Journal of Research in Pharmacy

Research Article

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LC-MS/MS simultaneous determination of 37 bioactive compounds in *Bunium crassifolium* Batt. and its biological activities

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Received: 18 December 2020 / Revised: 22 April 2021/ Accepted: 27 April 2021

ABSTRACT: *Bunium crassifolium* Batt. is an extremely rare species growing in Algeria. Its leaf or aerial part is used for flavoring and garnishing food. The aim of the current study was to determine 37 bioactive phytochemicals and biological evaluation of the methanol and methanol:water (70:30) extracts of *B. crassifolium* for the first time. A total of 10 phenolic acids and 8 flavonoids have been identified in the extracts of the aerial parts by LC–MS/MS. Chlorogenic, gallic and ferulic acids were the most abundant phenolic acids detected, while rhoifolin, quercitrin and rutin were the most abundant flavonoids. In addition, 3 non-phenolic organic acids (fumaric acids, quinic and malic) were detected and among them quinic and malic acids were the most abundant. Antioxidant activity was evaluted by six methods and the extracts showed significant activity. The total phenolic and flavonoid contents were determined and methanol:water (70:30) extract showed higher values which entails its greater antioxidant capacity. In addition, anticholinesterase (AChE and BChE) activity was evaluated for both extracts using Ellman method. Methanol extract showed a better anti-acetylcholinesterase activity than methanol:water (70:30) one. While, both extracts showed a weak anti-butyrylcholinesterase activity. Furthermore, the anti-tyrosinase activity was tested and the methanol:water (70:30) extract was promising and more potent. In conclusion, *B. crassifolium* could be used in food industries and pharmaceutical as a potential functional food ingredient.

KEYWORDS: *Bunium crassifolium;* LC-MS/MS; antioxidant activity; anticholinesterase activity; tyrosinase inhibition.

1. INTRODUCTION

The genus *Bunium* belongs to the family Apiaceae, subfamily Apioideae and tribe Apieae. This genus contains about 166 species distributed in southwest and central Asia, North Africa and Europe [1]. In Algeria, the *Bunium* genus is represented by seven species, four of which are endemic like *Bunium chaberti* Batt., *Bunium crassifolium* Batt., *Bunium elatum* Batt.; and *Bunium fontanessii* (Pers.) Maire [2]. *Bunium crassifolium* Batt. is an extremely rare and endemic species growing and distributed in the North-East of Algeria. It is a perennial plant 30-60 cm in height [3]. Due to rareness of *Bunium crassifolium,* our team is investigating the use of plant tissue culture or micropropagation technology for commercial production of this plant to ensure continuous supply of its plant material.

Some species of genus *Bunium* are important plants in economy [4]. The rhizomes of Turkish *Bunium pauciflorum* DC var. junceum (Boiss.) Wolff and those of Algerian *Bunium incrassatum* (Boiss.) Batt. are generally used as potatoes [4,5]. In Algeria, rhizome of the genus *Bunium* evoke for a remarkable food source

How to cite this article: Souilah N, Bendif H, Ullah Z, Hamel T, Djarri L, Öztürk M, Ertas A, Akkal S, Medjroubi K, Mustafa AM. LC-MS/MS simultaneous determination of 37 bioactive compounds in *Bunium crassifolium* Batt. and its biological activities. J Res Pharm. 2021; 25(4): 450-463.

ISSN: 2630-6344

https://dx.doi.org/10.29228/jrp.36

for some people, but for others, it is a symbol of misery reminiscent of the famine of years of food shortage especially through the second world war and the period of national revolution. During these periods, the population of the Atlas mountains consumed *Bunium incrassatum* as a flour by drying in the sun. The flour obtained was mixed with that of wheat or barley to make a couscous or bread and sometimes, this type of bread was made without any other flour. The people of the mountains of Serraidi (Algeria) consume our plant of interest *B. crassifolium* rhizome, raw, boiled or roasted, while its leaf or aerial part was used for flavoring and garnishing food like parsely [6].

Phytochemical studies previously performed on various species of the genus *Bunium* L. reported the presence of coumarins, sesquiterpenes and monoterpenes. Two coumarins (scopoletin and scoparone), oleic acid, sucrose and *β*-sitosterol were obtained from roots of *Bunium incrassatum* [4]. Genus *Bunium* includes interesting medicinal and aromatic plants, whose seeds and volatile oils were used in medicine and food in different parts of the world for long time. For example, *Bunium elegans, B. caroides*, *B. persicum, B. cylindricum* are rich in essential oils [7].

Numerous pharmacological effects have been attributed to many species of genus *Bunium*. For example, *B. incrassatum* is used as astringent, stop diarrhea, against hemorrhoidal inflammation and bronchitis [4], and *B. persicum* is famous for antioxidant, carminative, antidiarrheal and digestive properties [8]. Also, *B. paucifolium* is indicated in urinary inflammations [9]. Nowadays, rhizomes of the genus *Bunium* are of interest to certain Algerian herbalists for its therapeutic use against goitre and thyroid dysfunction [10].

To the simplest of our knowledge, the chemical profile and biological assays of *B. crassifolium* species have not been reported before. For this reason, the aim of the current study was to perform for the first time the phytochemical characterization and biological evaluation (antioxidant, antityrosinase and anticholinesterase activities) of the methanol and methanol:water (70:30) extracts of *B. crassifolium*. The current study is a trial to focus on and discover the health benefits of this forgotten plant, hoping to lead us to the development of functional food ingredients for the treatment and prevention of various ailments like free radical, neurodegenerative and hyperpigmentation disorders.

2. RESULTS AND DISCUSSION

2.1. Optimization of chromatographic conditions

Optimization of the LC–MS/MS conditions was performed by varying them in flow injection analysis (FIA) of the analytes (4 µl of 1 µg/ml individual standard solutions). For the accurate identification of the analysed compounds, the HPLC–MS/MS analysis was achieved with electrospray ionization (ESI) mode using multiple reaction monitoring (MRM) which monitor the transitions of the parent to daughter ions of all standards. Analytes were characterized by their MS/MS spectra and retention time. For optimum MS results, ionization was accomplished in negative ESI mode and the precursor ions were corresponding to the deprotonated $[M-H^+]^-$ adducts. Quantification of target compounds was achieved after optimizing the acquisition parameters (Table 1).

