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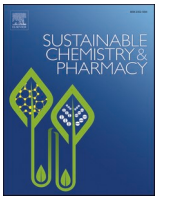

Pistacia vera L. leaves as a renewable source of bioactive compounds via microwave assisted extraction

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ABSTRACT

The production of pistachio in Tunisia generates a large amount of potentially valuable waste, such as leaves, that could be used as source of bioactive compounds. In this work, the extraction of phytochemicals from *Pistacia vera* L. leaves (male and female) by microwave-assisted extraction (MAE) has been investigated. A response surface methodology (RSM) was used for the optimization of bioactive compounds extraction, where the independent variables were temperature and extraction time, and the dependent variable was extraction yield. Total phenolic content (TPC), total flavonoid content (TFC), antioxidant activities (DPPH, ABTS, and FRAP) and antimicrobial activity of the extracts obtained at optimal conditions for both leaves were evaluated and compared not only between them, but also with the extracts obtained by maceration. The optimum extraction conditions were 70 °C and 61 °C, and 5.6 and 12 min for male and female leaves respectivily. The best results were achieved for female leaves by MAE with 36% of extraction yield, TPC of 196.35 mg gallic acid equivalents (GAE)/g dry weight (DW), TFC of 83.34 mg catechin equivalents (CE)/g dry weight (DW), and high antioxidant and antimicrobial activities. The evaluation of biological activity of the extracts showed that MAE provides extracts with better antioxidant and antimicrobial capacities then those obtained by maceration. Moreover, UPLC-DAD-ESI-MS was performed to confirm the presence of some phenolic compounds in MAE extracts, such as quercetin, apigenin and myricetin derivatives. The results revealed that MAE is an efficient technique for the extraction of active components from *Pistacia vera* L. leaves.

1. Introduction

Lignocellulosic biomass is a natural, renewable and cheap source of a wide variety of different compounds that can be used in different applications. Currently, there is an interest in the use of natural products obtained from different biomass, especially plants, in order to substitute, partially or totally, the use of petroleum-based compounds. *Pistacia vera* L. (from now on *P. vera*) is a deciduous

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leaves tree widely diffused in the Mediterranean area. It belongs to the Anacardiacea family (Magi et al., 2018), and these trees can be distinguished into male and female (Awwad and Albiss, 2015). Tunisia is the ninth producer of pistachio in the world, with an estimated production of 3,109 tons (FAO, 2019). The pistachio production generates an important quantity of biowaste such as leaves, hull and steam. These waste materials are underused resources that need to be managed within the framework of the circular economy.

The popularity of the biocompounds, together with the need to valorize the lignocellulosic wastes produced in the pistachio industry led to an increase in the number of studies related with the valorization of these residues. An example of this are the different studies related to the extraction of several bioactive compounds from different parts of *P. vera*, including phenolic compounds, fatty acids and terpenoids (Valasi et al., 2020). Oleic acid has been detected in *P. vera* fruit (Phillips et al., 2005) and in the oil extracted from *Pistacia terebinthus* fruits (Özcan, 2004). Prior studies revealed the pharmacological properties of the genus of *Pistacia*, due to their phytochemicals compounds. Essential oil gum of *P. vera* demonstrated antimicrobial effects (Alma et al., 2004), while *Pistacia atlantica* leaf extracts showed a significant antidiabetic activity (Ben Ahmed et al., 2018), and a strong antioxidant activity, as well as anticholinesterase, anti-atherosclerotic and anticancer, among others (Bozorgi et al., 2013; Sonmezdag et al., 2017). Furthermore, the anti-inflammatory effects of *Pistacia terebinthus* gall was also confirmed (Giner-Larza et al., 2002).

The use of natural components from vegetable sources to substitute synthetic colorants and antioxidants is rising due to their side effects on human health as well as on the environment. The recovery of phytochemicals from plants can be performed using several extraction methods (conventional and non-conventional techniques), and its selection mainly depends on the desired final product (Slimestad and Solheim, 2002). Therefore, extraction technique is an important step in the extraction of secondary metabolites such as phenolic compounds (Lapornik et al., 2005). Conventional extraction techniques have some disadvantages including high-solvent consumption, long extraction time, which can degrade bio-compounds, and low extraction yield (Prado et al., 2015). To overcome these drawbacks, non-conventional techniques have been widely used, such as microwave assisted extraction (MAE), ultrasound assisted extraction (UAE), and pulse electrical field (PEF), among others. MAE is employed as an alternative method for wide applications in order to decrease extraction time, energy consumption and the amount of used solvents (Alara et al., 2018).

In MAE technique, electromagnetic irradiation (microwave) is used to heat efficiently the solid-liquid mixture so that tissues and cells are destroyed and easily release the compounds of interest from the sample to the solvent (Pérez-Serradilla and Luque de Castro, 2011; Jain et al., 2013). Currently, MAE has been reported in different studies and successfully investigated to extract biomolecules from different biomasses (Sillero et al., 2018; Mishra and Aeri, 2016). The use of MAE as intensification process increases the extraction yield (Rodríguez-Pérez et al., 2016; Fiorini et al., 2020). The efficiency of the MAE can be affected by different parameters like temperature, irradiation time, solvent ratio and microwave power (Raut et al., 2015). To optimize these parameters, the response surface methodology (RSM) can be employed. This technique is effective for optimizing complex processes that are influenced not only by different variables, but also by their interactions (Alara et al., 2018). RSM is a widely employed methodology for the optimization of the extraction of biomolecules from lignocellulosic biomass as it has been reported in previous studies (Dahmoune et al., 2014; Rajaei et al., 2010; Alara et al., 2018).

In this study, MAE was optimized for the extraction of bioactive molecules from male and female *P. vera* leaves. A three-level two factor experimental design was used to study the effects of temperature and extraction time on extraction yield. Furthermore, total phenolic content, total flavonoid content and the biological activities of the male and female leaves extracts obtained by MAE at the estimated optimal conditions and by maceration were determined and compared. Finally, the optimum MAE extracts were studied by High Performance Size Exclusion Chromatography (HPSEC), and UPLC-DAD-ESI-MS in order to determine the composition of the extracts. Therefore, the main objective of this study is to optimize the MAE of bioactive compounds from *P. vera* leaves, and evaluate the existing differences between the extracts obtained from female and male leaves, which as far as we know has never been studied before.

