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Original Article

Antimicrobial and Resistance Reversal Activity of Ethanolic Extract of *Phoenix dactylifera (Dates)* on β-lactamase Producing Uropathogens

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ABSTRACT

Phoenix dactylifera fruits (Dates) are used as an important dietary supplement in the Arabian countries. Numerous claims of immense health benefits associated with this fruit along with its ease of availability worldwide point us towards a possible herbal source of remedy for complex infections like Urinary Tract Infections (UTIs). The current study was carried out to investigate the antibacterial activity of *P. dactylifera* against β-Lactamase producing uropathogens. For this purpose, solvent extracts of *P. dactylifera* fruits were prepared with the help of Soxhlet apparatus at 60°C to obtain a semi-solid mass. Different solvents used in our study were ethanol, methanol, petroleum ether and distilled water. The antibacterial activity of these solvent extracts was assessed by the agar well diffusion method, where ethanol extracts showed maximum antimicrobial activity with zones of inhibition in the range of 20-37mm and MBC of 20%. The reduction in the Minimum Bactericidal Concentration of ampicillin from 10mg/mL to 200-300µg/ml was also observed in presence of sub-inhibitory concentration of ethanol extract of *P. dactylifera.* A good anti-oxidant activity was suggested by the DPPH method as compared to standard ascorbic acid. Phytochemical screening further revealed the presence of tannins, alkaloid, flavonoids and carbohydrates in the fruits of *P. dactylifera* which was further confirmed by Gas Chromatography-Mass Spectroscopy analysis. Since UTIs are increasingly being reported due to multi-drug resistant pathogens, our study may aid in shifting our focus towards a safe herbal approach for treatment of the same.

Key words: *Phoenix dactylifera*, Antibacterial activity, Soxhlet, β-Lactamase, uropathogens.

1. INTRODUCTION

The persistence of antimicrobial resistance among pathogens is redefining the treatment strategies used to combat infectious diseases. The gradual challenges of single class drug resistance, multi-drug resistance as well as complete

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drug resistance have been faced by the medical communities globally, over the past few decades. Unlike general problems, elucidating the various causes of antimicrobial resistance and attempting to bypass it, has not helped the scientific communities to resolve the same[1]. This situation is increasingly becoming worse due to the emergence of multi-drug resistant strains of pathogens like ESBL (Extended spectrum β-lactamase) and MBL (Metallo β-lactamase) producers especially causing common infections like Urinary Tract infections (UTIs). [2, 3]

In this effect, the use of herbal remedies appears to be feasible given its safety with respect to drug overdose and decreased immunity that is associated with other treatment protocols like combination therapy[4]. In addition, the pathogens are unlikely to develop resistance against herbal medicines due to its composite blend of bioactive compounds.[5] Herbal medicines are the backbone of modern day drug discovery programs. They are not only antibacterial in nature but also show resistance-modifying properties[6,7]. Several studies have reported the antibacterial activities of plants like *Eriobotrya japonica*, *Zataria multiflora* and *Terminalia chebula* on ESBL and MBL producers in the past. [8-10]

Keeping the above scenario in mind, the current study was aimed at exploring the anti-bacterial and resistance reversal activity of *Phoenix dactylifera* fruit, commonly known as Dates, against drug-resistant pathogens. *P. dactylifera* is a monocotyledon, dioecious plant belonging to the Palmaceae family which includes around 220 genera and upto 2500 species. [11] It is a commercially important fruit tree, mainly cultivated in the Arabian Peninsula. Several published reports suggest that dates are effective in the treatment of several conditions like inflammation, fever, sore throat, paralysis, bronchial catarrh, nervous disorders, intestinal troubles and memory disturbances. [12, 13] Other studies also suggest the benefits of dates in the treatment of infections like gonorrhea and to counteract alcohol intoxication. [14, 15] There are also reports of the role of *P. dactylifera* pit extracts in reducing the side effect of methylprednisolone on some neurotransmitter content in the brain of male albino rats[16]. In addition, it is an excellent source of dietary fiber, essential micro-nutrients and natural antioxidants[17]. It is further claimed to function as a total substitute for food in terms of nutrition. [18]

Considering the increasing antibiotic resistance among pathogens, the objective of our study was to investigate the efficacy of *P. dactylifera* extract as a possible alternative source of medicine by exploring its antibacterial as well as resistance reversal activities against β-lactamase producing uropathogens.

