CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITY OF *PISTACIA VERA* L. LEAVES: BENEFICIAL EFFECTS OF FEMALE LEAVES EXTRACT ON FOOD PRODUCTS

MANEL ELAKREMI,[*,***] LEYRE SILLERO,[**] LAZHER AYED,[****] FATEN MANNAI,[*****] RIDHA BEN SALEM,[*] JALEL LABIDI[**] and YOUNES MOUSSAOUI[*,***]

[*]*Organic Chemistry Laboratory (LR17ES08), Faculty of Sciences of Sfax, University of Sfax, Sfax 3029, Tunisia*
[**]*Department of Chemical and Environmental Engineering, Biorefinery Processes Research Group, University of Basque Country (UPV/EHU), Donostia-San Sebastian, Spain*
[***]*Faculty of Sciences of Gafsa, University of Gafsa, Gafsa 2112, Tunisia*
[****]*Laboratory of Applied Fluids Mechanics and Modeling, National School of Engineers of Sfax, University of Sfax, Sfax 3029, Tunisia*
[*****]*Laboratory for Application of Materials to the Environment, Water and Energy (LR21ES15), Faculty of Sciences of Gafsa, University of Gafsa, Tunisia*
✉ *Corresponding author: Y. Moussaoui, y.moussaoui2@gmx.fr*

Received January 5, 2022

The aim of this work was to analyze the chemical composition of two types of leaves (female and male) of *Pistacia vera* L. and estimate their potential use in the bread industry. With that purpose, the total phenolic and flavonoid contents and the biological activities of their extracts were investigated. The extracts expressed high values of phytochemicals, as well as antioxidant and antimicrobial activities. The extract of female plant leaves, having shown the best results, was chosen to fortify flour bread. The fortified bread showed improvement in phytochemicals content and biological activities. The total phenolic content of bread samples varied from 0.09 to 7.96 mg gallic acid equivalent (GAE)/g bread, and the total flavonoid content varied from 0.06 to 5.78 mg catechin equivalent (CE)/g bread. The antioxidant activity of bread was in the range of 0.04-11.08 mg trolox equivalent (TE)/g bread, using DPPH tests. Thus, it was concluded that the extract of *Pistacia vera* L. female plant leaves could be added to baking formulations to improve bread properties and prolong its shelf life.

Keywords: *Pistacia vera*, chemical composition, antioxidant activity, antimicrobial activity, bread additive

INTRODUCTION

Pistacia vera L. (pistachio) is a dioecious tree that can reach heights of 3 to 10 meters.[1] It belongs to the Anacardiacea family, it is native to central and western Asia, and is widely distributed in the Mediterranean region, especially in North Africa.[1–3] *Pistacia vera* L. is cultivated in Tunisia and it is important for the national economy. The most important pistachio producing regions are Gafsa, Sidi Bouzid, Sfax and Kasserine. The region of Gafsa ranks first in the country in terms of plantation area and production. The number of trees in this region is about 2 million 114 thousand plants distributed over an area of 18 thousand 875 ha, which represents 65% of the overall arable land under pistachios in Tunisia. The production of pistachio nuts in the region of Gafsa was 1200 tons in 2019.[4]

Pistachios are used as dried nuts or as an additive in the food industry.[2,5,6] The production of pistachios generates a large amount of potentially valuable waste, such as leaves and husks that have been used as a valuable resource in traditional medicine.[7] Nowadays, scientific papers have confirmed the presence of desirable biological compounds or ingredients in different

parts of *Pistacia* sp., such as kernels, husks and leaves, as well as in its gums and oleoresins.[5,7–9] In addition, it has been demonstrated that the consumption of pistachios reduces the levels of the main risk factors for cardiovascular disease (CVD), prevents the appearance of cancer[10] and lowers LDL cholesterol.[11] Besides the above-mentioned human health benefits, other potential beneficial effects have also been studied. Barreca *et al.*[12] reported the antimicrobial capacity of pistachio shells, likewise, Erson *et al.*[13] and Grace *et al.*[14] have shown that *Pistacia vera* L. husk is provided with antioxidant activity. Pistachio gum has been used in the case of certain diseases of the stomach and the respiratory tract.[9] Additionally, the oleoresin from *Pistacia vera* demonstrated antinociceptive and anti-inflammatory activities.[5,15] In other studies, the extracts of *Pistacia vera* L. leaves have revealed an antiemetic effect in young chickens and a remarkable antioxidant effect.[8,9,16] Furthermore, the essential oils of *Pistacia vera* nuts have been reported to exhibit antimicrobial and antioxidant activities.[7]

Synthetic antioxidants obtained from fossil fuels have been widely used in industrial applications, such as foods, pharmaceuticals and cosmetics. According to Shahid *et al*.,[17] some of these compounds have side effects on human health, as well as on the environment. To decrease these effects, plants are being used to obtain natural bio-compounds, particularly phenolic compounds, as natural antioxidants.[18] Researchers and the food industry are seeking to improve the quality of final food products through an increase in the availability of bioactive compounds in some food preparations, such as bakery products, by adding components with nutraceutical and functional properties in order to obtain a more effective product in terms of nutritional value and longer shelf life. Bread is a basic food product consumed worldwide,[19] which is considered as a source of energy and can bring some nutrients essential for the human body.[20] As far as we know, there are no previous studies concerning bread fortified with extracts of *Pistacia vera* L. leaves.

In order to assess the suitability of leaves of *Pistacia vera* L. as bread additive, the chemical characterization of the leaves was performed. Also, the total phenolic content (TPC), total flavonoid content (TFC), antioxidant and antimicrobial activities of the extracts were determined to assess the potential use of the extracts in the food industry. In addition, the effects of adding extracts of *Pistacia vera* L. female leaves to the formulation of bread products on the characteristics of the latter, including their resistance to bacterial and fungal growth, were assessed.

EXPERIMENTAL

Reagents and chemicals

Ethanol and gallic acid were obtained from Scharlab S.L. Sigma-Aldrich supplied sulfuric acid, trolox, catechin, 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ). All solvents and chemicals were used without further purification.