2.2. Method validation

LC-MS/MS method was used for determining the quantity of 37 marker compounds (17 flavonoids, 14 phenolic acids, 3 non-phenolic organic acids, 1 phenolic aldehyde, 1 benzopyrene and 1 catechol) in the studied plant species. The developed method was fully validated in terms of linearity, accuracy (recovery), inter-day and intra-day precision (repeatability), detection and quantification limits (LOD/ LOQ) and relative standards uncertainty (U%) at 95% confidence level (k = 2) (Table 2). The method exhibited a good linearity of all standards ($R^2 \geq 0.990$) over a wide scale of concentrations (Table 2). The intraday precision of the HPLC-MS/MS method was validated with the injection of the standard mixture solution under the selected optimal conditions five times a day. For interday precision, measurements were conducted once a day on three consecutive days. All of the precision measurements were expressed as relative standard deviations (RSDs).The method demostrated a good precision as the relative standard deviations (RSDs %) of the inter- and intra-day studies ranged from 0.058 to 3.209 % and 0.076 to 2.605 %; respectively. Accuracy was evaluated by recovery study. For this purpose, known amounts of the standard mixture solution were added to the plant material, which was then extracted and assayed as described before. The percent of recovery was evaluated by calculating the ratio of detected amount versus the added amount. The extraction recuperated of the analyzed standards in the intra- and inter-day studies were got to be within the acceptable range (Table 1). The percentages of recoveries ranged from 98.47 to 104.09 %. Therefore, the matrix effect of the extracts was negligible for the assay. The LODs and LOQs were obtained by injecting serial dilutions of the corresponding

standard solutions, taking the signal-to-noise (S/N) ratio of 3 and 10 as criteria, respectively. This method was sensitive as LODs and LOQs ranged from 0.003 to 0.821 and 0.004 to 0.859 µg/ml; respectively (Table 2). High specificity was achieved using tandem mass spectrometry. The most abundant product ion of the precursor/product ion transitions was selected for quantification of each analyzed marker compound (Table 1) instead other transitions were given for qualtitative analysis. Table 2 shows that relative standard uncertainties were equal or less than 2.82% for all the analyzed compounds.

Table 1. LC–MS/MS acquisition parameters used for the analysis of the 37 marker compounds in the extracts of *B. crassifolium*.

No.	Compounds	Retention Time (min)	Scan mode	Polarity (ESI)	Precursor ion $[M-H]^-$ (*m/z*)	MS^2 fragments (*m/z*)
1	Quinic acid	1.13	MRM	Negative	190.95	85.3-93.3
2	Malic acid	1.23	MRM	Negative	133.00	115.2-71.3
3	Fumaric acid	1.48	MRM	Negative	115.00	71.4
4	Gallic acid	3.00	MRM	Negative	168.85	125.2-79.2
5	Protocatechic acid	4.93	MRM	Negative	152.95	108.3
6	Pyrocatechol	6.48	MRM	Negative	109.00	108.35-91.3
7	Chlorogenic acid	7.13	MRM	Negative	353.15	191.2
8	*p*-Hydroxybenzoic acid	7.39	MRM	Negative	136.95	93.3-65.3
9	Vanillic acid	8.57	MRM	Negative	166.90	152.3-108.3
10	Caffeic acid	8.80	MRM	Negative	178.95	135.2-134.3
11	Syringic acid	9.02	MRM	Negative	196.95	182.2-167.3
12	Vanillin	10.87	MRM	Negative	151.00	1363-92.2
13	Salicylic acid	11.16	MRM	Negative	136.95	93.3-65.3
14	*p*-Coumaric acid	11.53	MRM	Negative	162.95	119.3-93.3
15	Rutin	12.61	MRM	Negative	609.05	300.1-271.1
16	*tr*-Ferulicacid	12.62	MRM	Negative	192.95	178.3
17	Sinapic acid	12.66	MRM	Negative	222.95	208.3-149.2
18	Hesperidin	12.67	MRM	Negative	609.00	301.1
19	Isoquercitrin	13.42	MRM	Negative	463.00	300.1-271.1
20	Rosmarinic acid	14.54	MRM	Negative	359.00	161.2-197.2
21	Nicotiflorin	14.68	MRM	Negative	593.05	285.1-255.2
22	*a*-Coumaricacid	15.45	MRM	Negative	162.95	119.4-93.3
23	Rhoifolin	16.11	MRM	Negative	577.05	269.2-211.1
24	Quercitrin	16.41	MRM	Negative	447.15	301.1-255.1
25	Apigetrin	16.59	MRM	Negative	431.00	268.2-239.2
26	Coumarin	17.40	MRM	Negative	147.05	91.0-103.2
27	Myricetin	18.72	MRM	Negative	317.00	179.2-151.3
28	Fisetin	19.30	MRM	Negative	284.95	135.2-121.3
29	Cinnamic acid	25.61	MRM	Negative	147.00	103.15-77.3
30	Liquiritigenin	25.62	MRM	Negative	254.95	119.3-135.1
31	Quercetin	28.17	MRM	Negative	300.90	151.2-179.2
32	Luteolin	28.27	MRM	Negative	284.75	133.2-151.2
33	Naringenin	30.68	MRM	Negative	270.95	151.2-119.3
34	Apigenin	31.43	MRM	Negative	268.95	117.3-151.2
35	Hesperetin	31.76	MRM	Negative	300.95	164.2-136.2
36	Kaempferol	31.88	MRM	Negative	284.75	255.1-117.3
37	Chrysin	36.65	MRM	Negative	252.95	143.3-119.4

2.3. Application of HPLC–MS/MS method to the extracts of *B. crassifolium*

The LC-MS/MS method we developed was used for the simultaneous quantification of 37 bioactive compounds (Fig. 1A) in both extracts of *B. crassifolium*. Regarding the LC-MS/MS results (Table 3 and Fig. 1B & C), the analyzed extracts were characterized by high amounts of phenolic acids and flavonoids. A sum of 10 phenolic acids (chlorogenic, *p*-hydroxybenzoic, salicylic acid, *p*-coumaric acid, *tr*-ferulic, sinapic, gallic, protocatechic, cinnamic and caffeic acids) and 8 flavonoids (isoquercitrin, nicotiflorin, rhoifolin, quercitrin, hesperidin, apigetrin, rutin and apigenin) have been identified in the analyzed plant extracts. Chlorogenic (4,568.99 µg/g), gallic (480.69 µg/g) and ferulic (122.35 µg/g) acids were the most abundant phenolic acids detected, while rhoifolin (13,803.21 µg/g), rutin (3,868.02 µg/g) and quercitrin (1,015.6 µg/g) were the highest flavonoids. Furthermore, quinic, malic, and fumaric acids were detected as non-phenolic organic acids and among them quinic (210,804.68 µg/g) and malic (8,927.55 µg/g) acids were detected in huge amounts in both extract. LC-MS/MS analyses revealed that 7 flavonoids and 9 phenolic acids were detected in methanol extract. While 8 flavonoids and 8 phenolic acids were detected in methanol:water (70:30) extract. So, the number of total phenolic compounds detected in both methanol:H_2O (70:30) and methanol extracts appeared

to be the same (16 compounds). It was observed that quinic, malic, fumaric, protocatechic, *p*-coumaric, chlorogenic, *p*-hydroxybenzoic, sinapic and *tr*-ferulic acids were detected in larger quantities in methanol:water (70:30) extract, while gallic and caffeic acids were detected in larger quantities in methanol extract. Salicylic acid was observed only in methanol extract, and apigenin only in methanol:water (70:30) extract while the other compounds were detected in both extracts. Zengin *et al* [11], showed that the quinic acid, chlorogenic acid, isoquercitrin, rutin, apigenin were present in the *B. brachyactis*, *B. pinnatifolium*, *B. microcarpum*, and *B.sayai*.

Table 2. Concentration range, linearity (R^2), Limits of Detection (LODs), Limits of Quantification (LOQs) and percentages of recoveries of the analysed 37 compounds by LC–MS/MS.

