EXPLORING THE ANTI-LEISHMANIAL ACTIVITY OF OF PHOENIX DACTYLIFERA (DATE PALM)

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Summary

Infections due to protozoa of the genus Leishmania are a major worldwide health problem, with high endemicity in developing countries. The drugs of choice for the treatment of leishmaniasis are the pentavalent antimonials (SbV), which present renal and cardiac toxicity. Besides, the precise chemical structure and mechanism of action of these drugs are unknown till date. In order to find new drugs against leishmaniasis, we have been studying ethanolic extract of date palms. The date palm (*Phoenix dactylifera L.*) is known to be one of the oldest cultivated trees in the world. In the present study, we have evaluated the effectiveness of ethanolic extract of *Phoenix dactylifera* L., Palmae, against the extracellular forms promastigotes of *L. tropica.* Ethanolic extract of *Phoenix dactylifera* was much more effective against *L. tropica* IC_{50} value of 68.50 µg/ml. Phytochemical screening of ethanolic extract of *Phoenix dactylifera* showed the presence of phenols, flavonoids, tannins, and carbohydrates as the major chemical compounds. Attempts have been carried out to characterize tannins in respect to gallic acid content by HPTLC quantification. HPTLC analysis was carried out by using stationary phase as Silica Gel 60 F_{254}, Mobile Phase as Toluene : Ethyl acetate: Formic acid (5:4:1), Solvent front run up to 8 cm; Detection was carried out at 272 nm by using CAMAG make HPTLC. Derivatisation - 5% Ferric chloride reagent in Methanol at 110 ^{o} C for 5 mins. R_f value was found to be 0.32. Quantification of ethanolic extract of *Phoenix dactylifera* against the applied standard gallic acid was found to be 6.86%. Based on these in vitro results against *L. tropica,* new studies should be carried out to find the compounds with anti-leishmanial activity.

Key words: Date palm, *Phoenix Dactylifera,* antileishmanial activity, gallic acid, HPTLC.

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Introduction

Date palms (*Phoenix dactylifera* L., Palmae) have been cultivated in the Middle East over at least 6000 years ago (1). For the natives in this region, dates are considered a staple carbohydrate food (2). Date fruits are also used in the production of local beverages and spirits. In local medicinal practices, dates are considered a tonic and aphrodisiac, useful against ulcer. In fact, some communities believe that "*He who eats seven dates every morning will not be affected by poison or magic on the day he eats them*" (3). The pollen grains of date palms have been used in Egyptian local practices to improve fertility in women, and in some locations in Arabia date pits are roasted and used in lieu of coffee as a hot beverage.

Relatively only a few pharmacological studies have been conducted on dates. It has been shown that, depending on the type of extract used, date fruits and pit extracts significantly increase or decrease gastrointestinal transit in mice (4) and that date fruit extract has strong antioxidant and antimutagenic properties (5). Date palm kernels have been shown to exhibit antiaging properties and significant reduction in skin wrinkles in women (6) and natural fats from date palm has been reported to prevent irritant contact dermatitis (7). In animals, the pits have been included in the diet of chickens, sheep, fish, and rats and have been shown to enhance growth in these species (8).

Leishmaniasis is a tropical disease caused by protozoa of the *Leishmania* genus. These protozoa cause a disease with different clinical forms, among them cutaneous, hyperergic, mucocutaneous, and anergic diffuse leishmaniasis. Leishmania species are intracellular parasite haemoflagellates that infest macrophages of skin and visceral to produce disease in their vertebrate host. Three major manifestations of leishmaniasis are recognised: visceral, cutaneous, and muco-cutaneous leishmaniasis (9). According to recent estimates, 1.5 million new cases of cutaneous leishmaniasis (CL) occur each year. More than 90% of cases occur in five countries in the Old World (Afghanistan, Algeria, Iran, Iraq, and Saudi Arabia) and two countries in the New World (Brazil and Peru). CL in the Old World is caused by *Leishmania infantum, L. major*, *L. tropica* and *L. aethiopica,* which are found in southern Europe, the mediterranean basin, the Middle East, and Africa. CL in the New World is mainly caused by members of the *L. Braziliensis* complex *(L. braziliensis* and *L. peruviana), L. mexicana, L. amazonensis* and *L. guyanensis* complex *(L. guyanensis* and *L. panamensis)* (10). The disease presents as fever, weight loss, hepatosplenomegaly with biochemical abnormalities of hyper-y-globulinema and pancytopenia (11). It has increased attention in developing countries because of the growing number of cases seen in AIDS patients and viscerotropic Leishmania tropica diseases in patients (12). Prevelant antimicrobial drugs have remained as standard treatment for visceral Leishmaniasis. The drugs of choice for the treatment of leishmaniasis are the pentavalent antimonials, but they present renal and cardiac toxicity. Second choice for treatment of the disease is a diamidine (pentamidine isethionate) which also has serious side effects (13). However, already in some trials, alternative pharmaceutical formulations have been used to reduce the toxicity of these drugs (14).