2. Materials and methods

2.1. Chemicals and raw material

Ethanol, Folin-Ciocalteu's phenol reagent and gallic acid were purchased from Scharlau. DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)), TPTZ (4,6-tri(2-pyridyl)-s-triazine), catechin, Trolox and aluminium chloride were bought from Sigma-Aldrich. Iron (III) chloride hexahydrate were acquired from Acros Organics.

Male (ML) and female (FL) mature fresh leaves were collected from pistachio (*P. vera*) trees, from Mateur cultivar, grown in the south of Tunisia (Gafsa). The collection area is located between 34° 33′ and 45.385" North latitude and 8° 51′ and 29.992" East longitude, area that is characterized by an arid climate.

Collected leaves were dried at room temperature in the dark, and then they were ground using a domestic miller and sieved in particles size < 0.5 mm in order to reduce the size of the particles and increase surface area. The dried materials (ML and FL) were stored in darkness until further use.

2.2. Maceration

25 g of each sample (ML and FL) was immersed in 70 mL of EtOH:H_2O (70:30, v/v) mixture for 24 h at room temperature. The obtained extracts (MLm and FLm) were filtered and stored at 4 °C. The extraction yield was calculated gravimetrically and referenced to 100 g of dry weight of leaves (DW). Each assay was performed in triplicate.

2.3. Microwave assisted extraction (MAE)

4 g of powdered sample was placed in a round bottom flask and mixed with 40 mL of EtOH:H_2O (70:30, v/v) mixture. The

extraction was performed with an open vessel microwave (MILESTONE flexi WAVE), and the studied independent variables were extraction time (X_1) and temperature (X_2). The extractions were carried out using the conditions detailed in Table 1, and the studied response value was the extraction yield. The limits selected for each variable were based on the results obtained in previous experiments, which results are not shown in this work.

During the extraction process, samples were stirred using a magnetic stirring bar, and irradiation energy was fixed at 400 W. After the extraction, the extracts were recovered by vacuum filtration using a Whattman filter. Then, the liquid phases were centrifuged (4000 rpm, 10 min), and after that, the supernatants were stored at 4 °C for further analysis. The extraction yields were calculated gravimetrically and referenced to 100 g of dry weight of leaves (DW).

Two independent variables (temperature and extraction time) in 3 levels (−1, 0, 1) were used in order to evaluate their influence on the extraction yield (Table 1). The only output variable selected was the extraction yield because of the great potential in extracts of these raw materials (Elakremi et al., 2022). Furthermore, other authors have confirmed that the temperature and time ranges selected here do not strongly affect the TPC (Martínez-Patiño et al., 2019). 10 experiments and 2 more replicates of the central point were applied in randomized order. The optimization was carried out by the response surface methodology (RSM), using the Statgraphics Centurion XV.II software, applying the Box-Behnken model. The suitability of the optimization model was assessed using both the coefficient of determination (R^2) and the significance (*p*-value). The extraction yields obtained at the optimal extraction conditions, which were conducted in triplicate, were compared with those predicted in order to validate the model. An analysis of variance (ANOVA) was used with a confidence level of 95% in order to check the adequacy of the statistical significance of the regression coefficients. The extracts of ML and FL obtained at optimal conditions were denoted as OptML and OptFL, respectively.

2.4. Phenolic content of extracts

Total phenolic content (TPC), expressed in mg of gallic acid equivalent/g of dry leaves (mg GAE/g DW), was measured by Folin Ciocalteu assay following the methodology described by Taghizadeh et al. (2018), using gallic acid as standard. The extracts absorbance was measured at 760 nm. Total flavonoid content (TFC), expressed as mg of catechin equivalent/g dry leaves (mg CE/g DW), was determined by the aluminium chloride colorimetric assay according to the methodology described by Sillero et al. (2018). The absorbance of extracts was measured at 510 nm using catechin as standard. Each assay was performed in triplicate.

2.5. Bioactive extracts characterization

Fourier transform infrared (FTIR) spectroscopy was performed to identify the functional groups presents in maceration and MAE extracts. For that, a Perking Elmer Spectrum Two equipped with Universal Attenuated Total Reflectance accessory was used. The spectral range of 500–4000 cm^{-1}, with a resolution of 4 cm^{-1}, and 12 scans was used.

The surface morphology of *P. vera* leaves (ML and FL) was examined using a HITACHI. S–3400 N system scanning electron microscope.

High performance size exclusion chromatography (HPSEC) was performed by a Jasco LC-NetII/ADC equipped with a RI-2031Plus reflex index detector, PolarGel-M guard (50 × 7.5 mm) and two PolarGel-M columns in series (300 × 7.5 mm). The flow rate of the mobile phase was 0.7 mL/min, with an injection volume of 20 μL, using dimethylformamide with 0.1% of lithium bromide as eluent. For calibration, polystyrene standards were used (266–70,000 g/mol).

The Ultraperformance Liquid Chromatography-Diode Array Detector-Electrospray Ionization-Mass Spectrometry (UPLC-DAD-ESI-MS) analysis was performed on a UPLC instrument using C18 analytical column (Acquity Waters, 100 mm × 2.1 mm, 1.7 mm particle size) at 30 °C. The mobile phase consisted of 0.1% formic acid in water (v/v) (phase A), and methanol (phase B). A 0.3 mL/min constant flow rate was applied with the following elution gradient: 0 min 95% A up to 0.5 min, 16 min 1% A up to 18 min, and 18.5 min 95% A up to 20 min. 5 μL were used as injection volume in the UPLC system. The UV spectra were recorded from 190 to 500 nm, but only chromatograms at wavelengths 254, 320 and 350 nm have been studied. For the mass spectrometry analysis, a LCT Premier ESI-TOF (Waters) was used. The analyses were performed using scans from *m/z* 50 to 2000. The capillary and cone voltages were set at 2000 and 50 V, respectively, in positive and negative ionization mode.

