Phytochemical Study, Polyphenols Determination and Evaluation of Antioxidant Activity of *Satureja calamintha* from Morocco

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ABSTRACT

Introduction: The domestication of Satureja calamintha, either because of its superior tolerance to disease and environmental factors or because of its potential for larger quantities of useful chemicals. Materials and Methods: wild plants were harvested in the Ksar El Kabîr region, Morocco, and seeds were planted in open-air plots. Part of the cultivated plant was then harvested after one year of planting, while the other was harvested after two years. Results: The results show that the plant cultivated and harvested in the second year is characterized by a slightly higher polyphenol content for all three extraction methods than the same plant cultivated in the first year, and that the spontaneous plant, particularly the decoction, has the highest content (174.42±0.52 mg GAE/g extract), while the infused extract has the lowest content (123.242±5.64 mg GAE/g extract). Decoction and infusion remain suitable extraction extraction methods for total flavonoids for the plant grown in the second year, representing (86.23±0.12 mg EQ/g extract) and (76.45±0.06 mg EQ/g extract) respectively, followed by macerate of the spontaneous plant (57.7±1.46 mg EQ/g extract). In terms of antioxidant activity, the extract obtained by decoction and harvested in the second year showed high antioxidant activity against DPPH $(IC_{50}=1.174\pm0.141 mg/mL)$, FRAP $(EC_{50}=4.254\pm0.03)$ and ABTS $(IC_{50}=9.969\pm0.09 mg/mL)$, while maceration showed low activity against the same three tests. Conclusion: The aqueous extracts of the studied spontaneous and cultivated plants showed positive results for all activities and were characterized by an increase in phytochemicals, particularly in the extracts from the second year.

Keywords: *Satureja calamintha* (L), Wild plant, Cultivated plant, Flavonoids, Polyphenols, Antioxidant potential.

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INTRODUCTION

Since the 1980s, the interest of the cultivation of medicinal and aromatic plants has increased in the industrialized and developed countries. The domestication process allows a sustainable exploitation of the natural resources and improvement of the quality of plants.¹

Satureja, known by the common name Minta, belongs to the mint family (Lamiaceae) which includes about 4000 species and 210 genera.² It consists of about 200 species of herbs and



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shrubs, often aromatic. This genus is widely distributed in the Mediterranean region, in Asia and North of America.³ Satureja grows from 30 to 40 cm in size and is much branched from the base. It has a lanceolate leaf at the end. The stems are more or less hairy. The flowers bloom from white to purple in the leaf axils from July to September.^{4,5} Satureja is characterized by aerial parts with a distinctive taste, and it can be added to several culinary preparations or used in traditional medicine to treat various ailments. Thus, the species of calamintha is well represented and widespread throughout the Mediterranean region. This plant is known for its medicinal uses as a stimulant, tonic, antiseptic and antispasmodic. It is a fragrant plant and is therefore used in various culinary recipes as well as an infusion.^{6,7} In Morocco, this plant is used in infusion as refreshing and the leaf powder is used in sweet urination, colds, flu and broncho-pulmonary affections.8,9

The domestication of *Satureja calamintha* can contribute to the development of its cultivation and reduce the overexploitation of the wild plant. This work is one of the first studies of the effect of domestication on the intrinsic chemical and biological quality of the plant.

MATERIALS AND METHODS

Plant material

For the collection of *Satureja calamintha*, we made two trips: the first during the flowering period of 2018 in the Taza region to contact the gatherers and collect the aerial parts (leaves and flowers) for studies of the wild plant, and the second during the flowering period in April of the following year to collect a sufficient quantity for the domestication of this plant. We collected samples from the station in the Ksar El Kabîr region, located at an altitude of 30.53 m, latitude: 35°00'01" North, longitude: 5°54'13" West. Our professor deposited reference samples at the INRA Rabat herbarium with reference number RAB114271.

Acclimatization of the plant in the greenhouse is taking place at the Institut Technique Agricole in Taounate, where we have installed the crop on an experimental plot in the open field, using ordinary soil as the fertilizer.

Preparation of the aqueous extracts

The traditional methods of extraction of medicinal plant material for making an aqueous extract from leaves have been used according to methods described in ref.^{10,11} with a slight modification. From the powder of the plant leaves, the maceration is prepared by dispensing 50 g in 500 mL of distilled water under magnetic stirring during 24 hr at a room temperature (25°C); the same quantity of the leaf powder is dissolved in 500 mL of distilled water and heated under reflux during 15 min to perform the decoction. While the infusion has been prepared by boiling 50 g of the leaf powder in 500 mL of distilled water during 30 min until cooling.

