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Article

Antibacterial, Anti-Biofilm, and Antioxidant Activity of Date Seeds Extract from Babylon Palm Phoenix dactylifera L. in Iraq

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Abstract: Date seeds (Phoenix dactylifera L.) are a rich source of bioactive phytochemicals with anti-inflammatory, antioxidant, and antibacterial properties. Despite their potential, limited research has explored their antibacterial efficacy against pathogenic bacteria. This study aimed to evaluate the antibacterial and antioxidant activities of date seed extract obtained using 70% ethanol. High-performance liquid chromatography (HPLC) analysis confirmed the presence of Caffeic acid (44.6 µg/g) and Sinapic acid (76.2 µg/g). The extract demonstrated significant antibacterial activity, with inhibition zones of 15-34 mm against Gram-negative and 21-37 mm against Gram-positive bacteria. Antioxidant potential, assessed using DPPH free radical scavenging, reached 87.77% at 200 µg/ml. These findings suggest that date seed extract holds promise for developing natural antibacterial and antioxidant agents.

Keywords: Date seeds, Antibacterial activity, Antioxidant properties, HPLC analysis, Bioactive compounds

1. Introduction

Researchers have extensively studied the antibacterial properties of medicinal plants, which are used in botanically based treatments such as herbal remedies, industrial pharmaceuticals, phyto-pharmaceuticals, and traditional medicines (Al-Harrasi Aetal,2014). Medicinal plants can be a cheaper and more efficient source of medication than synthetic antibiotics (Ali Haimoud Setal,2016). The date palm tree, or Phoenix dactylifera L., is a member of the Arecaceae family and offers a variety of pharmacological and nutritional advantages. (Bouhlali E dine Tetal,2016). Strong anti-oxidant, anti-cancer, neuroprotective, hepatoprotective, nephroprotective, and gastrointestinal protective properties are exhibited by several secondary metabolites present in date palms (Ghnimi S,etal,2017).

Date seeds are considered the most important waste product of the date business, while being a rich source of economic resources. They might be hazardous to the environment if they are found in large concentrations in the wild. (Hamad I,2014). Recent studies have shown that dates' seeds contain higher levels of dietary fiber, flavonoids, phenols, and antioxidants than those found in fresh dates. Large concentrations of glutathione, ascorbic acid, and α-tocopherol are present, along with polyphenol components such caffeic acid and sinapic acid, as well as protocatechnic acid (Hong YJ,etal,2006).

Date seeds rich in multi-aromatic compound such as alcohols, citrates, aldehydes, Ketones, saturated and unsaturated hydrocarbonates(Jamal Metal,2018). The aim of this

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research to investigate the antioxidant activity of date seeds ethanolic extract ,investigates about the presence of some anti-oxidant and anti-baterial phytochemical compounds.

2. Materials and Methods

Getting Date seed powder in its raw form

After getting the Halawi date seed from the nearby Hilla market, the Phoenix dactylifera seeds were extracted from the dates. After that, the seeds were cleaned and baked at 45 degrees Celsius. An electric miller was used to grind them into a fine powder. They were then kept for further use in a closed, sterile cup (Juices F,2018) after that.

Method for making ethanolic extract from date seeds

Date seed powder was extracted in its unprocessed form using a 1:1 V/V methanol-water solvent as described by Al-Sultany (2018), with minor adjustments. Ten milliliters of solvent were combined with one gram of raw date seed powder to create the mixture. The combination was first shaken vigorously for an hour, and then it was submerged in water in a path below 40 degrees Celsius for two hours. The mixture was filtered using filter paper. The filtered liquid was concentrated by dryness below 45 C in the oven. The condensed and dried material was first processed in an electric grinder, and the resulting powder was sterilised in a dark, sterile cup before being stored for later use (Juices F,2018).

HPLC analysis for both quantitative and qualitative research on:

1. Gallic acid

The C18-ODS (25 cm x 4.6 mm x 5 μm) column was used for the HPLC procedure. The approach developed by Zubillaga and Maerker (1990) found the mobile phase, which comprised two distinct types of solutions. (A) Ethanol The gradient was 40% of A, 60% of B for 4 minutes, 50% of A, 50%

2. of B for 5 to 8 minutes, and 60% of A, 40% of B for 8 to 10 minutes. Acetic acid: Distilled water (10:2:88) and (B) Methanol: Acetic acid: Distilled water (90: 3: 7). The injection volume of each specimen and standard solution was 100 μL, the identification wavelength was 280 nm, and the column temperature was 25 °C Khan H,2010).