2. MATERIAL AND METHODS

Test organisms

Gram-negative uropathogens isolated and characterized for β-lactamase production in a previous study was used as test organisms. [19-21] A total of forty-three representative β-lactamase producing pathogens including *K. pneumoniae* (11), *E. coli* (17), *P. aeruginosa* (5), *Proteus mirabilis* (6) and *Citrobacter diversus* (4) were used in the current study. In addition, six common laboratory cultures including *Staphylococcus aureus* (2), Salmonella sp. (1) Bacillus sp. (1) Corynebacterium sp. (1) and Streptococcus sp. (1) were also used. The β-lactamase producing isolates were maintained on Nutrient Agar (NA) slants supplemented with 100µg/mL of ampicillin whereas the laboratory pathogens were maintained on regular NA slants. The cultures were stored at refrigerated conditions until further use.

Extraction of bioactive components

In our study, the antibacterial efficacy of *Phoneix dactylifera* i.e., Palm date fruit was determined against the test isolates. The fruit was collected from a local market in Mumbai and authenticated by an expert botanist from St. Xaviers College, Mumbai. The collected specimen was sorted to obtain damage free and uniform size dates for extraction of bioactive components. In order to prepare the fruit sample for the extraction process, it was de-seeded, washed with distilled water and shade dried for one week. Subsequently, it was crushed into a paste using mortar and pestle, and stored at -20ºC until the commencement of extraction procedure. [22] The extraction of bioactive compounds was carried out, from a 30g sample in 350mL solvents (viz., ethanol, methanol, petroleum ether and distilled water) using soxhlet apparatus, over a period of 48h. The obtained extracts were filtered using Whatman No. 1 filter paper (Whatman International Ltd, England) and concentrated at 40°C by using a rotary vacuum evaporator for 24h. The yellowish brown viscous mass thus obtained was stored at -20ºC in dark glass bottles until further use. These concentrates were prepared in large volumes, in order to avoid batch to batch variations in our study. For further analysis, *P. dactylifera* extracts were re-suspended in respective solvents to get the required concentration.

Sterility testing of plant extracts

The sterility of solvent extracts was confirmed by checking for bacterial or fungal growth after spot inoculating them on a sterile Nutrient Agar (NA) and Sabouraud's Agar (SAB) plate respectively[23]. The NA plates were incubated at 37°C and SAB plates at 30°C for an extended duration of 7 days to confirm the absence of contaminants.

A qualitative study of the inhibitory activity of *P. dactylifera* extracts against test organisms

The antibacterial effect of *P. dactylifera* extracts against test pathogens was determined by agar well diffusion method[14]. Sterile molten NA butt was seeded with 0.4mL of 24h old test pathogens (0.1 OD_{540nm}) and poured into sterile petri-plates. After solidification, wells were punched into the medium using a sterile cork-borer and 50µL of solvent extracts were added to the same. It was then allowed to diffuse through the wells during its incubation at 37°C for 24h, after which the resulting zones of inhibition were

measured. Control wells were also set up using 50µL of solvents used in our study for each isolate. Out of the four solvents used for extraction, the solvent extract showing a maximum zone of inhibition against test pathogens was selected for further study.

