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ORIGINAL ARTICLE

Assessment of polyphenol composition, antioxidant and antimicrobial properties of various extracts of Date Palm Pollen (DPP) from two Tunisian cultivars

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Kill-time assay

Abstract This study aimed to investigate and compare the total phenolic content (TPC) and total flavonoid content (TFC) and antioxidant and antibacterial activities of different extracts recovered from Date Palm Pollen collected from Tunisian cultivars of Kerkennah (DPP-K) and Tozeur (DPP-T) using various solvents, including hexane, chloroform, ethyl acetate, acetone, ethanol and water. The results revealed that DPP-T had higher TPC than DPP-K for all solvent extracts, except for hexane, with a value of 237.74 ± 9.58 mg GAE/g for the water extract. The highest level of TFC (75.10 ± 4.37 mg QE/g) was recorded in the acetone DPP-T extract, which was about twice as high as that of DPP-K. A total of eight phenolic compounds were identified and quantified in both extracts by HPLC, namely gallic acid, catechin, caffeic acid, epicatechin, vanillic acid, coumarin, quercitin and rutin. The antioxidant activities of the extracts were screened using DPPH and β-carotene bleaching assays. The results indicated that the DPP-T acetone extract showed the best DPPH scavenging activity (IC_{50} = 46.56 ± 0.28 μg/ml), with no activity being recorded for DPP-K. The DPP-T extract also showed significant effects in terms of the β-carotene test (28.12 ± 0.04 μg/ml) when compared to BHT as a reference standard. The extracts were screened for antimicrobial activity against 10 bacterial and 7 fungal strains, and the results showed that ethyl acetate DPP-K extract exhibited the strongest activity against *Listeria monocytogenes* and that the *Staphylococcus aureus* strain was most sensitive to DPP-T, with MIC and MBC values of

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0.98 mg/ml and 1.95 mg/ml, respectively. Both DPP-T and DPP-K showed strong inhibition effects on the growth of *oxysporum*. Further time kill assays demonstrated the potency of DPP-K and DPP-T ethyl acetate extracts to inhibit the growth of *L. monocytogenes* and *S. aureus*, respectively. Overall, the findings suggest that DPP could be considered a promising source of new natural antioxidant and antimicrobial agents for use in various food and pharmaceutical products and formulations. This suggested that the DPP extract is a good potential inhibitor of food spoiling microbial growth and could be a highly effective therapeutic choice for human and plant infections.

1. Introduction

Date palm (*Phoenix dactylifera* L.) is a dioecious, monocotyledon species belonging to the family Arecaceae, with male female and flowers occurring on separate plants (Zohary and Hopf, 1993). It is one of the oldest trees cultivated by mankind and is widely distributed in various arid and semi-arid regions of the world, including the Middle East, and North Africa, where it represents a focal point in crop production within the oasis system. It is a multipurpose tree with diverse nutritional, medicinal, socio-economic and ecological values. Several date-palm products, such date fruits and palm juice, have traditionally been used in folk medicine for the treatment of various health diseases and disorders, including memory disturbances, fever, inflammation, paralysis, and loss of consciousness (Al-Qarawi et al., 2004). Date palm fruit extracts were also reported to possess wide range of attractive properties, such as anti-fungal (Shraideh et al., 1998), antibacterial (Saddiq and Bawazir, 2010), antiparasitic (Metwaly et al., 2012), antiviral (Jassim and Naji, 2010), hepatoprotective activities (Saafi et al., 2011), and anti-coccidial, anti-inflammatory and anti-apoptotic activities (Metwaly et al., 2014). The antimicrobial effects of various extracts from different parts of *Phoenix daylifera* from Iran have been studied, and the results showed that this plant, particularly the pollen part, may be used in the treatment of infections, including gram positive bacteria (Shakibaa et al., 2011).

Date Palm Pollen (DPP) is a fine powder material produced by male flowering date palm plants. Plants are pollinated through the transfer of pollen from the stamen of a flower to the stigma of another. Fresh pollen consists of water (5–36%) and solids (64–95%). It contains mineral salts, vitamins, sugars, lipids, growth factors, certain antibacterial activities and over 100 kinds of enzymes and co-factors (Hassan, 2011). Pollen and pollen products have a long history of use in traditional herbal medicine and have been reported to display a wide range of antimicrobial (Baltrusaityte et al., 2007), anti-oxidative (Le-Blanca et al., 2009), anti-inflammatory (Choi, 2007), anti-toxicant (Eraslan et al., 2008), and hepato-protective (Uzbekova et al., 2003) activities. They have also been described to contain concentrations of photochemical and nutrients and to be rich in carotenoids and flavonoids (Broadhurt, 1999). Furthermore, the literature indicates that they are good source of protein, amino acid, vitamins (A, B, C, D, and E), dietary fiber, fatty acid, enzymes, hormones and minerals (Alferz and Campos, 2000). Several previous studies on DPP showed the presence of estrone, α-amirin, triterpenoidal saponins, flavonoids and a crude gonadotrophic substance (Mahran et al., 1976, 1985; Bennet et al., 1996). More recently, DPP has been noted to contain estrone, estradiol and estriol, in addition to five flavonoid compounds (Abbas and Ateya, 2011).

Due to the renewed interest in bioactive plant-derived medicinal compounds, pollen-derived products have gained increasing popularity as dietary supplements in various parts of the world. A variety of pollen-based food products and formulations, such as candy and chocolate bars, are currently marketed and commercialized throughout the world (Stanley and Linkens, 1974). Free radicals have previously been reported to be implicated in the human pathogenesis of at least 50 diseases (Halliwell, 1994; Aruoma, 1998). Accordingly, there has been a growing interest in plant-based dietary components to counteract oxidative stress-induced disease since it is involved in various diseases and may exacerbate their symptoms (Forsberg et al., 2001). Nowadays, much attention has been paid to health promotion related to the activity of photochemical, and increasing attention has been given to the isolation of novel bioactive compounds from medicinal plant as an effective strategy for the treatment of different diseases (Alinezhad et al., 2013; Nabavi et al., 2013).

Despite this large flow of data on the promising properties and attributes of DPP, no studies have so far been performed to explore the antioxidant and antimicrobial prosperities of DPP in various extracts. Accordingly, the present study was undertaken to evaluate and compare, for the first time, the phenolic and flavonoid compounds as well as the antioxidant (DPPH and β-carotene assays) and antimicrobial activities of different DPP extracts from two biotopes growing in Tunisia (Tozeur, designated as DPP-T and Kerkennah referred to as DPP-K). It also aimed to elucidate the possible mode of action responsible for their antimicrobial properties against sensitive strains by kill time analysis.

