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Therapeutic Potential of Selected Varieties of Phoenix Dactylifera L. Against Microbial Biofilm and Free Radical Damage to DNA

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Abstract

Phoenix dactylifera L. (Date palm) is the most widely consumed fruit around the world and is rich source of nutrients containing dietary fibers, minerals, vitamins, sugar, protein and antioxidants with potent bioactivities against various microbial pathogens. This study evaluated the therapeutic potential of 2 varieties of ethanolic extracts of *Phoenix dactylifera* i-e Ajwa and Khalas against bacterial biofilms. This study also investigated the protective effect of Ajwa and Khalas against hydroxyl radical damage to calf thymus DNA. Antioxidant potential through different antioxidant assays showed that Ajwa has higher antioxidant potential than Khalas. Both Ajwa and Khalas presented good antimicrobial activities against *Bacillus subtilis* and *Pasteurella multocida*. Biofilm inhibition assay showed that increasing concentration of Ajwa and Khalas exhibited higher percentage of bacterial biofilm inhibition. Microscopic examination revealed significant inhibition of microbial biofilm. Ajwa and Khalas protected the calf thymus DNA against damage caused by hydroxyl radicals produced by fenton reagent. Fourier Transform Infrared (FTIR) spectra confirmed the presence of O–H, C=C and C–O functional groups in tested extracts. The study concluded that tested varieties of Date palm have the potential to inhibit bacterial biofilms and can be used for therapeutic purposes against biofilm producing pathogens.

Keywords

biofilm, phoenix dactylifera, bacillus subtilis, pasteurella multocida

Introduction

Nowadays, the intake of fruits and vegetables are highly recommended for healthy life to fulfill the requirements of essential nutrients through these natural sources. However, numerous established epidemiological studies reported that increased intake of fruits and vegetables are associated with indisposition and mortality from numerous persistent diseases such as cancer, cardio-vascular diseases, coronary heart diseases, aging, atherosclerosis, neuro-degenerative disorders and inflammation.[1] World Health Organization (WHO) reported that 80 percent of global population rely on herbal medicines for certain feature of their primary health care requirements. According to WHO, large number of plants have the prospective for being used as remedial plants. The fruits are significant components of our routine diets that comprise of numerous bio-active nutraceuticals, which improve our body's power to combat various illnesses.[2] The composites believed to be accountable for the defensive effects, include vitamin E, carotenoids and also polyphenols.[3]

Phytochemicals are the chemicals derived from plants and are useful for health when taken as part of the routine diet or medicine. Dates are rich in phytochemicals such as polyphenols (flavonoids, lignans, isoflavones and phenolic acids), carotenoids, sterols and tannins.[4] Carotenoids are considered the major phytochemicals found in the lipid fractions of date fruits and are

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vitamin A precursors playing vital role in vision and act as antioxidants protecting the cells from deleterious effects of free radicals.[5] Another major phytochemicals found in date fruit are phytosterols.[6] Phenolic acids contain hydroxyl functional group on aromatic benzene ring having carboxylic acid groups. These act as free radical scavenger or captor, hence effective antioxidant.[7] The presence of significant amounts of phenolic acids in dates has been reported in various published studies from several research groups.[8,9] Mansouri et al. studied different varieties of date fruits cultivated in Algeria and identified the major phenolic acids such as sinapic acid, ferulic acid and *p*-coumaric acid.[10] Saudi date fruit varieties contain p-coumaric acid, gallic acid and ferulic acid derivatives as the major phenolic acids.[11] Flavonoids are the polyphenolic secondary metabolites found in vegetables and a variety of fruits act as antioxidant and anti-inflammatory agents with notable health benefits.[12]

Dates are used as the principal diet in Middle East for thousands of years. Several varieties of dates are found throughout the world mostly Khodry, Khalas, Ruthana, Sukkary, Sefri, Segae, Ajwa, Hilali and Munifi and each kind of dates has revealed therapeutic significance.[13] Date palm (*Phoenix dactylifera*) has a place in plant family *Arecaceae* that surrounds around 200 species with about 3,000 classes. *P. dactylifera* have been grown in the Central East since preceding 6,000 years.[14] Dates possess a large proportion of total sugars, protein, fat, crucial salts and minerals, vitMiamins and higher concentration of nutritional fibers. Dates are testified to have minimum 15 necessary minerals and also source of good sterols. Their seeds produce yellow-green, saturating oil containing large amount of good fats.[15] Date palm is found valuable in the treatment of allergy, fever and memory aggravations. It is utilized as astringent in intestinal inconveniences, treatment of sore throat, bronchial infection, fever, gonorrhoea, edema, liver and stomach diseases. Powder of Date palm seed is utilized in customary prescriptions to treat toothache.[16]

