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Article in *Journal of Research in Pharmacy* · January 2022

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# Antioxidant, anticancer activities, and HPLC-DAD analyses of the medicinal halophyte *Limoniastrum guyonianum* Dur. extracts

Sara ZERROUKI<sup>1\*</sup>, Samia MEZHOU<sup>1</sup>, Ayse SAHİN YAĞLIOĞLU<sup>2</sup>, Chawki BENSOUICI<sup>3</sup>, Mehmet NURİ ATALAR<sup>4</sup>, Ibrahim DEMİRTAS<sup>2</sup>, Souad AMEDDAH<sup>5</sup>, Ratiba MEKKIOU<sup>1,\*</sup>

<sup>1</sup> Unité de recherche: Valorisation des Ressources Naturelles, Molécules Bioactives et Analyses Physicochimiques et Biologiques (VARENBIOMOL), Université des Frères Mentouri Constantine, Constantine, Algérie.

<sup>2</sup> Technical Sciences Vocational School, Department of Chemistry and Chemical Process Technology, Amasya University, 05186/ Amasya, Turkey.

<sup>3</sup> National Center of Biotechnology Research, Ali Mendjli, Constantine, Algeria.

<sup>4</sup> Faculty of Science and Arts, Biochemistry Department, Şehit Bulent Yurtseven Campus, Iğdir University, 76000, Iğdir, Turkey.

<sup>5</sup> Laboratoire de Biologie et environnement, Faculté des sciences de la nature et de la vie, Université des Frères Mentouri Constantine 1, Constantine, Algeria.

\*Corresponding Author. E-mail: mekkiou\_ratiba@yahoo.fr; mekkiou\_ratiba@umc.edu.dz (R.M.); Tel. +213550201977

Received: 02 August 2021 / Revised: 28 February 2022 / Accepted: 28 February 2022

**ABSTRACT:** Although notable medicinal value and biological activities such as antioxidant, antibacterial, antimicrobial and antifungal of *Limoniastrum guyonianum* have been reported, identification and quantification of phytoconstituents of CHCl<sub>3</sub>, EtOAc, and *n*-BuOH extracts from the aerial parts of the halophyte medicinal plant from Algerian Sahara was not well investigated for biological and anticancer activity in spite of their great interest. Therefore, the aim of this study was to investigate the antioxidant and anticancer activities of *L. guyonianum*. The phytoconstituents of CHCl<sub>3</sub>, EtOAc, and *n*-BuOH extracts were screened using HPLC-DAD. Total phenolic and flavonoid contents were quantified, antioxidant activity, using DPPH radical scavenging activity, ABTS<sup>•+</sup> decolorization, cupric reducing power, and anticancer activity against human cervical adenocarcinoma (HeLa) cell line were investigated and quantified. The results showed the presence of chlorogenic, gallic, syringic, *p*-coumaric, trans ferulic, *o*-coumaric acids, and quercetin in significant varying quantities. Chlorogenic acid was present in the greatest quantity (344.027–422.711mg/g of extract) as well as quercetin in CHCl<sub>3</sub> extract. It is important to note the absence of caffeic acid and *o*-coumaric acid from EtOAc and CHCl<sub>3</sub> extracts, respectively. The content of phenolic and flavonoid were higher in EtOAc extract (934±4,38mgGAE and 218.33±6.36mgQE/g of extract), respectively. EtOAc extract possesses a strong antioxidant effect *in vitro*, while the CHCl<sub>3</sub> extract proved to be the most active against HeLa cell lines (IC<sub>50</sub>= 50.369±0.020µg/ml). The findings in this study demonstrate the potential use of these compounds in medicine and importance of this species, which have largely been used in folk medicine for several diseases' treatment.

**KEYWORDS:** *Limoniastrum guyonianum*; HPLC-DAD; chemical composition; anticancer activity; antioxidant activity.

## 1. INTRODUCTION

Plant secondary metabolites are plant compounds that are metabolically produced for plant survival and for defense mechanism against diseases, pests, and harsh environmental conditions under which they grow. Halophytes are plants that have the ability to thrive in salty areas. These extreme climatic conditions induce plants to develop defensive mechanisms by synthesizing primary and secondary metabolites that may be potentially useful new sources of natural antioxidants with interesting biological properties and several industrial and medical applications [1]. Recent data indicates that halophytes can also serve as a source of useful secondary metabolites with an assumed economic value [2].