The three extracts were passed through Whatman filter paper (No.1) in a Buchner funnel and concentrated in a rotary evaporator (BüchiRotavapor R-200). The remaining residue was kept at 0°C until further investigation.

The yield of the extraction is calculated by the following formula:

Where: R is the yield (%); M_{ext} present the mass of the extract after evaporation of the solvent (mg) and M_{ech} denotes the dry mass of the plant sample (mg).

Polyphenols assay Total polyphenols content

The assay is based on Folin-Ciocalteu method using the phosphomolybdic/phosphotungstic acid complexes using as yellow reagent.¹² In this work, 100 μ L of each extract was mixed with 500 μ L of a freshly prepared of Folin-Ciocalteu reagent and 400 μ L of 7.5% of the Sodium Carbonate (Na₂CO₃). The entire is incubated at room temperature during 60 min. The method relies on the transfer of electrons in alkaline medium from phenolic compounds to form a blue chromophore constituted by a phosphotungstic/phosphomolybdenum complex where the maximum absorption depends on the concentration of phenolic compounds. The reduced Folin-Ciocalteu reagent is detectable with a spectrophotometer in the range of 690 to 710 nm.¹³ Gallic acid is used as the reference standard compound.

Total flavonoids content

The total flavonoid content of the extracts is measured using the aluminum chloride colorimetric assay. Five hundred micro liters (500 μ L) of extract was mixed with 1.5 mL of the methanol followed by 100 μ L of 10% of aluminum chloride AlCl₃, 100 μ L of potassium acetate 1 M and 2.8 mL of the distilled water.

The samples are incubated at room temperature in the dark during 30min and the absorbance of the reaction mixture is measured at 415 nm versus a blank. Quercetin is used for the calibration curve and the total flavonoids are expressed as mg Quercetin Equivalents (QE)/g extract \pm Standard Deviation (SD).¹⁴

Antioxidant activity

The antioxidant potential of plant extracts was evaluated by three methods: scavenging of the free radical 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH), iron reduction (FRAP, for Ferric Reducing-Antioxidant Power) and the 2, 2'-Azino-bis (3-Ethylbenzothiazoline-6 Sulfonic Acid) assay (ABTS).

2,2-diphenyl-1-picrylhydrazyl free radical scavenging test

The anti-free radical activity of the different plant extracts is evaluated by the DPPH method.¹⁵ In brief, 250 μ L of various concentrations of each aqueous extract (0.05; 0.1; 0.2; 0.25; 0.5; 1 and 2 mg/mL) is mixed with 1.5 mL of a solution of DPPH (0.1mM) dissolved in the freshly methanol. After 20 min, the discoloration process is evaluated by absorbance measured at 517 nm with UV-1601 spectrophotometer (Shimadzu). The extract is replaced by ascorbic acid solution as control. The absorbance was measured under the same conditions as the samples and for each concentration. For each concentration, the test is repeated three times. The results are expressed as Inhibition Percentage (% IP):

% IP=(Abs control-Abs test)/(Abs control)*100

The median Inhibitory Concentrations (IC₅₀) are determined graphically by the linear regression and the results obtained from the mean of three independent experiments are expressed as percentage of the mean radical scavenging activity (%)±Standard Deviation (SD) and mean IC₅₀±SD.

Reducing power

The reducing power of the different extracts is evaluated by the spectrophotometric method of the transformation of (Fe3+)-(Fe²⁺).¹⁶ Different concentrations of 250 µL of aqueous extracts (0.05; 0.1; 0.2; 0.25; 0.5; 1 and 2 mg/mL) is mixed with 1250 µL of 0.2M phosphate buffer (pH=6.6) and 1250 μ L of 1% potassium ferricyanide [K₂Fe(CN)₆], and incubated in a water bath at T=50°C during 20 min. Then, 1250 µL of 10% of trichloroacetic acid is added to the cooled mixture that is centrifuged at 3000 revolutions during 10min. The supernatant (1250 µL) is mixed with 1250 µL of the distilled water and 250 µL of 0.1% of a fresh solution of Ferric Chloride (FeCl₂). The absorbance is measured at 700 nm after 10 min. The ascorbic acid is used as a positive control; the results are obtained from average of three independent experiments and are expressed by the mean of the absorbance values±the standard deviation in equivalent of the ascorbic acid.

