Ethnobotanical and Phytochemical Study of the Medicinal Plant *Atriplex Halimus* and Its Importance in the Traditional Algerian Pharmacopoeia.

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Atriplex halimus, is a medicinal plant widely used in Algerian phytotherapy. This study included an ethnobotanical and phytochemical survey on aqueous extracts (phytochemical screening, HPLC analysis, phenolic content and antioxidant activity). The ethnobotanical study on *A. halimus* showed that aqueous extracts are the most used therapeutic means. The phytochemical study has determined that the phenolic content varies significantly from one extract to another depending on the extraction method. In addition, HPLC analysis has revealed various bioactive compounds that mainly belong to the flavonoid category. *A. halimus* is a valuable source of nutraceutic for various diseases.

Introduction

Algeria's Mediterranean climate conceals an important reservoir of medicinal plants whose virtues have been proved for decades. *A. halimus* is a plant from the phytotherapeutic drawer that has always been used for the treatment of various diseases. In addition to its nutritional value for humans and to its richness in dietary fiber (cellulose) which facilitates digestion, it also contains proteins, vitamins (B and C) and mineral salts (sodium, calcium, potassium, magnesium, phosphorus). This species also has many other assets for human health, such as its hypoglycemic effect by an active ingredient of a mineral nature: the tissue chromium of this plant would indeed regulate blood glucose by activating the effect of insulin [1]? *A. halimus* also reduces inflammation of the urinary tract and treats lithiasis [2,3]. In addition, its high flavonoid content gives it an important antimicrobial, antifungal effect and an antioxidant, regenerative and cancer-protective activity [3]

Experimental part

Material and methods

Ethnobotanical Study Method

In order to find out the possible use of *A. halimus* in the traditional pharmacopoeia of

Mascara, an ethnobotanical survey among the populations (traditional practitioners, herbalists, healers, shepherds, old people, doctors and pharmacists) was carried out. A total of 200 informants, aged between 20 and 80 years, participated in the study. The survey was carried out by using a form filled in by oral questioning. The questionnaire focused on the information related to the respondents (age, sex and function) and their therapeutic habits (local name, used parts of the plant, therapeutic indications, forms of preparations) and the modes of administration as well as.

The data entered on cards were inserted and processed in Excel® spreadsheet software version 2013, allowing us to establish their values in the form of tables and histograms.

Collection, Authentication, and Preparation of Samples

A. halimus was collected in Mach 2015 in the region of Mascara. The authenticity of the plant species was confirmed by the Mascara Forest Protection Service and the Nature and Life Science Faculty, University of Mascara, Algeria.

According to the ethnobotanical study recommendations, three modes of preparation often used were quoted in the form of aqueous extracts (decocted, infused and macerated). The most used forms were the subject of the phytochemical study. The leaves were dried and reduced to powder. 50 grams of the powdered plant were used in 100 mL of hot water for 20 min. The mixture was filtered, concentrated and freeze-dried, and stored at +4°C until use.

Phytochemical study

Preliminary Phytochemical Screening

The aqueous extracts were subjected to phytochemical screening using standard procedures according to described methods [4,5].

Determination of Total Phenolic Content

The determination of total polyphenols content (TPC) was carried out according to the Folin-Ciocalteu (FC) method [6]: 100 µL of extract mixed with 500 µL of Folin-Ciocalteu reagent and 400 µL of 7.5% (m/v) Na_2CO_3 solution was added after 5 min. The mixture was stirred and incubated in the dark and at room temperature for 10 min. The absorbance was measured at 760 nm using a UV mini-1240-vis - SHIMADZU. The results were expressed in mg gallic acid equivalent/g of dry extract with reference to the gallic acid calibration curve.

Determination of Total Flavonoid Content

The total flavonoid content (TFC) of the extracts was determined using the aluminum chloride assay previously reported [7]: 500 µL of each extract to be analyzed was added to 1500 µL of 95% methanol, 100 µL of 10% (w/v) $AlCl_3$, 100 µl of 1 M sodium acetate and 2.8 mL of distilled water. The mixture was stirred and incubated in the dark at room temperature for 30 min. The blank was made by replacing the extract with 95% methanol and the absorbance was measured at 415 nm using a UV mini-1240-vis -

SHIMADZU. The results were expressed in mg quercetin equivalent/g of dry extract with reference to the quercetin calibration curve.