Zerrouki S, Mezhoud S, Sahin Yaglioglu A, Bensouici, Nuri Atalar M, Demirtas I and Mekkiou R. Antioxidant, anticancer activities and HPLC-DAD analyses of the medicinal halophyte *Limoniastrum guyonianum* Dur.Extracts.J Res Pharm. 2022; 26(3): 598-608.

The *Limoniastrum* genus, belonging to the Plumbaginaceae family, is a widespread and commonly grown genus in the Algerian Sahara [3]. The species of this genus are widely used in traditional medicine to treat several ailments [4-5]. *Limoniastrum guyonianum* Dur. was described for the first time by Boissier from eastern Algeria [6-7]. It is an endemic shrub from North Africa, growing on salty arid soils and sandy Saharan lands and locally known by "Zeïta" or "Zita" [3, 8]. Local communities have used this endemic species as forage for camels [9]. Many biological activities of *Limoniastrum guyonianum*, such as antioxidant, antibacterial, antimicrobial, and antifungal properties, as well as anticancer activity, have been reported in the literature in recent years [10-15].

Based on the above reports and available literature, and due to the notable medicinal value of *Limoniastrum guyonianum*, the objective of our current research was to further investigate the biological and cervical activities of this plant, and identify and quantify the phytoconstituents of  $\text{CHCl}_3$ , EtOAc, and *n*-BuOH extracts from the aerial parts of this halophyte plant from the M'Sila region. This plant was considered to be of interest as part of our ongoing survey of Algerian medicinal plants [16-21] and assessment of their antioxidant and antiproliferative activities.

## 2. RESULTS AND DISCUSSION

### 2.1. Total phenolic and flavonoid contents

The total phenolic and flavonoid contents of all extracts were investigated using the Folin-Ciocalteu and Aluminium chloride methods, respectively. As shown in Table 1, the contents of total phenolic and flavonoid considerably varied between different extracts and ranged from  $55.66 \pm 1.07$  to  $934 \pm 4.38 \mu\text{g GAE/mg}$  extract for total phenolic, and from  $10.33 \pm 2.84$  to  $218.33 \pm 6.36 \mu\text{g QE/mg}$  extract for total flavonoid. The highest amounts of both total phenolic and flavonoids were found in EtOAc extract, followed by *n*-BuOH extract. The lowest levels were recorded in the  $\text{CHCl}_3$  extract.

**Table 1:** Total phenolic content (TPC) and total flavonoid content (TFC) values of *L. guyonianum*.

Extract	TPC ( $\mu\text{gGAE/mg extract}$ )	TFC $\mu\text{gQE/mg extract}$
$\text{CHCl}_3$	$55.66 \pm 1.07$	$10.33 \pm 2.84$
EtOAc	$934 \pm 4.38$	$218.33 \pm 6.36$
<i>n</i> -BuOH	$257.00 \pm 6.57$	$32.00 \pm 2.29$

In comparison with other studies, our research findings showed that there is a wide range of phenolic and flavonoid contents in *L. guyonianum* polar extracts growing in Algeria. According to Ziani et al. (2015) [22], the total phenolic content of the aqueous extract of *L. guyonianum* was about  $262 \mu\text{g GAE/mg}$  extract. However, Belfar et al. (2015) [23] found that the amounts of flavonoids in ethyl acetate and *n*-butanol extracts from the aerial parts of *L. guyonianum* collected from the Ouargla and Oued Souf regions were about 2.85 and 13.44 mg EQ/100 g extract, respectively. The  $\text{CHCl}_3$  solvent showed less ability to extract the phenolic compounds, with the lowest concentrations for both total phenolic and flavonoid content. This can be explained by the lower solubility of phenolic compounds in solvents of low polarity [24]. It is important to note that the total polyphenol and flavonoid content varies significantly depending on the region of origin, the solvent of extraction, the climatic conditions, and the stage of maturation of the plant itself during harvest, and especially the salinity for the halophytes, which stimulate the biosynthesis of secondary metabolites such as polyphenols [25-26].

