Identification of some *Opuntia* spp. from two Algerian regions and ultrasound assisted extraction of their phenolic compounds

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ABSTRACT

Much attention has been paid to cacti for their distinctive characteristics. The nopal *Opuntia ficus-indica* and related species are found in arid and semi-arid regions around the world. *O. ficus-indica* shows considerable variability in response to environmental conditions through variation of areola and spine attributes, among other traits. Therefore, the present study sought to morphologically identify some cactus pear plants in two areas of Algeria (Bejaia and Souk-Ahras) in order to evaluate the cactus pears by optimizing the extraction conditions of the phenolic compounds of the cladode, to evaluate the antioxidant activity of these compounds, to measure the total flavonoid content, and to compare the yields among three *Opuntia* spp. (*O. ficus-indica*, *O. megacantha* and *O. amyclae*) from the sites within the study regions. These three *Opuntia* spp. were subjected to extraction of polyphenols using a conventional method, and an ecofriendly process using ultrasound-assisted extraction (UAE) technique. Once the extractions was completed, the total phenolic content and flavonoids of the extracts were characterized. In addition, the antioxidant capacity of the total phenolic compounds (TPC) was determined. The UAE was better than conventional extraction for the recovery of TPC. The use of a solvent mixture (50% water/ethanol, v/v) improved the TPC extraction efficiency. These results show that UAE was a useful tool for the extraction of phytochemicals from cactus pear cladodes. Other factors and conditions that influence the optimization of TPC extraction should also be studied, such as pH, ultrasound frequency as well as ecological factors that could possibly influence extraction efficiency.

Keywords: *Nopal, Opuntia* spp*., ultrasound assisted extraction, phenolic compounds.*

INTRODUCTION

A great deal of attention has been paid to cacti due to their distinctive characteristics, such as spiny, succulent cladodes that store water and their adaptation to arid conditions through CAM photosynthesis (Adli et al., 2017). Cactus pear or *Opuntia ficus-indica (L.)* Mill (Cactaceae) are among the highly abundant cacti in Algeria (Ksouri et al., 2007). The species originates Central and South America and was domesticated by the ancient Mexicans (Griffith, 2004). The *Opuntia* genus is widespread in Mexico and Central America, with 78 species native to Mexico, where many species are found, including wild and semi-domesticated cultivars (Astello-García et al., 2015). The domestication of *Opuntia ficus indica* (*OFI*) dates back from approximately 8,000 to 9,000 years ago (Bravo-Hollis and Sanchez-Merojada, 1991; Callen, 1967, 1965). Through the discovery of America, *O. ficus-indica* was brought to Spain for its anti-scurvy feature. It was subsequently spread to other parts of the world, mainly throughout the Mediterranean basin (Kiesling, 1998).

In Algeria, wild accessions of *O. ficus-indica* species showed considerable variability in response to environmental conditions through the occurrence of variation in areoles and spines (Adli et al., 2017). The commonly cultivated spineless form of *OFI*, resulted from a long cultural selection process and is lacking in wild populations. In 1919, Britton and Rose divided *Opuntia* in several series, emphasizing that, although the *Ficus-indicae* series (*O. ficus-indica*, *O. crassa* and *O. undulata*) has been closely linked to the Streptacanthae series (which includes 12 species, including *O. amyclae*, *O. megacantha*, and *O. streptacantha*), both were retained for convenience (Inglese et al., 1998). Both groups can be differentiated according to the size and the form of the cladode, number of areoles and the distance between them, length of the flower, number of spines per areole, as well as the color of the fruit peel (Reyes-Agüero et al., 2005).

The main identifying character of *O. ficus-indica* is its lack of spines. From the divergence of vegetative and reproductive characteristics, Reyes-Agüero et al., (2005) inferred that *O. ficus-indica* is taxonomically distinct from *O. megacantha* and *O. streptacantha*. Furthermore, Kiesling (1998) indicated that spiny and spineless varieties are individual forms of O. *ficus-indica*. *O. ficus-indica* has great economic and ecological importance as a fodder for livestock or as a medicinal plant, due to its health-promoting attributes (Angulo-Bejarano et al., 2014). In Mexico, young cladodes (nopalitos) are consumed as a human food and are prepared as a vegetable dish (Griffith, 2004). Cactus flowers, fruits and leaves are consumed as vegetables in different areas of the globe (Kamble et al., 2017). Cactus derivatives like pad juice and cladode mucilage are very effective components for water purification solely or in combination with wastewater treatment processes (Adjeroud et al., 2018, 2015; Djerroud et al., 2018). Some other studies have been performed in Algeria on the extraction and characterization of mucilage from cladodes of *O. ficus-indica* (Adjeroud-Abdellatif et al., 2020; Adjeroud et al., 2018, 2015; Djerroud et al., 2018; Felkai-Haddache et al., 2016; Lefsih et al., 2018). Another study reported the phytochemical content and physical characteristics as well as the antioxidant activity of Algerian cactus pear fruits grown in northeastern region of the country (Mazari et al., 2018). This study showed that the growing site markedly influenced the biometric parameters of the fruit, including size and weight, besides the content of phytochemical classes and the level of the antioxidant capacity of the fruit pulp (Mazari et al., 2018).

Several studies have examined and reviewed the flavonoids content of the various organs of the cactus pear plant. According to del Socorro Santos Díaz et al., (2017), the most common compounds present in *Opuntia* tissues from wild and cultivated species include kaempferol, quercetin, isorhamnetin, and isorhamnetin glucosides. *O. ficus-indica* flowers contain 3-glycosides of isorhamnetin and their yellow petals yield penduletin, kaempferol, luteolin, quercetin, and rutin (Brinker, 2009). The pharmacological activity of *O. ficus indica* flower extract could be associated with its abundance of isorhamnetin 3-O-robinobioside (De Leo et al., 2010). Pads are also a source of polyphenols. A recent comparative analysis of 15 *Opuntia* cultivars from *O. streptacantha, O. hyptiacantha, O. megacantha, O. albicarpa*, and *O. ficus-indica*, cultivated under the same environmental conditions and at the same developmental stage, showed that metabolite content in cladodes was independent of domestication grade. Thus, such differences depended upon the biochemical characteristics of each species (Astello-García et al., 2015).

Fruit skin tissue of *O. ficus-indica* appeared to be more enriched in flavonoids compared with pulp (Farag et al., 2017). El-Hawary et al., (2020), identified 37 secondary metabolites, mainly isorhamnetin glycosides, from extracts of different parts (cladodes, fruit peel and fruit pulp) of *O. ficus indica*. According to their findings, *O. ficus indica* extracts decreased $AlCl_3$-induced neuroinflammation in rat brain. This function was assigned to the antioxidant effect of flavonoids (as glycoside or free aglycones), which could cross the blood-brain barrier as demonstrated in cell models and *in vivo* experiments (Faria et al., 2010; Youdim et al., 2003).

The extraction of phenolic compounds from plant material is a very important step for their industrial applications. The effectiveness of extraction as well as the antioxidant potential could be significantly influenced by certain variables such as, solvent nature and concentration, temperature, and extraction time (Pradal et al., 2018). Conventional methods for bioactive compounds extraction, including extraction with solvents (solid-liquid and liquid-liquid extraction) backed by pressing, mechanical shaking, or heating systems are commonly used and generally require high solvent consumption, and high temperatures during prolonged treatment times. Therefore, the economic costs, together with an elevated risk of thermal sensitive components alteration, are important considerations when performing extractions (Pradal et al., 2018; Spigno et al., 2007). However, some new “eco-friendly” extraction methods, which, usually consume less solvent and energy, have been described to address these limitations (Chemat et al., 2012). Over the last years, several alternative methods have been used, including pressurized liquid extraction, supercritical fluid extraction, microwave-assisted extraction, and ultrasound-assisted extraction (Dahmoune et al., 2013). The latter is a well-adapted method for the extraction of polyphenols from plants (Pradal et al., 2018). Ultrasound-assisted extraction (UAE) of *OFI* cladode mucilage was used to enhance electrocoagulation-electroflotation (EC-EF) water treatment (Adjeroud-Abdellatif et al., 2020).

