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Solvent Fractionation and Bioguided Screening of Powerful Antioxidants from *Pelargonium denticulatum* Jacq. Aerial Parts

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Abstract:
Background*: In recent years, scientific communities have shown an increased interest in plant antioxidants which could be used as natural additives to replace synthetic ones. This upheaval involves a constant growing requirement of raw materials and new ingredients from natural sources. Phenolic compounds constitute one of the major groups of plant secondary metabolites, involved in the antioxidant defense. The present study consists of an exploration of the effect of solvent fractionation on the phenolic compound contents, their antioxidant activities and their chemical composition in P. denticulatum aerial parts.*
Materials and Methods*: P. denticulatum was harvested during the flowering stage. Plant material was air dried then subjected to a differential extraction using different polarity solvents. Comparison of the effect of this parameter on the contents of polyphenols, flavonoids and total condensed tannins as well as on the total antioxidant, anti-radical, iron-reducing and bleaching-inhibiting capacities of β-carotene was been carried out. In a second step, a qualitative and quantitative chromatographic study of the phenolic compounds of the most active fraction was carried out.*
Results*: The results obtained showed a close relationship between the composition of the aerial parts of P. denticulatum in terms of these metabolites as well as their antioxidant capacities and the factor studied. The aqueous fraction exhibited the highest contents of phenolic compounds and the strongest antioxidant activities. Chromatographic investigation of its chemical composition divulged the presence of three compounds. Although all of the peaks that appeared didn't match to the 47 used standards, these compounds represent 93% of the total mass of the fraction studied and 32% of the plant material in its raw state. Besides, peak (1) constituted the major part of the aqueous fraction (68%) as it may be one of the main major phenolic compounds of P. denticulatum (24% DM), or even the only one.*
Conclusion: *P. denticulatum parts are rich in phenolic compounds exhibiting high antioxidant activities. Solvent fractionation of these metabolites permitted the separation of an aqueous fraction enriched in polar polyphenols. Their contents testify to their importance at the scale of the whole plant as they reflect the interest of the solvent fractionation used method.*
Key Word*: Pelargonium denticulatum; Phenolic compounds; Solvent fractionnation; Antioxidant activities; RP-HPLC.*

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I. Introduction

The investigation of natural and safe antioxidants from natural origin is the fashion wave invading the world of food and pharmaceutical sciences, in attempt to be used as natural additives to replace synthetic ones, since it was proven that these latter have potential adverse health effects[1,2,3]. Phenolics are the most represented dietary antioxidants and have shown various beneficial effects on human health[4]. The structural plurality of these compounds due to their dual biosynthetic origin is responsible for their functional heterogeneity, which is expressed differently depending on the degree of complexity of the molecule concerned and the number of hydroxyl groups in each benzene ring[5]. Plants contain a complex mixture of phenolic compounds with different polarities. Therefore, an efficient extraction procedure is generally the analytical step for the precise separation and quantification of the phenolic compounds and the estimation of antioxidant activity of plants. The choice of extraction method and solvent has an important impact on the extraction of these metabolites. In fact, solvent polarity plays a key role in increasing the solubility of phenolics[3,6], since the capacity of extraction depends on the chemical structure, solubility, polarity and diffusion in the plant material. Thus, some parameters must be

taken in consideration when choosing the solvent; essentially the molecular affinity between the solvent selected and the solute for an efficient extraction of antioxidants[7].

P. denticulatum is a well branched strongly aromatic plant with sticky finely divided leaves, widely distributed in the Mediterranean basin and in South Africa. With its attractive, strongly scented leaves and pinkish purple flowers, it represents an interesting addition to the mixed herbaceous border, adding texture, fragrance and color. To the best of our knowledge, no previous quantitative or qualitative study has described the phenolic compounds and their related biological activities in this species, which constitutes an originality of our work.

