



In vitro antioxidant activity of essential oil of aerial parts of *Mentha pulegium* L.

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ABSTRACT

This work was conducted to evaluate the antioxidant activity of the essential oil obtained from the aerial part of *Mentha pulegium*. The antioxidant power of the essential oil obtained by hydrodistillation using a Clevenger-type apparatus was evaluated by using three methods: free radical-scavenging activity, reducing power and liver lipid peroxidation assay. Results showed that *Mentha pulegium* oil displayed good quality according to its physicochemical characteristics, and a higher yield $5.1 \pm 0.2\%$. The essential oil of *Mentha pulegium* showed a higher DPPH radical scavenging activity $90.54 \pm 1.5\%$ at a concentration of $1000 \mu\text{g/mL}$. This value was close to the results obtained with ascorbic acid $96.23 \pm 1.2\%$, and catechin $94.50 \pm 1.4\%$. This oil exhibited significant potential for reducing iron (the value observed by optical density was 1.8 ± 0.3), while ascorbic acid and catechin provided an OD of 2.069 ± 0.03 and 2.66 ± 0.016 at the same concentration of $1000 \mu\text{g/mL}$. The tested oil protected against lipid peroxidation induced by Fe^{+2} , and considerably increased the percentage of anti lipid peroxidation in a dose-dependent manner. The studied oil displayed a good degree of antioxidant activity and can be exploited in food and pharmaceutical industries

Keywords: Antioxidant activity, DPPH, essential oil, lipid peroxidation, *Mentha pulegium*.

ИЗВОД

Овај рад је спроведен за процену антиоксидативне активности есенцијалног уља добијеног из надземног дела биљке *Mentha pulegium*. Антиоксидативна снага есенцијалног уља добијеног хидродестилацијом помоћу апарата типа Clevenger процењена је применом три методе: активност уклањања слободних радикала, смањење снаге и тест пероксидације липида у јетри. Резултати су показали да је уље *Mentha pulegium* показало добар квалитет у складу са својим физичко-хемијским карактеристикама и да је имало већи принос од $5,1 \pm 0,2\%$. Есенцијално уље *Mentha pulegium* показало је већу активност уклањања DPPH радикала $90,54 \pm 1,5\%$ у концентрацији $1000 \mu\text{g/mL}$. Ова вредност била је близу резултата добијених са аскорбинском киселином $96,23 \pm 1,2\%$ и катехином $94,50 \pm 1,4\%$. Ово уље је имало значајан потенцијал за редукцију гвожђа, вредност уочена оптичком густином од $1,8 \pm 0,3$, док аскорбинска киселина и катехин дају OD од $2,069 \pm 0,03$ и $2,66 \pm 0,016$ у истој концентрацији $1000 \mu\text{g/mL}$. Тестирано уље штити од липидне пероксидације изазване Fe^{+2} јонима, знатно повећава проценат анти-липидне пероксидације на начин који зависи од дозе. Испитивано уље је показало добар степен антиоксидативне активности и може се искористити у прехранбеној и фармацеутској индустрији.

Кључне речи: антиоксидативна активност, DPPH, есенцијална уља, липидна пероксидација, *Mentha pulegium*.

1. Introduction

Plants are a real source of natural and medicinal products once serving as the source of all drugs (Balandrin et al., 1993). Actually, the use of aromatic and medicinal plants in herbal medicine has been developed intensively by exploiting different herbs, fruits and legumes. Many studies were focused on naturally bioactive products that can preserve human health from oxidative stress damage caused by reactive oxygen species. The imbalance between reactive oxygen species and antioxidant defense system may lead to the chemical modification of biologically relevant macromolecules (DNA, carbohydrates, proteins or lipids). These patho-biochemical mechanisms cause the development of different

diseases (Troszynska et al., 2002). Antioxidants are vital agents that possess the ability to protect the body from damage caused by free radical induced oxidative stress and retard the progress of several chronic diseases. Hence, the studies on natural antioxidants have gained increasingly greater importance. Many antioxidant substances naturally occurring in plant sources have been identified as potential free radical or active oxygen scavengers (Duh, 1998). Due to their richness in various bioactive compounds, plants have been used for food preservation, pharmaceutical treatment, alternative medicine and natural therapies (Lis-Balchin and Deans, 1997). It has long been acknowledged that some plant essential oils exhibit antioxidant activity and it is necessary to investigate those plants scientifically (Al-Bayati, 2008). *Mentha*