The lack of an effective anti-leishmanial drug led a renewed interest in the study of traditional remedies as sources for the development of new chemotherapeutic compounds with better activity and less toxicity (15). Several plants have been used for the treatment of parasitic diseases (16). In order to find new drugs against

leishmaniasis, we have studied ethanolic extracts of date palms (*Phoenix dactylifera* L., Palmae) against L.tropica and its characterization.

Material & Methods

Plant material:
Fresh fruits of dates (*Phoenix dactylifera* L., Palmae) were obtained from a local date manufacturing factory. Samples of these dates were kept frozen for future reference.

Preparation of plant extract:
The date fruits were manually separated from the pits and the latter were washed clear of any fruit, dried at room temperature and grounded to powder using a stainless steel blender. 400 g of powdered dates were used for extraction. Soxhlet apparatus was used to obtain an ethanol extract. The ethanol extracts were then concentrated to dryness (19%) and were stored at 4^oC for further use.

Parasite culture:
The promastigote culture of Leishmanial strain (*L. tropica*) was obtained from Jawarahalal Nehru University, New Delhi, India, maintained in Novy-Nicolle-McNeal (NNN) culture medium (10% of rabbit blood in 4% of Bactoblood agar base) and incubated at 24°C.

Phytochemical chemical screening;
The ethanolic extract was subjected to preliminary phytochemical testing (18) for the detection of major chemical groups like phenols, alkaloids, cardiac glycosides, tannins, terpenoids, steroids and flavanoids.

High performance thin layer chromatography (HPTLC)
Plate: Precoated silica gel 60F 254 HPTLC plate (E. Merck) (0.2 mm thickness). Spotter: CAMAG Linomat 5, Developing chamber: CAMAG glass twin trough chamber. Scanner: CAMAG TLC scanner 3 and WINCATS 4.0 integration software.

***In vitro* leishmanicidal activity:**
Leishmanial promastigotes were grown in NNN culture medium using normal saline. Parasites at log phase were centrifuged at 2000 rpm, washed three times with saline at same speed for 10 min. Parasites were diluted with fresh culture medium to a final density of 10^6 cell/ml. Subsequently, 100 µl of culture was added in all wells except first column which received 180 µl of culture. The last 2 rows were left for negative and positive controls. Negative control received medium with solvents while the positive control contained varying concentrations of the standard solution of Amphotericin B.

Serial dilutions of *Phoenix dactylifera* ethanolic extracts were performed in 96 well micro titre plates in triplicates. Total 20 µl of solubilized extracts were added into the first well and mixed well by micropipette. A total of 100 µl of sample was removed and added into the next well and repeated till the 8th well was reached. Remaining 100 µl was discarded. By doing this, the first well received a final concentration of 100 µg/ml while the last had 0.78 µg/ml of crude extract to be tested. The plates were incubated in dark at 22^oC for 72 hrs on orbital shaker. After 5 days exposure, drug activity (IC_{50}) was assessed microscopically using improved Neubauer-counting chamber programme (12).

HPTLC Analysis:
Preparation of standard solution of Gallic acid:
A stock solution of gallic acid was prepared by dissolving 10 mg of accurately weighed harmine in methanol and making up the volume to 100 ml with methanol. From this stock solution, standard solutions of 100 to 500ng/µL were prepared by transferring aliquots (1 to 6 mL) of stock solution to 10 ml volumetric flasks and adjusting the volume with methanol.

Preparation of sample solutions:
Accurately weighed 1 g amount of powder of fruits of *Phoenix dactylifera* was extracted for 12 hrs in 10 ml of ethanol and made upto 10 ml ethanol in a volumetric flask.

Estimation of gallic acid from methanolic extracts of *Phoenix dactylifera*:
A 10 µl volume of sample solution was applied in triplicate on a precoated silica gel G_{60} HPTLC plate (E. Merck) with the CAMAG Linomat V Sample spotter. The plate was developed and scanned. The peak areas were recorded. Amount of harmine present in the sample was calculated using the calibration curve for harmine.

Results

Phytochemical screening:
The results of Phytochemical screening of ethanolic extract of *Phoenix dactylifera* is shown in Table No. 1. The results indicate the presence of phenols, tannins, flavanoids, carbohydrates, and terpenoids.

***In vitro* leishmanicidal activity:**
Concentration of *Phoenix dactylifera* ranging from 0.78-100 µg/ml in triplicates was tested for their anti-leishmanial activity. The results of ethanolic extract of date palms are shown in Table No. 2 and were found to be Leishmanicidal at an IC_{50} value of 68.50 µg/ml. IC_{50} value $\leq$ 100 µg/ml for extracts was considered signficant (12).