Raw materials

Mature fresh leaves (male and female) of *Pistacia vera* L., Mateur cultivar, were collected from 15-year-old pistachio trees from a plantation far from pollution in the region of Ouled Ahmed Ben Saad – Gafsa (Tunisia) (34° 33' 45.385" N 8° 51' 29.992" E). The irrigated pistachio trees were planted at a spacing of 7 × 5 m. The region is characterized by an arid climate and an annual precipitation of 162 mm.

After harvesting, the collected raw materials were washed to remove debris and dust, and then they were dried at room temperature for 15 days in the dark. Then, the leaves were dried in an oven at 45 °C for 24 hours. Each type of dry leaves (male plant leaves (ML) and female plant leaves (FL)) were ground to a particle size of less than 0.5 mm and were stored in glass vials protected from light and moisture.

Chemical composition of dried material

The chemical composition of the leaves was analyzed by determining the contents of ash, extractives, acid soluble lignin, Klason lignin and polysaccharides. Each analysis was performed in triplicate. Moisture was evaluated according to the Technical Report of National Renewable Energy Laboratory (NREL) TP510-42621. One g of raw material (male plant leaves and female plant leaves separately) was heated at 105 ± 3°C for 24 h, and the residue was weighed. The ash content was determined according to NREL TP-510-42622. One g of raw material was incinerated at 575± 25 °C overnight and the combustion residue was weighed and reported as ash content of the dry sample.

The content of extractives was determined using sequential Soxhlet extraction of 5 g of each sample with ethanol and distilled water for 24 h for each solvent. After drying the solid residue at 105 °C, the solubilized extractives were determined and reported as a percentage of the original dry sample.

The determination of Klason lignin, acid soluble lignin and carbohydrates contents was carried out according to NREL TP-510-42618. The extractive-free raw material was subjected to acid hydrolysis with

72% sulfuric acid at 30 °C for 1 h, after which water was added to reduce the acid concentration to 4%, and the hydrolysis was completed at 121 °C for 1 h in the autoclave. The mixture was filtrated and the obtained solid residue was dried at 105 °C for 24 h and weighed, to be considered as Klason lignin and reported as a percentage of the original dry sample. Soluble lignin was quantified by measuring the absorbance of the obtained liquid phase at 240 nm using UV–Vis spectroscopy (Jasco V630 UV-Vis Spectrophotometer).

The polysaccharide content of the filtrate was estimated by High Performance Liquid Chromatography (HPLC), using a Jasco LC Net II/ADC chromatograph, equipped with a refractive index detector and an Aminex HPX-87H column, 300 × 7.8 mm (Bio-Rad Laboratories, USA). The mobile phase was H_2SO_4 0.005 M at a flow rate of 0.6 mL/min at 50 °C. Glucose, arabinose, xylose and galacturonic acid were the standard monosaccharides and their retention times were used to estimate the polysaccharide composition. The polysaccharides were reported as a percentage of the original dry mass.

Samples were analyzed by Pyrolysis-Gas chromatography-Mass spectrometry (Py-GC/MS) using a Pyroprobe Pyrolyzer (5150, CDS Analytical Inc, Oxford, PA) connected to an Agilent 6890 gas chromatograph coupled to an Agilent 5973 mass spectrometer (Agilent Technologies Inc., USA). Samples were degraded in an inert atmosphere. The pyrolysis was performed at 500 °C for 10 s (2 °C/ms). The GC oven program started at 50 °C and was held for 2 min, then it was raised to 120 °C (10 °C/min) for 5 min and after that the temperature was raised to 280 °C (10 °C/min) for 8 min and finally was raised to 300 °C (10 °C/min) for 10 min. The mass spectra were compared to data of the National Institute of Standards and Technology (NIST) mass spectra library.

Extraction technique

The extraction was performed according to the method described by Sillero *et al.*[21] in a temperature-controlled ultrasonic bath (Elmasonic 570 H, Elma) using ethanol-water (50/50 (v/v)) mixture as solvent. Extracts (male leaves extract (MLE) and female leaves extract (FLE)) were prepared using 4 grams of each dry material. The samples were placed in a glass bottle, and the used solid/liquid ratio was 1:10 (w/v). After 1 hour at 50 °C, the samples were filtrated under vacuum; the supernatant extracts were used to determine the amounts of total phenolic and flavonoid content, and to evaluate the antioxidant activity. The solid phase was dried at room temperature and then, the extraction yields were calculated gravimetrically and referenced to a 100 g of dried material. Each assay was performed in triplicate.

Determination of total phenolic and total flavonoid content

The total phenolic content (TPC) of the extracts and bread samples was determined according to the Folin–Ciocalteu method, following the methodology described by Sillero *et al.*[21] The absorbance was read at 760 nm using a Jasco V-630 spectrophotometer. Gallic acid was used as standard, and its calibration curve was determined with concentration ranging from 0 to 0.35 g L^{-1}. The equation of the calibration curve was: y = 0.1348x - 0.007; R^2 = 0.9991. The total phenolic content was expressed in mg of gallic acid equivalent/g of dry extract (mg GAE/g DE) for extracts and mg of gallic acid equivalent/g of bread (mg GAE/ g bread) for bread. Detections were done in triplicate.

The total flavonoid content (TFC) of each extract (MLE and FLE) and each bread type (0%, 1% and 3%) was determined by the aluminium chloride colorimetric assay, using the methodology detailed by Sillero *et al.*[21] The absorbance was read at 510 nm, and catechin was used as a standard. Solutions of different catechin concentrations, from 0 to 0.824 g L^{-1}, were used to plot the calibration curve. The total flavonoid content was expressed as mg catechin equivalent/g of dry extract (mg CE/g DE) for extracts and mg catechin equivalent/g of bread (mg CE/g bread) for bread. The catechin calibration curve equation was y = 1.1185x - 0.0141; R^2 = 0.9971. Detections were performed in triplicate.

Antioxidant capacity

The determination of the antioxidant activity of male leaves extract (MLE), female leaves extract (FLE) and bread samples was carried out using three methods. For all the methods, trolox was used as a standard and the results were expressed as mg of trolox equivalent/g of dry extract (mg TE/g DE) using a calibration curve with different trolox concentration solutions for each assay. The Jasco V630 UV-Vis Spectrophotometer was used for all the tests.