The objectives of this work were to (i) identify morphologically diverse cactus pear plants in two areas of Algeria (Bejaia and Souk-Ahras), (ii) valorize the cactus pears by optimizing the extraction conditions of the cladode phenolic compounds, (iii) evaluate the antioxidant activity of these compounds, (iv) measure the total flavonoids content and compare the yields among three *Opuntia* spp. (*O. ficus-indica*, *O. megacantha*, and *O. amyclae*) of the study region sites.

MATERIAL AND METHODS

Ecotype selection

The different ecotypes of the genus *Opuntia* were selected based on their morphology and the quality of their fruits. Over the prospected *Opuntia* accessions in Algeria, seven were selected from two regions for sampling: Bejaia (Bir-Essalam, Oued-ghir, Amizour and Taourirt), located within the sub-humid zone, with precipitation approaching 718 mm per year, and Souk-Ahras region (Merahna and Sidi-Fredj), which is within the semi-arid zone with precipitation of about 265 mm per year.

Table 1. Climatic and geographic distribution of the cactus pear accessions.

Location	T (°C)	Altitude (m)	Rainfall (mm)	Latitude	Longitude	Vernacular accession names	Accession Scientific names
Bejaia							
Bir-Essalam	13.87	28	875	36°43'20.95"N	5°03'23.81"E	Imeslem	(*OFI*)
Oued-ghir	14.15	99	864	36°43'08,7"N	4°58'51,2"E	Arrumi	(*OM*)
Amizour	18	204	832	36°38'54.3"N	4°55'57.0"E	Arrumi	(*OA*)
Taourirt	16.21	300	750	36°39'28.97"N	4°72'71.23"E	Imeslem	(*OFI*)
Souk-Ahras							
Merahna1	15.1	805	254	36°11'49.1"N	8°10'11.6"E	Chouak 1	(*OA*)
Merahna2	15.1	843	238	36°12'53,1"N	8°13'12,5"E	Chouak 2	(*OM*)
Sidi-Fredj	15	747	221	36°10'23,2"N	8°12'17,8"E	Imeslem	(*OFI*)

OA: Opuntia amyclae, OFI: Opuntia ficus-indica, OM: Opuntia megacantha.
T: Yearly Average Temperature.

Bejaia is located in the central north region of Algeria, as part of the Wadi Soummam watershed. It has a Mediterranean climate, which is usually wet with a slight change in seasonal temperature, whereas Souk-Ahras, which is situated in the interior eastern region of the country, is characterized by a warm and temperate climate. In Sidi-Fredj (Souk-Ahras), the annual average temperature is nearly 15 °C, with a pronounced amplitude being able to go down to -7 °C between December and March (very cold winter) and > 35 °C in summer months (hot summer). The climate in Bejaia region is warm and temperate. In summer, the rainfall is less than in winter, and the average temperature reaches > 25 °C in August, and > 10.1 °C in January. The collection sites were presented in Table 1 and the accessions were illustrated in Figure 1.

The introduction of the cactus to North Africa had two origins: First, it was favored by the Spanish expansion during the sixteenth and seventeenth centuries and by the return of the Moors to their homeland when they were finally expelled from Spain in 1610. They took with them the “Indian fig tree” with its succulent fruits and planted them around their villages (Inglese et al., 2018). On the other hand, Gonzalo Fernández de Oviedo (1478-1577) reported in his book (Natural and General History of the Indies, 1536) that the prickly pear plantations were used as hedges to defend themselves from the Spaniards in the midst of the war towards the end of the 14th century in North Africa. de Oviedo described the plant precisely for the first

time. He cited that his earliest traces in North Africa date from around 1505 to 1510 (Inglese et al., 2018). The second origin corresponds to projects during the 1930s and 1940s wherein plantations were carried out not only within the framework of FAO projects, but also by French settlers in Algeria.

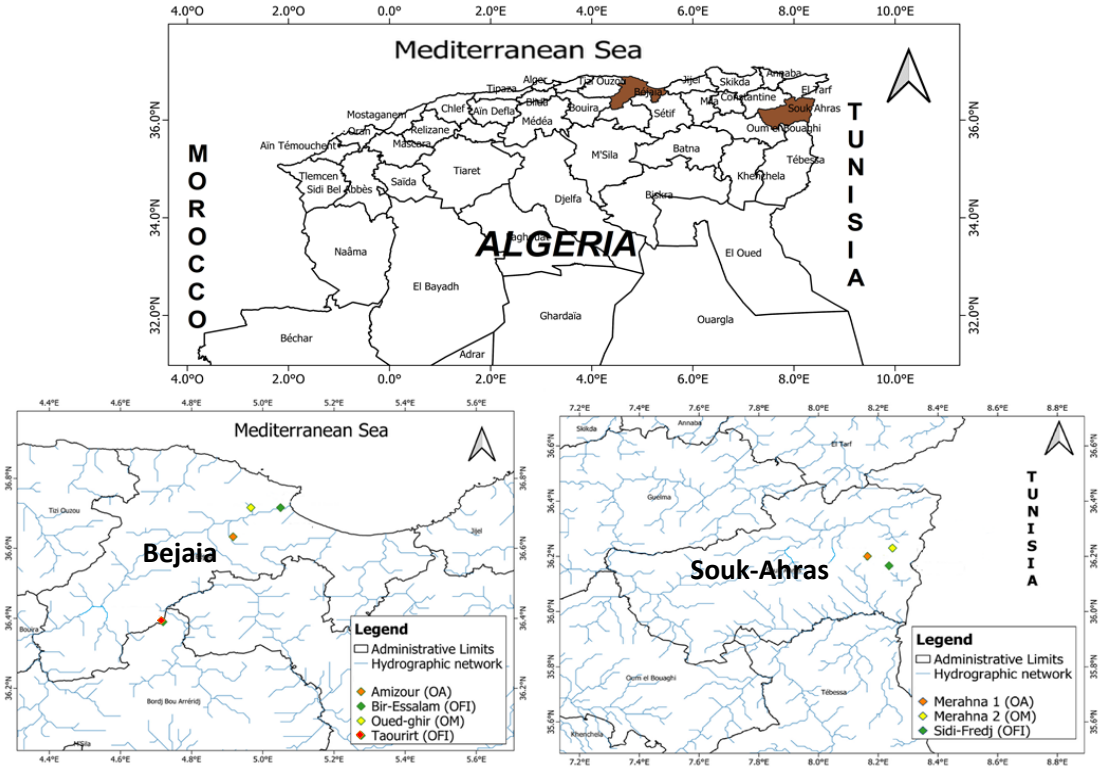

Figure 1. Enlarged view of cactus pear species collection areas in Algeria: from Souk-Ahras (east side): *Opuntia ficus-indica* (*OFI*) was collected from Sidi-fredj, *Opuntia amyclae* (*OA*) and *Opuntia megacantha* (*OM*) species were collected from Merahna 1 and Merhana 2 areas, respectively. Bejaia (west side): *OM* was collected from Oued-ghir, *OA* was collected from Amizour, and *OFI* species were collected from Bir-Essalam and Taourirt.

Plant material preparation

The plant samples (cladodes) were kept in a dry place at room temperature until used. Cladodes were cleaned, dark spots were removed, and then were cut into small strips. After sun-drying, cladode strips were placed in a ventilated oven at 56 ± 1°C for 48 h, according to the modified protocol (Barka et al., 2013b). An electric grinder (A11Basic, IKA, Retsch, Germany) was used to grind the dried samples into a powder. The latter was then sieved with < 200 µm pore diameter sifter (Retsch, Germany) in order to obtain a relatively fine powder (Msaddak et al., 2017). The powders were kept in tinted glass bottles to avoid compounds oxidization.

Polyphenols extraction

The extraction of polyphenols was first made by a conventional method (CE) and a more ecofriendly process using ultrasound-assisted extraction. In both processes, some factors were varied in order to improve the extraction yields. This preliminary optimization was made previously on the collected *O. ficus-indica* (*OFI*) species from the Bejaia region (Bir-Essalam). Then optimal conditions were applied to polyphenols ultrasound-assisted extraction of the other *Opuntia* spp. cladodes (Figure 2), considering that all collected accessions belonged to the *Opuntia* genus.

