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Original Article

Chemical Composition and Antioxidant Effect of *Mentha rotundifolia* Extracts

Ferdjioui Siham[1,*], Belhattab Rachid[1], Raed M Al-Zoubi [2]

ABSTRACT

Aim: This report was aimed to investigate both the chemical composition and the antioxidant activity of two extracts from *Mentha rotundifolia* aerial parts. **Methods:** Aqueous and acetonic extracts were obtained by decoction and Soxhlet apparatus, respectively. Total polyphenols contents were determined using Folin- Ciocalteu reagent whereas flavonoids were evaluated by $AlCl_3$ method. LC-MS/MS method was carried out to reveal the phytochemical composition of extracts. Some tests were used to evaluate the *in vitro* antioxidant activity of extracts such as DPPH free radical scavenging assay, $ABTS^+$ radical cation decolorization assay, $OH^•$ radical scavenging assay, ferrous ion chelating, reducing power and β-caroten/linoleic acid bleaching assay. **Results:** The results showed that the aqueous extract was richer on polyphenols and flavonoids comparing to the acetonic extract. The analysis by LC-MS/MS allowed to identify many phenolic compounds in extracts, predominated by rosmarinic acid. In addition, the extracts were found to possess a significant antioxidant activity. **Conclusion:** Accordingly, it can be concluded that *M. rotundifolia* is quite rich in phenolic compounds and has a good antioxidant activity.

Key words: *Mentha rotundifolia*, Extraction, Phenolic compounds, LC-MS/MS, Antioxidant activity.

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INTRODUCTION

It is admitted that chronic diseases, as well as neurodegenerative pathologies such as cancer, cardiovascular diseases, Alzheimer's and other physiological disorders, are associated with the oxidant stress caused by free radicals and other oxidant.[1] An antioxidant can be broadly defined as any substance that delays or inhibits oxidative damage to a target, herbal plants are considered as good antioxidants since ancient times.[2] Plants of the Lamiaceae family constitute one of the major sources of gastronomic and medicinal plants all over the world. One of the important genera belonging to this family is the genus *Mentha* which is composed of around 19 geographically widespread species and 13 named hybrids.[3] *M. rotundifolia* is one of these species and is largely used in Algerian as well as in Mediterranean traditional medicine.[4]

MATERIALS AND METHODS

Plant material

The aerial parts of *M. rotundifolia* were collected during 2015 in the region of Djemila, Wilaya of Setif (Algeria). The collected material was identified by Pr. Laouer H from the Laboratory of Botanical Sciences, F. A. Setif1 University. The plant material was then air dried at room temperature and away from humidity.

Chemicals

All solvents and reagents used in this study were obtained from Sigma-Aldrich (Germany), Fluka and Merck.

Extraction

The aqueous extract of *M. rotundifolia* was obtained by decoction of the grinded plant material (100g) for 15 min in distilled water, filtered and the solvent was evaporated to get the aqueous extract. The acetonic extract was obtained from 20 g of the plant material powder mixed with 200 mL of acetone, using Soxhlet extractor for 6 h. Thereafter, the extract was filtered and evaporated to dryness under vacuum at 40°C using a rotary evaporator.

Determination of total polyphenols

The amount of total phenolics in the extracts of *M. rotundifolia* was determined according to the method of Li *et al.* (2007).[5] Briefly, 200 µL of plant extracts solutions (1.0 mg/mL) were mixed with 1.0 mL of Folin- Ciocalteu reagent (10.0%) for 4 mins. 800 µL of aqueous Na_2CO_3 (7.5%) solution were added. The mixture was kept at room temperature for 2 h and then the absorbance was measured at 765 nm. The results were expressed as µg Gallic acid equivalent (GAE)/mg of extract.

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Determination of total flavonoids

Total flavonoids content in *M. rotundifolia* extracts was determined by aluminum chloride colorimetric method.[6] A volume of 1.0 mL of each extract was mixed with 1.0 mL of $AlCl_3$ solution in methanol (2.0%). After incubation for 30 min at room temperature, the absorbance of the reaction mixture was measured at 430 nm. The total flavonoids contents of the extracts were expressed as µg quercetin equivalent/mg of extract.