Determination of Condensed Tannins Content

The condensed tannins content (CTC) was determined by the acidic vanillin method described by Ba *et al.* [8]. The vanillin reagent was prepared by mixing 8% (v/v) HCl 37% (v/v) in methanol and 4% vanillin in methanol (m/v) at equal volume. The mixture was maintained at 30°C before the assay. 200 µL of each extract to be analyzed were added to 1000 µL of the vanillin reagent. The mixture was stirred and incubated in the dark at 30°C for 20 min.

The absorbance was measured at 500 nm by a using a UV mini-1240-vis -SHIMADZU vs a blank consisting of a mixture of methanol (37%) and HCl (8%) at equal volumes. Results were expressed in mg catechol equivalent /g of dry extract by referring to the calibration curve of catechol.

Determination of DPPH Radical Scavenging Activity

The antioxidant test was performed with the DPPH (2,2-DiPhenyl-1-PicrylHydrazyl) method [9]. 50µL of each methanol solution of extracts at different concentrations (0.0125 to 5 mg/mL) were added to 1.95 mL of the DPPH methanol solution (0.025g/L). The negative control was prepared by mixing 50 µL of methanol with 1.95 mL of the DPPH methanol solution. The absorbance reading was made against a blank prepared for each concentration at 515 nm after 30 min incubation in the dark and at room temperature. The positive control was a solution of a standard antioxidant, ascorbic acid, whose absorbance was measured under the same conditions as the sample and for each concentration as well. The test was repeated three times. IC_{50} values were determined graphically by linear fits.

Identification of phenolic compounds in Infusion Extract of A. halimus by HPLC

Qualitative analysis of the polyphenol compounds in the infusion extract was performed by high performance liquid chromatography (HPLC) on a Prominence-i LC 2030C System with a 15 cm x 4.6 mm, 5 m Ascentis C18 column (Supelco, Bellefonte, PA, USA).

Two (2) µL were injected into a gradient eluent system of hydro-organic solvents of methanol and acetic acid. The mobile phase was composed of 0.075% acetic acid/water (solvent A) and 0.075% acetic acid/methanol (solvent B) in a linear gradient mode studied, in order to provide a complete resolution of all components in a consistent analysis time: 0 min, 2% B; 5 min, 2% B; 60 min, 50% B; 70 min, 50% B, 71 min, 2% B, under thermostatic control at 30°C, with a flow rate of 0.08 mL/ min, the wavelength range of the chromatograms was 280 nm. The wavelength range of the chromatograms was 280 nm. Data acquisition was performed by the Shimadzu Lab Solution software version

5.10.153 and compared with literature data to identify the nature of the metabolites.

Data Analysis

The results were expressed as mean ± standard error of mean (SEM). Statistical analysis was performed by one-way analysis of variance (ANOVA) and $P \leq 0.05$ were considered as significant.

Results and discussion

The ethnobotanical study

The ethnobotanical studies allow the scientific research on the phytotherapeutic use of plants. The present study on *A. halimus* has enrolled 200 people, 56% of whom were men and 43% women. The age of the people questioned varies between 20 and 78 years old of whom 91% affirm to use the medicinal plants, of whom 65% specify to have used *A. halimus* (different parts).

The convergence of information collected concerning the therapeutic use of *A. halimus* shows its usefulness in the treatment of a range of very varied symptoms grouped in 10 categories of pathology summarized in **Figure 1A**, that translates the importance of the therapeutic use of this plant to solve the problems of primary health and to meet the needs for domestic medicine.

The estimation of the most frequent uses of the plant highlights a massive indication in digestive pathologies with 27%, as well as its wide use against metabolic diseases specifying diabetes with 22.5%, followed by the problems of hydrated cysts with 12%. More interesting is its use in digestive cancer pathologies with 11.5%.

The predominance of the use of *A. halimus* remains in the affections of the digestive tract. We will try thereafter through certain biological test to elucidate certain effect of this plant.

