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Total phenolic contents, *in vitro* antioxidant activity, enzymes inhibition and anti-inflammatory effect of the selective extracts from the Algerian *Lavandula multifida*

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ABSTRACT

The present research aimed to carry out the phytochemical analysis, *in vitro* antioxidant activity, enzymes inhibition and anti-inflammatory effect of the selective extracts of the Algerian *L. multifida* from two regions; Msila region and Constantine region. The total phenolic and flavonoids contents of crude extract (CE) and its solvent partition fractions: dichloromethane (DME), ethyl acetate (EAE) and n-butanol (BUE) were determined spectrophotometriclly. The antioxidant activity of extracts was achieved by the use of seven methods and the enzyme inhibitory activity of extracts was evaluated against α-amylase and

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butyrylcholinesterase. Moreover, the *in vivo* anti-inflammatory activity of the aqueous extract of *L. multifida* from Constantine region was evaluated using paw edema model. The M.EAE present the highest TPC and TFC, which were about 462.23±11.74µg GAE/mg extract and 125.90±0.16µg QE/mg extract, respectively. In addition, the M.EAE exhibited an excellent antioxidant activity, as it has a great ability to scavenging the DPPH, ABTS and galvinoxyl free radicals, as well as reducing power and metal chelating. However, the M.CE and C.CE showed the best inhibitory activity of the α-amylase butyrylcholinesterase (IC50=64.17±1.81µg/ml and 83.55±1.97µg/ml, respectively. The preliminary investigation reveals that the EAE has a good bio-pharmacological activity, which it possesses an interesting potential for pharmaceutical/nutraceutical applications.

Key words: Antioxidant activity, anti-inflammatory effect, enzymes inhibition, *Lavandula*.

INTRODUCTION

Since the beginning of human existence, human has become acquainted with plants and has used them in various ways and in different fields. Medicinal plants play an important role in the prevention and treatment of human diseases. This relationship between plants and human has developed and many plants have been used as a natural traditional remedy. In recent years, the pharmacological effects of medicinal plants have been considered as a potential source of future medicaments [1]. In biological systems, the reactive oxygen species (ROS) and reactive nitrogen species (NERs) can damage DNA and cause oxidation of lipids and proteins in cells. Naturally, an antioxidant system present in the human body can eliminate these free radicals, which would help maintain the balance between the production of reactive oxygen species and the antioxidant capacities of the body [2]. Studies on the toxicity of synthetic antioxidants have increased the demand for natural antioxidants, particularly from a plant source, in the food, pharmaceutical and cosmetic sectors, since they can be used as natural substituents for these synthetic antioxidants[3]. The presence of natural antioxidant such as phenolic compounds, flavonoids and tannins in plants may provide protection against a number of diseases, and their use has been inversely associated with mortality and morbidity due to degenerative disorders [4]. Lamiaceae, or Labiatae, is a family of angiosperms with 236 genera and more than 7000 species [5]. Lamiaceae are widely used in traditional and modern medicine worldwide, thanks to the many beneficial pharmacological effects they can exert [6]. The genus Lavandula is an important member of Lamiaceae, it comprises 39 species. *Lavandula multifida* is a small, semi-evergreen perennial shrub composed of several small leaves and aromatic

flowers. It grows commonly in the Mediterranean region and North Africa, where it is mainly distributed in pre-Saharan areas, more and more on rocky outcrops and on more or less drained limestone soils at the edge of temporarily drained rivers, between 800 and 2000 meters of altitude 7-[8]. The main aim of this work is the total phenolic contents estimation and of biological activities study of *L. multifida* from two Algerian geographical areas.

METHODOLOGY

Biological material

L. multifida aerial parts were collected in 2016 from the Maadid region of Msila wilaya (Algeria) in April 2018. The second region *L. multifida* plant was collected from Jebel El Ouahch region of Constantine Province (Algeria) in June 2019 (**Figure 1**). After drying in the shade, the aerial parts of the plant are crushed, the vegetable powder thus obtained was used for extraction.

Figure 1. The Geographic position of Msila (**1**) and Constantine (**2**) in Algeria.

The *in vivo* study was carried out on female Wistar rats, whose weight varies between 130 g and 170 g. These animals were obtained from the animal department of the natural sciences and life of the University of Mentouri Constantine Brothers. The rats were placed in polypropylene cages at room temperature (20-25 °C) where they had free access to water and food. These animals benefited from an adaptation period of one week before their use.

Extraction

The hydromethanolic extraction: The hydromethanolic extraction was carried out by maceration of 100 g powdered dry plant (of each region) in 1 L of methanol (80%) for 72 h at room temperature. After 72 hours of contact, the methanol mixture was filtered and concentrated at 40 °C under reduced pressure using a rotary evaporator (BUCHI) to give crude extract (M.CE: crude extract of the plant from Msila region; C.CE: crude extract of the plant from Constantine region). The crude extract of etch plant was subjected to liquid-liquid extraction (fractionation) using organic solvents with increasing polarity using dichloromethane giving dichloromethane extract labeled (DME), ethyl acetate giving ethyl acetate extract labeled (EAE) and *n*-butanol giving butanol extract labeled (BUE). All solvents were removed using rotary evaporator to give the respectively extracts: M.CE and their fraction M.DME; M.EAE and M.BUE; C.CE and their fractions C.DME; C.EAE and C.BUE.

