Phenolic composition, in vitro antioxidant effects and tyrosinase inhibitory activity of three Algerian Mentha species: M. spicata (L.), M. pulegium (L.) and M. rotundifolia (L.) Huds (Lamiaceae)

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Abstract

The leaves of three *Mentha* species harvested in Algeria, *M. spicata* L. (MS), *M. pulegium* L. (MP) and *M. rotundifolia* (L.) Huds (MR) were examined for their content in polyphenols and for some activities-linked biological properties these could impart. The contents in total phenolics (TPC) and flavonoids (TFC) were evaluated by the Folin-Ciocalteu and the aluminium chloride methods, respectively. Whereas MS showed the highest TPC (12.0 ± 0.3 mg gallic acid equivalents/g of dry weight), MR had the highest content in TFC (3.3 ± 0.1 mg quercetin equivalents of dry weight). The pharmacological properties of these extracts were evaluated by assessing *in vitro* their antioxidant and antityrosinase activities. The modulation of mushroom tyrosinase activity was measured by colorimetry of the melanins formed in the presence of tyrosine. MS exhibited the strongest radical scavenging activity (RSA) in all assays: *(i)* the IC_{50s} values to neutralize the 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) cation radicals ($ABTS^{\bullet+}$) and the 2,2'-diphenyl-1-picrylhydrazyl radicals ($DPPH^{\bullet}$) were 10.3 ± 0.9 and 16.2 ± 0.2 µg/mL, respectively; and *(ii)* its original electrochemically measured superoxide quenching index value is 188 ± 37 µg/mL (AI_{50}). MR however showed the highest tyrosinase inhibitory activity (IC_{50} = 108 ± 20 µg/mL). A silica gel thin-layer chromatography (TLC) technique revealed the presence of caffeic and rosmarinic acids and diosmin in all extracts. These results were confirmed by high performance liquid chromatography with diode array detection (HPLC/DAD).

Keywords: *Mentha* ssp, antioxidant activities; tyrosinase; phenolic compounds; TLC/HPLC.

1. Introduction

Aromatic plants produce active compounds (phytochemicals having a pharmacological effect on living organisms) that consist partially of essential oils (Curutchet et al., 2014). Some aromatic plants also contain polyphenols, compounds that have shown special interest over the last few years, due to the great variety of biological properties most of them display, notably for anti-carcinogenic, anti-inflammatory, antiviral, anti-allergic, estrogenic, and immune-stimulating effects (Atoui et al., 2005). From these, flavonoids have been extensively investigated for their possible roles in cancer chemoprevention (Charles et al., 2012). Many of these activities have been associated with the metal chelation and redox properties of polyphenols, which allow them to act as antioxidants, hydrogen donators, and reactive oxygen species quenchers (Kaisoon et al., 2011).

An antioxidant activity may also be essential in inhibiting tyrosinase (EC 1.14.18.1), an enzyme involved in melanin production. Alterations in melanin production might be responsible for a part of the pathological features unique to malignant melanoma (Prasad et al., 2010) and melanin may be part of the high resistance of melanoma to chemo- and radiotherapy (Farmer et al., 2003). Therefore, tyrosinase inhibitors may be clinically useful for the treatment of skin cancer (Jimbow et al., 1993). Also, recently, more attention is being paid to the use of natural plant extracts in the cosmetic industry as tyrosinase inhibitors for active depigmentation (Prasad et al., 2010); insecticide properties have also been associated with inhibition of insect tyrosinase (El Nagar et al., 2012).

The *Mentha* genus belongs to the subfamily *Nepetoideae* of *Menthae* tribe in the Lamiaceae family (Lawrence, 2007). It includes aromatic herbs of difficult taxonomic classification, due to a great variability in morphological characters and frequent hybridizations that occur, both in wild and cultivated populations. According to the latest taxonomic treatment, the genus *Mentha* comprises 61 species (Kew, 2010) placed into the four sections *Pulegium, Tubulosae, Eriodontes*, and *Mentha* (Saric-Kundalic et al., 2009).

This genus is one of the important members of the Lamiaceae family, represented in the flora of Algeria by 5 major species [*M. rotundifolia* (L.) Huds., *M. pulegium* L., *M. spicata* L., *M. aquatica* L., and *M. longifolia* (L.) L.] (Quezel and Santa, 1962).

M. spicata L., commonly known in the Algerian systems of medicine as ‘‘naana’’, has many culinary and medicinal uses in Maghreb. It is popularly consumed in the form of tea and added to several preparations as a flavor enhancer; the dry or fresh leaf of spearmint is added specially during the brewing of tea. Biliary disorders, menstrual cramps, stomach pain, constipation, gingivitis and ondotalgies are treated with the decoction of spearmint leaves (Brahmi et al., 2012). *M. pulegium* L., known in Algeria as ‘‘feliou’’, is used for the treatment of flatulent dyspepsia and intestinal colic due to its carminative and antispasmodic properties (Dellile, 2007). *M. rotundifolia* grows wildly in Algeria, is locally known as "timija", and used mostly as a condiment but also for medicinal properties, exhibiting tonic, stimulative, stomachic, antiemetic, carminative, choleretic, antispasmodic, antidiarrheal**,** anti-haemorrhoidal, anti-inflammatory, sedative, hypotensive, analgesic and insecticidal activities **(**Ladjel et al., 2011).

All these properties, typical of *Mentha* species, have been ascribed to the combination of the essential oil, essentially based on monoterpenoids (Brada et al., 2007), and of polyphenol derivatives **(**Dorman et al., 2003; Zaidi et al., 1998).

While the beneficial health properties of some *Mentha* species such as *M. spicata* have been extensively studied, much less attention has been given so far to *M. rotundifolia*, especially regarding its polyphenols and antioxidant properties. Furthermore, the literature data on the biological activities of *Mentha* species is frequently scattered and, to our knowledge, there is only one work comparing the antioxidant activity of these three plants (Nickavar et al., 2008); no report is available so far on their antityrosinase ability.

