phoenix dactylifera Extract

Phoenix dactylifera was obtained from Abadan county (located at Khuzestan province), southwest of Iran. The date pits were isolated manually from fruits and washed. Then methanolic extracts of date fruit and pit was prepared using percolation method as described previously.⁴

Leishmania major Promastigotes Culture

Leishmania major (MRHO/IR/75/ER) promastigotes was kindly gifted by Dr Mohebbi (Tehran University of Medical Sciences, Iran). Promastigotes were cultured in RPMI 1640 media (containing 25 mM HEPES, pH 7.2, 25°C ± 1°C) and supplemented with 10% to 15% heat-inactivated FCS, 2 mM L-glutamine and antibiotic (100 IU/mL penicillin and 100 µg/mL streptomycin) as described earlier.^{5,9} Flask cultures were checked every day in order to detection of microbial contamination.

Promastigote Viability Measurements Using Colorimetric MTT Assay

In this study, MTT assay was employed for obtaining value of IC₅₀ and IC₁₀₀ (50% and 100% inhibitory concentration, respectively).¹⁰ Briefly, cells in logarithmic phase (2×10^6 promastigotes/mL) were seeded in 96-well microplate and treated with methanolic extracts of date pit and fruit in different concentration. The cells were incubated at temperature of 25°C ± 1°C for 24, 48, and 72 hours. After reaching each time

points, MTT evaluation was performed in order to determine the IC₅₀ and IC₁₀₀ for extracts using enzyme-linked immunosorbent assay (ELISA) reader at 540 nm. Finally, findings were compared with both positive and negative control.^{4,5} All tests were repeated in triplicates.

Morphological Changes

For observation of cellular alterations, IC₅₀ value was used in exposed promastigotes as described earlier.^{5,9} Briefly the cells, were centrifuged at speed of $1000 \times g$ and after removing the supernatant, suspended in phosphate-buffered saline. Exposed and unexposed promastigotes at mentioned times were examined by light microscope under 100× magnification and 20 microscopic fields for each sample were observed. Eventually, morphological changes such as cells shape and motility in both control and experimental groups were compared together.

Data Analysis

Leishmanicidal activity of extracts known as IC₅₀ and IC₁₀₀, were calculated using SPSS software and linear regression test (IBM SPSS, Somers, NY, USA). Percentage of viable promastigotes in experimental and control groups was obtained by the following formula⁹:

$$\text{Viable promastigotes (\%)} = (A_T - A_B) / (A_C - A_B) \times 100$$

where A_B is absorbance of the blank, A_C is absorbance of the untreated promastigotes, and A_T is the absorbance of the treated promastigotes.

Results

Antileishmanial Activity In Vitro

In this survey, we assayed methanolic extracts of pit and fruit of *P. dactylifera* against *L. major* promastigotes. IC₅₀ and IC₁₀₀ values of the 2 extracts against mentioned *Leishmania* species at 3 time points (24, 48, and 72 hours) are shown in Table 1. Based on current findings, both extracts showed a dose- and time-dependent antiproliferative activity against *Leishmania* parasites; although, leishmanicidal activity of pit extract was dramatically higher than fruit extract.

Morphological Changes of Leishmania major Promastigotes

Survival alterations of treated cells with IC₅₀ (23 µg/mL) was observed at four time points (0, 24, 48, and 72 hours). At the beginning of proximity, the mean number of promastigotes were 2×10^6 cells/mL, which, 24 hours after treatment, slumped by half. This decreasing trend in number of alive cells

