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Antibiofilm, antimicrobial, and antioxidant activities of Moroccan *Phoenix dactylifera* L. pit extracts

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ABSTRACT

Multidrug-resistant bacteria represent a serious public health concern due to their reduced susceptibility to multiple antibiotics and their capacity to form biofilms, which complicates treatment and contributes to persistent infections. Total phenolic compounds and flavonoids were quantified using spectrophotometric methods. The antimicrobial and antioxidant properties of the extracts were evaluated using agar diffusion, microdilution, and various antioxidant assays, respectively. Antibiofilm activity was determined by measuring the inhibition of biofilm formation in the tested bacterial strains. Phytochemical analysis revealed high levels of polyphenols (1.973 ± 0.064 to 4.501 ± 0.082 g GAE/100 g DW) and flavonoids (0.150 ± 0.018 to 0.304 ± 0.031 g RE/100 g DW). These findings are consistent with the notable antioxidant activity observed, with IC_{50} values ranging from 30.8 to 491.3 µg/mL. Furthermore, the extracts exhibited significant antimicrobial activity against *Staphylococcus aureus* and *Staphylococcus epidermidis*, with inhibition zone diameters ranging from 10.31 to 14.13 mm. The minimum inhibitory concentration ranged from 0.23 to 0.94 mg/mL, while the minimum bactericidal concentrationvaried between 0.47 and 1.88 mg/mL. Moreover, all extracts significantly reduced biofilm formation compared to the untreated control, with inhibition rates ranging from 6.47 ± 0.5% to 72.17 ± 2.50%, highlighting their strong antibiofilm potential. This study highlights the potential of date seed extracts as a natural source of bioactive compounds capable of inhibiting biofilm formation and combating associated bacterial resistance.

Keywords: *Phoenix dactylifera* L., seeds valorization, biochemical composition, antibiofilm activities, antioxidant capacity, antibacterial activity.

INTRODUCTION

Microbial infections remain a major global public health concern and constitute a leading cause of morbidity and mortality worldwide (Ahmad et al., 2022). In this context, the emergence of antimicrobial resistance has intensified the search for alternative therapeutic strategies, particularly those based on natural products. Plant-derived compounds and herbal remedies have gained increasing attention due to their bioactive properties and reduced side effects compared to conventional drugs. Consequently, the identification, screening, and pharmacological evaluation of plant-derived phytochemicals have become essential approaches in the development of novel antimicrobial agents (El-Far et al., 2021). Among plant-based resources, fruit by-products such as seeds, stones, and pits are increasingly recognized for their therapeutic potential. These materials are rich in bioactive compounds that contribute to disease prevention, treatment, and stress reduction.

In particular, *Phoenix dactylifera* L. (date palm), a key agricultural species widely cultivated in arid and semi-arid regions, holds significant economic and nutritional importance. In Morocco, date production exceeds 113,000 tons annually, ranking the country among the major global producers. The Moroccan date sector is largely dominated by natural clones locally known as "Khalt," which represent a substantial proportion of production but remain poorly characterized (Canton, 2012). Date palm fruits consist of a fleshy pericarp and a seed, with seeds accounting for approximately 5–15% of the total fruit weight (Alharbi et al., 2021; Sayas-Barberá et al., 2020). These seeds are generated in large quantities as agro-industrial by-products during the processing of date-derived products such as paste, syrup, and pitted fruits. Although traditionally used as animal feed, date seeds have recently attracted interest for their potential applications in food, pharmaceutical, and cosmetic industries, contributing to a sustainable circular bioeconomy (Maqsood et al., 2020). Date palm seeds are characterized by a rich biochemical composition, including dietary fiber, proteins, lipids, and essential minerals such as calcium, potassium, magnesium, iron, and zinc (Al-Alawi et al., 2017; Platat et al., 2019). They also contain significant amounts of bioactive compounds, particularly phenolic acids and flavonoids, which are associated with strong antioxidant properties (Saryono et al., 2019). These compounds are known to exhibit various biological activities, including anti-inflammatory, antimicrobial, hypoglycemic, and anticancer effects (Gregorova et al., 2020). Recent studies have highlighted the antimicrobial potential of date seed extracts against a broad spectrum of pathogenic microorganisms, including both Gram-positive and Gram-negative bacteria such as *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* spp., and *Listeria monocytogenes* (Daoud et al., 2019). Furthermore, the efficacy of these extracts is strongly influenced by the extraction solvent, as variations in phenolic content play a key role in determining their biological activity (Platat et al., 2019). Despite these promising findings, studies focusing on Moroccan date seeds, particularly the Khalt variety, remain limited. Moreover, their potential antibiofilm activity, a critical aspect in combating persistent and resistant infections, has not yet been investigated.

Therefore, the present study aims to characterize the phytochemical composition of Moroccan *Phoenix dactylifera* L. seed extracts and to evaluate their antioxidant, antimicrobial, and antibiofilm activities. The findings of this work are expected to contribute to the valorization of date seed by-products and to provide new insights into their potential applications in pharmaceutical, food, and biomedical fields.

MATERIALS AND METHODS

Collection of plant materials

In November 2023, seeds from two date palm clones, Khalt E and Khalt Z, were collected from the Erfoud (31°27'18"N 4°15'02" W) and Zagora (30.332° N, 5.838° W) regions in southern Morocco, respectively. Seeds were extracted from the fruits, washed with distilled water, ovendried at 40 °C until completely dehydrated, ground to a fine powder, and stored in glass jars at 4 °C until further use (Djaoudene et al., 2019).

Date palm seed extraction technique

Ten grams of powdered seeds were separately macerated in 100 mL of methanol (80%) and acetone (70%) at room temperature (28 °C) with continuous stirring at 150 rpm. The resulting mixtures were filtered, and the supernatants were evaporated using a rotary evaporator at 45 °C, under vacuum conditions (El-Mergawi et al., 2016).

Extraction yield calculation

The percentage yield of extraction was determined according to the following equation:

$$Extraction\ yield = \left(\frac{A}{B}\right) \times 100 \quad (1)$$

where: *A* – weight of the extract residue after solvent evaporation, B – weight of the starting seed powder (Rima et al., 2022).

Bacterial strains

The antimicrobial assessments were performed using four pathogenic strains of bacteria, encompassing two Gram-positive and two Gram-negative organisms. Specifically, the tested microorganisms were the Gram-positive species (*S. aureus* ATCC 25923 and *S. epidermidis* IPM 24728) and the Gram-negative species (*E. coli* CIP 54127 and *S. typhimurium* CIP 5535).

Chemicals, solvents, and antibiotics

Reagents, solvents, and chemicals necessary for this investigation, including the Folin Ciocalteu reagent, aluminum chloride, 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, Trolox, β-carotene, potassium persulfate, resazurin, methanol, acetone, and dimethyl sulfoxide (DMSO), were purchased from Sigma-Aldrich (Deisenhofen, Germany). These suppliers also provided the standard antibiotics utilized in the testing. BIOKAR (France) furnished the microbiological culture media employed, specifically Mueller-Hinton agar and broth, tryptic soy agar, and Luria-Bertani agar.

Phytochemical analysis

For every crude extract, a biochemical analysis was done to look for any secondary metabolites that might be linked to the antioxidant and antibacterial and antibiofilm properties.

Total phenolic content assay

The total phenolic content (TPC) within the date palm seed extracts was quantified via the Folin-Ciocalteu technique, following the established procedure outlined by Al-Farsi and Lee (2008). The spectrophotometric reading was taken at 750 nm. Final data is presented as grams of gallic acid equivalents per 100 grams of dry material (g GAE/100 g DW).

Total flavonoid content assay

The total flavonoid content (TFC) was quantified using the colorimetric assay based on aluminum chloride, as previously reported (Brighente et al., 2007). Optical density readings were taken at 430 nm, and the final values are presented as grams of rutin equivalent per 100 g of dry matter (g RE/100 g DW).

