



Original Research Article

The effects of solvents polarity on the phenolic contents and antioxidant activity of three *Mentha* species extracts

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ABSTRACT

The present work had two objectives: the first, to compare the total phenolic contents (using Folin–Ciocalteu's reagent) and antioxidant activity (using three different in vitro test assays) of three *Mentha* species and second, to determine whether these parameters changed with change of solvents extract polarity. *Mentha spicata*, *Mentha pulegium* and *Mentha piperita* were successively extracted with solvents of increasing polarity (n-hexane, dichloromethane (DCM), methanol and distilled water) using a soxhlet extractor. Our results reported that water and methanol (polar solvents) extracts, which contained the highest amount of total phenolic contents exerted strong reducing power and scavenging DPPH radical activity, which are almost equal to positive control BHT. Hexane and DCM (non polar solvents) extracts were showed a negligible antioxidant activity with both of test assays. Thus, the results indicate that polar solvents were important for obtaining fractions with high antioxidant activity and total phenolic content. All extracts tested in this study were found to be moderate in antioxidant capacity in the β -carotene/linoleic acid test assay and none of the extracts showed activity as strong as BHT. A positive correlation was found between total phenolic level and DPPH radical scavenging ($r^2 = 0.82$) and reducing power ($r^2 = 0.86$) assays. Whereas, there was no correlation ($r^2 = 0.25$) between phenol contents and β -carotene/linoleic acid assay.

Keywords

Antioxidant activity,
Total phenolic content,
Mentha species,
Polar solvent extracts,
Non-polar solvent

Introduction

The processes of oxidation are intrinsic in the management of energy of all living organisms and are, therefore, kept under strict control by several cellular mechanisms (Halliwell and Gutteridge, 2007). However, reactive oxygen species (ROS), generated

during metabolism, are very unstable and highly reactive, due to the presence of unpaired electrons, which include superoxide anion radicals, hydroxyl radicals, hydroperoxyl radicals, peroxy and non-free radical species such as hydrogen peroxide,

ozone and singlet oxygen. The production excessive of ROS tend to initiate chain reactions which result in irreversible chemical changes in proteins, lipids and DNA (Wijeratne *et al.*, 2005), these deleterious reactions can result in cellular dysfunction and cytotoxicity. Thus, increasing evidence suggests that many human chronic diseases, such as cancer, cardiovascular disease and neurodegenerative disorders are the results of the oxidative damage by ROS (Aruoma, 1998; Lai *et al.*, 2010).

In order to lower the risk of oxidative deterioration, synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been added to many food products. These compounds are suspected of being carcinogenic and causing liver damage (Lin and Tang, 2007; Song *et al.*, 2010). Therefore, there is great interest in finding new and safe antioxidants from natural sources.

Recently, many in vitro studies indicate that phenolic compounds like flavonoids, coumarines, phenolic acid, lignans, hydroxycinnamates and stilbenes can have considerable antioxidant activity (Duthie and Crosier, 2000). In addition, a strong relationship was found between total phenolic content and antioxidant activity in fruits, vegetables, and medicinal plants (Dorman *et al.*, 2003). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Tuberoso *et al.*, 2010). Many researchers have shown a great interest in replacing synthetic antioxidants with natural plant-based alternatives (Devi *et al.*, 2008), because of their safety and related total antioxidant potential (Djeridane *et al.*, 2006;

Katalinic *et al.*, 2006). So plants containing high level of polyphenols have a great importance as natural antioxidants.

The genus *Mentha* (Lamiaceae), comprising more than 25 species, grows widely throughout the temperate regions of the world. Leaves, flowers, and the stems of *Mentha* species are frequently used due to their antiseptic properties and in herbal teas or as additives in commercial spice mixtures for many foods to offer aroma and flavor (Gulluce *et al.*, 2007; Moreno *et al.*, 2002). In addition, the plant oil has also proved to have different biological activities; anti-radical (Ahmad *et al.*, 2012), antimicrobial (İşcan *et al.*, 2002; Derwich *et al.*, 2010; Mahboubi and Haghi, 2008; Tassou *et al.*, 1995), antifungal (Adam *et al.*, 1998), and anticancer (Kumar *et al.*, 2004). However, there is only limited information in the literature on the use of *Mentha* organic extracts as an antioxidant. Kanatt *et al.* (2007) reported that *Mentha* extracts retarded lipid oxidation in radiation-processed lamb meat. Nickavar *et al.* (2008) observed that the ethanol extract from five *Mentha* species has a strong radical scavenging activity in both DPPH and ABTS^{•+} assays.