Determination of Minimum Bactericidal Concentration of *P. dactylifera* extracts against test cultures

The determination of Minimum Bactericidal Concentration (MBC) of *P. dactylifera* extract was carried out by the agar dilution method. Sterile molten BHI agar butts were supplemented with different concentrations (10%-50%) of the solvent extract of *P. dactylifera*. After solidification of the medium, test pathogens were spot inoculated and plates were incubated at 37°C for 24h. The lowest concentration of *P. dactylifera* that completely inhibited the growth of test culture was considered as its MBC. [24]

Determination of synergistic activity of *P. dactylifera* extracts and ampicillin against test cultures

The agar dilution method was similarly used to determine the synergistic activity between solvent extracts of *P. dactylifera* and ampicillin. The sub-lethal concentration i.e. 1/2MBC of *P. dactylifera* solvent extract was incorporated into molten BHI butt along with 100-500µg/mL of ampicillin with an interval of 100µg/mL. After solidification of medium, test pathogen was spot inoculated and plates were incubated at 37°C for 24h. [24]

Evaluation of antioxidant activity of *Phoenix dactylifera* by DPPH method

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay was carried out with an aim to measure the scavenging activity of any antioxidant towards DPPH, which is stable organic nitrogen radical. The electrons in DPPH pairs with a suitable antioxidant present in the plant extract and lose its color. The disappearance of the violet color of DPPH is proportional to the antioxidant activity of plant extract. In our study, a 2mL volume of various diluted aliquots of *P. dactylifera* solvent extracts and ascorbic acid (control) was mixed with 2mL of 0.2mM DPPH prepared in ethanol. The mixture was kept in dark for 30min after vigorous shaking. The absorbance of DPPH was measured at 520nm using a spectrophotometer. Ascorbic acid was used as a standard antioxidant. The capability to scavenge the DPPH radical of each solution was calculated using the following equation:

Scavenging effect (% inhibition) = [(Control – Test)/ Control)] × 100……(Eq.1)

The IC_{50} was calculated by plotting a graph of the %inhibition versus various concentrations of extract in µg/mL[25].

Qualitative analysis of phytochemical constituents of *P. dactylifera* fruit

The phytochemical screening of *P. dactylifera* fruit was carried out qualitatively with the help of simple tests described by Hidebert et al. (1996)[26]. It was screened for the presence of various phytoconstituents like alkaloids, flavonoids, phenols, saponins, tannins, terpenoids, sterols and glycosides.

Alkaloids: The appearance of reddish brown precipitate on treatment of *P. dactylifera* fruit extract with 3-5drops of Wagner's reagent indicated the presence of alkaloids.

Flavonoids: Intense yellow coloration of 2ml of extract on treatment with few drops of 20% sodium hydroxide solution, and subsequent decoloration on the addition of dilute hydrochloric acid was indicative of the presence of flavonoids.

Phenols: Appearance of blue or green color after addition of 2mL distilled water and few drops of 10% ferric chloride solution to 1mL of *P. dactylifera* extract indicated the presence of phenols.

Saponins: Formation of persistent foam after adding 6mL of distilled water to 2mL of *P. dactylifera* extract was indicative of the presence of saponins.

Tannins: The appearance of blue or green color after treatment of 2mL *P. dactylifera* extract with 10% alcoholic ferric chloride solution indicated the presence of tannins.

Terpenoids: Formation of a reddish brown layer at the interface of 2mL *P. dactylifera* extract treated with 2mL of chloroform and concentrated sulphuric acid solution confirmed the presence of terpenoids.

Sterols: A 2mL *P. dactylifera* extract was mixed with 2mL chloroform and then 1mL acetic anhydride along with 2 drops of concentrated sulphuric acid solution was added from the side of the test tube. The appearance of red color followed by blue and finally green color indicated the presence of sterols.

Glycosides: The appearance of reddish brown color at the junction of the two liquid layers formed after addition of 1mL of glacial acetic acid, one drop of 5% ferric chloride and concentrated sulphuric acid solution to 2mL *P. dactylifera* extract indicated the presence of glycosides.

Gas Chromatography-Mass Spectrophotometry analysis of ethanolic extracts of *P. dactylifera* fruit

The bioactive components from *P. dactylifera* fruit were analyzed by GC-MS HP 7890 system (Agilent technologies). The entire analysis was carried out at IIT Bombay, Mumbai 400076. The compounds of the *P. dactylifera* fruit were identified by comparison of their retention indices (RI) and mass spectra fragmentation with those on the stored library available with IIT, Bombay.