The aqueous extraction: For the evaluation of the anti-inflammatory activity *in vivo*, the 10 g dried and powdered aerial parts of *L. multifida* from Constantine region were extracted by maceration with 500 ml of distilled water. After 24 hours of contact, the whole is filtered with filter paper in order to recover the filtrate; the latter has been dried in an oven to obtain aqueous extract [8].

Total phenolic contents (TPC)

The total phenolic content of the extracts was determined using the Folin-Ciocalteu reagent (FCR) according to the method described by [9]. In the 96-well microplate, a volume of 20 µl of plant extract (1 mg/ml) was added to 100 µl of diluted FCR (1:10). Then 75 µl of sodium carbonate (7.5%) was added. The mixture was left in the dark for 2 hours at room temperature. The absorbance of different intensities of the resulting blue color was measured at 765 nm using a PerkinElmer 96-well microplate reader (USA). The TPC was expressed as µg gallic acid equivalent per mg of extract (µg AGE/mg E), using the linear regression equation of the calibration curve plotted by gallic acid (y=0.0034x+0, 1044) with R^2 = 0.9972.

Total flavonoid contents (TFC)

The total flavonoid content of the extracts was determined using aluminium nitrate ($Al(NO_3)_3$) reagent according to the method described by [10]. In the 96-well microplate, a volume of 50 µl of the extract was mixed with 130 µl of methanol, 10 µl of potassium acetate and 10 µl of aluminum nitrate. The mixture was incubated for 40 minutes and then the absorbance was measured at 415 nm using a PerkinElmer 96-well microplate reader (USA). The TFC was

expressed as μg quercetin equivalent per mg of extract (μg QE/mg E), using the linear regression equation of the calibration curve plotted by quercetin (y=0.0071x+0.0274; R^2 = 0.9985).

In vitro antioxidant activity

The antioxidant capacity of extracts was achieved through seven methods: DPPH free radical scavenging assay, galvinoxyl free radical scavenging test, ABTS assay, reducing power, cupric reducing antioxidant capacity (CUPRAC), Fe^{+2}-phenanthroline reduction and metal ions chelation.

DPPH radical scavenging activity

The DPPH antiradical activity of extracts was determined spectrophotometrically according to the method described by [11]. Briefly, a 160 μl of DPPH solution (0.6 mg/100 ml methanol) was mixed with 40 μl of the extract prepared in several concentrations. The mixture was kept in dark at room temperature. After 30 min of incubation, the absorbance was measured at 517 nm. The antiradical activity was expressed as EC_{50} the values, i.e., concentration of the studied extracts, which causes a 50% decrease in the absorbance at 517 nm as compared to the control. The percent inhibition was calculated according to the following equation: DPPH scavenging (%) = ((*A* of control – *A* of sample)/ *A* of control) × 100

Galvinoxyl radical-scavenging assay

The antiradical activity of the extracts against galvinoxyl radical was determined according to the method described by [12]. Briefly, 160 μl of galvinoxyl radical (10 μM) was mixed with 40 μl of extract at several concentrations. The reaction was carried out for 2 h at room temperature. The decrease in galvinoxyl radical concentration was determined spectrophotometrically by measuring the absorbance at 432 nm, the antiradical activity was expressed as EC_{50} the values, i.e., concentration of the studied extracts, which causes a 50% decrease in the absorbance at 432 nm as compared to the control sample. The percent inhibition was calculated according to the following equation: Galvinoxyl radical-scavenging (%) = ((*A* of control – *A* of sample)/ *A* of control) ×100

$ABTS^{\bullet+}$-scavenging assay

$ABTS^{\bullet+}$-scavenging capacity was determined according to the method described by [13]. The $ABTS^{+}$ radical was generated by the mixture of ABTS solution (7 mM) with potassium persulfate ($K_2S_2O_8$) solution (2.45 mM). The mixture was incubated in the dark at room temperature for 16-24 h. The standard solution of ABTS was diluted by the addition of methanol to have an absorbance of

0.700 (±0.02) at 734 nm. An aliquot of 40 µL of extract was mixed with 160 µL of ABTS and absorbance was recorded after 30 minutes.

The antiradical activity was expressed as EC_{50} the values, i.e., concentration of the studied extracts, which causes a 50% decrease in the absorbance at 432 nm as compared to the control. The percent inhibition was calculated according to the following equation: Galvinoxyl radical-scavenging (%) = ((*A* of control – *A* of sample)/ *A* of control) × 100.

The data were the concentration required for an effect of 50% (EC_{50}).

Cupric reducing antioxidant capacity

The cupric ion reducing antioxidant effect of the extracts was estimated according to the method of [14]. 40 µL of extract soultion was mixed with 50 µL of copper chloride solution (10 mM), 50 µL of neocuproine alcoholic solution (7.5 mM) and 60 µL of ammonium acetate buffer solution (1 M, pH 7.0) to make final volume of 200 µL. After one hour of incubation, the absorbance was measured at 450 nm against the reagent blank. Standard curve was prepared using different concentrations of Trolox and the results were expressed as $A_{0.50}$ (µg/ml) corresponding to the concentration giving an absorbance of 0.50.