Phytotherapeutic knowledge is the traditions left behind and perpetuated from generation to generation through customs and oral tradition, in which, knowledge of the properties and uses of medicinal plants are acquired through long-accumulated experience [10, 11, 12]. In the context of our scientific research this knowledge is today the subject of ethnobotanical or ethnomedical studies. They rely on methods of choice for the knowledge of medicinal plants and for their therapeutic use. They allow us to orient ourselves in order to target specific biological tests. Thus, in our study it was essential to begin our research with an ethnobotanical study on our plant *A. halimus*.

Floristic part used

Different parts of the plants are used for the preparation of different therapeutic recipes (leaves, stems, bark, seeds, roots, flowers, etc., **Figure 1B**).

In the case of *A. halimus* the leaves are the most used parts, followed by the stems. The other parts are used with frequencies lower than 10% **(Figure 1B)**. Effectively, leaves are mainly used because of their high concentration of active ingredients, since they are the sites of biosynthesis and the storage of secondary metabolites [13].

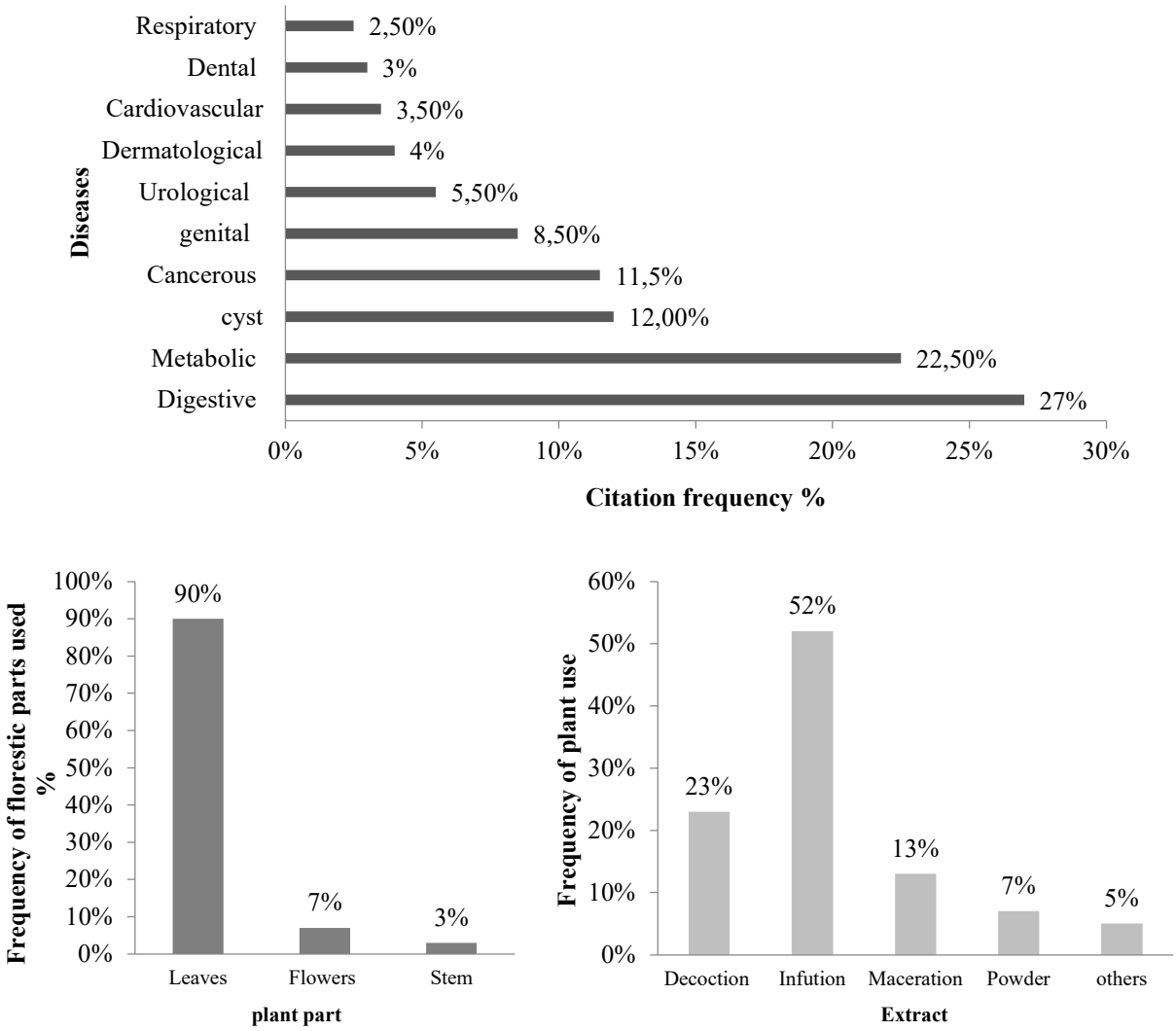

Figure 1. Ethnobotanical study of *A. halimus*: frequency of different diseases treated by the plant (%) **(A)**, frequency of floristic part used (%) **(B)**, frequency of plant use (%) **(C)**

Mode of preparation

The administration of the active principle of plants uses several modes of preparation, namely in the form of aqueous extracts (decoction, infusion, maceration), or extracts with other solvents (oil, vinegar etc.), powder, etc. Users are always looking for the simplest method to prepare herbal medicines. Infusion is the most common method of preparation. This mode is mainly applied to the delicate organs of the plant such as leaves, flowering tops and flowers, in order to preserve better the active ingredients [14, 15, 16].

According to our study **(Figure 1C)**, the most quoted recipe for the use of *A. halimus* recommends the ingestion of the aqueous infusion of about 10 g of plant powder three times a day for 30 days.

Phytochemical analysis

Extraction yields

According to the ethnobotanical study the most common way of using *A.halimus* was water.