Solvent extraction is frequently used for isolation of the antioxidants and all extraction yield, phenolic content and antioxidant activity of the extracts are strongly dependent on the solvent, due to the different antioxidant potentials of compounds with different polarity.

Lee *et al.* (2007) showed that water is the most suitable solvent for extraction of phenolic compounds from *Pleurotus citrinopileatus*. Ozsoy *et al.* (2008) showed that the highest content of total phenolic compounds in *Smilax excelsa* leaves was found in water extract and the lowest was

found in ethyl acetate. The reports of Hertog *et al.* (1993) and Yen *et al.* (1996) showed that methanol is a widely used and effective solvent for extraction of antioxidants and phenolic compounds.

Thus, this study was investigated the effect of several extracting solvents on the total phenolic compounds and antioxidant activity of the crude extracts derived from three *Mentha* species.

Materials and Methods

Chemical reagents

n-hexane, DCM, Folin-Ciocalteu phenol, sodium carbonate (Na₂CO₃), tannic acid, 2,2-diphenyl-1-hydrazyl (DPPH), potassium ferricyanide, trichloroacetic acid, linoleic acid and β-carotene were obtained from Sigma Aldrich (Germany). Dimethyl sulfoxide (DMSO), ferric chloride and tween 40 were purchased from Riedel-de Haën (Germany). Butylated hydroxytoluene (BHT) was procured from Fluka Chemie GmbH (Germany). All other reagents were of analytical grade.

Plant material

Plants were collected in October from the Northern of Morocco. The leaves were separated from the other parts and dried at room temperature. The dried leaves were reduced to a fine powder and used for solvent extraction.

Preparation of plant extracts

The air dried powdered plant samples were extracted in Soxhlet extractor successively with solvents of increasing polarity at 50°C: n-hexane, DCM, methanol and distilled water at 100°C. The extracts were concentrated by rotary vacuum evaporator

and then dried. The dry extract obtained with each solvent was weighed to determine the yield and kept at +4°C until tested. Before use, the hexane and dichloromethane extracts were dissolved in dimethylsulfoxide (DMSO), methanol and aqueous extracts were dissolved in methanol.

Calculation of the extract yields

The extract yields were calculated as follows:

$$\text{Yield (\%)} = A/A' * 100$$

Where A: weight of the extract and A': weight of the initial powdered plant.

Determination of total phenolic content

The total phenolic content was determined according to the method reported by Manian *et al.* (2008) using Folin-Ciocalteu reagent, with some modifications. 50 µl of aliquot of the extracts were taken in test tubes and made up to the volume of 500 µl with distilled water. Then 250 µl of Folin-Ciocalteu phenol reagent (1:1 with water) and 1.25 ml of sodium carbonate solution (20%) were added sequentially in each tube. Soon after vortexing the reaction mixture, the test tubes were placed in dark for 40 min and the absorbance was recorded at 725 nm against the reagent blank. A calibration curve was prepared using tannic acid and total phenols were calculated as tannic acid equivalents (TAE) and expressed as µg TAE/mg.

DPPH free radical scavenging assay

The free radical scavenging activity of the extracts was measured using DPPH by the method of Blois (1958). In brief, a 0.1 mM solution of DPPH in methanol was prepared and 1 ml of this solution was added to 3 ml

of various concentrations (0.125 to 1.0 mg/ml) of sample dissolved in methanol to be tested. After 30 min, absorbance was measured at 517 nm. Two controls were used for this test, a negative control (containing all reagents except the test sample) and positive control (using the antioxidant BHT). The scavenging activity was calculated as follows:

$$\text{Scavenging activity (\%)} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] * 100$$

Where A_{control} is the absorbance without extract and A_{sample} is the absorbance with the extract. The EC_{50} value (mg/ml) is the extract dose required to cause a 50% decrease of the absorbance at 517 nm.