Reducing power

The reducing power of the extracts was determined according to the methods of [15]. A volume of 10 µl of the extract at different concentrations was mixed with 40 µl of phosphate buffer solution (0.2M, pH=6.6) and 50 µl of a potassium ferricyanide [$K_3Fe(CN)6$] solution (1%), the mixture was incubated was incubated at 50 °C for 20 min. Then, 50 µl of trichloroacetic acid (10%) were added to stop the reaction and the whole was centrifuged at 3000 r/min for 10 min. Finally, 50 µl of the supernatant solution was mixed with 50 µl of distilled water and 10 µl of $FeCl_3$ (0.1%) and the absorbance was recorded at 700 nm after an incubation for 10 min. Increased absorbance of the reaction mixture. The positive control was represented by two standard antioxidant solutions; ascorbic acid and α-tocopherol, the absorbance of which was measured under the same conditions as the samples. The results were expressed as µg ascorbic acid equivalent/mg extract.

Reduction activity by phenanthroline method

Reduction activity by phenanthroline method was determined according to [16]. A volume of 10 µl of the extract at different concentrations was mixed with 50 µl (0.2%) of ferric chloride ($FeCl_3$), 30 µl of phenanthroline (0.5%) and 110 µl of methanol. The obtained solution was left at room temperature in a dark

for 20 min. After the incubation, the absorbance of an orangered solution was measured at 510 nm. BHA and BHT wre used as standards. The results were calculated as $A_{0.5}$ (µg/ml) corresponding to the concentration indicating an absorbance of 0.50.

Metal ions chelation activity

Iron (Fe^{+2}) chelating property of the extracts was determined using the method of [17]. A volume of 40 µl of extract solution at various concentrations was mixed with 40µl of $FeCl_2$ (0.2 mM) and of 40 µl of methanol. The reaction was initiated by the addition of 80 µl of the ferrozine (5 mM) to the whole. After an incubated in the dark for 10 min, the absorbance of the solution was measured at 562 nm against a similarly prepared blank.

The metal chelating activity of ethylenediamine tetra-acetic acid (EDTA) was also determined and the metal chelating activity was expressed as µg equivalent EDTA /mg extract.

Enzymes inhibition activity

Alpha-amylase inhibition

The α-amylase inhibitory activity of the extracts was assayed according to the procedure described by [18]. The extract solution (25 µL) was mixed with 25 µL of phosphate-sodium buffer (5 mM, pH 6.9) and 50 µL α-amylase solution (1U), the mixture was incubated at 37 °C for 10 min. Then, 50 µl of soluble starch (1%) were added and the mixture re-incubated for 10 min at 37 °C. After 10 min of incubation, the reaction was stopped by the addition of 25 µl of HCl (1M) and 100 µl of iodine-potassium iodide solution. The absorbance was measured at 630 nm and the percentage inhibition of α-amylase was calculated as follows:

α-amylase inhibition % = [(A of control –A of sample)/ A of control] × 100

The IC_{50} concentration required for inhibition of 50% of α-amylase was determined graphically and Acarbose was used as a positive control.

Butyrylcholinesterase (BuChE) inhibitory activity

Butyrylcholinesterase (BuChE) inhibitory activity was measured using Ellman's method as previously reported by [19]. Briefly, the plant extract (10 µL) was mixed with dithiobisnitro-benzoate (DTNB) (10 µL) and BuChE (10 µL) in 150 µl of sodium phosphate buffer (100 mM, pH 8.0) in a 96-well microplate. The reaction was initiated by the addition of 20 µl butyrylthiocholine chloride (0.2 mM). The solution mixture was maintained at 30 °C for 15 min using water bath. The hydrolysis of these substrates was followed spectrophoto-metrically

at 412 nm with the formation of a yellow color. The BuChE inhibitory activity was calculated as follows:

$$\text{BuChE inhibition \%} = [(\text{A of control} - \text{A of sample}) / \text{A of control}] \times 100$$

The IC_{50}, concentration required for inhibition of 50% of BuChE was determined graphically and Galantamine was used as a positive control.

Evaluation of anti-inflammatory activity *in vivo*

The *in vivo* study was carried out on female Wistar rats, whose weight varies between 130 g and 180 g. These animals were obtained from the animal department of the natural sciences and life of the University of Mentouri Constantine Brothers. The rats were placed in polypropylene cages at room temperature (20-25 °C) where they had free access to water and food. These animals benefited from an adaptation period of one week before their use. The experimental protocols were approved by Institutional Animal Ethics Committee (N°: 35/2017) in accordance with the guideline formulated by Committee for Purpose of Control and Supervision of Experiments on Animals, Algeria.

The anti-inflammatory activity of extracts of *L. multifida* (from Constantine region) was evaluated using formaldehyde induced paw edema method according to [20]. The rats were randomly divided into 3 groups based on their body weight; each group contains six animals. One hour before the formaldehyde injection, each group receives via intraperitoneal injection the following experimental solutions:

Group I: Negative control, received 200 µl of saline water (0.9%).

Group II: Positive control, received 200 µl of anti-inflammatory drug Diclofenac (CLOFENAL®) at dose of 25 mg/Kg dissolved saline water.

Group III: Treated group, received 200 µl of aqueous extract of *L. multifida* (200 mg/Kg) dissolved saline water.