The DPPH and ABTS assays were used to determine the rate of scavenging of free radicals; this assay is based on hydrogen atom transfer. The FRAP essay has been widely used to determine the ferric ion reducing power and it is based on electron transfer.

The DPPH radical scavenging assay was performed using the method described by Gullon *et al.*[22] Briefly, 3 mL of DPPH solution (6 x 10^{-5} mol L^{-1}) was added to 0.3 mL of ethanolic solution of each sample. The decrease in absorbance was measured at 515 nm after 15 min of incubation at room temperature. To plot the calibration curve of trolox, stock standard solutions in the range of 0 to 0.062 g·L^{-1} were used. The equation of the calibration curve of trolox was y = -0.1304x + 0.0696; R^2= 0.9983.

The trolox equivalent antioxidant capacity (TEAC) was assessed by the ABTS radical cation scavenging assay, following the methodology described by Sillero *et al.*[23] Briefly, 30 µL of each sample solution was mixed with 3 mL of the ABTS solution. The absorbance was recorded at 734 nm after 6 min. Trolox solutions, with different concentrations in the range of

0-0.712 g·L^{-1}, were used in order to plot the calibration curve. The equation of the calibration curve of trolox was y = -0.9604x + 0.7174; R^2 = 0.9961.

The ferric reducing antioxidant power (FRAP) assay was carried out using the method detailed by Sillero *et al.*[21] This test was based on the reduction of the complex ferric Fe(III)–2,4,6-tripyridyltriazine (Fe(III)–TPTZ) to ferrous Fe(II)–TPTZ.[22] Briefly, 100 µL of each sample solution was added to 3 mL of prepared reactive solution. The absorbance was measured at 593 nm after 6 min. The regression equation of the calibration curve of trolox was y = 0.3775x - 0.0137; R^2 = 0.9978, determined using different trolox solutions with concentrations in the range of 0-0.54 g·L^{-1}.

Antimicrobial activity of the extracts

The antimicrobial activity of male and female leaves extracts (MLE and FLE) was evaluated by the diffusion test according to Trabelsi *et al.*[24] and Oussaid *et al.*[25] The selected strains were as follows: bacteria: *Staphylococcus aureus* (+), *Enterococcus faecalis* (+), Group B *Streptococcus* (+), *Citrobacter freundii* (-), *Escherichia coli* (-) and *Klebsiella pneumoniae* (-); and yeast: *Candida albicans*.

Petri dishes on the surface of Muller-Hinton agar were inoculated with a bacterial suspension (10^6 CFU mL^{-1}), while the fungal (*Candida albicans*) suspension (10^6 CFU mL^{-1}) was seeded on the surface of Sabouraud Dextrose agar.

The extracts (MLE and FLE) were dissolved in dimethyl sulfoxide (DMSO) (1 mg mL^{-1}). Discs (Ø = 6 mm) were impregnated with 10 µL of MLE and FLE dissolved in DMSO, and then deposited on the surface of the inoculated culture. Next, the plates were stored for 2 hours at 4 °C to ensure the diffusion of the extracts into the agar, and then they were incubated for 24 h at 37 °C. The diameter around the discs was determined by a graduated ruler (in mm).

The antimicrobial activity was expressed as the diameter of the inhibition of growth of microbes (bacteria and yeast) by the extracts. The average of three measurements was taken. Selected antibiotics (levofloxacin and vancomycin for bacteria and fluconazole for fungi) were used as a positive control and DMSO as a negative control in each assay.

Preparation of bread and its characterization

Three different bread formulations were prepared for this study based on 0%, 1% and 3% substitution of wheat flour with female leaves extract (FLE) (Table 1). The formulation (0%) based on 50 g of wheat flour without FLE served as control bread. The bread samples were made from flour, dry yeast powder, salt, sugar, butter, milk powder, and female leaves extract (FLE). After mixing all the ingredients, the dough samples were fermented for 30 min, then baked at 150 °C for 20 minutes using a classic oven (Focus).

The color monitoring was determined as previously described.[26] The color of samples of slices (almost 5 g) of bread (0%, 1% and 3%) was measured using a colorimeter (Minolta CR 300, Ramsey, NJ, USA). The results were expressed as the three parameters a*, b* and L*. The parameter a* indicates the characteristics of red and green color, the a* value is from -100 (green) to +100 (red), the b* parameter represents yellowness and blueness and varies from -100 (blue) to +100 (yellow), and the L* value is a measure of brightness and varies between -100 (black) and +100 (white). Color was measured on three randomized sides of slices of bread, and the mean values of the three parameters were calculated. The measurements were made at room temperature in triplicate.

Table 1
Formulation of bread samples

Ingredients (g)	Control bread (0%)	Bread (1%)	Bread (3%)
Flour	50	49.5	48.5
FLE	0	0.5	1.5
Dry yeast powder	2	2	2
Salt	0.6	0.6	0.6
Sugar	2	2	2
Butter	4	4	4
Water	40	40	40
Milk powder	1.4	1.4	1.4

Bacterial growth in control bread and breads enriched with FLE was studied as described by Ijah *et al.*[27] and Krichen *et al.*[28] The microbial counts were carried out on the 1st, 3rd, 7th and 14th days of storage at ambient temperature. Bread samples were mixed with sterilized peptone solution (270 mL) and homogenized using a Stomacher (SCIENTZ-11L paddle blender). The mixture solutions were diluted decimally (10^{-2}, 10^{-3}) and 100 µL aliquots were diffused on different selective media. Total viable counts (TVC) were determined using the plate count agar (PCA) after incubation at 30 °C for 72 h. Coliform bacteria (CB) and fecal coliform were determined using violet red bile agar (VRBA) after incubation for 24 h at 37 °C and 44 °C, respectively. *Escherichia coli* were detected in MacConkey agar after incubation at 37 °C for 24 h.

Sulfite-reducing anaerobic bacteria were determined in roll tubes, using tryptone sulfite neomycin agar (TSN agar), after 48 h at 44 °C. Yeasts and mould counts were determined using potato dextrose agar (PDA) after incubation at room temperature for 5 days.

