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Original Article

Antioxidant Activity of Rosmarinic Acid Extracted and Purified from *Mentha piperita*

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Abstract

Rosmarinic acid was obtained from methanolic extract of *Mentha piperita L.* under a reflux condenser. The current study aimed to evaluate the *in vitro* antioxidant activities and rosmarinic acid levels of the methanol extracts of *M. piperita*. The analysis of the sample by high-performance liquid chromatography technique (HPLC) indicated that rosmarinic acid was present in high concentration 1.9 mg/mL in the extract. Purification was carried out by column chromatography to give 0.020 g from 1 g of crude extract, and then the antioxidant activity of purified rosmarinic acid was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH), H_2O_2 scavenging, and REDOX methods. It was revealed that the anti-oxidant potential of the rosmarinic acid extract was greater than 95% (at 100 µg/mL) for DPPH assay and 87.83% (at 100 µg/ml) for H_2O_2 scavenging assay. This study was performed by using a reflux methanolic extraction of *M. piperita*. This possible instructional technique proved to be a quick and successful method for retaining the antioxidant properties of rosmarinic acid. The rosmarinic acid content was determined using HPLC.

Keywords: Anti-oxidant, DPPH, H2O2, *M. piperita*, RA, Reflux

1. Introduction

Natural enzymatic and non-enzymatic anti-oxidants regulate the production and content of the reactive oxygen species (ROS) which include free radicals generated by a variety of metabolic reactions in the human body. However, any disruption in this area as a result of the imbalance of equilibrium reaction that increases the amount of ROS and oxidative stress causes harmful repercussions in the body. Many disorders, including cardiovascular illnesses (1, 2), Alzheimer's disease (3), diabetes mellitus (4), inflammatory illnesses (2), carcinogenesis (4), neurodegenerative (5), and pulmonary and hematological disorders (6), are linked to oxidative stress.

Antioxidants are substances that react with and neutralize free radicals. As a result, they prevent or lessen their harmful effects on the human body. It might come from synthetic or natural sources. Since ancient times, medicinal plants and their phytochemicals have been thought to have pharmacological value. Plants have been used in medicine since 60,000 years ago, long before civilization (7). Plants are responsible for more than 30% of all therapeutic medications (and their derivatives and analogs), and natural goods will continue to have a significant impact on human medicine.

The majority of synthetic bioactive medications are structurally identical to the phytochemicals extracted from plants. The Lamiaceae family is one of the most important medicinal plant families. The presence of phenolic chemicals in this plant, particularly rosmarinic acid (RA), which is renowned as a good antioxidant, is

primarily responsible for the medicinal qualities of this plant family.

In a previously published study by Scarpati (8), he showed the structure of RA, which is an ester of caffeic acid and 3, 4-dihydroxy phenyl lactic acid. The RA is a phenolic chemical found primarily in the Lamiaceae family of plants. Due to the abundance of RA in such plants, the existence of this component isolated from *Mentha piperita* L. is well known in the genus Mentha.

To stop the chain process of lipid peroxidation and limit the rate of lipid peroxidation, RA can compete with unsaturated fatty acids for binding to lipid peroxyl groups. Many other natural antioxidants cannot compete with the ability of RA to scavenge radiation-induced ROS and prevent free radical-induced cell damage.

Rosmarinic acid protects against the damaging effects of ionizing radiation by acting as a radioprotector (9). It has long been known that reactive oxygen metabolites produce some types of inflammatory tissue injury, and that, in addition to enhancing direct toxicity, they also promote indirect toxicity. Upregulation of numerous genes involved in the inflammatory response also initiates and/or amplifies inflammation (10).

The production of ROS can be prevented or reduced, which has been shown to minimize liver injury in ischemia-reperfusion models (11, 12). Due to the antioxidant and anti-inflammatory properties of rosmarinic acid, they may have protective effects on the harm caused by ischemia-reperfusion in the liver.

The extraction method and the identification of bioactive components are the most significant processes in assessing polyphenol chemicals. Due to the huge quantity and variety of natural phenolics in various classes, it is one of the most difficult tasks for analysts (13, 14).