Identification and Quantification of Individual Phenolic Compounds using Liquid Chromatography Mass Spectrometry (LC/MS/MS)

The analyses of extracts by LC-MS/MS were performed in Pharmaceutical Research Center - Jordan University of Science and Technology using an Agilent 1200 chromatography system (Agilent 1200, Agilent Technologies, Wilmington, DE, USA) equipped with mass detector API-3200.

The extracts and phenolic standard were dissolved in methanol at the concentration of (1.0 mg/mL) and injected onto a C_{18} column Agilent zorbax 150 mm X 4.6 mm. The analysis was performed using a gradient solvent system with aqueous-formic acid (0.10%) as solvent (A) and acetonitrile (100%) as solvent (B). A five-step linear gradient elution with a total run time of 35 min was employed, the gradient elution was realized by decreasing solvent A to 10% and increasing solvent B to 90%. An injection volume of 20 µL at a constant flow rate of 1.0 mL/min was used for each analysis.

The entire flow from the High-performance Liquid Chromatography (HPLC) was directed into a triple- quadrupole mass spectrometer (API 3200; MDS Sciex, Concord, ON, Canada). The mass spectral data were acquired in negative ion mode with a capillary voltage of 4500 V, an Electrospray Ionization (ESI) ion source, a cone voltage of 70 V, a collision energy of 35 eV, a drying temperature of 650°C, N_2 as the drying gas with a flow rate of 4.0 L/ min and Analyst software version 6.

A diode-array UV detector was used to scan between 200 and 400 nm to evaluate the contents of individual phenolic compounds and the eluted samples and standards were detected at 280 nm.

Antioxidant Activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) Radical Scavenging Assay

DPPH radical-scavenging activity of extracts was determined according to the method of Que *et al.* (2006).[7] Briefly, 1.0 mL of each plant extracts at different concentrations was added to 1.0 mL DPPH solution (0.01M in methanol). The mixture was shaken and left to stand for 30 min at room temperature in the dark. The absorbance was measured at 517 nm. The capability to scavenge the DPPH• radical was calculated using the following equation.

$$\text{Percentage of inhibition (\%)} = [(A_{control} - A_{sample})/A_{control})] \times 100.$$

OH$^{\bullet}$ Scavenging Activity Assay

Hydroxyl radical (OH$^{\bullet}$) scavenging ability was measured according to the literature procedure of Ates *et al.* 2008,[8] with slight modifications. The reaction mixture (3 ml) contained 1 ml of $FeSO_4$ (1.5 mM), 0.7 ml of H_2O_2 (6 mM), 0.3 ml of sodium salicylate (20 mM) and varying concentrations of extracts or standard antioxidant (BHA). This mixture was incubated at 37°C for 1h; afterwards, the absorbance of the hydroxylated salicylate complex was measured at 562 nm. The scavenging rate was calculated as percentage inhibition effect.

$$I\ (\%) = [(A_{control} - A_{sample})/A_{control})] \times 100.$$

ABTS^{+} Radical Cation(s) Decolorization Assay

The spectrophotometric analysis of ABTS radical scavenging activity was determined according to Re *et al.* (1999)[9] with some modification. ABTS was dissolved in water to a 7.0 mM concentration. ABTS radical cation (ABTS$^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (Final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before using. The radical was found to be stable in this form and for more than two days when stored in dark at room temperature. For the study, the ABTS$^{\bullet+}$ solution was diluted with ethanol, to an absorbance of 0.70 at 734 nm. 1.0 mL of ABTS$^{\bullet+}$ solution was added to 50.0 µL of the extract solution at different concentrations. After 30 min, percentage inhibition was calculated for each concentration at 734 nm. Appropriate solvent blanks were used in each assay. The ABTS^{+} scavenging ability as percentage was calculated as.

$$I\ (\%) = [(A_{control} - A_{sample})/A_{control})] \times 100.$$

Metal ion chelating assay

The estimation of iron ions chelation of the extracts was performed according to Le *et al.* (2007).[10] Briefly, an aliquot (500 µL) of different concentrations of extracts was mixed with 100 µL $FeCl_2$ (0.6 mM) and 900 µL methanol. After 5 min incubation, the reaction was initiated by the addition of 100 µL ferrozine (5 mM). The mixture was shaken vigorously and after 10 min incubation period the absorbance of the solution was measured at 562 nm. EDTA was used as positive control. The ratio of inhibition of ferrozine–Fe^{2+} complex formation was calculated as follows.