3. An HPLC analysis of sinapic acid was performed using a C18-ODS (25 cm x 4.6 mm x 5.0 μm) column. A liner gradient containing O-phosphoric acid 25% (A): acetonitril (B) was present during the movement phase. It started at 95:5 A:B for two minutes and changed to 90:10 A:B for five minutes, 85:15 A:B for three minutes, 80:20 A:B for thirteen minutes, 70:30 A:B for five minutes, and 50:50 A:B 23 for four minutes. A milliliter per minute was the inflow rate. For all specimen standard solutions, the injection volume was 100 μL, the column temperature was 25 °C, and the detection wavelength was 360 nm (Khan H,2010).

The antibacterial activities of Date seed extract

Using Agar well diffusion methods, the antibacterial properties of Date seed extract were evaluated against S. aureus, Klebsiella pnemonia, streptococcus mutanus, and E. coli. Fresh culture colonies were suspended in 5 milliliters of brain heart infusion broth and cultured for four to twenty-four hours at 37°C. Sterile broth was used to calibrate the turbidity that growing culture produced in order to reach an optical density that was equivalent to the 0.5 McFarland requirements.The suspension was dipped into a sterile cotton swab. The Mueller Hinton agar tray was streaked all over with the dipping cotton swab. After that, four walls were filled with distal water as a control and pores with a diameter of 4 mm were made and filled with Date seed extraction at three different

concentrations (2000, 1500, and 1000 μg/ml) using a sterile cork porer. After that, the Petri plates were incubated for 24 hours at 37°C. Antimicrobial activity was assessed by measuring the growth inhibition zones' diameter in millimeters (Vahid, 2020).

Anti- biofilm effect by Date seed extract

The method of using 96-well microtiter plates was used to measure the in vitro ant biofilm activity. A 100 molar hinton broth (M.H.B) medium containing 1% glucose and 100 μL of Date seed extract was added to the first well microtiter plate. The medium was then prepared at several diluted concentrations (1000, 1500, and 2000μg/ml). Subsequently, we introduced 100 μL of the diluted concentration into each well microtiter plate, up until the final one. This served as a control to verify that bacteria developed biofilm and that Date seed extract inhibited the production of biofilm.

Next, 10μL of isolated bacteria that had been cultivated overnight were put to each well microtiter plate. Thus, 110 μL of mixing suspensions will be placed in each well microtiter plate (Shakibaie, Alipour-Esmaeili-Anari, et al., 2019). At 37 °C, the 96-well microtitre plates were incubated for 24 hours. To exclude free-floating "planktonic" bacteria, the contents of each well were taken out and three times cleaned with 200 μL of phosphate buffer saline (PBS, pH 7.2).

'Planktonic' bacteria are eliminated by repeating this method three times. Biofilms formed in the plate well by sticky, "sessile" organisms were fixed with sodium acetate (2 percent w/v) and dyed with crystal violet dye (0.1 percent w/v). After rinsing away the excess stain with sterile distilled water, the plates were set aside to dry. 200 μL of 95% (v/v) ethanol were added to the wells after they had dried (Shakibaie, Hajighasemi, et al., 2019). Utilizing an ELISA reader (Multiskan EX, Thermo Scientific, Finland), the absorbance was determined at 620 nm. The findings were used as proof that bacteria were sticking to the wall's surface to form biofilms (Barapatre et al., 2016).

Antioxidant effect of PVD

An offline (DPPH) assay was utilized to evaluate antioxidant activity. The radical cation test known as DPPH (1,1-diphenyl-2-picrylhydrazyl) was modified to evaluate PVD's capacity to scavenge free radicals. DPPH was the reagent for DPPH. 1 mg/ml was generated by dissolving 0.1 mg/ml of the 1, 1-diphenyl 1-2-picrylhydrazyl (DPPH) Radical Stock solution in a 1:9 (v:v) DMSO: Methanol combination. Dissolving 0.1 mg/ml of ascorbic acid in a 1:9 (v:v) DMSO: Methanol mixture yielded the ascorbic acid solution, which served as the positive control. The DPPH solution, on the other hand, served as the negative control. For each extract, five different concentrations of μg/ml (62.2, 125, 250, 500, and 1000) were prepared. Next, 0.3 ml of methanolic DPPH was added to the mixture (three duplicates for each concentration). The tubes were incubated for 30 minutes in the dark at 37° C. then a measurement of the decreased absorbance was made at 517 nm. The following formula was used to quantify the samples' percentage inhibition of radicals (Lee et al., 2015).

Radical scavenging (%) = [(A)control − (A)sample/ (A)control] × 100.