Dates are reported to provide defense against oxidative destruction as a result of the phagocytic action of lymphocytes against pathogens and insects due to the presence of large quantity of dietary fibers, selenium, carotenoids, ascorbate and other essential antioxidants. Except the nutritious importance, date fruits contain large quantity of compounds like carotenoids and phenolics due to which they exhibit antioxidant and antimutagenic potential.[8] Dates and their components exhibited a part in diseases prevention due to their anti-oxidant, anti-inflammatory and anti-bacterial potential. The defensive influence of *P. dactylifera* against toxic elements present in the surrounding has been confirmed through various investigations.[14] Dates have significant role in glucose and lipid control metabolism of diabetic patients. Dates have additionally been recognized to diminish coronary diseases and tumor growth. The overall production, usage and industrialization of dates are expanding due to its dietary and health benefits.[17]

Therefore, the present research work was planned to study the therapeutic potential of 2 different varieties of *P. dactylifera* L. i-e Ajwa and Khalas in inhibiting the microbial biofilm produced by selected bacterial strains. The protective effect of tested extracts against the damaged DNA caused by hydroxyl radicals was also evaluated.

Materials and Methods

Sample Collection, Preparation and Extraction

Two varieties of *P. dactylifera* (Date palm) locally known as Ajwa and Khalas were obtained from the local market of Faisalabad. These particular Date palm varieties were identified, classified and approved from Botany Department, University of Agriculture, Faisalabad.

The collected Date palm varieties were shade dried, grinded and stored. The grinded pulp of Khalas and Ajwa were extracted using ethanol as solvent (1:5 w/v) for 3 days on orbital shaker (Gallenkamp, UK) at ambient temperature.[18] The extracts were filtered through whatmann filter paper no.1.[19] The excess of solvent was evaporated through rotary vacuum evaporator at 45°C to get concentrated extract, then lyophilized to remove the solvent effect and powdered extracts were stored at 4°C to avoid the loss of chemical compounds before further testing.[20]

Proximate Analysis

The dried powder of Date palm pulp were analyzed for crude protein, crude fibers, moisture, fiber and total carbohydrate content following the procedure described by Jadid et al.[21]

Antioxidant Potential

Antioxidant activities of the Date palm ethanolic extracts were evaluated through different antioxidant assays including DPPH radical scavenging assay for the determination of percentage inhibition of free radicals,[22] and reducing power assay was performed following the procedure described by Laouini et al.[23] Folin-Ciocaletu reagent method was used for the determination of total phenolic contents (TPC) using gallic acid as the standard[24] and the total flavonoids content (TFC) of the extracts was determined by aluminum chloride complex forming assay using catechin as standard.[18,25]

Hemolytic Activity

Spectrophotometric technique was performed for *in vitro* hemolytic activity for extracts of 2 different varieties of Date palm against red blood cells using PBS and Triton-X-100 as negative and positive controls, respectively.[26]

DNA Damage Protection Assay

The capability of different concentrations of Ajwa and Khalas to protect calf thymus DNA from damage caused by hydroxyl radicals produced through Fenton's reagent was evaluated using agarose gel electrophoresis.[27]

Antibacterial Activity and Minimum Inhibitory Concentration (MIC)

The antimicrobial activity was evaluated against 2 selected isolates: *Bacillus subtilis and Pasteurella multocida* using agar well diffusion method by adding 100 µL of 10 mg/mL extract solution. Ampicillin was used as positive control. The MIC was performed to find out the smallest concentration of Date palm extracts that inhibited the growth of bacteria.[28]

Biofilm Inhibition Potential

The potential of ethanolic extracts of Date palm to inhibit microbial biofilm formation was assayed following the method of O'Toole.[29] Varying concentrations of Date palm extracts were used to assess the microbial biofilm inhibition. The microtiter plate assay was used to quantify the biofilm inhibition potential by measuring the absorbance of the solubilized crystal violet at 550 nm in microtiter plate reader using 30% acetic acid in water as the blank. All the tests were carried out in triplicates.