$$I\% = [(A_{control} - A_{sample}) / A_{control}] \times 100.$$

Reducing power assay

The reducing power of extracts was determined according to the method cited by Beyhan *et al.* (2010).[11] 1.0 mL of extracts at different concentrations was mixed with 2.5 mL phosphate buffer (pH 6.6) and 2.5 mL of 1.0 % potassium ferricyanide [$K_3Fe(CN)_6$] solution. The mixture was incubated at 50°C for 20 min, 2.5 mL of 10% trichloroacetic acid was added to the mixture. After vigorous agitation, 2.5 mL of this solution was mixed with 2.5 mL of distilled water and 0.5 mL $FeCl_3$ (0.1%), the absorbance was measured at 700 nm. The BHA was used as positive control. The EC_{50} for this test is defined as the effective concentration at which the absorbance is 0.5.

β-Carotene/ Linoleic Acid Bleaching Assay

The ability of extracts to prevent bleaching of β-carotene was assessed as described by Miraliakbari and Shahidi (2008).[12] A stock solution of β-carotene/linoleic acid was prepared by dissolving 0.5 mg of β-carotene in 1.0 mL of Chloroform, 25 µL of linoleic acid and 200 mg of Tween 40. The Chloroform was completely evaporated under vacuum in a rotatory evaporator at 40°C, 100 mL of distilled water saturated with oxygen were added, 2500 µL of this reaction mixture were dispensed into test tubes 350 µL of the various extracts, prepared at 2.0 mg /mL concentrations were added and the emulsion system was incubated for 48 h at room temperature. The same procedure was repeated with the positive control (BHA) and blanks (MEOH and H_2O). The covered tubes were incubated at 50°C and the absorbance was measured at 470 nm each 15 min during 120 min. The antioxidant activity was calculated as following equation.

$$AA\% = A_t / A_0 * 100.$$

A_0: Absorbance of the sample at t_0.

A_t: Absorbance of the sample after 120 min of incubation.

Statistical analysis

The results were presented as the mean of three repetitions (± Standard deviation (SD)). Statistical analysis of data was performed by using the GraphPad Prism 5 program. The data were analyzed by one-way Analysis of variance (ANOVA). Significant differences between means and standards were determined by Dennett's test, the level of significance was set at $p<0.05$.

RESULTS AND DISCUSSION

Phytochemical analyses

The extraction of phenolic compounds from *M. rotundifolia* arial parts was realized by two methods. The aqueous extract was obtained by decoction at a yield of 14.2 %, whereas the acetonic extract obtained by soxhlet apparatus showed a yield of 3.08 %. The amount of polyphenols and flavonoids was determined using Folin Ciocalteu and aluminium chloride method respectively. The results shown in Table 1 indicated that the aqueous extract was richer in polyphenols and flavonoids than the acetonic extract.

LC-MS/MS Analysis

The qualitative and quantitative analysis of phenolic compounds in aqueous and acetonic extracts of *M. rotundifolia* was conducted by using LC-MS/MS analysis. Phenolic compounds were identified by comparison of retention times and spectra of each peak with those of known standards analyzed in the same conditions.

(Apigenin, isoquercetin, catechol, epicatechin, gallic acid, procyanidin B2, quercetin 3-O- galactoside, luteolin, chlorogenic acid, epigallocatechin-gallate, cyaninchlorid, myrtillin, quercetin, rutin, caffeicacid,ellagicacid, ferulic acid, hydroxybenzoic acid, p-coumaric acid, rosmarinic acid, syringic acid, transcinamaldehyde acid, vanilic acid and hypericin) (Figure 1).

Among the 24 standards used, the analysis led to the identification of 15 and 16 phenolic compounds (Flavonols, phenolic acids etc.) in the aqueous and the acetonic extracts respectively (Table 2), the predominant compound was rosmarinic acid at a rate of 6610 ng/mL and 6370 ng/mL in aqueous and acetonic extract respectively. This phenolic acid was the subject of several scientific studies which made it possible to determine its therapeutic effect as anticancer,[13] antioxidant[14] and anti-Alzheimer.[15] A large diversity of molecules was observed in these extracts as phenolic acids (Chlorogenic acid, ferulic Acid, vanilic Acid), flavones and flavonols (Apigenin, luteolin, quercetin 3-O-galactoside). It is worth to note that the quantities of these compounds are different between extracts, these differences may be due to the method of extraction and the type of solvents.