2.6. Antioxidant activity

DPPH, ABTS and FRAP spectrophotometric methods were used for the antioxidant activity measurement. The determinations were carried out using a Jasco V630 UV-Vis spectrophotometer, and in the three determinations the antioxidant activity was expressed as mg of Trolox equivalent/g of dry leaves (mg TE/g DW). All assays were performed in triplicate.

The total equivalent antioxidant capacity (TEAC) using DPPH was performed following the methodology described by Sillero et al. (2018), determining the absorbance at 515 nm. ABTS radical scavenging test was performed using the method used by Sillero et al.

Table 1
Selected parameters for the experimental design.

	Factors	Min (−1)	Max (+1)	Medium (0)
ML	Time (min)	2	20	11
	Temperature (°C)	50	70	60
FL	Time (min)	1	20	10.5
	Temperature (°C)	50	70	60

ML: male leaves of *P. vera*.; FL: female leaves of *P. vera*.

(2018), measuring the absorbance at 734 nm. Finally, ferric reducing antioxidant power (FRAP) assay was determined using the methodology described by Gullon et al. (2017), and determining the absorbance at 593 nm.

2.7. Antimicrobial activity

The leaves extracts were tested for antimicrobial activity against four bacterial strains and one yeast. Selected bacteria to evaluate the antimicrobial properties of pistachio leaves extracts were: *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Klebsiella pneumoniae* ATCC 9997 and *Pseudomonas aeruginosa* ATCC27853. The selected yeast was *Candida albicans* ATCC 10231.

2.7.1. Diffusion disc method

The antimicrobial activity of leaves extracts, obtained by maceration and MAE, was evaluated by a diffusion disc method according to the methodology described by Larrazabal-Fuentes et al. (2019). Briefly, sterile discs (diameter 6 mm) were impregnated with 10 μL of each solution samples (MLm, FLm, OptML and OptFL) diluted in DMSO (10%) and deposited on the agar surface. Depending on the strain or yeast being studied, different media were used: Mueller-Hinton agar inoculated with a bacterial suspensions (10^6 CFU mL^{-1}), Mueller Hinton agar with sheep blood (5%, v/v) inoculated with an *E. faecalis,* and Peptone Dextrose agar inoculated with *C. albicans*. The antimicrobial activity was evaluated measuring the diameter of inhibition zone expressed in millimeter (mm). All the plates were incubated for 24 h at 37 °C. DMSO (10%) was used as negative control agent, and the positive control agents were levofloxacin (5 μg/disc), used for Gram (+) bacteria, gentamycin (10 μg/disc), tested against Gram (−) bacteria, and miconazole (20 μg/disc), used for yeast. All experiments were conducted in triplicate. The results were expressed as the mean ± standard deviation (SD) of the inhibition diameter zone (mm).

2.7.2. Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentrations (MICs) were determined following the methodology described by CLSI (CLSI, 2007). Briefly, using 96-well microplates, in each well 100 μl of Müller-Hinton broth, 40 μl of extract solution (20–0.01 mg/mL) and 10 μl of suspension of the tested microorganism were added. After that, the plates were covered and incubated at 37 °C. For the test, successive dilutions of extracts were prepared and tested, varied from 20 to 0.01 mg/mL. DMSO (10%) was used as negative control agent, and the strains without samples were employed as positive control. The last dilution with no noticeable growth (turbidity) was considered as the MIC. All assays were done in triplicate.

2.8. Statistical analysis

Statistical analysis was conducted using SPSS statistical software (version 24, Inc. Chicago, IL, USA). This statistical analysis was carried out by ANOVA, estimating significant differences using Duncan's multiple range test. Results are given as mean ± standard deviation, and p-values < 0.05 were considered statistically significant.

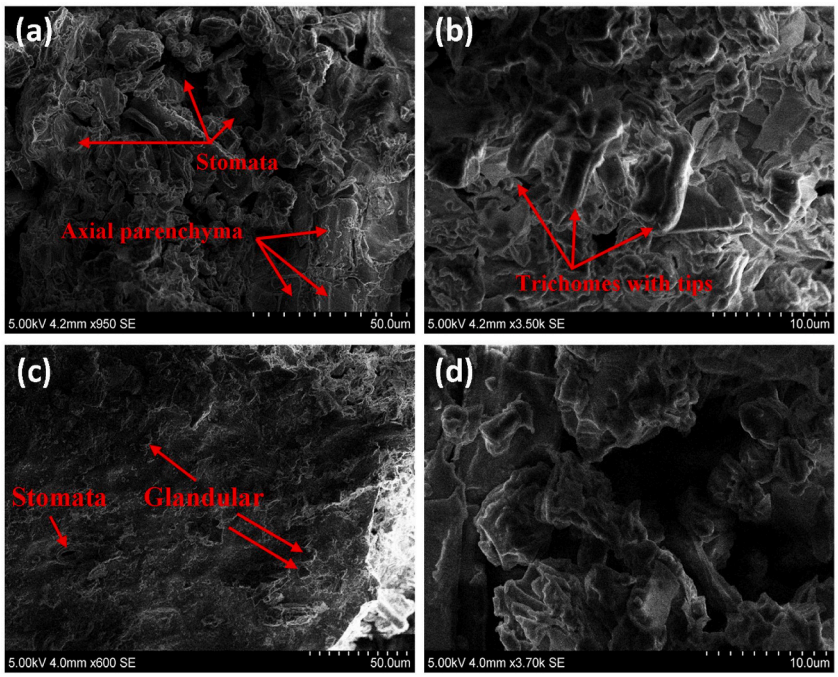

Fig. 1. SEM micrographs of the dorsal surface of *P. vera* leaves: (a and b) male leaves; (c and d) female leaves.

3. Results and discussion

3.1. SEM analysis

SEM is widely used to collect information about the surface, physiological state, and morphology of plant leaves (Kim, 2018). The male and female leaves of *P. vera* were examined by SEM analyses to investigate their structural differences (Fig. 1).