The biofilm inhibition potential of Date palm ethanolic extracts was also observed through Phase contrast microscopy. For this, few drops of the overnight culture of *Bacillus subtilis* and *Pasteurella multocida* was added on separate glass slides and incubated at 37°C for 14 h. Using phosphate buffer saline, the slides were washed and supplemented with Date palm extracts. Then the slides were rinsed, stained and the biofilms were dissolved using 30% glacial acetic acid. Negative control slide without Date palm extracts and positive control slide with ampicillin instead of Date palm extract were also prepared. All the prepared smears on glass slides were examined microscopically.[30]

Scanning Electron Microscopy

Extracts of Date palm sample were characterized using scanning electron microscope (FEI Quanta 250, Czech Republic) for surface morphology following the standard procedure.[31]

Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectroscopy was performed by mixing grinded forms of Ajwa and Khalas with KBr to find out the active functional groups.[32]

Statistical Analysis

All the experiments were conducted in triplicate and the data were presented as mean ± standard deviation (SD) of replicate measurements. Analysis of Variance (ANOVA) was applied to determine the statistical significance of experimental data and $p < 0.05$ was considered as statistically significant.

Table 1. Proximate Composition of Studied Variety of *P. Dactylifera.*

Parameters	Khalas	Ajwa
Ash content (%)	5.09 ± 0.171	1.60 ± 0.04
Moisture content (%)	13.81 ± 0.3	15.80 ± 0.46
Crude fiber content (%)	27.01 ± 0.017	29.60 ± 0.20
Crude fat content (%)	4.25 ± 0.187	2.47 ± 0.10
Crude protein content (%)	3.86 ± 0.052	1.60 ± 0.30
Total Carbohydrate content (%)	45.98	48.93

Results and Discussion

Proximate Analysis

Proximate analysis determines the nutritional value of test samples and the contents of primary nutrients like carbohydrates, proteins and fats. The results of the proximate analysis of tested varieties of *P. dactylifera* i-e Ajwa and Khalas are given in Table 1. The results of present study indicated that Ajwa has higher moisture content (15.80 ± 0.46%) than Khalas (13.81 ± 0.3%). Percent crude fat content and crude protein were found higher in Khalas compared to their contents in Ajwa. Ajwa has higher total carbohydrate content than Khalas. Total ash is a measure of the presence of inorganic compound in the sample. Lower value of acid-insoluble ash is indicative of the presence of a small amount of non-physiological components like silica and silicates. Higher value of acid-soluble ash showed larger amount of acid soluble compounds like oxalates, carbonates, phosphates, and oxides, respectively. Ajwa has lower ash content which showed that Ajwa has lower inorganic compounds. The high nutritive value suggests that *Phoenix dactylifera* can be used as formulation in various dietary supplements.[33]

Results are expressed as mean percentages ± standard deviation (SD) of triplicate measurements on dry matter basis.

Antioxidant Potential

Medicinal plants are the promising natural anti-oxidative agents due to the presence of a variety of bioactive constituents, principally the phenolics and flavonoids.[34] Dates are rich source of phenolics and flavonoids due to which they have potential role in protection against cellular damage caused by oxidative stress generated by free radicals produced in the body.[7] Some varieties of *P. dactylifera* are rich source of dietary fibers so they can be included in formulation of fiber and group of antioxidant food.[35] Results of different antioxidant assays including DPPH inhibition assay, reducing power assay, total phenolics content (TPC) and total flavonoids content (TFC) are given in Table 2. Results showed that TPC in Khalas and Ajwa ranged from 47.8 ± 0.04 to 121 ± 0.16 and 89.6 ± 0.05 to 140.4 ± 0.18 mg/100 g, respectively. It proved that sample containing higher phenolic content has higher antioxidant activity.[36] Total flavonoids content (TFC) in Khalas and Ajwa varied from 20.65 ± 0.03 to 121 ± 0.16 and 23.81 ± 0.04 to 83.28 ± 0.68 mg/100 g, respectively. Table 2 showed

Table 2. Antioxidant and Cytotoxic Potential of Ethanolic Extracts of Selected Varieties of Date Palm.

Samples	Extract Conc. (mg/mL)	TPC (mg GAE/100 g)	TFC (mg CE/100 g)	DPPH inhibition (%)	Reducing power (%)	Lysis of RBCs (%)
Khalas	10	47.8 ± 0.04	20.65 ± 0.03	59.29 ± 0.21	4.95 ± 0.06	1.14
	50	82.6 ± 0.10	45.65 ± 0.34	71.74 ± 0.10	6.89 ± 0.04	2.18
	100	121 ± 0.16	74.34 ± 0.17	81.84 ± 0.11	13.25 ± 0.11	2.95
Ajwa	10	89.6 ± 0.05	23.81 ± 0.04	79.65 ± 0.09	9.32 ± 0.10	3.01
	50	104.8 ± 0.2	58.28 ± 0.15	87.51 ± 0.36	15.11 ± 0.18	3.35
	100	140.4 ± 0.1	83.28 ± 0.68	97.67 ± 0.07	19.3 ± 0.09	3.45
Positive Control		-	-	77.92	-	90.05

