

Antimicrobial and Synergistic Activity of Date palm pits Extract (*Phoenix dactylifera* L.) against Pathogenic Bacterial Isolates

Alugaili Dhafar N.*, Mhawesh Ahmed A. and Mohsien Rana A.

Department of Molecular and Medical Biotechnology, College of Biotechnology, Al-Nahrain University, IRAQ

*dhafar7778@yahoo.com

Abstract

The date palm (*Phoenix dactylifera* L.) is considered the most important source of food for humans in both arid and semiarid regions. The present work aimed to study the antibacterial effect of date palm pits crude extracts (aqueous and alcoholic) alone and in combination (synergistic) with the antibiotics that some pathogenic bacterial species (*staphylococcus aureus* and *Pseudomonas aeruginosa*) show resistant to it. HPLC analysis revealed that tested date pit aqueous extract contains several phenolic compounds (Caffeic acid, Ferulic acid, Chlorogenic acid, p-coumaric acid, Sinapic acid, Genstic acid, p-hydroxy benzoic acid, Gallic acid and Protocatechuic acid). No antibacterial effect was detected for the extracts against *staphylococcus aureus*.

In contrast, both extracts show an excellent inhibition activity against *Pseudomonas aeruginosa*. Synergistic activity was tested; four concentrations of date palm pit extract (100, 75, 50 and 25 mg/ml) were prepared using dimethylsulfoxide. The most active concentrations that gave synergistic antibacterial activity with vancomycin against *Pseudomonas aeruginosa* were (100 and 75 mg/ml) with alcoholic extract showing less effect on the *pseudomonas aeruginosa* than the aqueous extract. On the other hand, no synergistic activity was observed for the extracts and the vancomycin against *staphylococcus aureus*.

Keywords: *Phoenix dactylifera* L., Antimicrobial Activity, Synergistic Activity, *staphylococcus aureus* and *Pseudomonas aeruginosa*.

Introduction

Dates, the staple food in the valleys of the Tigris, Euphrates and Nile rivers have an important role in the sustainable agriculture in many countries around these areas since the dawn of history²². The date palm (*Phoenix dactylifera* L.) is considered as one of the oldest cultivated fruit trees and was well-known in Babylon, Iraq 4000 B.C. The palms were celebrated during that period for their strength and majesty¹⁸. In addition, the date palms trees have a long history of cultivation and utilization in Middle East and North Africa for more than five thousand years⁸. In the Arabian world, one

of the most important commercial crops which represents a vital element of the daily diet is the date palm fruits (dates)³¹.

The edible part of the date palm tree has been recognized to possess many medicinal properties when consumed alone or in mixture with other medicinal herbs²⁹. The treatment of infectious diseases became more difficult due to the emergence of drug resistance phenomenon among subpopulations of bacteria that developed resistance to one or more antibiotics and consequently the emergence of multidrug resistant (MDR) or superbugs that spread at an alarming rate in different countries²⁸.

The antibiotic resistance in bacteria in general is a natural adaptation phenomenon for antimicrobial agents. Once bacteria become resistant to some antibiotic, they transfer this trait to their progeny through horizontal or vertical transfer¹⁴.

The (MDR) bacteria are a global problem which has led to increase efforts focused upon the development of new antimicrobial drugs that can act successfully against this type of pathogenic bacteria. The interest was focused towards traditional medicines instead of the conventional treatment depending on published reports by the World Health Organization that confirm the utilization of medicinal plants as a good source for a variety of drugs depending upon their safer action (in terms of tolerance and side effects)³.

Date fruit is considered a high nutritional value food that represents a good source of carbohydrates and high concentrations of vitamin B complex such as thiamine (B1), riboflavin (B2), niacin (B3), pantothenic (B5), pyridoxine (B6), folate (B9) in addition to dietary fibers, proteins and minerals⁷. In addition it is rich in phytochemicals (which are ascribed to bioactive non-nutrients) such as carotenoids, polyphenols (e.g. phenolic acids, isoflavons, lignans and flavonoids), tannins and sterols²⁰. These phytochemicals have gained increased clinical interest due to potential in the prevention of diabetes, cardiovascular diseases in addition to other health benefits such as chemoprevention of cancer, antioxidant activity and cholesterol-lowering properties^{7,29}.

Several parameters including date variety, stage of fruit picking, storage, post-harvest processing, the geographical origin of the dates and soil conditions may affect the concentration and composition of these constituents very widely¹⁰.