2. Materials and methods

2.1. Materials

2,2-Diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), linoleic acid, β-carotene, polyoxyethylenesorbitan monolaurate (Tween-40), ascorbic acid, gallic acid, quercitin, Folin–Ciocalteu's and dimethyl sulfoxide (DMSO) were purchased from Sigma (Sigma, France). For antimicrobial tests, Mueller Hinton Agar (MHA) and Potato Dextrose Agar (PDA) were purchased from Bio-Rad (Bio-Rad, France).

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2.2. Plant material

DPP was collected from two regions in Tunisia (Tozeur and Kerkennah) in April 2014. After collection, the pollen was air-dried and ground to fine powder using a grinder. The powdered material was stored at +4 °C until further use. Its botanical identification and authentication were confirmed at the Department of Biology, University of Sfax, Tunisia.

2.3. Extraction of plant material

Samples of powdered plant material (200 g) were extracted twice (800 ml) for 24 h using the following solvents with increasing polarity: hexane, chloroform, ethyl acetate, acetone, ethanol and water. The macerates were, then, filtered through filter paper (Whatman) in a Buchner funnel. The filtered solution was evaporated in a rotary evaporator under vacuum at 45 °C. The dry extract and stock solutions were kept at +4 °C until further analysis.

2.4. Determination of total phenolic and flavonoid contents

The total phenol content of the extracts was analyzed using a slightly modified version of the Folin–Ciocalteu's method as described by Li et al. (2008). The absorbance of the samples was measured at 760 nm, and their total phenol contents were expressed as mg of gallic acid equivalents per g of dry plant extract (mg GAE/g). Total flavonoid contents were quantified spectrophotometrically at 430 nm using the method described by Quettier-Deleu et al. (2000) and expressed in mg of quercetin equivalent per g of dry plant extract (mg QE/g).

2.5. Analysis by high performance liquid chromatography (HPLC)

The phenolic compounds in the DDP samples were analyzed by an HPLC system using a Varian Prostar HPLC equipped with a ternary pump (model Prostar 230) and a Prostar 330 diode array detector. Active compounds were separated on a C-18 reverse phase HPLC column (Varian, 150 mm × 4.6 mm, particle size 5 µm) and elution gradient at 25 °C. Eluent A was pure methanol and eluent B was a 0.05% acetic acid aqueous solution. Gradient conditions were initial = 35% A and 65% B, 30 min = 50% A and 50% B, and 40 min = 90% A and 10% B. The flow rate was 1 ml/min and the injection volume was 20 µl. Gallic acid, catechin, caffeic acids, epicatechin, vanillic acid, coumarin, rutin and quercitin were used as standards. Phenolic and flavonoid content analyses were monitored at 230 nm and 365 nm, respectively.

2.6. Antioxidant activity

2.6.1. DPPH free radical scavenging activity

DPPH free radical scavenging activity was determined according to a slightly modified version of the method of Barros et al. (2007). In brief, 1 ml DPPH (0.1 mM) was dissolved in methanol and added to a 0.5 ml solution of DPP extracts at different concentrations. Samples were kept in the dark for 30 min at room temperature, and decrease in absorption was measured at 517 nm against a blank using a spectrophotometer (Bio-Rad SmartSpecTM plus). The blank was prepared from the reaction mixture without DPPH solution. BHT and vitamin E were used as positive controls. The experiment was performed in triplicate.

The decrease in the absorption of the DPPH solution was calculated by the following equation:

$$\text{DPPH radical scavenging activity } (\%) = \left[\frac{A0 - A1}{A0}\right] \times 100$$

where $A0$ refers to the absorbance of the control reaction containing all reagents except the tested compound, and $A1$ to the absorbance of the test compound.

The results were expressed as IC_{50} (µg/ml) values, referring the amount of sample necessary to decrease the absorbance of DPPH radicals by 50%. All measurements were performed in triplicate.

2.6.2. β-carotene–linoleic acid bleaching assay

The antioxidant activity of the extracts under investigation was evaluated using a β-carotene–linoleate model system following a slightly modified version of the method described by Siddhuraju and Becker (2003). In brief, 2 mg of β-carotene was added to 10 ml of chloroform. After that, 20 µl linoleic acid and 200 mg Tween 40 were mixed with 1 ml of the chloroform solution. After evaporation of the chloroform under vacuum at 45 °C, 50 ml oxygenated water was added, and the mixture was vigorously shaken. Four milliliters of aliquot of this mixture and 0.2 ml of each extract at different concentrations or BHT (control antioxidant) were placed in test tubes and mixed thoroughly. The tubes were immediately incubated at 50 °C for 120 min. The oxidation of the β-carotene emulsion was monitored spectrophotometrically by measuring absorbance at 470 nm. The antioxidant activity (Inhibition %) of the samples was calculated using the following equation:

$$AA\ (\%) = \left[1 - \frac{A0 - At}{A'0 - A't}\right] \times 100$$

where $A0$ and $A'0$ refer to the absorbance values measured at zero time for the test sample and control, respectively, and At and $A't$ refer to the corresponding absorbance values of test sample and control measured after incubation for 120 min, respectively. All tests were carried out for three sample replications.

2.7. Antimicrobial screening

2.7.1. Microbial strains and growth conditions

The antibacterial activities of DPP were tested against 10 strains of bacteria. These included Gram-positive bacteria: *Bacillus subtilis* JN 934392, *Bacillus cereus* JN 934390, *Staphylococcus aureus* ATCC 6538, *Enterococcus faecalis*, *Micrococcus luteus*, *Listeria monocytogenes*, and Gram-negative bacteria: *Salmonella enteric serotype* Enteritidis ATCC43972, *Salmonella enteric serotype* Typhimurium, *Escherichia coli* ATCC 25922, and *Klebsiella pneumoniae*. Antifungal activities were tested using four fungal strains: *Aspergillus niger*, *Fusarium phyllophilum* AB587006, *Fusarium* sp. JX391934, *Fusarium oxysporum* AB586994, *Penicillium* sp., *Pythium catenulatum* AY598675, and *Rhizoctonia solani*.

The test bacteria were cultured on Petri dishes containing Mueller Hinton Agar (MHA) and incubated for 18–24 h.