3. RESULTS AND DISCUSSION

Sterility testing of plant extracts

The solvent extracts obtained from *P. dactylifera* were found to be free of any bacterial or fungal contamination as confirmed by the absence of growth on NA and SAB plates respectively. The extended incubation time of 7 days confirmed the absence of slow-growing contaminants and stressed cells that may have survived the processing of solvent extracts.

A qualitative study of the inhibitory activity of *P. dactylifera* extracts against test organisms

Data shown in Table 1 represents the antibacterial activities of methanol and ethanol extracts of *P. dactylifera* carried out by agar well diffusion method. It showed zones of inhibition in the range of 20-36mm for ethanol extracts against both β-lactamase producers as well as laboratory cultures. Methanol extracts showed very less antibacterial activity against the test isolates as suggested by the observed zones of inhibition (07-11mm). The Petroleum ether and distilled water extracts did not show any activity against the test cultures. Consequently, no further studies were carried out using these extracts. The solvent controls used in our study also did not show any zone of inhibition against test cultures.

In a similar study, the antibacterial activity of ethanolic extract of *P. dactylifera* was tested against *E. coli* cultures. It showed zones of inhibition of 13mm, 15mm and 20.44mm in presence of 10µg/mL, 100µg/mL and 1000µg/mL concentration of extracts respectively[27]. In contrast to our findings, *S. typhi* was found to be extremely susceptible to methanolic extracts of *P. dactylifera* fruits with zones of inhibition in the range of 10-38mm. However, *P. mirabilis* (0-18mm), *K. pneumoniae* (0-26mm) and *E. coli* (0-24mm) cultures showed comparatively less or no zones of inhibition in their study[28]. The methanolic extracts of *P. dactylifera* fruits were also found to be effective on *E. coli* and *S. aureus* cultures in another study that reported their zones of inhibition of 20mm and 22mm respectively[29].

Determination of Minimum Bactericidal Concentration of *P. dactylifera* extracts against test cultures

The ineffectiveness of petroleum ether, methanol and distilled water extracts, and the antibacterial activity of ethanol extracts of *P. dactylifera* against the test cultures were further confirmed by determination of it MBCs. The data shown in table 1 represents the MBC of the ethanol extracts of *P. dactylifera* carried out by agar dilution method. It was found to be 20%. Other solvent extracts of *P. betel* leaves showed very high MBC compared to that of ethanol extract and hence not represented in the current study.

A similar study reported the MBC of methanolic extracts of *P. dactylifera* fruit to be 1mg/mL against *E. coli*[27]. However, the MBC of ethanolic extracts of *P. dactylifera* fruit extracts was found to be 5mg/mL against *S. typhi* and *K. pneumoniae* isolates and 10mg/mL against *P. mirabilis* in another study[28]. Several studies carried out using a similar setup and protocols also show differences in activity, as observed in the zones of inhibition and MBCs in our study and other published data. This is because, the antibacterial activities of any plant extract depends on several biological, growth and environmental factors that may vary from one experimental setting to another. In case of our study, the potency of the crude extracts obtained may be further increased by either modifying the extraction protocols or considering varied polarity of solvents.

Determination of synergistic activity of *P. dactylifera* extracts and ampicillin against test cultures

Table 2 represents the synergistic effect of ethanol extract of *P. dactylifera* fruit and ampicillin against the test cultures. The most interesting finding of our study was the reduction in MBC value of ampicillin from 10mg/mL to 200-300µg/mL when used in combination with ethanol extract of *P. dactylifera* fruit. Similar to our findings, a synergistic activity has been reported between solvent extracts of *Thymbra spicata* L. and common antibiotics like ampicillin, cefotaxime, amikacin and ciprofloxacin against multidrug-resistant strains of *S. aureus* and *K. pneumoniae.* In their study, the *T. spicata* extracts were found to enhance the activity of cefotaxime by 8- to 128-fold against *S. aureus* strains. [30]