In this research, rosmarinic acid was extracted from peppermint (*M. piperita*) by efflux extraction method. Extraction of reflux is a solid-liquid extraction technique that takes place at a consistent temperature with reproducible solvent evaporation and condensation over a set amount of time with no solvent loss. The technique is commonly utilized in the herbal industry since it is efficient, simple, and inexpensive (15).

2. Materials and Methods

2.1. Plant Materials

M. piperita plants were cultivated in a private orchard at Abu Al Khaseeb district, Basra, and authenticated by Asst. Prof. Dr. Ula Al-Mousawi, Pharmacognosy Department, Pharmacy College, Basra University, Basra, Iraq. In September 2020, fresh peppermint leaves were collected, cleaned, washed, shade dried, homogenized to a fine powder, and kept in an airtight bottle.

2.2. Chemicals

The rosmarinic acid standard was provided by Sigma Aldrich, USA while trichloroacetic acid was obtained from SDFCL, India, and acetonitrile, methanol, and ethanol absolute were provided by BDH, India. Sigma-Aldrich provided the 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Steinheim, Germany). The H_2O_2, ascorbic acid (vitamin-C) ferric chloride, trichloroacetic, acid, chloroform formic acid, and ethyl acetate.

2.3. Extraction Procedure

Powdered of the dried leaves of *M. piperita* L. were defatted with hexane by Soxhlet extractor equipment for 24 h. The resultant extract was allowed to stand for 24 h to evaporate all the hexane used. Afterward, the sample was extracted with 90% methanol (1:10) under a reflux condenser for 45 min and the resultant extract was filtered by using filter paper. Moreover, the solvent was evaporated by using a rotary evaporator. The resultant extract was stored in a dark glass container at 4 ^{o}C, then high-performance liquid chromatography technique (HPLC) and thin-layer chromatography (TLC) were used for identification and column chromatography was used for purification of rosmarinic acid.

2.4. Chromatographic Analysis for the Detection of Rosmarinic Acid Thin-Layer Chromatography

The TLC examination of the extract in relation to the rosmarinic acid standard was performed using a mobile

phase of chloroform: ethyl acetate: formic acid (5: 4: 1, v/v/v). The compounds were identified using UV detection at 254 nm after the standard solution and the samples were spotted on silica gel 60 F_{254} as the stationary phase.

2.5. High-Performance Liquid Chromatography-Ultraviolet Analysis of Rosmarinic Acid

The HPLC analysis was performed for detection and estimation of rosmarinic acid in peppermint extract (1 g/10 mL) of crude extract. The extract of peppermint leaves was analyzed by the HPLC method with UV detection. The HPLC analysis was carried out by a prominence HPLC system (shimadzu) with a degasser (DGU-20A) (3.9×30 CM×5 micro C18 columns at the temp 45 ^{o}C). Next, the mixture was passed through 0.45 µm disposable filters and then after aliquots of 5 µL of each sample was injected into the HPLC system.

The detection was done by elution with a mixture of Mobile phase including solutions A and B (20:80%) in which Solution A consisted of 0.5 ml of trifluoroacetic acid in 1 L of acetonitrile after degassing for at least 10 min. The solution consisted of B 0.5 ml of trifluoroacetic acid in 1 L of water after degassing for at least 10 min. A flow rate was set at 1.0 ml/min, detected by UV at 230 nm. Run time was the duplicate of the retention time of the test (about 30 min) in the sample solution.

The rosmarinic acid was detected according to the retention time of the standard rosmarinic acid which was prepared by weighing about 20 mg of rosmarinic acid standard in 100 ml of the volumetric flask with 70 ml 0f methanol and shaken vigorously for about 5 min. Next, the volume was completed by the same solvent, mixed, and then injected (rosmarinic acid conc 0.2 mg/ml) as a standard solution.

2.6. Isolation and Purification of Rosmarinic Acid by Column Chromatography

The selected solvent systems produced in TLC were used to isolate rosmarinic acid from *M. piperita* crude extract using column chromatography. To generate rosmarinic acid-rich extract with minimal solvent usage, the percentages of solvent systems and their volume, the height of the packed bed column, and the stability of the absorbent were tuned.

A 3-16 cm column chromatography packed with 50 g silica gel 60 (60-200 mesh) was used to fractionate the crude extract. Solvent systems chloroform: ethyl acetate: formic acid (5: 4: 1, v/v/v) was selected from TLC experiments as the best solvent system to separate rosmarinic acid by Column Chromatography.