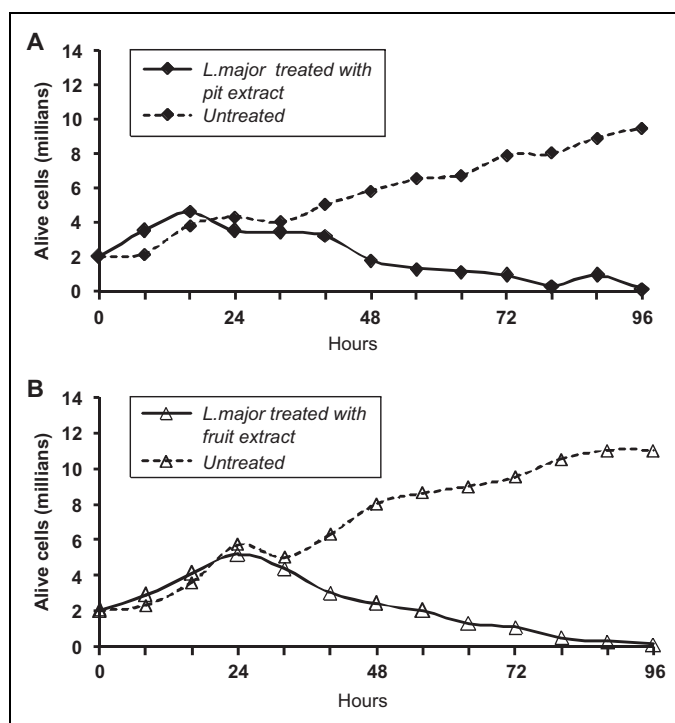


Figure 1. Number of promastigotes treated with or without *Phoenix dactylifera* pit and fruit extracts at different time points. (A) Treated *Leishmania major* (MRHO/IR/75/ER) promastigotes with pit extract and (B) *Leishmania major* (MRHO/IR/75/ER) promastigotes with fruit extract.

was continuous in other intervals up to 72 hours. So that after 72 hours, a significant decline was seen in total promastigotes counts compared with 0 h. Observations in control group (untreated) were in contrast (Figure 1A and B). At the 48-hour time point, some visible changes were observed in treated cells, including immobility, cell shrinkage, cytoplasmic condensation, and rounded-shaped cells. It should be noted that in unexposed group no certain changes happened (Figure 2).

Discussion

In the present investigation, we found the methanolic extracts of date fruit and pit showed antileishmanial activity against *L. major* promastigotes with IC_{50} value of 500 mg/mL and 23 μ g/mL, respectively. Majority of investigations have focused on date fruit and the antiparasitic activity of date pit is poorly studied; accordingly, there is not enough information about this issue. Hence, the current study is the first survey about methanolic extract of both fruit and pit of *P. dactylifera* against *L. major* promastigotes. Date pit is almost always thrown away after consuming the fruit; whereas, based on reports, various therapeutic properties have been mentioned for it.^{8,11,12} Based on our findings, antileishmanial activity of date pit was significantly higher than fruit (Table 1). In contrast to our findings, in the study by Al-Daihan and Bhat,¹² extract of date fruit showed better efficacy against some selected bacteria. This discrepancy between results could be justified with

kind of *P. dactylifera* and differences among protozoa and bacteria mechanisms.

Antibacterial activity of 3 different extracts (aqueous, methanolic, and acetonetic) of fruit, seed, leaf, and bark of *P. dactylifera* were examined against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, and *Escherichia coli* by the disk diffusion technique. The findings showed that all extracts possess acceptable antibacterial effects against the mentioned pathogens; although, the aqueous extract was found to be less effective than the methanolic and acetonetic extracts. The reason might be the increased capability of acetone and methanol in isolation and extraction of wide range of chemical ingredients compared with aqueous extraction. Fruits extract illustrated better antimicrobial effect compare with leaves, barks, and pits extracts.¹² Also, recently it has been reported the antifungal activity of 3 various extracts (aqueous, methanolic, and acetonetic) of pits and leaves of *P. dactylifera* against 7 pathogenic fungi using agar well diffusion and agar dilution methods.¹¹ Phenolic contents present in *P. dactylifera*, considered as a potential antimicrobial ingredient that is able to prevent growth of wide range of pathogens, as well as alkaloids and tannins found in *P. dactylifera* have similar activity.^{12,13}

Harsha et al¹³ investigated the ethanolic extract of date fruit against *L. tropica* promastigotes in vitro. IC_{50} obtained was 68.5 μ g/mL. Also, phytochemical analysis of ethanolic extract using high-performance thin layer chromatography method confirmed the presence of tannins, phenols, and flavonoids. According to high-performance thin layer chromatography results, they attributed the leishmanicidal activity of date fruit to the existence of tannins, particularly gallic acid¹³; this has been shown in other studies as well.^{14,15} It is worth mentioning that tannins also show extensive pharmacological and biochemical effects in vitro such as antitumor and anticancer activities, and acting as antimicrobial, antioxidant, enzyme inhibitor, and free radical scavenger.¹⁴ Accordingly, antioxidant activity and free radical scavenging effect of gallic acid might be liable for probable efficacy mechanism against *L. major* promastigotes in the present study. Further studies to clarify this issue is recommended. In the study by Al-Musayeb et al,¹⁶ IC_{50} of methanolic extract of *P. dactylifera* against *L. infantum* amastigotes was estimated as 32.5 ± 4.3 μ M/mL. In the current study, antileishmanial activity of date pit and fruit against *L. major*, were confirmed; hence, according to the study by Harsh et al¹³ on *L. tropica* (the agent of dry cutaneous sores), the study by Al-Musayeb et al¹⁶ on *L. infantum* (the agent of visceral form), and our study on *L. major* (the agent of wet cutaneous sores), it can be concluded that *P. dactylifera* is effective against all forms of leishmaniasis forms, and shows potential as a candidate drug.