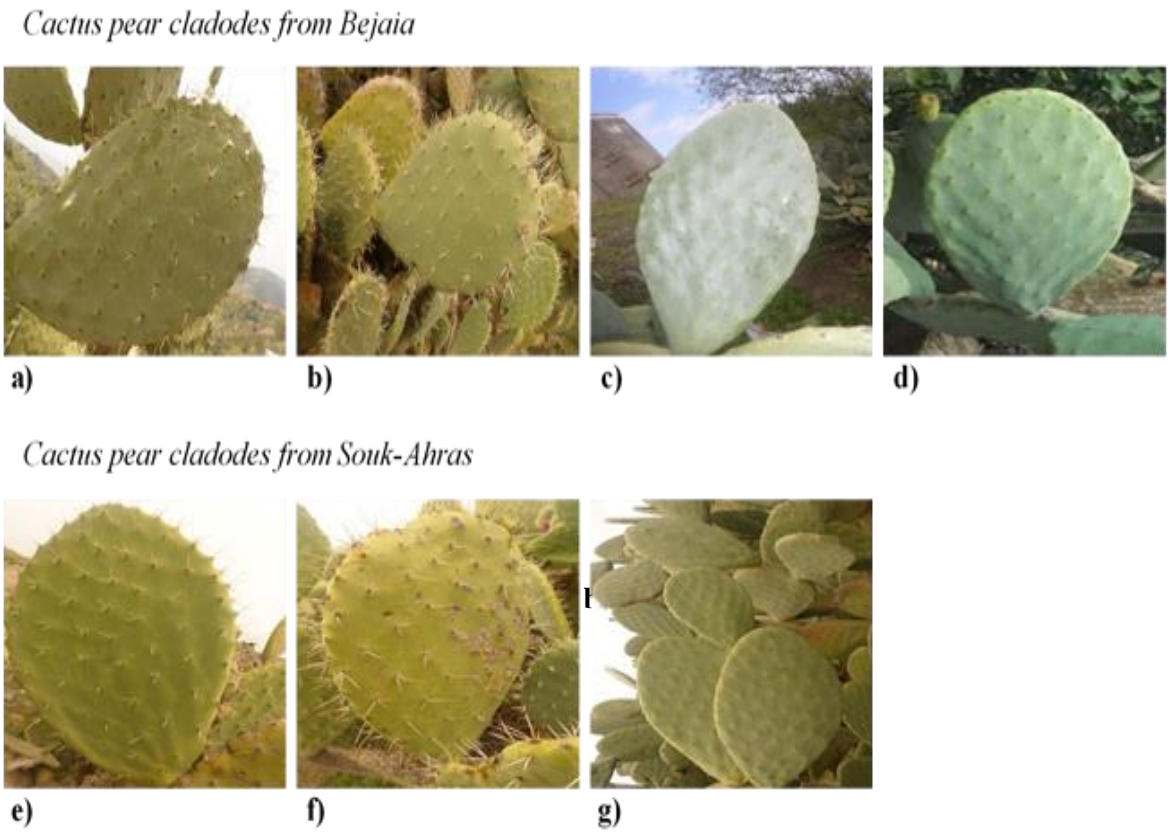

Figure 2. Cactus pear cladodes from different accessions in Bejaia and Souk-Ahras regions: *Opuntia amyclae* (**a** and **e**), *Opuntia megacantha* (**b** and **f**), *Opuntia ficus-indica* (**c** (Taourirt), **d** (Bir-Essalam), and **g** (Sidi-Fredj)).

Conventional extraction

Total phenolic compounds (TPC) were extracted following the conventional solid-liquid extraction (CE) described by Guevara-Figueroa et al., (2010) and Dahmoune et al., (2013). One of the parameters that could affect extraction yield and phytochemical content is solvent concentration. In this context, as an initial experiment, three concentrations were tested to select the optimal solvent concentration. The sample, 0.5 g of Bir-Essalam's *OFI* powder, was added to 50 mL of ethanol at the concentrations 20, 50, and 96% (v/v). The mixtures were stirred at room temperature (20~22 °C) for 2 h 50 min, then centrifuged at 5000 x *g* (TD5-1C

Benchtop low speed centrifuge, China). Each extraction was repeated three times. The supernatants were recovered and stored at 4 °C until use.

Ultrasound-assisted extraction

A sample of Bir-Essalam's *OFI* powder (0.5 g) was extracted with 50 mL of ethanol-water (50%, v/v) and subjected to ultrasonication (42 ± 2.5 KHz) using an ultrasonic bath (2.8 L tank capacity, Bransonic, 2510E-DTH, Mexico) at 20°C. Ultrasound-assisted extraction (UAE) was carried out at various extraction times (1, 5, 10, 15, 20, 30, 40, 50, 60, 70, and 80 min). Further, after determining the optimal extraction time, similar conditions were applied for the optimization of the best extraction temperature. All experiments were carried out in triplicates for each parameter. The best time and temperature for extraction were selected based on the maximum TPC value.

Determination of total phenolic content

The TPC was quantified using the protocol of Georgé et al., (2005). An aliquot of 0.25 mL of extract was dissolved in 1.25 mL diluted Folin–Ciocalteu reagent. The resulting solution was mixed and incubated at room temperature for 5 min. Then, 1 mL of sodium carbonate solution (Na_2CO_3; 7.5% w/v) was added. After incubation 15 min at 50 °C in a water bath, the absorbance was measured at 760 nm using a UV-Vis spectrophotometer (Spectroscan 50, Japan) against a blank. The calibration curve was set using gallic acid as standard. The polyphenol content was then calculated from the calibration curve. The results were reported as mg GAE/g DW.

Determination of total flavonoids

In accordance with the method defined by Djeridane et al., (2006), total flavonoids (TF) content of extracts was determined. A 1 mL of extract was added to 1 mL of aluminum chloride solution ($AlCl_3$; 2% w/v). The mixture was assessed for absorbance against a blank at 430 nm after being incubated in the dark at room temperature. The calibration curve was prepared using quercetin as standard. TF content was expressed as milligrams quercetin equivalents (QE) per gram dry weight (mg QE/g DW).

Evaluation of the antioxidant capacity

The ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical cation assay is frequently used method for determining the concentration of free radicals. The antioxidant capacity of samples was evaluated by the method of Re et al., (1999) using an ABTS radical inhibition test. Exposing the ABTS solution to an antioxidant reduces cationic radical activity and the absorbance rate at 734 nm. A previously prepared (12~16 *h* before use) radical ABTS solution composed of ABTS (7 mM) and potassium persulfate (6.62 mg) was diluted with 96% ethanol until an initial optical density of 0.7 ± 0.02 was obtained (A_{Blank}) at 734 nm. Then, 0.2 mL of the sample extract to be tested were added to 1.8 mL of the ABTS solution. After 6 min of incubation in the dark the reduced $ABTS^+$ solution was measured at 734 nm, to determine the optical density of the reacted extract ($A_{Extract}$). The antioxidant capacity of the tested extracts was expressed to the relative concentrations of Trolox standard in milligram equivalent Trolox antioxidant capacity per gram of dry weight (mg TEAC/g DW). The antioxidant capacity (AOC) was calculated as percentage absorbance decrease as in (Eq. (1)):

$$AOC = \frac{A_{Blank} - A_{Extract}}{A_{Blank}} . 100 \quad (1)$$

Fourier-transform Infrared (FTIR) spectroscopy

The cladode powders of three species (Bejaia and Souk-Ahras's *Opuntia ficus indica* (*OFI*), *O. megacantha* (*OM*), and *O. amyclae* (*OA*)) were analyzed using FTIR. Each sample was prepared in the form of pellets made from a fine mixture of 2 g of cladode powder and 80 mg of potassium bromide KBr. The mixture was then pressed (60 bar) in a pelletizer for 1 min. Spectra were recorded in the absorbance mode at room temperature, in the mid-infrared range of 400 to 4000 cm^{-1}, using a Thermo Nicolet spectrometer (Avatar 370 FT-IR) coupled to a computer loaded with IR-solution software.