Salmonella spp. was evaluated in three steps. Each sample mixed with buffered peptone water was incubated at 37 °C for 24 h for pre-enrichment. In the second step, the homogenate was transferred to Rappaport Vassiliadis medium (RV) for selective enrichment at 44 °C for 24 h. After incubation, the mixture of each tube was inoculated with hectoen enteric agar and was incubated for 24 h at 35 °C. The results of microbial counts were counted and expressed as colony forming units per gram of bread (CFU/g). All counts were done in duplicate using the Galaxy 230 colony counter (ROCKER).

Statistical analysis

Means and standard errors were calculated and shown as means ± S.D., experimental data were subjected to analysis of variance (ANOVA). All analyses were carried out with three replications (n = 3) and differences were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

Raw material characterization

The chemical composition of male leaves (ML) and female leaves (FL) of *Pistacia vera* L. were presented in Table 2. In terms of the total ash content, FL had lower ash content (8%), compared to ML (10%). Water and ethanol were used in order to determine the total extractives content in the leaves. The total extractives in FL were higher than those in ML. The extractives content is related to the solubility of the different compounds in the used extraction solvent. Water extractives were 32% and 25% for FL and ML, respectively. The amounts of ethanol extractives were lower than those measured for water (Table 2).

The highest ethanolic extractives content was found for ML (13%). The total lignin content ranged from 20 to 22% of dry leaves. The Klason lignin content of the samples varied between 14% and 16% of dry leaves, while the acid soluble lignin reached about 6.5% of dry leaves. From the monosaccharides measured in the acid hydrolysis, the glucose was attributed to the cellulose content, while the hemicelluloses content was provided by the sum of galactose, arabinose and xylose.[29] The cellulose contents were found to be 8.48% and 9.02% for ML and FL, respectively. Likewise, the hemicelluloses content found for FL (10.28%) was slightly higher than that found for ML (9.67%). In the literature, Dahmoune *et al*. reported 10.77% of cellulose, 14.89% of hemicelluloses and 36.07% of lignin for *Pistacia lentiscus* L. leaves.[30]

Table 2
Dry matter chemical composition of male leaves (ML) and female leaves (FL) of *Pistacia vera* L. (expressed in % of dry mass of leaves)

Samples	ML	FL
Ash (%)	10.40±0.07	8.89±0.02
Total extractives (%)	39.27±0.95	43.17±0.91
Water	25.05±1.78	32.18±0.68
Ethanol	13.93±0.65	10.98±0.84
Soluble lignin (%)	6.55±0.17	6.45±0.21
Klason lignin (%)	16.48±0.62	14.51±0.12
Cellulose (%)	8.48±0.24	9.02±0.33
Hemicelluloses (%)	9.67±0.06	10.28±0.15

Data are expressed as mean ± SD from three replications

In order to get deeper knowledge of the chemical composition of ML and FL, pyrolysis coupled to GC/MS was carried out. The identified compounds are tabulated in Table 3. The major component identified was 1-methyl-1H-Pyrrole (17.80% in ML), which is a nitrogen-containing compound. Pyrrole and derivatives were formed from the pyrolysis of chlorophyll pigments.[32] Indole, styrene and phenol were also formed during pyrolysis from proteins and amino acids present in the samples.[33] In addition, furfural was identified as a product; this compound could be generated in the pyrolysis of residual carbohydrates.[34] Limonene, a monocyclic monoterpene, was detected with amounts of 15.28% and 17.46% for ML and FL, respectively. This compound contributes to the flavor of the samples. Phenol, 2-methylphenol, 3-methylphenol,

4-ethylphenol, 2-methoxy-4-(1-propenyl)-phenol, 2,6-dimethoxy-4-(2-propenyl)-phenol, 2-methoxy-4-vinylphenol, 1,2-benzenediol and 4-hydroxy-3,5-dimethoxy-benzaldehyde detected in the pyrolysis of the leaves (ML and FL) are compounds derived from lignin. Among the fatty acids released from the pyrolysis of the samples, n-hexadecanoic and octadecenoic acids were the dominant fatty acids in ML. Alpha-tocopherol (vitamin E) and sitosterol were only detected in ML. The results suggested that male and female leaves of *Pistacia vera* L. are rich in natural bioactive compounds.

Table 3
Peak assignments from PY-GC/MS chromatograms, with relative amounts (%), retention times (RT) and main fragments (m/z) of male and female *Pistacia vera* L. leaves

Compound	RT (min)	Main fragments (m/z)	Relative amounts (%)	
			ML	FL
2,5-Dimethylfuran	3.497	74/96/57	1.26	1.35
1-Methyl-1H-pyrrole	3.999	81/80/53	17.80	14.75
3,3-Diethylcyclopropene	4.242	67/81/66	---	0.5
Pyridine	4.282	87/73/77	---	---
Toluene	4.496	91/92/57	----	5.12
5-Heptyn-3-ol	5.125	84/54/55	---	1
3-Methyl-1H-pyrrole	6.361	91/92/65	0.50	--
Furfural	6.096	96/95/82	2.08	5.12
1,3-Dimethylbenzene	7.303	91/106/86	3.01	1.00
Styrene	8.14	104/107/103	1.31	0.53
5 Methylfurfural	11.449	110/109/105	1.44	2.53
Phenol	12.605	94/66/65	5.34	0.66
1-Methyl-4-(1-methylethyl)-benzene	14.776	97/95/94	0.65	3.26
Limonene	15.03	68/67/93	15.28	17.46
Benzyl alcohol	15.434	97/67/68	0.68	0.63
2-Methylphenol	16.797	108/107/77	0.74	1.48
4-Methylphenol	18.131	107/108/77	2.78	0.60
2,4-Dimethylphenol	22.746	70/83/82	0.66	---
4-Ethylphenol	23.999	107/122/121	2.39	1.77
1,2-Benzenediol	26.679	110/64/63	4.00	5.76
4-Ethyl-3-methylphenol	28.66	121/91/69	-	0.73
3-Methoxy-1,2-benzenediol	30.023	140/125/97	1.44	0.56
Indole	32.033	117/90/89	1.79	0.64
2-Methoxy-4-vinylphenol	33.367	150/135/77	1.53	1.00
2,6-Dimethoxyphenol	35.879	154/139/96	2.93	1.43
Vanillin	38.969	151/152/81	-	0.61
2-Methoxy-4-(1-propenyl)-phenol	42.711	168/164/153	1.92	0.54
6,11-Dimethyl-2,6,10-dodecatrien-1-ol	43.704	69/81/95	-	0.56
1-(3,4-Dimethoxyphenyl)-ethanone	47.007	180/165/137	-	0.97
4-Hydroxy-3,5-dimethoxybenzaldehyde	49.006	178/99/156	0.86	17.46
2,6-Dimethoxy-4-(2-propenyl)-phenol	49.665	194/91/179	1.65	0.83
1-(4-Hydroxy-3,5-dimethoxyphenyl)-ethanone	50.184	181/196/153	2.69	0.86
Tetradecanoic acid	50.525	73/60/55	1.40	2.70
Tridecanedial	51.478	68/95/82	1.59	2.15
n-Hexadecanoic acid	52.939	73/60/55	10.45	1.28
Octadecenoic acid	54.891	73/60/55	6.63	0.54
Tridecylphenol	55.983	108/107/276	-	0.70
Benzeneacetic acid, 4-hydroxy-3-methoxy-, methyl ester	56.555	137/151/138	0.93	0.49
4-Methoxy-4',5'-methylene	59.182	272/273/211	1.72	-
Hexadecamethylheptasiloxane	60.031	221/147/73	-	0.62
α-Tocopherol	65.599	165/430/164	0.57	-
Sitosterol	68.914	414/57/55	1.29	-