II. Material And Methods

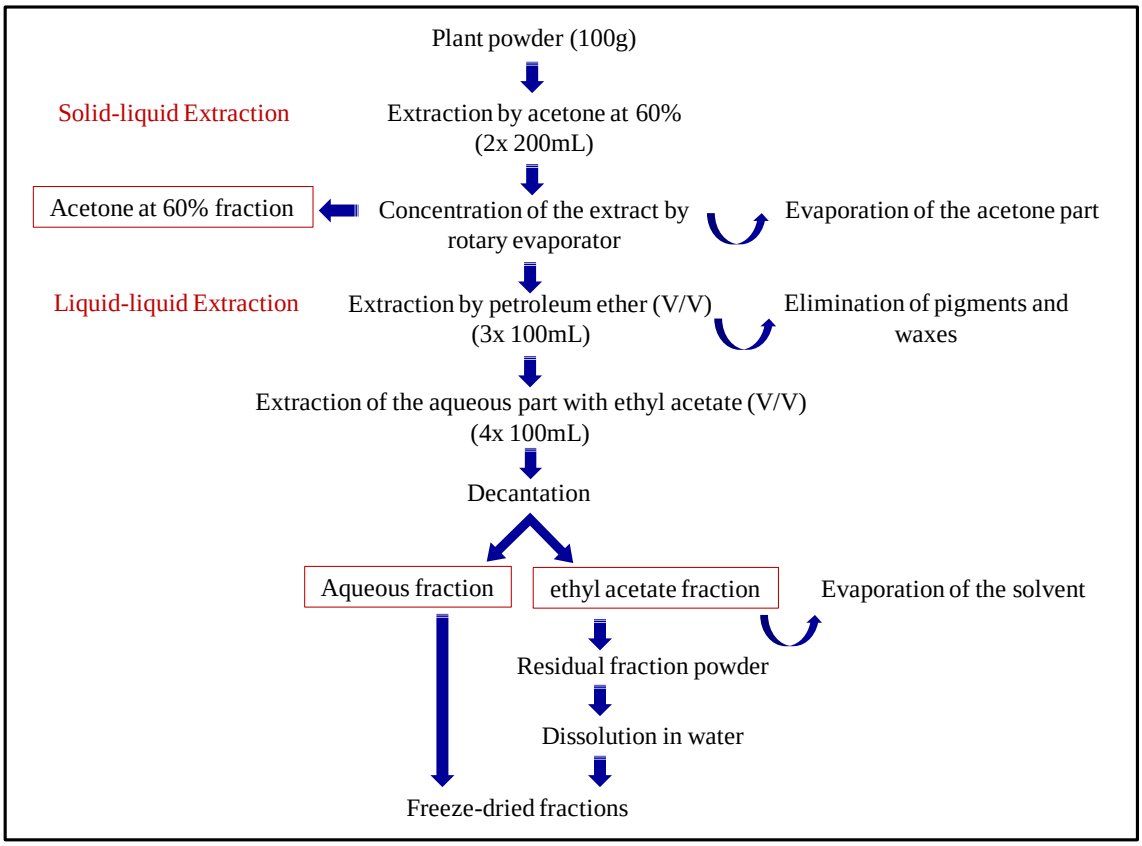
Plant sampling

P. denticulatum was collected in the region of Soliman (governorate of Nabeul) in the North-East of Tunisia (N36°42', E10°29'), belonging to the Upper Semi-Arid bioclimatic stage (average temperatures 18°C, annual rainfall between 500-600 mm)[8]. The harvested plant was identified at the Biotechnology Center of Borj Cedria. A voucher specimen (PD-CBBC-07) was deposited in the herbarium of the Laboratory of Aromatic and Medicinal Plants (LPAM). Aerial parts were air dried at the shadow then grounded to a fine powder.

Extraction of phenolic compounds

Aerial part powder was subjected to a differential extraction using diverse polarity solvents (petroleum ether, ethyl acetate, acetone mixed with water) as described by Jallali et al.[2] and as illustrated in Figure 1. A solid/liquid extraction of 100 g of sample powder by 60% aqueous acetone (v/v) (2 x 200 mL) was carried out. Mixture was kept in frequent agitation at ambient temperature for two hours then filtered through a Whatman N°4 filter paper. After filtration, the aqueous acetone extracts were combined and concentrated at 35 °C under reduced pressure. The residual aqueous phase (100 mL) was extracted by petroleum ether (3 x 100 mL) for two hours to eliminate pigments and waxes then subjected to a liquid/liquid extraction by ethyl acetate (4 x 100mL). Mixture was transferred in a funnel and left to stand until two phases were observed. Aqueous phase was discarded and the organic phase was collected and evaporated under vacuum at 35 °C to dryness then dissolved in water to be freeze-dried. Dry residue was stored in the darkness at 4 °C until analysis.