In vitro antioxidant activity

Our investigation employed three distinct in vitro methods to quantify the antioxidant capacity of the extracts. These techniques were the β-carotene test, which measures the extract's ability to inhibit the oxidation of linoleic acid and the subsequent formation of conjugated hydroperoxides. The DPPH and ABTS assays, conversely, assess the antioxidants' free radical scavenging power by measuring their efficacy against the 1,1-diphenyl-2-picrylhydrazyl and 2,2'-azino-bis (ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cations. The IC_{50} value, defined as the concentration required to neutralize 50% of the DPPH radicals, was derived from a plot of the inhibition percentage versus the extract concentration. Crucially, the concentration of active antioxidant components within the extract is inversely related to the IC_{50} value. Consequently, a lower IC_{50} reading corresponds to a higher level of antioxidant activity.

Assessment of radical scavenging activity

The radical scavenging potential was evaluated using the DPPH methodology, adapted from the protocol published by Bondet et al. (1997). For the test, 1 mL of the extract (at various concentrations) was mixed with 1 mL of a 0.2 mM methanolic DPPH solution. The resulting solution was then stored in darkness for a 30-minute incubation period at ambient temperature. Following incubation, the optical density was measured at 517 nm. Ascorbic acid was included as a reference standard. The scavenging efficiency percentage was calculated using the equation below:

$$\frac{(Ab^{517}.control\ nm - Ab^{517}.sample\ nm)}{Ab^{517}.control\ nm} \times 100 \tag{2}$$

where: $Ab_{517}.control$ – represents the absorbance of the control, $Ab_{517}.sample$ – corresponds to that of the sample.

Carotene bleaching assay

The β-carotene bleaching assay was employed to determine the antioxidant capacity of the extracts, which measures their ability to prevent β-carotene oxidation, following the procedure of Boros et al. (2010). An emulsion was prepared using a mixture of 1 mL of chloroform in which 0.5 mg of β-carotene, 25 µL of linoleic acid, and 200 mg of Tween 20 were dissolved. After removing the chloroform under vacuum, 100 mL of distilled water was incorporated while continuously stirring to achieve a uniform and stable emulsion. For the assay, 4.5 mL of the working emulsion was mixed with 0.5 mL of every extract within reaction vessels. The absorbance at 490 nm was determined instantaneously following the addition of the samples. The vessels were subsequently held at 50 °C, and a second reading of the absorbance at 490 nm was taken after a two-hour period. Ascorbic acid served as the primary positive standard for the entire test. The percentage inhibition of lipid peroxidation (LPI %) was derived using the equation (A).

$$A = \frac{Ab^{490\,nm}\ after\ 2h}{Ab^{490\,nm}\ initial} \times 100 \tag{3}$$

where: $Ab^{490\ nm} initial$ – absorbance measured at time zero (initial absorbance),
$Ab^{490\ nm} after\ 2h$ – absorbance measured after 2 hours of incubation at 50 °C.

ABTS radical neutralization assay

The ABTS radical scavenging capability was evaluated according to the protocol detailed by Re et al. (1999). The ABTS working solution was prepared by combining equivalent volumes of a 2.45 mM potassium persulfate solution and a 7 mM ABTS solution. This resulting mixture was subsequently stored in the dark at ambient temperature for a duration of 12 to 16 hours to facilitate the generation of the radicals. Prior to commencing the assay, this solution was diluted with methanol to achieve a target absorbance of 0.70 ± 0.02 at 734 nm.

Next, a 25 µL aliquot of each extract, which was prepared in either water or methanol based on its solubility profile, was added to 2 mL of the ready-to-use ABTS solution. The reaction vessels were then incubated at room temperature in the absence of light for five to ten minutes. A control blank was simultaneously prepared by introducing 25 µL of distilled water to 2 mL of the ABTS solution. The absorbance measurement at 734 nm followed the incubation period. Trolox served as the reference positive control. Subsequently, the percentage of radical scavenging activity was calculated using the formula (B).

$$B = \frac{(Ab^{734\,nm}\ control - Ab^{734\,nm}\ sample)}{Ab^{734\,nm}\ control} \times 100 \tag{4}$$

where: $Ab^{734\ nm}\ control$ – absorbance of the control (ABTS solution without extract),
$Ab^{734\ nm}\ sample$ – absorbance of the extract solution.

The antimicrobial activity

Agar diffusion method

The antimicrobial activity of the extracts was assessed using the disc diffusion method (Rota et al., 2004). Sterile distilled water and commercial antibiotics (Augmentin 30 µg and ciprofloxacin 5 µg) served as negative and positive controls, respectively. Bacterial suspension was spread onto Mueller Hinton agar plates using sterile cotton swabs. Sterile 6 mm discs were impregnated with 100 mg/mL extract, placed on the agar, and prediffused at 4 °C for 1 hour. Plates were incubated at 37 °C for 18–24 hours, and inhibition zones were measured with a caliper.

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of date palm seed extracts were determined using the microdilution method. Following preliminary screening for antimicrobial activity, only the extracts exhibiting the strongest antibacterial effects were selected for further evaluation. MIC values were assessed using a broth microdilution assay with resazurin as a bacterial growth indicator (Cherrat et al., 2014). Briefly, serial dilutions of each extract (180 µL per well) were prepared in sterile 96-well microplates. Subsequently, each well received 20 µL of a bacterial suspension adjusted to 10^6 CFU/mL, followed by incubation at 37 °C for 24 hours. After incubation, 5 µL of 0.01% resazurin (v/v) was added to each well, and the MIC was defined as the lowest extract concentration at which no color change occurred, indicating complete inhibition of bacterial growth (Gaamouche et al., 2021).

Minimum bactericidal concentration

Following the 24-hour incubation period, samples were taken from the wells exhibiting no detectable color alteration (signifying inhibition of bacterial proliferation). These aliquots were then inoculated onto Mueller-Hinton agar medium and placed in the incubator at 37 °C for 18 to 24 hours. The MBC was established as the smallest concentration of the extract that prevented any discernible bacterial colony formation on the agar plates (Cherrat et al., 2014; Gaamouche et al., 2021).

Antibiofilm activity of date palm seed extracts

Biofilm inhibition was evaluated according to the method described by Ruiz-Duran et al. (2023), with slight modifications. Different concentrations of date palm seed extracts were prepared, including the minimum inhibitory concentration and its serial dilutions (MIC/2, MIC/4, and MIC/8), by dilution in tryptic soy broth (TSB). Then, 100 µL of each extract concentration was dispensed into the wells of a microplate. An equal volume (100 µL) of bacterial suspension (~10^6 CFU/mL) was added, resulting in a final volume of 200 µL per well. Biofilm formation control was performed by incubating the bacterial suspension in TSB medium only. All experiments were carried out in triplicate. The microplates were incubated at 37 °C for 24 h without agitation. After incubation, the wells were washed three to six times to remove non-adherent cells. The plates were then dried at 60 °C for 30 min, and the wells were stained with 0.10% crystal violet solution (200 µL per well) for 10–15 min. After staining, the wells were rinsed three to six times to remove excess dye. The crystal violet bound to the biofilm was solubilized by adding 200 µL of ethanol to each well. The optical density (OD) was measured at 570 nm using a microplate ELISA reader.