No.	Compounds	Conc. Range (µg/ml)	R^2	LOD (µg/ml)	LOQ (µg/ml)	Interday (n=3) RSD (%)	Intraday (n=3) RSD (%)	Recovery % (n = 3)		U (%)
								Interday	Intraday	
1	Quinic acid	0.250-10	0.996	0.075	0.079	0.259	0.274	100.28	98.77	0.82
2	Malic acid	0.250-10	0.999	0.055	0.067	0.477	0.527	101.26	99.83	1.13
3	Fumaric acid	0.10-5	0.997	0.028	0.034	0.536	0.460	99.74	99.86	1.24
4	Gallic acid	0.250-10	0.998	0.095	0.106	1.601	01.443	100.00	100.45	2.82
5	Protocatechic acid	0.100-5	0.995	0.028	0.031	1.236	1.296	99.40	101.07	0.04
6	Pyrocatechol	1-20	0.996	0.261	0.278	1.313	1.339	99.98	99.93	2.35
7	Chlorogenic acid	0.025-1	0.998	0.006	0.008	0.058	0.076	100.80	99.96	0.69
8	*p*-Hydroxybenzoic acid	0.250-10	0.998	0.033	0.038	1.284	1.538	99.66	100.05	2.89
9	Vanillic acid	0.1-20	0.999	0.122	0.139	0.528	0.619	100.09	104.09	0.50
10	Caffeic acid	0.025-1	0.998	0.018	0.022	1.454	1.469	100.91	98.82	0.35
11	Syringic acid	0.1-20	0.996	0.021	0.023	1.049	1.345	99.92	99.97	2.38
12	Vanillin	0.250-10	0.998	0.044	0.053	0.696	0.793	99.67	99.61	2.80
13	Salicylic acid	0.025-1	0.989	0.005	0.006	1.016	1.242	100.98	99.01	0.32
14	*p*-Coumaric acid	0.025-1	0.992	0.007	0.009	1.820	1.727	100.61	101.22	0.56
15	Rutin	0.025-1	0.997	0.005	0.006	0.473	0.624	100.99	98.01	1.59
16	*tr*-Ferulic acid	0.250-10	0.997	0.036	0.042	0.708	0.619	99.98	100.28	0.49
17	Sinapic acid	0.250-10	0.992	0.078	0.086	1.446	1.517	100.16	99.96	2.81
18	Hesperidin	0.025-1	0.998	0.003	0.004	0.945	1.126	101.73	101.26	2.62
19	Isoquercitrin	0.025-1	0.999	0.005	0.006	0.682	0.515	100.59	100.72	1.33
20	Rosmarinic acid	0.100-5	0.994	0.006	0.008	2.014	1.751	99.20	103.43	0.71
21	Nicotiflorin	0.100-5	0.991	0.022	0.025	0.737	0.875	102.55	100.97	2.76
22	*a*-Coumaric acid	0.025-1	0.999	0.003	0.009	2.730	2.566	98.34	99.06	0.53
23	Rhoifolin	0.100-5	0.999	0.023	0.027	0.747	1.528	101.04	101.73	0.94
24	Quercitrin	0.100-5	0.999	0.022	0.025	1.528	2.320	99.72	100.62	2.07
25	Apigetrin	0.025-1	0.993	0.005	0.006	1.797	1.607	101.39	100.41	0.55
26	Coumarin	1-20	0.994	0.208	0.228	1.306	1.239	99.94	100.08	2.37
27	Myricetin	0.250-10	0.999	0.053	0.057	0.652	0.711	99.98	100.04	1.26
28	Fisetin	0.250-10	0.991	0.054	0.051	0.557	0.820	99.87	100.03	1.48
29	Cinnamic acid	5-20	0.996	0.821	0.859	0.648	0.816	100.05	99.92	1.43
30	Liquiritigenin	0.025-1	0.996	0.005	0.006	1.849	1.738	100.33	99.95	0.34
31	Quercetin	0.100-5	0.990	0.023	0.028	1.589	1.360	98.47	100.10	0.54
32	Luteolin	0.025-1	0.997	0.005	0.006	0.575	0.696	100.77	99.52	1.74
33	Naringenin	0.025-1	0.995	0.005	0.006	2.054	2.019	99.88	101.00	0.52
34	Apigenin	0.025-1	0.990	0.005	0.006	2.304	2.204	101.44	101.33	0.65
35	Hesperetin	0.025-1	0.997	0.005	0.006	3.209	2.605	98.85	99.43	0.56
36	Kaempferol	1-20	0.992	0.206	0.214	1.436	1.070	99.97	99.85	2.09
37	Chrysin	0.025-1	0.993	0.005	0.006	0.490	0.630	100.33	100.43	2.08

RSD %: relative standard deviation.
U (%): uncertainty percent at 95% confidence level (k = 2).

The highest content flavonoid, rhoifolin (apigenin 7-O-neohesperidoside, a dihydroxyflavone and a disaccharide derivative) was detected in methanol and methanol:water (70:30) extracts with values of 12,885.08 and 13,803.21 µg/g extract; respectively, while the lowest content flavonoid aglycon, apigenin (a trihydroxyflavone) was detected only in methanol: water (70:30) extract (11.19 µg/g extract). It is noteworthy that the methanol:water (70:30) extract was more rich in rhoifolin and tetrahydroxyflavone, rutin (a disaccharide derivative). Generally, flavonoids linked mainly to disaccharides (i.e. more polar compounds) were detected in larger quantities in the methanol: water (70:30) extract, than those detected in the methanol extract (a flavone glucoside, a flavone, and a flavanone).

According to the results we obtained, the methanol:water (70:30) extract was the richest one in the analyzed compounds except gallic acid, caffeic acid, isoquercitrin (a tetrahydroxyflavone and a

monosaccharide derivative) and apigetrin (apigenin 7-O-glucoside) which were detected in higher amounts in methanol extract (Table 3). The identified phenolic compounds in both extracts of *B. crassifolium* were reported to possess a positive effect on health and could be used for several applications in pharmacy [12].

Table 3. Quantitative determination of 37 phenolic compounds in the extracts of *B. crassifolium* (µg/g extract) by LC-MS/MS, relative standard deviations (RSDs %) were in a range from 0.55 to 2.25%.

No.	Compounds	Methanol extract	Methanol:water (70:30) extract
1	Quinic acid	188,783.28	210,804.68
2	Malic acid	7,530.00	8,927.55
3	Fumaric acid	27.62	28.09
4	Gallic acid	480.69	21.05
5	Protocatechic acid	18.04	22.86
6	Pyrocatechol	N. I	N. I
7	Chlorogenic acid	4,281.65	4,568.99
8	*p*-Hydroxybenzoic acid	38.66	46.12
9	Vanillic acid	N. I	N. I
10	Caffeic acid	105.01	99.79
11	Syringic acid	N. I	N. I
12	Vanillin	N. I	N. I
13	Salicylic acid	N. I	N. I
14	*p*-Coumaric acid	58.8	64.46
15	Rutin	3,049.74	3,868.02
16	*tr*-Ferulic acid	114.08	122.35
17	Sinapic acid	7.26	10.7
18	Hesperidin	94.4	147.9
19	Isoquercitrin	235.75	220.65
20	Rosmarinic acid	N. I	N. I
21	Nicotiflorin	325.33	371.4
22	*a*-Coumaric acid	N. I	N. I
23	Rhoifolin	12,885.08	13,803.21
24	Quercitrin	880.57	1,015.6
25	Apigetrin	201.26	183.29
26	Coumarin	N. I	N. I
27	Myricetin	N. I	N. I
28	Fisetin	N. I	N. I
29	Cinnamic acid	N. I	N. I
30	Liquiritigenin	N. I	N. I
31	Quercetin	N. I	N. I
32	Luteolin	N. I	N. I
33	Naringenin	N. I	N. I
34	Apigenin	N. I	11.19
35	Hesperetin	N. I	N. I
36	Kaempferol	N. I	N. I
37	Chrysin	N. I	N. I
Total no. of detected compounds		19	19

N.I: Not Identified.
The omitted metabolites were not detected.