SEM micrographs of male leaves clearly showed tightly axial parenchyma cells associated with leaf venation and indicated several small openings on the surface, which are mainly representation of stomata (Fig. 1b). These rough perforated structures of *P. vera* male leaves are mainly responsible for the control of gas exchange. Mature simple trichomes with tips were observed in male leaves. These trichomes protect the leaves from microbial organisms and insects because the availability of secondary metabolites such as flavonoids. Female leaves have a smooth surface with the disposition of irregular glandular trichomes, stomata with thin waxy layer which reduce the rate of water loss from the leaf surface. Porous, wave-let and spongy microstructures were clearly shown in the female leaves surface (Fig. 1d). These amorphous morphologies make the material accessible to solvent diffusion into the solid matrix.

3.2. Optimization of the microwave assisted extraction (MAE)

A response surface method (RSM) was performed in order to optimize the extraction yield (%) in order to maximize it. For the experimental design, two factors at three level (−1, 0, 1) were used, with three replicates of the central point. Table 2 summarizes the parameters for each experiment (time (X_1) and temperature (X_2)), as well as the results obtained for male and female leaves.

Resulting from the experimental design, a quadratic regression equation for the female and male leaves microwave-assisted extractions were determined using the regression coefficients defined by the models (Eq. (1) and Eq. (2)).

$$Yield\ ML\ (\%) = 31.0712 - 0.7315\ X_1 + 1.721\ X_2 - 2.69862\ {X_1}^2 - 2.51975\ X_1X_2 + 0.872\ {X_2}^2 \quad (1)$$

$$Yield\ FL\ (\%) = 34.0312 + 0.3115\ X_1 + 0.6413\ X_2 - 0.846625\ {X_1}^2 - 0.2435\ X_1X_2 - 2.4191\ {X_2}^2 \quad (2)$$

In order to verify the interaction effect between the two extraction parameters (temperature and time) on the male and female leaves extraction yield, the three-dimensional (3D) response curves and contour plots are shown in Fig. 2.

The highest value of extraction yield from FL (36.26 ± 0.13%) was reached at 61 °C, and an extraction time of 12 min (Fig. 2b and d). The highest yield from ML (35.26 ± 0.49%) was achieved with temperature of 70 °C, and with extraction time of 5.6 min (Fig. 2a).

The time (X_1) and temperature (X_2) have a significant effect on ML extraction yield, as can be seen in Fig. 2a and c. To release the analytes dissolved in the solvent, ML need high temperature to reach the maximum value; in fact, solvent has the ability to dissolve more components at high temperature (Hemwimon et al., 2007; Shi et al., 2003).

Both extraction yields obtained under optimal conditions were comparable, but the optimal conditions were different. It should also be noted that there are significant differences between the results of the two optimizations (Table 3). For ML, when both temperature and time increase, the extraction yield decrease, so, to enhance the performance of extraction, a shorter extraction time was needed (less than 6 min), but higher temperature (70 °C). However, for FL, the maximum extraction yield was reached at medium temperature and time. The diffusion of extractable compounds through the leaves tissues to the solvent need longer extraction time and lower temperature than for ML.

Extraction time and temperature contribute to the efficiency of MAE yield as can be seen in Fig. 2c and Fig. 2d. According to the values in Table S1, only temperature has a significant influence on the FL optimization. While in the case of ML optimization, both variables have a significant influence on the extraction yield. The extraction yield was favored by the temperature increase for ML, and extending the extraction time for FL. Prior studies reported that temperature may enhance the recovery of bioactive compounds, because it increases their solubility, which facilitates their diffusion to the release medium (Shi et al., 2003). However, higher temperature might degrade the compounds especially when it reaches above 75 °C (Carrera et al., 2012). Irradiation time is also a critical factor because it influences the extraction rate. During the MAE process, the solutes are in contact with the solvent so the increase of

Table 2
Experimental design parameters and results of ML and FL microwave-assisted extraction (MAE).

Experiment	X_1	X_2	ML			FL		
			Time	T (°C)	Yield (%)	Time	T (°C)	Yield (%)
1	−1	1	2	70	33.81	1	70	31.30
2	0	0	11	60	30.50	10.5	60	34.90
3	0	0	11	60	30.07	10.5	60	34.46
4	0	−1	11	50	29.90	10.5	50	30.26
5	1	0	20	60	26.80	20	60	32.51
6	1	1	20	70	28.02	20	70	31.90
7	0	0	11	60	32.23	10.5	60	33.54
8	−1	0	2	60	29.70	1	60	32.70
9	0	0	11	60	31.74	10.5	60	33.58
10	−1	−1	2	50	25.54	1	50	29.70
11	1	−1	20	50	29.89	20	50	31.25
12	0	1	11	70	33.74	10.5	70	31.90
Optimal conditions			5.6	70	35.25	12	61	36.02

X_1: extraction time; X_2: temperature; ML: *P. vera* male leaves; FL: *P. vera* female leaves.