Date seeds (also called pits, stones, or kernels) representing 10- 15 % of the whole fruit depending on the variety¹⁷ are valuable byproduct of date fruit processing industries. The pits of *Phoenix dactylifera* contain different chemical compounds such as saturated and unsaturated fatty acids, Zinc (Zn), Cadmium (Cd), Calcium (Ca) and Potassium (K)⁹. Saturated fatty acids include stearic and palmitic acid and unsaturated fatty acids contain linoleic and oleic acids which could inhibit the 5- α reductase enzyme. The chemical composition of date pits depending on the dry weight was found to consist of 5–10% moisture, 5–7% protein, 7– 10% oil, 10–20% crude fiber, 55–65% carbohydrates and 1–2% ash. The carbohydrates, as the largest component of the dry weight, are usually comprised of neutral detergent fiber 75%, acid detergent fiber 57.5%, hemicelluloses 17.5%, lignin 11%, cellulose 42.5% and ash 4%¹⁶.

Many investigations reported the medical benefits of date pits phenolic compounds (phenolic acids and flavonoids) depending on the observed positive effects on human health, attributed mainly to their antioxidant activities, anti-carcinogenic, antimicrobial, antimutagenic, anti-inflammatory activities and the reduction of cardiovascular disease²¹.

The date pit extract was found to have the ability to restore the normal functional status of the poisoned liver in addition to its action to protect against subsequent carbon tetrachloride hepatotoxicity on the liver in rats²⁶. The date pits antimicrobial action against pathogenic bacteria have been poorly investigated. However, the ethanolic date pits extract showed low antibacterial activity when it was tested against several strains of microorganisms. This study was primarily aimed to evaluate the synergistic antimicrobial activity of date palm pits *Phoenix dactylifera* L (aqueous and ethanolic) extract with antibacterial drugs against pathogenic bacterial isolates.

Material and Methods

Collection of the sample: The seeds of date (*Phoenix dactylifera* L.); (Barhee) were removed manually from the fruits that were freshly collected from one of the gardens in Baghdad, Iraq, at the beginning of the 2017 harvest season. Tap water was used to wash the seeds; then we removed the adhering date flesh with sterilized distilled water and let the seeds to dry in air. High temperature (50 °C) for 5 hrs. was used to get further dried. 100 g of seeds were ground in grinder into a fine powder and then kept at 4 °C up to analysis.

Microorganism isolates: The pathogenic bacteria (*staphylococcus aureus* and *Pseudomonas aeruginosa*) used in this study were obtained from the Dept. of Molecular and Medical Biotechnology, Al-Nahrain University.

Preparation of Aquatic and ethanolic extracts of *Phoenix dactylifera* L. pits for antibacterial activity: Pits of

Phoenix dactylifera L. were ground into free powder using grinding machine².

Aqueous extract: A quantity of 50 g of the pits powder was mixed with 500 ml double distilled water. A shaker incubator was used to incubate the mixture at 37°C for 24 hrs. and then filtered by a filter paper (Whatmann no. 1). The filtrate was concentrated using rotary evaporator at 40°C until dryness. 2 ml of distilled water was used to reconstitute the dried crude extract in Eppendorf tubes and stored at -18°C till we examine the antimicrobial activity. Most of the antioxidants and active components found in dates are successfully extracted in water. Therefore, aqueous extract was selected⁶.

Ethanolic extract: A quantity of 50 g of seeds powder was extracted with 500 ml of 70% ethanol in flask and put on magnetic stirrer for 72 hrs. at room temperature and then filtered through filter paper (Whatmann no.1) The solution was then evaporated to dryness using a rotary evaporator at 40°C and filtrate was concentrated using rotary evaporator at 40°C until dryness. then 2 ml of distilled water was used to reconstitute the dried crude extract in Eppendorf tubes and stored at -18°C till we examine the antimicrobial activity.

Detection of some phenolic compounds of date palm

aqueous seeds extract: High performance liquid chromatography was used to analyze phenolic compounds in the date palm extracts.