A=absorbation

3. Results and Discussion

Collocation of date seed

The current study included collectiong of (50 kilo from date and from were obtained 3 kilo seeds

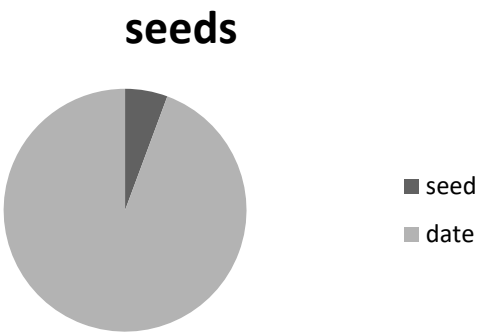

Figure 1. The current study included collectiong of (50 kilo from date and from were obtained 3 kilo seeds

Collocation of pathogenic bacteria

Four isolates of pathogenic bacteria were obtained, two Gram-positive and two Gram-negative, from the Advanced Microbiology Laboratory in the College of Science, Department of Life Sciences, Babylon University.their diagnosis was confirmed using the Vitek device as show in the table (1)

Table 1. Probability of pathogenic bacteria in Vitek2 system

Isolates	Source	Probability in Vitek2 system
S. aureus	AdvancedMicrobiology Laboratory in the College of Science	95%
S.mutanus		97%
K. pnemoniae		99%
E.coli		98%

Quantitative and qualitative identification of Caffeic acid and Sinapic acid in date seeds extracts by HPLC.

Investigation about Caffeic acid , Sinapic acid presence and concentration in date seeds extract HPLC analysis was performed Fiugers (2.3) , the results show appearance of retention time of the extreact in (4.28 , 6.80)min respectively and area was (90224.59 , 54182.69 , 65748.90) mAU.s respectively ,and by compared with standers retention times and areas results indicate the presence of Caffeic acid, Sinapic acid and Gallic acid in the respectively after doses (42.65 , 66.29 ,80.22) ppm. Many studies was performed to investigate phytochemical composition of different type of date seeds extracts such as Bouhlali,; et al (2015) and Adeosun,; et al (2016) and all confirmed the presence of many important phytochemical compounds in ethanolic date seeds extract including Caffeic acid, Sinapic acid and Gallic acid (18 and 19).

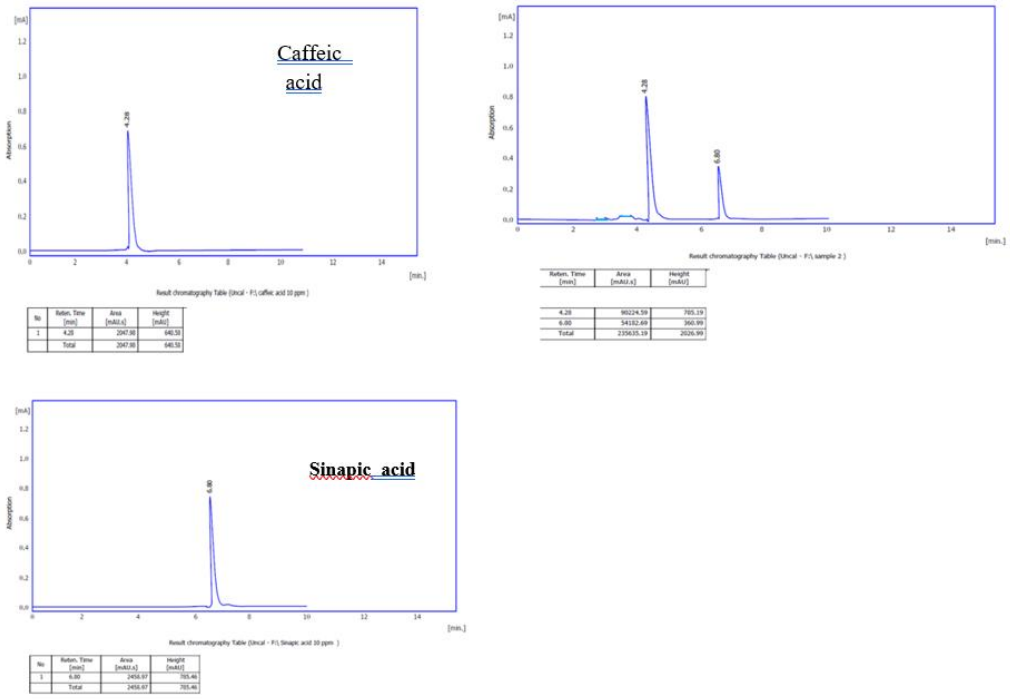

Figure 2. HPLC detection of Sinapic acid and Caffeic acid in date seeds ethanolic extract