No previous work illustrates the chemical composition of aqueous and acetonic extracts of *M. rotundifolia*. However, there essential oil has been the subject of numerous phytochemical investigations belonging to different regions in the world.[4,16]

Antioxidant activity

Phenolic compounds have been shown to be responsible for the antioxidant effect of plant. The antioxidant activity of polyphenols was attributed to their redox properties, which allows them to act as reducing agents, hydrogen donators and singlet oxygen quenchers, some show metal chelation properties.[17,18] Antioxidants can also prevent free radicals from forming, protect cells from free radical damage and enhance the body's defense system and work to protect, lipids from peroxidation by radicals.[19] In order to determine the mode of action of extracts; six different assays were used.

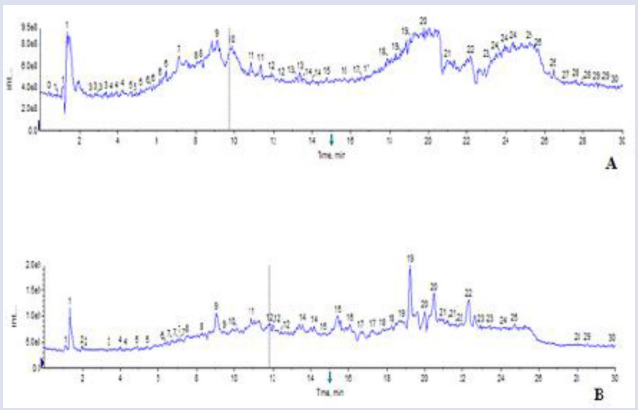

Figure 1: LC/MS Chromatogram of *M. rotundifolia* extracts (A): Aqueous extract, (B): Acetonic extracts.

Table 1: Amounts of Total Polyphenols and Flavonoids in *M. rotundifolia* Extracts.

Extracts	Polyphenols (µg GAE/ mg extract)	Flavonoids (µg QE/mg extract)
Aqueous extract	154.15±1.95	29.358± 2.009
Acetonic extract	113.7±2	16.275±0.375

DPPH· free radical scavenging assay

Substances which are able to donate hydrogen or an electron to DPPH·, can be considered as antioxidants and therefore radicals scavengers.[20] To determine the efficacy of extracts concentration providing 50% inhibition (IC_{50}) was calculated from the plot of inhibition percentage against the extract concentration (Figure 2), noting that lower IC_{50} value indicates higher antioxidant activity. The results reveal that the DPPH radical-scavenging activity of acetonic extract was more marked than that of aqueous extract with IC_{50} of 15.066± 0.449 µg/mL and 97.862 ± 0.218 µg/ mL for acetonic and aqueous extracts respectively. However, the effect of extracts was lower ($p < 0.001$) than that of BHA (IC_{50} of 5.742±0.206 µg/mL).

$ABTS^+$ Radical Cation Scavenging Activity

The ABTS radical scavenging action is known to be one of the various mechanisms for measuring antioxidant activity of food. This method was not only a rapid and reliable test of antioxidant capacity but also an advantageous assay applicable to both hydrophilic and lipophilic antioxidants/systems.[21] As shown in Figure 3, the extracts have a good antioxidant effect with IC_{50} of 5.868± 0.487µg/mL and 22.026± 0.759 µg/mL for aqueous and acetonic extract respectively. To evaluate the potency of extracts, the activity was compared with the synthetic antioxidant BHT, which presents the best potency (IC_{50}=2.745± 0.180 µg/mL).

OH· Scavenging Activity Assay

Among the Reactive Oxygen Species (ROS), hydroxyl radicals are the most reactive and are the predominant radicals generated endogenously

Table 2: Phenolic Profile of *M. rotundifolia* Aqueous and Acetonic Extracts Identified by LC- MS/MS.