2.4. Biological activities

2.4.1. Antioxidant activity, total phenolic and flavonoid contents

The plant extracts usually showed chemical complexity, often a combination of compounds with various chemical classes and polarity, which may lead to scattered results, according to the type of the assay. Therefore, assessment of the antioxidant potential of plant extracts with numerous tests would be more informative and even necessary [13]. It is well known that phenolic acids, flavonoids, and tannins as phenolic ingredients are free radical scavengers and may contribute straight forwardly to the antioxidant action, so the

connection level between the phenolic content and the antioxidant activity is a fascinating aspect. For this reason, the contents of total phenolic and flavonoid contents of the extracts were additionally assessed.

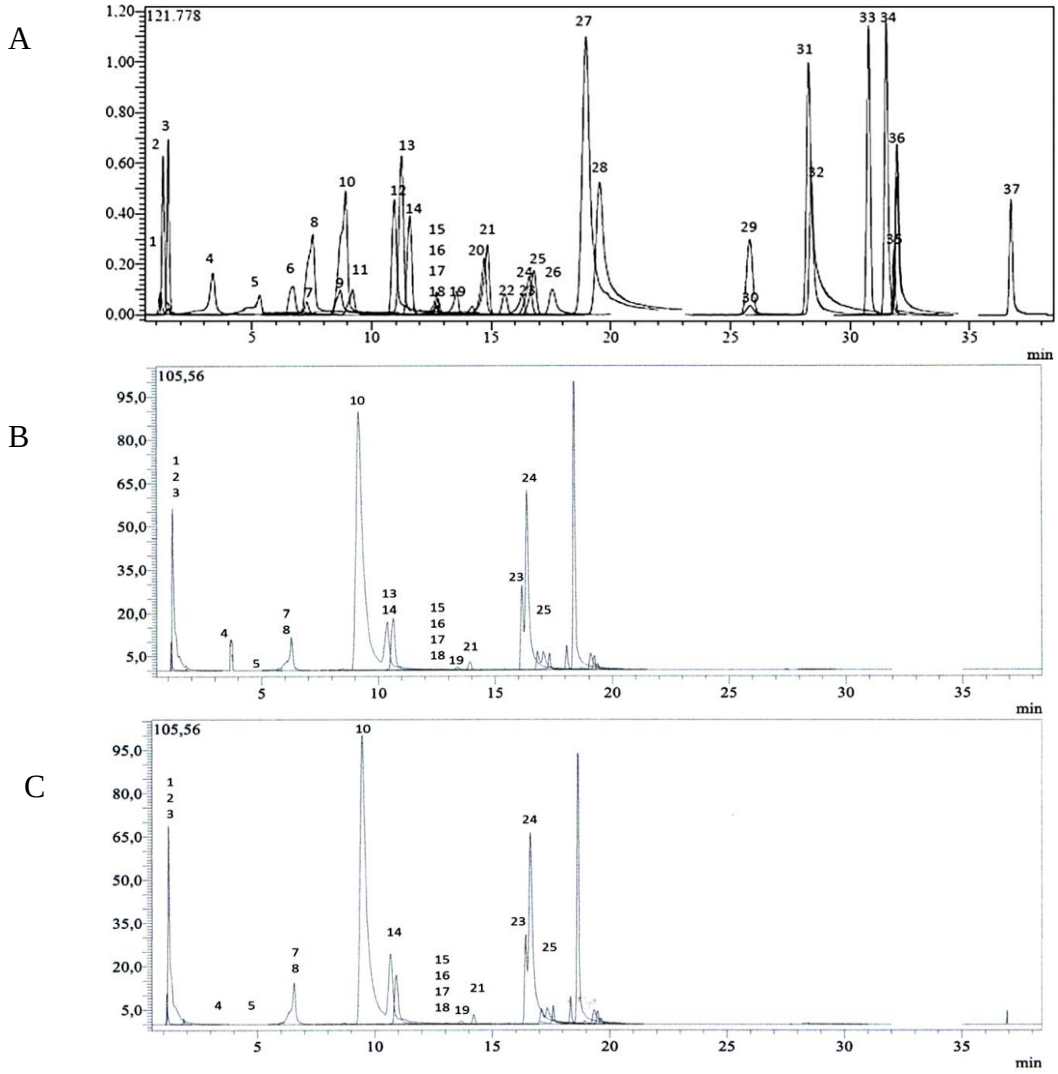

Figure 1. LC-MS/MS chromatograms: (A) TIC chromatogram of the standards mixture (1µg/ml); (B) Chromatogram of methanol extract of *B. crassifolium*; (C) Chromatogram of methanol:water (70:30) extract of *B. crassifolium*. Legend**:** (1) quinic acid, (2) malic acid, (3) fumaric acid, (4) gallic acid,(5) protocatechic acid, (6) pyrocatechol, (7) chlorogenic acid, (8) 4-OH-benzoic acid, (9) vanillic acid, (10) caffeic acid, (11) syringic acid, (12) vanillin, (13) salicylic acid, (14)*p*-coumaric acid, (15) rutin, (16)*tr*-ferulic acid, (17) sinapic acid, (18) hesperidin, (19) isoquercitrin, (20) rosmarinic acid, (21) nicotiflorin, (22)*α*-coumaric acid, (23) rhoifolin, (24) quercitrin, (25) apigetrin, (26) coumarin, (27) myricetin, (28) fisetin, (29) cinnamic acid, (30) liquiritigenin, (31) quercetin, (32) luteolin, (33) naringenin, (34) apigenin, (35) hesperetin, (36) kaempferol and (37) chrysin.

The results of the total phenolic contents (Table 4) of the two extracts of *B. crassifolium* demonstrated that the methanol:H_2O (70:30) extract possessed the highest value of 174.07 ± 3.00 mg GAE/g extract, in comparison with methanol extract (130.21 ± 6.36 mg GAE/g extract). Also, methanol:water (70:30) extract (49.72 ± 3.32 mg EQ/g of extract) was greater than that of the methanol extract (38.84 ± 1.24 mg EQ/g extract) according to total flavonoid content (Table 4). It was reported that addition of water to organic solvents increases the solubility of polyphenols by changing the polarity of the organic solvent [14].