Antibacterial effects of date seeds ethanolic extract

The antibacterial activity date seeds ethanol extract was determined using the agar well diffusion technique (Alvi et al., 2021). The effectiveness of the extract(date seeds ethanolic extract) was tested against four types of pathogenic bacteria, two gram positive(S. mutans ,S. aureus,) and two gram negative all of studied of isolated bacteria were inhibited by PVD at different concentration as show in table(2) , figure(3)

Table 2. Antibacterial activity of date seeds ethanolic extract

Bact. / con.	Inhibition Diameter µg\ml ± SD			
	E. coli	***K. pneumonia***	***S. mutans***	***S. aureus***
control	Zero	Zero	Zero	Zero
2000 µg\ml	30±1.3	34±1.5	34±1.6	37±1.4
1500 µg\ml	28±2.45	30±0.9	24±0.9	25±2.02
1000 µg\ml	15± 1.34	16±2.3	21±0.8	21±0.9

The result showed that both positive and negative bacteria were affected by the date seeds ethanolic extract. The finding in the current study show S. aureus is the species that is most sensitive to the inhibitory pigments effectiveness. Whereas E. coli is the species that is less sensitivity to the inhibitory of date seeds ethanolic extract effectiveness

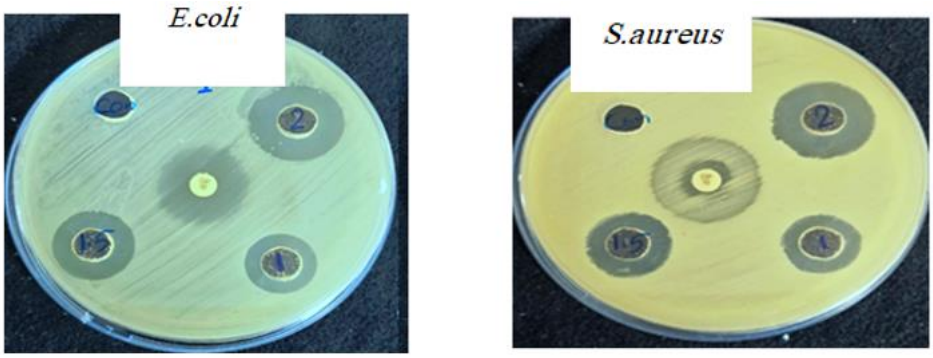

Figure 3. Inhibitory efficacy of date seeds ethanolic extract

There are a few reasons why positive bacteria are more affected by date seeds ethanolic extract than negative bacteria. Cell wall structure: Gram-negative bacteria have a thin outer membrane that is more permeable to date seeds ethanolic extract than the thick peptidoglycan cell wall of Gram-positive bacteria. This allows date seeds ethanolic extract to more easily reach and damage the inner membrane of Gram-negative bacteria. Lipopolysaccharide (LPS): The outer membrane of Gram-negative bacteria contains LPS, a molecule that is involved in many of the bacterium's essential functions. date seeds ethanolic extract can bind to LPS and disrupt its structure, which can lead to cell death.)while positive bacteria, their wall is thicker, consisting of a peptidoglycan layer and a Tiechoic acid layer in addition to lipopolysaccharide.

It is believed that this thick wall prevents date seeds ethanolic extract from reaching the lipopolysaccharide effectively.(Cumont,2023). Thes results agreed the findings of (Vollenweider., et al 2023),who reported that date seeds ethanolic extract have inhibitory activity against pathogenic bacteria such as S. mutans ,S. aureus. E. . coli and K. pneumonia.

Date seeds ethanolic extract 's antibiofilm activity

The most important characteristic of bacteria that appears to enhance their adherence to surfaces of prostheses and medical equipment appears to be their biofilm. Using the micro titer plate technique, the antibiofilm effect of date seeds ethanolic extract produced by P. aeruginosa was examined against four strains of bacteria, including Gram positive and Gram negative bacteria, including Staphulococcus aureus, Streptococcus mutanus, Klebsiella pnemoniae, and E. coli. The doses of Date seeds ethanolic extract used were 2000µg/ml, 1500µg/ml, and 1000µg/ml.