HPTLC analysis:
Analysis was carried out using Stationary phase as Silica Gel 60 F_{254}, Mobile Phase as Toluene : Ethyl acetate: Formic acid (5:4:1), Solvent front run up to 8 cm, Detection was carried out at 272 nm. Instrument used - CAMAG make HPTLC. Applicator used was Linomat V, Derivatisation - 5% Ferric chloride reagent in Methanol at 110^{o}C for 5 mins. HPTLC plates were scanned at different wavelengths (Figure No. 1). R_f Table of ethanolic extract of *Phoenix dactylifera* and standard gallic acid (Table No. 3). Quantification of the active principle against the applied standard gallic acid (Table No. 4). Densitometric scan at 272 nm in 3D representation is shown in Figure No. 2.

Table No. 1: Phytochemical screening of ethanolic extract of *Phoenix dactylifera*

Sl. No.	Phytoconstituents	Presence/ Absence +++ / ----
1.	Alkaloids	----
2.	Terpenoids	----
3.	Flavanoids	+++
4.	Phenols	+++
5.	Carbohydrates	+++
6.	Tannins	+++

Table No. 2: Antileishmanial activty of ethanolic extract of *Phoenix dactylifera*

Plant extract/ Standard	Concentration µg/ml	Inhibition (%) IC_{50}µg/ml.*
Ethanolic extract	10 100 1000	68.50 ± 0.3
Amphotericin B		0.14 ± 0.1

*values are Mean ± SEM (n=3), p 0.05 (Students t-test), Assay in 96 well micro titer plates

Table No. 3: R_f Table of ethanolic extract of *Phoenix dactylifera* and standard gallic acid.

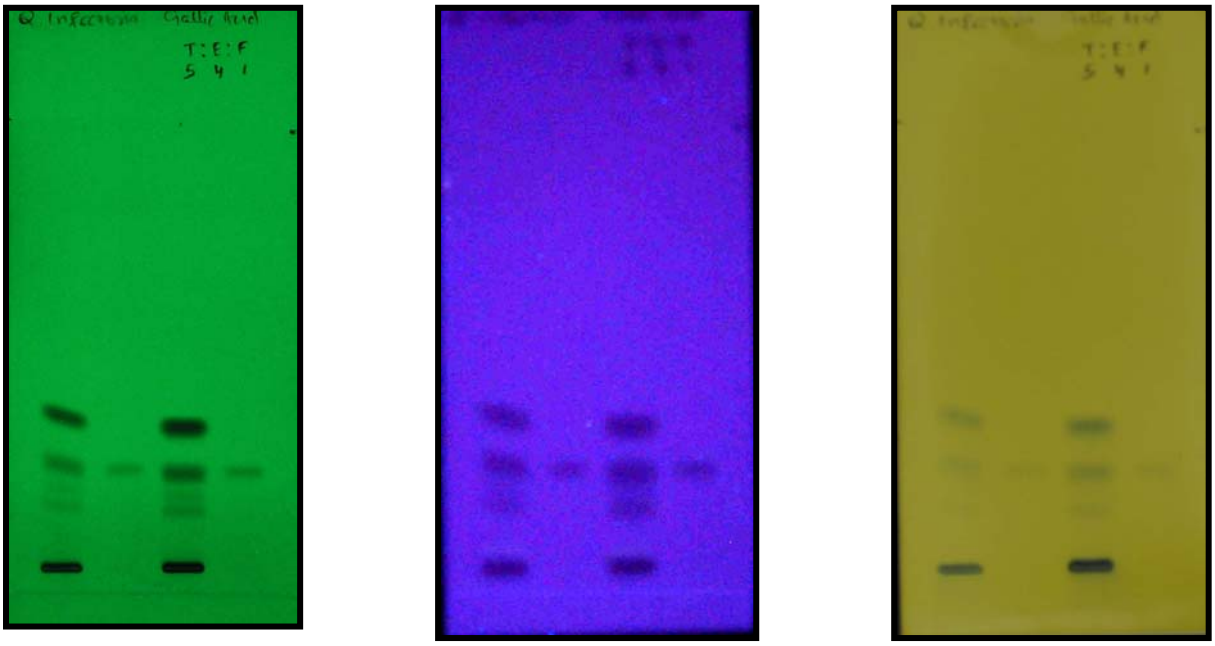
ID/Track	Sample Applied	Volume Applied (µL)	R_F values at 254NM (Black)	R_F values at 366 nm (Blue)	R_f values After 5% ferric chloride reagent in methanol (Green)
T1 & T3	Ethanolic extract of *Phoenix dactylifera*	5 & 10	0.32, 0.22, 0.17 and 0.13	0.32, 0.22, 0.17 and 0.13	0.32, 0.22, 0.17 and 0.13
T2 & T4	Gallic acid	2.5 & 3.0	0.32	0.32	0.32

Table No. 4: Quantification of the active principle against the applied standard

Sl. No.	ID/ Sample applied	Volume applied on HPTLC plate	Amount of Extract on HPTLC Plate µg	Total Area	Percentage of gallic acid content.
1	Std. Gallic acid	2.5 µL	0.245	4548.90	-
2	Ethanolic extract of *Phoenix dactylifera*	10µL	40	12999.48	6.86%

Figure No. 1: HPTLC Plates of ethanolic extract of *Phoenix dactylifera* and standard gallic acid.