Phoenix dactylifera with immunostimulatory effects, increases nonspecific immune responses against pathogen agents as well as augmenting both humoral and cellular immune responses. This action might be provoked by changes in cytokine secretion pattern or direct stimulation of T and B cells.¹⁷ So far there is no information about immunomodulatory activity of *P. dactylifera* against *Leishmania* spp. Hence, in

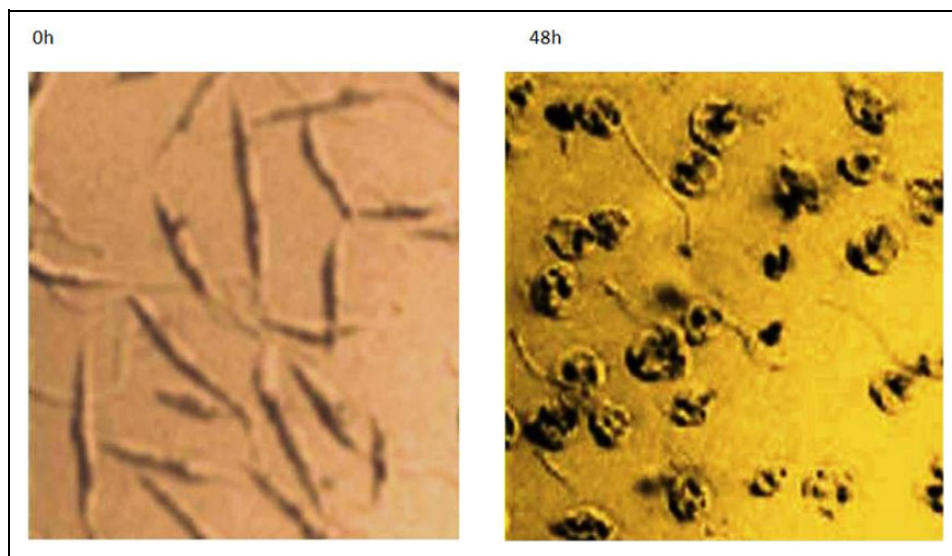


Figure 2. Analysis of morphology in light microscopy (magnification, 100×) of *Leishmania major* promastigotes following treatment with *Phoenix dactylifera* pit extract (23 µg/mL). (A) *Leishmania major* 0 hours after treatment and (B) *Leishmania major* 48 hours after treatment.

order to better understanding this issue, further studies are recommended. Some ingredients of *P. dactylifera*, such as phenolic contents as well as alkaloids and tannins, are liable for antimicrobial activity. Therefore, purification of these components and then determination of their efficacy on *Leishmania* spp will increase our knowledge and could be helpful.

Conclusion

Natural compounds that earlier have been utilized in traditional medicine, nowadays have created new hopes for the treatment of leishmaniasis. We evaluated the efficacy of methanolic extracts of fruit and pit of *P. dactylifera* against *L. major* in vitro. Value of IC_{50} for them were calculated as 500 mg/mL and 23 µg/mL, respectively, which indicates that leishmanicidal effects of date pit is significantly higher than that of the fruit. This lethal activity may be happening as a result of apoptosis induction in promastigotes. Accordingly, in future determination of occurrence or nonoccurrence of apoptosis event should be considered. Also, investigations based on immunostimulatory and immunomodulatory activity of *P. dactylifera* in vivo are recommended.

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Author Contributions

SA contributed to the literature search, manuscript preparation, and experimental studies. SK contributed toward concept, design, definition of intellectual content, and manuscript review. MDi edited and reviewed the manuscript. MF-R edited and reviewed the manuscript.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical Approval

As this study did not involve any animals or humans, ethical approval was not needed.

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