Reducing power assay

The reducing power of the extract was determined according to the method of Oyaizu (1986) as described by Amarowicz *et al.* (2010). Briefly, 1 ml of different concentration sample (0.125 to 1.0 mg/ml) was mixed with 2.5 ml of a 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of a 1% (w/v) solution of potassium ferricyanide. The mixture was incubated in a water bath at 50°C for 20 min. Afterwards, 2.5 ml of a 10% (w/v) trichloroacetic acid solution was added and the mixture was then centrifuged at 3000 rpm for 10 min. A 2.5 ml aliquot of the upper layer was combined with 2.5 ml of distilled water and 0.5 ml of a 0.1% (w/v) solution of ferric chloride, and absorbance was measured at 700 nm. Increase in the absorbance of the reaction mixture indicated increase in the reducing power. EC_{50} value is the effective concentration giving an absorbance of 0.5 for reducing power.

β -Carotene-linoleic acid assay

This test was evaluated using β -carotene/linoleic acid model system, as

described by Miller (1971). Two ml of a solution of β -carotene in chloroform (1 mg/ml) was pipetted into a flask containing 40 mg of linoleic acid and 400 mg of Tween 40. The chloroform was removed by rotary vacuum evaporator at 45°C for 4 min, and 100 ml of distilled water was added slowly to the semisolid residue with vigorous agitation to form an emulsion. A 5 ml aliquot of the emulsion was added to the tubes containing 0.2 ml of various concentrations (0.125 to 1.0 mg/ml) of sample, and the absorbance was measured at 470 nm, immediately, against a blank, consisting of the emulsion without β -carotene. The tubes were placed in a water bath at 50°C, and the absorbance was measured again at 120 min. The antioxidant activity (AA) was calculated as percentage of inhibition relative to the control using the following formula:

$$AA = (A_t - A_t') / (A_0' - A_t') * 100$$

Where A_0' is the absorbance of values measured at zero time of the incubation for control and A_t and A_t' are the absorbance measured in the test sample and control, respectively, after incubation for 120 min. The EC_{50} is the effective concentration at which the β -carotene bleaching inhibited by 50%.

Statistical analysis

Data were expressed as the mean \pm standard deviation of three independent experiments. Correlation coefficients and EC_{50} values were obtained from linear regression analysis in the EXCEL program.

Results and Discussion

Extract yield and total phenolic content

The extraction with solvents of increasing polarity involves of separating compounds

of a plant according to their degree of solubility. The yield percent and total phenolic content of hexane, DCM, methanol, and water extracts obtained of the leaves of *M. spicata*, *M. pulegium* and *M. piperita* were shown in Table 1. The maximum recovery percentage and extractable total phenolic content was recorded in Water extracts followed by methanol extracts. For the three *Mentha* species studied, the highest yields corresponded to Water extracts and the weakest yields corresponded to DCM extracts; Water extract yield of *M. spicata* was the highest (29.4 %) followed by that of *M. piperita* (22.4 %).

M. pulegium showed the lowest yields compared to the other *Mentha* species. The phenolics content increased with the polarity of the solvent; the highest amount of total phenolic content corresponded to water and methanol extracts (167.2 to 305.4 µg TAE/mg) followed by DCM extracts (111.8 to 133.8 µg TAE/mg). However, the total phenolic content of the hexane extracts were found to be the lower (0.2 to 23.4 µg TAE/mg). So, the amount of total phenolics was higher in polar solvents extract and weaker in non-polar solvents extracts. This result is in agreement with the report of Lee *et al.* (2007) who showed that water is the most suitable solvent for extraction of phenolic compounds from *Pleurotus citrinopileatus*. Ozsoy *et al.* (2008) showed that the highest content of total phenolic compounds in *Smilax excelsa* leaves was found in water extract and the lowest was found in ethyl acetate.

Variations in the yields and phenolic contents of various extracts are attributed to polarities of different compounds present in the leaves and such differences have been reported in the literature by Jayaprakasha *et al.* (2001).