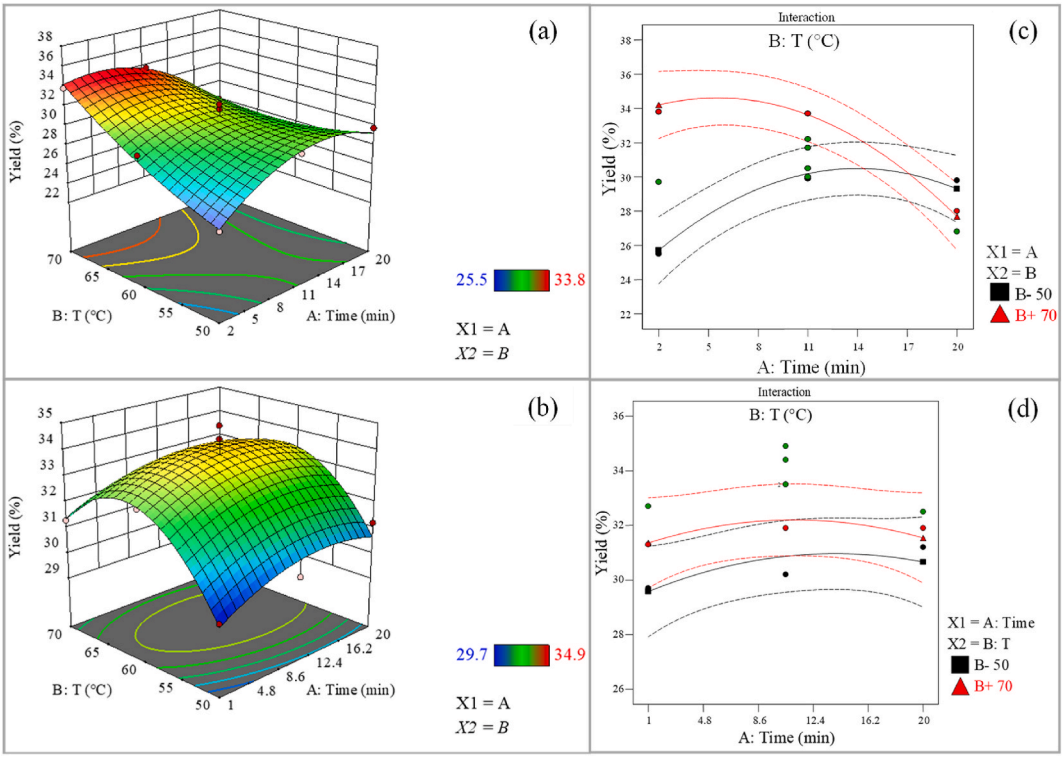

Fig. 2. RSM plots for MAE of male (a) and female (b) leaves of *P. vera*, and main effect plots showing the effect of each factor (X_1 and X_2) on the extraction yield from male (a) and female (b) leaves of *P. vera*.

Table 3
Extraction yield, TPC and TFC of MAE and maceration extracts of *P. vera* leaves.

Extracts	Extraction yield (%)	TPC (mg GAE/g DW)	TFC (mg CE/g DW)
OptML	35.25 ± 0.49^{a}	171.68 ± 3.01^{a}	82.16 ± 6.60^{a}
OptFL	36.26 ± 0.13^{b}	196.35 ± 10.74^{b}	83.34 ± 2.10^{a}
MLm	3.10 ± 0.14^{c}	1.60 ± 0.01^{c}	1.21 ± 0.04^{b}
FLm	3.20 ± 0.10^{c}	1.62 ± 0.01^{c}	1.15 ± 0.01^{b}

Values are given as mean ± SD from triplicate determinations. Different letters in the same column indicate significant differences between leaves extracts (Duncan's test, $p < 0.05$).
MLm: male leaves extracts obtained by maceration.
FLm: female leaves extracts obtained by maceration.
OptML: male leaves extracts obtained by MAE.
OptFL: female leaves extracts obtained by MAE.

irradiation time might enhances the solubility of the compounds, and their diffuse from cells to solvent. The quantity of extracted analytes reaches the maximum when the extraction time was 5.6 and 12 min for ML and FL, respectively.

To validate the model adequacy, three experiments were carried under the estimated optimal extraction conditions. The achieved experimental yields for OptML and OptFL were 35.25% and 36.06% (Table 2), respectively, while the predicted values were 34.5% for OptML and 34.9% for OptFL. Thus, the predicted values were comparable to the experimental results obtained by MAE under optimum extraction conditions, which confirmed the suitability of the models. The significance of the model was measured by R^2, being 93.02 for OptML, and 88.93 for OptFL.

3.3. Phenolic content of leaves extracts

MAE technique was compared with maceration extraction in terms of extraction yield, total phenolic content (TPC), total flavonoid content (TFC) and antioxidant capacities (DPPH, ABTS and FRAP) of male and female leaves extracts (Table 3).

The extraction yield of ML and FL obtained by MAE were higher compared with those obtained by maceration. By MAE, the 35–36% of extraction yield was reached, while by maceration, only 3.10–3.20% was achieved. It has been confirmed that there are no significant differences in the maceration extraction yield, while there are significant differences between the MAE, and between MAE and maceration. The increase of extraction yield confirmed the efficiency of the intensification process. The MAE technique achieves the highest extraction yields from leaves using less solvent and shorter extraction time. Extraction time was reduced from 24 h to 5.6

min and 12 min for ML and FL, respectively. The amount of used solvent was also decreased, from 70 mL to 40 mL. These results are in accordance with previous work. Rodríguez-Pérez et al. (2016) reported an extraction yield of 26 ± 2% from *Moringa oleifera* leaves with MAE (20 min, 42% ethanol and 158 °C). Moreover, Mubarak et al. (2015) investigated the effect of the microwave on the extraction yield from castor beans and reported optimum yield of 44.34% for 280 W and 120 s.

The TPC of the different extracts varied between 1.62 and 196.35 mg GAE/g DW, being the highest values those of MAE extracts. It has been confirmed that there are significant differences between the results of the two MAE optimizations, as well as with maceration. However, the results of the maceration of female and male leaves do not show significant differences. Regarding the TFC, the difference between the results from the extract obtained by maceration and by MAE remains large, with the best values calculated for OptML and OptFL, which are similar (Table 3). In this case, there are only significant differences between the two extraction methods, but no differences are seen when the same methods are used with either male or female leaves.