Therefore, the present study aims at characterizing polar extracts of *M. spicata* L. (MS), *M. pulegium* L. (MP) and *M. rotundifolia* (L.) Huds (MR) collected from the North-Eastern Bejaia region of Algeria for their: *(i)* phenolic composition, in both quantitative (total phenolics content (TPC), total flavonoids content (TFC) and major phenolics content) and qualitative (silica gel thin-layer chromatography (TLC), high performance liquid chromatography with diode array detection (HPLC/DAD)) aspects; *(ii)* antioxidant properties [quenching of the radicals 2,2'-azinobis (3-ethylbenzothiazoline-6-sufonic acid ($ABTS^{\bullet+}$), 2,2'-diphenyl-1-picrylhydrazyl($DPPH^{\bullet}$) and electrochemically-generated superoxide($O_2^{\bullet-}$)]; and *(iii)* tyrosinase inhibitory activity.

2. Material and methods

2.1. Material

2.1.1. Herbal material

The fresh leaves and herbarium samples of *M. spicata* (MS), *M. pulegium* (MP) and *M. rotundifolia* (MR) were collected just before flowering from the region of Bejaia, Algeria from June to August 2009. The cultivated MS was harvested in Tichy (36°40'0"N, 5°10'0" E; voucher specimen, BR 0000006946227), the wild MP in Chemini (36°36'0"N, 4°37'0"E; BR 0000006946043) and the wild MR in Smaoun (36°37'0"N, 4°48'0"E; BR 0000006946197). The collected plant material was dried at room temperature under shade and powdered to 250 mesh. The herbarium samples were authenticated by Professor J. Lejoly in the Laboratory of Systematical Botany and Phytosociology, Free University of Brussels (ULB), Belgium. Voucher specimens were deposited in the Herbarium of the National Botanical Garden of Meise (Belgium).

2.1.2. Chemicals

Standards. Gallic acid was purchased from BDH, Prolabo (Linars del Vallés, Spain); rosmarinic acid, 4-hydroxybenzoic acid and kaempferol from Carl Roth (Karlsruhe, Germany); *p*-coumaric acid, chlorogenic acid, diosmin, luteolin and naringenin from Sigma-Aldrich (St. Louis, USA); caffeic acid from Janssen Chimica (Beerse, Belgium); ferulic acid and hesperidin from Fluka (Buchs, Switzerland); rutin and naringin from Alfa Aesar (Ward Hill, MA). All standards were prepared as stock solutions in methanol (1 mg/mL).

The mushroom tyrosinase (MT; 5.370U/mg), L-tyrosine, L-3,4-dihydroxyphenylalanine (L-DOPA) and kojic acid, were purchased from Sigma-Aldrich (St. Louis, USA); ABTS and $DPPH^{\bullet}$ from Fluka Biochemika (Buchs, Switzerland); Folin-Ciocalteus's phenol reagent and sodium carbonate from Biochem Chemopharma (Montreal, Quebec); N,N-dimethylformamide extra dry ([H_2O]≤0.005%, stored over molecular sieve 3Å) and tetrabutylammonium hexafluorophosphate of electrochemical grade from Fluka Chemie (Buchs, Switzerland); Trolox®[(±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) from Acros Organics (Morris Plains, NJ, USA). All other solvents or chemicals used in the study were of analytical grade.

2.2. Preparation of plant extracts

The leaves powder (25 g) was defatted by stirring with 600 mL n-hexane (room t°, 130 rpm, 3 h), dried (RT, 2 h) and then extracted with 400 mL ethanol (room t°, 130 rpm, 24 h). The crude extract was filtered on cellulose, concentrated with a rotary vacuum evaporator (40°C), lyophilized and maintained in the dark at +4 °C until tested (Max. 90 days). The final amount of dry ethanolic extracts of MS, MP and MR were 3.14 g (15.7%), 0.12 g (2.5%), and 1.43 g (7.1%), respectively (Table 1).

2.3. Determination of total phenolics and flavonoids content

The total phenolics content of the extracts was determined spectrophotometrically according to the Folin-Ciocalteu colorimetric method (Dorman et al., 2003). One hundred µL of extract (1mg/mL) or of gallic acid (1 to 10 µg/mL) were transferred to a 10.0 mL volumetric flask containing 6.0 mL of water, to which were subsequently added 500 µL of undiluted Folin-Ciocalteu reagent; after 1 min, 1.5 mL of 20% aqueous Na_2CO_3 were added, and the flask was brought to volume with water. After 2 h incubation at 25°C, the absorbance was measured at 760 nm versus a blank prepared without extract. The total phenolics content was expressed as mg gallic acid equivalents (GAE)/g dry weight using the following equation based on the calibration curve: $y = 0.1116x+0.0118$ ($R^2 = 0.998$), where x was the absorbance and y the gallic acid concentration (µg/mL). Data presented are the average of three independent measurements, expressed in dry weights of leaves.

The flavonoids content in the extracts was determined spectrophotometrically using the method reported by of Brahmi et al. (2012) based on the formation of a complex flavonoid-aluminum. An amount of 1.5 mL of a 2% $AlCl_3$ ethanol solution was added to 1.5 mL of extract (250µg/mL) or of quercetin (5 to 25 µg/mL). After 15 min at RT in the dark, the absorbance was measured at 430 nm versus a blank prepared without extract. The total flavonoids content was calculated as quercetin (mg/g) equivalents using the following equation based on the calibration curve: $y = 0.0552x+0.0019$ ($R^2 = 0.999$) where x was the absorbance and y was the quercetin concentration (µg/mL). Data presented are the average of three independent measurements, expressed in dry weights of leaves.