Extraction yield and phenolic content

Total phenolic content (TPC) and total flavonoid content (TFC) in the leaves extracts, as well as the extraction yield, are summarized in Table 4. The extraction yields of MLE and FLE were 28% and 29%, respectively (Table 4). In the same context, Ozbek *et al.*[38] studied the effect of the ethanol–water ratio on the extraction yield from pistachio hull, reporting the highest yield (32.9%) at an ethanol–water ratio of 1/1 (v/v). Moreover, Miyataka *et al*. and Sun *et al.*[39,40] reported the highest extraction yields of Brazilian propolis (60%) and Beijing propolis (51%) using ethanol/water as solvent.

The total phenolic contents were 450.4 mg GAE/g DE and 533.4 mg GAE/g DE for MLE and FLE, respectively (Table 4). These values are higher than those reported for pistachio nuts,[37] as well as compared to those reported for hull of *Pistacia vera* L.[34] The TPC values obtained are lower than that provided by Tamaino *et al.*[36] for *Pistacia vera* L. skins (116.3 mg GAE/g DE). The flavonoid content obtained for FLE was 397.5 mg CE/g DE, which was higher than that observed for MLE (373.1 mg CE/g DE).

The contents of TPC and TFC were different in male and female leaves. It is because different parts of the same plant may synthesize and accumulate different amounts of phenolic content due to their differential gene expression. The leaves of *Pistacia vera* L. can be considered to represent a potential source of phenolic compounds and flavonoids.

Antioxidant activity of extracts

The antioxidant capacities of the extracts of the raw materials were studied in order to know their possible benefits. In this study, three chemical assays were used to study the antioxidant activity of *Pistacia vera* L. leaves extracts (MLE and FLE). The results obtained are presented in Table 5, confirming the antioxidant activity of MLE and FLE.

The values of trolox equivalent obtained by the DPPH assay were in the range of 704-644 mg TE/g DE, and similar values were reached by the ABTS assay. The highest values for both assays were recorded by FLE. The FLE, which was the extract with the highest TPC, was the most effective radical scavenger.

Table 4
Extraction yield (expressed as % of dry mater), total phenolic content (TPC), total flavonoid content (TFC) of male and female *Pistacia vera* L. leaves extracts, as well as different results reported by other authors

Samples	Extraction yield (%)	TPC (mg GAE/g DE)	TFC (mg CE/g DE)	Data source
MLE	28.87±0.55	450.43±20.33	373.18±39.74	This work
FLE	29.28±0.45	533.41±15.07	397.58±10.18	This work
Pistacia vera L. hull		182.11±7.21		[34]
Pistacia vera L. nuts		4.94±0.02–10.47±0.13		[35]
Pistacia vera L. skins		116.32±8.54	70.27±5.41	[36]
Pistacia vera L. nuts		16.20±0.40	7.2±0.38	[37]

Data are expressed as mean ± standard deviation of three experiments; DE: dried extract; GAE: gallic acid equivalent; CE: catechin equivalent

Table 5
Antioxidant activity of MLE and FLE (analyzed by DPPH, ABTS and FRAP methods), as well as different results reported by other authors

Samples	(mg TE/g DE)			
	DPPH	ABTS	FRAP	Data source
MLE	644.36±31.20	1394.95±5.49	808.24±18.13	This work
FLE	704.53±14.16	1334.35±14.16	521.52±4.11	This work
Pistacia palaestina leaves	86±2.75	13.275±1.65	-	[41]
Pistacia lentiscus leaves	490±13.10	336±10	400±14.10	[42]
Pistacia vera L. nuts	7.656±0.19	-	33.125±0.50	[37]

Mean value of three replicates ± standard deviation; TE: trolox equivalent, DE: dried extract

According to the results of the FRAP assay, the extracts achieved excellent results, between 521 and 808 mg TE/g DE. This test highlighted the high reducing power of MLE, compared to FLE of *Pistacia vera* L. The reducing power of the leaves extracts is a good property, as it can be of interest in the process of catalyzing LDL oxidation,[43] among others. The antioxidant activities of leaves extracts from different species have been reported in different studies (Table 5). Botsaris *et al.*[42] reported the values of DPPH, ABTS and FRAP for *Pistacia lentiscus* leaves aqueous extract. The measured values were 490, 336 and 400 mg TE/g DE, respectively, which are lower than the values obtained in this work.

Antimicrobial activity of extracts

The antimicrobial activities of the leaves extracts were evaluated against gram positive bacteria, gram negative bacteria and yeast. These activities were influenced by strain sensibilities (Table 6).