In previous work, TPC and TFC values of different plants have been reported, which are similar to those obtained in the present study using MAE. Dahmoune et al. investigated the use of MAE in different raw materials reporting TPC values of 185.69 ± 18.35 mg GAE/g for *Pistacia lentiscus* leaves extracts, and 162.49 ± 16.95 mg GAE/g for *Myrtus communis* leaf extracts (Dahmoune et al. 2014, 2015). Benamar et al. (2018) reported higher TPC (421.01 ± 8.92 mg GAE/g DW) obtained from aqueous extract of *Pistacia atlantica* subsp. leaf. Other studies reported lower TPC and TFC values for different leaves compared to those of OptML and OptFL. Dadi et al. (2019) reported 43.00 ± 0.50 mg GAE/g DW of TPC, and 17.20 ± 0.80 mg CE/g DW of TFC for *Moringa stenopetala* leaf extracts. Moreover, Grassino et al. (2016) found 21.56–47.52 mg GAE/g DW of TPC and 19.30–32.60 mg CE/g DW of TFC for tomato pectin. Rajaei et al. (2010) reported 61.02 ± 1.43 mg GAE/g DW for pistachio shell extracts obtained by MAE (20:1 (v/w), 65 °C, 45 min). The study of Elez Garofulić et al. (2021) presented different experimental concentrations of TPC in leaves (108.14 ± 2.12 mg GAE/g) and fruits (41.14 ± 0.76 mg GAE/g) extracts of *P. lentiscus* L. obtained by MAE under optimal conditions. The optimal conditions for leaves were 69 °C, 512 W and 12 min; while for fruits, 75 °C, 602 W and 15 min were employed. Butsat and Siriamornpun (2016) determined higher TPC and TFC values for *Amomum chinese* leaf extracts obtained by maceration than those achieved in this work with the same technique (8.33 mg GAE/g DW and 9.04 mg CE/g DW, respectively).

These results suggest that *P. vera* L. leaves extracts obtained by MAE can be considered as an interesting source of phenolics compounds with a potential valorization as food additive.

3.4. Biological activities

3.4.1. Antioxidant activity

The results of the antioxidant capacity of the extracts obtained by MAE and maceration evaluated by three techniques, DPPH, ABTS scavenging activity and ferric reducing assay power (FRAP), are illustrated in Table 4.

Each of the antioxidant capacity measurement methods provides different information. DPPH is an assay that measures the ability of hydrogen donation, while FRAP assay measures the ability of antioxidants present in extracts to act as reducing agents (Sillero et al., 2019). However, ABTS radical cation assay is an excellent way to determine the antioxidant activity of the lost electron of the ABTS radical (Dahmoune et al., 2015). For all the tests (DPPH, ABTS and FRAP), there are significant differences between the values calculated for the extracts obtained under the optimal conditions of MAE, and those obtained by maceration (Table 4). OptML and OptFL showed greater antioxidant activity than MLm and FLm. For the three tested methods, it is concluded that there are significant differences for the results calculated for female and male leaf extracts obtained by MAE, as well as for these in comparison with the ones obtained by maceration.

Different values of antioxidant capacity of extracts from various plants have been reported in the literature, but the comparison of these results with those obtained in this work should be made with caution. The scavenger effect of free radical DPPH of *Pistacia palaestina* leaf extracts obtained by maceration determined by Abu-Lafi et al. (2020) was 86 ± 2.75 mg TE/g DW, lower than MAE extracts studied in this work. In a study conducted by Liao et al. (2012) for the evaluation of the antioxidant capacity of *Abelmoschus esculentus* L. flower, lower DPPH value was reported (233.01 ± 2.46 mg TE/g DW). Botsaris et al. (2015) evaluated the scavenging activity of *Pistacia lentiscus* leaves extracts referring higher value for ABTS assay of 336 ± 10 mg TE/g DW compared to the values found in the present study. Whereas, for extracts from *Pistacia palaestina* leaves, lower ABTS antioxidant activity value (13.27 ± 1.65 mg TE/g DW) was reported by Abu-Lafi et al. (2020).

Regarding FRAP assay, Mohammad et al. (2019) reported the highest values of Trolox equivalents (143.70 ± 1.84 mg TE/g DW) for mangosteen pericarp extracts obtained by MAE. In a study where the antioxidant capacity of *Curcuma longa* L. root extracted by MAE is

Table 4
Antioxidant activity of MAE and maceration extracts from *P. vera* leaves.

Extracts	DPPH (mg TE/g DW)	ABTS (mg TE/g DW)	FRAP (mg TE/g DW)
OptML	277.32 ± 19.96^{a}	198.70 ± 9.57^{a}	162.44 ± 4.40^{a}
OptFL	300.64 ± 14.40^{b}	226.38 ± 5.03^{b}	187.38 ± 14.48^{b}
MLm	0.89 ± 0.07^{c}	11.24 ± 0.02^{c}	3.79 ± 0.14^{c}
FLm	0.91 ± 0.01^{c}	11.35 ± 0.14^{c}	4.59 ± 0.05^{c}

Data are given as mean ± SD from triplicate tests. Different letters in the same column indicate significant differences between leaves extracts (Duncan's test, $p < 0.05$).
MLm: male leaves extracts obtained by maceration.
FLm: female leaves extracts obtained by maceration.
OptML: male leaves extracts obtained by MAE.
OptFL: female leaves extracts obtained by MAE.

evaluated, a FRAP value of 255.66 ± 0.24 mg TE/g DW was reported (Fernández-Marín et al., 2021), which is higher than that found in the present study. FRAP values of 41.70 mg TE/g DW and 22.10 mg TE/g DW were reported for *Ginkgo biloba* leaf extracts obtained by MAE and maceration, respectively (Kaur et al., 2012).

Similar studies demonstrated the high ability of MAE method in extracting valuable bioactive compounds from different biomass compared to the conventional extraction process, such as Cherry laurel leaves (Karabegovic et al., 2014), *Pistacia lentiscus* leaves (Dahmoune et al., 2014) and *Melastoma sanguineum* fruit (Zhao et al., 2018).

3.4.2. Antimicrobial activity

The antimicrobial activity of leaves extracts from *P. vera* was tested against 2 Gram (+) bacteria, 2 Gram (−) bacteria, and one yeast. As shown in Table 5, leaves extracts obtained by maceration and MAE displayed various inhibitory zone diameters of the tested strains. The MAE extracts were more active against microorganisms than extracts obtained by maceration. Furthermore, female leaves extracts obtained by MAE exhibited the highest diameters of inhibition zones ranging from 18.20 ± 0.14 to 26.10 ± 0.51 mm, except against *P. aeruginosa* (Table 5).