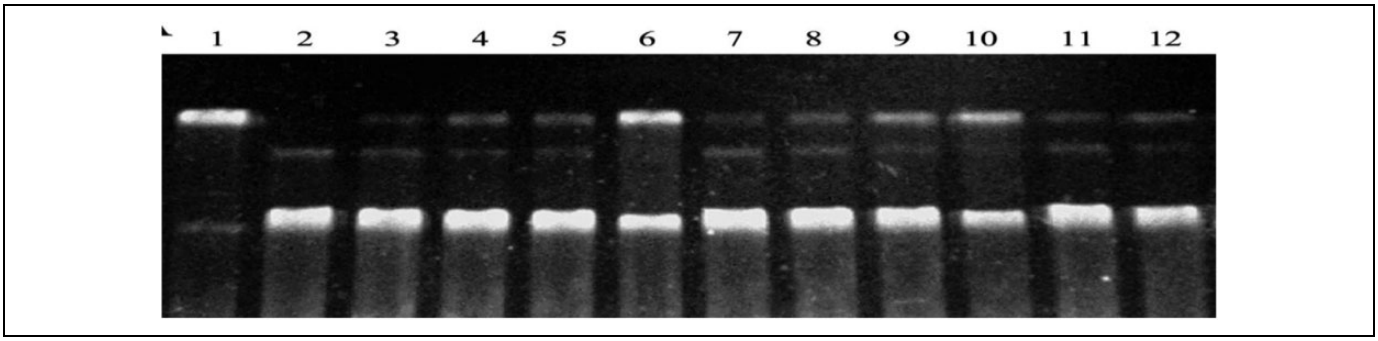

Figure 1. DNA damage protection assay of Khalas and Ajwa extracts. 1: DNA of calf thymus + H_2O_2+ $FeSO_4$, 2: DNA of calf thymus, 3-5: (10, 50 and 100 mg/mL) Concentrations of Khalas extract + calf thymus DNA + H_2O_2, 6-8: (10, 50 and 100 mg/mL) Concentrations of Ajwa extract + calf thymus DNA+ H_2O_2.

that TFC varies by changing the concentration and also by using different Date palm varieties. The ferric reducing power potential was confirmed by the change of yellow color of the test solution to various shades of green and blue depending on the concentration of the plant extract, the highest reducing power was obtained by using 100mg/mL concentration of Ajwa (19.3 ± 0.09) and lowest was (4.95 ± 0.06) by using 10mg/mL concentration of Khalas.

The advantage of using DPPH radical scavenging assay to evaluate the antioxidant potential is its more stable radical than hydroxyl or superoxide radicals. The DPPH assay usually involves a hydrogen atom transfer reaction. The decrease in absorbance of DPPH caused by antioxidants is due to the reaction between antioxidant molecules and the radicals, which results in scavenging the radical by hydrogen donation. This is visualized as a discolouration from purple to yellow. The results indicated that the antioxidant activity of this plant extract was equivalent to 97.67 ± 0.07% and 81.84 ± 0.11% radical inhibition *in vitro* for Ajwa and Khalas, respectively. These effects proposed that scavenging capabilities of various extracts of Date palm was dependent on the concentration against DPPH. So, DPPH scavenging potential increases by increasing the quantity of various extracts of Khalas and Ajwa.[37] Previous studies reported the highest concentration of polyphenols in date fruits among the dried fruits because of the extreme temperature and sunlight exposure for date fruits.[7] Antioxidant potential of 28 fruits routinely used in China has been studied by Guo et al. and found that the date fruits have the second highest antioxidant activities following Hawthorn.[38] In another study, Saafi et al. investigated the protective effect of date fruit extract against oxidative damage along with dimethoate induced hepatotoxicity on rat liver. Their study results showed that the extract treatment repaired the liver damage.[39]

Cytotoxic Activity

Cytotoxic activity of tested varieties of Date palm was carried out through hemolytic assay using human red blood cells (RBCs). The percentage hemolysis of different extract and concentrations of Ajwa and Khalas is given in Table 2. The highest percentage of red cell lysis was observed in ethanolic extract of Ajwa (3.45%) at 100 mg/mL concentration while lower percentage of red cells lysis was observed in ethanolic extract of Khalas at 10 mg/mL concentration. The Triton X-100 used as positive control showed 90.05% lysis of red cells. The mechanical stability of the erythrocytic membrane is a better sign of the effect of various *in vitro* studies by chemical constituents for the screening of cytotoxicity. So in this study, the cytotoxic effect of fruits extract and concentrations against human red cells was evaluated. The percentage lysis of human erythrocytes was below 5.0%, so it was concluded that the extract and concentrations have less cytotoxic effects.