Figure no 1: Experimental protocol of the differential extraction of phenolic compounds from the aerial parts of *P. denticulatum*



Determination of total polyphenol content

Colorimetric quantification of total polyphenol was determined using the Folin–Ciocalteu reagent[9]. Briefly, 125 µL of suitable diluted sample extract was dissolved in 500 µL of distilled water and 125 µL of the Folin–Ciocalteu reagent. The mixture was shaken before adding 1250 µL sodium carbonate anhydrous (Na_2CO_3) (70 g L^{-1}), then adjusted with distilled water to a final volume of 3 mL, and mixed thoroughly. After incubation for 90 min at 23 °C in the dark, the absorbance versus prepared blank was read at 760 nm (LABOMED, INC. UV/Vis apparatus). Total phenolic content of plant aerial parts was expressed as mg gallic acid equivalents per gram of dry weight (mg GAE g^{-1} DW) through the calibration curve with gallic acid ranging from 0 to 400 µg.mL^{-1} (r^2 = 0.99). All samples were analyzed in three replications.

Estimation of total flavonoid content

Total flavonoids were measured according to Dewanto et al.[9]. An aliquot of diluted sample or standard solution of (+)-catechin was added to a 75 µL of sodium nitrite solution ($NaNO_2$), and mixed for 6 min before adding 0.15 mL of aluminum chloride hexahydrate solution ($AlCl_3$, $6H_2O$) (100 g.L^{-1}). After 5 min, 0.5 mL of sodium hydroxide NaOH (1M) was added. The final volume was adjusted to 2.5 mL with distilled water and thoroughly mixed. Absorbance was determined at 510 nm against the same mixture, without the sample, as a blank. Total flavonoid content was expressed as milligram catechin equivalent per gram dry weight (mg CE.g^{-1} DW), through the calibration curve of (+)-catechin ranging from 0 to 400 µg.mL^{-1} (r^2 = 0.99). All samples were analyzed in three replications.

Quantification of total condensed tannins

Proanthocyanidins were measured using the modified vanillin assay[10]. To 50 µL of properly diluted sample, 3 mL of methanol vanillin solution and 2.5 mL of sulfuric acid (H_2SO_4) were added. The absorption was measured at 500 nm against extract solvent as a blank. The amount of total condensed tannins is expressed as mg CE.g^{-1} DW. The calibration curve range 0-400 µg.mL^{-1} (r^2 = 0.99). All samples were analyzed in three replications.

RP-HPLC identification of *P. denticulatum* phenolic compounds

The identification of *P. denticulatum* phenolic compounds was done using Agilent 1260 HPLC system consisting of a vacuum degasser, an autosampler, and a binary pump with a maximum pressure of 600 bar (Agilent technologies), equipped with a reversed-phase C18 analytical column of 4.6 × 250 mm and 5µm particle size (Zorbax Eclipse XDB C18). The DAD detector was set to a scanning range of 200 to 400 nm. Column temperature was maintained at 25°C. The injected sample volume was 2 µL and the flow rate of mobile phase was 0.4 mL.min^{-1}. Mobile phase B consisted of 0.1% formic acid Milli-Q water and mobile phase A was acetonitrile. The elution program was as follows: 0 to 6 min: 35% B; 6 to 9 min: 35% to 60% B; 9 to 14 min: 60% to 80% B; 14 to 25 min: 80% to 100% B; 25 to 30 min: 100% to 35% B. Each sample was directly injected and chromatograms were monitored at 280 nm.

Antioxidant activities of *P. denticulatum* extracts

Evaluation of total antioxidant capacity

The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. An aliquot (0.1 mL) of plant extract was combined to 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate), then incubated in a thermal block at 95 °C for 90 min. After the mixture had cooled to room temperature, the absorbance of each solution was measured at 695 nm against a blank. The antioxidant capacity was expressed as mg GAE.g^{-1} DW[11]. The calibration curve range was 0–500 µg.mL^{-1}. All samples were analyzed in triplicate.

DPPH assay

DPPH quenching ability of plant extracts was measured according to Hanato et al.[12]. One mL of the plant fraction extracts properly diluted was added to 0.25 mL of a DPPH methanolic solution. The mixture was shaken vigorously and left standing at room temperature in the dark. The DPPH radical-scavenging capacity was reported after 30 min reaction time for each diluted sample by the decrease in absorbance at 517 nm. IC_{50} value (mg. mL^{-1}), defined as the concentration of antioxidant required for 50% scavenging of DPPH radicals in this specified time period, is a parameter widely used to measure antioxidant activity; a smaller IC_{50} value corresponds to a higher antioxidant activity of the plant extract. The ability to scavenge the DPPH radical was calculated using the following equation:

DPPH scavenging effect (%) = [(A0-A1)/A0]*100 (1)

Where A0 is the absorbance of the control at 0 min, and A1 is the absorbance of the sample at 30 min. All samples were analyzed in three replications.