### 2.2. Composition of extracts

All extracts,  $\text{CHCl}_3$ , EtOAc, and *n*-BuOH of *L. guyonianum* were analyzed by the HPLC-DAD method. The identification and quantification of phenolic acids and flavonoids have been performed on the basis of their retention times and by comparison with those of different standards. As shown in Table 2, many compounds are present in varying amounts.

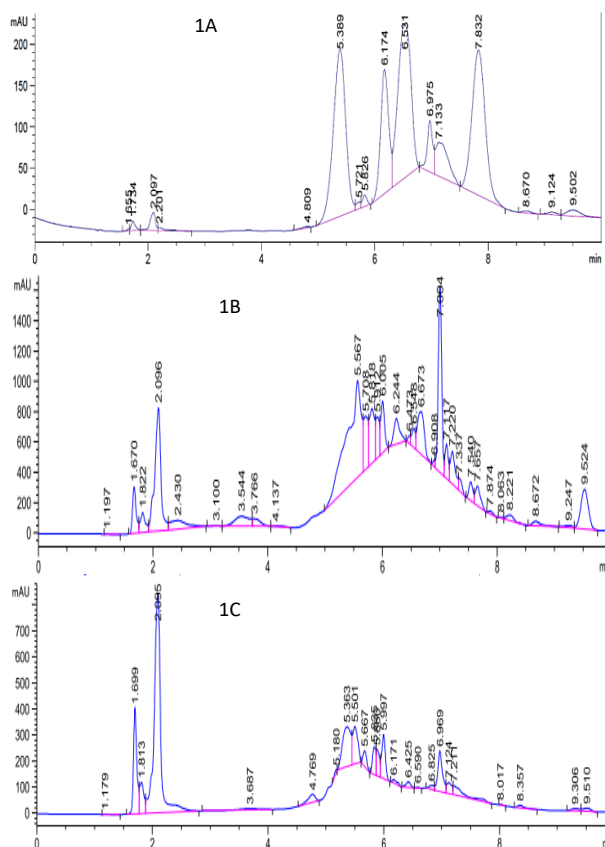
**Table 2:** HPLC-DAD analysis of phenolic acids and flavonoids from *L. guyonianum* extracts.

Rt	Names of compounds	Amounts mg/g extract		
		CHCl <sub>3</sub>	EtOAc	n-BuOH
1.71	chlorogenic acid	351.32	344.03	422.71
2.09	Gallic acid	2.49	113.27	122.52
3.42	Catechin	Nd	Nd	Nd
4.25	Syringic acid	0.83	12.20	7.92
4.77	Caffeic acid	0.15	Nd	3.14
5.67	<i>p</i> -Coumaric acid	0.34	17.22	2.34
5.88	Trans ferulic acid	1.39	20.86	6.87
6.59	<i>o</i> -Coumaric acid	Nd	27.276	0.18
8.02	Quercetin	40.338	1.3747	0.105

Figure 1 shows the chromatographic profiles of *L. guyonianum* extracts. In general, eight compounds including seven phenolic acids and one flavonol, were detected and identified with a retention time varying between 1.07 and 8.017 minutes (min.). The first chromatogram (Figure 1A) represents the CHCl<sub>3</sub> extract and shows a major peak at a retention time of 1.73 min. identified as chlorogenic acid (351.32 mg/g extract). The other part of the chromatogram, ranging from 2 to 8 minutes, is characterized by the presence of small peaks, more or less resolved. Five phenolic acids (gallic, syringic, caffeic, *p*-coumaric, transferulic acids), and one flavonol identified as quercetin with an important amount (40.34mg/g extract) were detected.

An overview of the second chromatogram (Figure 1B) indicates a much greater richness of the EtOAc extract in phenolic compounds than the third one (Figure 1C), which represents the *n*-BuOH extract. Both chromatograms show two major peaks at retention times of 1.73 and 2.09 min. identified as chlorogenic acid (344.03; 422.71 mg/g of extract) and gallic acid (113.27; 22.52 mg/g of extract), respectively.