One hour after the extract (drug) administration, 0.1 ml of 1% v/v formaldehyde solution was injected into the subplantar aponeurosis of the left hind limb of the rat. The follow-up of the evolution of the inflammation was done by measurement of the paw volumes before and after induction of the edema at 0, 30, 60, 180 and 360 minutes. The paw volumes measured using a graduated tube and were expressed as a percentage change in paw volume at various time intervals in comparison to the initial values. The anti-inflammatory activity was expressed as paw volume reduction, which was given by the following formula:

The anti-inflammatory activity %= [1-(paw volume of treated group at time n - paw volume of treated group control at time 0)/(paw volume of control group at time n - paw volume of control group at time 0] ×100.

Statistical analyses

The experiments of the *in vitro* study were done in triplicates and all data were shown as mean±standard error of the mean (SD). The IC_{50} (50% inhibition concentration) and A0.50 (the concentration indicating 0.50 absorbance) values are calculated by the linear regression method.

The data were recorded as mean±SD in *in vitro* study, while as mean±SEM in *in vivo* study. The Graphpad Prism 7 was used to analyzed the data. The statistical differences between the experimental groups were analyzed using two-way analysis of variance (ANOVA) followed by a Tukey post-test. The differences are considered statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

Extraction, TPC and TFC

The TPC and TFC of the different extracts were analyzed and presented in **Table 1**. As shown, the results indicate that the M.EAE exhibited higher TPC and TFC comparatively to the other extract, which were about 462.23±11.74 µg GAE/mg E and 125.90±0.16 µg QE/mg E, respectively. However, the C.DME had the lowest TPC and TFC, with 29.2±1.11 µg GAE/mg E and 3.98±0.08 µg QE/mg E, respectively.

Extraction is the main step for recovering and isolating phytochemicals from plant materials, extraction yield can be affected by the chemical nature of phytochemicals, the method used, the solvent used, as well as the presence of interfering substances [21].

The difference in the polyphenol and flavonoid content of the crude extracts and their fractions results from the difference in polarity of the organic solvents, the extraction time and temperature, the solid-liquid extraction ratio as well as the chemical and the physical characteristics of the samples [22]. By comparing the results obtained, we note that the *L. multifida* plant from the Msila region contains more polyphenols and flavonoids than that of Constantine region with an amount up to 462.23±11.74 µg EAG/mg extract of polyphenols and (125.90±0.16 µg EQ/mg extract) of flavonoids in the case of EAE. This variability in the polyphenol and flavonoid contents of the two *L. multifida* plants is probably due to the phenolic composition of the extracts, the nature of the soil and the type of microclimate, and the bioclimatic stages

where this plant grows [23].

Other studies on the phytochemical hydromethanolic extracts of *L. multifida* collected from different regions of Morocco, demonstrated that the quantity of polyphenols and flavonoids of the plant from south-west of Morocco are 29.87±0.57 µg EAG/mg of extract and 5.51±0.19 µg EQ/mg of extract, respectively. In other hand, the extracts of the plant from the south of Morocco are less rich of polyphenols flavonoids (16.38 µg EAG/mg extract and 14.31 µg EQ/mg extract) [24; 25].

In vitro antioxidant activity

DPPH radical scavenging activity

In this study, the free radical scavenging activity of the different extracts of *L. multifida* harvested from two regions was evaluated by DPPH radical scavenging test. The obtained results showed that the EAE has the highest free radical scavenging activity for the two plants. Moreover, the C.EAE free radical scavenging activity compared to all extracts with an effective concentration (EC_{50}) (EC_{50}=12.32±0.82 µg/ml), this activity is two times lower than that of BHA (EC_{50}=5.73±0.41 µg/ml). While, the M.DME and C.DME showed the lowest activity with an EC_{50}>100 µg/ml (**Table 1**). Having analyzed the obtained results, *L. multififida* from the Msila region showed better free radical scavenging activity compared to that of Constantine region. The activity of extracts its poverty in polyphenols can be justified according to [26], by the fact that the inhibiting activity of the DPPH radical is not dependent on the total content polyphenols but polyphenols which have specific chemical structures. In other words, by the high selectivity of DPPH because it only reacts with flavonoids containing hydroxyl groups in the B ring, as well as aromatic acids containing more than one hydroxyl group [27].

The study of the antioxidant activity of the hydromethanolic extract of the *L. multifida* plant collected from different regions of Morocco has found a percentage of inhibition of the order of (74.10±0.14%) in the south-west of the country, while in the south of the country this extract gave a percentage inhibition equal to 90.35% [11; 12]. For their part, Messaoud *et al*., (2012) [28] found a great capacity of methanolic extract of Tunisian *L. multififida* to scavenge the DPPH radical with an EC_{50} value of 19.3±1.2 µg/ml.

Galvinoxyl radical scavenging capacity (GOR)

The galvinoxyl radical scavenging activity of extracts was determined, and the results were expressed as $EC_{50.}$ However, the results showed that the M.EAE

exhibited the highest activity (EC_{50}=9.60±0.06 µg/ml), this result is eight times greater than the activity of C.EAE (EC_{50} =72.04±4.33 µg/ml) and three times lower than that of BHT (EC_{50}=3.32±0.18 µg/ml) (**Table 1**).