Anti-free radical activity

Antiradical activity was estimated by using (ABTS.⁺) Free radical decolorization assay developed by Chakraborty with some modifications.¹⁷ Briefly, the preformed radical monocation of ABTS was generated by reacting ABTS solution (7 mM) with (2.45 Mm) Potassium persulfate ($K_2S_2O_8$). The mixture was incubated in the dark at room temperature during 15 hr. To

obtain an absorbance of 0.7 ± 0.2 units at 734 nm, the solution was diluted with the ethanol. The samples were separately dissolved in ethanol to obtain various concentrations: 0.0312, 0.0625, 0.125, 0.25, 0.50 and 1.00 mg/mL. In order to estimate the antioxidant activity of the samples, 10 µL of each extract, at various concentrations, was added to 990 µL of a diluted ABTS.⁺. After 6 min, the absorbance was measured by spectrophotometer at 734 nm. All measurements were performed in triplicate. The antioxidant potential of the tested samples was expressed as inhibition percentage. The percentage of the trapping of ABTS.⁺ was calculated by the following formula:

Activity of trapping (%)=(Ao-Ax)/Ao×100 Ax

Where: A_0 and A_x are the absorbance at 734nm of the samples without extract and with extract, respectively.

Data analysis

All analysis was done at least in triplicate, and these values were then presented as average values along with their Standard Derivations (SD). The comparison of the groups with the control is performed using the Graph pad prism 8.0.2 software and the values are considered to be significantly different for p<0.05.

RESULTS

Yield of extracts

The yield values of the different types of extracts are higher in the *Satureja calamintha* after two years of cultivation (Figure 1). The cultivated plant shows the highest extraction yields than the spontaneous. The infusion a better yield (18.22%), then the yield extract is significant in the decoction from the spontaneous (17.15%).

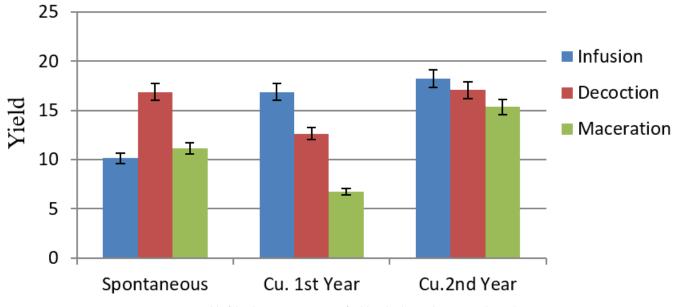


Figure 1: Yield of the three types extracts of wild and cultivated Satureja calamintha.

Table 1: Total phenol content of the different extracts.

	Content (mg EAG/g)		
Different extraction methods	Spontaneous	Cu. 1 st Year	Cu.2 nd Year
Infusion	123.242±5.64	123.226±0.586	126.625±0.127
Decoction	163.034±2.902	158.835±1.354	174.423±0.523
Maceration	131.173±2.902	150.192±0.895	160.682±0.221

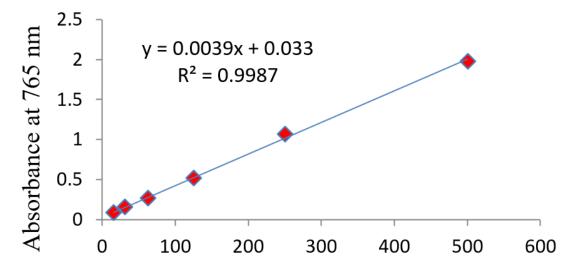


Figure 2: Gallic acid calibration curve.

Total phenols and flavonoids content Phenols content

Evaluation of the antioxidant activity Determination of DPPH radical scavenging capacity

The concentrations of total phenolic content (mg EAG/g dry material) are shown in Table 1 and Figure 2.

The results show that the extracts from the plant cultivated for two years are richer in polyphenols than those extracted from the wild and cultivated plants for one year. The decoction from cultivated plant for two years shows the highest content of polyphenols (174.423 ± 0.523 mgEAG/g) than the other types of extracts obtained from spontaneous plant (123.242 ± 5.64 ; 163.034 ± 2.902 and 131.173 ± 2.902 mgEAG/g).

Flavonoid content

The flavonoid contents are expressed as mg quercetin equivalents (QE)/g extract± Standard Deviation (SD). The results are given in Table 2 and Figure 3.

The infusion and decoction obtained from the plant cultivated for 2 years are considered the richest extract in flavonoids (Table 2). The highest rate of flavonoids is detected in the decoction obtained from plant cultivated for two years ($86.233\pm0.121 \text{ mgEQ/g}$ of extract), whereas the flavonoids content is $67.919\pm0.840 \text{ mgEQ/g}$ of extract in the case of the wild plant.