Values are mean ± standard deviation (SD) of triplicate measurements. Powdered extract dissolved in dimethyl sulfoxide (DMSO). Vitamin C was used as positive control in DPPH assay and Triton X-100 as positive control for lysis of RBCs.

Table 3. Antibacterial Activities of AJWA and Khalas Extracts Against Selected Bacterial Strains.

	P. multocida (-)		*B. subtilis* (+)	
Samples	IZ	MIC	IZ	MIC
Ajwa	33 ± 3.1	15 ± 1.50	45 ± 4.3	26 ± 0.50
Khalas	31 ± 2.8	11 ± 1.10	42 ± 3.8	23 ± 0.10
Positive control (Ampicillin)	35 ± 3.4	16 ± 1.21	48 ± 4.6	28 ± 0.23

IZ: Inhibition zone in mm, MIC: Minimum inhibitory concentration in μg/mL. Data is represented as mean ± standard deviation of triplicate measurements. IZ: Inhibition zone, MIC: Minimum inhibitory concentration, **Note:** Different capital letters in superscripts along rows and columns indicate significant differences in mean values.

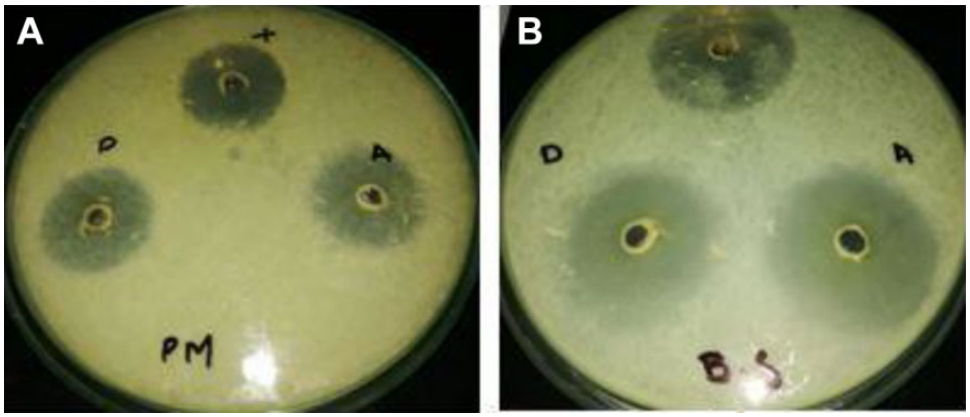

Figure 2. Antibacterial activities of Ajwa and Khalas against *P. multocida* (A) and *B. subtilis* (B).

DNA Damage Protection Potential

The assay was performed by using DNA of calf thymus. The test relies on potential of Date palm extracts to protect calf thymus DNA from damage caused by hydrogen peroxide. The protective effects of ethanolic extracts of Date palm through DNA damage protection assay are shown in Figure 1.

Pure calf thymus DNA formed a complete and clear band in second lane that was taken as standard of negative control and lane 1 is the positive control in which calf thymus DNA is treated with H_2O_2 and $FeSO_4$. In the presence of calf thymus DNA and Fenton reagent in lane 3 to 8 showed no smear formation. This protection of calf thymus DNA in the presence of Fenton reagent has proven the oxidative damage protection potential of extracts.

Antibacterial Activity

Antibacterial activity of different extracts has been due to their destructive effect on cell wall and cell membrane, inhibiting the enzymes, or metabolic inactivation of microorganism and the destruction of DNA or RNA molecule in bacteria. Results of the antibacterial activities of studied varieties of *P. dactylifera* namely Ajwa and Khalas by using 100mg/mL concentration against selected bacterial strains i-e *P. multocida* and *B. Subtilis* are given in Table 3 as inhibition zone (IZ; mm) and minimum inhibitory concentration (MIC; μg/mL). The inhibition zones (mm) as a measure of antibacterial activity of tested varieties of *P. dactylifera* are shown in Figure 2.

The study results showed significant antibacterial activities of the tested varieties of *P. dactylifera* against both the tested bacterial strains namely *P. multocida* and *B. Subtilis*. Largest inhibition zones (mm) and lower MIC values (μg/mL) indicate higher antimicrobial activities of the sample. In present study, ethanolic extract of both Ajwa and Khalas exhibited largest inhibition zones and lowest MIC values against *B. Subtilis* whereas lower inhibition zone against *P. multocida*. These activities of extracts against microorganism is because of their chemical constituents due to which extracts chemically react with cell membrane or cell wall of bacteria entering into the cell and destroy their cellular proteins and DNA structures.