FRAP assay

The iron (III) reductive capacity of the plant extracts was assessed as described by Oyaizu[13]. Briefly, 1mL of extract was mixed with 2.5 mL phosphate buffer (0.2 mol.L^{-1}, pH 6.6) and 2.5 mL (1%) $K_3Fe(CN)_6$ solution. After 20 min at 50°C, 2.5 mL (10 %) trichloroacetic acid was added and the mixture was centrifuged for 10 min at 650 x *g*. Finally, a 2.5 mL aliquot was mixed with 2.5 mL ultra-pure water and 0.5 mL (0.1 g.100 mL^{-1}) $FeCl_3$ and the absorbance was recorded at 700 nm. Ascorbic acid was used as a positive control. A higher absorbance indicates a higher reducing power. Results are expressed as Effective Concentration at which the absorbance was 0.5 (EC_{50} in mg.mL^{-1}) obtained from linear regression analysis.

***β*-Carotene bleaching test (BCBT)**

To assess this activity, a slightly modified method of Koleva et al.[11] was employed. *β*-carotene (2 mg) was dissolved in 20 mL chloroform and to 4 mL of this solution, linoleic acid (40 mg) and Tween 40 (400 mg) were added. Chloroform was evaporated under vacuum at 40°C and 100 mL of oxygenated ultra-pure water was added, then the emulsion was vigorously shaken. Extract samples and reference compounds (BHT and BHA) were prepared in ethanol. An aliquot (150 µL) of the *β*-carotene: linoleic acid emulsion was distributed in each of the wells of 96-well microtitre plates and methanolic solutions of the test samples (10 µL) were added. Three replicates were prepared for each of the samples. The microtitre plates were incubated at 50°C for 120 min, and the absorbance was measured using a model EAR 400 microtitre reader (Labsystems Multiskan MS) at 470 nm. Readings of all samples were performed immediately (t=0 min) and after 120 min of incubation. The antioxidant activity (AA) of the extracts was evaluated in term of *β*-carotene blanching using the following formula:

AA (%) = [(A0-A1)/A0]*100 (2)

Where A0 is the absorbance of the control at 0 min, and A1 is the absorbance of the sample at 120 min. All samples were analyzed in three replications. The results are expressed as IC_{50} values (µg.mL^{-1}).

Statistical analysis

Means were statistically compared using the STATISTICA program. A one-way analysis of variance (ANOVA) and Duncan's multiple range tests were carried out to perceive any significant differences between parameters at $P< 0.05$.

III. Result

Contents of polyphenols, flavonoids and total condensed tannins

Total polyphenol content

The results of total polyphenol quantification from the different fractions of the aerial parts of *P. denticulatum* showed significant variability in the levels of these secondary metabolites depending on the extraction solvent (Table 1). Indeed, the comparison of the contents of phenolic compounds of the different extracts showed that the aqueous fraction contained high amounts of polyphenols, reaching 581.2 mg GAE.g^{-1} DM. These levels were up to almost twice those of the other two fractions with ethyl acetate fraction coming second, followed by that of acetone at 60%.

Total flavonoid content

Flavonoids represent one of the most studied classes of polyphenols today. The flavonoid contents of the aerial part fractions of *P. denticulatum* are shown in Table 1. As for the total polyphenols, the flavonoid contents of the aqueous fraction far exceed those of the other two fractions. Their amounts were about 231.1 mg CE.g^{-1}DM and represented a little more than the double (89.9 mg CE.g^{-1}DM) and the quadruple (50.2 mg CE.g-^{1}DM) of those obtained in the ethyl acetate and acetone at 60% fractions, respectively.

Quantification of total condensed tannins

The results of the total condensed tannins quantification (Table 1) revealed a clear distinction of the aqueous fraction with a very high content of these metabolites reaching 492.5 mg CE.g^{-1}DM. On the other hand, we noted that contrary to the contents of polyphenols and total flavonoids, proanthocyanidins are present in greater quantities in the acetone at 60% fraction than the ethyl acetate one.

Antioxidant activity of *P. denticulatum* fraction extracts

To evaluate antioxidant efficiency, three complementary methods were chosen due to their simplicity, stability, and accuracy.