In this work, the antioxidant activity was assessed by six assays, namely DPPH radical scavenging activity, phosphomolybdenum, metal chelating activity, *β*-carotene bleaching method, ABTS cation radical scavenging activity and cupric reducing antioxidant capacity. In fact, the methanol:water (70:30) extract was more potent as antioxidant than the methanol extract in all the used methods except in case of *β*-carotene bleaching method (Table 4). Noteworthy, the higher total phenolic and flavonoid contents in the methanol:water (70:30) extract entails its greater antioxidant capacity. Therefore, a good linear connection between the total phenolic and flavonoid contents and the antioxidant activity was observed.

Table 4. Total phenolic and flavonoid contents; and antioxidant activity of the extracts of *B. crassifolium* by the β-carotene-linoleic acid, $DPPH^{\cdot}$, $ABTS^{\cdot+}$, Phosphomolybdenum, CUPRAC and Fe^{+2} chelating assays.

Samples	Total Phenols[b]	Total Flavonoids[c]	DPPH IC_{50} (µg/ml)	Phospho-molybdenum IC_{50} (µg/ml)	Fe^{+2} Chelation IC_{50} (µg/ml)	β-carotene IC_{50} (µg/ml)	$ABTS^{+}$ IC_{50} (µg/ml)	CUPRAC $A_{0.50}$ (µg/ml)
Methanol extract	130.21±6.36	38.84±1.24	206.81 ± 7.78	311 ± 6.67	> 800	69.03 ± 7.39	16.06 ± 3.30	12.46 ± 0.95
Methanol:H_2O (70:30) extract	174.07±3.00	49.72±3.32	30.93 ± 5.31	276 ± 9.83	28.68 ± 0.06	71.60 ± 1.48	14.99 ± 5.72	9.37 ± 0.91
(+)-Catechin[a]	-	-	4.32 ± 0.15	NT	NT	8.79 ± 0.89	1.16 ± 0.02	NT
Quercetin[a]	-	-	2.07 ± 0.10	250.09 ± 0.87	NT	1.81 ± 0.11	1.18 ± 0.03	NT
α-Tocopherol[a]	-	-	7.31 ± 0.17	NT	NT	2.10 ± 0.08	4.31 ± 0.10	10.20 ± 0.01
BHT[a]	-	-	45.4 ± 0.47	NT	NT	1.34 ± 0.04	4.10 ± 0.06	3.80 ± 0.00
EDTA[a]	-	-	NT	NT	6.50 ± 0.07	NT	NT	NT
Ascorbic acid[a]	-	-	NT	7936.48 ±0.07	NT	NT	NT	NT

[a] Standard compounds, [b] mg gallic acid equivalent/g extract ;[c]mg quercetin equivalent/g extract. NT: Not Tested.

The results of β-carotene bleaching method (Table 4) showed that both methanol (IC_{50}: 69.03 ± 7.39 µg/ml) and methanol:water (70:30) (IC_{50}: 71.60 ± 1.48 µg/ml) extracts were less potent as lipid peroxidation inhibitors than the tested standards BHT (IC_{50}: 1.34 ± 0.04 µg/ml), quercetin (IC_{50}: 1.81 ± 0.11 µg/ml), catechin (IC_{50}: 8.79 ± 0.89 µg/ml) and *a*-tocopherol (IC_{50}: 2.10 ± 0.08 µg/ml).

In DPPH radical scavenging test, the methanol:water (70:30) extract (IC_{50}: 30.93 ± 5.31 µg/ml) revealed a better antiradical activity than BHT standard (IC_{50}: 45.4 ± 0.47 µg/ml), while the methanol extract (IC_{50}: 206.81 ± 7.78 µg/ml) showed lower activity in comparison to BHT, *a*-tocopherol (IC_{50}: 7.31 ± 0.17 µg/ml), catechin (IC_{50}: 4.32 ± 0.15 µg/ml) and quercetin (IC_{50}: 2.07 ± 0.10 µg/ml). Zengin *et al* [11], showed that the methanolic extract of *B. pinnatifolium* and *B. microcarpum* were the strongest DPPH scavangers, while the least effective ones were *B. sayai* and *B. brachyactis*. The team of Mariot (2009) [15], demostrated that anti-radical activity is associated with the level of flavonoids and polyphenols in medicinal plant extracts. The higher total phenolic and flavonoid contents in the methanol:H_2O (70:30) extract entails its greater antioxidant capacity. Therefore, a good linear association between percent inhibition of DPPH and the total polyphenols content was observed.

Both extracts (methanol:water (70:30) extract 14.99 ± 5.72 µg/ml and methanol extract 16.06 ± 3.30 µg/ml) were less active than catechin (IC_{50}: 1.16 ± 0.02 µg/ml), quercetin (IC_{50}: 1.18 ± 0.03 µg/ml), *a*-tocopherol (IC_{50}: 4.31 ± 0.10 µg/ml) and BHT (IC_{50}: 4.10 ± 0.06 µg/ml) standards in $ABTS^{\cdot+}$ assay. Zengin *et al* [11], demonstrated that the methanolic extract of *B. pinnatifolium* and *B. microcarpum* were the most effective anioxidant in ABTS assay, while *B. sayai* and *B. brachyactis* were the least effective ones.

The phosphomolybdenum assay depends of the reduction of Mo (VI) to Mo (V) by the antioxidant components present in the plant extracts. In the present study, both methanol:water (70:30) extract (IC_{50}: 276 ± 9.83 µg/ml) and methanol extract (IC_{50}: 311 ± 6.67 µg/ml) were more potent in reduction of Mo (VI) to Mo (V) than the standard ascorbic acid (IC_{50}: 7936.48 ± 0.07 µg/ml) and less effective than quercetin (IC_{50}: 250.09 ± 0.87 µg/ml). This proposed that the extracts of *B. crassifolium* have a better antioxidant activity than ascorbic acid. This action may be due to the high amounts of phenolic ingredients in the studied extracts which are referred to assume a critical job as an antioxidant through various mechanisms of action [16]. The team of Zengin [11], showed, the strongest effective antioxidant in the phosphomolybdenum were *B. pinnatifolium* and *B. brachyactis*, while the least antioxidant ones were *B. sayai* and *B. microcarpum*.

According to results of CUPRAC assay, the methanol:water (70:30) extract ($A_{0.50}$: 9.37 ± 0.91 µg/ml) exhibited a higher antioxidant activity than *a*-tocopherol standard ($A_{0.50}$: 10.20 ± 0.01 µg/ml) and a lower activity than BHT ($A_{0.50}$: 3.80 ± 0.00 µg/ml). While methanol extract ($A_{0.50}$: 12.46 ±0.95 µg/ml) was less active than the same antioxidant standards. The research of Zengin [11], showed that the methanolic extract of *B. pinnatifolium* was the highest effective anioxidant in CUPRAC assay, while the least effective were *B. sayai*, *B. brachyactis* and *B. microcarpum*. Prior *et al.* (2005) [17] classified the CUPRAC antioxidant method as an electron transfer technique, and advocated the superiority of this method over other antioxidant tests.