2.4. Determination of antioxidant activity

2.4.1. DPPH$^{\bullet}$ radical scavenging assay

The DPPH$^{\bullet}$ radical-scavenging activity was determined using the method proposed by Blois (1958). A stock solution of DPPH$^{\bullet}$ (1.10^{-3} M) in pure ethanol (96%) was prepared fresh

daily. One mL of the extracts (10 to 80 µg/mL) was added to 1.0 mL of ethanol and 500 µL of $DPPH^{\bullet}$ solution, maintained in the dark at room temperature for 1h and the absorbance was measured at 517 nm versus a blank prepared without extract. Gallic acid (0.5 to 1.5 µg/mL), Trolox® (2.0 to 8.0 µg/mL) and quercetin (0.5 to 2.5 µg/mL) standards were used as positive references. The percentage inhibition of the $DPPH^{\bullet}$ radical by the samples was calculated by the following formula:

$$DPPH\ scavenging\ effect\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Extract concentration providing 50% inhibition (IC_{50}) was obtained by plotting the inhibition percentage against extract solution concentrations. All determinations were performed in triplicate.

2.4.2. Trolox® equivalent antioxidant capacity (TEAC) assay

The TEAC determination is based on the ability to scavenge the stable radical cation $ABTS^{\bullet+}$ (Re et al., 1999). $ABTS^{\bullet+}$ was daily produced by incubating ABTS 7 mM with 2.45 mM potassium persulfate in the dark at RT for 12-16 h; the solution was then diluted to obtain an absorbance of 0.70 ± 0.02 at 734 nm. Ten microliters of extracts (10-60 µg/mL) or of Trolox® (5 to 20 µM) were added to 990 µL of $ABTS^{\bullet+}$ solution and the decrease in absorbance at 734 nm was monitored for 30 min. Inhibition free radical $ABTS^{\bullet+}$ in percent was determined using the same equation previously used for the DPPH method. The TEAC of gallic acid (2 to 6 mM) and quercetin (5 to 10 mM) were also measured for comparison. TEAC were calculated from the concentration response curves obtained with Trolox®. All determinations were performed in triplicate.

2.4.3. Electrochemical measurement of antioxidant capacity

2.4.3.1. Electrochemical material

Cylic voltammetry experiments were performed on dual potentio-galvanostat PGSTAT 100 (Autolab instrument, Eco Chemie B.V., Utrecht, The Netherlands). All measurements were carried out on a three-electrode thermostated cell. A glassy carbon disk working electrode (diameter 2 mm), a platinum wire counter electrode and a reference electrode, Ag/AgCl in EtOH saturated by LiCl, were used for all electrochemical experiments. The reference electrode was separated from the solution by a salt bridge containing 0.5 M Bu_4NPF_6 in N, N-dimethylformamide (DMF). The glassy carbon disk working electrode was polished using silicon carbide 4000 paper with a laboPol-5 (Struers, Ballerup, Denmark), washed with distilled water and then dried. For all measurements, the temperature was maintained at 20±0.02 °C with a Julabo heating circulator MP-5 (Julabo, Seelbach, Germany).

2.4.3.2. Measurement of the antioxidant capacity

The methodology developed by Le Bourvellec et al. in 2008 is based on the reaction kinetics of the antioxidant substrate, either polyphenols or flavonoids, with the superoxide radical ($O_2^{\bullet -}$). A voltammetric technique was used to generate $O_2^{\bullet -}$ by reduction of molecular oxygen in an aprotic medium (N, N-dimethylformamide (DMF) extra dry). The reactivity of the radical $O_2^{\bullet -}$ was directly measured in cyclic voltammetry at the backward scan by the anodic current decay from its oxidation in the presence of extract.

A solution of 10 mL of an extra dry DMF containing the supporting electrolyte 0.1 M Bu_4NPF_6 was saturated by dry air during 10 minutes. In these conditions, the solubility of oxygen was assumed to be $C_{O_2} \approx 9.4 \times 10^{-4}$ mol L^{-1}, this value corresponding to a partial pressure of 0.2 bar at 20°C (Dapremont-avignon et al., 1991). The cyclic voltammogram (CV) of the oxygen reduction was then recorded at a scan rate 0.1 $V.s^{-1}$, with the initial potential at 0 V and the reverse one at -1.5 V *vs.* Ag/AgCl (potential corresponding to about 350 mV after the peak potential of O_2 reduction).

Aliquots of stock solutions (5 mg/mL) were successively added to the 10 mL oxygen solution in order to obtain an extract concentration in the range of [50 – 600 µg/mL]. CV of oxygen without phenolic compounds or extract is first recorded in order to get the initial cathodic and anodic peak currents. Then, successive aliquot additions are performed and CV recorded at a scan rate 0.1 V s^{-1} after each aliquot addition.

The measurement of the antioxidant activity is estimated by an antioxidant index value AI_{50}, defined as the concentration needed to consume respectively 50% of the electrogenerated superoxide anion radical (corresponding to $Ip_a^0 - Ip_a^S)/Ip_a^0 = 0.5$ where $Ip_a^0$and Ip_a^S are the intensities of the anodic peak current of $O_2^{\bullet-}$ respectively in absence and presence of a concentration of the polyphenolic sample).With this characterization, the lower the AI_{50} value, the more the substrate has a strong reactivity toward the superoxide anion radical.

2.5. Measurement of tyrosinase inhibition

Tyrosinase inhibition was determined as described (Tomita et al., 1990) with minor modifications (Kamagaju et al., 2013). In 96-well plates, 80 µL of base 2 logarithmic dilutions of a fresh solution of tested extracts (50, 100 and 500 µg/mL) or reference compound (kojic acid, 142 and 1421 µg/mL) were mixed with 80 µL of 4 mM L-dopa – 7 mM L-tyrosine solution [aq.] and 90 µL of mushroom tyrosinase (MT) (50 U/mL in PBS). Kojic acid was the positive control and the negative control was performed without plant extract. Plates were maintained in the dark at 37°C and absorbances were measured at 450 nm every 20 min over 180 min with a Multi-Mode Microplate Reader (Multiskan Ex., USA). Tyrosinase inhibition was calculated with the following equation:

$$\%\ inhibition = \frac{A_{sample} - A_{negat.control}}{A_{negat.control}} \times 100$$

The IC_{50s} were determined by fitting experimental data to a *"four parameters logistic"* curve (GraphPad Prism 3.0).