Total antioxidant activity

The results of the total antioxidant activity shown in Table 1 revealed a very marked effect of the extraction solvent. Indeed, the aqueous fraction exhibits a very high activity equal to 1938.7 mg GAE.g^{-1}DM. It

represented almost three times the activities of the ethyl acetate and acetone at 60% fractions, which was also very significant and reached 662.3 and 635.7 mg GAE.g^{-1}DM, respectively.

Antiradical activity (DPPH test)

The antiradical power of the different extract fractions of *P. denticulatum* aerial parts was estimated by the test of the inhibiting capacity of the synthetic radical DPPH. The comparison of the various 50% inhibition concentrations (IC_{50}) showed a clear difference in the results obtained as compared to the other tests and with respect to the solvents used (Table 1). Indeed, the ethyl acetate fraction expressed the lowest IC_{50} value (5.1 µg.ml^{-1}) thus reflecting the strongest antiradical activity followed by the aqueous fraction, while the acetone at 60% fraction came last. The anti-radical activity of the ethyl acetate fraction was very interesting. It even exceeded that of the positive control BHT.

Ferric Reducing Antioxidant Power (FRAP)

In order to evaluate the reduction capacity of transition metal ions such as iron, the measurement of the reducing power of *P. denticulatum* extract fractions was performed. The effective concentrations (EC_{50} expressed in µg.mL^{-1}) corresponded to the concentration of the extract at an absorbance equal to 0.5.

By analogy to the TTA capacity and differently to the DPPH test, the comparison of the EC_{50} of the various fractions revealed a tangible effect of the solvent on the reducing potentialities of iron (Table 1), with the prepotency of the aqueous fraction expressing the greatest reducing power with an EC_{50} value of around 172 µg.mL^{-1}, followed by that of ethyl acetate fraction, and finally the 60% acetone fraction.

***β*-Carotene Bleaching Inhibition Test (BCBT)**

The results of the *β*-carotene bleaching inhibition activity expressed the same tendency as TAA and FRAP tests. Indeed, the aqueous fraction exhibited an interesting antioxidant capacity with an IC_{50} = 196 µg.mL^{-1}. The ethyl acetate fraction was second, while that with acetone at 60% exhibited very low activity (IC_{50} = 1837 µg.mL^{-1}), as in the two previous tests.

Table no 1: Phenolic compound contents and *In vitro* antioxidant activities of *P. denticulatum* extracts. Means followed by the same letter in the row are not significantly different at P < .05 (means of 3 replicates).
Abbreviations: TPC: total polyphenols content; TFC: total flavonoid content; CTC: condensed tannins content; TAA: total antioxidant activity; DPPH test: 2,2-diphenyl-1-picrylhydrazyl test; FRAP test: ferric reducing antioxidant power; BCBT: *β*-carotene bleaching test; mg CE.g^{-1} DW: milligram catechin equivalent per gram dry weight; mg GAE.g^{-1} DW: milligram gallic acid equivalent per gram dry weight; AsA: Ascorbic Acid; BHA: butylated hydroxyanisole; BHT: butylated hydroxytoluene.

	Acetone at 60% Fraction	Ethyl acetate fraction	Aqueous fraction	Positive controls		
				BHT	BHA	AsA
TPC (mg GAE.g^{-1} DW)	284±4.5 C	303±7.7 B	581±10.0 A			
TFC (mg CE.g^{-1} DW)	50.2±2.6 C	90±2.3 B	231±3.1 A			
TCT (mg CE.g^{-1} DW)	235±2.3 B	91±2.1 C	492.5±9.0 A			
TAA (mg GAE.g^{-1} DW)	636±9.5 C	662±6.5 B	1939±11.4 A			
DPPH (µg.mL^{-1})	65.3±3.5 A	5.1±0.13 C	18.5±2.0 B	11.5±0.4	6.1±0.3	-
FRAP (µg.mL^{-1})	992±14 A	194±9.0 B	172±4.0 C	-	-	37.3±0.3
BCBT (µg.mL^{-1})	1837± 28 A	371±10 B	196±12 C	75±0.1	48±0.2	-

According to the results of the various analyzes carried out, it was concluded that the aqueous fraction of the aerial parts of *P. denticulatum* is the richest in phenolic compounds and the most active among the three analyzed fractions. For this, it was retained for the identification of its chemical composition by HPLC-PR as well as the determination of the amounts of the various products contained in.

Identification and quantification of the phenolic compounds of the most active fraction of *P. denticulatum* by HPLC-RP

The chromatographic profile of the main phenolic compounds obtained from the aqueous fraction of *P. denticulatum* aerial parts (Figure 2) revealed the presence of a large major peak (1) followed by two peaks of lesser magnitude (2) and (3), in addition to the peak corresponding to BHT (4) co-injected with the extract.