In this context our research will be based on aqueous extraction. The extraction yield of the three methods used, depicted in **Figure 2A**, does not reveal any significant difference ($p>0.05$). However, the infusion seems to be the best extraction method with a yield of 25.8% against 25.1% for decoction, and 24.4% for maceration.

Phytochemical screening

The analyses carried out showed the presence of polyphenols, flavonoids, alkaloids, saponins, terpenoids, and the absence of coumarins in all the extracts. The results are listed in **Table 1**.

Table 1. Phytochemical Constituents of *A. halimus* of Infusion Extract

Secondary Metabolites	Leaves
Polyphénols	++
Tannins	+
Flavonoids	+
Coumarins	-
Alkaloids	+
Terpenoids	+
Saponins	+++

(-) not detectable, (+) low quantities, (++) high quantities, (+++): very high quantities

Total phenolic content of the extracts

Phenolic compounds are highly sought-after bioactive molecules because they are known for their valuable biological properties (antioxidants, antimicrobials, etc.). For these reasons, the crude extracts, obtained by aqueous extraction, were analyzed quantitatively by spectrophotometry for their content in total polyphenols, flavonoids and condensed tannins. The results of the assay are displayed in **Figure 3**.

Examination of the results showed that the aqueous extracts of *A.halimus* had significantly different levels of total polyphenols in close relation with the extraction method **(Figure 2B)**. The infusion had the highest level (12 ± 0.015 mg GAE/g DW) while the macerate had the lowest level (9.5 ± 0.009 mg GAE/g DW).

The results revealed abundance of flavonoids in the extracts **(Figure 2C)**. These compounds reached a content of 225 ± 0.05 mg CATE/g DW by the decoction method which gave the richest extracts, as well as 223 ± 0.05 and 224 ± 0.040 mg CATE/g DW for the infused and the macerate, respectively. In addition, the tannin dosage showed significant differences **(Figure 2D)**.

The decoction method had the highest content (142 ± 0.11 mg QE/g DW). However, the infusion and macerate methods unveiled low contents with 20 ± 0.007 and 24 ± 0.014 mg QE/g DW, respectively. The variability in the amounts of polyphenol compounds between extracts can be attributed to the extraction method through the used boiling temperature.

Determination of DPPH radical scavenging activity

The antioxidant activity in this study was evaluated by the DPPH scavenging activity (**Table 2**).