The results demonstrated that *Staphylococcus aureus* (Gram (+) bacteria) is more sensitive to the OptML and OptFL extracts. In addition, to confirming that there are significant differences between these two extracts, it has been confirmed that both extracts perform significantly better than the blank. The antimicrobial activity of extracts can be attributed to their flavonoids content. Ulanowska et al. (2006) reported that flavonoids are good inhibitors of the enzymes that make up the cytoplasmic membrane of Gram (+) bacteria. In this context, Magiatis et al. (1999) reported that the antibacterial activity of *P. vera; P. terbinthus* and *P. lentiscus* was stronger against Gram (+) bacteria compared to Gram (−) bacteria, which was also confirmed in the present study. Another study conducted by Benabderrahmane et al. (2009) confirmed the inhibitory power of *P. atlantica* resin against *S. aureus, P. aeruginosa* and *K. pneumonia* bacterial growth.

All extracts obtained from *P. vera* inhibited the growth of *C. albicans*, with a diameters of inhibition zone ranged from 12 to 25 mm (Table 5). The extracts obtained by MAE were more efficient than those extracts obtained by maceration. Benabdallah et al. (2017) evaluated the antibacterial activity of essential oils from *P. atlantica* Desf. Leaves, determining lower inhibition values against *C. albicans* (12–14 mm) in comparison with those obtained by OptML and OptFL.

In order to evaluate the antimicrobial effects of obtained extracts, the minimum inhibitory concentration (MIC) were determined and results are showed in Table 6.

The values of MIC are inversely proportional to the inhibition diameters; leaves extracts with significant inhibition diameters have the lowest MIC. While *S. aureus* and *K. pneumoniae* were the most sensitive with MIC ranging from 0.04 to 0.01 mg/mL for MAE extracts, and from 0.60 to 0.10 mg/mL for macerated extracts, *P. aeruginosa* was the less sensitive bacteria to leaves extracts (Table 6).

This confirms *P. vera* leaves as a potential source of phenolic compounds with antioxidant and antimicrobial properties, as well as the extraction efficiency of these compounds by MAE. These extracts could be used in a variety of applications, including their use as an additive in bread baking. The use of the extracts as an additive in bakery industry will extend the shelf life of the food, thanks to their antioxidant and antimicrobial properties (Elakremi et al., 2022).

3.5. Infrared spectroscopy

The leaves extracts were characterized by FTIR in order to study their principal chemical functional groups. Fig. 3 illustrated the different bands and the corresponding wave numbers in the region between 4000 and 500 cm^{-1} of the leaves extracts obtained by maceration and MAE. All the spectral profiles of the extracts, which correspond to chemical structures of components from *P. vera* leaves, were very similar. The greatest differences are observed between extracts from different techniques rather than between male and female leaves. The assignments of the peaks were done according to the literature.

The large band between 3000 and 3500 cm^{-1} and the band at 1603 cm^{-1} were associated to the OH stretching vibration (Awwad and Albiss, 2015; Herrera et al., 2014). The fingerprint region (2900–2950 cm^{-1}) was attributed to the alkyl chains (Awwad and Albiss,

Table 5
Antimicrobial activity of leaves extracts from *P. vera* obtained with maceration and MAE.

Strains	Diameter of inhibition zone (mm)				
	OptML	MLm	OptFL	FLm	Antibiotic
Gram (+) bacteria					levofloxacin
Staphylococcus aureus	23.30 ± 0.20^{a}	18.20 ± 0.14^{b}	26.10 ± 0.51^{c}	19.10 ± 0.31^{d}	20.10 ± 0.13^{e}
Enterococcus faecalis	18.50 ± 0.51^{a}	14.10 ± 0.14^{b}	19.20 ± 0.15^{c}	14.20 ± 0.13^{b}	20.10 ± 0.08^{d}
Gram (−) bacteria					gentamycin
Klebsiella pneumoniae	20.10 ± 0.22^{a}	18.20 ± 0.66^{b}	20.20 ± 0.11^{a}	18.10 ± 0.22^{b}	21.10 ± 0.03^{c}
Pseudomonas aeruginosa	19.20 ± 0.55^{a}	16.20 ± 0.33^{b}	18.40 ± 0.88^{a}	14.30 ± 0.11^{c}	23.10 ± 0.11^{d}
Yeast					micronazole
Candida albicans	22.20 ± 0.32^{a}	12.20 ± 0.17^{b}	25.10 ± 0.15^{c}	15.20 ± 0.51^{d}	30.20 ± 0.04^{e}

Antibiotic: Levofloxacin (5 μg), Gentamycin (10 μg), Micronazole (20 μg). Data are given as mean ± SD. Different letters in the same row indicate significant differences between leaves extracts (Duncan's test, $p < 0.05$).
MLm: male leaves extracts obtained by maceration.
FLm: female leaves extracts obtained by maceration.
OptML: male leaves extracts obtained by MAE.
OptFL: female leaves extracts obtained by MAE.

Table 6
Minimum inhibitory concentrations (MIC) (mg/mL) of leaves extracts from *P. vera* obtained with maceration (MLm and FLm) and MAE (OptML and OptFL).

Strains	MIC (mg/mL)			
	MLm	FLm	OptML	OptFL
Gram (+)				
Staphylococcus aureus	0.6	0.1	0.02	0.01
Enterococcus faecalis	2.5	2.5	0.1	0.10
Gram (−)				
Klebsiella pneumoniae	0.6	0.3	0.04	0.04
Pseudomonas aeruginosa	2.5	2.5	0.30	0.20
Yeast				
Candida albicans	2.5	2.5	0.50	0.40

MLm: male leaves extracts obtained by maceration.
FLm: female leaves extracts obtained by maceration.
OptML: male leaves extracts obtained by MAE.
OptFL: female leaves extracts obtained by MAE.