It was noted that only the methanol:water (70:30) extract (IC_{50}: 28.68 ± 0.06 µg/ml) exhibits a good metal chelating effect compared to EDTA (IC_{50}: 6.50 ± 0.07 µg/ml). According to IC_{50} values, the methanol:water (70:30) extract was 4 times only less active than EDTA standard as a ferrous ions chelating agent. While the methanol extract (IC_{50}: > 800 µg/ml) showed a negative activity. Zengin *et al* [11], showed that the *B. pinnatifolium* exhibited the highest ferrous ions chealating test, while, the three species of *Bunium* (*B. sayai*, *B. brachyactis* and *B. microcarpum*) exhibited the least activity. Chelation of iron plays an an essential job for

evaluation the antioxidant activity of therapeutic plants. The high chelating activity of the aqueous-alcoholic extract of *B. crassifolium* relative to the non-aqueous extract can be explained by the solubility of the chelating agents in water. It was reported that chelating effects of plant extracts directly proportional to the polarity of their solvents [18]. While, Carrër (2005) [19] demonstrated that the capture and release of iron (III) was fast and effective with quercetin, rutin and apigenin. The results obtained explained the antioxidant capacity of the methanol:water (70:30) extract by the presence of high total flavonoid content especially rutin amount (3,868.02 µg/g of extract) relative to the methanol extract.

2.3.2. Acetylcholinesterase and butyrylcholinesterase inhibitory activity

Plants are still viewed to be the most important source of potential new acetylcholinesterase (AChE) and butyryl-cholinesterase (BChE) inhibitor drugs which could be utilized for curing neurodegenerative diseases such as Alzheimer's disorder [20]. In the current study, the methanol and methanol:water (70:30) extracts of *B. crassifolium* were tested for anticholinesterase (AChE and BChE) activity and results are mentioned in Table 5. The methanol extract demonstrated quite good acetylcholinesterase inhibitory activity with IC_{50} value of 352.57 ± 8.72 µg/ml, while methanol:water (70:30) extract demonstrated a weak activity (IC_{50}: 1,014.05 ± 9.79 µg/ml) in comparison with galantamine positive control (7.39 ± 0.80 µg/ml). Regarding the results of butyrylcholinesterase inhibitory activity test, both methanol:water (70:30) and methanol extracts demonstrated weak activity with IC_{50} values of 5,983.20 ± 6.20 and 6,201.67 ± 0.00 µg/ml; respectively. Zengin *et al* [11], observed that the *B. sayai* and *B. brachyactis* were effective in inhibition of AchE and the most effective BchE was *B. brachyactis*. Generally, the extracts displayed a superior action against AChE enzyme, and methanol extract was the most active one. It was reported that consumption of flavonoids in food is inversely proportional to the dementia disorder [21] and high dietary intake of vegetables may be related to slower rate of occurence of neurodegenerative diseases in older age [22]. The potentiation of antioxidant defenses including enzymatic and non-enzymatic antioxidants by plant nutrients (e.g. polyphenolics) is the main mechanism proposed for the beneficial effect of vegetables and fruits. Neuronal protein misfolding, membrane dysfunction, and glial cell activation that are associated with normal ageing or certain neurodegenerative diseases are linked to oxidative stress [23]. Although the mechanisms underlying this action are still unknown and need more investigation, *B. crassifolium* seems to help in prevention of cognitive decline during aging as it revealed a competitive inhibitory activity of acetylcholinesterase with that of galantamine.

Table 5. Acetylcholinesterase, butyrylcholinesterase and tyrosinase inhibitory activities of the extracts of *B. crassifolium.*

Samples	AChE IC_{50} (µg/ml)	BChE IC_{50} (µg/ml)	Tyrosinase IC_{50} (µg/ml)
Methanol extract	352.57 ± 8.72	6201.67 ± 0.00	5.72 ± 0.30
Methanol:water (70:30) extract	1014.05 ± 9.79	5983.20 ± 6.20	3.49 ± 3.63
Galantamine[a]	7.39 ± 0.80	50.90 ± 0.90	NT
Kojic acid[a]	NT	NT	0.67
L-Mimosine[a]	NT	NT	0.64

[a] Standard compounds
NT: Not Tested.

2.3.3. Tyrosinase inhibitory activity

Melanin is the source of the pigmentation of the hair and skin but its production in excess amounts may lead to hyperpigmentation or vitiligo disease. Tyrosinase is an important enzyme in melanin production, so tyrosinase inhibitors have attracted attention last years due to hyperpigmentation [24]. The look for new natural tyrosinase inhibitors is necessary because of the side effects of synthetic inhibitors currently used. We tested the tyrosinase inhibitory activity for both extracts of *B. crassifolium* and they showed good activity (Table 5). The methanol:water extract (IC_{50}: 3.49 ± 3.63 µg/ml) and methanol extract (IC_{50}: 5.72 ± 0.30 µg/ml) showed quite good tyrosinase inhibitory activity in comparison with kojic acid (IC_{50}: 0.67 µg/ml) and L-mimosine (IC_{50}: 0.64 µg/ml) standard compounds. It has been reported that tyrosinase enzyme can be inhibited by aromatic aldehydes and acids, flavonoids and copper chelators [25,26] and this is may explains why the methanol:water (70:30) extract was more potent than methanol extract because it was more rich in phenolic acids (e.g. chlorogenic acid) and flavonoids especially rhoifolin, rutin and apigenin according to the obtained LC-MS/MS results. Furthermore, rutin was reported to be a potent antipigment agent due to its tyrosinase inhibitory activity [27]. The group of Zengin [11], showed that the higest activity was exerted by *B. brachyactis,*

while the lowest activity was demonstrated by *B. microcaprum*. Several compounds identified in *B. crassifolium* are known to be strong enzyme inhibitors. Apigenin previously showed significant inhibition against mushroom tyrosinase and appeared to competitively inhibit the polyphenol oxidase activity of tyrosinase [28]. Our outcomes demonstrated that *B. crassifolium* might be a promising candidate for hyperpigmentation disorders.

3. CONCLUSION

The present study investigated the phenolic compounds composition in methanol and methanol:water (70:30) extracts of *Bunium crassifolium* which is a rare and endemic species of Algeria by LC-MS/MS technique. A sum of 19 compounds were quantified in each extract. Quinic acid, malic acid, chlorogenic acid, rutin and apigetrin were the major phenolic compounds detected. The outcomes demonstrated that both extracts were rich in phenolic and flavonoid contents, and the methanol:water (70:30) extract indicated higher values than those of the methanol extract. In addition, the tested extracts showed a noteworthy antioxidant action. Furthermore, the methanol extract demonstrated a good anti-acetylcholinesterase activity, while methanol:water (70:30) extract demonstrated a weak activity. So, methanol extract revealed a competitive acetylcholinesterase inhibitory action with that of galantamine standard. Regarding the antibutyrylcholinesterase test, both extracts demonstrated a weak activity. Finally, the extracts showed quite good tyrosinase inhibitory activity and methanol:water (70:30) extract was more potent. In conclusion, this forgotton *B. crassifolium* plant is promising and could be exploited in food and pharmaceutical industries as a potential functional food ingredient.

4. MATERIALS AND METHODS

4.1. Plant material and extraction method

Samples of the plant (*Bunium crassifolium* Batt.) were collected in full bloom in Sérraïdi (Annaba, Algeria), during May 2015. The plant was identified and confirmed by Dr. Tarek Hamel (Department of Plant Biology and Environment, Badji Mokhtar University, Annaba, Algeria). A voucher specimen was inserted in the herbarium under the code, ChifaDZUMCAPBC000037. Samples were shade dried, then they were cut into smaller pieces.