pulegium L. belonging to the Labiatae family is one of the *Mentha* species commonly known as pennyroyal. This aromatic herb is native to Europe, North Africa, Minor Asia and the near East (Chalchat et al., 2000). The flowering aerial parts of *Mentha pulegium* have been traditionally used in food preparation and also as antimicrobials (Mahboubi and Haghi, 2008), in the treatment of cold, sinusitis, cholera, food poisonings, bronchitis and tuberculosis (Zargari, 1990). It is also used as antispasmodic, carminative, anti-inflammatory, diuretic, antitussive, antifatulent and menstruation agent (Bouyahya et al., 2017a; Nickavar and Jabbareh, 2018). It is used as an aromatic stimulant, analgesic, and abortifacient (Gordon and Khojasteh, 2015), as an alternative pesticide and insect repellent (Cheraghi Niroumand et al., 2016; Domingues and Santos, 2019), as an antioxidant (El-Ghorab, 2006) and as a cytotoxic agent (Shirazi et al., 2004). The purpose of the present work was to determine the in vitro antioxidant activity of the essential oil extracted from the aerial part of *Mentha pulegium*.

2. Material and Methods

Chemicals substances

All chemicals and solvents were purchased from Sigma Aldrich (Munich, Germany), unless otherwise specified.

Plant material

The aerial parts of *Mentha pulegium* L. were collected during April 2018 in the region of Mascara (North-West of Algeria). The species was identified by botanists at SNV Faculty, University Mustapha STAMBOULI of Mascara. The collected plants were washed with tap water to remove all impurities and then with distilled water. The samples were dried in darkness at room temperature and chopped into small particles to increase the surface of diffusion.

Isolation of essential oils

A 100 g sample was submitted to hydro-distillation in 500 mL of distilled water for 3 hours, using a Clevenger-type apparatus (ST15 OSA, Staffordshire, UK) until total recovery of oil. The extracted essential oil was dried over anhydrous sodium sulfate. In order to preserve its original quality, the oil was stored at 4 °C until tested in an opaque glass bottle sealed to protect it from air and light (main agents of degradation). The essential oil yield was determined by the gravimetric method and expressed in terms of % w/w (ratio between the weight of the obtained oil and the weight of the sample to be treated). The purity of oil was assessed by measurement of physicochemical indices. The physical indices (pH, density at 20 °C, relative density, refractive index at 20 °C, rotatory power and miscibility with ethanol at 96%) and chemical indices (acid, ester, carbonyl, saponification, peroxide and iodine number) were evaluated according to the European Pharmacopoeia (2000).

Evaluation of the antioxidant activity DPPH free radical-scavenging activity

The antioxidant activity of *Mentha pulegium* essential oil was evaluated in terms of hydrogen donating or radical-scavenging activity, using the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a reagent. The ability of the oil to scavenge DPPH was determined by using the method described by Kirby and Schmidt (1997) with some modifications. The essential oil to be tested for its antioxidant potential was prepared in methanol to achieve the concentration of 1 mg/mL. Dilutions were made to obtain concentrations of 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, 1.99, and 0.97 µg/mL. A volume of 50 µL of these solutions was added to 1950 µL of methanol solution of DPPH (6.10⁻⁵ M) as a free radical source. The mixtures were stirred for 30 seconds and then incubated in the dark for 30 min at room temperature. The absorbance was measured using a UV/Vis spectrophotometer model Hitachi 4-2000 at 517 nm against pure methanol (Shimada et al., 1992). Ascorbic acid and catechin were used for comparison as positive controls. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity. The inhibition percentage of DPPH was calculated using the equation:

$$\% \text{ of inhibition} = \left(\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \right) * 100$$

Where: Absorbance of the control containing all reagents except the oil and absorbance of the sample (presence of the essential oil). IC₅₀ value (concentration of substrate that inhibits 50% of the DPPH radicals present in the reaction medium) was determined from the % inhibition versus concentration plot, using a non-linear regression algorithm.