The packed bed column was loaded with 1 g of crude extract dissolved in 10 mL methanol. At a flow rate of 1.5 mL/min, the chosen solvent solution was gently introduced and passed through the packed column. The plant fraction was collected from every 10 mL of the solvent that ran out of the packed column.

2.7. 2, 2-Diphenyl-1-Picrylhydrazyl Free Radical Scavenging Assay

The DPPH method established by Krings and Berger (16)was used to determine the radical scavenging activity of rosmarinic acid *in vitro*. For the purposes of the research, 2.5, 5, 10, 25, and 100 µg/ml of rosmarinic acid was added to a solution of 0.002% of DPPH in methanol. The combination was incubated at room temperature for 30 min in the dark, and the absorbance was measured at 517 nm using a quartz cell in a blank. Each concentration was tested in triplicate, and the percentages of inhibitory action to DPPH of each concentration was calculated using equation 1 and plotted against the tested concentrations to estimate the amount of rosmarinic acid required to reduce the starting concentration of DPPH by 50% (IC_{50}):

% AA=(DPPH-sample)/DPPH×100)……. Equation 1

Here, AA stands for antioxidant activity, ADPPH for DPPH absorption against the blank, and A sample for extract or control absorption against the blank. The positive control was ascorbic acid.

2.8. Hydrogen Peroxide Scavenging Assay

Ruch, Cheng (17) measured the ability of rosmarinic acid to scavenge hydrogen peroxide (H_2O_2). A 5, 10, 25, 50, and 100 µg/ml of rosmarinic acid was placed into Eppendorf tubes, which were then filled with 50

mM phosphate buffer (pH 7.4) to reach a volume of 2 mL which was followed by the addition of 0.1 mm of H_2O_2 solution. After that, the mixture was incubated at room temperature for 10 min, and its absorbance at 230 nm was measured. Positive control was employed, which was ascorbic acid. The following equation was used to calculate the ability of rosmarinic acid to scavenge H_2O_2:

H_2O_2 scavenging activity percentage=[(A0-A1)/A0]×100

where A_0=Absorbance of control and A_1=Absorbance of the sample.

2.9. Ferric Reducing Antioxidant Power Assay

The reducing power was evaluated using the Oyaizu (18) technique with minor modifications, in which different doses of rosmarinic acid (50, 25, 10, 5, 2.5, 1.25, and 0.625 µg/ml) were added to 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide [$K_3Fe(CN)_6$] solution.

The reaction mixture was then vortexed well and incubated for 20 min at 50 °C. Following the incubation, 2.5 mL of 10% trichloroacetic acid was added to the mixture and centrifuged for 10 minutes at 3,000 rpm. The supernatant (2.5 mL) was combined with 2.5 mL deionized water and 0.5 mL ferric chloride (0.1 %). The UV was used to measure the colored solution at 700 nm against a blank and compared to a standard.

3. Results and Discussion

Reflux extraction of peppermint with methanol yielded extraction yields of 0.9%. This extract was obtained in 45 min at 70 °C, demonstrating that reflux is a quick extraction process. The obtained extract was evaluated using HPLC, and rosmarinic acid was determined by comparing its retention time to a rosmarinic acid standard (Figure 1 and Table 1). The typical rosmarinic acid solution has a retention period of 3.283 min.

On the basis of the chromatogram of peppermint extract, a peak with a retention time corresponding to rosmarinic acid standard (Figure 2 and Table 2) can be identified, indicating the existence of this component in the peppermint reflux methanolic extract.