Antibacterial activity showed reduction in bacterial growth in terms of increased zone of inhibition and decreased MIC indicating that extracts exhibited excellent antimicrobial activity. The strong antibacterial activity of Ajwa and Khalas ethanolic extract may be attributed to large amounts of phenolics and flavonoids. The potent antibacterial action of ethanolic extract can be correlated with its high phenolic content because of phenolic toxicity to microorganisms.[40] Abdullah et al. studied the antibacterial activity of hot aqueous and methanol extracts of Ajwa date fruit against Gram negative bacteria including *S. typhi, E. coli, V. Cholera* and *S. flexneri* through well diffusion method. They found that methanol extract

Table 4. Biofilm Inhibition Potential of Selected Varieties of *P. Dactylifera* L. (Date Palm).

Varieties of date palm	Sample concentrations (mg/mL)	Percentage inhibition of biofilm formation	
		P. multocida	*B. subtilis*
Kkalas	10	28.34 ± 0.07	35.03 ± 0.96
	50	33.47 ± 0.23	39.64 ± 0.36
	100	37.01 ± 0.12	42.01 ± 0.04
Ajwa	10	30.55 ± 0.09	40.94 ± 0.38
	50	35.76 ± 0.19	49.57 ± 0.02
	100	40.44 ± 1.23	53.78 ± 0.16
Ampicillin (positive control)		60.86 ± 0.51	

Data is presented as mean ± SD of triplicate measurements.