From these results, it can be said that the *L. multifida* plant from the Msila region showed a good scavenging activity for the Galvinoxyl radical compared to that from the Constantine region. This variability in extract activity is due to the types of polyphenols contained in the extract. Indeed, it is well known that the hydrogen-donating antioxidant reacts quickly with the Galvinoxyl radical. The latter can be used to measure the stoichiometric number of phenolic hydrogens in an antioxidant. So, this method can be used for the determination and comparison of the antioxidant activity of hydrogen-donating compounds, whether in pure substances or in mixtures [29]. This study is the first in evaluation of the scavenging activity of the Galvinoxyl radical of the *L. multifida* plant. On the other hand, a study on the methanolic extract of *L. stoechas* from Algeria, a species of the same genus as *L. multififida*, gave a scavenging activity of the Galvinoxyl radical equal to (227 µg of Trolox equivalent/mg of extract) [30].

ABTS radical cation scavenging activity

The antiradical activity of extracts was measured using ABTS scavenging assay. In this test, the antioxidant reacts with $ABTS^{\bullet+}$ blue/green in color by electron transfer to restore the colorless $ABTS^{\bullet+}$. This transformation was followed by measuring the absorbance and determining the EC_{50} of different extracts in comparison with the BHA and BHT standards. The results obtained were showed that the M.EAE exhibited the highest antiradical activity (EC_{50}=4.89±0.20 µg/ml), this activity was five times lower than the BHA standards (EC_{50}= 1.03±0.01 µg/ml) and three times than the BHT (EC_{50}=1.59±0.03 µg/ml) (**Table 1**). These results of the free radical scavenging activity of the *L. multifida* plant from the Msila region confirm the strong antioxidant capacity of these extracts compared to those of *L. multifida* from the Constantine region. In this test, the extracts showed a better free radical scavenging effect than in the DPPH test. This difference can be justified by the ability of the cation radical ABTS to be more versatile than DPPH, which is soluble in water and organic solvents and allows an evaluation of free radical scavenging activity for hydrophilic and lipophilic compounds [31]. Our study is the first carried out using the ABTS method to evaluate the free radical scavenging of *L. multifida* extracts plant. However, a study on the essential oils of three plants of *L. stoechas*, a species of the same genus as *L. multifida*, collected in different regions of Spain revealed a scavenging activity ranging between 175.3±3.3 and 14.8±0.6 µmol Trolox equivalent/ml of essential oils against ABTS radical [32]. In addition, Amira *et*

al. (2012) [30] noted that the methanolic extract of *L. stoechas* from Algeria gave a scavenging activity equal to 457 µg of Trolox equivalent/mg of extract. In study released by Nikolic *et al.* (2019) [33] on the hydromethanolic extract of *L. angififolia* from Serbia gave a scavenging activity equal to 2.54±0.2 µg of Trolox equivalent/mg of extract.

Reduction activity of the copper-neocuproin complex (CUPRAC)

The reducing activity of extracts was determined using CUPRAC method, which used to measure the ability of antioxidant to reduce ferric Fe (III) and cupric Cu (II) ions to their respective lower valency state [34]. The results showed that the M.EAE has an excellent reducing activity ($A_{0.5}$ =5.8±0.50 µg/ml), compared to the BHA ($A_{0.5}$= 3.6±0.19 µg/ml).

The reducing activity of M.EAE was eight times stronger than the activity of C.EAE ($A_{0.5}$ =49.65±5.42 µg/ml) (**Table 1**). On the other hand, the extracts M.DME, C.CE and C.DME showed a low reduction activity ($A_{0.50}$> 100 µg/ml). This study allowed us to confirm the high activity of the *L. multifida* plant from the Msila region in which the EAE gave an excellent reduction of the copper-neocuproin complex compared to BHA standard and compared to extracts of *L. multififida* from the Constantine region. The difference in the reducing activity of the extracts may be due to several criteria, which polyphenols have such as the number and the position of hydroxyl groups as well as the degree of conjugation of the entire molecule (double bond) [35]. The carbonyl group in position 4 (oxo) and the ortho-dihydroxy structure on the B ring (catechol group) which are important for the easy transfer of electrons [36]. The evaluation of the antioxidant activity by CUPRAC method is made for the first time for *L. multifida* plant. A study on the hydromethanolic extract of *L. angififolia* from Serbia, a species of the same genus as *L. multifida* gave a reduction activity of the copper-neocuproin complex equal to 0.07±0.00 mg of Trolox equivalent/g of extract [33]. Another study on the methanolic extract of *L. stoechas* from Turkey gave a reduction activity of the copper-neocuproin complex equal to 369.66±6.73 mg of ascorbic acid equivalent/g of extract [37].

Ferric reducing/antioxidant power (FRAP)

The reducing power of the extracts was determined. The results obtained showed that the M.EAE have the strongest reducing capacity with values 1181.50±8.64 µg AA equ/mg E. Then, this activity was followed by the activity of M.BUE with 626.27±7.29 µg AA equ/mg E (**Table 1**). While the extracts M.DME, C.CE, C.DME and C.BUE showed the lowest reducing power compared to the other extracts with an activity lower than 50 µg AA equ/mg E. For this activity, the

presence of reducers compounds in plant extracts makes it possible to reduce the Fe^{+3} to Fe^{+2}. The reducing power of plant extracts was dependent on their concentration, where polyphenol-rich extracts have a higher reducing power [38; 39]. The reducing power of *L. multifida* extracts (Msila) is probably due to the presence of phenolic compounds containing hydroxyl groups which can serve as electron donor, which can react with free radicals and convert them into more stable products, thus putting an end to chain reactions of free radicals [40; 41]. The study carried out by Ramchoun *et al.* (2009) [42], on the aqueous extract of *L. multififida*, harvested in Morocco recorded a reducing power with a value of (12.76±0.48 mmol of Trolox equivalent/g of extract), this activity is low compared to our results. In addition, the work carried out by Amri *et al.* (2015) [24] on the hydromethanolic extract of the *L. multifida* collected from different regions of Morocco gave the value of (325.65±3.20 µmol/g extract), this value is similar to our results.