	Compounds	C_1 (ng/mL)	C_2 (ng/mL)	RT	Q_1	Q_3	MWT
1	Gallic acid	/	/	1.53	169	124.6	170.12
2	Catechol	/	/	5.5	109	109	110.11
3	Chlorogenic acid	77.4	197	5.94	353.155	190.4/84.8/93.1	354.31
4	Vanilic acid	67.8	260	6.38	166.66	107.8/151.2/151.7	168.15
5	Procyanidin B2	/	/	6.4	577.1	407/ 289	578.52
6	Hydroxybenzoic acid	29.9	77.7	6.48	134.779	88.4/106.8/89.1	135.12
7	Syringic acid	95.1	197	6.48	196.718	120.4/120.7/152.3	198.17
8	Caffeic acid	23.4	50.8	6.5	178.465	134.2/106.4/89.1	18.16
9	Epicatechin	/	/	6.57	289.1	108.8	290.3
10	Epigallocatechin gallate	/	/	6.66	456.579	168.2/ 168.5/124.8	458.372
11	Rutin	64.8	139	6.84	609.419	299/299.9/270.9	610.52
12	Quercetin3-O-galactoside	45.6	32.7	7	463	301	464.379
13	Isoquercitrin	20	/	7	464.9	300	464.4
14	Myrtillin	0.414	18.1	7.01	462.178	299.8/270.8/254.7	500.8
15	P-Coumaric acid	75.8	262	7.06	162.756	118.8/118.1/92.7	164.16
16	Ferulic acid	93	99.2	7.2	192.807	133.9/133.4/177.8	194.18
17	Rosmarinic acid	6610	6370	7.38	358.319	160.7/161/132.7	360.32
18	Transcinamaldehyde acid	/	171	7.95	131.797	103.7/102.8/101.9	132.16
19	Luteolin	40.9	263	8	285	217	286.24
20	Cyanin chloride	59.9	438	8	286.198	133.4/132.8/150.6	287.1
21	Ellagic acid	/	/	8.01	300.703	149.7/150.6/149.9	302.197
22	Quercetin	/	8.09	8.01	300.604	150.4	302.2
23	Apigenin	9.12	139	8.4	269	151	270.12
24-	Hypericin	/	/	10.53	503	405	504.45

C_1: Concentration of phenolic compound in aqueous extracts; C_2: Concentration of phenolic compound in acetonic extract, Q_1: Compound molecular weight, Q_3: Fragment Molecular Weight, MWT: Molecular Weight.

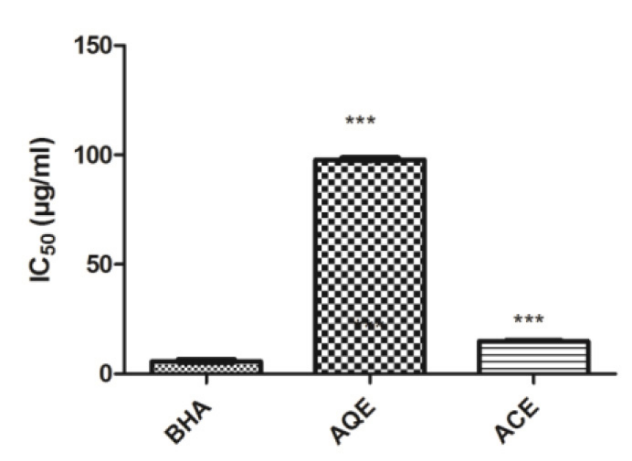

Figure 2: DPPH Scavenging Activity of *M. rotundifolia* Extracts Expressed as IC_{50} Values, AQE: Aqueous extract, ACE: Acetonic Extract. Values are the mean ± SD (n = 3). Comparisons are made with BHA. ***: $p \leq 0.001$.

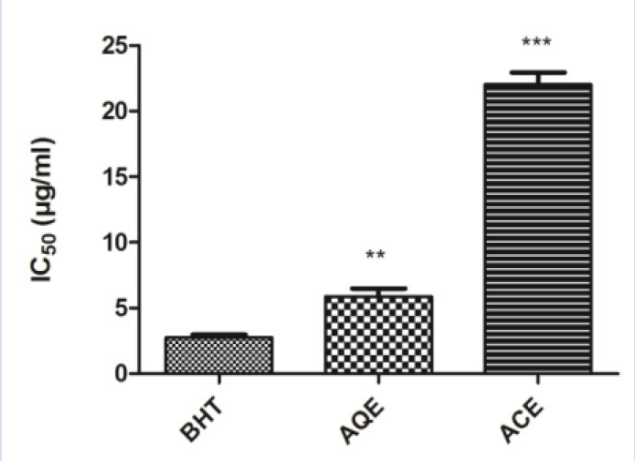

Figure 3: ABTS$^+$ Scavenging Activity of *M. rotundifolia* Extracts Expressed as IC_{50} Values. AQE: Aqueous Extract, ACE: Acetonic Extract. Values are the mean ± SD (n= 3). Comparisons are made with BHT **p<0.01,***: $p \leq 0.001$.