Both extracts have an antibacterial activity against all the strains, the highest activity was recorded against *Staphylococcus aureus* – the diameter of the inhibition zone was 26 mm for FLE. FLE exhibited a strong activity against this strain, compared to the antibiotic. The lower activity was against Group B *Streptococcus* at higher concentration. *Klebsiella pneumonia* showed a maximum zone of inhibition of 20 mm for both leaves extracts. The FLE and MLE exhibited good activity against the rest of the strains. This difference in sensitivity between Gram (-) and Gram (+) could be attributed to differences in morphological constitution of these microorganisms.[24,44] It is known that the morphological constitution is different for Gram (+) bacteria and Gram (–) bacteria, which could explain the highest activity of the leaves extracts against Gram (+) bacteria. The extracts exhibited considerable antifungal activity against *Candida albicans*, with a diameter of the inhibition zone of 22 and 25 mm for MLE and FLE, respectively.

Effects of FLE on bread quality

The volumes of the breads (0%, 1% and 3%) were significantly different. The bread with 3% FLE has the highest volume (Fig. 1). This increase may be due to the presence of phenolic compounds, which can interact with the protein and polysaccharides in the dough. These interactions increased the molecular weight of the gluten, which affects the resistance of extensibility of the dough.[45–47]

The changes in bread color redness (a*), yellowness (b*) and brightness (L*) were analyzed by the colorimeter. FLE levels of fortification influenced the color index of the bread. The increase of the FLE from 0% to 3% led to changes in the color of the bread from white to green (Fig. 1).

Table 6
Inhibition zone diameter (mm) of male and female leaves extracts (MLE and FLE) of *Pistacia vera* L.

Strains	Diameter of inhibition zone (mm)		
	MLE	FLE	Antibiotic
Staphylococcus aureus	24	26	25
Enterococcus faecalis	18	19	20
B *Streptococcus*	17	19	24
Citrobacter freundii	25	24	30
Escherichia coli	20	22	29
Klebsiella pneumoniae	20	20	21
Candida albicans	22	25	29

Figure 1: Bread 0% FLE (left), bread 1% FLE (middle) and bread 3% FLE (right)

Table 7
Effect of FLE addition on characteristics of bread samples

Samples	Bread (0%)	Bread (1%)	Bread (3%)
Weight of bread (g)	88.5±0.5	90.5±0.5	92.3±0.26
a*	1.62±0.12	2.39±0.188	6.66±0.09
b*	26.76±1.10	32.55±0.74	46.15±0.21
L*	89.47±0.30	77.17±0.71	70.83±0.19
Moisture (%)	20.10±0.6	17.7±0.2	16.03±0.32
Ash (%)	1.36±0.07	1.44±0.09	1.66±0.08
TPC (mg GAE/g)	0.09±0.01	2.68±0.11	7.96±0.14
TFC (mg CE/g)	0.06±0.01	2.07±0.10	5.98±1.18
DPPH (mg TE/g)	0.04±0.01	3.75±0.21	11.08±0.27

Data are expressed as mean ± standard deviation of three experiments; GAE: gallic acid equivalent; CE: catechin equivalent; TE: trolox equivalent

The presence of pigments in the extracts can influence the color of the bread. The value of a* increases with the increase in the added amount of FLE. The bread containing 3% FLE was the redder sample, with the highest value of a* (Table 7). Also, it has the highest value of b*, followed by the bread with 1% and the least – for the control bread 0% (Table 7). The addition of FLE increased the yellow color of the bread. The control bread 0% has the highest value of L* (89.47), while the lowest value (70.83) was noted for the bread with 3% FLE, which explains the dark color of the bread. A slight increase in the ash content is also observed, which could be explained by the contribution of the preventive minerals of the extract.

The bread enriched with 3% FLE had higher total phenolic and total flavonoid content, compared to the bread with 1% and the control bread (Table 7). This increase was mainly due to the rich phenolic and flavonoid content of FLE.

The antioxidant activity of the breads (0%, 1% and 3%) was evaluated using the DPPH assay (Table 7). The level of antioxidant activity increases with increasing the percentage of FLE.

Table 8
Microbial growth in bread samples (0%, 1% and 3% FLE) during storage

	Sample	Storage days			
		Day 1	Day 3	Day 7	Day 14
Total viable counts (UFC/g)	0%	Absence	$4.35x10^2$±0.5	$6.87x10^5$±0.9	$2.34x10^7$±0.66
	1%	Absence	Absence	$4.64x10^3$±1.2	$8.86x10^5$±0.87
	3%	Absence	Absence	Absence	$3.54x10^2$±0.73
Total coliforms (UFC/g)	0%	Absence	Absence	Absence	Absence
	1%	Absence	Absence	Absence	Absence
	3%	Absence	Absence	Absence	Absence
Fecal coliforms (UFC/g)	0%	Absence	Absence	Absence	Absence
	1%	Absence	Absence	Absence	Absence
	3%	Absence	Absence	Absence	Absence
Sulfite reducing bacteria (SRB)	0%	Absence	Absence	Absence	Absence
	1%	Absence	Absence	Absence	Absence
	3%	Absence	Absence	Absence	Absence
Escherichia coli (UFC/g)	0%	Absence	Absence	Absence	540 10^2±0.3
	1%	Absence	Absence	Absence	Absence
	3%	Absence	Absence	Absence	Absence
Salmonella spp. (UFC/g)	0%	Absence	Absence	Absence	Absence
	1%	Absence	Absence	Absence	Absence
	3%	Absence	Absence	Absence	Absence
Yeasts and molds counts (UFC/g)	0%	Absence	5.67 10^2±0.57	3.67 10^5±0.92	8.24 10^7±0.6
	1%	Absence	Absence	Absence	2.11 10^2±0.45
	3%	Absence	Absence	Absence	Absence

Data are expressed as mean ± standard deviation of duplicate experiments; UFC: unit forming colony

The lowest value was found for the control bread (0.04 mg TE/g bread), whereas the bread with 3% FLE had 11.08 mg TE/g bread.

The results indicated that enriched bread exhibited a high amount of phenolic compounds and good scavenging activity. Therefore, FLE can be used as a food additive in bread.