The aerial parts (leaves, stems and flowers) of *B. crassifolium* were extracted with two solvents like methanol and methanol:H_2O (70:30). The naturally dried plant materials were ground by a 2 mm pore size electric mill. The powdered plant material (200 g) was extracted with 1000 ml pure methanol and methanol:water (70:30) by magnetic stirrer (200 rpm for 24 h, at room temperature) till exhaustion. After filtration, the extracts were concentrated by rotary evaporation (Rota Vapor, Büchi R-200, Germany) at 40 °C and a thick semisolid pastes were obtained. The percentages yield of pure methanol and methanol:water (70:30) extracts were found to be 23.55 and 28.50 % (w/w); respectively. The extracts were stored in a refrigerator at 4°C until LC-MS/MS analyses and biological studies.

4.2. Chemicals for LC-MS/MS analysis

The analytical standards, HPLC-grade ammonium formate, acetonitrile and formic acid were purchased from Sigma–Aldrich (Milano, Italy).

4.3. Chemicals for biological studies

Quercetin, pyrocatechol, ferrous chloride, ferric chloride, copper (II) chloride, ethylenediamine tetraacetic acid (EDTA), potassium persulfate and boron trifluoride-methanol complex (BF_3:MeOH) were obtained from E. Merck (Darmstadt, Germany). *β*-Carotene, polyoxyethylene sorbitan monopalmitate (Tween-40), linoleic acid, 3-(2-pyridyl)- 5,6-di(2-furyl)-1,2,4-triazine-5′,5′′-disulfonic acid disodium salt (Ferene), neocuproine, Folin–Ciocalteu's reagent, ammonium acetate, butylated hydroxytoluene (BHT), DPPH dye, Electric eel acetylcholinesterase (AChE, Type- VI-S, EC 3.1.1.7, 425.84 U/mg), horse serum butyrylcholinesterase (BChE, EC 3.1.1.8, 11.4 U/mg), acetylthiocholine iodide, butyrylthiocholine chloride, galantamine and 5,50-dithiobis (2-nitrobenzoic) acid (DTNB), were purchased from Sigma Chemical Co. (Sigma–Aldrich GmbH, Sternheim, Germany). 2,20-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was obtained from Fluka Chemie (Fluka Chemie GmbH, Sternheim, Germany). Analytical grade chemicals, reagents and solvents were consumed throughout the work. All other reagents unless indicated were purchased from Sigma (St. Louis, MO, USA).

4.4. Preparation of standards

The standard stock solutions were prepared in methanol (50 µg/ml) except isoquercitrin and hesperidin, that were dissolved in dimethylsulfoxide (50 µg/ml). Working solutions were prepared from the stock solutions by dilution in methanol. All solutions were stored in a refrigerator at 4°C until analysis.

4.5. LC-MS/MS Analysis

4.5.1. Sample preparation for LC-MS/MS

The extracts (1 mg/ml) were prepared and filtered with a 0.2 µm syringe filter prior to LC-MS/MS analysis [29]. Each sample was analyzed in triplicates.

4.5.2. Chromatographic instruments and conditions for LC-MS/MS

The quantitative study of 37 bioactive compounds was evaluated using a Nexera Shimadzu UHPLC model coupled to an MS tandem instrument. The chromatographic instrument was coupled to a SIL-30AC autosampler, LC-30AD binary pumps, a CTO-10ASvp column oven and a DGU-20A3R degasser. Chromatographic separation was performed on a RP-C18 Insertil ODS-4 analytical column (100 mm x 2.1 mm, 2 µm). Ultra-high performance reverse phase liquid chromatography has been optimized to achieve optimal separation of phytochemicals. The column temperature was kept at 35 °C. The elution gradient consisted of eluent A (10 mM ammonium formate, water and 0.1% formic acid) and eluent B (acetonitrile). The following gradient elution program was applied: 5-20% B (0-10 min), 20% B (10-22 min), 20-50% B (22-36 min), 95% B (36-40 min), 5% B (40-50 min). The solvent flow rate was maintained at 0.25 ml/min and the injection volume was set at 4 µl.

MS detection was done using a Shimadzu brand LC-MS 8040 model tandem mass spectrometer coupled to an ESI source. LC-ESI-MS/MS data was collected and shipped by LabSolutions Software (Shimadzu). The working ESI conditions of the mass spectrometer were set as follows: interface gas temperature 350 °C; DL temperature 250 °C; temperature of the thermal block 400 °C; nebulization gas flow (nitrogen), 3 ml/min; and drying gas stream (nitrogen) 15 ml/min.

4.6. Quantification of total phenolic content

The total phenolic content was evaluated by the method of Djeridane *et al.* (2006) [30]. Briefly, 300 µl of the extract was additioned to 1.5 ml of the Folin-Ciocalteu reagent (10 times diluted). After 4 minutes, 1.2 ml of sodium carbonate solution (7.5%) was added. Then, the solution obtained was kept in the dark for 2 hours and was measured at 750 nm absorbance. The concentration of total phenols was calculated from a calibration graph established with gallic acid. The results were expressed in mg gallic acid equivalent per g dried extract (mg GAE/g extract).

4.7. Quantification of total flavonoid content

The content of total flavonoids was evaluted by Djeridane *et al.* (2006) [30] method's. 500 µl of 2% aluminum chloride was added to 500 µl of the extracts. After 10 minutes of incubation the absorbance of the solution was measured at 430 nm. The concentrations of flavonoid were deduced from a calibration graph by mg quercetin equivalent per g dried extract (mg QE/g extract).

4.8. Antioxidant capacity

4.8.1. Evaluation of antioxidant activity by β-carotene bleaching test

The *β*-carotene-linoleic bleaching test was descrideb by the method of the team of Öztürk in 2011 [13]. A 0.5 mg of *β*-carotene dissolved in 1 ml of chloroform was mixed with 200 mg of Tween 40 and 25 µl of linoleic acid. After, the chloroform was evaporated and was added to the mixture 100 ml of distilled water saturated with oxygen under vigorous stirring. Then, 4 ml of the previously prepared solution were added to the extracts at different concentrations in ethanol. The absorbance of the mixture was measured immediatly at 470 nm at zero time. Then, the mixture was incubated for 2 h at 50 °C and the absorbance was measured again. A negative control, free of *β*-carotene was used. The bleaching rate (R) of *β*-carotene was determined from the equation 1:

$$R = \ln a/b \ / \ t. \qquad \text{(Eq. 1)}$$

Where ln is the natural log, a is the absorbance at zero time, b is the absorbance at time t (120 min). The antioxidant activity (AA) was calculated as percent inhibition by the following equation 2:

$$\% \text{ inhibition} = [R \text{ control} - R \text{ sample} \ / \ R \text{ control}] \times 100 \qquad \text{(Eq. 2)}$$

Quercetin, catechin, BHT and *a*-tocopherol antioxidant standards have been used for the comparison.