In addition, the interval of both chromatograms, ranging from 2.4 to 4.5 min., is characterized by the presence of small peaks, which are less resolved. Another comparison between the intervals of 4.5 to 10 min. indicates the presence of a larger and more or less resolved number of peaks in the EtOAc extract, from which syringic, *p*-coumaric, trans ferulic, and *o*-coumaric acids were found to have the highest values in the EtOAc extract. The whole chromatogram of this later shows that other polyphenolic compounds are present but could not be identified due to the lack of standards. This observation allows to predict the possibility of the presence of other compounds belonging to various polyphenol groups.



**Figure 1** : Chromatogram of extracts of *L. guyonianum* obtained on an HPLC chromatograph equipped with diode array detector. **1A**: CHCl<sub>3</sub> extract; **1B**: EtOAc extract, and **1C**: *n*-BuOH extract.

Previous phytochemical studies, carried out on *L. guyonianum* polar extracts, indicated that the findings vary considerably. For example, the quantitative analysis of the aqueous extract of *L. guyonianum* from the M'Sila region (Algeria) using HPLC showed the presence of low quantities of gallic, fumaric, gentisic, 4-hydroxybenzoic, vanillic, syringic acids, catechin, naringin, diosmin, and eupatorin [27]. Another study reported the presence of catechin, gallic acid, epigallocatechin, epigallocatechin-3-O-gallate, as well as p-coumaric and sinapic acids [28]. Other researchers identified unusual compounds such as limoniastramide, a new dimer of phenolic acid amide, together with two natural monomers, N-E-caffeoyl tyramine and N-E-feruloyl tyramine, that have been isolated from this plant [29]. Therefore, our findings differ from previous studies and illustrate the presence of large amounts of chlorogenic acid as well as quercetin and the complete absence of catechin for the first time in *L. guyonianum* extracts.

### 2.3. Antioxidant activity

The literature review on antioxidant activity showed that several studies have examined and reported the antioxidant properties of phenolic compounds. In this report, three extracts from the aerial parts of *L. guyonianum* were investigated and evaluated for their antioxidant activity using DPPH, ABTS, and CUPRAC assays. In this study, the antioxidant activity, expressed as IC<sub>50</sub> (μg/ml) values, of all extracts of *L. guyonianum* was compared to three standards: BHT, BHA, and ascorbic acid (Table 3). As shown in Table 3, the EtOAc extract revealed the best capacity to neutralize DPPH radicals with a concentration of 244.39±41.41 μg/ml compared with the positive control antioxidants: BHA (IC<sub>50</sub> 6.14±0.41 μg/ml), BHT (IC<sub>50</sub> 12.99±0.41 μg/ml) and ascorbic acid (IC<sub>50</sub> 13.94±2.82 μg/ml). Similarly, the EtOAc extract demonstrated the greatest ABTS<sup>•+</sup> scavenging activity, with the lowest value of IC<sub>50</sub> (41.97±1.05 μg/ml), followed by the *n*-BuOH extract (198.44±19.39 μg/ml) when compared to the standards: BHA (IC<sub>50</sub> 1.81±0.10 μg/ml), BHT (IC<sub>50</sub> 1.29±0.30 μg/ml) and ascorbic acid (IC<sub>50</sub> 1.74±0.10 μg/ml). The CHCl<sub>3</sub> extract was less active in the DPPH and ABTS assays. On the other hand, a strong increase in the antioxidant activity was noted with the EtOAc extract in the CUPRAC assay. Indeed, the IC<sub>50</sub> values (μg/ml) were ordered as follows: BHA > BHT > Ascorbic acid > EtOAc > *n*-BuOH > CHCl<sub>3</sub>.