2.6. Profiling of phenolic compounds

2.6.1. TLC analysis

The qualitative analysis of investigated extracts was performed on Silica gel 60 F254 plates; (Merck, Germany), 0.25 mm, using ethyl acetate–formic acid–water (90:6:6, v/v/v) as a solvent system; plates were sprayed with a 1% MeOH solution of aminoethanol diphenylborate then a 5% MeOH solution of macrogol 400 and visualized under UV 365 nm (Wagner and Bladt, 1996). Extracts were analyzed before and after acidic hydrolysis (hydrolysis with HCl 1.2 M, 100°C, 1h).

2.6.2. HPLC analysis

High performance liquid chromatography with diode array detection (HPLC-DAD) allowed profiling the extracts and identifying major low-molecular weight polyphenols. The peaks were identified by comparison with authentic standards of retention times and UV spectra.

Chromatographic conditions were as follows: column, Alltima C18 5 µm (250 x 4.6 mm i.d.; Alltech, Deerfield, USA); mobile phase, gradient of acetonitrile, water, formic acid (19/80/1, v/v/v) (phase A) and acetonitrile, methanol, formic acid (59/40/1, v/v/v) (phase B) (0 min: 0% B; 5 min: 0% B; 15 min: 15% B; 20 min: 15% B; 40 min: 60% B, 45 min: 100% B); injection, 10 µL; flow rate, 1.2 mL/min; column t°, 40°C; detection, 280, 320 and 350 nm with scan 190 to 800 nm.

3. Results and discussion

3.1. Extract yield, total phenolics and flavonoids content

Table 1 lists the yields of ethanol extraction for the 3 *Mentha*; very similar weight extractives were obtained, in the range of 4.6%. The total phenolic contents (TPC) of the 3 Algerian mints are presented in Table 1. Whereas MS showed the highest total phenolics content (12.0 ± 0.3 mg GAE/g), MR presented the highest flavonoids content (3.3 ±0.1 mg QE/g). This relative poorness of MS in flavonoids is clearly confirmed by our TLC data (cf *infra*).

Literature data on the phenolics content of *Mentha* species is frequently scattered throughout the papers and the available data are often difficult to compare because of methodological differences. Mata et al. (2007), studying five plants used as Portuguese food spices, found that *M. spicata* contains more total phenolics than *M. pulegium*. However, Nickavar et al. (2008), who studied the total phenolics content of five *Mentha* species from Iran, found that *M. rotundifolia* had the highest TPC content and *M. spicata* the lowest content.

3.2. Antioxydant activity

In this work, three *in vitro* antioxidant tests were carried out, a $DPPH^{\bullet}$ radical scavenging assay, a Trolox® equivalent antioxidant capacity (TEAC) assay and a rapid electrochemical method for antioxidant capacity based on the reactivity of electrogenerated superoxide. This recently developed (Blanc et al., 2011; Le Bourvellec et al., 2008) technique is applied to mint extracts for the first time, comparing with conventional photometric antioxidant assays.

3.2.1. Scavenging activity towards $DPPH^{\bullet}$

The $DPPH^{\bullet}$ stable purple radicals react with suitable reducing agents (A–H), during which the electrons become paired off, yielding a stable diamagnetic molecule (yellow-

colored diphenyl picrylhydrazine); the solutions stoichiometrically loses color, depending on the number of electrons taken up (Blois, 1958; Mata et al., 2007).

The scavenging effect of tested extracts and reference standards on $DPPH^{\bullet}$ radical compare as follows: gallicacid > quercetin >Trolox® > MS > MP > MR (Table 2). A highly positive correlation was found between anti-$DPPH^{\bullet}$ activity [expressed as $1/IC_{50(DPPH^{\bullet})}$] and total phenolics content (R^2 = 1.00; $P < 0.05$). However, no similar relationship was observed between flavonoids content and $DPPH^{\bullet}$ scavenging, meaning that the quality of polyphenols is at least as important as their amounts.

Antioxidant studies with ethanol extracts have already been reported for the three *Mentha* species from different origins (Table 3). For species grown in Tehran province (Iran) during the flowering period in summer 2006, the sequence of activity is also in direct relationship with TPCs, confirming that phenolics are responsible for a significant part of their antioxidant and free radical scavenging effects (Nickavar et al., 2008). Similar relationships have also been observed by Dorman et al. (2003) on aqueous extracts.

3.2.2. Scavenging activity towards $ABTS^{\bullet+}$

As expected, an excellent correlation was observed between the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ results (R^2 = 0.96); this is due to the similarity of the two methods that both measure the ability of antioxidants to donate an H atom. Lower IC_{50s} values are observed for the $ABTS^{\bullet+}$ tests (Table 2); this may be due to differences in redox potentials, in reaction stoichiometry or/and in steric effect of the two radicals (Nickavar et al., 2008), depending on the extract's compounds involved.

The literature for $ABTS^{\bullet+}$ scavenging activity is rather scarce for *Mentha* species. In line with the present work, Arumugam et al. (2006) have shown that the antioxidant activities

against $ABTS^{\bullet+}$ of MS solvent fractions are closely related to their content in total phenolics. Kumar and Chattopadhyay (2007) measured, for methanolic extracts of MS from India, an $ABTS^{\bullet+}$quenching IC_{50} (11.9 µg/mL) close to the Algerian MS with a highly positive correlation between $1/IC_{50s(ABTS^{\bullet+})}$ values and total phenolics content ($R^2 > 0.98$, $P < 0.05$). Curiously, Nickavar et al. (2008) measured considerably higher IC_{50s} for $ABTS^{\bullet+}$ quenching than reported in the present study and found no correlation between TPC and $ABTS^{\bullet+}$ data. A detailed comparison of polyphenolics between Iranian and Algerian samples may help in understanding such conflicting data.