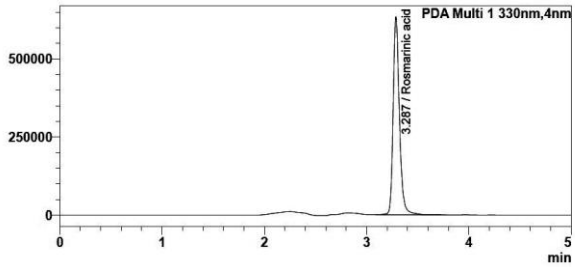

Figure 1. High-performance liquid chromatography technique chromatogram of standard rosmarinic acid solution

Table 1. Retention time and peak area of high-performance liquid chromatography technique analysis for rosmarinic acid standard

ID#	Name	Retention Time	Area	Tailing Factor	Resolution
1	Rosmarinic acid	3.287	2705192	1.259	--
Total			2705192		

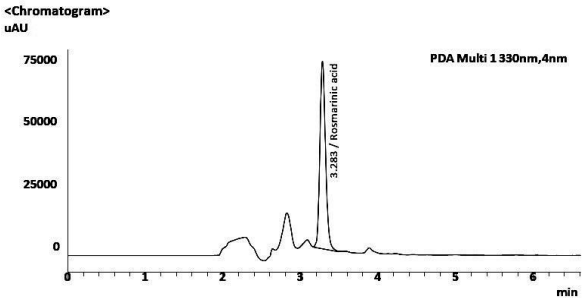

Figure 2. High-performance liquid chromatography technique chromatogram of reflux extraction peppermint with methanol

Table 2. Retention time and peak area of high-performance liquid chromatography technique analysis for rosmarinic acid extracted from peppermint leaves

ID#	Name	Retention Time	Area	Tailing Factor	Resolution
1	Rosmarinic acid	3.283	360942	1.188	--
Total			360942		

The isolated compound from the column chromatography purification method was subjected to different identification methods, namely analytical TLC and HPLC analysis. Moreover, it was compared with

the RA standard to confirm that the isolated compound is rosmarinic acid.

An isolated Rosmarinic acid that was purified from column chromatography was detected by analytical TLC under UV light at 254 nm, with reference to rosmarinic acid standard, as shown in figure 3 show the presence of one spot with the same R_f value, compared to rosmarinic acid standard which means that purified compound is rosmarinic acid with good purity (Table 3).

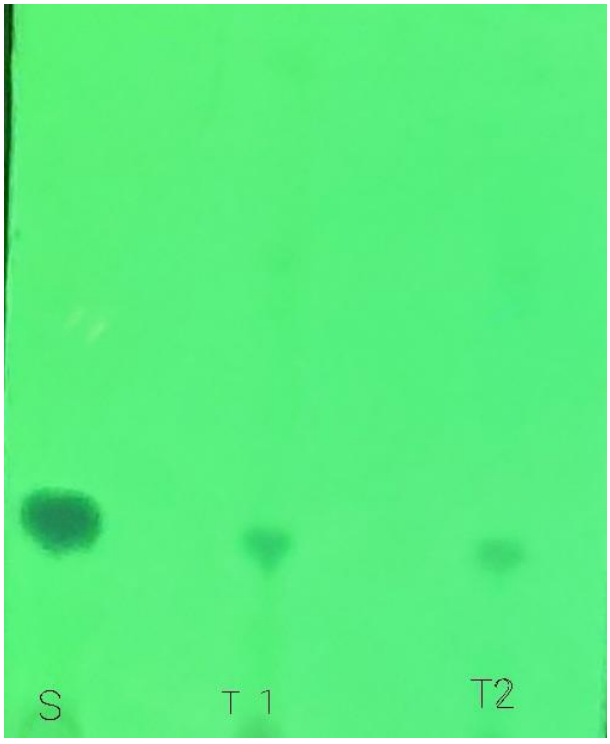

Figure 3. Analytical thin-layer chromatography for isolated rosmarinic acid under UV light at 254 nm. T=test sample, S=standard

Table 3. R_f of rosmarinic acid (RA) standard and RA isolated from methanolic extract of peppermint leaves in thin-layer chromatography

Solvent system	R_f value of RA standard	R_f value of RA in peppermint
Chloroform:ethyl acetate: formic acid (5:4:1)	0.34	0.32

Isolated rosmarinic acid was also detected by HPLC, with reference to rosmarinic acid standard, as shown in figures 4 and 5. The retention time of rosmarinic acid standard is 3.02 while the retention time of isolated rosmarinic acid is 2.97 with a single peak that supports the isolated compound is pure rosmarinic acid.