- **Estimation of phenols date palm pit by HPLC:** The FLC (Fast Liquid Chromatography) column was used to separate the main compound under optimum conditions.
- **Column:** Phenomenex C-18, 3 μ m particle size (100x 4.6 mm I.D) column.
- **Mobile phase:** Linear gradient of solvent A 0.1 % trifluoro acetic acid (TFA acid) in deionized water: solvent B was acetonitrile gradient program from 0% B to 100 % B for 10 minutes.
- **Flow rate:** 1.3 ml/min.
- **Detection:** UV at 280 nm.
- **Calculation:** Concentration of sample μ g/ml = (area of sample / area of standard) x conc. of standard x dilution Factor.
- **Extraction:** 10 mg of the extraction phenolic compounds was dissolved in 10 ml methanol HPLC grade subjected to ultra – sonication (Branson sonifier, USA) at 60 % duty cycles for 25 min. at 25 °C followed by centrifugation at 7500 rpm for 15 min. Charcoal treatment was applied to remove any pigments in the clear supernatant of the sample prior to evaporation under vacuum (Buchi Rotary evaporator Re Type). The dried samples were re-suspended in 1.0 ml HPLC grade methanol and mixed by vortex. Then, mixture was filtrated using 2.5 μ m disposable filter and stored at 4°C for further analysis. Finally, 20 μ l of the sample was injected into HPLC system according to the optimum separation condition²³.

Antimicrobial activity of the plant extract and preparation of microorganisms for sensitivity test - Disc agar diffusion (DAD) test:

The disc diffusion assay was performed using Kirby-Bauer method. All the bacterial isolates were plated by streaking on Muller-Hinton agar medium (with a turbidity equivalent to 0.5 McFarland tube; containing approximately 1 to 2×10^8 cfu/ml) by a sterile cotton swab and rotating the plate between streaking to ensure even distribution of the inoculum, the inoculated plates were placed at room temperature for 10 minutes to allow absorption of excess moisture. Gently each antibiotic disc (Penicillin, Vancomycine, Erythromycin, Ceftriaxone and Ciprofloxacin) was pressed down with sterile forceps to ensure complete contact with agar. Discs were arranged so as to avoid the development of overlapping of inhibition zones and within 15 minutes of applying discs, the inverted plates were aerobically incubated at 37°C for 24 hours.¹⁵

After incubation period, the inhibition zone diameter which represents the area of bacterial growth inhibition surrounding the disc (including the disc), was measured and compared with a standard of each drug according to CLSI published data. The zone margin should be considered the area which has no obvious, visible growth that can be detected with the unaided eye. This gave a profile of drug susceptibility vis-à-vis antibiotic resistance¹⁵.

Antimicrobial activity of date pits extracts: Antimicrobial activity of the crude extracts was screened against the tested bacterial isolate by agar well-diffusion method. Diluted solutions were prepared by diluting extract with DMSO for both alcohol and aqueous extracts. Muller-Hinton agar medium plates (prepared previously) were swabbed with 0.1 ml of bacterial suspension (*P. aeruginosa* and *Staph. aureus*) using sterile cotton swabs that were dipped in the suspension and streaked equally over the whole surface of the agar plate to obtain uniform inoculums. Holes were punched out from the Muller-Hinton agar using opposite side of the sterilized pasture pipette. 50 μl of the crude extract was poured in respective wells with the help of micropipette. The negative control was performed using dimethylsulfoxide (DMSO).

Antimicrobial activity of date pits extracts in combination with antibiotic:

The combined antimicrobial effect of the crude extracts and the antibiotic were determined using agar well-diffusion method. Different concentrations (25, 50, 75 and 100 mg/ml) of extracts were added to the wells in combination with antibiotic and allowed to diffuse at room temperature for 20 minutes. The plates were incubated at 37°C for 24 hrs. Each one of the extracts was tested against each microorganism in replicate. The antimicrobial activities of pits extract were recorded as the mean diameter of the resulting inhibition zones of growth measured in millimeter.

Results and Discussion

Plants are source of different chemical substances and phytochemicals are one of these chemicals which may give

health benefits when consumed as a part of daily diet or taken as a medicine drug. Therefore, they are employed by herbalists of different cultures, anciently and recently, to treat peoples of their sicknesses¹⁹.

The outcome of the HPLC chromatogram for water date palm extracts is represented in table 1. The results in the table showed the detected phenolic compounds and their concentrations in respect to standard. It is obvious that tested date pits extract contains a significant amount of some important phenolic compounds with higher concentrations of Caffeic acid, Ferulic acid, Chlorogenic acid and p-coumaric acid as described in table 2. These results are in accordance with the previous study by Al-Alawi and his colleagues⁴ and Saleh with his colleagues²⁷

Phenolic acids are considered as one of the main aromatic secondary plant metabolites, containing hydroxyl function located on aromatic benzene ring with one or more carboxylic acid groups⁴.

The susceptibility of *Staph aureus* and *P. aeruginosa* isolates against five different types of antibiotics, each of them acting to inhibit the bacteria by a different mechanism, was tested. The results in table 3 showed that, the two bacterial isolates were found to exhibit high degree of resistance against vancomycin in comparison with the other antibiotics that act to produce an obvious inhibition zone. So, vancomycin has no effect upon the bacteria used, a fact that is confirmed by published data of Tiwari et al²⁹ in which they demonstrated that the emergence of vancomycin resistant strains in the hospital environment may occur faster than expected.