3.2.3. Scavenging activity towards electrochemically generated $O_2^{\bullet-}$

This assay measures the decrease of the oxidation peak current value of the $O_2^{\bullet-}$ which is produced electrochemically in cyclic voltammetry from the dissolved oxygen reduction. This recently developed electrochemical method has been applied successfully to seaweed extracts (Audibert et al., 2010; Blanc et al., 2011). Here, the method to determine the peak currents is different than in these previous works. Also the cyclic voltammograms were exploited by using convolution time semi-derivative transformation (Oldham and Spanier, 1973) for the peak current measurements. The resulted convolution curves are much better resolved compared to the asymmetric voltammetric curves. It is easier to measure the oxidation current before and after polyphenolic extract additions because the baselines were easier estimated (Figure 1). The measurement of the antioxidant activity by using the electrochemical method is estimated by an antioxidant index value AI_{50} (see 2.4.3 in the experimental part for details). Figure 1 (B) presents a typical determination of the antioxidant index values (AI_{50s}) for a MR extract.

For the standard polyphenols compounds (gallic acid, quercetin, Trolox®) there is a very good correlation between AI_{50s} values ($O_2^{\bullet-}$quenching) and IC_{50} values (DPPH and

$ABTS^{\bullet+}$). The antioxidant index values (AI_{50}) obtained for mint extracts correlate in their sequence with the IC_{50s} measured in the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays **(**Table 2). It appears that the electrochemical method gives similar results for antioxidant capacity measurement than the two spectrometric methods but with the advantage to be no disturbed by cloudy or colored extract solutions.

From the AI_{50s} values it appears that the antioxidant capacity is higher for MS extract and with the following sequence: MS > MP > MR. This is in accordance with their total phenolic contents (TPC values).

3.3. Tyrosinase inhibition

The antityrosinase activity was measured by a late end-point (pigmentation), corresponding to the formation of melanins (Kamagaju et al., 2013). The *Mentha* extracts potency (IC_{50s}) ranged in the sequence MR (108± 20 µg/mL) > MS (223±41 µg/mL) > MP (286±45 µg/mL), values to be compared to the IC_{50} measured for kojic acid, a reference potent tyrosinase inhibitor (23±1 µg/mL). These activities are in the lower range of those obtained by Kamagaju et al. (2013) who, in their study of five Rwandese herbal medicines traditionally used for skin treatment, measured, for raw extracts, IC_{50s} ranging from 0.8 to 285 µg/mL.

The inhibition of tyrosinase activity might be dependent *(i)* on the hydroxyl group of phenolic compounds that could form a hydrogen bond to a site of the enzyme, resulting in steric hindrance or modified conformation (Prasad et al., 2010); or *(ii)* on the scavenging of the free radicals implied in the tyrosinase reaction and in the formation of melanins.

MR is characterized by the predominance of flavonoids, the compounds that are the most abundant and documented plant polyphenols with such activities; some flavonoids

effectively inhibit the tyrosinase by active site chelation, while others act as cofactor and/or substrate of tyrosinase (Fu et al., 2005).

To the best of our knowledge there are no previous studies on the antityrosinase activity of the 3 *Mentha* species investigated in the present study. Previous works on *Mentha piperita* indicate that its essential oil also inhibits the mushroom tyrosinase (IC_{50}, 240 µg/mL) (Fiocco et al., 2011) and may cause inactivation or inhibition of the insect tyrosinase which results in incomplete cuticle hardening and darkening (El Nagar et al., 2012).

Fiocco et al. (2011) concluded that *Mentha* essential oils are promising herbal ingredients for developing depigmenting agents in clinical, cosmetic and industrial processes; our data indicate that total extracts (polyphenols) may be even more interesting ingredients.

3.4. Identification and quantification of polyphenolic compounds

There are many reports dealing with phenolic constituents of *Mentha* species (Table 4) that include cinnamic acids, aglycon, glycoside or acylated flavonoids, and steroidal glycosides (Dorman et al., 2003). Nevertheless, data about species of the Algerian flora are quite limited; moreover, the phenolic composition of MR has been rarely reported.

TLC and HPLC with DAD detector were combined to identify the phenolic profiles from Algerian *Mentha*, thereby reducing the possibility of misidentification (Sherma, 2000).

3.4.1. TLC characterization

After chromatographic separation and chemical derivation with the aminoethanol diphenylborate/macrogol reagent, flavonoids were detected as orange-yellow zones with

distinct yellowish-orange fluorescences in UV light at 365 nm, while phenolic acids showed bright blue fluorescence under the same conditions (Figure 2) (Wagner and Bladt, 1996).

The extracts of the 3 *Mentha* species showed TLC chromatograms characteristic for each species (Figure 2), with flavonoids predominating in MR and phenolic acids in MS. Caffeic acid (Rf 0.80) and high concentrations of rosmarinic acid (Rf 0.77) appeared in all extracts; rosmarinic acid was particularly abundant in the MS extracts. In our previous study, using a TLC densitometric quantitative method, the MS extract was found to contain significantly higher concentrations of rosmarinic acid compared to MP and MR (Brahmi et al., 2014). Concerning flavonoids we detected diosmin (Rf 0.43) especially in hydrolysed extracts. MR showed principally the presence of flavonoid glycosides, some of them described before (Zaidi et al., 1998), with naringenin (Rf 0.83), being the most abundant. MP presents both flavonoids and phenolic acids with characteristic spots at 0.63 and 0.83, which are not present in MS.

In the hydrolysed extracts, we noted the appearance of additional spots. The flavonoid glycosides in the MR and MP extracts were effectively hydrolysed but the resulting aglycones could not be identified. Phenolic acids were freed by acid hydrolysis; a significant amount of caffeic acid was released in all extracts by hydrolysis of phenolic derivatives. Other phenolic acids which do not correspond to the used reference compounds also appeared upon hydrolysis.

Many flavonoids (eriocitrin, luteolin glucoside, apigenin, acacetin, thymusin, thymonin, sideritoflavone and diosmetin) have previously been identified from different extracts of *M. spicata* (Yamamura et al., 1998); in the absence of reference compounds, we could not confirm or infirm their presence in our Algerian samples.

The 3 *Mentha* investigated can be readily differentiated from their botanical and morphological characteristics, but also by their TLC profiles.