The percentage of biofilm inhibition was calculated from the mean absorbance values using the following equation (Srikanth et al., 2020):

$$\text{Percentage of inhibition} = \frac{(OD\ Negative\ Control^{570\ nm} - OD\ Experimental^{570\ nm}\ sample)}{OD\ Negative\ control^{570\ nm}} \times 100 \quad (5)$$

Statistical analysis

All statistical analyses were performed using SPSS software. Results are expressed as mean ± standard deviation (SD) of three independent replicates. A parametric analysis of variance (ANOVA) was performed after verifying the assumptions of normality (Shapiro-Wilk test) and homogeneity of variances (Levene test). Where appropriate, a logarithmic transformation of the data was applied to improve their distribution. Differences between groups were assessed using a two-way ANOVA for biochemical parameters, antioxidant activity, and antimicrobial activity, and a three-way ANOVA with interaction terms for antibiofilm activity, taking into account solvent type, concentration, and geographic origin. In the case of significant effects, multiple comparisons were conducted using Tukey's and Bonferroni post hoc tests to identify differences between groups. Statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Phytochemical analysis

As shown in Figure 1, extraction yields ranging from 11.53 ± 0.5% to 23.92 ± 0.38%. The two-factor ANOVA revealed that the overall model was highly significant (F = 423.011; $p < 0.001$), explaining 99.4% of the variance in yield ($R^2 = 0.994$). The results show a significant effect of the solvent (F = 7.144; $p = 0.028$; $\eta^2 = 0.472$) and a highly significant effect of the region (F = 1261.509; $p < 0.001$; $\eta^2 = 0.994$), indicating that geographic origin is the primary factor influencing extraction yield. No significant interaction between solvent and region was observed ($p = 0.555$), suggesting that the effect of the solvent is independent of the region. Post hoc comparisons (Bonferroni) revealed a significant difference between regions ($p < 0.001$), with a higher yield in Zagora (23.383 ± 0.229) than in Erfoud (11.867 ± 0.229), confirmed by a 95% confidence interval [10.769 – 12.264]. With regard to the solvent, the estimated means indicate a slightly higher yield for acetone (18.058 ± 0.229) compared to methanol (17.192 ± 0.229), with a significant difference ($p = 0.028$). However, this effect remains moderate compared to that observed in the region. The combined analysis confirms that the extraction yield is consistently higher in Zagora than in Erfoud, regardless of the solvent used, while acetone yields slightly higher results in both regions. These results confirm that the variation in yield is primarily related to geographic origin, with a secondary effect of the solvent and no interaction between the two factors. These results are confirmed by those published by Ourradi et al. (2022), who observed extraction yields of 23.90 ± 0.28% and 15.25 ± 0.21% for acetone extracts from the Khalt Z and Khalt E clones, respectively. Similarly, another study revealed that extraction yields of date palm (*Phoenix dactylifera* L.) seed powder were 9.21%, 8.35%, and 6.58% using methanol, water, and n-hexane solvents, respectively (Olaniyi et al., 2022). Ouahioune et al. (2020) also reported a yield of 4.6 ± 0.03% for the aqueous extract of the Degla-Baïda cultivar (*Phoenix dactylifera* L.). The variations observed in extraction yield may be attributed to a several factors, including climatic conditions, geographical origin, soil composition, and the nature and polarity of the solvent used for extraction.

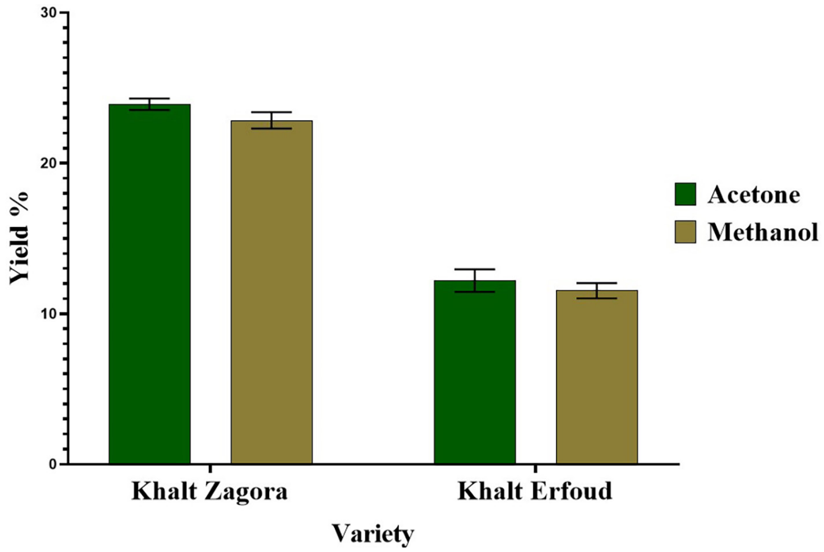

Figure 1. Extraction yields of seed extracts from two date palm clones (Khalt Zagora and Khalt Erfoud)

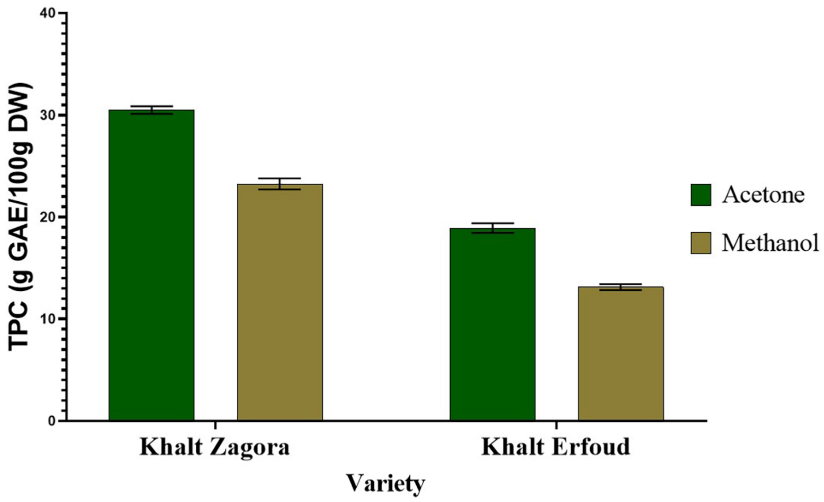

Figure 2. Total phenolic content of seed extracts from two date palm clones (Khalt Zagora and Khalt Erfoud)

The total phenolic and flavonoid contents of the two date palm clones are presented in Figures 2 and 3. Total phenolic content ranged from 13.15 ± 0.4 to 30.5 ± 0.52 g GAE/100 g DW. Two-way ANOVA revealed a highly significant effect of the model ($p < 0.001$), with significant effects of solvent and region, as well as a significant interaction between these factors. Samples from Zagora exhibited significantly higher TPC values than those from Erfoud ($p < 0.001$), while acetone extraction yielded higher TPC than methanol ($p < 0.001$). The highest values were recorded in Zagora using acetone, whereas the lowest were observed in Erfoud with methanol. These findings indicate that TPC variation is mainly driven by geographic origin, but also influenced by the extraction solvent, with an interaction-dependent effect.