DPPH free radical scavenging activity

Free radicals are known to be a major factor in biological damages. The DPPH radical scavenging assay is a widely used method to evaluate the ability of plant extracts to scavenge free radicals generated from DPPH reagent (Chung *et al.*, 2006). DPPH, a stable free radical with a purple color, changes into a stable yellow compound on reacting with an antioxidant. As can be seen from the Figure 1, at concentration above 1mg/ml methanol extracts of all the *Mentha* species were showed activity nearly equal to that of the positive control BHT (90.61%) with values 86.11%, 89.38 % and 92% for *M. piperita*, *M. spicata* and *M. pulegium*, respectively. A similar report (Ahmad *et al.*, 2012) found that methanolic extracts of nine *Mentha* species were showed a significant higher free radical scavenging (DPPH) potential. The next highest activity was for the Water extracts with 77.54%, 90.32% and 57.26% for *M. piperita*, *M. spicata* and *M. pulegium*, respectively, followed by the DCM extracts with 31.36%, 38.45% and 46.31%, respectively. The weakest radical scavenging activity was exhibited by the hexane extracts with values included with 4.71% and 15.95%.

From the EC₅₀ values of the extracts (Table 1), the results clearly indicated that methanol and water extracts which contained the highest amount of total phenolics, were the stronger radical scavenger. Romero-Jimenez *et al.* (2005) were reported that the level of activity was strongly associated with phenolic content of the extracts. It is well known that the antioxidant activity of plant extracts containing polyphenol components is due to their capacity to be donors of hydrogen atoms or electrons and to capture the free radicals (Shon *et al.*, 2003). The lower activity was showed by Hexane and DCM

extracts with EC₅₀ values superior to 1 mg/ml. EC₅₀ value of positive control was 0.13 mg/ml. The antioxidant activity in DPPH assay correlated well with phenolic content ($r^2 = 0.89, 0.88, 0.66$ for *M. piperita*, *M. spicata* and *M. pulegium* respectively). Many researchers have reported positive correlation between free radical scavenging activity and total phenolic compounds (Zheng and Wang, 2001; Wangenstein *et al.*, 2004; Kanatt *et al.*, 2007; Sarikurkcu *et al.*, 2008).

Reducing power

Actually, reducing power is a very important aspect for the estimation of the antioxidant activity. Different studies have indicated that the antioxidant effect is related to the development of reductones, which are terminators of free radical chain reactions (Dorman *et al.*, 2003; Singh and Rajini, 2004). Siddhuraju *et al.* (2002) have reported that the reducing power of bioactive compounds was associated with antioxidant activity.

The reducing power of hexane, DCM, methanol, and water extracts of different *Mentha* species is shown in Figure 2. Similar to the DPPH radical assay, at 1mg/ml, the highest reducing power was shown by methanol and water which are almost superior to the BHT (1.601) reducing power with values varied from 1.662 to 2.717 (except the water and methanol extracts of *M. pulegium* with 0.66 and 1.508 respectively). The Hexane and DCM extracts were showed the weakest reducing power with values included with 0.057 and 0.198.

It can be observed from these results; changes on solvent polarity alter its ability to dissolve antioxidant compounds. However, in this test assay and in the DPPH

test assay, methanol and water extracts were found to be the most efficient solvent to have antioxidant activity for all three *Mentha* species. This is in agreement with the reports of Hertog *et al.* (1993) and Yen *et al.* (1996) that methanol is a widely used and effective solvent for extraction of antioxidants and phenolic compounds.

A high correlation was observed between reducing power and total phenolics content in this test system too, with correlation coefficients $r^2 = 0.93, 0.89$ and 0.67 for *M.spicata*, *M.piperita* and *M. pulegium*, respectively. A strong correlation between the content of total phenolics and the reducing power was found in the extracts of some plant species (*Glycyrrhiza lepidota*, *Echinacea angustifolia*, *Polygala senega*, *Arctostaphylos uva-ursi* and *Equisetum spp.*) from the Canadian prairies as reported by Amarowicz *et al.* (2004). According to the results obtained by Baskan *et al.* (2007), water and methanol are the best solvents for extracting rosmarinic acid. In addition, it was reported that there is a strong correlation between the level of rosmarinic acid and the antioxidant activity potential (Tepe *et al.*, 2007).

β -Carotene-linoleic acid assay

This method is based on the loss of the orange color of β -carotene due to its reaction with radicals formed by linoleic acid oxidation in an emulsion.