The antimicrobial activity of the crude pits extract (ethanolic and aqueous) was evaluated; both extracts were found to almost have a negligible effect on *Staph. aureus* which did not show any inhibition zone against the two types of the extracts used in this study. On the other hand, the two-extract showed effective antibacterial action against *P. aeruginosa* which considered as one of the important pathogenic microorganisms responsible for wide variety of infections difficult to be controlled with antibiotics or disinfectants. The data for the inhibition zone indicates that these extracts were able to check the growth of the tested *P. aeruginosa* isolate as illustrated in table 4.

The inhibition of bacterial growth by this extract could be due to the presence of some active compounds which may act alone or in combination to inhibit bacterial growth. These results were found to be in accordance with earlier studies^{5,24} who recorded positive antibacterial effects for the date pits extract against gram negative bacteria.

Many studies reported the antimicrobial activity of phenolic acids¹¹ and of caffeic acid²⁵. In addition to the previous study by Ammar and his colleagues¹² who suggested that the flavonoids present in the date seed extract are responsible for

the inhibitory action of these extracts upon the tested bacterial isolates. On the other hand, the resistance of *Staph. aureus* to the pit extract may refer to the presence of virulence factors that *Staph. aureus* possess and make bacteria show some antibiotics resistance. Differences in resistance of bacteria to anti-tested materials may be due to change in membrane permeability of cells, thereby hindering the entry of enzymes or excretions by the change in the chemical composition of the constituent chemical or by changing the nature of some of their components¹.

Depending upon the obtained results of antibacterial activity for the date pits extract; the *in vitro* test for the determination of antibacterial efficacy of vancomycin (the less active antibiotic) and the two pits extract was investigated against *Staph. aureus* and *P. aeruginosa*, the antibacterial activity was expressed as the mean diameter of inhibition zones (mm) produced by the antibiotic alone and when used in combination with different concentrations of the extract.

As presented in fig. 1, the combination of the vancomycin with the aqueous extracts gave a positive effect due to the presence of clear inhibition zone against *P. aeruginosa* and the increase in the inhibition zone diameter as concentration increase which might be due to the selective or synergistic action of various chemicals present in date seed and the vancomycin antibiotic.

The combination of the ethanolic extracts with the vancomycin enhanced the antibacterial activity of the antibiotic moderately as we recorded (fig. 2) a clear inhibition zone that unfortunately overcame with the bacterial growth which may be related to the composition of the extract. As reported, water is called the universal solvent because more substances dissolve in water than in any other chemical. This has to do with the polarity of each water molecule. The hydrogen side of each water (H₂O) molecule carries a slight positive electric charge while the oxygen side carries a slight negative electric.

This helps water dissociate ionic compounds into their positive and negative ions. Ethanol is the second most important solvent after water; due to the presence of hydroxyl (OH) group which makes it a very polar molecule with the high electronegativity of oxygen allowing hydrogen bonding to take place with other molecules. Therefore, it attracts polar and ionic molecules.

From data obtained above it is clear that gram-negative bacteria appear more sensitive to the pits extracts than the Gram-positive bacteria.

We can conclude that seeds of date palm consider a good source of natural polyphenolic compounds with an obvious antibacterial potentials and so further research work is needed for the isolation and identification of active ingredients present in the extracts which may be a basic for

further improvement of antibacterial drug design and the possible exploitation in pharmaceutical use.

Table 1
Sequences of the eluted material of the standard
(Concentration 25 µg / ml).

Seq.	Subjects	Retention time/min.	Area µvolt
1	Gallic acid	1.28	34652
2	Genstic acid	2.12	70529
3	Cafeic acid	3.04	95867
4	Chlorogenic acid	5.53	81401
5	p-coumaric acid	6.35	106120
6	Ferulic acid	7.43	118751
7	p-hydroxy benzoic acid	8.52	98922
8	Sinapic acid	9.24	84973
9	Protocatechuic acid	10.39	119875

Table 2
Estimation of some active compounds
(phenolic compounds) in Palm pit extract

Phenolic compounds	Concentration µg/ml
Caffeic acid	276.33
Ferulic acid	235.12
Chlorogenic acid	164.26
p-coumaric acid	145.16
Sinapic acid	95.09
Genstic acid	91.42
p-hydroxy benzoic acid	91.11
Gallic acid	78.57
Protocatechuic acid	19.44