Unfortunately, these peaks were not identified. They did not match any phenolic standard compound from the list in Table 2, used for the identification. Nevertheless, we tried to quantify the contents of these three products in relation to the totality of the aqueous fraction and the plant material initially used (Table 3).

Table no 2: Retention time of 47 standards of phenolic compounds. Components are listed in order of elution in ZORBAX ECLIPSE XDB-C18 column.

N°	Compound name	Retention time (mn)
1	Tannic acid	3.69
2	Gallic acid	3.67
3	Catechin hydrates	4.24
4	Procatechic acid	4.16
5	3,4-dihydroxybenzoic acid	4.33
6	Resorcinol	6.12
7	Chlorogenic acid	6.52
8	methyl gallate	6.68
9	Apicatechin	6.38
10	vannilic acid	6.02
11	Genistic acid	5.48
12	Caffeic acid	5.56
13	Syringic acid	9.65
14	2, hydroxyphenyl acetic acid	6.73
15	Phloretic acid	9.82
16	trans-4-hydroxycinnamic acid (p-coumaric)	14.66
17	Taxifolin	14.8
18	Ferulic acid	16.87
19	Naringin	17.68
20	Naringenin-7-o-glucoside	17.84
21	Coumarin	18.22
22	Rutin hydrates	16.75
23	n-propyl-3,4,5-trihydroxybenzoate	18.4
24	Methyl-4-hydroxybenzoate acid	18.6
25	Myrictin	14.45
26	Fistein hydrates	14.7
27	Morine	18.62
28	Daidzein	-
29	3,4-methoxyphenyl propinoic acid	18.37
30	Quercetin	18.46
31	Naringenin	19.16
32	Luteolin	19.06
33	Trans-cinnamic acid	19.8
34	Hesperetin	19.81
35	Genistein	19.91
36	Trans methyl cinnamate	-
37	betelunic acid	-
38	Warfarin	22.39
39	Flavone	22.64
40	5,7 dihydroxyflavone (chrysin)	23.19
41	Kaemperide	22.64
42	Flavonose	23.78
43	Chalcone	24.39
44	Flavonol	23.68
45	BHT	28.06
46	Rosmarinic Acid	13.78
47	Carnosoic acid	25.29

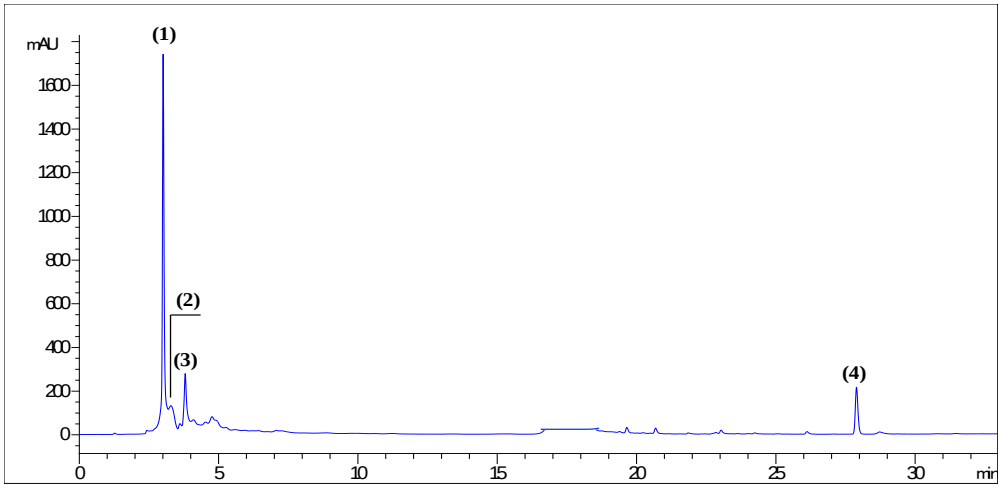


Figure no 2: Chromatographic profile of the main phenolic compounds of the aqueous fraction of the aerial parts of *P. denticulatum* analyzed at 280 nm. Peaks (1), (2) and (3) correspond to the products contained in the extract, while peak (4) relates to the internal standard BHT.