4.8.2. DPPH free radical scavenging test

The anti-radical activities against DPPH were determined by the DPPH test described by Öztürk *et al*. (2011) [13]. Briefly, 1 ml of the different concentrations of the extracts were added to 4 ml of 0.1 mM solution of DPPH in methanol. Then, the mixture was incubated for 30 minutes in the dark at room temperated and the absorbance was measured at 517 nm. The antioxidant activity was calculated as a percentage of DPPH radical inhibition, from the following equation 3:

$$\% \text{ inhibition} = [A \text{ control} - A \text{ sample} \ / \ A \text{ control}] \times 100 \qquad \text{(Eq. 3)}$$

The IC_{50} value was calculated from a calibration curve constructed at different concentrations of each extract. Quercetin, catechin, BHT and *a*-tocopherol antioxidant standards have been used for the comparison of activity.

4.8.3. ABTS radical cation reduction test

The anti-radical activities against the $ABTS^{\cdot+}$ radical were evaluted by the method of Öztürk *et al*. (2011) [13] with slight modification. Briefly, the $ABTS^{+}$ was generated by 7 mM of ABTS mixing in 2.45 mM of potassuim persulfate and water. The mixture was incubated for 12 hours in the dark at room temperature. Then, 2 ml of the $ABTS^{\cdot+}$ solution was additioned to 1 ml of the extracts at different concentrations (5-50 mg/ml). After 30 minutes, the percent inhibition at 734 nm was calculated for all concentration. The $ABTS^{\cdot+}$ scanning capability was calculated using the following equation 4:

$$\% \text{ inhibition} = [Abs \text{ control} - Abs \text{ sample} \ / \ Abs \text{ control}] \times 100 \qquad \text{(Eq. 4)}$$

Where the Abs control is absorbance of $ABTS^{\cdot+}$ plus methanol, and the Abs sample is absorbance of $ABTS^{\cdot+}$ plus extract or standard. The IC_{50} value was calculated for each sample and compared with catechin, BHT, quercetin and *a*-tocopherol antioxidant standards which were used for activity comparison.

4.8.4. Total antioxidant capacity test

The total antioxidant capacity test was evaluated by the the method of the phosphomolybdenum described by Ramalakshmi *et al*. (2008) [31]. A 100 µl of each extract was added to 1 ml of the phosphomolybdate reagent (0.6 M of sulfuric acid, 28 mM of sodium phosphate and 4 mM of ammonium molybdate). Then, the mixture was incubated for 90 minutes in water bath at 95 °C. Then, the absorbance was recorded at 695 nm. Percent inhibition was deduced by the equation 5:

$$\% \text{ inhibition} = (1 - \text{absorbance of sample/absorbance of control}) \times 100 \qquad \text{(Eq. 5)}$$

Quercetin and ascorbic acid antioxidant standards were used for activity comparison.

4.8.5. Cupric reducing antioxidant capacity (CUPRAC) test

The cupric reductive antioxidant capacity was evaluated by the method of Öztürk's group [13] with a slight modification. A 50 µl of 7.5 mM neocuprone, 60 µl of NH_4Ac buffer (1 M, pH 7.0) and 50 µl of 10 mM

Cu (II) solution were added to obtain a mixture. Then, to obtain a final volume of 200 µl we added 40 µl of different concentrations of the extracts and the absorbance was measured after 1 hours at 450 nm. The results were given as $A_{0.50}$ (µg/ml) which corresponds to the sample concentration giving 0.50 absorbance. $A_{0.50}$ was obtained from the graph of the absorbance of cupric reductive antioxidant capacity. The antioxidant standards such as *a*-tocopherol and BHT were used for comparison of the activity.

4.8.6. Ferrous ions chelating test

The ferrous ion chelating test was measured by the using of ferrin according to the method described by Öztürk *et al*. (2011) [13]. A 40 µl of 0.2 mM $FeCl_2$ was additioned to extract solution (80 µl diluated in different concentrations of ethanol). Then, 80 µl of 0.5% ferene was added to the mixture and kept at room temperature for 10 minutes and stirred. The absorbance was measured at 593 nm. The ferrous ions chelating activity was deduced from the following equation 6:

$$\% \text{ of metal chelation activity} = [A \text{ control} - A \text{ sample} / A \text{ control}] \times 100 \qquad \text{(Eq. 6)}$$

Where A control is the absorbance of the sample-free control and A sample is the absorbance of the sample in the presence of the chelator. The IC_{50} of metal chelation activity was deduced from the curve of the percentage of Fe^{2+} chelation effects with the different concentration of extract. EDTA antioxidant standard was used for comparison of activity.

4.9. Acetylcholinesterase and butyrylcholinesterase inhibitory activities

The inhibitory activities of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were evaluated by the method mentioned by Öztürk *et al*. (2011) [13]. A 10 µl of the sample dissolved in ethanol at different concentrations, 150 µl of 100 mM sodium phosphate buffer (pH 8.0) and 20 µl of AChE solution (5.32×10^{-3} U) or BChE (6.85×10^{-3} U) were mixed and incubated at 25 °C for 15 minutes. After, 10 µl of 0.5 mM DTNB and 10 µl of acetylthiocholine iodide (0.71 mM) or butyrylthiocholine chloride (0.2 mM) were added to the mixture. The absorbance was measured at 412 nm and the percent inhibition of AChE or BChE was obtained using the equation 7:

$$\% \text{ inhibition} = (E - S) / E \times 100 \qquad \text{(Eq. 7)}$$

Where E is the enzyme activity without the test extract, and S is the enzyme activity with the tested extract. We performed the assays three times and galantamine was used as the reference compound.

4.10. Tyrosinase inhibitory activity

The tyrosinase inhibitory activity of the extracts relative to kojic acid and L-mimosine standards was determined using fungal tyrosinase according to Khatib *et al*. (2005) [32]. A mixture of 0.07 ml of potassium phosphate buffer (50 mM) at pH 6.5, 0.3 ml of tyrosinase (333 units/ml) and 2 µl of the tested extracts (0.5 to 500 µM) dissolved in ethanol and was incubated for 5 minutes at room temperature. After, 12 mM L-DOPA was added and incubated for another 20 minutes. The absorbance of the extracts and control were measured at 492 nm. The percent inhibition of the enzyme and the IC_{50} values of the extracts were calculated using equation 8:

$$\% \text{ inhibition} = [A - B / A] \times 100 \qquad \text{(Eq. 8)}$$

Here A and B are the absorbances of the control and samples; respectively.

4.11. Statistical analysis

All data of antioxidant, anticholinesterase and antityrosinase activities tests were the mean of three analyses. The data were recorded as mean ± standard deviation.

Acknowledgements: The authors are grateful to the Departments of Chemistry in University of Constantine 1 (Algeria), University of Mugla (Turkey) and University of Dicle (Turkey) for providing support to perform the present research.

Author contributions: Concept – N.S., H.B., L.D.; Design – L.D, M.Ö., K.M.; Supervision – M.Ö., S.A., K.M.; Resources – T.H., E.A., M.Ö., Z.U.; Materials – L.D., T.H., E.A.; Data Collection and/or Processing – N.S., Z.U., T.H., K.M.; Analysis and/or Interpretation – A.M.M., Z.U., S.A.; Literature Search – N.S., H.B., A.M.M., T.H.; Writing – N.S., H.B., A.M.M.; Critical Reviews – N. S., H.B., Z.U., T.H., L.D., M.Ö., A.E., S.A., K.M., A.M.M.

Conflict of interest statement: "The authors declared no conflict of interest" in the manuscript.

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