**Table 3:** IC<sub>50</sub> (µg/ml) values of antioxidant activities of *L. guyonianum* extracts.

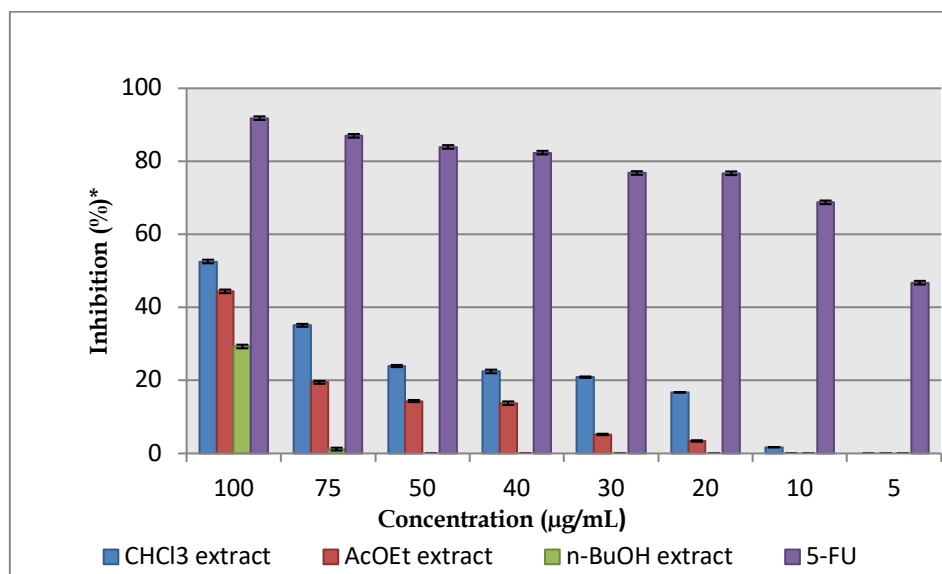
	Antioxidant activity		
	DPPH	ABTS	CUPRAC
CHCl <sub>3</sub>	>800	>800	345.5±3.82
EtOAc	244.39± 41.41	41.97±1.05	34.44±4.30
<i>n</i> -BuOH	>800	198.44±19.39	375±19.09
BHA	6.14±0.41	1.81±0.10	5.35±0.71
BHT	12.99±0.41	1.29±0.30	8.97±3.94
Ascorbic Acid	13.94±2.82	1.74±0.10	12.43±0.09

Footnote: DPPH: 2,2-diphenyl 1-picrylhydrazyle; ABTS: acide 2,2'-azino-bis(3-éthylbenzothiazoline-6-sulphonique)  
CUPRAC: CUPric Reducing Antioxidant Capacity. The results are considered to be significant when  $p < 0.05$ .

The comparison of the different extracts of *L. guyonianum* indicated that the EtOAc was more active than the CHCl<sub>3</sub> and *n*-BuOH extracts. EtOAc extract showed the most potent activity, suggesting that this solvent of medium polarity extracted compounds had the highest antioxidant activity. Several studies have shown that the ethyl acetate extract of many plants has a strong ability to act as an antioxidant when compared with other extracts [18, 30].

## 2.4. Anticancer activity

We investigated herein the anticancer activity against HeLa (human cervix carcinoma) cell lines of CHCl<sub>3</sub>, EtOAc, and *n*-BuOH extracts obtained from the aerial parts of the halophyte *Limoniastrum guyonianum* compared with 5-FU used as a standard (Figure 2). The IC<sub>50</sub> and IC<sub>75</sub> values of the extracts were even in Table 4.



**Figure 2:** The antiproliferative activity of CHCl<sub>3</sub>, EtOAc, and *n*-BuOH extracts of *L. Guyonianum* against HeLa cells.  
\*Each substance was tested twice in triplicates against cell lines. Data show average of two individual experiments;  $p < 0.01$  was used as level of significance.



**Table 4:** IC50 and IC75 ( $\mu\text{g/ml}$ ) values of antiproliferative activity of *L. guyonianum* extracts.

Extracts of <i>L. guyonianum</i>	Against HeLa cell	
	IC <sub>50</sub> ( $\mu\text{g/ml}$ )	IC <sub>75</sub> ( $\mu\text{g/ml}$ )
CHCl <sub>3</sub> extract	50.369 $\pm$ 0.020	76.367 $\pm$ 0.054
AcOEt extract	63.611 $\pm$ 0.025	89.408 $\pm$ 0.035
<i>n</i> -BuOH extract	87.628 $\pm$ 0.036	*
5-FU	*	18.522 $\pm$ 0.025