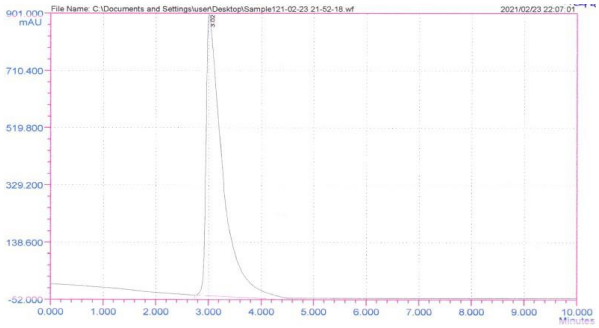

Figure 4. Analytical high-performance liquid chromatography technique for rosmarinic acid standard

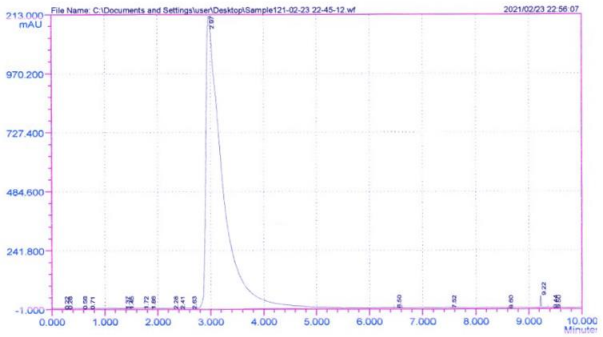

Figure 5. Analytical high-performance liquid chromatography technique for isolated rosmarinic acid

3.1. *In vitro* Antioxidant Effect of Rosmarinic Acid

The DPPH redox H_2O_2 test methods were used to test the antioxidant effect of rosmarinic acid. This approach gives an idea of the ability of rosmarinic acid to scavenge.

3.2. DPPH (2, 2-Diphenyl-1-Picrylhydrazyl) Free Radical Scavenging Assay

The findings revealed that rosmarinic acid produced yellowish color solutions on a purple backdrop (Figure 6) indicating that it had antioxidant capacity in the DPPH experiment.

Figure 6. DPPH color changes induced by rosmarinic acid

The investigation of rosmarinic acid's radical-scavenging capacity was quantified by detecting the reactivity of DPPH with RA at 517 nm, as previously described. The ability of RA to scavenge free radicals was demonstrated (IC_{50}:0.160). The percentages of free radical scavenging are provided as 95.58% and 88,67% at conc. 100, 25 µg/mL (Table 4, Figure 7), compared to the reference standard vitamin C, which shows 96.36% 95.62%, and 88.522% at the same dose as rosmarinic acid (100, 25, 10 µg/mL) (Table 5, Figure 8).

Table 4. Inhibition percentage of DPPH free radical by rosmarinic acid

Concentration (µg/ml)	Inhibition (%)
100	95.58
25	88.67
10	67.72
5	20.15
2.5	17.32
1	12.79
0.5	9.39

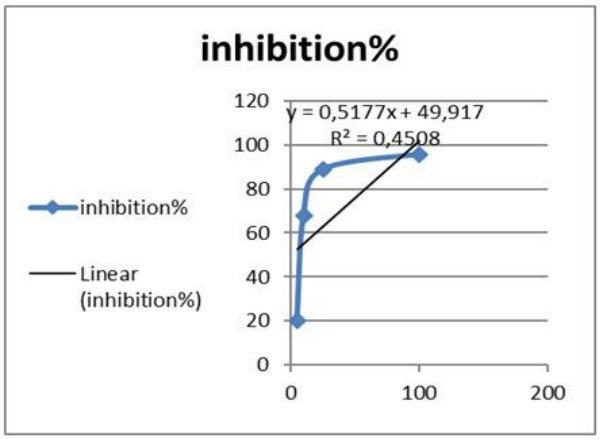

Figure 7. Inhibition percentage of DPPH free radical by rosmarinic acid

Table 5. Inhibition percentage of DPPH free radical by standard vitamin C

Concentration (µg/ml)	Inhibition (%)
100	96.36
25	95.62
10	88.522
5	68.181
2.5	26.13
1	22.76
0.5	3.409