The quantification of the amounts of the three compounds which appeared in the chromatographic profile of *P. denticulatum* aqueous fraction (Figure 2) revealed that the major product (1) represents 68 % of the total fraction studied and almost 24% of the dry plant matter initially used (Table 3), which implies that it is also the major one in *P. denticulatum* aerial parts. The compounds (2) and (3) showed close contents representing 12 and 13% of aqueous fraction, respectively, and almost 4% of the dry vegetable matter. These levels are quite significant but remain very low compared to the magnitude of the major product (1).

Table n 3: Relative contents of phenolic compounds in the aqueous fraction and in the dry matter initially used of *P. denticulatum* aerial parts. Compound proportions were calculated from the chromatograms obtained on the ZORBAX ECLIPSE XDB-C18 column.

Pick N°	$mg.g^{-1}$ Aqueous Fraction	$mg.g^{-1}$ Dry Matter
(1)	681	237
(2)	129	45
(3)	121	42

IV. Discussion

Effect of solvent fractionation on phenolic compound contents and antioxidant capacities of *P. denticulatum extracts*

The examination of the influence of the extraction solvent on the phenolic compound contents in *P. denticulatum* aerial parts revealed the marked effect of this post harvesting factor on these metabolites. The phenolic compound contents of the crude fraction (acetone at 60%) and of the two fractions derived from it (ethyl acetate and aqueous ones) are high and significantly variable. This variability is related to the physico-chemical properties of the solvent used as well as to the chemical class of the phenolic compounds extracted. Indeed, the solubility of these secondary metabolites is, in reality, governed by the type of solvent used, the degree of polymerization of these molecules as well as their interaction with other constituents of the plant[14,15]. Solvent polarity is a definitive parameter in the extraction of different classes of phenolic compounds. In this context, previous work showed that total polyphenol contents increase with increasing solvent polarity[15,16]. On the other hand, results of the phenolic quantification showed a preferential accumulation of these metabolites in the aqueous fraction. The latter contains 2 to 4 times more of these compounds than the other two fractions. For total polyphenol and total flavonoid contents, the order of superiority is as follows: aqueous fraction > ethyl acetate fraction > acetone at 60% fraction. However, the total condensed tannins tended to migrate towards the fractions with higher polarity (acetone at 60% and aqueous fractions) where they were concentrated at very important amounts, especially at the aqueous fraction. Our results corroborated previous works demonstrating that a combination of water and organic solvents may help improve the efficiency of extracting phenolic compounds from plant matrix[3,5,17].

In addition to the phenolic compound quantification, estimation of the antioxidant activities of the different fractions confirmed the important effect of the extraction solvent on these parameters. In fact, the three

fractions studied showed a very interesting antioxidant potential, considerably variable depending on the solvent and the reaction principles of the different tests evaluated. By analogy to its high levels of phenolic compounds, the aqueous fraction expressed the strongest antioxidant activities, excepting the anti-DPPH activity where the ethyl acetate fraction was the best. In fact, the results showed that the aqueous fraction exhibits a very interesting total antioxidant activity up to more than three times that of the acetone at 60% and ethyl acetate fractions. The distribution of antiradical molecules between these three fractions was illustrative and highlighted the interest of solvent fractionation of secondary metabolites. The liquid/liquid extraction of the acetone at 60% crude fraction permitted to recover an ethyl acetate fraction with a very high activity followed by an aqueous fraction with a fairly interesting antiradical power. This phenomenon can be explained by the fact that the majority of the antiradical molecules of the crude extract have a high affinity to ethyl acetate. This hypothesis suggests that these molecules belong to the class of flavonoids since other studies have shown that they are preferentially extracted by this low to medium polarity solvent[2,18]. In addition, we founded that aqueous fraction has the most interesting antioxidant potentialities. However, several other works have shown that ethyl acetate extracts of several plants have the ability to act as powerful antioxidant compared to other extracts[2,18,19,20]. This divergence from our results is logically due to the interspecific variability of the phenolic repertoire and its solubility in different solvents and suggests that active phenolics of *P. denticulatum* are rather polar compounds with high molecular weight, probably belonging to the phenolic acids or condensed tannins, as shown by the results of phenolic compounds quantification. In fact, other studies showed that the phenolic acids, which are very polar, cannot be completely extracted with pure organic solvents and the use of alcohol–water or acetone–water mixtures was suggested[21,22].These results affirm that the selective extraction of bioactive molecules from a natural source is important in the context where we want to end up with fractions with greater biological activities that can be used in pharmaceutical and agri-food industries. Indeed, the ethyl acetate and aqueous fractions come from a liquid/liquid separation from the crude fraction. This separation made it possible to expose the real antioxidant potentialities of the molecules enclosed therein, thus highlighting the beneficial effect of the separation of phenolic extracts into several fractions with a view to improve their biological activities. On the other hand, these antioxidant activities do not only depend on the concentration of phenolic compounds in the plant but also on their structure (simple and polymerized forms) and their interactions with each other or with other compounds of the plant[6,14,23]. It should be noted that in the presence of complex mixtures of phenolic compounds, the molecules can be rearranged by changing their structure and orientation, thus leading to a decrease in their biological activity[24]. The fractionation of phenolic compounds with respect to their properties (molecular weight, solubility and polarity) is a suitable approach which allows the separation of complex mixtures of these molecules, thus eliminating the antagonistic effect between them and facilitating the study of their activities.