3.6.2. HPLC-DAD analysis

The typical chromatogram of the MR extract is presented in Figure 3. Compounds were identified by comparison of retention times and UV spectral characteristics with standard reference compounds. Quantitative data were obtained from their respective calibration curves.

Similar phenolic derivatives were detected in the HPLC-DAD profiles of the 3*Mentha* species, even though differences in concentration of each individual compound were observed (Table 5). In the group of phenolic acids, rosmarinic acid was the major compound detected in all extracts (MS: 6.7±1.1%, w/w; MP: 4.03±0.66%, w/w; MR: 2.5±0.4%, w/w), followed by chlorogenic acid with a particularly high concentration in *M. pulegium* extract (2.88±0.04%, w/w).

Amongst flavonoids, luteolin and diosmin were the major identified compounds. MP extract was characterized by its significant amount of diosmin (4.5±0.9%, w/w).

Qualitative TLC and HPLC analysis revealed the same compounds in the samples, indicating that diosmin and rosmarinic acid are the major phenolic compounds in *Mentha* species. This is consistent with previous reports, notably the studies by Dorman et al. (2003) and Sankara Subramanian and Nair (1972).

Mentha species are then a possible source for rosmarinic acid, a compound that has attracted a great deal of attention due to its reported health benefits. The data obtained in our previous study suggest, for the studied *Mentha* species, a direct correlation between total antioxidant capacity and rosmarinic acid content, indicating that this phenolic acid significantly contributes to this biological activity (Brahmi et al., 2014). According to Psotova et al. (2006) the supplementation of skin care cosmetics with rosmarinic acid may offer protection against UVA-induced oxidative stress.

4. Conclusion

Mentha species bioactive constituents have attracted the interest of scientists seeking to prevent disease and promote health. In this context the present study evaluated the antioxidant and antityrosinase properties of 3 *Mentha* harvested in Algeria. The *Mentha* extracts were found to contain a high amount of total phenolics, notably *Mentha spicata*, which harbors both a high phenolic content and a superior anti-radical activity. Flavonoids proved to be the main compounds of *M. rotundifolia*, which probably contribute to the observed antityrosinase activity.

Mentha species find wide applications in traditional medicine in Algeria, often ascribed to their content in essential oils. The present work further validates the usefulness of *Mentha* for the treatment and prevention of human diseases by showing that polyphenols probably largely contribute to reported uses. *Mentha* polar extracts represent an easily accessible item of food rich in natural antioxidants, and could become a valuable food supplement but also an active ingredient agent in the cosmetics industry.

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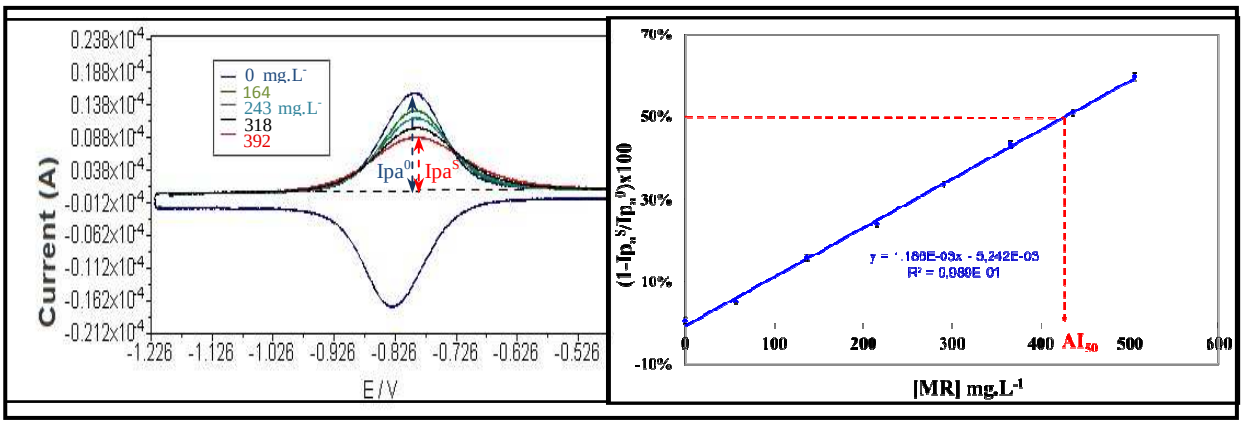
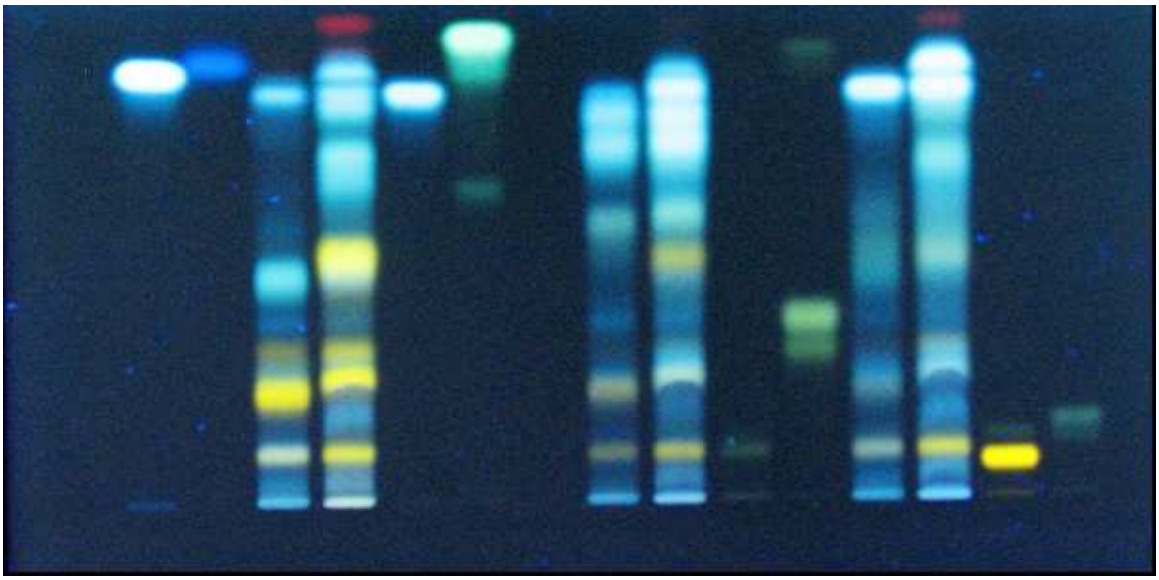