Statistical study

All data were reported as the average of three replicate tests. An analysis of the variance (ANOVA), followed by Fischer's LSD test, were applied using XLSTAT (v. 2014.5.03) software to display significant differences between varied parameters in conventional or ultrasound-assisted extractions. The degree of significance of the data was considered at the probability of $p < 0.05$. The results were reported as mean ± standard deviation (S.D.).

RESULTS AND DISCUSSION

Ecotypes morphological identification

From the available morphological data of cactus pear plants of both regions, physical characters were observed and were sufficient to their classifications. The names of the plants were those of the traditional classification (Anderson and Brown, 2001; Bravo Hollis and Sánchez Mejorada, 1978; Britton and Rose, 1963), as well as that of Kiesling (1998).

Some characteristics of the collected accessions and plant cladodes were presented in Table 2. The descriptor of the International Union for the Protection of New Varieties of Plants (UPOV) was used for morphological characteristic determination (UPOV, 2004). The color of cladode was ascertained using a Munsell Color Chart for Plant Tissues.

O. ficus-indica (*sensu stricto*) is a plant without spines; however, in *sensu lato* the designation spiny plants are accepted (Arreola Nava and Portillo Martinez, 1994; Kiesling, 1998), as this character helps to identify subspecies forms. According to Kiesling, (1998) there are only two forms, both belonging to *O. ficus-indica*; one with spines: *amyclae* (Figures **2 a** and **e**) and *megacantha* forms (Figures **2 b** and **f**), and the second spineless one *ficus-indica* form (Figures **2 c**, **d** and **g**). In literature, both forms are synonyms of *O. megacantha* and *O. ficus-indica*, respectively (Bravo Hollis and Sánchez Mejorada, 1978), and are present in Algeria.

Table 2. Characteristics of collected cactus pear plants.

Accession name	Plant		Cladode	
	Growth habit	Shape	Thickness	Color
Bejaia				
Opuntia amyclae	upright	narrow obovate	thick	medium green (2,5 G 5/4)
Opuntia ficus indica (Bir-Essalam)	upright	medium elliptic	medium	dark green (5 G 5/4)
Opuntia ficus indica (Taourirt)	upright	medium elliptic	medium	dark green (5 G 5/4)
Opuntia megacantha	spreading	narrow obovate	thick	yellow green (7,5 GY 6/2)
Souk-Ahras				
Opuntia amyclae	spreading	medium elliptic	medium	yellow green (7,5 GY 6/4)
Opuntia megacantha	spreading	rhombic	medium	green (2,5 G 6/6)
Opuntia ficus indica	spreading	broad elliptic	medium	yellow green (7,5 GY 6/4)

Extraction of total phenolic content

In order to optimize phenolic compounds extraction from cactus pear cladodes, some parameters were taken into consideration including method of extraction, solvent concentration, and time and temperature of extraction.

Conventional extraction (CE)

Solid-liquid extraction with stirring was carried out at ambient temperature (20~22 °C) on Bir-Essalam's *OFI* cladode powder. The effect of different concentrations of ethanol (20, 50, 96% v/v) on extraction efficiency was monitored. The results were given in Figure 3. The highest content was recorded using 50% ethanol (24.88 ± 2.74 mg GAE/g DW) followed by 20% ethanol (17.75 ± 1.77 mg GAE/g DW). Ethanol 96% resulted in the lowest amount (6.59 ± 1.58 mg GAE/g DW) of extracted total phenolic compounds (TPC), after 2 h 50 min extraction time.

Our results are in agreement with those of several authors whom revealed that mixed aqueous-organic solvents were very effective for extraction of phenolic compounds. The use of aqueous-organic solvents resulted in a highly enriched phenolic compounds extracts (Mohammedi and Atik, 2011; Zhao et al., 2006; Zhou and Yu, 2004). This is due to the increase of the solubility of the phenolic compounds in the extracts compared to the use of pure solvent (Trabelsi et al., 2010). This may be due to the increase in the basicity and ionization of polyphenols in such solutions. Previous studies have shown that the use of very pure organic solvents can cause damage to plant cells through the denaturation of the cell wall proteins and dehydration and degradation of the cells, making it difficult for phenolic compounds to be extracted (Amendola et al., 2010; Galvan d'Alessandro et al., 2012; Garcia-Castello et al., 2015; Librán et al., 2013).

In this context, ethanol (50%, v/v) was shown to be the optimal solvent concentration for the highest extraction yield of phenolic compounds (Şahin and Şamlı, 2013). The authors emphasize a synergistic effect between solvents, where water behaves as a swelling agent for the sample matrix, while ethanol causes bond breakage between the solutes and the matrix. The 50% ethanol solvent ratio was ideal for TPC extraction; therefore, this concentration was used for further extractions.

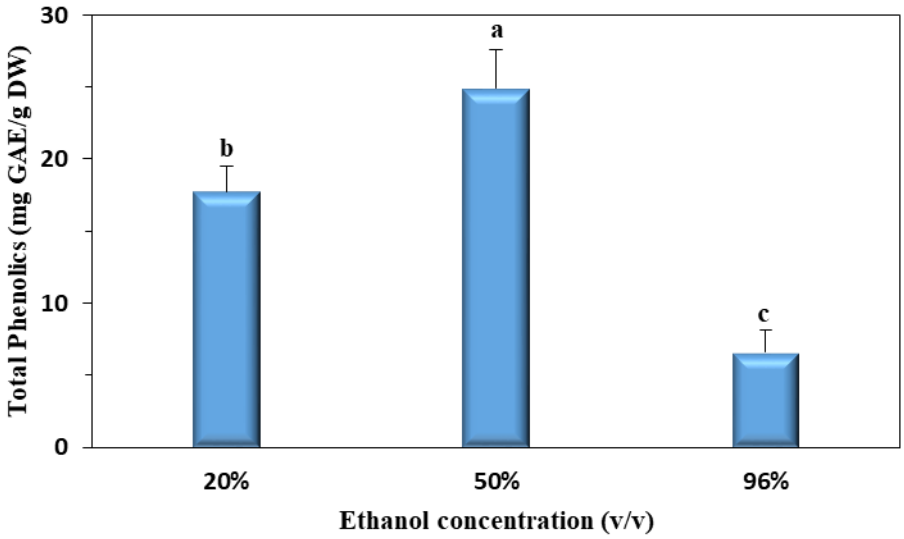

Figure 3. Effect of solvent concentration on the level of total phenolic content (TPC) of Bir-Essalam's *Opuntia ficus-indica* (*OFI*) cladode powder treated with conventional extraction (CE). Values were means ± s.d. of three measurements. The different letters indicate that the samples were significantly different ($p < 0.05$).

Ultrasound-Assisted Extraction

Determination of the optimal extraction time

Ultrasound-assisted extraction was applied for TPC extraction from Bir-Essalam *OFI* cladode powder using 50% ethanol, at room temperature (20~22 °C) and at different extraction time (from 1 to 80 min). When extraction time was increased, TPC initially increased and reached maximum values of 34.84 ± 1.70 and 36.41 ± 1.33 mg GAE/g DW at 30 and 60 min, respectively (Figure 4). However, TPC yield declined with increased sonication times and the lowest levels were observed at 70 and 80 min.

The phenolic compounds content extracted by ultrasound has been reported to increase time-dependent manner following a process involving two main stages (Şahin and Şamlı, 2013). The first one called a washing step, which covers the first 10 to 20 min of extraction. Soluble components are dissolved on the matrix surfaces. In this step, up to 90% of the overall content of phenolic compounds can be recovered. The mass transfer undertaken by diffusion in the second stage of this process, which is called slow extraction, can last from 60 to 100 minutes (Şahin and Şamlı, 2013). Several studies have shown that a prolonged contact time between the solvent and the plant material can lead to the degradation of certain active ingredients. The long duration of the ultrasound treatment can degrade the phenolic compounds, thus reducing the extraction yield (Carrera et al., 2012; Odabaş and Koca, 2016). Also, long-term ultrasonic irradiation might impair the stability of heat-sensitive compounds (Ma et al., 2008).