From these dishes, a bacterial culture was prepared in 3 ml MH broth with agitation (200 rpm) for 24 h at 37 °C, except for *Bacillus* species, which were incubated at 30 °C. Optical density read at 625 nm ranged from 0.08 to 0.10, a density equivalent to 10^7 CFU/ml (Mohammedi, 2006).

For fungal strains, growth was carried out at 30 °C for 4 days on Sabouraud agar until mycelia growth covered the entire dishes, from which, a spore suspension was obtained in 10 ml sterile water containing 0.1% Tween 80. Optical density was also adjusted to 0.08–0.10 to obtain a solution equivalent to 10^6 spores/ml (Ben Hsouna et al., 2011).

2.7.2. Antimicrobial activity detection by agar diffusion method

Antibacterial and antifungal activities were detected by the agar well diffusion test using a slightly modified version of the method described by Ben Hsouna et al. (2011). In brief, a cavity (wells) of 6 mm was created in the MHA using a sterile Pasteur pipette. A freshly prepared bacterial suspension or spore solution (100 µl) adjusted to 10^7 CFU/ml for bacteria and 10^6 spores/ml for fungus was inoculated onto the surface of agar plates using a sterile swab. Each well was then filled with 80 µl of each extract (125 mg/ml DMSO). A negative control was carried out simultaneously with the DMSO. The plate was left at +4 °C for 2 h to facilitate the diffusion of the extracts in the agar (Trigui et al., 2013), and then incubated at 37 °C for 24 h for bacterial strains and at 30 °C for 4–7 h for fungal strains. Antimicrobial activity was determined by measuring the diameter of inhibition zone around the well.

2.7.3. Determination of MIC and MFC by micro-dilution well method

The MIC values, representing the lowest extract concentrations that prevented the visible growth of microorganisms, were determined by the method of Gulluce et al. (2007) in a sterile 96-well microplate, with a final volume of 200 µl per well. A stock solution of each extract (125 mg/ml) was prepared in DMSO, which is known to have no strong antimicrobial activity (Gachkar et al., 2007). Twofold serial dilutions of the extracts were prepared in the microplate wells over the range 0.98–125 mg of extract/ml DMSO. Each well was supplemented with 100 µl of each extract dilution, and 10 µl of cell suspension to a final inoculum concentration of 10^7 CFU/ml and 10^6 spores/ml for bacteria or 90 µl of MH broth and PDB broth for fungi. The last well, which contained only bacteria or fungi in the adequate medium without the addition of extract, was considered as a positive growth control. The one containing DMSO without extract was used as a negative control. After content homogenization, the plates were covered with sterile plate covers and incubated for 24 h at 37 °C for bacterial strains and for 3 days at 30 °C for fungal strains.

Microorganism viability assays involved the addition of 25 µl of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2 H-tetrazolium bromide) (0.5 mg/ml sterile distilled water), as an oxidation reduction indicator, to each well and subsequent incubation of the mixture for 30 min at 37 °C. In this assay, the wells involving microbial growth inhibition stay clear after incubation with MTT (Ben Hsouna et al., 2011). The MIC was defined as the lowest concentration of the compounds to inhibit the growth of the microorganisms. The MBC values were interpreted as the highest dilution (lowest concentration) of the sample, which showed clear fluid with no turbidity development and without visible growth of microorganisms after incubation for 48 h at 37 °C (Diao et al., 2014; Mighri et al., 2010).

Minimum fungicidal concentrations (MFC) were defined by the first wells with no visible growth and determined by serial subcultivation of 10 µl in Potatoes dextrose agar (PDA) plates and incubation for 3–4 days at 30 °C. The MFC was considered as the lowest concentration that prevented mycelium growth (Zarrin et al., 2010).

2.8. Kill-time analysis

The bactericidal effects of the extracts were investigated by the kill-time curve assay method according to the technique described by Joray et al. (2011). This involved the monitoring of the decrease of bacterial cell growth, due to the effect of a definite extract concentration, over time. Accordingly, a standardized bacterial suspension, containing 10^6–5×10^6 CFU/ml, was prepared and inoculated with four extract concentrations (MIC, 2MIC, 4MIC and 8MIC). The cultivation with the extracts was performed using the same procedures described for MIC assays above, and controls containing only DMSO were simultaneously run (Joray et al., 2011). The obtained suspension was maintained under shaking at 37 °C. Aliquots of 100 µl of the suspension were withdrawn at different time intervals (0, 10, 20, 40 and 60 min), diluted 10 folds in a sterile physiological saline solution (0.9% NaCl), and spread on the surface of PCA agar. Incubation was then performed at 37 °C for 24 h (Ben Hsouna et al., 2011). A positive control was simultaneously prepared without the addition of extract. Total viable bacterial count was determined as colony forming units, with 10^2 CFU/ml being considered as the lower limit of detection. Each test was performed in duplicate.

2.9. Statistical analysis

All assays were performed in triplicate, and the results were calculated as the mean ± standard deviation. The statistical significance between phenolic content and antioxidant activity values of the extracts was carried out using the Excel program. The differences between measurements were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Total phenolic and flavonoid contents

Polyphenols are plant secondary metabolites and a class of antioxidant agents acting by chelating redox-active metal ions, inactivating lipoxygenase and preventing hydroperoxide conversions into reactive oxyradicals. The total phenolic content (TPC) and total flavonoid content (TFC) of various solvent extracts of DPP-K and DPP-T were investigated. The results presented in Table 1 revealed that DPP-T exhibited higher TPC and TFC than DPP-K for all solvent extracts except hexane. The TPC and TFC values recorded for DPP-T ranged from 5.40 ± 0.87 to 237.74 ± 9.58 mg GAE/g and from 4.67 ± 0.96 to 75.10 ± 4.37 mg QE/g; however, for DPP-K, they ranged from 13.42 ± 0.95 to 197.62 ± 7.41 mg GAE/g and from 3.79 ± 0.26 to 30.85 ± 1.98 mg QE/g for DPP-K,

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Table 1 A total phenolic, flavonoid content and IC_{50} values of the DPPH free radical scavenging and the β-carotene–linoleate model of both Date Palm Pollen extracts (DPP-T and DPP-K). BHT and vitamin E were used as standards.