Total flavonoid content ranged from 0.150 ± 0.018 to 0.304 ± 0.031 g RE/100 g DW. Two-way ANOVA indicated a highly significant model (F = 70.291; $p < 0.001$), explaining 96.3% of the variance ($R^2 = 0.963$). Both solvent (F = 66.777; $p < 0.001$; $\eta^2 = 0.893$) and region (F = 121.183; $p < 0.001$; $\eta^2 = 0.938$) exerted highly significant effects, with geographic origin identified as the primary determinant of TFC variability. A significant interaction between solvent and region was also observed (F = 22.913; p = 0.001; $\eta^2 = 0.741$), indicating that the influence of the extraction solvent on TFC varies depending on the geographic origin of the samples. Estimated means showed that samples from Zagora exhibited significantly higher TFC values than those from Erfoud ($p < 0.001$). Similarly, acetone extracts showed higher values than methanol extracts ($p < 0.001$), reflecting greater extraction efficiency. The combined analysis revealed that the highest TFC values were obtained with acetone extracts from Zagora (0.304 ± 0.031), whereas the lowest corresponded to methanol extracts from Erfoud (0.150 ± 0.018). In both regions, acetone yielded higher TFC values than methanol, and Zagora samples consistently exhibited higher levels than those from Erfoud. These findings confirm that total flavonoid content is strongly influenced by geographic origin and extraction solvent, with a significant interaction between these two factors. Numerous studies have reported considerable variability in the phytochemical composition of date seeds. For instance, Ourradi et al. (2022), reported TPC and TFC values ranging from 0.72 to 3.59 g GAE/100 g DW and from 0.1 to 1.11 g RE/100 g DW, respectively. Similarly, Radfar et al. (2019) found total phenolic contents between 1.48 and 3.38 g GAE/100 g DW. Abuelgassim et al. (2020) reported TFC values of 0.084 ± 0.001 and 0.095 ± 0.0014 g QE/100 g DW, and TPC values of 2.014 ± 0.212 and 2.060 ± 0.176 g GAE/100 g DW for Sukkari and Khalas cultivars, respectively. In addition, Bouhlali et al. (2015) observed TFC values ranging from 1.224 to 1.844 g RE/100 g DW and TPC values from 2.697 to 5.342 g GAE/100 g DW. However, the values obtained in the present study were higher than those previously reported. This discrepancy can likely be attributed to differences in cultivar, geographic and climatic conditions, fruit developmental stage, harvest period, soil composition, fertilization practices, post-harvest handling, and extraction methodology, including the solvent used. Date seeds are widely recognized as an important source of phenolic compounds and flavonoids, which exhibit notable biological and pharmacological activities (Djaoudene et al., 2019). These compounds can accumulate in substantial amounts in date seeds (Ourradi et al., 2022; Bouhlali et al., 2017; Alahyane et al., 2019).

Antioxidant activity

The DPPH radical scavenging assay results (Figure 4) showed IC_{50} values ranging from 30.77 ± 1.1 to 39.57 ± 2.51 µg/mL. These results exceeded those obtained for ascorbic acid, which had an IC_{50} value of 5.7 µg/mL. Two-way ANOVA revealed that the overall model was significant ($F = 7.228$; $p = 0.011$), explaining 73% of the variance in antioxidant activity (DPPH). Significant effects of solvent ($F = 15.185$; $p = 0.005$; $\eta^2 = 0.655$) and region ($F = 6.215$; $p = 0.037$; $\eta^2 = 0.437$) were observed, with solvent being the most influential factor. No significant interaction between these factors was detected ($p = 0.609$), indicating independent effects. Estimated means showed that extracts from Zagora had significantly lower DPPH IC_{50} values (30.77 ± 1.1) than those from Erfoud (39.57 ± 2.51), indicating higher antioxidant activity ($p = 0.037$). Similarly, acetone extracts exhibited significantly lower IC_{50} values than methanol extracts ($p = 0.005$), reflecting

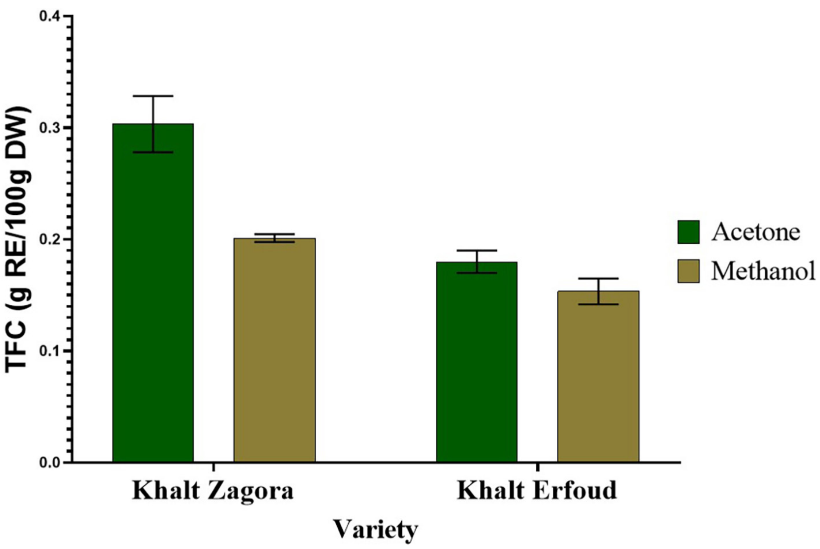

Figure 3. Total flavonoid concentration in seed extracts derived from two date palm cultivars (Khalt Zagora and Khalt Erfoud)

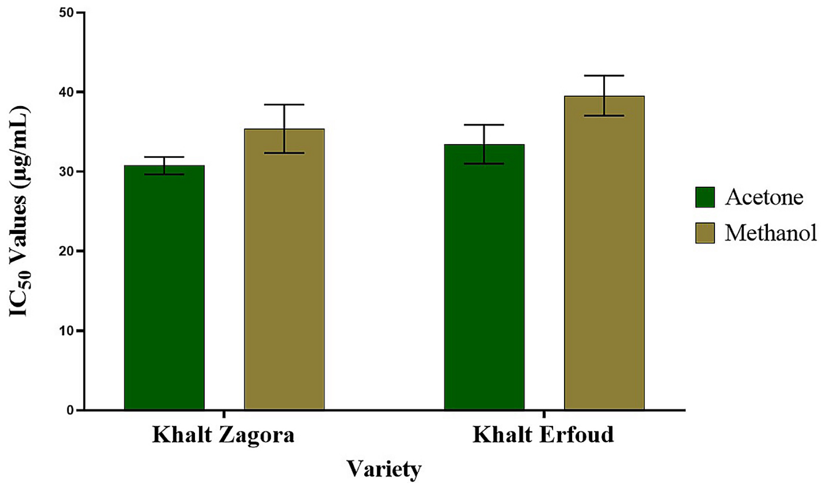

Figure 4. Radical scavenging capacity of seed extracts from two date palm cultivars (Khalt Zagora and Khalt Erfoud), evaluated via the DPPH method and presented as IC_{50} levels

greater antioxidant efficiency. Combined analysis indicated that the lowest IC_{50} values, corresponding to the highest antioxidant activity, were obtained with acetone extracts from Zagora, whereas the highest values were observed for methanol extracts from Erfoud. Overall, acetone yielded higher antioxidant activity than methanol in both regions, and Zagora samples consistently exhibited greater activity than those from Erfoud. These findings indicate that antioxidant activity is primarily influenced by the extraction solvent and, to a lesser extent, by geographic origin, with no interaction between the two factors. The antioxidant activity demonstrated by our extracts is generally consistent with prior investigations. Djaoudene et al. (2019) previously published IC_{50} data falling between 37.3 and 68.0 µg/mL. Separately, Bouhlali et al. (2015) determined IC50 levels in the 122−166 µg/mL range, while Ourradi et al. (2020) reported values ranging from 179.6 to 235.72 µg/mL, whereas Ouamnina et al. (2024) recorded markedly lower values for Moroccan dates (13.190–54.830 µg/mL), suggesting relatively higher antioxidant activity in the latter. Furthermore, Bouhlali et al. (2015; 2017) documented wide variations between tissues: IC_{50} figures of 5.250 µg/mL for pulp and 133 µg/mL for Majhoul seeds; 4,790 µg/mL for pulp and 122 µg/mL for Bousthammi seeds; and 342 µg/mL for pulp and 166 µg/mL for Boufgous seeds. Similarly, Anwar et al. (2022) reported IC50 measures of 1,272.61 µg/mL for seeds and 1,580.36 µg/mL for pulp, whereas Salamon-Torres et al. (2019) established 79 µg/mL for pulp and 4.6 µg/mL for seeds. Taken together, these data indicate that date seed kernels possess a substantially greater concentration of antioxidant components compared to the date fruit flesh. This finding underscores the seeds' viability as a potent source of naturally occurring antioxidants.