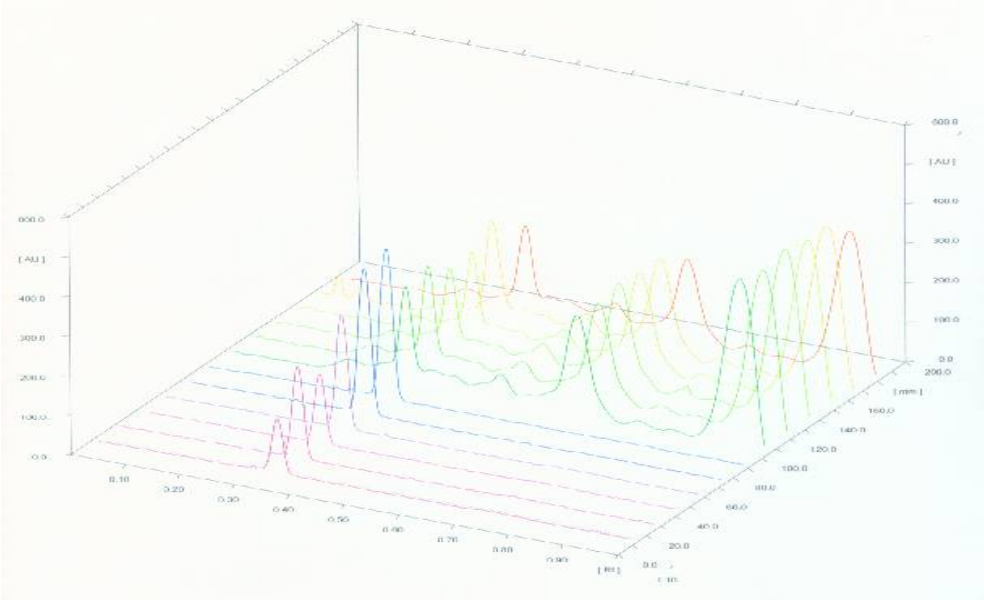
@ 254nm @ 366 nm After derivatisation



T1 T2 T3 T4 T1 T2 T3 T4 T1 T2 T3 T4

T1 *Phoenix dactylifera* ethanolic extract
T2 Standard gallic acid
T3 *Phoenix dactylifera* ethanolic extract
T4 Standard gallic acid

Figure No. 2: Densitometric scan of standard and ethanolic extract of *Phoenix dactylifera* at 272 nm in 3D representation.



Discussion

Phytochemical screening of ethanolic extract of *Phoenix dactylifera* showed the presence of phenols, flavanoids, tannins, and carbohydrates. Ethanolic extract of *Phoenix dactylifera* was screened against *L.tropica* and was found to be Leishmanicidal at an IC_{50} value of 68.50 µg/ml. Attempts have been to characterize the extract by HPTLC analysis for quantification of tannins by using gallic acid standard. Rf value was noted at 0.32, Plates were scanned at 272 nm and ethanolic extract of *Phoenix dactylifera* was quantified in terms of gallic acid to 6.68%.

Phoenix dactylifera is widely used in various traditional and herbal formulations worldwide. It's potential utility in the treatment of ulcer, diarrhoea, used as antioxidant and antimutagenic (8). To the best of our knowledge, there is no previous reports on the leishmanicidal activity of *Phoenix dactylifera.* Our decision was based on the observation of their use in the treatment of chronic skin diseases like ulcers, sores, swelling, and itching (19). Tannins, the main components of many plant extracts, act as free radical scavengers (20-21). Tannins, specially gallic acid is eralier reported to have leishmanicidal activity (22-25). Our results also show the presence of tannins by chemical test which was further quantified by HPTLC analysis in respect to gallic acid which could be used as regular quality control tool for quantification of gallic acid in ethanolic extract of *Phoenix dactylifera*. Gallic acid is reported to be antioxidant and also possess antimicrobial effect against various micro-organisms (26). Hence, gallic acid would be the probably chemical compound responisble for leishmanicidal activity and free radical scavengers could be one of the possible mechanisms of action against *L.tropica*. Further invetigations are underway to isolate responsible tannin and to estabilsh exact mechanism involved in leishmanicidal activity.

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