The later table (Table 4) shows that the IC<sub>50</sub> values were 50.369 $\pm$ 0.020 $\mu\text{g/ml}$ , 63.611 $\pm$ 0.025 $\mu\text{g/ml}$  and 87.628 $\pm$ 0.036  $\mu\text{g/ml}$  for the CHCl<sub>3</sub>, EtOAc, and *n*-BuOH extracts, respectively. The resultant cytotoxicity of extracts against HeLa (cervical carcinoma) cells was concentration-dependent. In addition, all extracts were observed to have moderate anticancer activity relative to the standard compound at a concentration of 100 $\mu\text{g/ml}$ . The potency of inhibitions against HeLa cells at this concentration was on the order of: 5-FU > CHCl<sub>3</sub> extract > EtOAc extract > *n*-BuOH extract. Previous research has reported that natural products derived from plants have cancer chemopreventive and chemotherapeutic properties [31]. In the present study, the anticancer effect of CHCl<sub>3</sub> extract from *L. guyonianum* was found to be more pronounced against HeLa cell lines than EtOAc and *n*-BuOH extracts. The differences between the different extracts can probably be explained by the presence of more cytotoxic active substances such as chlorogenic acid and quercetin, which were most concentrated in the CHCl<sub>3</sub> extract. Our results are in agreement with several previous studies investigated by Krifa et al., that identified *L. guyonianum* as a potential cytotoxic agent and gave the best cytotoxicity against various cancer cell lines. These studies reported that the flavonoids present in the aqueous gall extract of *L. guyonianum* exert apoptotic effects *in vitro* and may stimulate immune responses [32]. The aqueous extract from *L. guyonianum* induced apoptosis in human cervical cancer cells, involving expression of p16INK4A and down regulation of DNMT1 [33]. The same extract exhibited an anticancer effect against mouse melanoma cells (B16F10) via modulation of the immune response [34]. Moreover, this extract induced apoptosis via DNA damage, PARP cleavage, and down regulation of UHRF1 in human glioma U373 cells [35]. Another study reported by Ziani et al. (2015) revealed that *L. guyonianum* showed better cytotoxicity against human tumor cell lines. It should be noted here that CHCl<sub>3</sub> extract is characterized by the presence of important amounts of chlorogenic acid and quercetin, which are considered to be some of the most active anti-tumor cells [22]. We hypothesized that these compounds might be the major contributors to the cytotoxic capacities. In addition, we believe that the anticancer effects of CHCl<sub>3</sub> extract may be due to the presence of mixtures of different compounds, among which minor components that can act synergistically. These compounds can interfere with specific stages of the carcinogenic process [36]. Both chlorogenic acid and quercetin, which are the main active agents in the CHCl<sub>3</sub> extract, have attracted great interest as their cytotoxic effects can potentially be enhanced against multiple cell lines. The cytotoxic activity of chlorogenic acid against human oral tumor cell lines [37] and its antitumorigenic properties in breast cancer [38] have been reported. A number of studies have investigated the anticancer activity of quercetin and found that various concentrations suppressed tumor growth of various cancer cell lines, including breast [39], lung [40], ovarian [41], melanoma [42], and leukemia [43]. This suggests a close relationship between the chemical properties of phenolic compounds and cancer cell growth.

### 3. CONCLUSION

The investigation of the three extracts, obtained from the aerial parts of *Limoniastrum guyonianum*, revealed the presence of chlorogenic acid as a major component in all extracts, while quercetin was present in a high quantity in the CHCl<sub>3</sub> extract. However, EtOAc extract possesses a strong antioxidant effect *in vitro* compared to both CHCl<sub>3</sub> and *n*-butanol extracts. These results are confirmed by high polyphenol and flavonoid contents as well as high amounts of phenolic compounds. On the other hand, CHCl<sub>3</sub> extract presents the most important cytotoxic effect against HeLa cell lines. Both antioxidant ability and antiproliferative effects involve complex combinations of several biochemical processes and different phytochemical compounds affecting biochemical pathways. Our findings provide the scientific community with further knowledge and understanding of the antioxidant and anticancer activities of the medicinal halophyte *Limoniastrum guyonianum* Dur. extracts under Algerian Sahara conditions.