Identification and quantification of phenolic compounds in the aqueous fraction of the aerial parts of *P. denticulatum*

The phenolic repertoire of plants is made up of a variety of structures representing the majority of phenolic classes[25]. The combination of the different structures, their relative proportions as well as their interactions (synergism or antagonism) is the origin of the diversity of their biological activities[26]. A modification of the phenolic repertoire, under the influence of one factor or another, could have a direct effect on a characteristic biological property of a plant. Results of the first part of this work showed that the aqueous fraction expressed the best antioxidant potential. It was so retained to be the subject of a qualitative and quantitative identification, by RP-HPLC, of its phenolic composition. This identification is an originality of this work since no previous study has reported it in this species.

The peaks of phenolic compounds are identified according to their retention time by co-injection of pure controls of phenolic acids and flavonoids under the same experimental conditions (Table 2). The quantification of these identified compounds was done using an internal standard which is BHT. Analysis of the chromatographic profile of the aqueous fraction of *P. denticulatum* revealed the presence of three compounds. However, they couldn't be identified because they didn't correspond to any one of the standards (consisting essentially in phenolic acid and flavonoids). Gathering this results to previous ones showing the richness of this fraction on phenolics, especially the spectacular amounts on condensed tannins, and their relative important antioxidant activities; and taking in consideration that the water is a polar solvent having specific extraction preferences on polar molecules with high molecular weight, we can suppose that the three picks on the chromatogram correspond to hydrolysable or condensed tannins.

In addition, the dosage of the proportions of these three compounds relative to the mass of the dry residue of the aqueous fraction as well as to the dry plant matter showed that, from one hand, these compounds represent 93% of the total mass of the fraction studied and 32% of the plant material in its raw state. On the other hand, peak (1) constituted the major part of the aqueous fraction (68%) as it may be one of the main major phenolic compounds of *P. denticulatum* (24% DM), or even the only major one. The analysis of this

chromatographic profile in relation to the amount assay results and the antioxidant activities of the phenolic compounds mentioned above showed that there is a close relationship between the chemical composition of the aqueous fraction and its very interesting antioxidant potential. It remains to be proven whether these potentialities are due to compound (1) only or it is the result of a synergism between the three products available. Further studies using more efficient separation and identification techniques, such as preparative HPLC and NMR, will allow the structural elucidation of these compounds as well as the study of their interactions and biological activities.

From another side, the reduced number of phenolic compounds in this fraction, as well as their amounts, are very significant and reflect the importance of solvent fractionation in the pre-purification and the concentration of the active principles of an extract. Indeed, the experimental process followed during the extraction is an easy technique for separation and pre-purification of the target molecules. Washing the crude extract with petroleum ether is essential to get rid of the pigments and lipids which constitute a significant part of the dry residue and are reflected by an overload of peaks at the level of the chromatographic profiles which camouflages, in certain cases, the target molecules to be identified[5]. Subsequently, the liquid/liquid extraction of the depigmented extract with a solvent of medium polarity, such as ethyl acetate, having an affinity towards a very specific class of molecules[15] allows the separation of phenolic compounds by respecting their properties (molecular weight, solubility and polarity) thus allowing a better study of their biological activities[24]. The presence of only three peaks in the chromatographic profile of the *P. denticulatum* fraction is a good indicator of the effectiveness of this method.

V. Conclusion

P. denticulatum is an aromatic plant having important phenolic amounts with high antioxidant potential, easily recoverable by a simple, but adapted extraction method based on the separation of these compounds according to their polarities and affinities to the used solvents. This step is crucial when arguing about the biological effectiveness of plant secondary metabolites and may play a key role in the valorization of this species as a source of valuable antioxidant molecules that may be used in food, cosmetic, and pharmaceutical industries.

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