Table 2. DPPH Scavenging Activity of *A. halimus*

Extracts	IC_{50} (mg/mL)
Decoction	0.29 ± 0.01**
Infusion	0.85 ± 0.02**
Maceration	1.52 ± 0.002**
Ascorbic acid	0.005 ± 0.001

IC_{50} values are expressed are means ± SEM. The values showed significant differences between the samples and ascorbic acid at $P < 0.001$.

The results brought to light a moderate antioxidant activity, although less potent than the reference antioxidant ascorbic acid (IC_{50} = 0.005 ± 0 .001 mg/mL), the decoction giving the highest DPPH radical scavenging activity with IC_{50}= 0.29 ± mg/mL, followed by the infusion extract (IC_{50}= 0.85 ± 0.02 mg/mL), the lowest activity being that of the maceration extracts (IC_{50}= 1.52 ± 0.02 mg/mL).

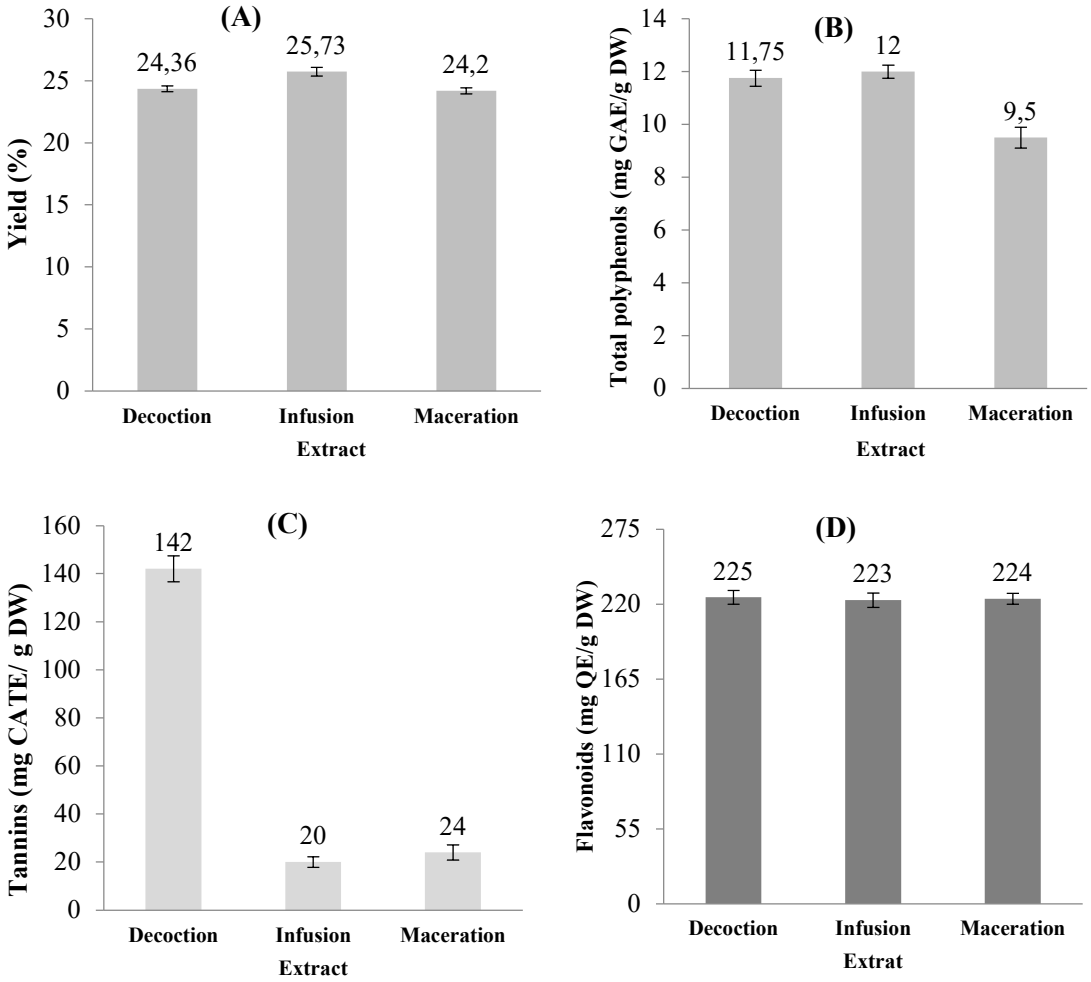

Figure 2. Yield of aqueous extraction (Decoction, Infusion and Maceration) **(A)**, Total polyphenols content **(B)** expressed on (mg GAE/g DW), Total flavonoids content **(C)** expressed on (mg QE/g DW) and Tannins content **(D)** expressed on (mg CATE/g DW)