The results showed no substantial difference between sonication times of 1~20 min and 80 min. However, the best TPC yields were obtained at 30 and 60 min. Thus, 30 min was used as optimal UAE time for the rest of the study. Excessive sonication times can increase the

amount of energy required to release the target molecules, while altering the phenolic compounds (Carrera et al., 2012).

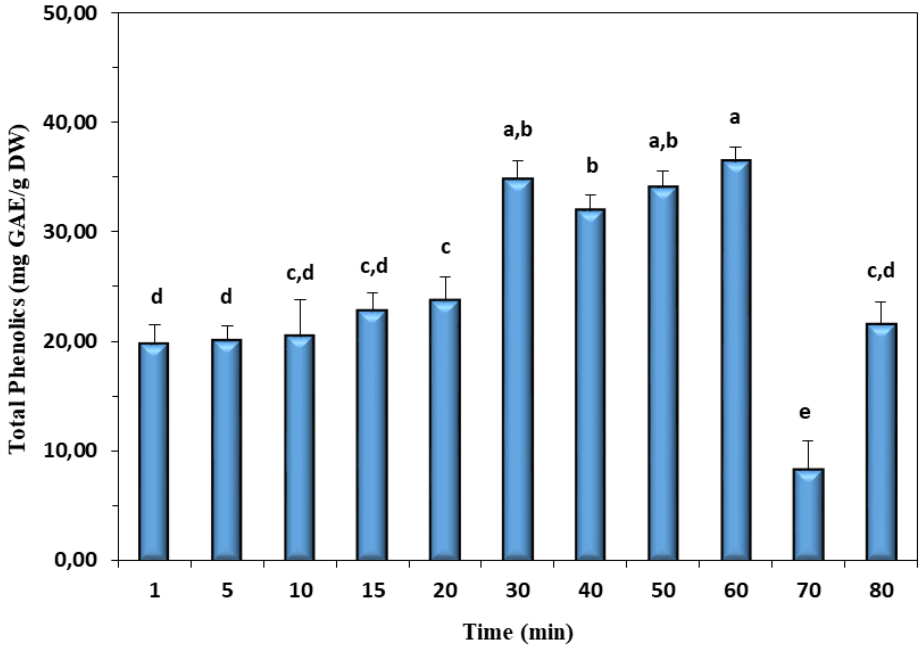

Figure 4. Effect of ultrasound-assisted extraction (UAE) treatment time on the total polyphenol content of Bir-Essalam's *Opuntia ficus indica* cladode powder. UAE conditions: 50% ethanol concentration at 20 °C. Values are means ± s.d. of three measurements. The different letters indicate that the samples are significantly different ($p < 0.05$).

Determination of the optimal extraction temperature

The effect of temperature on TPC was evaluated between 30 and 70 °C, with the fixed parameters: extraction time of 30 min and 50% ethanol concentration. The results indicated that TPC yields increased with increasing temperatures (Figure 5). Indeed, polyphenol concentration reached a desorption and solubility equilibrium at 60 °C with a TPC yield of 39.15 ± 1.78 mg GAE/g DW, while the lowest value was obtained at 30 °C (28.02 ± 1.86 mg GAE/g DW). TPC fell to 32.12 ± 2.83 mg GAE/g DW at 70 °C. Consequently, the temperature of 60°C was considered as the best temperature for polyphenols extraction. Jaramillo-Flores et al., (2003) reported that thermal treatment for 30 minutes increased the extractability of carotenoids and phenolic compounds of cactus pear cladodes.

TPC yield increased along with the rise in the temperature of extraction. The increase of the temperature could modify the structure of the plant matrix, and therefore, facilitate the extraction process (Prasad et al., 2009). Thus, high temperatures can speed up the softening and swelling of the raw materials, increasing the solubility of the extracted compounds (Vilkhu et al., 2008) and their diffusivity in the solvent and improves material transfer. However, if the temperature continues to rise, it could alter the properties of ultrasonic cavitation and oxidize the phenolic compounds (Tchabo et al., 2015; Vilkhu et al., 2008) causing denaturation of the products to be extracted, which may affect the solubility of the phenolic compounds. In addition, a high temperature will lead to high-energy consumption (Medina-Torres et al., 2017).

Ma et al., (2008) observed a reduction in the extraction yield of polyphenols at elevated temperatures. This has also been demonstrated by Carrera et al., (2012), when temperatures above 75°C resulted in denaturation, which potentially decreased the phenolic content. High temperatures application involves the limitation of reducing the cavitation intensity, subsequent to the decreased surface tension and enhanced vapor pressure of the cavitation bubbles (Chemat et al., 2017; Tao and Sun, 2015). Moreover, Angulo-Bejarano et al., (2014) reported several TPC levels: 562-905 µg of GAE/g, 0.57-2.3 mg GAE/g, and 2.7-3.7 g GAE/100g DW for *OFI* nopal flour (dw), dehydrated nopal, and nopal by-products, respectively.

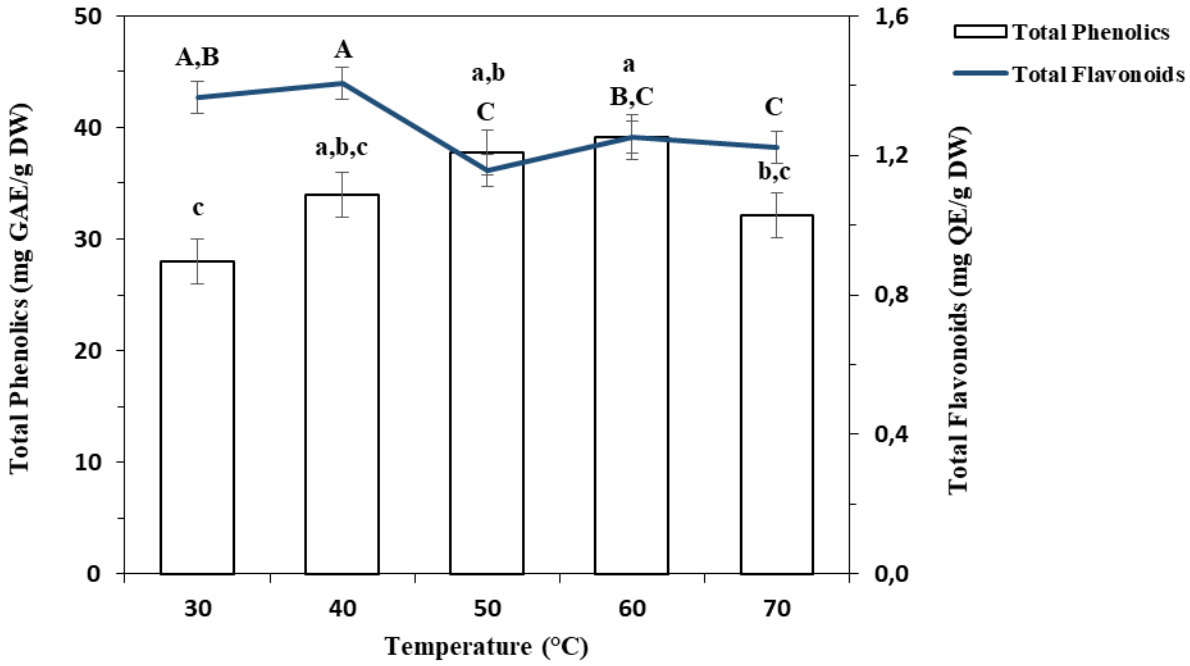

Figure 5. Effect of temperature on the total polyphenols and total flavonoids contents of the extracts of *O. ficus indica* cladodes powder from Bir-Essalem subjected to ultrasound-assisted extraction (UAE). Conditions of UAE: 50% ethanol, 30 min sonication time. Values are means ± s.d. of three measurements. The different letters indicate that the samples are significantly different ($p < 0.05$).

Extracts obtained with different solvents might behave differently. According to Allai et al., (2017) the phenolic content of ethanolic extract of *O. ficus-indica* cladode was in the range of 111.2 ± 5.8 mg of gallic acid in 1 g of extract lyophilizate; whereas, the acetone extract provided a content of 73.1 ± 2.1 µg gallic acid/mg lyophilisate extract. The nature of the solvent used, the plant part, the extraction method, the geographical location, and the stage of maturation of the cactus sample at the time of collection might lead to different results.