The free radical scavenging potential of the seed extracts, as evaluated by the ABTS assay, is summarized in Figure 5. The IC_{50} levels for the extracts spanned from 208.8±14.47 to 336.7±5.1 µg/mL, a span consistently exceeding the value of the Trolox standard (IC_{50} =5 µg/mL). Two-way ANOVA revealed a highly significant model (F = 155.295; $p < 0.001$), explaining 98.3% of the variance in antioxidant activity measured by ABTS. Significant effects of solvent (F = 10.194; $p = 0.013$; $\eta^2 = 0.560$) and a very highly significant effect of region (F = 403.866; $p < 0.001$; $\eta^2 = 0.981$) were observed, indicating that geographic origin is the dominant factor. A significant interaction between these factors was also detected (F = 51.826; $p < 0.001$; $\eta^2 = 0.866$), suggesting that the effect of the solvent depends on the region. Estimated means showed that extracts from Zagora exhibited significantly lower ABTS IC_{50} values than those from Erfoud, indicating higher antioxidant activity ($p < 0.001$). In addition, acetone extracts showed significantly lower IC_{50} values than methanol extracts ($p = 0.013$), reflecting greater antioxidant efficiency. The combined analysis highlighted marked variations depending on the solvent–region combination. In Zagora, acetone extraction yielded higher antioxidant activity than methanol, while in Erfoud, methanol showed slightly higher activity than acetone. These findings confirm that ABTS antioxidant activity is strongly influenced by both geographic origin and extraction solvent, with an interaction-dependent effect. These IC_{50} values exceeded those reported by Djaoudene et al. (2019) (13.80–32.31 µg/mL) and Ourradi et al. (2022) (48.55–68.21 µg/mL). When comparing the antioxidant activity of date seeds and pulp, seeds consistently exhibited higher activity. Salamón-Torres et al. (2019) recorded an IC_{50} of 238 µg/mL for seeds and 13,720 µg/mL for pulp. Similarly, Bouhlali et al. (2015; 2017) reported the following IC_{50} values for seeds and pulp: Majhoul – 5.287 and 0.553 mmol TE/100 g DW, respectively; Bousthammi – 8.021 and 0.619 mmol TE/100 g DW; and Boufgous – 4.807 and 0.564 mmol TE/100 g DW. These results confirm that date seeds are richer in antioxidant compounds than the pulp, highlighting their potential as a valuable natural source of antioxidants.

The outcomes of the β-carotene/linoleic acid test, presented in Table 1 and Figure 6, demonstrate that the date seed extracts effectively blocked lipid peroxidation (LPI %). The LPI percentages spanned from 29.74±0.35% to 69.63±0.22%, a range below the 85.9% inhibition recorded by the ascorbic acid positive standard. Two-way ANOVA revealed a highly significant model (F = 106.439; $p < 0.001$), explaining 97.6% of the variance in antioxidant activity measured by the β-carotene assay ($R^2 = 0.976$). Highly significant effects of solvent (F = 141.569; $p < 0.001$; $\eta^2 = 0.947$) and region (F = 176.880; $p < 0.001$; $\eta^2 = 0.957$) were observed, indicating that both factors strongly influence antioxidant activity. No significant interaction between solvent and region was detected ($p = 0.379$), suggesting independent effects. Estimated means showed that samples from Zagora exhibited significantly lower IC_{50} values than those from Erfoud ($p < 0.001$), indicating higher antioxidant activity. Similarly, acetone extracts displayed lower IC_{50} values than methanol extracts ($p < 0.001$), reflecting greater antioxidant efficiency. The combined analysis revealed that the highest antioxidant activity was obtained with acetone extracts from Zagora (IC_{50} = 144.5 ± 12.11), whereas the lowest activity corresponded to acetone extracts from Erfoud (IC_{50} = 491.3 ± 34.18). Overall, Zagora samples consistently showed greater activity than those from Erfoud. These findings confirm that antioxidant activity measured by the β-carotene assay is strongly influenced by geographic origin and extraction solvent, with no interaction between these factors. These findings are consistent with previous studies. Ourradi et al. (2022) reported LPI % values ranging from 36.13 ± 1.45 to 67.88 ± 1.86%. Similarly, Salamón-Torres et al. (2019) reported antioxidant activities of 65.5% for the pulp and 47.75% for the seeds of Medjool dates.

A head-to-head comparison of the three in vitro assays used to quantify the radical scavenging capacity of date palm seeds was performed. The DPPH method yielded the superior activity, with IC_{50} concentrations extending from 30.77 to 39.57 µg/mL. In contrast, the ABTS assay provided IC_{50} readings between 208.8 and 336.7 µg/mL. Conversely, the β-carotene bleaching assay exhibited the lowest effectiveness, with IC_{50} data spanning 144.5 to 491.3 µg/mL. For comparison, Ouarradi

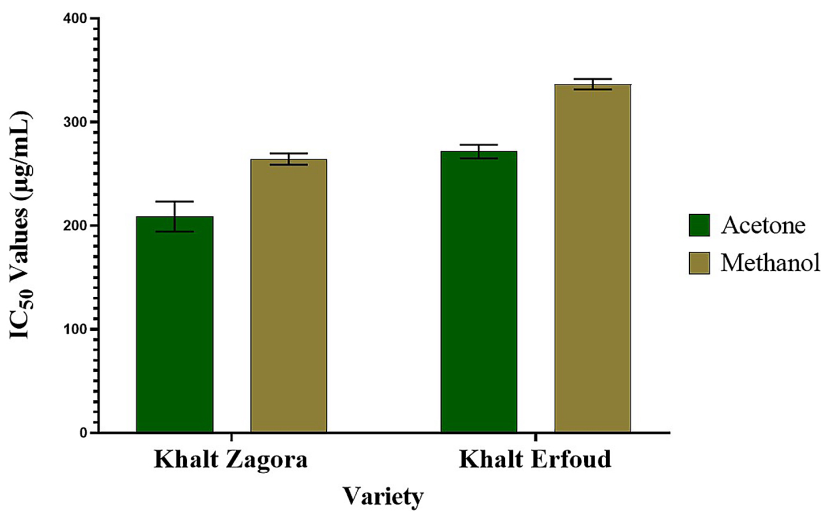

Figure 5. Free radical scavenging potential of the two date palm varieties (Khalt Zagora and Khalt Erfoud) as evaluated by the ABTS method, with results displayed as IC_{50} level

Table 1. Antioxidant capacity of date seeds (*Phoenix dactylifera* L.) evaluated by the β-carotene bleaching method

Extracts	Percentage of lipid peroxidation inhibition (%)
Khalt A acetone extract	38.01±4.50
Khalt A methanol extract	29.74±0.35
Khalt Z methanol extract	50.03±4.92
Khalt Z acetone extract	69.63±0.22

et al. (2022) reported IC_{50} values of 48.55 to 68.21 µg/mL for the ABTS test and 179.66 to 235.72 µg/mL for the DPPH assay, while the β-carotene assay results ranged from 36.13 % to 67.88 % LPI. Similarly, Salamón-Torres et al. (2019) observed IC_{50} values of 0.0045 mg/mL (DPPH) and 0.238 mg/mL (ABTS), with 47.75% inhibition in the β-carotene assay. Additionally, Bouhlali et al. (2015) reported ABTS values between 4.807 and 8.021 mmol TE/100 g DW, whereas DPPH IC_{50} values ranged from 0.112 to 0.166 g/L. Overall, these results indicate that the DPPH test is the most sensitive method for detecting antioxidant activity in date seed extracts, followed by the ABTS test, while the β-carotene test generally produces lower activity values.