The microbial growth on the 3 samples of bread (0%, 1% and 3%) during the days of storage at ambient temperature was determined and the results were summarized in Table 8. The major microbes (total coliforms, fecal coliforms, *Escherichia coli*, sulfite reducing bacteria and *Salmonella* spp.) were absent from all bread types (Table 8). Contrariwise, *Escherichia coli* was detected in the control bread after 14 days. The absence of fecal and *Salmonella* contamination makes the bread fortified with the leaves extract safe for consumption. The control bread (0%) exhibited high *E. coli* and fungal population (viable counts), compared to the fortified bread, which increased with storage time at ambient temperature. Only the bread with 3% FLE showed lower microbial counts and absence of fungal growth for 14 days. Indeed, the stability of bread can be influenced by moisture.[48] The control bread had the highest moisture content (20.1%), compared to the fortified breads (1% and 3%), which may explain its short shelf life. The retarding effect of microbial growth in fortified bread might be explained by the antimicrobial activity of FLE. Thus, the addition of FLE to the dough prolonged the shelf life of the bread to 14 days, compared to 3 days for the control one.

CONCLUSION

In this study, leaves of *Pistacia vera* L. were characterized. This chemical characterization showed a high content of extractives and a significant amount of phenolic compounds, which explain their good antioxidant activities. FLE had the highest antioxidant potential, as demonstrated by DPPH and ABTS, while MLE had higher antioxidant potential given by FRAP. The use of FLE as an ingredient in bread formulations improves the nutritional value and storage stability of bread products. FLE increased the polyphenols content and the antioxidant activity of the bread and prolonged its shelf life, even after storage for 14 days at room temperature.

Pistacia vera L. leaves can be considered as an interesting resource, taking into consideration the biological potential of the extracts. They could be used as a natural antioxidant or an antimicrobial agent, instead of the synthetic ones in different applications in the food, cosmetic and pharmaceutical industries.

ACKNOWLEDGMENTS: The authors greatly acknowledge the financial support of the Ministry of Higher Education and Scientific Research of Tunisia. L.S. would also like to thank the Department of Economic Development and Infrastructure of the Basque Government (scholarship of young researchers training).

REFERENCES

[1] M. Talebi, M. Akbari, M. Zamani and B. E. Sayed-Tabatabaei, *J. Genet. Eng. Biotechnol.*, **14**, 31 (2016), https://doi.org/10.1016/j.jgeb.2016.04.002

[2] K. Arena, F. Cacciola, D. Mangraviti, M. Zoccali, F. Rigano *et al*., *Anal. Bioanal. Chem*., **411**, 4819 (2019), https://doi.org/10.1007/s00216-019-01649-w

[3] J. J. Rodríguez-Bencomo, H. Kelebek, A. S. Sonmezdag, L. M. Rodríguez-Alcalá, J. Fontecha *et al*., *J. Agric. Food Chem*., **63**, 7830 (2015), https://doi.org/10.1021/acs.jafc.5b02576

[4] Webmanagercenter, 2020, https://www.webmanagercenter.com/2020/05/18/450562/

[5] G. Magi, E. Marini, A. Brenciani, D. Di Lodovico, D. Gentile *et al*., *Arch. Oral Biol*., **96**, 208 (2018), https://doi.org/10.1016/j.archoralbio.2018.09.013

[6] N. G. Taş and V. Gökmen, *Curr. Opin. Food Sci*., **14**, 103 (2017), https://doi.org/10.1016/j.cofs.2017.03.001

[7] H. Mahmoudvand, F. Kheirandish, E. S. Dezaki, S. Shamsaddini and M. F. Harandi, *Biomed. Pharmacother*., **82**, 393 (2016), https://doi.org/10.1016/j.biopha.2016.05.012

[8] M. Aouadi, M. T. Escribano-Bailón, K. Guenni, A. S. Hannachi and M. Dueñas, *J. Food Meas. Charact*., **13**, 2448 (2019), https://doi.org/10.1007/s11694-019-00165-w

[9] H. Hosseinzadeh, S. A. S. Tabassi, N. M. Moghadam, M. Rashedinia and S. Mehri, *Iran. J. Pharm. Res*., **11**, 879 (2012), http://www.ncbi.nlm.nih.gov/pmc/articles/pmc3813125/

[10] S. G. West, S. K. Gebauer, C. D. Kay, D. M. Bagshaw, D. M. Savastano *et al*., *Hypertension*, **60**, 58 (2012), https://doi.org/10.1161/HYPERTENSIONAHA.111.182147

[11] E. Yanni, G. Mitropoulou, I. Prapa, G. Agrogiannis, N. Kostomitsopoulos *et al*., *Metab. Open*, 100040 (2020), https://doi.org/10.1016/j.metop.2020.100040