The rate of β -carotene bleaching can be slowed down in the presence of antioxidants (Kulisic *et al.*, 2004). The antioxidant activity in terms of ability to prevent the bleaching of β -carotene of extracts of *M. spicata*, *M. pulegium* and *M. piperita* were investigated and results are presented in Figure 3. At a concentration of 1 mg/ml, all the extracts inhibited less (6.35% to 34.8%)

the oxidation of β -carotene than BHT (75.94%). EC₅₀ values of all extracts tested were superior to 1 mg/ml compared with the EC₅₀ value of positive control (0.31 mg/ml). The control, without addition of sample, oxidized rapidly and the absorbance at 470nm tends to zero. The results indicated that the extracts had acted as a modest antioxidant in a β -carotene linoleic acid model system.

There are many reports in the literature which correlate the total phenolics content of a plant extract with its antioxidant activity (Tuberoso *et al.*, 2010; Kanatt *et al.*, 2007). However, as mentioned by some authors (Hinneburg *et al.*, 2006; Lakić *et al.*, 2010), this relationship is not always present or depends on the method used for evaluating the antioxidant ability. In this work there was no correlation ($r^2 = 0.25$) between phenolics content and the ability to prevent the bleaching of β -carotene. A similar report (Amarowicz *et al.*, 1993; O'Sullivan *et al.*, 2011) demonstrated previously that there was no correlation between total phenolic content and the results of β -carotene bleaching assay. According to Chew *et al.* (2008), the total phenolic content measurement indicates the level of both lipophilic and hydrophilic compounds, however the β -carotene bleaching assay only provides an indication of the level of lipophilic compounds because the β -carotene bleaching test bleaching test is similar to an oil-in-water emulsion system.

Our results suggest that the phenolic compounds present in the *Mentha* leaves played an important role in antioxidant activity. Triantaphyllou *et al.* (2001) and Areias *et al.* (2001) reported that the extracts of *Mentha* species contained many different kinds of phenolic compounds. The components caffeic acid, eriocitrin, luteolin-7-O-glucoside, naringenin-7-O-glucoside,

isorhoifolin, rosmarinic acid, eriodictyol, luteolin, and apigenin were identified in water-soluble extracts from different *Mentha* species by Dorman *et al.* (2003).

Recently, Caffeic acid (3,4-dihydroxycinnamic acid) is found to be a potent free radical scavenger and antioxidant (Ozguner *et al.*, 2006).

Eugenol is a naturally occurring allyl benzene and an active component of leaves of *M. piperita* (Ambika *et al.*, 2007). Eugenol has also been reported to be an antioxidant and hepatoprotectant (Parasakthy *et al.*, 1996).

The antioxidant activity of phenolics is mainly due to their redox properties which make them act as reducing agents, hydrogen donors and singlet oxygen quenchers. They also may have a metallic chelating potential (Rice-Evans *et al.*, 1995).

From the estimated EC₅₀ values (Table 2), it can be seen that the polar (methanol and water) extracts of *Mentha* species studied were more effective than their non polar (Hexane and DCM) extracts in the DPPH and reducing power test assays. Recently, Hajji *et al.* (2010) revealed that water extract of *Mirabilis jalapa tubers* had significant antioxidant activity and free radical-scavenging activity followed by methanol and dichloromethane extracts.

When compared EC₅₀ values of Methanol extracts studied, in the DPPH and reducing power assays *M. spicata* was the most active (EC₅₀ = 0.16 and 0.17, respectively), followed by *M. piperita* (EC₅₀ = 0.4 and 0.27, respectively) and *M. pulegium* (EC₅₀ = 0.49 and 0.3, respectively).

When compared EC₅₀ values of Water extracts, the same order was showed (*M.*

spicata > *M. piperita* > *M. pulegium*). The EC₅₀ values of DCM and Hexane extracts of all three *Mentha* species in all three test assays were superior at 1 mg/ml. In the β-

carotene/linoleic acid assay, all extracts were showed EC₅₀ values superior at 1 mg/ml.