Extracts	DPP-T				DPP-K			
	Total phenolic (mg GAE/g)	Total flavonoid (mg QE/g)	IC_{50} (μg/ml)		Total phenolic (mg GAE/g)	Total flavonoid (mg QE/g)	IC_{50} (μg/ml)	
			DPPH	β-carotene			DPPH	β-carotene
Hexane	5.40 ± 0.87	4.67 ± 0.96	ND	170.86 ± 0.02	48.29 ± 2.81	8.85 ± 0.83	ND	233.40 ± 0.07
Chloroform	133.14 ± 6.53	7.69 ± 1.13	ND	43.25 ± 0.07	53.53 ± 5.30	3.79 ± 0.26	ND	46.30 ± 0.03
Ethyl acetate	100.36 ± 4.69	69.72 ± 3.76	67.50 ± 0.95	34.50 ± 0.03	31.93 ± 1.62	9.48 ± 1.51	ND	41.23 ± 0.04
Acetone	213.36 ± 5.72	75.10 ± 4.37	46.56 ± 0.28	28.12 ± 0.04	197.62 ± 7.41	30.85 ± 1.98	ND	36.75 ± 0.03
Ethanol	211.11 ± 10.02	22.25 ± 2.86	144.86 ± 0.54	ND	13.42 ± 0.95	4.29 ± 0.31	ND	ND
Aqueous	237.74 ± 9.58	73.59 ± 5.62	534.37 ± 0.05	60.75 ± 0.06	180.04 ± 6.72	27.05 ± 1.84	ND	64.52 ± 0.05
BHT	–	–	32.17 ± 0.42	36.82 ± 0.53	–	–	32.17 ± 0.40	36.82 ± 0.53
Vitamin E	–	–	21.86 ± 0.57	–	–	–	21.86 ± 0.57	–

The data are expressed as mean ± S.D. (n = 3).
(mg GAE/g): mg of gallic acid equivalent per g of dry plant extract.
(mg QE/g): mg of quercitin equivalent per g of dry plant extract.
IC_{50} (μg/ml) values corresponding to the amount of extract required to scavenge 50% of radicals present in the reaction mixture.
–: not tested.
ND: not detected.

respectively. The highest TPC and TFC recorded for both DPP-T and DPP-K were achieved by the aqueous and acetone solvents. The good TPC and TFC extraction yields obtained with acetone can be attributed to its good solubility, low toxicity, medium polarity, and high extraction capacity (Horiuchi et al., 2007).

The results revealed that the DPP-T extracts generally exhibited higher polyphenolic contents than the DPP-K extracts, with this content increasing with the increase in solvent polarity. This behavior could be attributed to the wide range of solubility displayed by the various polar compounds within the DPP solvents, the degree of polymerization of phenols and their interaction, genetic factors, geographical variations, and climatic changes. Overall, the findings indicated that both DPP-T and DPP-K are rich in phenolic and flavonoid contents, which could be the major contributor to their antioxidative properties. These results suggest that both varieties of DPP offer promising sources of beneficial bioactive compounds for human health and nutrition.

3.2. Identification of phenolic compounds

To the authors' knowledge, the present study is the first to investigate and identify the phenolic compounds of DPP from Tunisian origins. Accordingly, the results so far obtained by the Folin–Ciocalteu method needed to be further complemented by HPLC analysis so as to further qualify and quantify the phenolic constituents in the DPP extracts under investigation. The results, expressed as percentage of content, are summarized in Table 2. The findings revealed the presence of eight phenolic compounds, namely gallic acid, catechin, caffeic acid, epicatechin, vanillic acid, coumarin, quercitin and rutin. Those compounds were partially identified by the comparison of their retention times and UV spectra to those of authentic standards analyzed under identical conditions. Quantitative data were determined from their respective calibration curves. The results revealed that those compounds could be classified as phenolic acids and flavonoids. The most common phenolic acids were cinnamic acids (coumarin and caffeic acid) and benzoic acids (gallic and vanillic acids), and the most common flavonoids were epicatechin, catechin, rutin, and quercitin. Phenolic acid contents were noted to vary from one extract to another. The most abundant compounds were recorded for DPP-K, including caffeic acid (84.35%) in the aqueous extract and vanillic acid (56.23%) in the ethanol extract. DPP-T, on the other hand, was richer in phenolic acids, showing higher amounts of coumarin in the ethanol (55.76%), acetone (42.08%) and ethyl acetate (30.50%) extracts and significant amounts of caffeic acid (41.98%) in the acetone extract. Among the flavonoid compounds, epicatechin was the most abundant in both DPP-K and DPP-T independently of the extraction solvent used.

The highest content of phenolic acids recorded in the DPP-K extracts was obtained with ethanol (89.04%) and aqueous (88.61%) solvents, followed by acetone (60.29%) and ethyl acetate (43.37%). However, the highest content of phenolic acids registered in the DPP-T extracts was achieved with acetone (91.71%), followed by the ethanol (66.10%), aqueous (55.95%) and ethyl acetate (43.60%) solvents, respectively. The highest flavonoid contents observed in DPP-K were obtained with the ethyl acetate (57.92%) extract, followed by the acetone (46.26%), ethanol (12.98%) and aqueous (11.98%) extracts, respectively. The DPP-T extracts, however, showed the following order of richness in flavonoid contents: ethyl acetate (56.31%), aqueous (44.25%), ethanol (34.16%) and acetone (8.28%).

The variability in phenolic and flavonoid compounds observed for the DPP-T and DPP-K cultivars could be attributed to several of biological factors, including genotypic and agronomic differences, as well as other edaphic and environmental parameters, such as maturation stages, salinity, temperature, water stress, and light intensity conditions. The results presented in this study are important because they are the first to provide insights on the composition of DPP from Tunisian

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Table 2 Percentage of phenolic compounds in the Date Palm Pollen extracts from Tozeur (DPP-T) and from Kerkennah (DPP-K).

Percentage of the content of polyphenol and flavonoids								
	Ethyl acetate		Acetone		Ethanol		Aqueous	
Phenolic acids	DPP-T	DPP-K	DPP-T	DPP-K	DPP-T	DPP-K	DPP-T	DPP-K
Gallic acid	09.33	13.72	02.73	11.57	05.16	23.17	27.43	00.92
Caffeic acid	00.10	12.00	41.98	25.15	–	09.64	10.36	84.35
Vanillic acid	03.67	03.69	04.92	–	05.08	56.23	05.91	02.10
Coumarin	30.50	13.96	42.08	23.57	55.76	–	12.25	01.24
Total	43.60	43.37	91.71	60.29	66.10	89.04	55.95	88.61
Flavonoids								
Quercitin	00.11	01.21	–	05.35	00.11	–	00.17	00.55
Rutin	–	00.09	–	01.20	00.06	02.02	00.03	00.03
Catechin	03.63	03.46	–	–	04.04	03.06	04.76	–
Epicatechin	52.57	53.16	8.28	39.71	29.95	07.90	39.29	11.40
Total	56.31	57.92	8.28	46.26	34.16	12.98	44.25	11.98

cultivars and on the variability of their phenolic and flavonoid compounds.