## 4. MATERIALS AND METHODS

### 4.1. Plant material

The aerial parts of the plant material were collected in March 2016 from the M'Sila region (Algeria) and identified by Dr. SARRI Djamel, Department of Biology, M'Sila University, according to Quezel and Santa, 1963. A voucher specimen was deposited in the herbarium of the unit of research VARENBIO MOL under the code PLG 03/16 at Constantine University, Constantine, Algeria.

### 4.2. Extraction procedure

The dried aerial parts (1900g) were firstly macerated in 100% petroleum ether followed by 100% chloroform to remove chlorophyll, then in a hydroalcoholic mixture (EtOH/H<sub>2</sub>O; 80: 20) for 72 hours three times. After filtration, the crude extract was concentrated at room temperature, and diluted with 800 ml of distilled water. The remaining aqueous solution was extracted with EtOAc and *n*-BuOH, respectively. All extracts were filtered, dried with anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated to give 18g of PE; 18g of CHCl<sub>3</sub>; 2.5g of ethyl acetate (EtOAc) and 9g of *n*-BuOH extracts, respectively.

### 4.3. Total phenolic and flavonoid contents

The total phenolic content of the three extracts was determined by the method of Singleton et Rossi, 1965 [44] using gallic acid (GA) as a standard. The results are expressed as gallic acid equivalents per g of dry weight (mg GAE/g DW of extract), while the total flavonoid content was determined using the method of Topçu et al. 2007 [45] with quercetin (Q) as a standard. The results were expressed as Quercetin equivalent per g of dry weight (mg GAE/g DW extract).

### 4.4. Quantitative analysis

A 1260 Infinity II LC System model of high-performance liquid chromatography (HPLC) coupled with a DAD detector was used to accomplish the quantitative evaluation of the compounds. The reversed-phase HPLC was equipped with a column oven (1260 TCC), binary pumps (1260 Bin Pump), and a degasser (1260 Degasser). The chromatographic conditions were optimized in order to achieve optimum separation for the compounds and overcome the suppression effects. Thus, the chromatographic separation was performed on a reversed-phase Agilent Poroshell 120 EC-C18 model (150 mm × 4.6 mm, 2.7 μm) analytical column. The column temperature was set to 25°C. The elution gradient was composed of eluent A (% 0.1 phosphoric acid in water) and eluent B (acetonitrile), with the solvent flow rate and injection volume adjusted to 0.8 mL/min and 20 μL. The following gradient elution profile was used: 17% B (0–2 min), 30% B (2–4 min), 40% B (4–10 min), and 17% B (10–15 min). Diode-array detection was set to collect data in the range of 300–200 nm.

Finally, sample preparation and the working plant extracts with the proper concentration were prepared by appropriate dilutions of the stock solution with 5% methanol and 45% acetonitrile in water. All working standards were filtered through a 0.45 μm nylon-membrane syringe filter (Acrodisc, Sigma-Aldrich, Bulgaria) and put into the 2 ml vials.

### 4.5. Antioxidant activity

The antioxidant activity using three different assays, including DPPH free radical scavenging activity, ABTS cation radical decolorization, and cupric reducing antioxidant capacity (CUPRAC) assay, was determined.

#### 4.5.1. DPPH scavenging activity

The antioxidant activity was established according to the method described by Blois [46] using the DPPH assay. Briefly, in a 96-well plate, 160 μl of DPPH solution at 0.1 mM was added with 40 μl of sample solutions in methanol at different concentrations. At room temperature, the plate was kept in the dark for 30 min of incubation. The absorbance was read at 517 nm. BHA, BHT, and ascorbic acid were used as antioxidant standards. The scavenging activity of the extract was calculated using equation 1:



$$DPPHscavenging\ effect\% = \left( \frac{A_{control} - A_{sample}}{A_{control}} \right) * 100$$

Where  $A_{control}$  and  $A_{sample}$  are the absorbances of the reference and sample, respectively.