Antimicrobial activity

Antimicrobial activity was evaluated using a panel of four clinical strains, including two Gram-positive bacteria (*S. aureus* and *S. epidermidis*) and two Gram-negative bacteria (*E. coli* and *S. typhi*). As shown in Figures 7 and 8, the disk diffusion test revealed variability in inhibition zones depending on the bacterial species, the geographic origin of the samples, and the extraction solvent used. The Gram-positive bacteria showed moderate susceptibility, with inhibition zone diameters ranging from 10.31 ± 0.19 mm to 14.13 ± 0.38 mm, while the Gram-negative bacteria were found to be completely resistant to the tested extracts. However, the inhibition zones obtained remain smaller than those observed with the reference antibiotics, ciprofloxacin and amoxicillin-clavulanic acid (30 µg), whose diameters ranged from 13 mm to 28.75 mm. A comparative analysis of antibacterial activity against *Staphylococcus aureus* and *Staphylococcus epidermidis* revealed consistent patterns across both strains. Two-way ANOVA showed that the models were highly significant ($p < 0.001$ and $p = 0.001$, respectively), with geographic origin emerging as the primary driver of variability ($p < 0.001$ for both), followed by a secondary but significant effect of the extraction solvent ($p = 0.036$ and $p = 0.004$, respectively). No significant interaction between factors was detected ($p > 0.05$), indicating independent effects. Across both bacterial strains, extracts from Zagora consistently exhibited greater antibacterial activity than those from Erfoud ($p < 0.001$), while acetone extracts outperformed methanol extracts. The highest inhibitory effects were observed for acetone extracts from Zagora, whereas the lowest were recorded for methanol extracts from Erfoud. Notably, *S. aureus* displayed higher susceptibility compared to *S. epidermidis*, as reflected by larger inhibition zones, suggesting a differential sensitivity of the

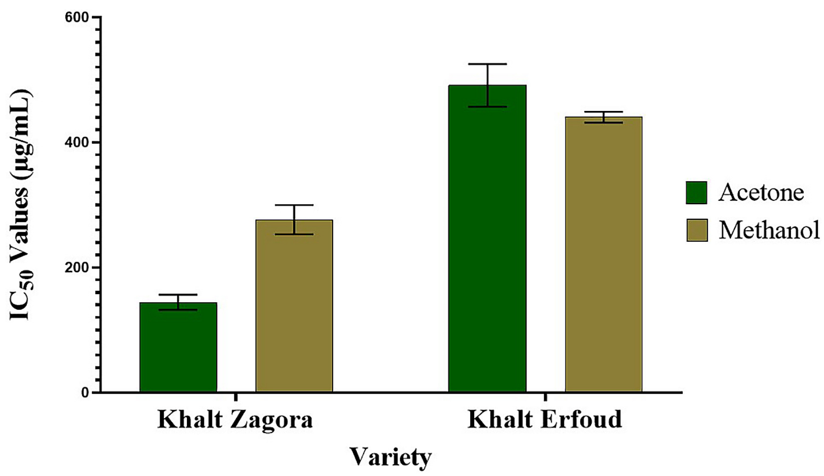

Figure 6. Antioxidant activity of seed extracts from two date palm clones, measured by the β-carotene test, expressed as percentage lipid peroxidation inhibition (LPI %)

strains to the bioactive compounds. Overall, these findings highlight the predominant role of geographic origin, with a secondary contribution of solvent type, in shaping the antibacterial potential of the extracts, independently of their interaction.

Our results are consistent with those of Radfar et al. (2019), who reported significant antibacterial activity of extracts from four different varieties of date seeds against *S. aureus*, but no effect on *E. coli* (O157:H7). In a similar study, Qadoos et al. (2017) found that extracts from date fruits and leaves were effective against Gram-positive bacteria, including *S. aureus* and *B. subtilis*, but had no inhibitory effect on Gram-negative strains such as *E. coli* and *P. aeruginosa*. In contrast, a more recent study reported remarkable antibacterial properties of methanolic date seed extracts against various human pathogenic microorganisms, with the highest inhibition zone diameter (IZD) of 17.6 mm recorded for *E. coli* and the lowest, 14.3 mm, for *B. subtilis* (Barakat et al., 2020). Similarly, Abuelgassim et al. (2020) found that methanolic extracts from Khalas and Sukkari varieties exhibited significantly higher inhibitory activity against Gram-positive bacteria (IZDs: 20–28 mm) compared to Gram-negative bacteria (IZDs: 13–20 mm). In another study, Gomaa et al. (2024) showed that ethanolic extracts of *Phoenix dactylifera* L. kernels displayed notable antimicrobial activity, with the highest IZD observed against *S. aureus* (35 mm) and the lowest against *S. typhi* (19 mm). The differences observed in terms of sensitivity between Gram-positive and Gram-negative bacteria can be attributed to variations in cell wall structure. Gram-positive bacteria have a thick layer of peptidoglycan surrounding the cytoplasm, while Gram-negative bacteria have an additional outer membrane rich in lipopolysaccharides (Alzoreky et al., 2003; Fattouch et al., 2007). The antimicrobial effects of phenolic compounds on G-positive bacteria are related to their ability to interact with the peptidoglycan layer, disrupting cell integrity and increasing vulnerability to osmotic and ionic stress (Papuc et al., 2017) (Table 3).

Table 2 presents the results of the minimum inhibitory concentration and minimum bactericidal concentration assays. The strongest antibacterial activity against *Staphylococcus aureus* was observed with the acetone extract of Khalt Z, which exhibited MIC and MBC values of 0.23 mg/mL and 0.47 mg/mL, respectively. In contrast, the methanolic extract of Khalt Z, as well as the acetone and methanolic extracts of Khalt E, showed higher values, with MICs ranging from 0.47 to 0.94 mg/mL and MBCs from 0.94 to 1.88 mg/mL. For *Staphylococcus epidermidis*, all extracts exhibited MIC and MBC values of 0.47 mg/mL and 0.94 mg/mL, respectively. These findings align with reports in the literature. Gomaa et al. (2024) found that *P. dactylifera* kernel extracts displayed MIC/MBC measurements between 0.25 and 1.0 mg/mL when tested against various bacterial species (*S. aureus*, *E. coli*, *B. cereus*, *E. faecalis*, *S. typhi*, and *S. sonnei*). Likewise, Abuelgassim et al. (2020) cited MIC readings between 0.25 and 0.75 mg/mL for Khalas and Sukkari pit extracts against *S. aureus* and *B. subtilis*.

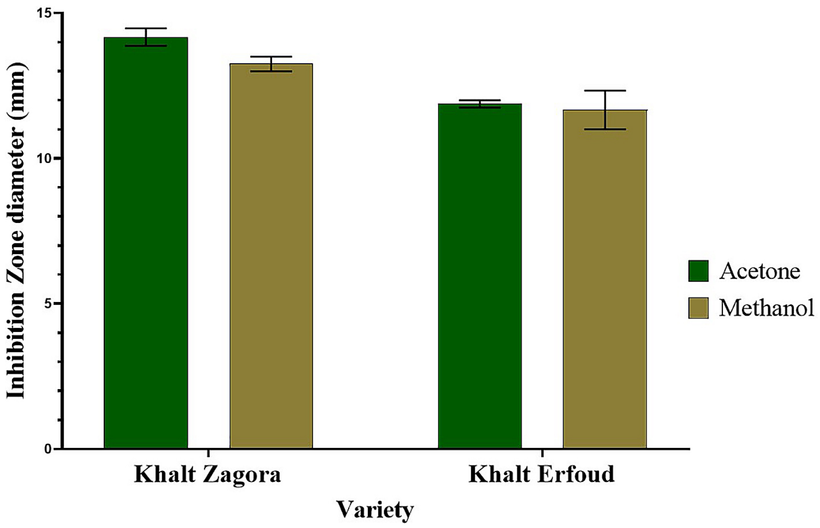

Figure 7. Antimicrobial efficacy of date palm seed extracts against *S. aureus*, quantified by the diameter of the inhibition zones (mm)