Reducing activity using Fe(II)-phenanthroline complex

According to the **Table 1**, the results demonstrated that the extracts; M.EAE, M.CE and M.BUE showed the greatest reducing power ($A_{0.5}$=10.92±3.31; 13.85±1.16 and 15.91±2.11 µg/ml; respectively). However, the two standards, BHA and BHT, have the strongest reducing activities ($A_{0.50}$=0.93±0.07 and 2.24±0.17 µg/ml, respectively). In addition, the extracts of *L. multifida* from Constantine region showed the lowest reducing power compared to the extracts of *L. multifida* from Msila region. In this activity, the presence of reducers in the plant extracts makes it possible to reduce Fe^{+3} to Fe^{+2}. Consequently, the latter forms a stable complex with phenanthroline, which is orange-red in color. However, to the best of our knowledge, there was no reference on application of the reaction between ferrous ions and 1,10-phenanthroline for determination of antioxidant capacity of edible oils and the other foodstuffs. Only, Berker *et al.* (2010) [43] used 1,10-phenanthroline method for assay of antioxidant capacities of different antioxidants and their mixtures. Besides that, Phen method was applied for measuring the total antioxidant capacity of plasma, pleural effusion and antioxidants defense system.

This difference in the reducing power may be due to the reducing capacity of polyphenols as antioxidants which depends on the degree of hydroxylation and the degree of conjugation of phenolic compounds [44]. Specifically for flavonoids, it has been suggested that the capacity for eliminating free radicals increases when the following conditions are present: the presence of a 3′, 4′-dihydroxy structure in the B cycle, the presence of a double bond (C2-C3) in conjunction with the 4-oxo group in the heterocycle and the presence of 3- and 5-hydroxyl

groups in ring A with a 4-oxo function in rings A and C [45]. Our study of the iron reduction activity by the formation of the Fe^{+2}-phenantroline complex was the first carried out on the *L. multifida* plant. A study on the extract of *L. pedunculata* collected from the south of Portugal, gave percentages of the Fe^{+2}-phenantroline complex of the order of 5.9±1.27% for the aqueous extract and 50.1±0.14% for the hydroethanolic extract. The activity is low compared to our results [46].

Metal ion chelation activity

Bivalent ferrous ions play an important role as catalysts of oxidative processes, leading to the formation of superoxide anion radicals and hydroxyl radicals via Fenton reactions. It was reported that the generated free radicals would cause the production of oxyradicals, lipid peroxidation and DNA damage [47]. These processes can be delayed by iron chelation or an antioxidant. In this assay, ferrozine can quantitatively form complexes with Fe^{+2}. In the presence of other chelating agents or antioxidants, the complex formation is disrupted with the result that the purple color of the complexes decreases. Monitoring this activity by determining the EC_{50} values and in comparison, with the EDTA standard. As shown in **Table 1**, all the extracts of *L. multifida* from Msila and Constantine showed a very low chelating power (EC_{50}> 800 µg/ml) compared to that of the EDTA standard (EC_{50}=8.80±0.47 µg/ml). However, the weak chelation activity of *L. multififida* extracts despite the great richness of that harvested from Msila in polyphenols can be justified according to [48], by the fact that the chelation capacity is not dependent on the total polyphenol content but on the type of polyphenols having hydroxyl groups in the vicinity or in the ortho-position of the benzene ring.

Among the phenolic compounds which have a good chelating capacity are the flavonoids, specifically those containing a ring B catechol nucleus, 3-hydroxyl and 4-oxo groups of the C ring, and 4-oxo and 5-hydroxyl groups between cycles A and C [49; 50]. A study carried out by Messaoud *et al.*, (2012) [28], showed that the methanolic extract of *L. multifida* exerted a capacity to chelate iron of EC_{50} of the order of (0.8±0.1 mg/ml).

Table 1. Total phenolic and flavonoids content and antioxidant activity of *L. multifida* extracts