3.3. Assessment of antioxidant activities

The antioxidant activities of the DPP extracts were investigated by two complementary colorimetric methods, namely the DPPH and β-carotene bleaching assays and compared to those of butylated hydroxyl toluene (BHT) and vitamin E used as reference standards.

3.3.1. DPPH free radical scavenging activity

The DPPH radical scavenging assay is an easy, rapid, and sensitive method commonly used for the screening and evaluation of the antioxidants activity and free radical scavenging ability of plant extracts. This test provides information on how capable an antioxidant is in inhibiting oxidative cell damages by preventing reactive radical species from attacking key biomolecules, such as lipoproteins, and polyunsaturated fatty acids. The results presented in Table 1 are expressed in IC_{50} values (extract concentrations required to scavenge DPPH radical by 50%); thus, a lower IC_{50} value would reflect greater antioxidant activity of the sample. The findings revealed that all extracts exhibited dose-dependent scavenging effects, with DPP-T showing higher antioxidant activity than DPP-K. Among all extracts, the acetone extract displayed the highest free radical scavenging activity (IC_{50} = 46.56 ± 0.28 μg/ml), followed by ethyl acetate (IC_{50} = 67.50 ± 0.95 μg/ml). The scavenging activities of the other extracts were, however, significantly low when compared to the BHT reference standard (IC_{50} = 32.17 ± 0.42 μg/ml) used at the same dose. These results implied that ethyl acetate and particularly acetone led to the extraction of more compounds, with possibly more antioxidant activity. The differences observed between the DPPH scavenging ability of the DPP-T and DPP-K extracts could presumably be attributed to differences in their polyphenolic contents. As mentioned earlier, the antioxidant activities of the acetone and ethyl acetate extracts could be ascribed to their high of phenolic contents. These findings confirm the earlier obtained data on the DPPH scavenging activity of Egyptian Date Palm Pollen methanolic extract with IC_{50} value of 0.8 μg/ml (Khider et al., 2013).

The antioxidant activity of phenolic compounds is due to the presence of a hydroxyl group which donates protons to free radicals and scavenges them (Fukumoto and Mazza, 2000). It was previously reported that epicatechin, vanillic, coumarin and caffeic acid have a great potential of antioxidant activity (Konyaloglu et al., 2005). Although free phenolic acids exist in lower amounts than the bound ones, they affect antioxidant properties. Epicatechin and catechin, a polyphenol very abundant in tea plants, have been reported to represent a strong radical scavenger and metal chelator in model chemical systems, and these effects correlate well with the present study (Lambert and Elias, 2010)

The diversity observed in chemical composition could present a principal factor in negative synergism when antioxidant results are compared since phenolic compounds and flavonoid aglycones are considered to possess more potent antioxidants than their corresponding glycosides (Gao et al., 1999).

3.3.2. β-carotene–linoleic acid bleaching assay

The antioxidant activities of the DPP extracts were evaluated through the measurement of percent inhibition of peroxidation in a linoleic acid system using the β-carotene bleaching test. The presence of antioxidants is known to avoid the destruction of the β-carotene conjugate system and minimize its oxidation by hydroperoxides (must be neutralized by the presence of antioxidants in the extracts). The results presented in Table 1 show that the DPP-T and DPP-K extracts were able to inhibit lipid peroxidation in a dose-dependent pattern. The concentrations providing 50% inhibition were expressed in IC_{50} values. The antioxidant potential of the DPP-T extracts in terms of inhibition percentages was noted to vary with the extraction solvent according to the following order: acetone > ethyl acetate > BHT > chloroform > aqueous > hexane extracts. However, the antioxidant potential of the DPP-K varied according to the following order: BHT ≈ acetone > ethyl acetate > chloroform > aqueous > hexane. The inhibitory concentrations required for each extract to provide 50% inhibition were compared to BHT and vitamin E as reference

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standards. The results indicated that DPP-T exhibited higher antioxidant activity than DPP-K, with the acetone extract showing the highest rate of activity, which was nearly similar to that of BHT. The variability observed for the antioxidant activities of both DPP extracts could presumably be attributed to differences in extraction procedures, sample processing or drying. During the processing of extracts, some active volatile compounds may have been destroyed or evaporated (Naczk and Shahidi, 2006).

As regards the antioxidant activity of some plants, we demonstrated that the antioxidant activity of DPP could be attributed to the differences in their chemical composition and primarily related to their hydroxylation and methylation patterns, to the presence of many phenolic compounds, such as flavonoids and polyphenols and others molecules such as proteins, ascorbate, β carotene, α-tocopherol and lycopene which enhance the antioxidant activity. Caffeic acid, catechin, and epicatechin have previously been reported for their abilities to provide stronger protective benefits against lipid oxidation, which may be helpful for oxidation-related disease prevention (Liao and Yin, 2000). Overall, these results are of significant importance, given the scarcity of data on the antioxidant activity of DPP in the literature.

3.4. Antimicrobial activities

In the present study, the antimicrobial activities of the DPP-T and DPP-K extracts were screened against 17 microorganisms, including 10 bacteria and 7 fungi, and their potency was qualitatively and quantitatively analyzed by the diameters of the inhibition zones (DDs), minimum inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC), and minimum fungicidal concentrations (MFC). The observed antibacterial activities were classified as follows: sensitive-inhibition zone, >18 mm; intermediate-inhibition zone, 13–17 mm; and resistance-inhibition zone, <13 mm (Okonko et al., 2009), and then compared to the growth inhibition results obtained for the controls (chloramphenicol for bacteria and cyclodextrin for fungi). The findings revealed that DPP-T exhibited higher antimicrobial behavior than DPP-K, with Gram positive bacteria being more susceptible than Gram negative bacteria and fungi except for *F. oxysporum*. The results given in Table 3 show that both DPP-T and DPP-K displayed generally moderate antimicrobial activity against a large panel of microorganisms, particularly Gram-positive bacteria. The largest inhibition zone recorded for DPP-K was produced by the ethyl acetate extract, with values ranging from 14.0 ± 2.0 (*K. pneumonia*) to 20 ± 1.0 mm (*S. aureus*). For DPP-T, the largest inhibition zones were produced by the ethyl acetate and acetone extracts, with values ranging from about 10 ± 1.0 (*K. pneumonia*) to 15.5 ± 0.5 mm (*S. aureus*). Among all microorganisms, *F. oxysporum* was the most sensitive, with DDs values of 42 ± 2.0 mm and 29 ± 1.0 mm being observed for the ethyl acetate extracts of DPP-K and DPP-T, respectively. These values were higher than the positive control (chloramphenicol, 20 ± 2.0 mm). However, the other DPP-K and DPP-T extracts generally showed no activity toward *A. niger*, *F. phyllophilum*, *Penicillium* sp., *P. catenulatum*, *R. solani* and *Fusarium* sp. The antimicrobial activity of DPP extracts could be attributed mainly to the high content of flavonoids, such as quercetin, which was previously reported to offer promising antibacterial activity (Loizzo et al., 2004).