Figure 1: Electrochemical measurement of antioxidant activity of the *Mentha rotundifolia* extract (A) Cyclic voltammograms of dissolved oxygen in DMF electrolyte at a Cv disk electrode (ϕ=2mm) for successive MR extract additions. Time semi-derivative convoluted curves at scan rate 0.1 V.s^{-}1. (B) Determination of the antioxidant index values (AI_{50}) from the Plot of ($1\text{-}Ipa^{S}/Ipa^{0}$) in function of the added MR extract concentration where Ipa^{0} and Ipa^{S} are the $O_2^{\bullet-}$ oxidation peak currents respectively in absence and in presence of MR extract.



Tracks: (1) caffeic acid ; **(2)** ferulic acid ; **(3)** *M. rotundifolia* extract; **(4)** *M. rotundifolia* hydrolysed extract ; **(5)** rosmarinic acid; **(6)** kaempferol; **(7)** apigenin; **(8)** *M. pulegium* extract; **(9)** *M. pulegium* hydrolysed extract; **(10)** diosmin; **(11)** apigenin-7-glucoside; **(12)** *M. spicata* extract; **(13)** *M. spicata* hydrolysed extract; **(14)** rutin; **(15)** naringenin

Figure 2: TLC of analyzed mint extracts and reference standards. Silica gel 60 F254 plates; elution with ethyl acetate–formic acid–water (90:6:6, v/v/v); detection under UV light at 365 nm after spraying of an aminoethanol diphenylborate/macrogol reagent.

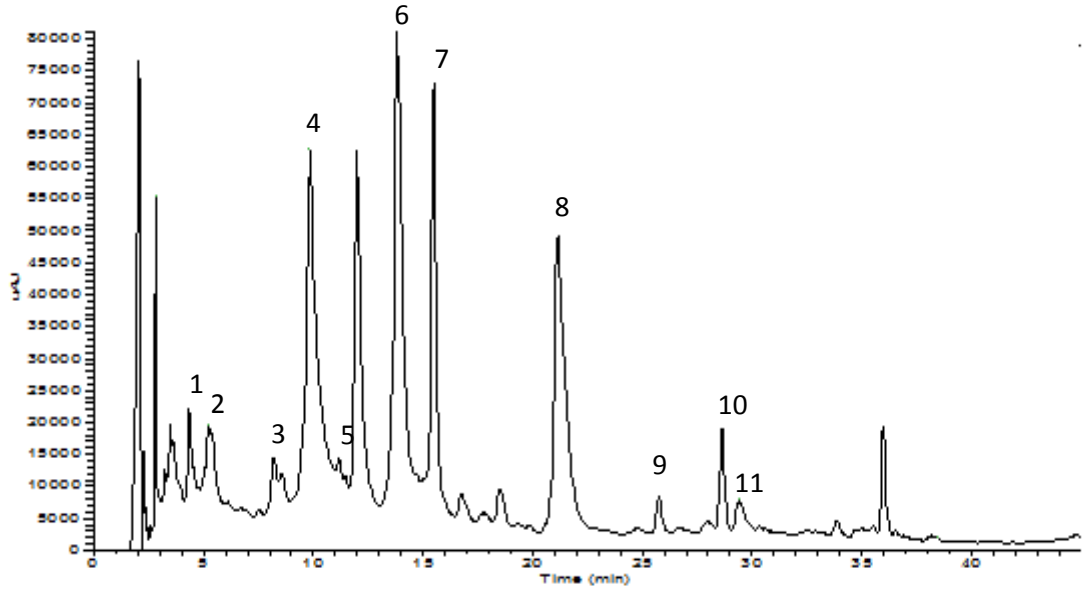


Figure 3: HPLC profile of phenolic compounds from *M. rotundifolia* extract.

Identified peaks: (1) 4-OH benzoic acid; (2) caffeic acid; (3) rutin; (4) chlorogenic acid; (5) naringin; (6) rosmarinic acid; (7) diosmin; (8) luteolin; (9) naringenin; (10) apigenin; (11) diosmetin

Table 1. Ethanol extraction yields, total phenolic content (TPC) and flavonoids content (TFC).

Extracts	Extraction yield (%)	TPC (*) (Eq. mg gallic acid/g)	TFC (*) (Eq. mg quercetin/g)
MS	4.8	12.0 ± 0.3^{a}	2.45 ± 0.02^{b}
MP	4.6	6.1 ± 0.5^{b}	0.85 ± 0.01^{c}
MR	4.6	4.6 ± 0.1^{c}	3.3 ± 0.1^{a}

(*)Data from 3 independent experiments; all contents are expressed versus dry plant material. Values in the same column sharing different letters are significantly different ($P < 0.05$).

Table 2. Comparison of antioxidant properties of *Mentha sp* extracts and reference standards

Sample	Quenching of $DPPH^{\bullet}$ (*)	Quenching of $ABTS^{\bullet+}$		Quenching of electrogenereted $O_2^{\bullet -}$
	IC_{50} (µg/ml)	IC_{50} (µg/mL)	TEAC (mM)	AI_{50} (µg/mL)
MS	16.2 ± 0.2^{d}	10.3 ± 0.9^{d}	0.90 ± 0.07^{c}	188 ± 37
MP	42.7 ± 2.3^{e}	30.2 ± 0.6^{e}	0.17 ± 0.02^{d}	210 ± 24
MR	71.3 ± 2.6^{f}	40.4 ± 0.7^{f}	0.20 ± 0.01^{d}	389 ± 52
Gallic acid	0.82 ± 0.05^{a}	3.00 ± 0.01^{a}	2.9 ± 0.1^{a}	88 ± 5