The infusion, decoction and maceration obtained from *Satureja calamintha* showed an antiradical power toward the DPPH which means that they have an antioxidant potential, especially in the case of cultivated plant for two years (Table 3). The anti-radical activity of the three extracts is higher than that of ascorbic acid with IC_{50} values of 3.514 ± 0.234 mg/mL, 1.174 ± 0.14 mg/mL and 3.504 ± 0.113 mg/mL, respectively. This activity is statistically similar to the activities performed by all extracts (*p*>0.05), along with the aqueous extracts of spontaneous *Satureja*, The difference in the anti-radical activity of classes of polyphenols that can have different antioxidant activities.

Ferric Reducing/Antioxidant Power (FRAP) assay

The Ferric-Reducing Antioxidant Power (FRAP) assay measures directly the reducing capacity of antioxidants. The IC_{50} concentration is determined to evaluate the reducing activity of the different extracts (Table 4). The results showed a correlation between the reducing capacity and the concentration of the extracts. The decoction and infusion obtained from plant cultivated for two years present respectively important reducing capacity (4.254±0.03 and 5.009±0.01 mg/mL), these values are

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Table 2: Flavonoid content of the different extracts.

	Flavonoid content mgEQ/g		
Different extraction methods	Spontaneous	Cu. 1 st Year	Cu.2 nd Year
Infusion	70.280±1.308	69.88±0.89	76.456±0.060
Decoction	67.919±0.840	69.88±1.08	86.233±0.121
Maceration	57.700±1.467	20.67±1.13	35.695±0.215

Table 3: Antiradical activity $\rm IC_{_{50}}$ (mg/mL) of the different plant extracts.

	IC ₅₀		
Different extraction methods	Spontaneous	Cu. 1 st Year	Cu.2 nd Year
Infusion	4.867±0.092	4.542±0.092	3.514±0.234
Decoction	3.112±0.181	2.080±0.191	1.174±0.141
Maceration	4.435±0.132	3.552±0.155	3.504±0.113
Ascorbic acid		0.124±0.040	

Table 4: IC₅₀ reducing activity (mg/mL) of extracts and standard.

	IC ₅₀		
Different extraction methods	Spontaneous	Cu. 1 st Year	Cu.2 nd Year
Infusion	8.021±0.01	7.798±0.05	5.009±0.01
Decoction	5.820±0.04	5.133±0.07	4.254±0.03
Maceration	7.221±0.03	6.252±0.05	6.342±0.03
Ascorbic acid		0.145±0.02	

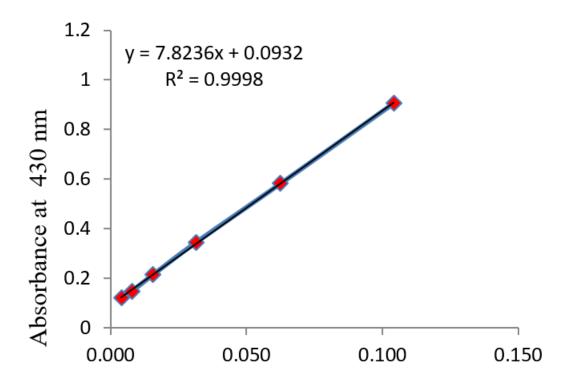


Figure 3: Quercetin calibration curve.

	IC ₅₀		
Different extraction methods	Spontaneous	Cu. 1 st Year	Cu.2 nd Year
Infusion	21.726±1.920	16.461±0.173	14.585±0.83
Decoction	13.228±0.24	11.978±0.12	9.969±0.09
Maceration	28.835±1.22	27.883±3.156	15.212±0.21
Ascorbic acid		2.159±0.081	

Table 5: Antioxidant activity IC₅₀ (mg/mL) of extracts and standard.

higher than that of ascorbic acid ($0.145\pm0.02 \text{ mg/mL}$). The values showed a significant difference (*p*<0.05).

Determination of ABTS radical cation scavenging capacity

The results of the analysis show that the different extracts show more important antioxidant activities than ascorbic acid (Table 5). The decoction obtained from cultivated plant shows highest values ($9.969\pm0.09 \text{ mg/mL}$ respectively) compared to that of ascorbic acid ($2.159\pm0.081 \text{ mg/mL}$). A significant difference was noted (p<0.05).