Table 3. Antibiofilm efficacy of Date seeds ethanolic extract against pathogenic bacteria

Pathogenic bacteria	PVD concentration µg\ml	Absorbance	Inhibition ratio
E.coli	2000	0.0046	99.26%
	1500	0.0399	93.63%
	1000	0.0469	92.50%
	control	0.6266	
K.pneumonia	2000	0.0036	99.10%
	1500	0.0137	96.77%
	1000	0.039	90.83%
	control	0.4254	
S.aureus	2000	0.0074	98.75%

	1500	0.0369	93.78%
	1000	0.0488	91.77%
	control	0.5933	
S.mutanus	2000	0.0049	98.62%
	1500	0.0401	88.75%
	1000	0.0552	84.52%
	control	0.3567	

Significant antibiofilm effect was demonstrated by bioactive Date seeds ethanolic extract at concentrations of 200 μg/ml, 1500 μg/ml, and 1000 μg/ml. Date seeds ethanolic extract, exhibited antibiofilm action at a concentration level at 2000μg/ml with an inhibition ratio of 99% for E. Coli, 99% for Klebsiella, 98% for Staph. aureus, and 99% for PVD. At the same concentration, the lowest inhibition ratio was 88.75% in S. mutans. The highest Date seeds ethanolic extract inhibition ratio for the 1000 μg/ml concentration was 92.50% in E. Coli, while the lowest was 88.75% in S. mutans at the same concentration.

A biofilm is a complex matrix made up of proteins, polysaccharides, and other organic materials that is formed when microbes bind together to develop strong adhesion to either biotic or abiotic surfaces [Mm et al,2016]. Microbes that adhere to a surface can survive in the face of challenging circumstances, such as natural host defenses and antimicrobial chemicals, thanks to biofilms [Jamal etal,2018]. Thus, one of the indirect ways that bacteria become resistant to antibiotics is through the creation of biofilms.

The effects of plant extracts with strong anti-cell attachment qualities on stopping or reducing the formation of new biofilms in biofilms that have already been established in 24 and 48 hours(Tiwari et al.2018). Antibacterial agents may be able to lessen the amount of microorganisms that colonize surfaces and the mucosa of the epithelium by inhibiting the creation of or destruction of biofilms.(Bavington et al,2005).

Antioxidant activity

By reducing DPPH free radicals and measuring the absorbance at 517 nm, the DPPH (2,2-Diphenyl-1-picryl-hydrazyl) study of radical scavenging was used to evaluate the antioxidant ability of the Date seeds ethanolic extracin vitro. The results, which indicated that the microtiter plate's original purple hue of DPPH changed to yellow, demonstrated PVD's capacity to scavenge DPPH free radicals (Lee et al., 2015). With a rise in Date seeds ethanolic extrac concentration came an increase in its capacity to reduce DPPH. From Methanol PVD, 1000μg/ml, 500μg/ml, 250μg/ml, 125μg/ml, and 62.2μg/ml.

As the concentration of Date seeds ethanolic extrac grew, so was its capacity to reduce DPPH. Methanol Date seeds ethanolic extrac yields 1000μg/ml, 500μg/ml, 250μg/ml, 125μg/ml, and 62.2μg/ml measurements. There was a significant differences in activity of scavenging in level ($p \leq 0.01$) between all concentrations

Table 4. Antioxidant activity of Date seeds ethanolic extrac

Extracts	Concentrations of extracts (µg/ml) Scavenging%(Mean ± stDev.)					F** - test
	1000	500	250	125	62.2	
F1	87.767±3.204 a	72.633±4.550 b	29.300±4.952 c	20.767±3.272 d	11.167±1.266 e	295.94
control = 85.133±2.159 a ** = (p≤0.01) Similar letters mean the absence of significant differences						

The most widely consumed fruit in the Middle East and North Africa is the date palm (Phoenix dactylifera L.). It is extensively consumed and has long been employed in traditional medicine. Rich in vitamins, minerals, sugar, protein, dietary fiber, flavonoids, and phenolic compounds, the fruits are nutrient-dense. Date palm fruits have strong antibacterial properties and are rich in antioxidants since they contain phenolic components.(Al-Shwyeh ,2019). 6.5%–11.5% total dietary fiber (up to 90% insoluble and 10% soluble), around 1% fat, 2% proteins, and 2% ash have all been found to be present in date fruits. Additionally, it has a lot of phenolic antioxidants(Hong et al,2006).

4. Conclusion

The plant has an effective role in biological control and can be used to eliminate many pathogens such as antibiotic-resistant bacteria. Date seed extract has the ability to eliminate pathogenic bacteria and is also highly effective in suppressing free radicals as an effective antioxidant. The extract has proven its effectiveness as an anti-biofilm and thus has the ability to eliminate pathogenic bacteria that form biofilm. Dates have an important biological effect that can be used in many medical fields

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