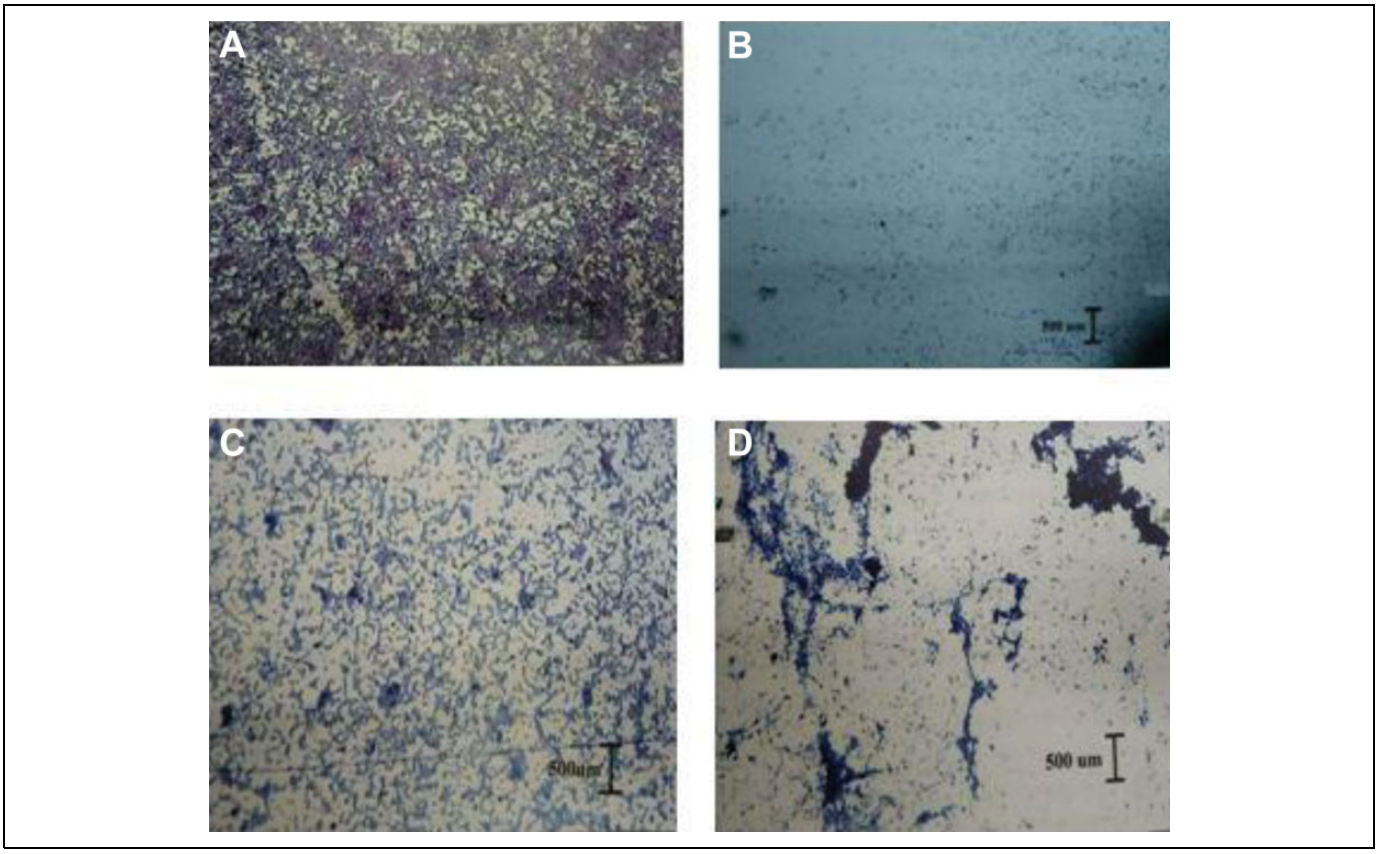

Figure 3. Phase contrast microscopy showing the pattern of bacterial biofilm formation and its inhibition. (A) Negative growth slide of *B. Subtilis,* (B) Positive control i-e Ampicillin treated, "(C & D) Khalas and Ajwa extract treated slides against *B. Subtilis* biofilm, respectively." The positive control and Date palm extract treated slides depicted the inhibition of bacterial biofilm.

showed higher antibacterial activity than aqueous extract suggesting that different extraction methods yields different phytochemicals producing the bactericidal effect.[41]

Biofilm Inhibition Potential

The formation of bacterial biofilm depends on bacterial cells interaction, the attachment surface and the medium around.[30] Potential of the 2 tested varieties of *P. dactylifera* L. including Ajwa and Khalas to inhibit the growth of bacterial biofilm was evaluated against *P. multocida* and *B. subtilis.* Both extracts at varying concentrations inhibited the bacterial biofilm formation. By increasing the concentration of extract sample, the potential of biofilm inhibition was increasing. At 100mg/ml concentration of Ajwa and Khalas extracts exhibited higher percentage of biofilm inhibition against *B. subtilis* and 10mg/ml concentration of Khalas and Ajwa showed lower percentage of biofilm inhibition against *P. multocida.* The results of biofilm inhibition assay of Ajwa and Khalas at varying concentrations are given in Table 4 and Figure 3 below. Phase Contrast Microscopy showed the compact bacterial growth on slide of negative control in the absence of any antibacterial

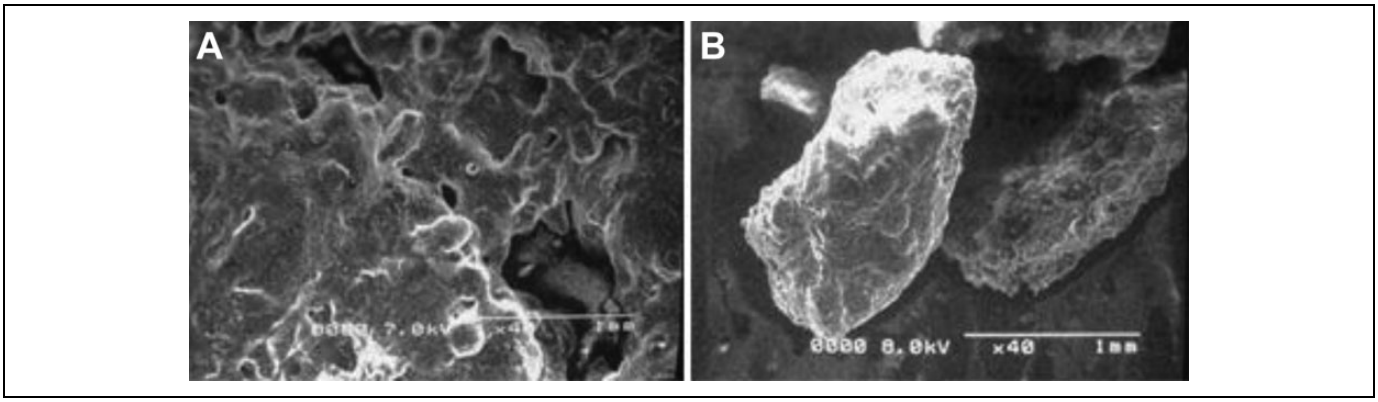

Figure 4. Morphological analysis of Ajwa (A) and Khalas (B).