	L. multifida from Msila region				*L. multifida* from Constantine region				Reference
	M.CE	M.DME	M.EAE	M.BUE	C.CE	C.DME	C.EAE	C.BUE	
TPC (µg GAE/ mg E)	204.29±6.57	52.72±7.94	462.23±11.74	291.94±11.22	60.66±26.05	29.2±1.11	178.21±47.45	43.60±13.45	
TFC (µg QE/mg E)	50.31±3.39	21.58±1.30	125.90±0.16	87.17±4.97	33.61±5.74	3.98±0.08	34.16±3.83	32.22±0.44	
Antioxidant assays									
DPPH (EC_{50} µg/mL	21.29±0.50	>100	16.46±0.35	34.73±0.38	42.74±1.22	>100	12.32±0.82	43.48±3.36	
BHA									5.73±0.41
Galvinoxyl (EC_{50} µg/mL)	14.37±0.29	>100	9.60±0.06	12.02±0.06	34.65±0.58	>100	72.04±4.33	36.29±2.34	
BHT									3.32±0.18
BHA									5.38 ±0.06
ABTS (EC_{50} µg/mL)	13.80±0.33	>100	4.89±0.20	9.88±0.60	33.71±0.24	>100	9.28±0.20	28.59±2.33	
BHT									1.59±0.03
BHA									1.03±0.01
CUPRAC ($A_{0.5}$, µg/mL)	16.59±0.26	>100	5.87±0.50	11.16±1.03	>100	>100	49.65±5.42	55.48±1.88	
BHT									9.62±0.87
BHA									3.64±0.19
RP (µg AA equ/ mg E)	356.50±9.64	<50	1181.50 ±8.64	626.27±7.29	<50	<50	77.75±5.97	<50	
RP phenanthroline ($A_{0.5}$ µg/mL)	13.85±1.16	>100	10.92±3.31	15.91±2.11	>100	>200	26.82±1.82	85.95±4.28	
BHT									0.93±0.07
BHA									2.24±0.17
Metal chelation (EC_{50} µg/mL)	>800	>800	>800	>800	>800	>800	>800	>800	
EDTA									8.80±0.47

RP: Reducing power; AA: Ascorbic acid; E: Extract

Enzymes inhibitory activity

Alpha-amylase inhibition

The anti-diabetic activity of extracts of *L. multififida* from the two regions was evaluated by measuring their capacities to inhibit the alpha-amylase enzyme.

In this study, the inhibitory activity of extracts on the alpha-amylase was estumated by determining the inhibitory concentrations IC_{50} compared to the standard acarbose based on the ability of a substance to inhibit this enzyme.

From the results obtained (**Table 2**), it was noted that the alpha-amylase inhibitory activity has been recorded only at the level of the M.CE with an (IC_{50}=64.17±1.81 µg/ml). This activity is fifty-six times higher than that of the standard acarbose (IC_{50}=3650.93±10.70 µg/ml), the latter reacts *in vivo* better than *in vitro*. By the comparison, we can see that *L. multififida* from the Msila region showed excellent anti-diabetic activity compared to that from the Constantine region. This difference in alpha-amylase inhibitory activity can be justified by the presence in the M.CE of a class other than flavonoids

because the latter only account for 24.62% of the total polyphenol content. This class was probably tannins, one of the main classes of phenolic compounds characterized by the presence of non-specific inhibitory molecules of various hydrolytic enzymes such as α-amylases, α-glucosidases and lipases. This inhibition is perhaps associated with their ability to bind strongly to proteins and carbohydrates whose interaction between tannins and proteins is the result of multiple hydrogen bonds and hydrophobic associations. As a result of this interaction, the catalytic sites of enzymes are blocked and their activity is therefore inhibited [51]. The inhibitory activity of the alpha-amylase enzyme of the *L. multifida* plant is the first carried out on this species and its genus. In addition, a study on the aqueous extract of *Ocimum basilicum* from Saudi Arabia (a species of the Lamiaceae family) gave an alpha-amylase inhibiting activity equal to 42.50 mg/ml [52], this result is similar to our result.

BuChE inhibitory activity

Butyrylcholinesterase (BuChE) is an enzyme which has been shown to be involved in the patho-genesis, treatment and prognosis of Alzheimer's disease [53]. The BuChE inhibition activity of the various extracts of the *L. multifida* plant was determined according to the method of [54]. Ellman's test was based on the cleavage of butyrylthiocholine by the BChE to produce thiocholine. The latter will react with 5,5'-dithiobisnitrobenzoate (DTNB) to form a yellow anion. In the presence of an enzyme inhibitor the yellow color will decrease, which makes it possible to evaluate the reaction, and subsequently to quantify the inhibition of the enzyme. This inhibition was followed spectrophotometrically by the measurement of the absorbance at 412 nm, to determine the inhibitory concentration (IC_{50}) of the different extracts in comparison with the standard galantamine.

In this study, it was shown that the C.CE exhibited the strongest inhibitory activity (IC_{50}=83.55±1.97 µg/ml), when the BuChE inhibitory activities of the extracts were compared among themselves. The activity of C.CE is twice lower than that of the standard galantamine (IC_{50}=34.75±1.99 µg/ml). In contrast, the C.DME slightly inhibited the activity of BuChE (IC_{50}=152.44±0.63 µg/ml). While, the extracts M.CE, M.DME, M.EAE, M.BUE and C.EAE have shown a weak inhibitory activity against BuChE (IC_{50}>200 µg/ml) and they are far from being compared to galantamine (**Table 2**). However, C.BUE was inactive for the different concentrations. From these results, it can be said that *L. multififida* from the Constantine region exhibited a moderate activity of inhibition of BuChE compared to that of the Msila region.

Table 2. Inhibition of α-amylase and BuChE by extracts. The results were presented as IC_{50} values in μg/mL.