The results obtained in terms of MIC, MBC and MFC values against all tested microorganisms were noted to depend on the extraction solvents and were generally consistent with those recorded for DDs with some slight irregularities (Table 4). The MIC (0.98 mg/ml) and MBC (1.95 mg/ml) values recorded for the DPP-K ethyl acetate extract indicated the significant inhibition growth of *L. monocytogenes*. These results prompted further analyses on the antibacterial properties and mechanisms of action of the ethyl acetate DPP-K extract against *L. monocytogenes* and of the ethyl acetate DPP-T extract against *S. aureus* using kill time assays. Furthermore, and in accordance with the classification reported elsewhere, DPP-K was found to exert a higher bactericidal and fungicidal effect than DPP-T.

The results indicated that the DPP-T and DPP-K extracts had different degrees of antimicrobial activity against the tested bacteria, particularly in terms of their abilities to inhibit the growth of *S. aureus*. The differential sensitivity of Gram-positive and Gram negative bacteria to plant extracts may be explained by the morphological differences between the microorganisms. The higher resistance of Gram-negative bacteria could be attributed to the differences in their cell membranes, since their outer membrane carries the structural lipopolysaccharide components and renders their surfaces highly hydrophilic (Smith-Palmer et al., 1998). This constitutes a selective barrier to the hydrophilic solutes with an exclusion limit of about 600 Da (Nostro et al., 2000). The Gram-positive bacteria should be more susceptible since they have only an outer peptidoglycan layer, which is not an effective permeability barrier and may facilitate the infiltration of hydrophobic compounds (Burt, 2004). According to Khider et al. (2013), Date Palm Pollen from various extracts exhibited antimicrobial activity against several strains, with *S. aureus* and *E. coli* being the most sensitive strains, followed by *L. monocytogenes*, *S. enteritidis* and *P. aeruginosa*.

F. oxysporum is a soil borne fungus, with some strains being pathogenic to plants and difficult to control. Interestingly, the results revealed a potent inhibition activity of DPP-K and DPP-T against *F. oxysporum*, which could be attributed to the richness of DPP in cinnamic derivatives, flavonoids, and gallic acids, previously reported to have potent antifungal activity on *Fusarium* (Sá et al., 2009).

The low to moderate antimicrobial activity observed for the other extracts can be explained by the weaker concentration of minor polarity compounds due to the mode of preparation of the tested extracts that did not contain metabolites, such as unsaturated fatty acids, which have been reported to possess good antimicrobial activity (Nazif, 2002).

3.5. Kill times analysis

3.5.1. Effect of DPP-T ethyl acetate extract on viable counts of *S. aureus*

Time-kill kinetic assays provide more accurate description of the antibacterial activity of antimicrobial agents than MIC assays. Accordingly, the effect of DPP-T ethyl acetate extract on the viable counts of selected bacteria such as *S. aureus* was investigated to further confirm its effect and clarify its

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Table 3 Antimicrobial activities of DPP-T and DPP-K against fungal and bacterial strains.