In this report, TPC varied according to the extraction techniques (CE and UAE). For both extraction methods ethanol (50%) was used as the extracting solvent. The extraction method significantly influenced the yield of phenolic content. Extractions yielded 24.88 ± 2.74 and 39.15 ± 1.78 mg GAE/DW for CE and UAE, respectively. The optimization of extracting

conditions of UAE improved phenolic content yield by 57% compared to CE. Similar results were also reported by other authors (Rodríguez-Pérez et al., 2015). In addition, UAE reduced the extraction time compared to the required time for the CE. UAE has the advantage of considerably reduced extraction times while increasing the levels of extracted phenolic compounds (Bourgou et al., 2016). UAE reduced the extraction time of grape phenolic compounds where it required one-tenth time compared to maceration (Carrera et al., 2012). This improvement was likely due to the ability of ultrasound waves to rupture cell walls, which subsequently increases solvent penetration and accelerates molecular diffusion (Medina-Torres et al., 2017).

Determination of Total Flavonoids

Flavonoids constitute a major group of *OFI* phenolic compounds (Djeridane et al., 2006). The flavonoid assay was performed on ultrasound extracts obtained at 30 min at different temperatures (Figure 5). The temperature of 60 °C was considered as the best temperature for TPC extraction; however, total flavonoids (TF) content could change according to temperature changes. The effect of temperature on the flavonoid content is shown in Figure 5.

The results indicated that the optimal concentration of flavonoids (1.40 ± 0.08 mg QE/g DW) was obtained at 40 °C. These results are consistent with the work of Tiho et al., (2017), where the maximum extraction yield was achieved at significantly different temperatures for each matrix compound (polyphenols and flavonoids). In addition, as flavonoids are denatured by heat, the best-assessed values were provided by the lowest drying temperature (40 °C) of the product extracted by maceration (0.5083 g QE/l; 101.66 mg QE/g), whereas, temperatures reaching 80 °C were used for total phenolic content extraction (Tiho et al., 2017). *OFI* cladode is very rich in various polyphenols (Angulo-Bejarano et al., 2014; Astello-García et al., 2015), which might explain the low flavonoids content obtained at 40°C, indicating that the ethanolic extracts contain other phenolic compounds such as tannins and phenolic acids, which have other chemical structures than those of flavonoids.

In another report, flavonoid content of *OFI* cladodes was found to be equal to 5.4 mg of flavonoids/g dry sample of ethanolic extract (Allai et al., 2017). However, Medina-Torres et al., (2011) obtained a flavonoid content of 23.40 ± 1.83 mg/g cladode. According to Allai et al., (2017), the flavonoid content of lyophilized ethanolic extract of *O. ficus-indica* cladode was around 27.0 ± 4.0 mg rutin equivalent/g, whereas in 1 mg of lyophilized acetone extract, the content was 22 ± 2.0 µg rutin equivalent. In a comparative study, *OFI* and *OM* cladodes showed low flavonoid content (Astello-García et al., 2015), with the quantitative determination of flavonoids of 19.4 µmol and 16.8 µmol QE/g sample in *OFI* and *OM* cladodes, respectively. The collection site, the extraction solvents, and methods reduce comparison reliability between studies.

Furthermore, the conditions under which the extraction is carried out, can affect the total content of phenols and flavonoids, and consequently the biological activities mediated by these metabolites (Lee et al., 2003). Our results were consistent with those of Ma et al., (2008), who observed a decrease in the UAE polyphenols yields at hotter temperatures for a prolonged period. This is probably due to the thermal reactions of alteration or polymerization of the phenols themselves. Thus, an increase in temperature decreases the effectiveness of ultrasound-assisted extraction. In the presence of these antagonistic effects, an optimum

temperature is observed. In addition, the stability and synthesis of flavonoids and phenolics in plants could be affected by several environmental factors such as light intensity, pH, temperature, solvent nature, enzymes, oxidants, metal ions promoting flavonoids degradation, CO_2 concentration, leaf maturity, and plant age (Hemm et al., 2004).

Evaluation of the antioxidant activity (ABTS test)

The reducing efficiency of a molecule can be a predictor of its potential antioxidant activity. This activity was evaluated through the ABTS test within the phenolic content extraction temperatures (30~70 °C). Results were summarized in Table 3. According to the results, ABTS inhibition rates were consistent, with no significant differences ($p < 0.05$) in inhibition levels and TEACs.

The Bir-Essalam's *OFI* extract induced no less than 77% inhibition of $ABTS^+$ radicals at the applied temperature range (30~70 °C). Despite increasing temperatures, the antioxidant activity remained stable. The antioxidant activity was likely the result of a synergistic effect of a matrix of various antioxidants including phenolic compounds.

In a study about phenolic compounds of cactus pear cladode, the antioxidant activity was related to the concentration of carotenoids (Jaramillo-Flores et al., 2003). The occurring TPC and flavonoids (Figure 5) may have contributed to the inhibition rates obtained at the monitored temperature range. Moreover, at 40 °C, the extract exhibited the highest inhibition rate (80.44 ± 1.02 %) corresponding to the highest flavonoids content phase. Antioxidant properties of a plant product are strongly related to its polyphenol content (Li et al., 2007).

Table 3. Antioxidant capacity of Bir-Essalam's *Opuntia ficus indica* cladode extract as ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) inhibition rate and Trolox equivalent (TEAC) at varied temperature range.

Temperature (°C)	ABTS inhibition rate* (%)	Antioxidant capacity* (mg TEAC/g DW)
30	79.26 ± 0.53	2.45 ± 0.04
40	80.44 ± 1.02	2.45 ± 0.22
50	80.14 ± 1.08	2.52 ± 0.08
60	77.30 ± 2.30	2.31 ± 0.17
70	79.49 ± 1.79	2.47 ± 0.13

*Means ± s.d. of three measurements.

Polyphenols possess great antioxidant potential, and act as hydrogen or electron donors. Cai et al., (2004) reported a strong correlation between total phenol content, estimated by the Folin-Ciocalteu method, and the antioxidant activity estimated by the ABTS test. However, in our case, the results suggested a participation of other compounds such as polysaccharides because at 70 °C the rate of inhibition remained high, while the levels of TPC and flavonoids were relatively low. According to Jaramillo-Flores et al., (2003), the extractability of the carotenoids is constrained by mucilage present in the cactus pear stems. This led us to conduct infrared analyses of the extracts. An interesting observation was the degree of correlation between phenolic content and antioxidant activity, but these compounds might diffuse differently in the analysis, and the phenolic fraction do not necessarily include all the antioxidants that might be present in the extract (Athamena et al., 2010). The relationship

between antioxidants and the antioxidant activity of a plant is difficult to explain based upon quantitative analysis alone, because of not only with the level of antioxidants, but also with the interaction among them and with other constituents (Yoo et al., 2008). Hence, the chemical composition and the structures of the active compounds of the extract can modulate the effectiveness of the antioxidant activity.

Total polyphenols content among *Opuntia* species of both regions

The optimal extraction conditions obtained in UAE (50% ethanol, 30 min sonicating time, at 60 °C) for the Bir-Essalam's *OFI* were applied to the cladode of the studied *Opuntia* spp. collected at the other sites. The results of the total polyphenol assay were shown in Table 4. The highest TPC yield was recorded for Bir-Essalam's *OFI* species, followed by *OM* and *OA* species. The TPC yield of Bir-Essalam's *OFI* was significantly higher than those of Taourirt and Souk-Ahras's *OFI* (Table 4). Moreover, the TPC yield of Oued-ghir's *OM* were significantly higher than Souk-Ahras's *OM*. No significant difference was observed between the yields produced by Oued-ghir's *OM* and Taourirt and Souk-Ahras's *OFI*. TPC levels of Amizour and Souk-Ahras's *OA* were not significantly different. The TPC values of Bejaia's Bir-Essalam *OFI* and Oued-ghir's *OM* were significantly higher than the equivalent species of Souk-Ahras.