Table.1 Yield percent and total phenolics in the extracts of *M. spicata*, *M. pulegium* and *M. piperita*

Extract	Yield (%)			Total phenolics (µg TAE/mg)		
	<i>M. spicata</i>	<i>M. pulegium</i>	<i>M. piperita</i>	<i>M. spicata</i>	<i>M. pulegium</i>	<i>M. piperita</i>
n-Hexane	3.32	2.83	4.85	23.4 ± 1.22	0.2 ± 0.65	20 ± 1.01
DCM	2.7	1.78	2.92	111.8 ± 0.73	133.8 ± 2.32	125 ± 1.19
Methanol	5.25	3.41	4.05	302.8 ± 2.05	167.2 ± 0.41	266.2 ± 0.79
Water	29.4	6.42	22.4	305.4 ± 1.32	238.4 ± 1.29	239.6 ± 2.53

Table.2 Antioxidant activity of solvent extracts of tree species of *Mentha* by three different test assays

Sample	EC ₅₀ (mg/ml)		
	DPPH free radical scavenging	Reducing power	β-carotene bleaching inhibition
BHT (positive control)	0.13	< 0.125	0.31
Hexane extract			
<i>M. spicata</i>	> 1	> 1	> 1
<i>M. pulegium</i>	> 1	> 1	> 1
<i>M. piperita</i>	> 1	> 1	> 1
DCM extract			
<i>M. spicata</i>	> 1	> 1	> 1
<i>M. pulegium</i>	> 1	> 1	> 1
<i>M. piperita</i>	> 1	> 1	> 1
Methanol extract			
<i>M. spicata</i>	0.16	0.17	> 1
<i>M. pulegium</i>	0.49	0.3	> 1
<i>M. piperita</i>	0.4	0.27	> 1
Water extract			
<i>M. spicata</i>	< 0.125	< 0.125	> 1
<i>M. pulegium</i>	0.61	0.59	> 1
<i>M. piperita</i>	0.3	0.24	> 1

EC₅₀: The effective concentration at which the DPPH radicals were scavenged by 50%, the effective concentration at which the DO700nm was 0.5, the effective concentration at which the β-carotene bleaching inhibited by 50%.