Sample		α-amylase inhibition	BuChE inhibition
L. multifida from Msila	M.CE	64.17±1.81	>200
	M.DME	>400	>200
	M.EAE	>400	>200
	M.BUE	>400	>200
L. multifida from Constantine	C.CE	>25	83.55±1.97
	C.DME	>25	152.44±0.63
	C.EAE	>25	>200
	C.BUE	>25	Inactive
References	Acarbose	3650.93±10.70	/
	Galantamine	/	34.75±1.99

The BuChE inhibitory activity by the *L. multifida* plant may be due to the presence of polyphenols. It is also due to alkaloids, terpenes, and coumarins, which are compounds with anticholinesterase properties [55]. A study on three extracts of *L. viridi* from Portugal, gave percentages of inhibition of BuChE of around 32.34±3.03%, 63.01±1.84%, and 51.19±1.52% for the aqueous extract, the hydroethanolic extract and the ethanolic extract respectively [56], these results are similar to those recorded in our study. In the present study, fraction method and methanol solvent were the most suitable solvent and method to get the strongest anticholinesterase activity. To the best of our knowledge, there have been no reports in literature on the anticholinesterase activity for this species. Therefore, in this study, the anticholinesterase activity of plant's extracts was examined for the first time.

In vivo anti-inflammatory activity

According to the results obtained, which represents the evolution of the edema after the intraperitoneal injection of formaldehyde, the inflammation was more pronounced in the control group who not received the treatment.

Moreover, the results showed that, the administration of aqueous extract of *L. multififida* (200 mg/kg) prevented formaldehyde-induced paw edema in rats, with an anti-inflammatory activity of 10.02%, 21.83%, 37.20%, and 43.71% at 30, 60, 180 and 360 min, respectively. While the Diclofenac showed a good anti-inflammatory effect, and the activity was as follows: 27.22%, 36.83%, 53.26%, and 80.28% at 30, 60, 180 and 360 min, respectively (**Figure 2**).

This moderate capacity of the aqueous extract of *L. multififida* to inhibit edema and therefore to inhibit the synthesis of pro-inflammatory substances such as cytokines and prostaglandins can be justified by the low content of our extract in flavonoids, the latter have a capacity to inhibit cyclooxygenase and therefore causing inhibition of inflammation [57].

Formaldehyde-induced paw edema model is a suitable experimental animal model for evaluating or screening the anti-inflammatory effects from natural products. We can also observe two intervals of evolution of the edema explained by the two-phase nature of the inflammatory response by formaldehyde, the first phase results mainly from the concomitant release of inflammation mediators such as serotonin, histamine and kinin. The second phase is characterized by the release of prostaglandins produced by macrophages [58].

In study of anti-inflammatory activity of *L. multifida* collected from southern of Morocco, showed that the ethanolic extract has an edema reduction capacity of up to 62%, while the aqueous extract has shown a weak anti-inflammatory activity with an edema reduction capacity equal to 33% in mice [47; 59].

It has been said that presence of certain flavonoids exerts profound anti-inflammatory activity by stabilizing the lysosomal membrane [60]. The outcome of our study of and from the previous database on this plant, it can be predictable that the anti-inflammatory effect exerted is because of flavonoid content. Sometimes it happens that the crude plant extracts are extra pharmacologically lively than their isolated active compounds [61]. The targeted mechanism of action for the anti-inflammatory activity of studied specimen just is not identified, but the extract may be intercepting the construction of inflammatory mediators dependable for inflammation, either COX pathway or different specific enzymatic mechanism.

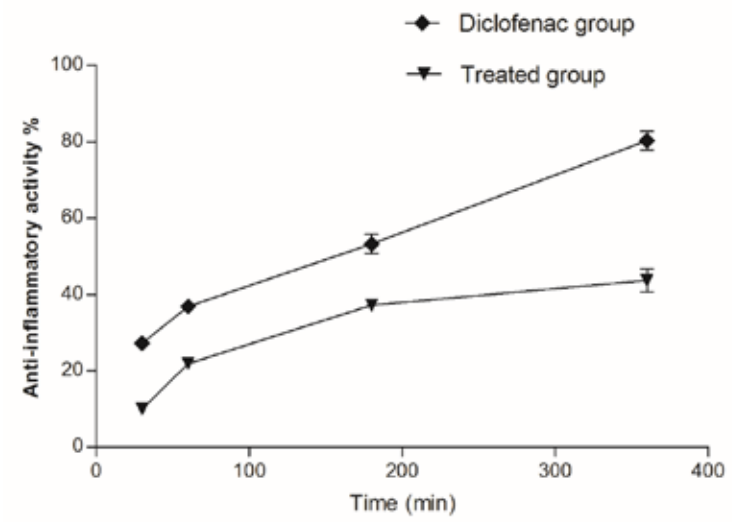

Figure 2: Anti-inflammatorty activity of the aqueous extract of *L. multifida* from Msila

Herbal remedies have been used therapeutically for thousands of years to naturally treat a variety of diseases. This property has been confirmed by modern scientific research, which has ensured the effectiveness of these plants thanks to their richness in secondary metabolites which give them diverse biological properties. In this context, in this study, we have extracted and phytochemical analyzed the phenolic components of the medicinal plant *L. multifida* from two different regions. When different extracts were obtained, we examined the *in-vitro* antioxidant, enzymes inhibition and anti-inflammatory effect the extracts. We examined the effect of the region and extraction methods on the biological activity. In addition, the evaluation of the biological activities of the *L. multifida* from Msila region showed an interesting *in-vitro* antioxidant potential, a remarkable *in-vitro* anti-diabetic activity, and moderate *in-vivo* anti-inflammatory activity and a weak anticholinesterase activity. This biological activity will be probably linked to the richness of the species, in particular that of Msila, in secondary metabolites, in particular in flavonoids.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication and dissemination of the information provided here in.

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