This result supports those obtained using the DPPH and FRAP assays for the evaluation of antioxidant activity in the various extracts.

DISCUSSION

The yield of Satureja extraction by decoction is comparable to that of the *S. montana* species (18.70%).¹⁸ Therefore, our study's yield pales in comparison to Bougendoura's yield of 22.19%. Generally, Satureja grew in the second year with a high yield compared to other plant types. The difference in yields between the extracts would be due to a number of factors, such as soil, science, the organ used in extraction and the extraction method, drying conditions, the metabolite content of each species, the nature of the solvent used in extraction and the extraction methods applied, as well as the rate of domestication.¹⁸ Thus, this difference is due to variations in factors of intrinsic or extrinsic origin, namely geography, harvesting period, and harvesting stage.

In Bougandoura's study, the aqueous extract had higher phenol content (12.6 ± 0.775 mg/g) than the total methanolic extract (2.968 ± 0.809). We found the flavonoid content of each extract using the aluminum trichloride method. We expressed the value in mg of catechin equivalent per gram of dry plant material. The results show that both extracts have moderate contents. Flavonoids represent 43.24% of the total phenols in the methanolic extract. This level does not exceed 24.84% in the aqueous extract.¹⁹

According to a reference study,²⁰ the spontaneous type 9.78 ± 0.028 mg EQ/g extract of the same Satureja species from the Ouazzane region had a very low flavonoid content. The flavonoid content of plant extracts depends mainly on their origin and composition. Some intrinsic factors are genetic, and some are extrinsic:

climatic conditions (growing environment), maturity at harvest (plant age and harvest season), the various diseases that can affect the plant,²⁰ or abiotic stress such as water stress.²¹ Consequently, solvent polarity plays an essential role in increasing the solubility of phenolic compounds. The level of phenolic compounds present in our extracts is very high. This may be useful when considering the study of biological activities.

The results of the DPPH free radical scavenging experiment demonstrate that the antioxidant compounds found in Satureja extracts, such as flavonoids and polyphenols, have the ability to release hydrogen, reduce DPPH, and alter its color. This involves moving one or more electrons from the outer orbital of the DPPH. The radical and antioxidant molecule will undergo a complete reaction. Because the electrical layers of the radical are already filled, the antioxidant activity remains constant even at higher concentrations (Roussy *et al.*, 2015).²² The results are comparable to those of Bougandoura because the antioxidant power results of the extracts tested show that at concentrations of approximately 4.624 and 5.0 mg/mL for aqueous and methanolic extracts, respectively, the percentage inhibition of *Satureja calamintha* is greater than 90%.¹⁹

The reducing power of *Satureja calamintha* ranged from 4.254 ± 0.03 mg/mL to 8.021 ± 0.01 mg/mL. Bougandoura discovered that the methanolic extract of *Satureja calamintha* had a much higher reducing power (OD=0.484) than the aqueous extract but a much lower reducing power than ascorbic acid. It is likely that *Satureja calamintha*'s reducing power comes from the phenolic compounds' hydroxyl groups, which can give away electrons. Consequently, antioxidants are considered to be oxidant reducers and inactivators.²³

CONCLUSION

In this work, polyphenols content and evaluation of the antioxidant activity the spontaneous and cultivated *Satureja calamintha* has been studied.

According to the results of this study, the decoction made from 15% to 18% of plants that were grown for two years has more polyphenols (ranging from 126.625 ± 0.1278 mg EGA/g to 174.423 ± 0.523 mg EGA/g) and works better as an antioxidant than the reference reactive. Extracts from the leaves of the cultivated plant are richer in polyphenols and contain more antioxidant activities. The results obtained confirm that domestication and cultivation affect slightly the chemical and biological quality of the plant. It is worthwhile to mention that these potential sources of the natural antioxidants could be exploited in the food, pharmaceutical and cosmetic industry.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

GAE/g DM: Milligrams of gallic acid equivalent per gram of dry weight; EQ: Quercetin equivalent; Ref: Reference; hr: Hour; min: Minute; no: Number; DPPH: 2,2-Diphenyl-1-picrylhydrazyl free radical scavenging test; FRAP: Ferric reducing-antioxidant power; ABTS: 3-Ethylbenzothiazoline-6 Sulfonic Acid; IC₅₀: Median inhibitory concentration; Cu. 1st Year: Cultivated for The First Year; Cu. 2nd Year: Cultivated for the Second Year; INRA: The French National Institute for Agronomic Research.

ETHICAL CONSIDERATIONS

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, and redundancy) have been completely observed by the authors.

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