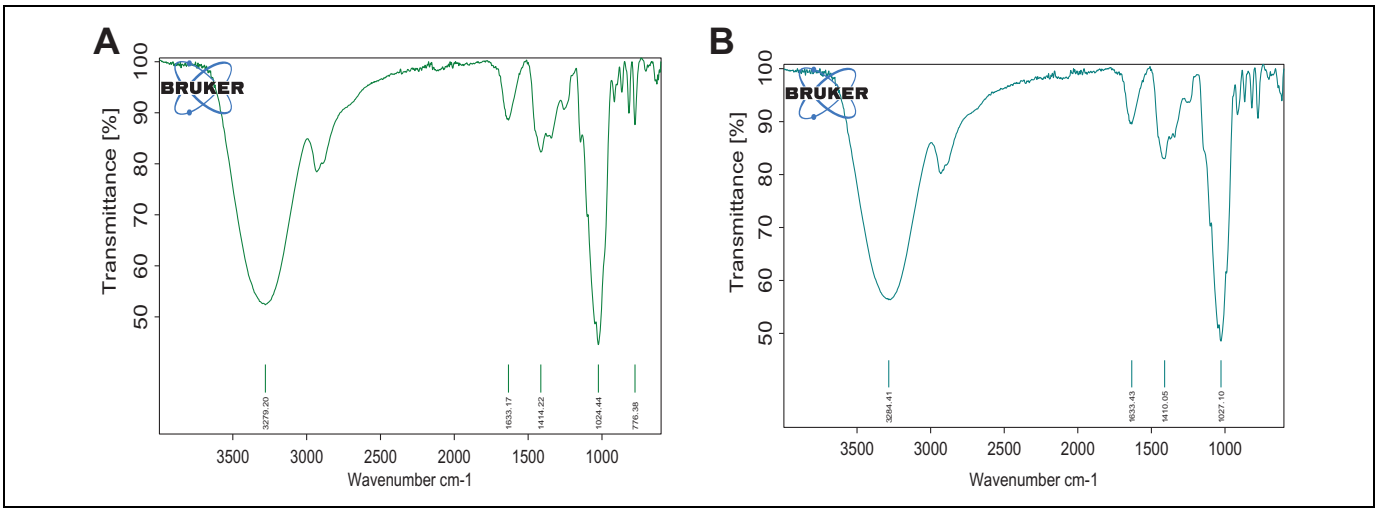

Figure 5. FT-IR spectra of Ajwa (A) and Khalas (B) extract.

agent. Condensed growth of bacteria was observed because of crystal violet. Positive control that contains antibiotic, Khalas and Ajwa extracts inhibited the growth of bacterial colonies which were grown in nutrient broth.

Scanning Electron Microscopy

The external structure of pulp of Ajwa and Khalas showed morphology which has importance for cultivators to distinguish and recognize the potential modification among species and cultivars within the species.[42] Scanning electron microscopic picture of Ajwa and Khalas are presented in Figure 4.

Fourier Transform Infrared Spectroscopy (FTIR)

For determining detailed data about the arrangements of different functional groups present in sample, FTIR spectroscopy was utilized and the FTIR spectra of tested samples are shown in Figure 5.

The spectrum for Ajwa and Khalas displayed a prevailing peak at 3284.41 cm^{-1} and 3219.20 cm^{-1} recognized as O–H functional group. The peaks observed at 1633.11 cm^{-1} for Ajwa and 1633.43 cm^{-1} for Khalas is consigned to C=C functional group. The peak at 1410.05 entimetre^{-1} for Ajwa and 1414.22 cm^{-1} for Khalas may represent C=C (aromatic groups). The peaks at 1027.10 cm^{-1} for Ajwa and 1024.44 cm^{-1} designated to C–O functional group.[32]

Conclusion

P. dactylifera has a long history as a medicinal plant with diverse therapeutic applications. The present study data revealed *P. dactylifera* as rich source of antioxidants that play vital role in controlling the imbalance between reactive oxygen species and body's ability to detoxify free radicals to repair the damage. The extracts and concentrations against selected bacterial strains showed significant antimicrobial activities. Due to the minor cytotoxicity the dates can be used as herbal medicine. The extracts of the selected varieties of *P. dactylifera* i-e Ajwa and Khalas showed excellent inhibition potential against bacterial biofilm, hence successful antibiofilm agent inhibiting the growth of bacterial biofilms. FTIR provided an excellent mean to visualize the chemical composition of different Date palm varieties. It would be worthwhile embarking on an intensive scientific experimentation and investigation on this apparently

valuable medicinal plant and to promote its large-scale utilization.

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Declaration of Conflicting Interests

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