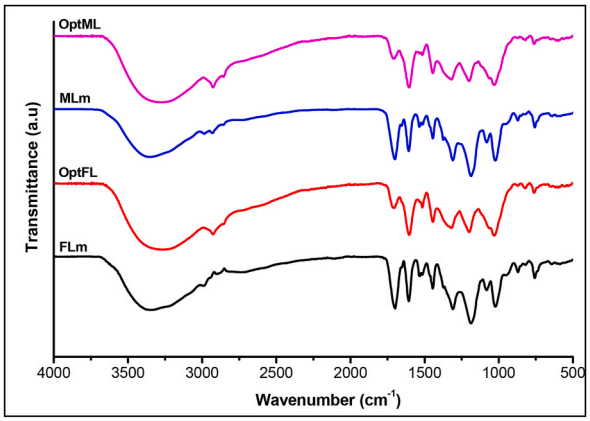

Fig. 3. FT-IR spectra of leaves extracts obtained by MAE (OptML and OptFL) and maceration (MLm and FLm).

2015). The peak at 1703 cm^{-1} was associated to the presence of carbonyl groups (Grassino et al., 2016). The band at 1515 cm^{-1} was assigned to the C–C aromatic compounds (Wahyono et al., 2019). The peak at 1444 cm^{-1} was attributed to C–H stretching vibrations. The absorption bands in the range of 1319, 1199 and 1030 cm^{-1} were attributed to CO stretching vibrations; these peaks were due to the presence of carbohydrates (Mahmoudvand et al., 2016). The bands from 900 to 720 cm^{-1} were assigned to C–H out of plane. Based on the above, we conclude that male and female leaves extracts had similar structural configurations.

3.6. Chromatography analysis

Finally, the extracts obtained by MAE under the optimum conditions were characterized by HPSEC and UPLC-DAD-ESI-MS. This study was carried out in order to identify the compounds that constitute the extracts and that provide their antioxidant and antimicrobial properties. The results are summarized in Tables 7 and 8.

The HPSEC study (Table 7) shows that there are differences between the sizes of the molecules present in the extracts of the female and male leaves. It can be said that the male extracts have a higher average molecular weight than the female ones, and that the dispersion index is higher. Nevertheless, it must be said that in both cases the dispersion index is high, which indicates that the extracts are constituted by a complex mixture of compounds.

In the compounds identification, essentially phenolic and polyphenolic compounds have been detected for OptML and OptFL (Table 8). The existence of flavonoid compounds is also confirmed by the presence of their derivatives (quercetin, myricetin and apigenin derivatives). The presence of dimers and trimers was also confirmed, demonstrating the degree of polymerisation discussed in the analysis of the molecular weight of the extracts (Table 7).

Based on the aforementioned, it can be concluded that male and female leaves are rich sources of phenolic compounds. The resulting extracts had strong antioxidant capacity as well as good antimicrobial activity, so the MAE extracts could be used in different applications such as medicine, cosmetic and foods. In addition, this study has also confirmed MAE as a suitable technique for the extraction of biomolecules from *P. vera* leaves.

4. Conclusions

In this study, a MAE method was optimized in order to maximize the extraction yield from *P. vera* leaves. The extracts obtained

Table 7
Weight average (Mw), number average (Mn) and polydispersity index (Mw/Mn) of OptML and OptFL.

	Weight average (Mw)	Number average (Mn)	Polydispersity index (Mw/Mn)
OptML	9,417	716	13.15
OptFL	6,416	656	9.78

Table 8
Tentative identification of the compounds of OptFL and OptML by UPLC-DAD-ESI-MS analysis.

R_t (min)	λ_{max} (nm)	Molecular ion [M − H] (m/z)	Peaks identification	OptFL	OptML	Ref.
7.226	254	197	Xanthoxylin	x	x	Zihad et al. (2021)
7.425	254	270	Apigenin		x	Zihad et al. (2021)
8.037	350	495	Digalloyl quinic acid	x	x	(Aouadi et al., 2019; Erşan et al., 2016)
8.037	350	517	Caffeic acid derivatives	x	x	Zhou et al. (2017)
8.652	350	446	Quercetin-hexoside		x	Baldan et al. (2017)
8.717	350	463	Quercetin-3-O-β-d-glucoside	x	x	(Aouadi et al., 2019; Erşan et al., 2016)
9.002	350	479	Myricetin 3-glucoside	x	x	Aouadi et al. (2019)
9.023	350	477	Quercetin glucuronide	x	x	(Aouadi et al., 2019; Erşan et al., 2016)
9.023	350	608	Quercetin-3-O-rutinoside	x	x	Aouadi et al. (2019)
9.177	254	631	Myricetin galloyl hexoside	x		(Aouadi et al., 2019; Erşan et al., 2016)
9.155	254	301	Quercetin hexoside	x	x	Aouadi et al. (2019)
9.637	254/350	617	Myricetin	x	x	Aouadi et al. (2019)
10.733	350	301	Quercetin hexoside	x	x	Aouadi et al. (2019)
10.733	350	515	Caffeic acid derivatives	x	x	Clifford et al. (2007)

under MAE optimum conditions exhibited higher values of TPC, TFC, antioxidant activities and antimicrobial activity compared to maceration extracts. MAE was shown to be more effective than the conventional extraction method for bioactive compounds recovery. MAE, apart from having better extraction yield and being richer in phenolic compounds, it has other advantages, such as the reduction of the extraction time and solvent consumption.

Regarding the possible differences between the extracts of the two genders of the leaves, it can be said that phenolic compounds are more abundant in female leaves. However, this cannot be confirmed since in the case of MAE extractions, there are significant differences between genders, but not, which raises the possibility of managing them together. *P. vera* leaves are thus confirmed as a rich source of phenolic compounds, natural antioxidants and natural preservative against pathogens that can be used in different applications. In particular, these extracts, due to their properties, could be used as food additives in order to extend the lifetime of food, such as bread. The results obtained in this work are promising, and confirm the leaves as a source of phenolic compounds. Nevertheless, this work is a preliminary approach, so further research will be necessary to investigate both, the potential applications of these compounds, and the possibility of scaling up the described process.

Author statement

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Leyre Sillero: Methodology, Validation, Writing- Original draft preparation, Writing- Reviewing and Editing.
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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scp.2022.100815.

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