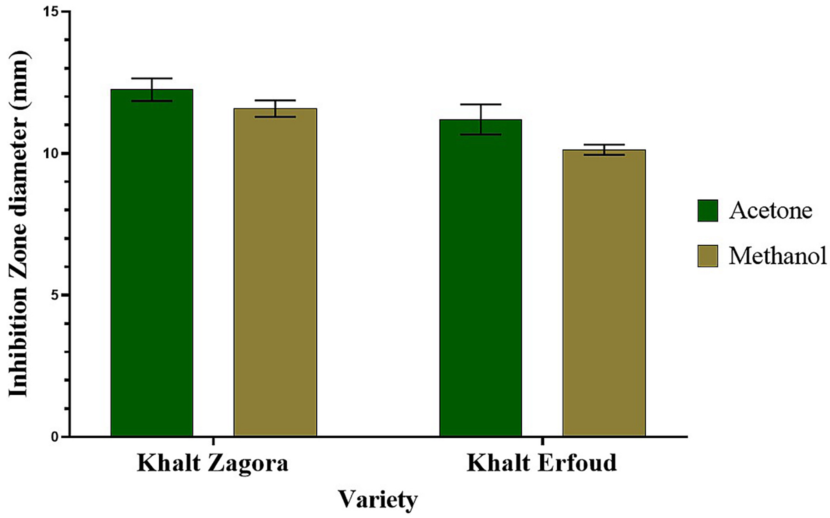

Figure 8. Efficacy of date palm seed extracts in inhibiting *S. epidermidis*, quantified by the diameter of the zone of inhibition (mm)

Table 2. Determination of MIC and MBC (mg/mL) of date seed extracts (*Phoenix dactylifera* L.) against *S.aureus* and *S.epidermidis*

Bacteria	Solvants/Variaty	Khalt Z		Khalt E	
		MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
S. aureus	Methanol	0.47 ± 0.00	0.94 ± 0.00	0.47 ± 0.00	0.94 ± 0.00
	Acetone	0.23 ± 0.00	0.47 ± 0.00	0.47 ± 0.00	0.94 ± 0.00
S. epidermidis	Methanol	0.47 ± 0.00	0.94 ± 0.00	0.47 ± 0.00	0.94 ± 0.00
	Acetone	0.47 ± 0.00	0.94 ± 0.00	0.94 ± 0.00	1.88 ± 0.00

Note: Results are shown as the average ± standard error (n = 3).

Table 3. Antibacterial activity of standard antibiotics against Gram$^+$ and Gram$^-$ bacteria

Inhibition zone diameter (mm)				
	S. aureus	*S. epidermidis*	*E.coli*	*S.thyphimirum*
Ciprofloxacin 5 ug	21.5	24.5	-	18.25
Amoxycillin + Clavulanate 30 µg	28.75	13.00	19.00	19.5

Radfar et al. (2019) also documented MIC and MBC data for ethanol extracts across four date seed types, spanning 1.56 to 3.125 mg/mL and 3.125 to 12.5 mg/mL, respectively, against *S. aureus*. Furthermore, Elkahoui et al. (2024) published methanol extract MIC and MBC figures of 1.4 and 5.6 mg/mL, respectively, for *P. dactylifera* against *S. aureus*. Conversely, higher MIC and MBC values have been reported in other studies compared to those observed in this study. Adedayo et al. (2020) for example, recorded 20 mg/mL and 80 mg/mL for aqueous and ethanolic *P. dactylifera* seed extracts, while Olaniyi et al. (2022) observed MIC/MBC concentrations that varied from 50 to 200 mg/mL against foodborne pathogens. This broad disparity is typically attributed to methodological factors (e.g., extraction solvent, concentration technique, microbiological assay modifications) and biological variability (e.g., date cultivar, maturity stage, and geographical origin). Similarly, according to Hussain et al. (2019) the MIC values of date kernel extracts prepared with different organic solvents ranged from 2.5 to 10 mg/mL when tested against *S. aureus* and *E. coli*. Numerous studies have highlighted the antibacterial properties of phenolic compounds in date extracts (Amensour et al., 2009; Ahmed et al., 2015). For instance, Selim et al. (2021) evaluated the antimicrobial effects of phenolic acids extracted from date pits against four bacterial strains. Their results showed that gallic acid was effective against Gram-positive and Gram-negative bacteria, producing inhibition zones of 11 to 22 mm and MIC/MBC values between 0.25 and 0.5 mg/mL. Similarly, Barkat et al. (2020) reported that ethanolic extracts of date seeds, rich in phenolic compounds, exhibited strong antimicrobial activity, with inhibition zones between 16 and 21 mm. In addition, date seed extracts showed particularly low MIC values, ranging from 1.1 to 1.7 mg/mL. Variations in the antimicrobial activity of date extracts can be attributed to differences in phenolic compound content, date varieties, extraction methods, solvents used, and geographical origin.

Antibiofilm activity of date palm seed extracts

Biofilm formation is a complex and dynamic process that occurs through several successive stages. The initial phase involves the adhesion of bacterial cells to a surface, marking the onset of biofilm development and the formation of a primary cellular layer. This stage is followed by cell–cell interactions leading to an accumulation phase, during which bacteria proliferate and organize into microcolonies. These microcolonies progressively develop into a multilayered three-dimensional structure, resulting in the formation of a mature and structured biofilm (Mack, 1999; Qu, 2016; Jing, 2017). In this context, the antibiofilm activity of date palm kernel extracts was evaluated by measuring their effect on the biofilm biomass of the studied bacteria. Overall, all extracts induced a significant reduction in biofilm formation compared to the untreated control, with inhibition rates ranging from 6.47 ± 0.5% to 72.17 ± 2.50%. Three-way analysis of variance (ANOVA) revealed that the model was highly significant ($p < 0.001$), with extract concentration identified as the main factor influencing antibiofilm activity. A progressive and significant decrease in activity was observed with decreasing concentration, confirming a strong dose-dependent effect. Furthermore, a significant effect of geographical origin was observed, with samples from Erfoud exhibiting higher antibiofilm activity than those from Zagora, whereas the type of solvent did not show a significant influence ($p = 0.065$). No significant interaction effects were detected among the factors, indicating that the effect of concentration remained consistent regardless of solvent type and geographic origin. Several studies have demonstrated the ability of various phytochemicals to inhibit the formation and maturation of bacterial biofilms (Packiavathy et al., 2014; Al-Monofy et al., 2025; Liu et al., 2017). Nevertheless, research on the anti-biofilm activity of date pit extracts remains limited. The results of the present study suggest that date pit extracts could be promising candidates for inhibiting the formation of bacterial biofilms. In this context, Qasim et al. (2020) evaluated the anti-biofilm activity of pulp extracts from two date varieties, Ajwa and Khalas, against *Pasteurella multocida* and *Bacillus subtilis*. The authors reported inhibition rates ranging from 30.55 ± 0.09% to 53.78 ± 0.16% for the Ajwa variety and from 28.34 ± 0.07% to 42.01 ± 0.04% for the Khalas variety. Furthermore, Gomaa et al. (2024) also demonstrated the anti-biofilm efficacy of date pit extracts against various pathogenic bacteria. At a concentration of 1000 μg/mL, high inhibition rates were observed, reaching 88.00%, 87.69%, and 74.46% against *Salmonella typhi*, *Shigella sonnei*, and *Escherichia coli*, respectively (Figure 9).

Correlation between different variables

To assess the influence of different compounds particularly total phenols and flavonoids on the antioxidant, antimicrobial, and anti-biofilm activities of date pit extracts, Pearson's correlation coefficients were calculated (Table 4).