[12] D. Barreca, G. Laganà, U. Leuzzi, A. Smeriglio, D. Trombetta *et al*., *Food Chem*., **196**, 493 (2016), https://doi.org/10.1016/j.foodchem.2015.09.077
[13] S. Erşan, Ö. Güçlü Üstündağ, R. Carle and R. M. Schweiggert, *Food Chem*., **253**, 46 (2018), https://doi.org/10.1016/j.foodchem.2018.01.116
[14] M. H. Grace, D. Esposito, M. A. Timmers, J. Xiong, G. Yousef *et al*., *Food Chem.*, **210**, 85 (2016), https://doi.org/10.1016/j.foodchem.2016.04.088
[15] H. Hosseinzadeh, E. Behravan and M. M. Soleimani, *Iran. J. Pharm. Res*., **10**, 821 (2011), https://www.ncbi.nlm.nih.gov/pubmed/24250418
[16] N. Seifzadeh, M. A. Sahari, M. Barzegar and H. A. Gavlighi, *Food Sci. Nutr*., **66**, 1741 (2018), https://doi.org/10.1002/fsn3.692
[17] F. Shahidi and P. Ambigaipalan, *J. Funct. Foods*, **18**, 820 (2015), https://doi.org/10.1016/j.jff.2015.06.018
[18] V. Lobo, A. Patil, A. Phatak and N. Chandra, *Pharmacogn. Rev*., **4**, 118 (2010), https://www.ncbi.nlm.nih.gov/pubmed/22228951
[19] A. Bagdi, B. Tóth, R. Lorincz, S. Z. Szendi, A. Gere *et al*., *LWT – Food Sci. Technol*., **65**, 762 (2016), https://doi.org/10.1016/j.lwt.2015.08.073
[20] R. Różyło, *Food Bioprocess. Technol*., **7**, 774 (2014), https://doi.org/10.1007/s11947-013-1100-1
[21] L. Sillero, R. Prado, M. A. Andrés and J. Labidi, *Ind. Crop. Prod*., **137**, 276 (2019), https://doi.org/10.1016/j.indcrop.2019.05.033
[22] B. Gullón, G. Eibes, M. T. Moreira, I. Dávila, J. Labidi *et al*., *Ind. Crop. Prod*., **107**, 105 (2017), https://doi.org/10.1016/j.indcrop.2017.05.034
[23] L. Sillero, R. Prado and J. Labidi, *Chem. Eng. Trans*., **70**, 1369 (2018), https://doi.org/10.3303/CET1870229
[24] N. Trabelsi, H. Falleh, I. Jallali, A. Ben Daly, H. Hajlaoui *et al*., *Acta Phys. Plant*., **34**, 87 (2012), https://doi.org/10.1007/s11738-011-0807-8
[25] S. Oussaid, M. Chibane, K. Madani, T. Amrouche, S. Achat *et al*., *J. Food Sci. Technol*., **86**, 635 (2017), https://doi.org/10.1016/j.lwt.2017.08.064
[26] K. L. Nyam, S. Y. Leao, C. P. Tan and K. Long, *J. Food Sci. Technol*., **51**, 3830 (2014), https://doi.org/10.1007/s13197-012-0902-x
[27] U. J. J. Ijah, H. S. Auta, M. O. Aduloju and S. A. Aransiola, *Int. J. Food Sci*., **2014**, 671701 (2014), https://doi.org/10.1155/2014/671701
[28] F. Krichen, M. Hamed, W. Karoud, H. Bougatef, A. Sila *et al*., *J. Food Meas. Charact*., **14**, 3020 (2020), https://doi.org/10.1007/s11694-020-00546-6
[29] L. Bertrand, B. Chabbert, B. Kurek and S. Recous, *Plant Soil*, **281**, 291 (2006), https://doi.org/10.1007/s11104-005-4628-7
[30] F. Dahmoune, G. Spigno, K. Moussi, H. Remini, A. Cherbal *et al*., *Ind. Crop. Prod*., **61**, 31 (2014), https://doi.org/10.1016/j.indcrop.2014.06.035
[31] M. F. Dignac, S. Houot, C. Francou and S. Derenne, *Org. Geochem*., **36**, 1054 (2005), https://doi.org/10.1016/j.orggeochem.2005.02.007
[32] R. T. Nguyen, H. R. Harvey, X. Zang, J. D. H. Van Heemst, M. Hetényi *et al*., *Org. Geochem*., **34**, 483 (2003), https://doi.org/10.1016/S0146-6380(02)00261-9
[33] S. Constant, H. L. J. Wienk, A. E. Frissen, P. Peinder, R. DeBoelens *et al*., *Green Chem*., **18**, 2651 (2016), https://doi.org/10.1039/C5GC03043A
[34] K. Elhadef, S. Akermi, H. Ben Hlima, K. Ennouri, M. Fourati *et al*., *J. Food Qual*., **2021**, 9953545 (2021), https://doi.org/10.1155/2021/9953545
[35] H. Kelebek, A. S. Sonmezdag, G. Guclu, N. Cengiz, T. Uzlasir *et al*., *J. Food Process. Preserv*., **44**, e14605 (2020), https://doi.org/10.1111/jfpp.14605
[36] M. Tomaino, T. Martorana, D. Arcoraci, C. Monteleone, C. Giovinazzo *et al*., *Biochimie*, **92**, 1115 (2010), https://doi.org/10.1016/j.biochi.2010.03.027
[37] E. Tsantili, K. Konstantinidis, M. V. Christopoulos and P. A. Roussos, *Sci. Hortic*., **129**, 694 (2011), https://doi.org/10.1016/j.scienta.2011.05.020
[38] N. H. Özbek, F. Halahlih, F. Göğüş, D. K. Yanik and H. Azaizeh, *Waste Biomass Valoriz*., **11**, 2101 (2020), https://doi.org/10.1007/s12649-018-0512-6
[39] H. Miyataka, M. Nishiki, H. Matsumoto, T. Fujimoto, M. Matsuka *et al*., *Biol. Pharm. Bull*., **20**, 496 (1997), https://doi.org/10.1248/bpb.20.496
[40] H. Sun, Z. Wang and H. Zhang, *Evidence-Based Complementary and Alternative Medicine*, **2015**, 595393 (2015), https://doi.org/10.1155/2015/595393
[41] S. Abu Lafi, F. Al-Rimawi, J. Abbadi, S. A. Naser and K. Qabaha, *J. Med. Plants Res*., **14**, 317 (2020), https://doi.org/10.5897/JMPR2020.6969
[42] G. Botsaris, A. Orphanides, E. Yiannakou, V. Gekas and V. Goulas, *Food Technol. Biotechnol*., **53**, 472 (2015), https://www.ncbi.nlm.nih.gov/pubmed/27904382
[43] A. Smeriglio, M. Denaro, D. Barreca, A. Calderaro, C. Bisignano *et al*., *Int. J. Mol. Sci*., **18**, 1212 (2017), https://www.ncbi.nlm.nih.gov/pubmed/28587291
[44] B. Uma, C. W. Ho and W. M. Wan Aida, *Sains Malaysiana*, **39**, 119 (2010), https://www.ukm.my/jsm/pdf_files/SM-PDF-39-1-2010/18.pdf
[45] P. Y. Lim, Y. Y. Sim and K. L. Nyam, *J. Food Meas. Charact*., **14**, 2425 (2020), https://doi.org/10.1007/s11694-020-00489-y
[46] T. Ozdal, E. Capanoglu and F. Altay, *Food Res. Int*., **51**, 954 (2013), https://doi.org/10.1016/j.foodres.2013.02.009
[47] P. R. Shewry, S. M. Gilbert, A. W. J. Savage, A. S. Tatham, Y. F. Wan *et al*., *Theor. Appl. Genet*., **106**, 744 (2003), https://doi.org/10.1007/s00122-002-1135-6
[48] S. Sharma, S. Kaur, B. N. Dar and B. Singh, *J. Food Sci. Technol*., **51**, 583 (2014), https://www.ncbi.nlm.nih.gov/pubmed/24587536