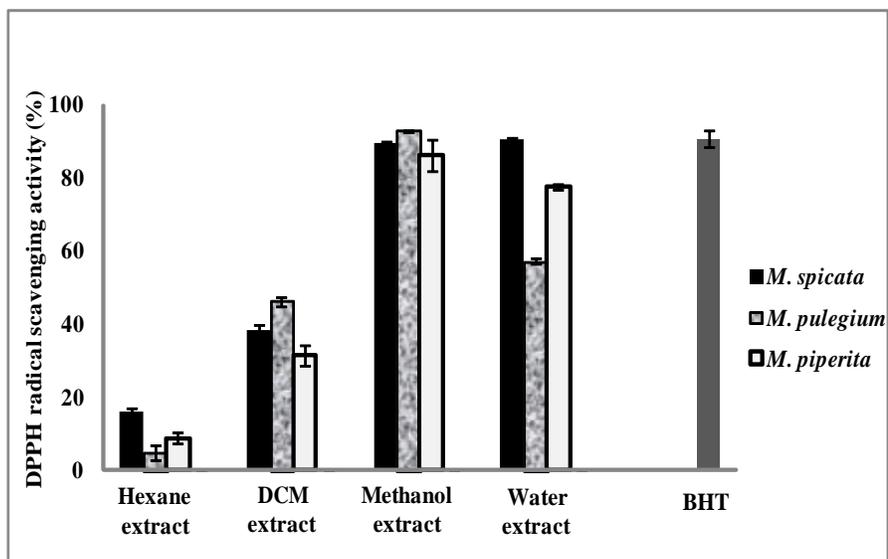


Fig.1 Antioxidant activity of extracts of *Mentha* species by DPPH test assay

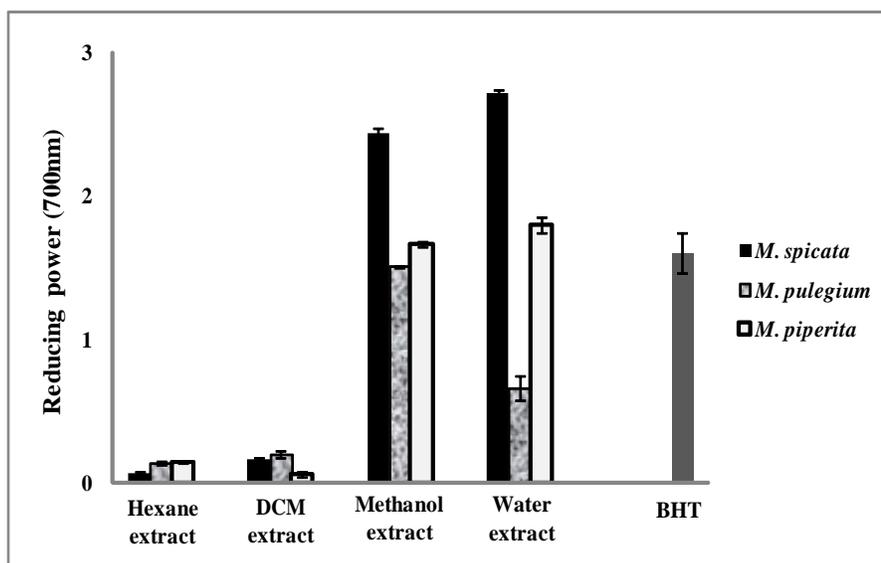


Fig.2 Antioxidant activity of extracts of *Mentha* species by reducing power test assay

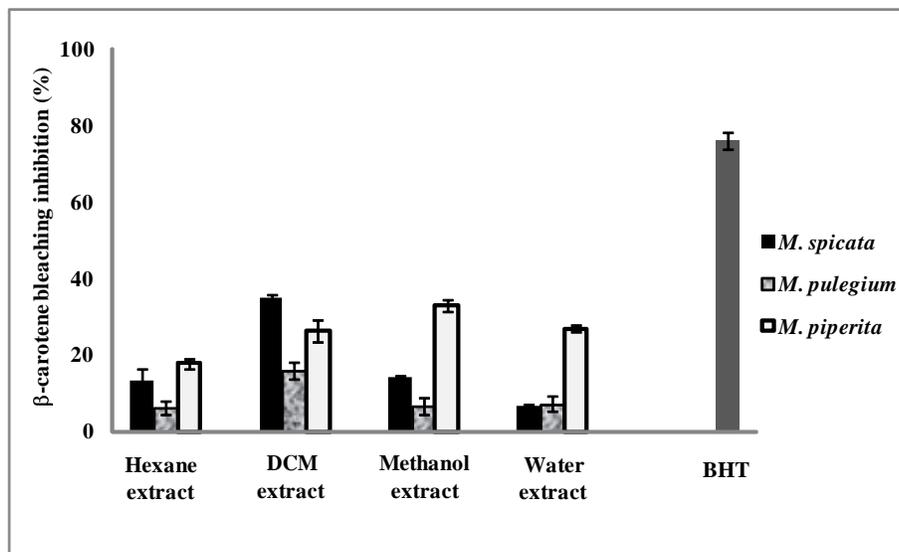


Fig.3 Antioxidant activity of extracts of *Mentha* species by β -carotene/linoleic acid test assay

Conclusions

To explore the suitability of different extracting solvents with different polarity, we have compared the yield, total phenol antioxidant properties of hexane, DCM, methanol and water extracts. We have demonstrated that the maximum recovery percentage and extractable total phenolic content, for all *Mentha* species, was recorded in water extracts followed by methanol extracts. *M. pulegium* showed the lowest yields compared to the other *Mentha* species.

When compared different *Mentha* extracts to the antioxidative potentials of the standard compound in this study (BHT), our results showed that polar extracts from all three *Mentha* species studied, which contained the highest amount of total phenolic contents exerted strong reducing power, excellent radical scavenging activity (almost equal to positive control BHT) and modest β -carotene bleaching inhibition. However, their non polar extracts were showed a weak activity in all

the test assays. Thus, the results indicate that selective extraction from natural sources, by an appropriate solvent, is important for obtaining fractions with high antioxidant activity. As mentioned above, our results clearly showed that a higher content of polyphenols was obtained with an increase in the polarity of the solvent used. Similar results were reported by Hayouni *et al.* (2007). In the present study, a good correlation was found between total phenolic contents and reducing power ($r^2 = 0.86$) and radical scavenging activity ($r^2 = 0.82$) assays. However, there was no correlation ($r^2 = 0.25$) between phenol levels and β -carotene/linoleic acid assay.

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