The same species from different sites of the same region can have different phytochemical composition in the case of Bir-Essalam and Taourirt's *OFI*, both of which are from the Bejaia region. Therefore, for one *OFI* species, the culture site could influence the content of phenolic compounds within the same region. Moreover, TPC of Bir-Essalam's *OFI* is higher than that of *OM* and *OA* species regardless of the collection region (Table 4). The difference in TPC levels among species might be attributed to the influence of several factors. Recent studies have shown that phenolic content is strongly influenced by extrinsic factors namely, geography and climate, genetic characteristics, stage of maturation, and the storage period (Cheurfa and Allem, 2016).

Phenotypic diversity analysis of *O. ficus-indica* in Algeria showed that there are variations among species at the level of spines and areoles that seem to be a response to climatic variations (Adli et al., 2017). However, the presence and absence of spines in different species affects the rate of TPC regardless the geographical origin. Astello-García et al., (2015) showed that *OFI* contained more phenolic compounds than *OM* species, which is consistent with our results, although, in the same study, *OM* had a higher polysaccharide level than *OFI*.

Table 4. Total polyphenols and flavonoids contents of cactus pear cladodes, treated with ultrasound-assisted extraction (UAE) at 60 and 40°C, 50% solvent concentration and 30 min extraction time, according to species and to the collection site.

Opuntia species	Location	Total Phenolics* (mg GAE/g DW)	Total Flavonoids* (mg QE/g DW)	
		Extraction Temperature (°C)		
		60	60	40
O. ficus indica	Bir-Essalam	39.15 ± 1.78^{a}	1.25 ± 0.05^{e}	1.40 ± 0.15^{f}
	Taourirt	30.25 ± 1.52^{b}	2.34 ± 0.08^{b}	2.49 ± 0.13^{b}
	Souk-Ahras	30.17 ± 1.26^{b}	2.88 ± 0.04^{a}	2.73 ± 0.09^{a}
O. megacantha	Oued-ghir	31.79 ± 2.25^{b}	2.08 ± 0.12^{c}	2.28 ± 0.07^{c}

	Souk-Ahras	25.62 ± 3.30^{c}	1.69 ± 0.03^{d}	1.64 ± 0.18^{e}
O. amyclea	Amizour	18.15 ± 1.35^{d}	2.31 ± 0.02^{b}	2.05 ± 0.06^{d}
	Souk-Ahras	19.74 ± 1.59^{d}	1.68 ± 0.07^{d}	1.34 ± 0.01^{f}

*Means ± s.d. of three measurements.

Total Flavonoids among *Opuntia* species of both regions

The total flavonoid (TF) assay was performed on extracts of UAE obtained under the optimized conditions (50% ethanol, 30 min time of sonication). The TF yield was monitored at two temperatures (40 and 60 °C). The results showed that Souk-Ahras's *OFI* exhibited the highest TF content among the studied species and sites (Table 4). No significant difference was observed between Souk-Ahras's *OM* and *OA*. The lowest TF content was noted for Bir-Essalam's *OFI*. The TF content assay, performed at 40°C (Table 4), showed that Souk-Ahras's *OFI* contained the significantly highest content of flavonoids, followed by Taourirt's *OFI*, Oued-ghir's *OM*, and Amizour's *OA*.

Astello-García et al., (2015) reported that *OFI* slightly exceeded *OM* in flavonoid levels. Reducing temperature of extraction slightly affected TF yields of Souk-Ahras's *OFI*. This effect was more pronounced for *OA* of both study regions. However, the lower extraction temperature affected positively the TF values of Bejaia species. TF content was enhanced in Bir-Essalam and Taourirt's *OFI* and Oued-ghir's *OM* (Table 4). This might suggest that species from Souk-Ahras might exhibit better heat adaptation, arising in a semi-arid environment. Therefore, it is important to control the temperature parameter to ensure an efficient extraction process. Indeed, phenolic compounds are usually classified as typical functional cell wall components of plant cells, and they play a key role in the defense mechanisms toward most of the abiotic stresses, including UV-irradiation, water stress and high temperature (Cheynier et al., 2013; Chinnici et al., 2004).

Al-Huqail et al., (2020) observed an increase in total phenolic and flavonoid concentrations in response to water-deficit stress in basil leaves; flavonoids also increased under high climate temperature conditions. Pinheiro et al., (2021) reported an increased flavonoids content in water stress sorghum genotypes. Moreover, Mayer et al., (2021), considered the metabolic profiling of epidermal and mesophyll tissues in *O. ficus indica*. They reported that the flavonoids kaempferol, naringenin, and quercitin-3-O-glucoside were higher in water-deficit stressed plants. Water deficiency induces the generation of reactive oxygen species, which causes oxidative damage to the plant. Plants have several adaptive methods to minimize this damage, such as a set of antioxidants that limits the chain of oxidative processes from spreading (Sánchez-Rodríguez et al., 2011). Thus, phenolic compounds are one of the major groups of antioxidants capable of detoxifying free radicals (Ksouri et al., 2007).

Furthermore, plants exposed to extreme heat-shock conditions, might activate certain adaptive and protective mechanisms (Ghorbanli et al., 2013). The mechanisms of acclimation of *Phillyrea latifolia* to high solar radiation were investigated and flavonoids secretion by glandular trichomes was shown to be a central part of the acclimation mechanism to excess light (Tattini et al., 2000).

Antioxidant activities among *Opuntia* species of both regions

The ABTS test was performed on *OFI* samples extracted with UAE under optimized conditions of 50% ethanol concentration, 60 °C and 30 min sonication temperature and time, respectively. According to Table 5, Oued-ghir's *OM* showed the significantly highest antioxidant activity, followed by Taourirt's *OFI* and Souk-Ahras's *OM*, Souk-Ahras and Bir-Essalam's *OFI*. Souk-Ahras and Amizour's *OA* provided the lowest ABTS inhibition values. Thus, *OM* and *OFI* showed the highest values contrary to *OA* species either in the Bejaia or Souk-Ahras regions (Table 5).

Table 5. Antioxidant capacities of cactus pear cladode extracts as ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) inhibition rate and Trolox equivalent (TEAC).

Opuntia spp.	Location	ABTS inhibition rate* (%)	Antioxidant Capacity* (mg TEAC/g DW)
O. ficus indica	Bir-Essalam	77.30 ± 2.30^{b}	2.31 ± 0.17^{c}
	Taourirt	$80.85 \pm 3.37^{a,b}$	$2.57 \pm 0.15^{a,b}$
	Souk-Ahras	79.67 ± 0.54^{b}	$2.51 \pm 0.04^{b,c}$
O. megacantha	Oued-ghir	83.51 ± 0.94^{a}	2.76 ± 0.07^{a}
	Souk-Ahras	$80.32 \pm 0.35^{a,b}$	2.53 ± 0.03^{b}
O. amyclae	Amizour	54.71 ± 2.78^{d}	0.67 ± 0.11^{e}
	Souk-Ahras	73.13 ± 1.90^{c}	2.01 ± 0.14^{d}

*Means ± s.d. of three measurements

In a comparative study of five *Opuntia* spp., *OFI* and *OM* showed similar antioxidant capacities (Astello-García et al., 2015). The same tendency for antioxidant activities of *OFI* and *OM* species of Souk-Ahras region could be observed. This tendency was observed to a lesser extent for plants from the Bejaia region (Table 5). For *OA* species, the inhibition rate was higher for plants from Souk-Ahras, than that of plants from Amizour, which is consistent with the TPC result of *OA* species (Table 4). Low levels of TPC might reflect better the inhibition rates and the presence of other antioxidants, which might interfere with the antioxidant activity. Higher TPC values of *OFI* and *OM* species might explain higher inhibition rates than that of *OA*. The TEAC values of the extracts were consistent with the inhibition rates (Table 5).