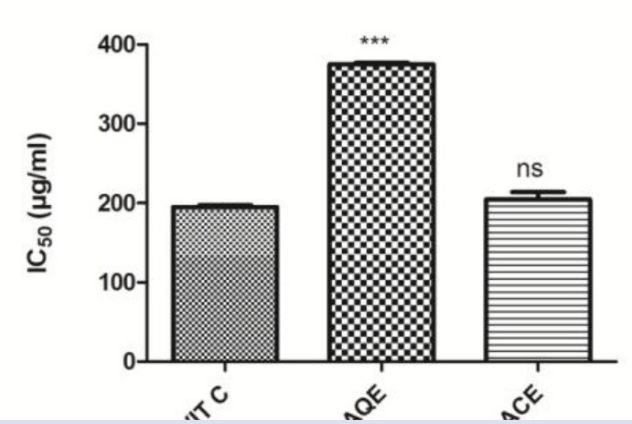

Figure 4: IC_{50} Values of *M. rotundifolia* Extracts for OH^- Scavenging Activity. AQE: Aqueous Extract, ACE: Acetonic extract. Values were Expressed as the mean ± SD ($n = 3$). Comparisons are made with Vitamin C, ns: No significant difference,***: $p \leq 0.001$.

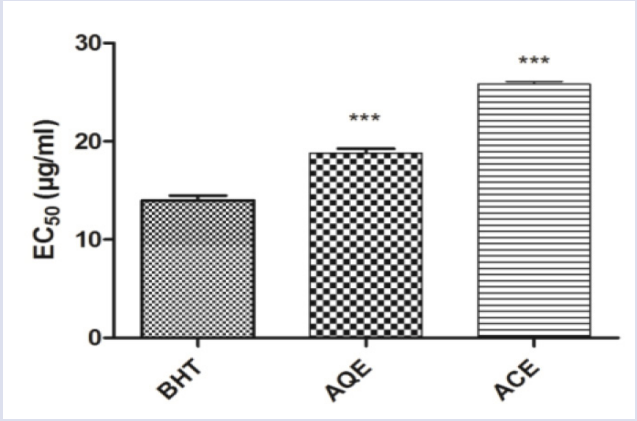

Figure 5: EC_{50} Values of *M. rotundifolia* Extracts for Reducing Power Test. AQE: Aqueous Extract, ACE: Acetonic Extract. Values were Expressed as the mean ± SD ($n = 3$). Comparisons are made against BHT. ***: $p \leq 0.001$.

during aerobic metabolism to initiate cell damage *in vivo*.[22] The results (Figure 4) indicate that the most potent scavenger of OH^- Radical was observed with vitamin C (IC_{50} = 194.868± 1.836 µg/mL) followed by ACE (IC_{50} = 204.355 ± 3.925 µg/mL) and AQE (IC_{50} = 377. 232± 4.995µg/mL).

Metal Chelating Activity

Iron is essential for life as it is required for oxygen transport, respiration and for activity of many enzymes. Chelating agents are effective as secondary antioxidants since they decrease the redox potential, thereby, stabilizing the oxidized form of the metal ion. Decrease in the red color of the complex ferrozine-Fe^{2+} indicates high radical scavenging activity of the compound.[23] To compare the effect of aqueous, acetonic extracts and standard (EDTA), the values of IC_{50} were calculated, which demonstrated that EDTA possess the most powerful chelating activity (IC_{50}= 6.273± 0.288 µg/mL) followed by the aqueous extract (IC_{50}= 128.155±5.887 µg/mL). However, the acetonic extract didn't reach 50% chelating activity and presents maximal activity of 41.433±2.518 % at 468.75 µg/mL.

Reducing Power

The presence of reductants such as antioxidant substances in the samples causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, Fe^{2+}can be monitored by measuring the formation of Perl's Prussian blue at 700 nm.[10] In this test, The EC_{50} values indicated that the BHA showed the good reducing power (EC_{50}= 13.997± 0.408µg/mL), followed by the aqueous extract (EC_{50}= 18.809± 0.369µg/mL) and finely the acetonic extract (EC_{50}= 25.83± 0.208 µg/mL) (Figure 5).