The increased occurrence of multi-drug resistance among pathogens has necessitated the search for new, efficient and cost-effective ways to control infectious diseases. Moreover, mere discovery of new antimicrobials are definitely not the solution to the current problem; since microbes are practically evolving every-day to adapt to any adverse environmental conditions including exposure to antibiotics. To this effect, our current study holds immense impact by suggesting combination therapy by use of a common herbal component and antibiotic to reverse the existing resistance. Also to our knowledge, there is no published data that represents the synergistic activity between ethanolic extracts of *P. dactylifera* fruits and ampicillin against β-lactamase producing uropathogens. Hence our findings are unique to that effect.

Evaluation of antioxidant activity of *Phoenix dactylifera* by DPPH method

Figure 1 represents the DPPH radical scavenging activity of ethanolic extracts of *P. dactylifera* fruits and ascorbic acid. It was observed that the scavenging activity of the extract increased with its increasing concentration. It was found to be 15.75% at 20µg/mL concentration and increased to 83.92% at 200µg/mL concentration. The DPPH radical scavenging activity of the standard ascorbic acid, on the other hand, was found to be 23.73% at 20µg/mL concentration and 98.07% at 200µg/mL concentration. The IC_{50} value was calculated to be 298.79µg/mL for the *P. dactylifera* extracts and 187.75µg/mL for the ascorbic acid. Hence indicating that the *P. dactylifera* leaves possess good antioxidant activity as compared to the control. In another study, four different species of Tunisian date palm fruits were characterized and Khouet Kenta was found to possess the highest antioxidant activity when compared to other types i.e., Kentichi which was followed by Deglet Nour and Allig variety[31]. In another study, the antioxidant activity of 6 different varieties of dates imported from different countries in the USA was assayed by FRAP and showed activities in the range of 3.29 to 5.22 µmol TE/g dw [32].

Qualitative analysis of phytochemical constituents of *P. dactylifera* fruit

The phytochemical screening of ethanolic extract of *P. dactylifera* fruit showed the presence of alkaloids, flavonoids, phenols, saponins, tannins, terpenoids, sterols and glycosides. All the identified phytochemicals are antibacterial in nature. In addition, they also contribute to and enhance other properties like anti-inflammatory, analgesic, anti-diarrhoeal, anti-oxidant, immune-modulatory, anti-helminthic, anti-tumor as well as insecticidal activities.[33]

Gas Chromatography-Mass Spectrophotometry analysis of ethanolic extracts of *P. dactylifera* fruit

The GC-MS chromatogram (Figure 2) of ethanolic extract of *P. dactylifera* fruit showed the presence of 2 major and 7 minor peaks. Table 3 represents the bioactive compounds identified by GC-MS analysis in our study. The highest peak observed at retention time 1.879mins followed by another major peak observed at retention time 6.552 was identified as 2,3-butanediol and hexanol respectively making them the major constituents of ethanolic extract of *P. dactylifera* fruit. A similar study carried out using 13 date palm fruit varieties from Egypt identified 89 volatile compounds where lipid-derived and phenylpropanoid derivatives were found to be the major components of date fruit aroma.[34] A study has also reported the presence of 40 and 27 different phytoconstituents in n-hexane and methanol fractions of Ajwa date seeds respectively. [35] Another study reported that the acetone extract majorly contains aliphatic molecules and chloroform extract contains aromatic molecules indicating a significant effect of solvents on the bioactivity of plants[36].