Quercetin	1.96 ± 0.04^{b}	4.06 ± 0.04^{b}	1.95 ± 0.05^{b}	141 ± 6
Trolox	4.6 ± 0.1^{c}	8.8 ± 0.2^{c}	-	493 ± 17

(*) Data are given as mean ± SD (n = 3). The data marked with the different letters of each sample category share significant differences at $P < 0.05$

Table 3. Reported antioxidant activity of *Mentha* species ethanol extracts from different origins

Origin	Method	IC_{50} (µg/mL)	References
M. spicata			
Portugal	$DPPH^{\bullet}$	65.2 ± 0.1	Mata et al., 2007
Iran	$DPPH^{\bullet}$	87.9	Nickavar et al., 2008
	$ABTS^{\bullet+}$	173.8	
M. pulegium			
Portugal	$DPPH^{\bullet}$	24.9 ± 0.2	Mata et al., 2007
Iran	$DPPH^{\bullet}$	17.9	Nickavar et al., 2008
	$ABTS^{\bullet+}$	152.6	
M. rotundifolia			
Iran	$DPPH^{\bullet}$	21.7	Nickavar et al., 2008
	$ABTS^{\bullet+}$	158.9	

Table 4. Phenolic compounds previously reported in different *Mentha* species

Class of compounds	Detected polyphenols	Origin of the herb	Reference
M. spicata			
Phenolic acids	Caffeic, chlorogenic, rosmarinic acids	*Finland*	Dorman et al., 2003
	Gallic acid	*Greece*	Proestos et al., 2005
	Gallic, chlorogenic, caffeic, vanillic, syringic, *p*-coumaric, ferulic, rosmarinic acids	*Finland*	Kivilompolo and Hyotylainen, 2007
	Gallic, 3,4-dihydroxybenzoic, vanillic, caffeic, ferulic, rosmarinic acids	*Greece*	Papageorgiou et al., 2008
Flavonoids	Diosmetin, diosmin, diosmin-7-glucoside	*India*	Sankara Subramanian and Nair, 1972
	Thymonin 5,6,4'-trihydroxy-7,3'-dimethoxyflavone	*Spain*	Tomas-Barberan et al., 1988
	Eriocitrin, luteolin glucoside, isorhoifolin, luteolin, apigenin	*Finland*	Dorman et al., 2003
	Apigenin, rutin, catechin	*Greece*	Proestos et al., 2005

	Rutin, quercetin, luteolin	*Greece*	Papageorgiou et al., 2008
	Rutin, scopoletin	*Czech Republic*	Adam et al., 2009
	Catechin, epicatechin, rutin, myricetin, luteolin, apigenin, naringenin	*Malaysia*	Bimakr et al., 2011
M. pulegium			
Phenolic acids	caffeic, vanillic, ferulic acids	*Greece*	Proestos et al., 2005
Flavonoids	Thymonin, jaceosidin, pectolinaringenin, ladanein, sorbifolin, pedalitin, 5,6,4'-trihydroxy-7,3'-dimethoxyflavone, 5,6-dihydroxy-7,3',4'-trimethoxyflavone, 5-hydroxy-6,7,3',4'-tetramethoxyflavone, apigenin, luteolin, chrysoeriol	*Algeria*	Zaidi et al., 1998
	Apigenin, luteolin, naringenin, catechin	*Greece*	Proestos et al., 2005
M. rotundifolia			
Phenolic acids	Caffeic, *p*-hydroxybenzoic, ferulic, *p*-coumaric acids	*Spain*	Marin Pares, 1983
Flavonoids	Apigenin, luteolinidin, pelargonidin, cyanidin, delphinidin, petunidin, luteolin	*Spain*	Marin Pares, 1983
	Thymonin, thymusin, 5,6-dihydroxy-7,8,3',4'-tetramethoxyflavone, jaceosidin, hispidulin, ladanein, sorbifolin, nodifloretin, apigenin, luteolin, genkwanin	*Algeria*	Zaidi et al., 1998
	Esculetin	*Czech Republic*	Dobiáš et al., 2010

Table 5: Phenolic acid and flavonoids identified and quantified by HPLC

Column, Alltima C18, 5 µm, 250 x 4.6 mm i.d.; mobile phase, gradient of acetonitrile, water, formic acid (19/80/1, v/v /v) and acetonitrile, methanol, formic acid (59/40/1, v / v / v); injection, 10 µL; flow rate, 1.2 mL/min; column t°, 40°C; detection, 280, 320 and 350 nm with scan 190 to 800 nm.

	Identified components[a]												
	Phenolic acids					Flavonoids							
Sample	4-OH benzoic acid (4.83) [b]	caffeic acid (4.95)	*p*-coumaric acid (8.33)	chlorogenic acid (9.90)	rosmarinic acid (13.70)	rutin (7.22)	naringin (11.47)	luteolin (21.98)	diosmin (14.00)	naringenin (25.42)	apigenin (28.25)	kaempferol (28.42)	diosmetin (29.97)
MS	0.20 ± 0.00	0.19 ± 0.02	0.10 ± 0.00	0.41 ± 0.00	6.7 ± 1.1	0.10 ± 0.01	2.8 ± 0.1	1.45 ± 0.06	0.44 ± 0.07	0.10 ± 0.00	n.d.	0.03 ± 0.00	0.04 ± 0.00
MP	0.20 ± 0.00	0.09 ± 0.00	0.06 ± 0.00	2.88 ± 0.04	4.0 ± 0.7	n.d.	n.d.	0.10 ± 0.00	4.5 ± 0.9	n.d.	0.04 ± 0.01	0.02 ± 0.02	n.d.
MR	n.d.[c]	0.42 ± 0.02	0.10 ± 0.00	2.33 ± 0.03	2.5 ± 0.4	n.d.	n.d.	0.35 ± 0.01	1.8 ± 0.6	0.20 ± 0.00	0.03 ± 0.01	0.09 ± 0.00	0.10 ± 0.00

[a]Values (% w/w) are expressed as mean ± standard deviation (n = 3).

[b]Retention time (min)

[c] Not detected