Strains	Inhibition zones diameter (mm)[a]						
	Extracts	Hexane	Chloroform	Ethyl acetate	Acetone	Ethanol	Aqueous	Chloram[c]
DPP-T								
Gram +								
Bacillus cereus		9.5 ± 0.5	9 ± 1.0	13 ± 1.0	14 ± 0.0	10 ± 0.0	8.5 ± 0.5	26 ± 1.00
Bacillus subtilis		9.5 ± 0.5	9 ± 1.0	14 ± 1.0	14.5 ± 0.5	11 ± 1.0	9.5 ± 0.5	24 ± 0.0
Enterococcus faecalis		9.5 ± 0.5	11.5 ± 0.5	15 ± 0.0	14 ± 0.0	10.5 ± 0.5	9.5 ± 0.5	12 ± 1.0
Staphylococcus aureus		9.5 ± 0.5	9 ± 0.0	15.5 ± 0.5	14.5 ± 0.5	12 ± 0.0	8 ± 0.0	16.5 ± 0.5
Micrococcus luteus		10.5 ± 0.5	10 ± 1.0	15 ± 1.0	13.5 ± 0.5	10 ± 1.0	11.5 ± 1.5	20 ± 2.0
Listeria monocytogenes		–[b]	9 ± 0.0	13 ± 1.0	10 ± 1.0	–	–	12 ± 0.0
Gram –								
Salmonella Enteritidis		9.5 ± 0.5	9 ± 0.0	13 ± 1.0	11.5 ± 0.5	8 ± 0.0	8 ± 0.0	16 ± 0.0
Salmonella Typhimurium		–	8 ± 0.0	12.5 ± 0.5	–	–	8 ± 0.0	17 ± 1.0
Escherichia coli		10 ± 1.0	–	13.5 ± 0.5	12 ± 1.0	10 ± 1.0	8 ± 0.0	23.5 ± 0.5
Klebsiella pneumoniae		–	–	10.5 ± 0.5	10 ± 0.0	–	8 ± 0.0	22 ± 1.0
Fungal strains								Cyclo[d]
Aspergillus niger		–	–	–	–	–	–	17 ± 1.0
Fusarium phyllophilum		–	–	–	–	–	–	14.5 ± 0.5
Penicillium sp.		–	–	–	–	–	–	14 ± 1.0
Fusarium oxysporum		22 ± 2.0	11 ± 1.0	29 ± 0.0	32.5 ± 2.5	24 ± 1.0	10 ± 0.0	20 ± 2.0
Pythium catenulatum		–	–	–	–	–	–	17.5 ± 1.5
Rhizoctonia solani		–	–	–	11 ± 1.0	–	–	15.5 ± 2.0
Fusarium sp.		–	–	–	–	–	–	18 ± 1.5
DPP-K								
Gram +								
Bacillus cereus		8.5 ± 0.5	8 ± 0.0	19.5 ± 1.5	10 ± 1.0	10 ± 0.0	8 ± 0.0	26 ± 1.00
Bacillus subtilis		10.5 ± 0.5	9.5 ± 0.5	18.5 ± 0.5	9.5 ± 0.5	13 ± 0.0	8 ± 0.0	24 ± 0.0
Enterococcus faecalis		10 ± 0.0	8.5 ± 0.5	19.5 ± 0.5	10 ± 1.0	10 ± 1.0	8.5 ± 0.5	12 ± 1.0
Staphylococcus aureus		11.5 ± 0.5	8.5 ± 0.5	20 ± 1.0	13 ± 1.0	14 ± 2.0	8 ± 1.0	16.5 ± 0.5
Micrococcus luteus		12.5 ± 0.5	11.5 ± 0.5	15.5 ± 0.5	14 ± 0.0	10 ± 1.0	8.5 ± 0.5	20 ± 2.0
Listeria monocytogenes		10.5 ± 0.5	8.5 ± 0.5	17 ± 1.0	10.5 ± 0.5	10 ± 0.0	–[b]	12 ± 0.0
Gram –								
Salmonella Enteritidis		11 ± 1.0	8.5 ± 0.5	16 ± 0.0	10.5 ± 0.5	9 ± 1.0	–	16 ± 0.0
Salmonella Typhimurium		–	–	15.5 ± 0.5	10.5 ± 0.5	8 ± 0.0	8 ± 0.0	17 ± 1.0
Escherichia coli		–	–	16 ± 1.0	8 ± 0.0	10.5 ± 0.5	8 ± 0.0	23.5 ± 0.5
Klebsiella pneumoniae		8.5 ± 0.5	8 ± 0.0	14 ± 2.0	9.5 ± 0.5	8 ± 0.0	8 ± 1.0	22 ± 1.0
Fungal strains								Cyclo[d]
Aspergillus niger		–	–	–	–	–	–	17 ± 1.0
Fusarium phyllophilum		–	–	–	–	–	–	14.5 ± 0.5
Penicillium sp.		–	–	–	–	–	–	14 ± 1.0
Fusarium oxysporum		16 ± 1.0	14 ± 0.0	42 ± 2.0	19.5 ± 1.5	23 ± 1.0	18.5 ± 0.5	20 ± 2.0
Pythium catenulatum		–	–	17 ± 1.0	–	–	–	17.5 ± 1.5
Rhizoctonia solani		–	–	13 ± 0.0	–	–	–	15.5 ± 2.0
Fusarium sp.		–	–	14 ± 2.0	–	–	–	18 ± 1.5

The data are expressed as mean ± S.D. (n = 3).
[a] Diameter of inhibition zones of extract including diameter of well 6 mm.
[b] No inhibition.
[c] Chloramphenicol was used as a standard antibiotic at a concentration of 15 µg/well.
[D] Cycloheximide was used as a standard antibiotic at a concentration of 20 µg/well.

mechanism of action. The results presented in Fig. 1 indicate that, compared to the control, the *S. aureus* strain treated with the DPP-T ethyl acetate extract at the MIC and 2MIC values (0.98 and 1.95 mg/ml, respectively) showed a decrease in the number of viable cells over the 60 min period of the test, reaching 3 and 2 log CFU/ml, respectively. At higher concentrations of 4MIC and 8MIC (3.92 and 7.84 mg/ml), no viable cells were observed after 10 min. As a consequence, the bactericidal concentration required for DPP-T to kill *S. aureus* should be less than 3.92 mg/ml (4MIC). The above results confirm the postulation that the antimicrobial substances in the tested species might be destroyed by the peptidoglycan layer of the cell wall, thus suggesting that the mode of action of the DPP-T extracts was cell-wall related (Okemo et al., 2001). Accordingly, DPP-T could be considered a promising candidate for application as natural preservative additive in various food and pharmaceutical products and formulations.

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Table 4 Determination of MIC, MBC and MFC (mg/ml) of DPP-T and DPP-K.

Strains	Extracts	Hexane		Chloroform		Ethyl acetate		Acetone		Ethanol		Aqueous	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
DPP-T													
Gram +													
Bacillus cereus		00.98	7.81	15.62	31.25	7.81	15.62	3.9	15.62	15.62	62.5	–	
Bacillus subtilis		07.81	62.5	15.62	31.25	7.81	15.62	1.95	7.81	15.62	62.5	3.9	15.62
Enterococcus faecalis		00.98	7.81	7.81	31.25	3.9	15.62	1.95	15.62	15.62	62.5	7.81	15.62
Staphylococcus aureus		15.62	62.5	15.62	31.25	0.98	1.95	1.95	7.81	7.81	62.5	–	
Micrococcus luteus		00.98	7.81	15.62	31.25	3.9	15.62	1.95	15.62	15.62	62.5	1.95	15.62
Listeria monocytogenes		–		7.81	31.25	3.9	15.62	1.95	7.81	–		–	
Gram −													
Salmonella Enteritidis		7.81	62.5	15.62	31.25	3.9	7.81	1.95	7.81	–		–	
Salmonella Typhimurium		–		–		3.9	15.62	–		–		–	
Escherichia coli		7.81	62.5	–		7.81	15.62	1.95	7.81	7.81	31.25	–	
Klebsiella pneumoniae		–		–		3.9	15.62	1.95	15.62	–		–	
Fungal strains													
		MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Aspergillus niger		–		–		–		–		–		–	
Fusarium phyllophilum		–		–		–		–		–		–	
Penicillium sp.		–		–		–		–		–		–	
Fusarium oxysporum		0.98	7.81	0.98	7.81	3.9	7.81	7.81	15.62	15.62	31.25	15.62	31.25
Pythium catenulatum		–		–		–		–		–		–	
Rhizoctonia solani		–		–		–		15.62	31.25	–		–	
Fusarium sp.		–		–		–		–		–		–	
DPP-K													
Gram +													
Bacillus cereus		–		–		0.98	3.9	15.62	31.25	7.81	15.62	–	
Bacillus subtilis		1.95	15.62	7.81	31.25	0.98	3.9	15.62	31.25	3.9	7.81	–	
Enterococcus faecalis		1.95	15.62	–		0.98	3.9	15.62	31.25	7.81	15.62	–	
Staphylococcus aureus		1.95	15.62	–		0.98	3.9	7.81	15.62	3.9	15.62	–	
Micrococcus luteus		1.95	15.62	7.81	15.62	0.98	3.9	15.62	31.25	7.81	15.62	–	
Listeria monocytogenes		1.95	15.62	–		0.98	1.95	15.62	31.25	3.9	15.62	–	
Gram −													
Salmonella Enteritidis		3.9	31.25	–		0.98	3.9	7.81	31.25	7.81	15.62	–	
Salmonella Typhimurium		–		–		1.95	3.9	15.62	31.25	–		–	
Escherichia coli		–		–		0.98	3.9	–		15.62	31.25	–	
Klebsiella pneumoniae		–		–		0.98	3.9	15.62	31.25	–		–	
Fungal strains													
		MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Aspergillus niger		–		–		–		–		–		–	
Fusarium phyllophilum		–		–		–		–		–		–	
Penicillium sp.		–		–		–		–		–		–	
Fusarium oxysporum		1.95	3.9	3.9	7.81	7.81	15.62	3.9	7.81	1.95	7.81	1.95	7.81
Pythium catenulatum		–		–		15.62	31.25	–		–		–	
Rhizoctonia solani		–		–		15.62	31.25	–		–		–	
Fusarium sp.		–		–		15.62	31.25	–		–		–	