Table 1: Antibacterial activity of ethanol extracts of *P. dactylifera* against β-lactamase producers and common laboratory cultures

Test organisms	No. of isolates	Zones of inhibition of solvent extracts in mm		Mean MBC in %
		Methanol	Ethanol	
β-lactamase producers				
K. pneumoniae	11	08.84-10.48	28.67-32.23	20
E. coli	17	09.84-11.75	20.33-25.87	20
P. aeruginosa	05	07.94-10.74	20.33- 29.67	20
P. mirabilis	06	09.66-10.65	26.67-32.33	20
C. diversus	04	08.34-10.33	29.50-36.67	20
Laboratory cultures				
E. coli	01	07.84	28.67	20
S. typhi	01	07.84	30.66	20
S. paratyphi A	01	07.33	32.33	20
S. paratyphi B	01	08.89	27.45	20
S. aureus 6538	01	08.73	31.58	20
Shigella sp.,	01	07.67	29.72	20
V. cholera	01	10.85	32.82	20

Table 2: Synergistic activity of ethanol extract of *P. dactylifera* fruit and ampicillin

Test Pathogens	MBC of Ampicillin	MBC of ethanol extract of *P. dactylifera*	Sub-lethal concentration used	MBC of ampicillin in presence of ethanol extract of *P. dactylifera* (µg/mL)
β-lactamase producers				
K. pneumoniae	More than 10mg/mL	20%	10%	200-300
E. coli				300
P. aeruginosa				200
P. mirabilis				200-300
C. diversus				300

Table 3: Bioactive constituents identified in the ethanol extract of *P. dactylifera* fruit by GCMS analysis

Sr. No.	Retention Time (min)	Peak Area (10^3)	Name
1.	1.879	24876.596	2,3-butanediol
2.	2.568	1838.831	2,4,6-cycloheptatrien-1-one, 3,5-bis-trimethylsilyl-
3.	5.065	2964.852	2-butanol, 1-(dimethylamino)-2-methyl-, benzoate
4.	6.552	18572.988	Hexanol
5.	8.098	2822.965	9-oxononanoic acid
6.	8.663	1637.100	Hexanal
7.	9.331	5197.989	3-ethyl-6-trifluoroacetoxyoctane
8.	10.436	1908.002	Pentanoic acid
9.	14.876	917.481	Cinnamaldehyde

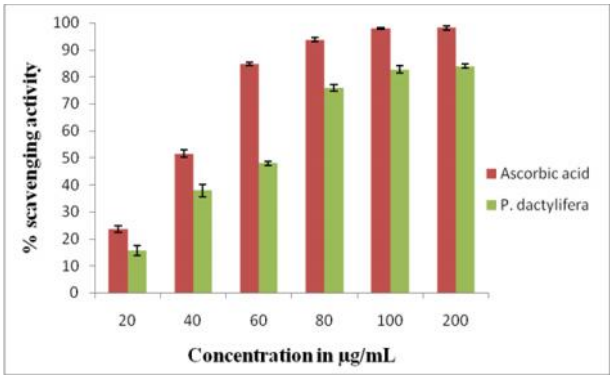

Fig 1: The DPPH radical scavenging activity of ethanol extract of *P. dactylifera* fruit and ascorbic acid

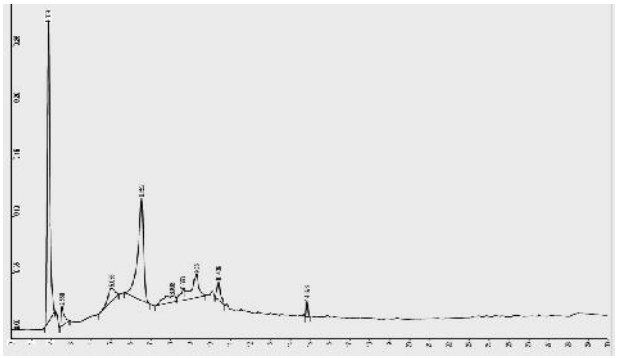

Fig 2: GC-MS chromatogram of ethanol extract of *P. dactylifera* fruit

4. CONCLUSION

The antibacterial and resistance reversal potential of *P. dactylifera* fruit extracts can be clearly observed in the current study. In particular, the choice of herbal extracts to overcome the developed resistance for antibiotics may have a significant impact on the clinical settings to help fight complex infectious diseases like UTIs.

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