Ferric-reducing power

The ferric reducing power of essential oil was determined by using the potassium ferricyanide-ferric chloride method described by Yildirim et al. (2001). One mL of essential oil at different concentrations was mixed with 2.5 mL of 0.2 M phosphate buffer pH 6.6 and 2.5 mL of potassium ferricyanide solution K₃Fe(CN)₆, 1 %. After incubation for 20 min at 50 °C, 2.5 mL of trichloroacetic acid 10% was added and the reaction mixture was centrifuged for 10 min at 3000 rpm (Sigma laborzentrifugen D-37620 Osterode am Harz, Germany). An aliquot of 2.5 mL of the supernatant from each mixture was mixed in a test tube with 2.5 mL of distilled water and 0.5 mL of ferric chloride solution (0.1%) prepared freshly in distilled water. After 20 min of reaction time at 35 °C, the absorbance was recorded at 700 nm against a blank that contained all reagents except the essential oil solutions and ferric chloride. The control was achieved by different concentrations of ascorbic acid and catechin. Higher absorbance of the reaction mixture indicated higher reducing power. Tests were carried out in triplicate. The concentration providing 0.5 of absorbance (IC₅₀) was calculated by plotting absorbance at 700 nm against the corresponding sample concentration.

Liver Lipid peroxidation assay

Lipid peroxidation assay in the liver tissues was evaluated using the thiobarbituric acid reactive substances (TBARS) method as described by Tatiya and Saluja (2010). The solution contained 0.5 mL of homogenate 10 %, 1 mL of KCl (0.15 M) and 0.5 mL of different dilutions of essential oil. The lipid peroxidation was initiated by adding 100 μ L of ferric chloride 1 mM. After incubation for 30 min at 37 °C, the reaction mixture was stopped by addition of 2 mL of iced HCl 0.25N containing 15% TCA trichloroacetic acid (tissue homogenate was deproteinized by TAC), 0.38% thiobarbituric acid and 0.2 mL of Butylated Hydroxyl Toluene (BHT, 0.05 %). The mixture was heated for 60 min at 80 °C, cooled and centrifuged at 6900 rpm for 15 min. The absorbance of the supernatant was measured at 532 nm using a spectrophotometer against a blank containing all reagents except the liver homogenate and essential oil. Identical experiments were carried out to determine the normal (without essential oil and FeCl₃) and the level of lipid peroxidation in the tissues (with FeCl₃ and without essential oil). For preparation of the homogenate, the liver was quickly removed after dissection of the rats, rinsed with physiological saline and homogenized at 4 °C in a solution of 0.15M KCl by 10%. The homogenate was centrifuged at 800 rpm for 15 min to remove cellular debris; the supernatant was recovered to examine the in vitro lipid anti peroxidation (Singh et al., 2007). The percentage of anti-lipid peroxidation effect (% ALP) was calculated by the following formula:

$$\% ALP = \left(\frac{A \text{ of FeCl}_3 - A \text{ of sample}}{A \text{ of FeCl}_3 - A \text{ of normal}} \right) * 100$$

Data analysis

All determinations were conducted in triplicates and results for each measured parameter were expressed as mean \pm SD. Data were statistically determined by the analysis of variance ANOVA at the significance level ($P < 0.05$) using Microsoft Excel and SPSS statistics software 8.1.

3. Results and discussion

Results of physic-chemical characterization

The essential oil of the aerial part of *Mentha pulegium* presents a mobile liquid aspect, light yellow color and herbaceous minty odor. This oil was characterized by a higher yield $5.1 \pm 0.2\%$. According to the literature, the essential oil yield of *Mentha pulegium* varies from 0.1% (Oliveira et al., 2011) to 5.4% (Bouyahya et al., 2017b). According to Marzouk et al. (2008), the plant polymorphism, seasonal, geographical variation and nutritional availability of plant affect extraction oil yields, which are higher when plants are extracted during the flowering period. *Mentha pulegium* showed greater yield compared with the cited research such as $1.45 \pm 0.01\%$ reported by Abdelli et al. (2016) and 1.8% cited by Benabdallah et al. (2018). The physico-chemical analysis (pH 4.56 ± 0.1 , density at 20 °C 0.86 ± 0.01 g/mL, relative density 0.908 ± 0.01 g/g, refractive index at 20 °C $1.486 \pm$

0.002 , rotatory power $+2.25 \pm 0.10$, miscibility with ethanol at 96% 1/10 (v/v), acid index 9.537 ± 0.01 mg KOH/g EO, ester index 46.573 ± 0.01 mg KOH/g EO at 20 °C, saponification index 56.11 ± 0.01 , peroxide index 8.0 ± 0.3 meq. O₂/Kg EO, carbonyl index 283.305 ± 0.02 , and iodine number 1.5 ± 0.1) showed good quality of the studied oil.