3.5.2. Effect of DPP-K ethyl acetate extract on viable counts of L. monocytogenes

According to the sensitivity of the tested microorganisms on DPP-K, *L. monocytogenes* was selected as a model to confirm the antibacterial effects of the DPP-K ethyl acetate extract and investigate its mode of action. As shown in Fig. 2, the number of *L. monocytogenes* viable cells decreased progressively from 6.7 to 2.61 log CFU/ml within 60 min after the addition of 0.98 mg/ml (1MIC) of extract. However, no viable cells were detected after 60 min with the 2MIC (1.95 mg/ml) concentration. No viable cells were also detected after 5 min with the addition of higher concentrations of 4MIC and 8MIC (3.92 and 7.84 mg/ml). However, the control (without extract) revealed approximately the same CFU/ml value throughout the 60 min, and no *L. monocytogenes* growth inhibition was observed. As a consequence, the bactericidal concentration required to kill *L. monocytogenes* should be less than 1.95 mg/ml (2MIC).

Taken together, the results showed the effect of time and DPP-K extract on bactericidal behavior. The comparison of the results presented in this work (5 min and 3.92 mg/ml) and the findings previously reported elsewhere for the *T.*

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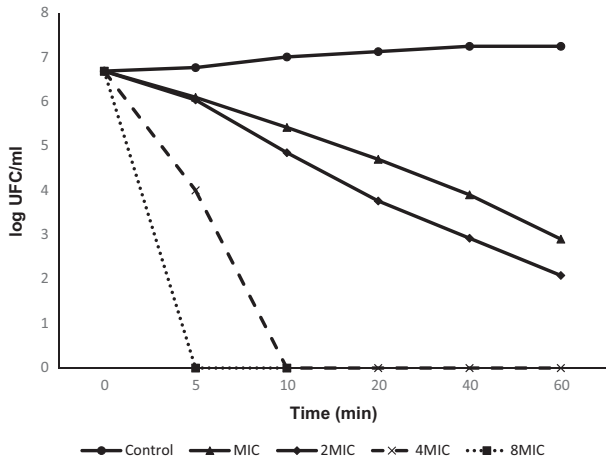

Figure 1 Bactericidal effect of Date Palm pollen ethyl acetate extract from Tozeur on *Staphylococcus aureus* strain. Samples were taken at different incubation times and viability was determined by the plate colony count procedure (CFU: colony forming unit).

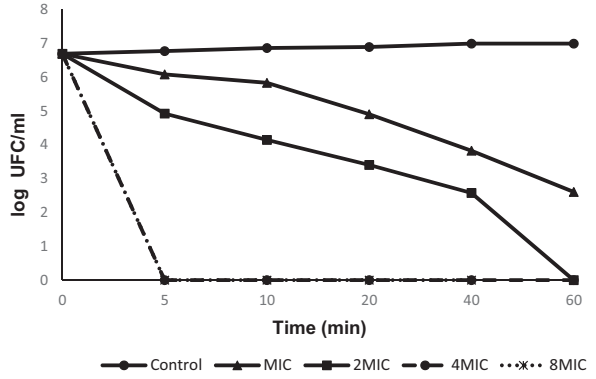

Figure 2 Bactericidal effect of pollen ethyl acetate extract from Kerkennah on *Listeria monocytogenes* strain. Samples were taken at different incubation times and viability was determined by the plate colony count procedure (CFU: colony forming unit).

hirsuta extract (10 min and 7.50 mg/ml) using the same strain pointed to the potent antibacterial effect of the DPP-K extract to inhibit the growth of *L. monocytogenes* (Trigui et al., 2013).

This suggested that the ethyl acetate DPP-K extract is a good potential inhibitor of food spoiling microbial growth and could be a highly effective therapeutic choice for human and plant infections.

4. Conclusion

To the authors' knowledge, this study is the first to report on the chemical composition, antioxidant and antimicrobial properties, and time kill analysis of DPP extracts from two different Tunisian cultivars (DPP-K and DPP-T). The findings clearly indicated that all the tested extracts exhibited significant phenolic and flavonoid contents and displayed good antioxidant and antibacterial activities. The results show also that the ethyl acetate DPP extract can be used as a potent antifungal agent against *F. oxysporum*. Considering the promising properties and activities of DPP, further studies are needed to explore the isolation, identification, and structural characterization of potential bioactive and functional compounds. The results of this study offer a scientific basis that can be further enriched so as to support the efficacy traditionally associated with DPP extracts. This extract might open new promising opportunities for the development of more efficient, safe, and cost-effective food preservatives against food deterioration, antioxidant agents for the alleviation of oxidation-induced cell damages and fungal plant diseases, or natural antimicrobial agents for the control of various pathogens in the food, pharmaceutical industry and new clinically effective antibacterial compounds. We recommend that future studies on DDP extract should focus on the examination of the *in vivo* antioxidant activity, antidiabetic and hypertensive activities.

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