The matrix uses the following abbreviations and their corresponding definitions: TPC: total phenolic compound content; PFC: total flavonoid content; IC_{50} DPPH: 50% inhibitory concentration obtained by the DPPH test; IC_{50} ABTS: IC_{50} measurement determined by the ABTS procedure; IC_{50} β-carotene: IC_{50} data from the β-carotene method; *S.a* IZD: diameter of the growth inhibition zone for *S. aureus*; *S.e* IZD: diameter of the growth inhibition zone for *S. epidermidis; S.a* Antibiofilm activity (MIC): Antibiofilm effect of the MIC concentration against *S. aureus*; *S.a* Antibiofilm activity (MIC/2): Antibiofilm effect of the MIC/2 concentration against *S. aureus*. *S.a* Antibiofilm activity (MIC/4): Antibiofilm effect of the MIC/4 concentration against *S. aureus*. *S.a* Antibiofilm activity (MIC/8): Antibiofilm effect of the MIC/8 concentration against *S. aureus*. *S.e* Antibiofilm activity (MIC): Antibiofilm effect of

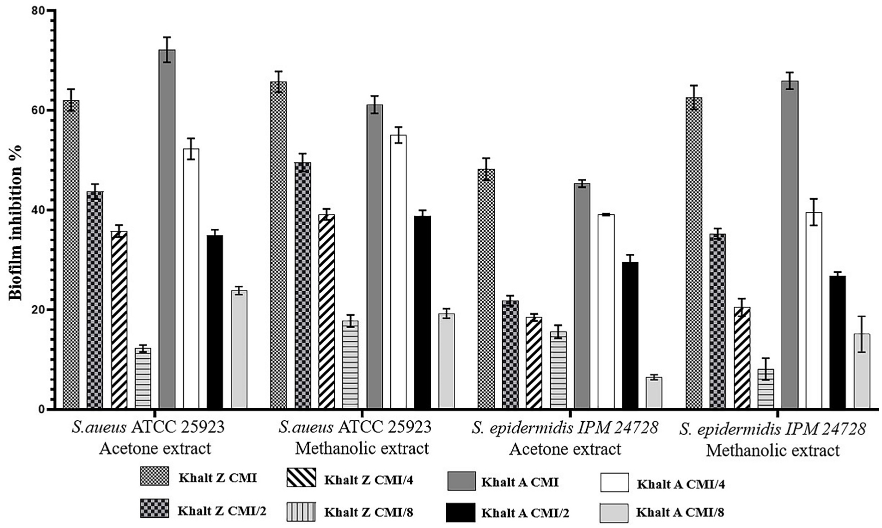

Figure 9. Inhibitory effect of date seed extracts on biofilm formation in *Staphylococcus aureus* and *Staphylococcus epidermidis*

Table 4. Pearson's correlation coefficients between biochemical parameters and the antimicrobial, antioxidant, and antibiofilm activities of Moroccan date palm (*Phoenix dactylifera* L.) pits

Parameter	Yield	TPC	TFC	IC_{50} DPPH	IC_{50} β-carotene	IC_{50} ABTS	*S.a* IZD (mm)	*S.e* IZD (mm)	*S.a* Antibiofilm activity (CMI)	*S.a* Antibiofilm activity (CMI/2)	*S.a* Antibiofilm activity (CMI/4)	*S.a* Antibiofilm activity (CMI/8)	*S.e* Antibiofilm activity (CMI)	*S.e* Antibiofilm activity (CMI/2)	*S.e* Antibiofilm activity (CMI/4)	*S.e* Antibiofilm activity (CMI/8)
Yield	1															
TPC	,891	1														
TFC	,801	,962*	1													
IC_{50} DPPH	-,595	-,882	-,852	1												
IC_{50} β-carotene	-,945	-,893	-,892	,584	1											
IC_{50} ABTS	-,791	-,976*	-,930	,962*	,774	1										
S.a IZD (mm)	,966*	,961*	,928	-,718	-,979*	-,879	1									
S.e IZD (mm)	,852	,979*	,901	-,925	-,798	-,990*	,903	1								
S.a Antibiofilm activity (CMI)	-,300	-,106	-,259	-,282	,516	-,090	-,331	,092	1							
S.a Antibiofilm activity (CMI/2)	-,879	-,993**	-,986*	,857	,918	,956*	-,967*	-,949	,204	1						
S.a Antibiofilm activity (CMI/4)	,091	-,363	-,418	,744	-,034	,537	-,104	-,435	-,539	,351	1					
S.a Antibiofilm activity (CMI/8)	-,804	-,744	-,819	,402	,949	,595	-,870	-,597	,743	,804	-,125	1				
S.e Antibiofilm activity (CMI)	-,084	-,508	-,524	,851	,100	,674	-,255	-,591	-,562	,483	,980*	-,041	1			
S.e Antibiofilm activity (CMI/2)	-,793	-,920	-,986*	,756	,921	,858	-,922	-,827	,414	,960*	,316	,895	,408	1		
S.e Antibiofilm activity (CMI/4)	-,962*	-,817	-,785	,450	,979*	,675	-,944	-,725	,549	,835	-,222	,922	-,076	,822	1	
S.e Antibiofilm activity (CMI/8)	,142	,137	,365	,102	-,441	,001	,277	-,063	-,910	-,253	,172	-,700	,240	-,508	-,391	1

Note: * The correlation is significant at the 0.05 level. ** The correlation is significant at the 0.01 level.

the MIC concentration against *S. epidermidis*. *S.e* Antibiofilm activity (MIC/2): Antibiofilm effect of the MIC/2 concentration against *S. epidermidis*. *S.e* Antibiofilm activity (MIC/4): Antibiofilm effect of the MIC/4 concentration against *S. epidermidis*. *S.e* Antibiofilm activity (MIC/8): Antibiofilm effect of the MIC/8 concentration against *S. epidermidis*.

Overall, the results highlight a significant influence of geographic origin and extraction solvent on the biochemical and biological properties of the extracts. Total phenolic content (TPC) and total flavonoid content (TFC) showed a very strong positive correlation with each other (r = 0.962; $p < 0.01$), as well as with extraction yield (r = 0.801–0.891; $p < 0.01$), indicating their major contribution to the chemical richness of the extracts. In addition, significant negative correlations were observed between TPC/TFC and IC_{50} values of antioxidant assays (up to r = −0.976 ; $p < 0.01$), confirming their key role in antioxidant activity. Furthermore, strong positive correlations were found among the different antioxidant assays (r > 0.85), indicating good consistency between the methods used. Regarding antimicrobial activity, inhibition zones against *Staphylococcus aureus* and *Staphylococcus epidermidis* showed strong positive correlations with TPC and TFC (up to r = 0.961; $p < 0.01$), suggesting a major contribution of these compounds to antibacterial activity. In contrast, antibiofilm activity exhibited more complex relationships, with correlations varying depending on concentration and bacterial strain, ranging from strong negative correlations (r = −0.993) to high positive correlations with certain antioxidant parameters (r ≈ 0.858–0.949). These findings suggest that phenolic compounds are not the sole determinants of antibiofilm activity. Overall, these results emphasize the central role of phenolic compounds and flavonoids in antioxidant and antibacterial activities, while highlighting the multifactorial and complex nature of antibiofilm activity, likely involving other bioactive metabolites or synergistic effects.

CONCLUSIONS

The results of this study highlight the high potential of Moroccan date pits (*Phoenix dactylifera* L., Khalt variety) as a rich source of phytochemical compounds, particularly polyphenols and flavonoids. The high content of these compounds confers remarkable antioxidant activity to the extracts, as confirmed by their ability to scavenge free radicals in various in vitro assays (ABTS, DPPH, and β-carotene). In addition, the extracts exhibited significant antimicrobial activity, particularly against Gram-positive bacteria, emphasizing their potential as natural antimicrobial agents. Furthermore, the extracts demonstrated notable antibiofilm activity, although this effect was concentration-dependent and could not be solely attributed to phenolic compounds, suggesting the involvement of other bioactive metabolites or synergistic effects. However, further investigations, including identification of active compounds and in vivo validation, are necessary to support their practical applications.

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