β-Carotene/ Linoleic Acid Bleaching Assay

In this test, the antioxidants give hydrogen molecules to the media which stops the peroxidation of linoleic acid, the hydrogen also scavenge singlet oxygen responsible of linoleic acid peroxidation.[23] The lowest β -carotene discoloration rate exhibited the highest antioxidant activity, Figure 6 shows the lipid peroxidation inhibitory activity of *M. rotundifolia* extracts by the β carotene-linoleic acid assay. The inhibition ratios of the oxidation of linoleic acid by the acetonic and aqueous extracts, were giving approximately the same percentage of inhibition ratio, 79.778± 1.812% and 84.666± 0.963 % respectively. However, the activity of BHA is more effective (89.479± 0.055 %).

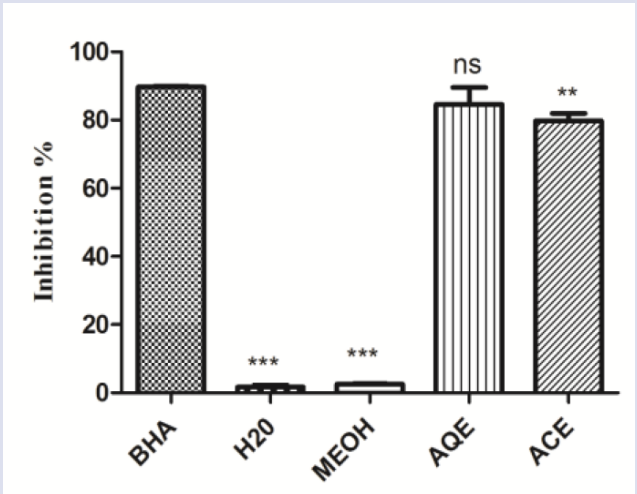

Figure 6: : Relative antioxidant activities of *M. rotundifolia* extracts and BHA in β-carotene/linoleic acid system after 2h. AQE: Aqueous Extract, ACE: Acetonic Extract. Values were expressed as the mean ± SD (n = 3). Comparisons are made against BHA, ns: No significant difference, **p<0.01, ***: $p \leq 0.001$.

Our results demonstrate the good antioxidant effect of *M. rotundifolia* extracts with al tests, this effect may be due to abundance of different flavonoids and phenolic compounds. The antioxidant activity of flavonoids depends on the functional group arrangement of nuclear structures. Configurations, substitutions and amounts of hydroxyl groups substantially affect some mechanisms of antioxidant activity.[24] a synergistic or antagonistic effect between the major and minor compounds also proposed to explain the effect of the extracts.

CONCLUSION

In this research we studied the chemical composition and the antioxidant activity of *M. rotundifolia* aqueous and acetonic extracts. The LC-MS/MS technique is used to identify numerous phenolic compounds, which are

known for their therapeutic effects. Moreover, a good antioxidant activity of extracts was confirmed by many tests. According to our results, it is clear that this plant is good for human. Further studies *in vitro* and *in vivo* are necessary to identify the biological roles of individual compounds of extracts and other biological properties of this plant.

ACKNOWLEDGMENT

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

DPPH: 2,2-diphenyl-1-picrylhydrazyl; **ABTS:** 2.2′-Azino-bis(3-ethylbenzenothiazoline 6-sulfonic acid); **BHT:** 2,6-di-tert-butyl-4-hydroxycarboxylic acid; **BHA:** Butylatedhydroxy anisole.

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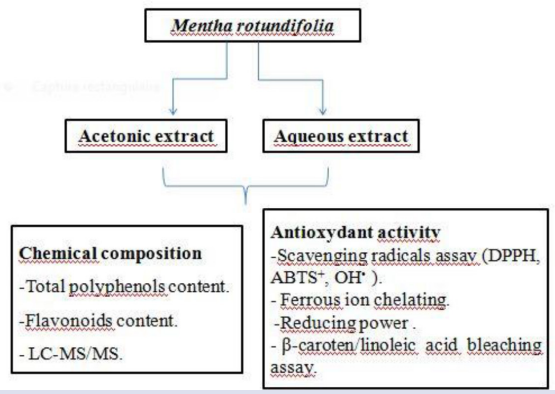
GRAPHICAL ABSTRACT



SUMMARY

- The present study provides information about the chemical composition and the antioxidant activity of *M. rotundifolia* extracts

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