Identification of phenolic compounds by HPLC

The identification of polyphenolic compounds was performed by high performance liquid chromatography (HPLC) on a 2030C Prominence-i LC system. The molecules detected are shown in **Figure 3** and **Table 3**.

A total of seven components were identified in the infusion extract of *A. Halimus*: Three phenolic acid (gallic acid, protocatechuic acid, *p*-coumaric acid) and flavonoids: catechin, epicatechin, rutin and isorhamnetin-3-O-glucoside.

In fact, the class of flavonoids is in the majority in the extract with important quantities of catechin (717.86 µg/g DM), rutin (360.84 µg/g DM) followed by isorhamnetin-3-O-glucoside (83.33 µg/g DM) and epicatechin (35.12 µg/g DM). On the other hand, among phenolic acids, p-coumaric acid presents the most important quantity (59.41µg/g DM). The other components are minor, with 27.9 and 13.59 µg/g DM for protocatechuic acid and gallic acid, respectively.

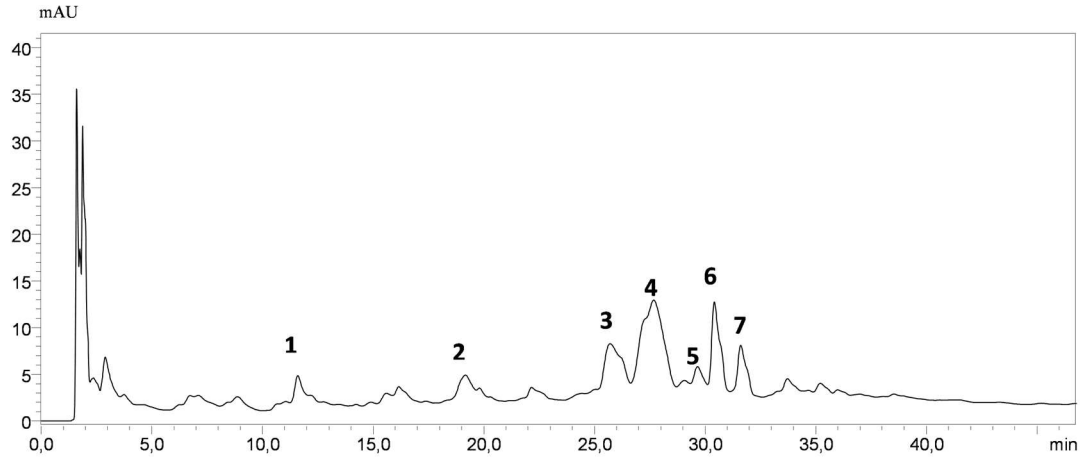

Figure 3. Identification of polyphenols and flavonoids compounds in infusion extracts of *A. halimus*. 1: gallic acid, 2: protocatechuic acid, 3: *p*-coumaric acid, 4: catechin, 5: epicatechin, 6: rutine, 7: isorhamnetin-3-O-glucoside.

Table 3. Phenolic compounds in *A.halimus* of infusion extract

Peak	Retention Time	λ max	Quantity (µg/g DM)	Compounds
01	11.596	215-270	13.59	Gallic acid
02	19.170	261	27.9	Protocatechuic acid
03	25.709	230-311	59.41	*p*-Coumaric acid
04	27.678	278-325	717.86	Catechin
05	29.675	252-345	35.12	Epicatechin
06	30.417	255-354	360.84	Rutine
07	31.609	252-353	83.33	Isorhamnetin glycoside

Conclusion

The analysis of the aqueous extracts of the aerial parts of *A. Halimus* has revealed that this species is a potential source of phenolic compounds and has a remarkable antioxidant effect. Hence, it represents a promising phytotherapeutic treatment for different diseases.

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