Table 3
Antibiotic susceptibility profile of the tested bacterial isolates to different types of antibiotics

Antibiotic	Inhibition zone diameter mm	
	<i>Staph. aureus</i>	<i>Pseudomonas spp.</i>
Penicillin (10 units)	30	24
Kanamycine (30 µg)	21	18
Erythromycin (15 µg)	13	26
Ceftriaxone (30 µg)	23	19
Vancomycin (30 µg)	-	-

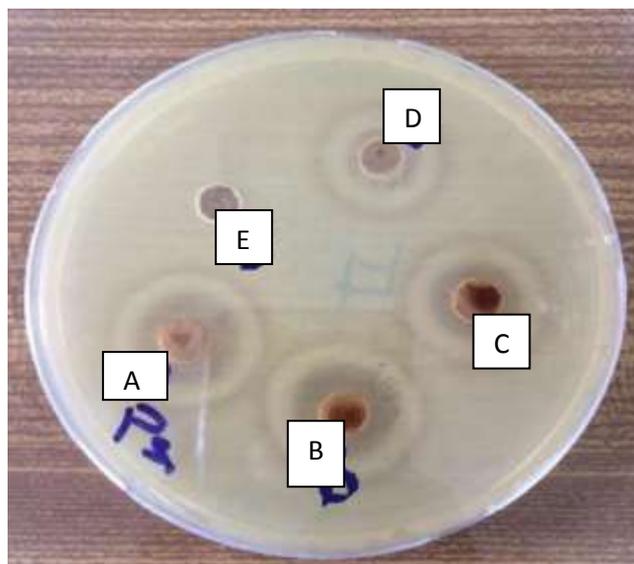


Figure 1: Synergistic effect of vancomycin (30µg) and aqueous extract on *pseudomonas* spp.
A- 100 mg/ml Vancomycin + extract, B- 75 mg/ml Vancomycin + extract, C- 50 mg/ml Vancomycin + extract and D- 25 mg/ml Vancomycin + extract.
E- Vancomycin

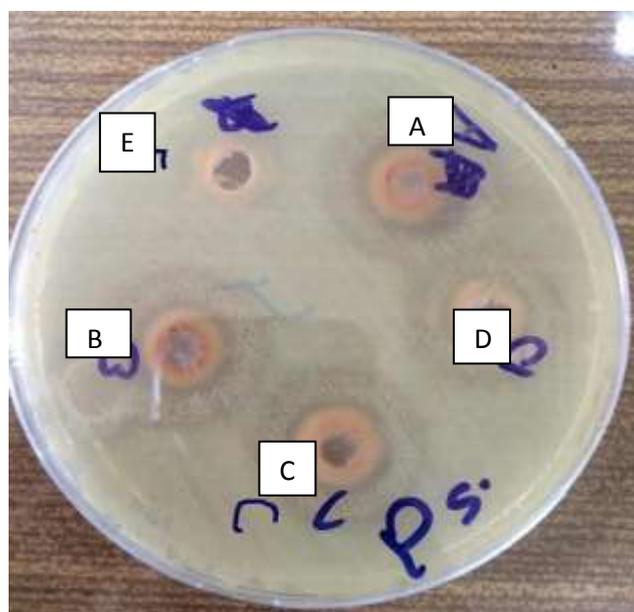


Figure 2: Alcoholic extract with vancomycin (30µg) showed less effect than aqueous extract.
A- 100 mg/ml Vancomycin + extract, B- 75 mg/ml Vancomycin + extract, C- 50 mg/ml Vancomycin + extract, D- 25 mg/ml Vancomycin + extract and E- Vancomycin

Conclusion

Our work may confirm or disagree the previous studies depending upon the fact that like many natural products, variation due to differences such as geographical location, plant nutrition and cultivar can influence the composition of the extract. Furthermore, it can be assumed that collection site affects antimicrobial activity due to the composition of soil; also, the age of the tree may affect the outcome results.

Acknowledgement

We would like to extend my thanks and gratitude to the College of Biotechnology at Al-Nahrain University for providing all the research requirements and harnessing all laboratory capabilities to support scientific research.

Table 4

Diameter of inhibition zone caused by the date palm pits extracts against *Staph. aureus* and *P. aeruginosa*

	Aqueous Extract mg/ml				Alcoholic Extract mg/ml			
	100	75	50	25	100	75	50	25
<i>Staph. aureus</i> inhibition zone diameter	-	-	-	-	-	-	-	-
<i>P. aeruginosa</i> inhibition zone diameter	16	13	9	7	14	11	8	-

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