Fourier transform Infrared (FTIR) Analysis

The generated FTIR spectra are shown in Figure 6. The data of FTIR spectra range between 500 to 4000 cm^{-1} of the analyzed cladode powders. Bejaia and Souk-Ahras's *Opuntia* species were used to gain an insight about the nature of their functional groups. The spectra show wide and strong bands in the range of 3647~3000 cm^{-1}, which is due to the presence of OH groups, which are characteristic of polyphenols and carbohydrates. The wide and convergent bands around 3600~3200 cm^{-1} pointed out the overlap of the hydroxyl and amine stretching vibrations (Barka et al., 2013a). The absorption bands at 2921 and 2843 cm^{-1} were due to the asymmetric stretching vibration of CH_2 and the symmetrical stretching vibration of CH_3 of aliphatic acids, respectively (Farinella et al., 2007).

The absorption band around 1606 cm^{-1} may be due to the vibration of elongation of the C=C bond of aromatic acids. The absorption band at 1614 cm^{-1} might be due to the vibration

of elongation of the C=O bond of ketones. The peaks about 1630 cm^{-1} relates to the C=C stretching referred to the C–C aromatic bonds and COO- asymmetric stretchings (Farinella et al., 2007). The peak at 1432 cm^{-1} is that of the phenolic stretch -OH (Barka et al., 2013a). The peaks observed at 1370 cm^{-1} reflect the stretching vibrations of symmetrical or asymmetric ionic carboxylic groups (-COOH) of pectins (Farinella et al., 2007).

The peaks observed at 1379 and at 1313 cm^{-1} prove the reappearance of the OH groups of the phenols and/or the presence of the OH groups of the tertiary alcohol. The peak at 1382 cm^{-1} might be due to the presence of the CH_3 group of alkanes (deformation in the symmetrical plane). The peaks at 1309 cm^{-1} might be due to the presence of nitrogen compounds from the class of nitro-aromatic compounds.

The Bir-Essalam *OFI* species had intense and wider absorption bands, especially in the interval from 1000 to 1500 cm^{-1} corresponding eventually to polyphenols; followed by Taourirt *OFI*. However, all the spectra clearly showed the phenolic nature of all analyzed powders. These results suggest that there are a wide range of functional groups (aldehydes, carboxyl, hydroxyl, ketones, phosphate, sulfate, etc.) constituting the key elements of the chemical properties of various cladodes-derived biomolecules. Perhaps, this could be at the origin of some inconsistencies in TPC content of *Opuntia* spp. and their relevant antioxidant activities.

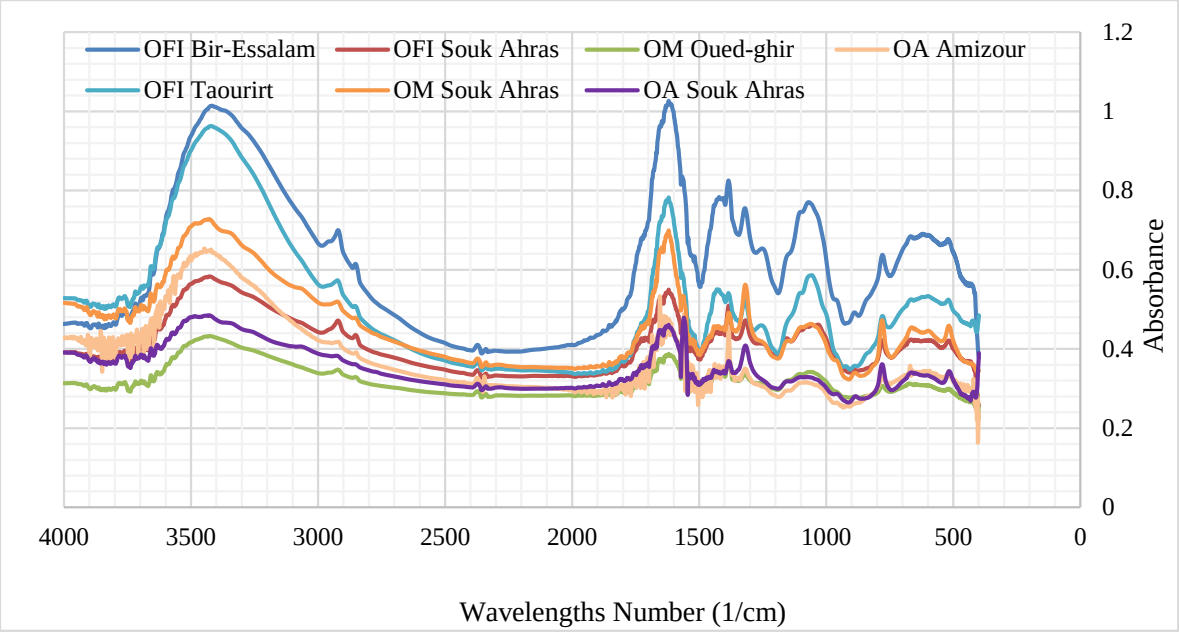

Figure 6. Infrared spectra analysis of the cladodes powder of the investigated cactus pear species (from Bejaia: Bir-Essalam's *Opuntia ficus indica* (*OFI*), Taourirt's *OFI*, Amizour's *Opuntia amyclae* (*OA*), and Oued-ghir's *Opuntia megacantha* (*OM*), from Souk-Ahras: *OFI* (Sidi-Fredj), *OA* (Merhana 1), and *OM* (Merhana 2)).

CONCLUSIONS

In the context of valorization of the Algerian cactus pear plant and its products, *Opuntia* species of two growing regions were identified. The morphological study revealed the existence of three species: *Opuntia ficus-indica* (*OFI*), *O. megacantha* (*OM*), and *O. amyclae* (*OA*).

Then, we investigated the optimal conditions for the best yield of total phenolic compounds (TPC). The use of mixed solvent (50% water/ethanol, v/v) improved the TPC extraction yield.

In order to increase the extraction yield, an innovative ecofriendly extraction process was used. Ultrasound assisted extraction (UAE) was better than conventional extraction (CE) for TPC recovery, wherein the best extracted yield (39.15 ± 1.78 mg GAE/g DW) was obtained with the optimal conditions in 30 min. In contrast, the CE yielded only 24.88 ± 2.74 mg GAE/g DW after 2h 50 min extraction time. The UAE process was faster and resulted in a 57.3% enhancement of extracted TPC. The variation of extracting temperature revealed that at 60°C the TPC yield was equal to the optimal value for the highest TPC yield. TF content recovery showed that the lower temperature (40 °C) gave rise to the highest TF yield for Bejaia's species, whereas the highest yields of TF were recovered at 60°C for Souk-ahras species.

While comparing the TPC, TF and antioxidant activities of the studied cactus pear species under the same optimal conditions of UAE, we concluded that Bir-Essalam's *OFI* showed the highest TPC yield, followed by Taourirt and Souk-Ahras's *OFI*, then *OM*, and finally *OA*. Globally, we observed a decreasing order of TPC yield (*OFI* > *OM* > *OA*) regardless the growth region or site. However, within the same region of Bejaia and for the same species (*OFI*), Bir-Essalam and Taourirt showed varied TPC yields. Thus, the growth site could influence the composition of the cactus pear cladodes for a single species even though Bejaia is a wet region, while noting that the Taourirt area is warmer than Bir-Essalam's area. The comparison of TF content among these species showed that cactus pear species of Souk-Ahras could have adapted to high temperatures than Bejaia species.

For the antioxidant activity, which was carried out through the ABTS test, whether in Bejaia or in Souk-Ahras, *OM* and *OFI* species exhibited the highest activities, which was the opposite to *OA* which had the weakest antioxidant activity. This is almost consistent with their respective TPC values.

The high levels of bioactive compounds observed in the powders by infrared specta analysis, was certainly a result of the inconsistency between the recovered TPC contents and the corresponding antioxidant activities. Infrared spectra analyses showed the varied composition of cactus pear cladodes with multiple functional groups, which make them an attractive source of secondary metabolites that could be used as potential additive in the food and nutraceutical industries.

Overall, these results illustrate the importance of UAE as in innovative tool for cactus pear cladode phytochemicals extraction. Other factors and conditions that influence the optimization of the TPC extraction required further investigation with consideration of other factors such as the response surface methodology, pH, the frequency of ultrasound, as well as the ecological factors that might possibly improve the extraction efficiency.

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