Results of antioxidant activity

DPPH free radical-scavenging activity

As shown in Fig. 1, the essential oil of aerial part of *Mentha pulegium* showed a higher DPPH radical scavenging activity ($90.54 \pm 1.5\%$) at a concentration of 1000 μ g/mL

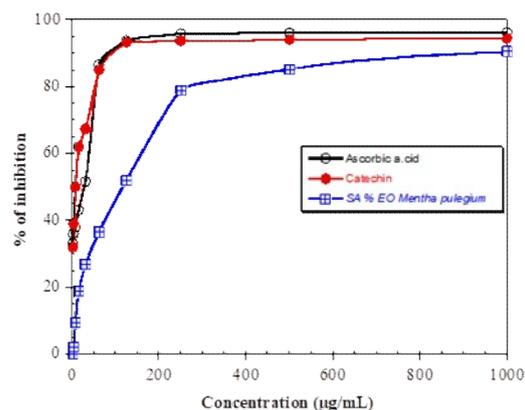


Figure 1. Free radical-scavenging activities of positive controls (ascorbic acid, catechin) and essential oil of *Mentha pulegium* measured by DPPH method. Values represent Mean \pm SD; n=3; Confidence level $p \leq 0.05$.

This result was close to the values found with ascorbic acid $96.23 \pm 1.2\%$ and catechin $94.50 \pm 1.4\%$. Result showed that the antioxidant activity of the essential oil was directly dependent on the concentrations used. According to Derwich et al. (2011), the antiradical activities of essential oils could be associated with their chemical composition, especially with their major compounds. The richness of *Mentha pulegium* essential oil in ketones, particularly pulegone, menthone, menthol (Ntalli et al., 2010), piperitone, and oxygenated monoterpenes (Pavela, 2008; Benomari et al., 2018), and neo-menthol (Abdelli et al., 2016), explains the antioxidant effect of this oil. The scavenger effect of active compounds on free radicals depends on the presence of free OH groups, in particular 3-OH, with a configuration 3',4'-rthodihydroxy (Heim et al., 2002). The obtained results are in agreement with the findings of several authors who reported that the efficiency of an antioxidant to reduce DPPH essentially depends on its hydrogen donating ability, which is directly related to the presence of phenolic compounds (Hazzit et al., 2009) and the abundance of monoterpenes hydrocarbons (Ruberto and Baratta, 2000) and oxygenated monoterpenes (Tepe et al., 2004). The IC₅₀ parameter commonly used to measure the antioxidant activity is necessary for each essential oil to reduce 50% of DPPH radical concentration in a defined period of time; a low IC₅₀ value corresponds to a higher antioxidant activity. The positive controls catechin and ascorbic acid displayed lower values of IC₅₀ 20 ± 0.2 and 32.5 ± 0.5 μ g/mL, followed by the essential oil of *Mentha pulegium* 130 ± 0.4 μ g/mL. The obtained IC₅₀ of

Mentha pulegium was lower than the value 69.60 ± 1.72 mg/mL cited by Abdelli et al. (2016). According to the IC50 values, the antioxidant activity of the essential oil was lower than the antioxidant effect of catechin and ascorbic acid and higher than the value cited in the literature.

Reducing power

The reducing power assay is often used to estimate the ability of a natural antioxidant to donate an electron or hydrogen to form a more stable product (Shimada et al., 1992). The determination of the ferric reducing antioxidant was based on the reduction of Fe³⁺/ferricyanide complex to the ferrous form in the presence of antioxidants in the tested samples. The Fe²⁺ were then monitored by measuring the formation of Perle's Prussian blue at 700 nm. In fact, it is widely accepted that higher absorbance at 700 nm is correlated with reducing power (Shimada et al., 1992). This author reported that the reductive potential may be related to the presence of phenolic compounds, such as isothymol and carvacrol, due to the hydroxyl substitutions in the aromatic ring, which possess potent hydrogen-bonding abilities. The reducing capacity of the essential oil of *Mentha pulegium* increases in a concentration-dependent manner (Fig. 2).