#### 4.5.2. ABTS<sup>•+</sup> scavenging activity

The ABTS<sup>•+</sup> scavenging activity was evaluated according to the method of Re et al. [47]. The ABTS<sup>•+</sup> solution was prepared by the mixture of 7mM ABTS with 2.45mM potassium persulfate using water as the solvent. The ABTS<sup>•+</sup> solution was diluted in ethanol or water to get an absorbance of  $0.708 \pm 0.025$  at 734 nm. In each well, the reaction mixture contained 40µL of sample solution in methanol at different concentrations and 160 µL of ABTS<sup>•+</sup> solution. After incubation, the absorbance was measured at 734 nm using a 96-well microplate reader. Each assay for all samples was carried out in triplicate. BHA, BHT and ascorbic acid were used as antioxidant standards. Percentage inhibition was calculated using the same equation (equation 1).

#### 4.5.3. Cupric reducing antioxidant capacity

The CUPRAC was determined according to the method of Apak et al. [48]. 40µl of each extract solution at different concentrations and 50µl of  $CuCl_2$  solution (10 mM) were added into a 96-well round-bottomed plate. Then, 50µl of neocuproine solution (7.5 mM) and 60µl of  $NH_4Ac$  buffer (1 M, pH 7.0) solution were added to each well. After 60 min, the absorbance was measured at 450nm. Results were given as  $A_{0.5}$  corresponding to the concentration indicating 50% absorbance intensity compared with the absorbance of BHA, BHT, and ascorbic acid, which were used as antioxidant standards.

#### 4.5.4. Statistical analysis

Statistical analysis was performed using the ANOVA package (SPSS, version 11.5 for Windows 2000, SPSS Inc.). All the experimental results are reported as a mean  $\pm$  standard deviation of three trials (replications).

#### 4.6. Anti-proliferative activity

The anti-proliferative activity of all extracts was investigated on HeLa (human cervix carcinoma) cell lines using the proliferation BrdU ELISA assay [49-50]. The cells were plated in 96-well culture plates (COSTAR, Corning, USA) at a density of 30.000 cells per well. The activities of extracts were investigated at 100, 75, 50, 40, 30, 20, 10, and 5µg/mL. 5-fluorouracil (5-FU) was used as a standard. The cells were then incubated overnight before applying the BrdU Cell Proliferation ELISA assay reagent (Roche, Germany) according to the manufacturer's procedure. By using a microplate reader (Awareness Chromate, USA), the amount of cell proliferation was determined at 450nm. Results were reported as a percentage of cell proliferation inhibition, where 100% of proliferation was considered, the optical density measured from vehicle-treated cells. The percentage of inhibition of cell proliferation was calculated as follows:

$$\%Inhibition = [1 - (A_{treatments} / A_{vehicle\ control})] * 100.$$

The stock solution of the extracts was prepared in dimethyle sulfoxide (DMSO) and diluted with Dulbecco's (Modified Eagle's Medium High Glucose (DMEM), Sigma, Germany). Final DMSO concentration was below 0.1% in all tests. In the paper, the  $IC_{50}$  and  $IC_{75}$  values were determined using ED50 plus v1.0. The results of the investigation *in vitro* are the means  $\pm$  SD of six measurements. Differences between groups were tested with ANOVA. p values of  $<0.01$  were considered as significant and analyzed by SPSS (version 11.5 for Windows 2000, SPSS Inc.).

**Acknowledgements:** This research project was supported by the Algerian Ministry of Higher Education and Scientific Research (MESRS) and the Algerian PRFU (B00L01UN250120180002) program.

**Author contributions:** Concept – R.M., A.S., I.D.; Design – R.M., S.Z., S.M.; Supervision – R.M., I.D., A.S., C.B.; Resources – R.M., S.Z., A.S., S.M.; Materials – R.M., S.Z., S.M., C.B., M.N.A.; Data Collection and/or Processing – A.C.Y., M.N.A., C.B.; Analysis and/or Interpretation – M.N.A., A.C.Y., C.B., R.M., S.Z.; Literature Search – S.Z., R.M.; Writing – S.Z., R.M.; Critical Reviews – R.M., I.D., A.S., S.Z., S.M., A.C.Y., M.N.A., C.B.

**Conflict of interest statement:** The authors declared no conflict of interest.

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