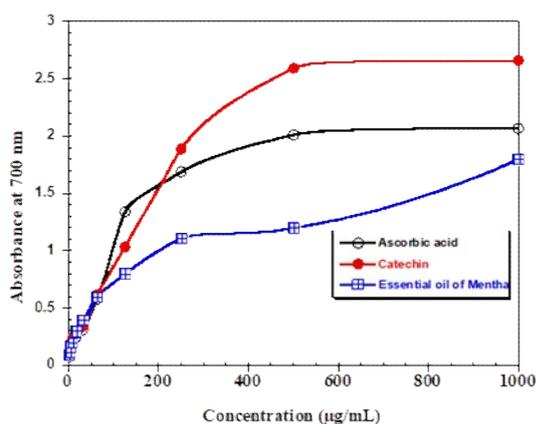


Figure 2. Antioxidant capacities of positive control (ascorbic acid, catechin) and essential oil of *Mentha pulegium* using ferric reducing power method. Values represent Mean \pm SD; n=3; Confidence level $p \leq 0.05$.

We noted significant activity for reducing iron (the value observed by optical density was 1.8 ± 0.3), while ascorbic acid and catechin provided an OD of 2.069 ± 0.03 and 2.66 ± 0.016 at the same concentration of 1000 µg/mL. We can classify power reduction of iron as follows: catechin, ascorbic acid, and essential oil of *Mentha pulegium*. The positive controls (catechin and ascorbic acid) displayed lower values of IC50 53 ± 0.3 and 58.3 ± 0.4 µg/mL, followed by the essential oil of *Mentha pulegium* 71.5 ± 0.3 µg/mL.

Lipid peroxidation

The inhibitory effect of the positive controls and essential oil of *Mentha pulegium* on Fe²⁺ induced lipid peroxidation in rat homogenates is shown in Fig. 3. The tested essential oil protected against lipid peroxidation induced by Fe²⁺, considerably increased the percentage of anti lipid peroxidation in a dose-dependent manner, and was close to those found by

ascorbic acid and catechin with a percentage more than $76 \pm 2\%$ and $73 \pm 1\%$ at 1000 µg/mL. This oil is effective in inhibiting the lipid peroxidation induced by the Fe²⁺ ascorbate system in rat homogenates. Lipid peroxidation is associated with a loss of membrane fluidity and an increase in membrane permeability, causing a decrease in physiological performance (Balu et al., 2005). The chemical structure of iron and its capacity to drive one electron reactions make iron a key factor in the formation of free radicals (Fraga and Oteiza, 2002). The generation of malondialdehyde (MDA) and related substances which react with thiobabaturique acid is inhibited by pulp oil (Tatiya and Saluja, 2010). This indicates significant activity of inhibiting the lipid peroxidation of oils. Phenolic substances are excellent nucleophiles and are able to scavenge radicals and inhibit lipid peroxidation, acting as breakers generated through lipid peroxidation. In addition, phenolic substances act as chelators of metal ions that induce oxidation (Han and Baik, 2008). The antioxidant capacity of essential oil may be associated with multiple systems, as they possess a chemical mixture with diverse functional groups, polarity and chemical behavior (Tepe et al., 2006).

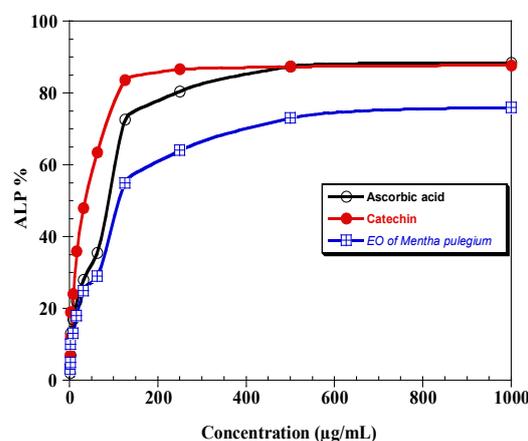


Figure 3. Antioxidant activity of positive control (ascorbic acid, catechin) and essential oils of *Mentha pulegium*. Values represent Mean \pm SD; n=3; Confidence level $p \leq 0.05$.

4. Conclusion

This study indicated that the essential oil of the aerial part of *Mentha pulegium* showed greater yield, good quality and a higher DPPH radical scavenging activity (percentage of inhibition $90.54 \pm 1.5\%$) at a concentration of 1000 µg/mL. This result was close to the values found with ascorbic acid $96.23 \pm 1.2\%$ and catechin $94.50 \pm 1.4\%$. The studied oil showed a lower value of IC50 130 ± 0.4 µg/mL. This oil displayed a significant activity for reducing iron (IC50 71.5 ± 0.3 µg/mL) and was effective in inhibiting the lipid peroxidation induced by the Fe²⁺ ascorbate system in rat homogenate. It may provide a valuable source of natural antioxidants for commercial exploitation.

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