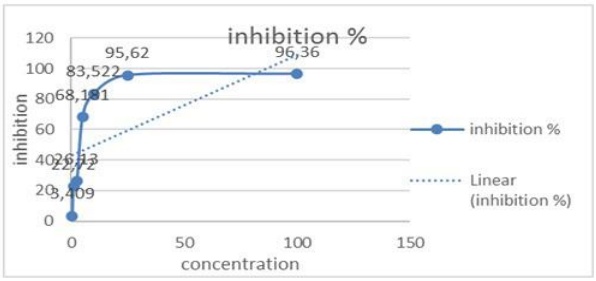

Figure 8. Inhibition percentage of DPPH free radical by vitamin C

3.3. Hydrogen Peroxide Scavenging Assay

The effect of scavenging of different concentrations for rosmarinic acid on H_2O_2 was concentration-dependent. The results showed that it was developed turbidity for the solutions which confirmed the presence of strong H_2O_2 scavenging for rosmarinic acid. As previously stated, the analysis of rosmarinic acid's radical-scavenging capability was quantified and calculated by detecting the response of H_2O_2 with RA at 230 nm.

It was clear that RA has good free radical scavenging activity of 87.83%, 76.7%, and 62.2 % at concentrations of 100, 50, and 25 µg/mL, respectively, as shown in table 6 and figure 9 with *IC_{50}28.12*. In comparison with reference standard vitamin C which showed the result of 89%, 67%, 62.34%, and 55% for the same concentration that was used for rosmarinic acid (Table 7 and Figure 10).

The H_2O_2 is found in low concentrations in the air, water, human body, plants, bacteria, and food. When it decomposes into oxygen (O_2) and water (H_2O), hydroxyl radicals (OH) are produced, which can cause lipid peroxidation and DNA damage. The capacity of RA to scavenge hydrogen peroxide may be attributed to the presence of phenolic groups, which can give electrons to H_2O_2, neutralize it, and convert it into H_2O.

Table 6. Inhibition percentage of H_2O_2 free radical by rosmarinic acid

Concentration (µg/ml)	Inhibition (%)
100	87.83
50	76.7
25	62.226
10	36.6
5	18.66

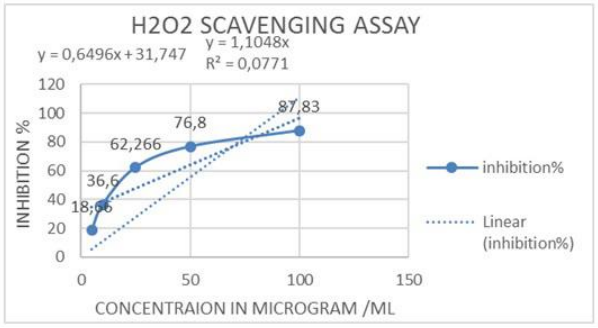

Figure 9. Inhibition percentage of H_2O_2 free radical by rosmarinic acid

Table 7. Inhibition percentage of H_2O_2 free radical by vitamin C

Concentration (µg/ml)	Inhibition (%)
100	89.67
50	62.34
25	55
10	47.67
5	19

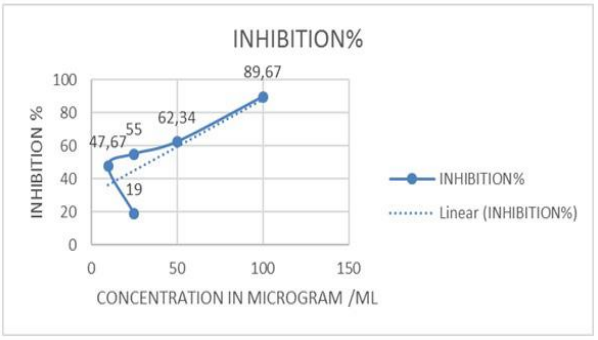

Figure 10. Inhibition percentage of H_2O_2 free radical by vitamin C

3.4. Ferric Reducing Antioxidant Power Assay

In this experiment, the presence of electron-donating chemicals caused Fe^{3+} (ferric) to be reduced to Fe2+ (ferrous). The results showed that it led to a change in the color of the solutions which confirmed that RA is a strong anti-oxidant (Figure 11). When the reducing potential of rosmarinic acid was evaluated at concentrations up to 50 µg/mL, it demonstrated general rise inactivity as the concentration was raised with absorbance (3) at 700 nm, RA had the highest lowering capability of free radicals scavenging at 25 and 50 µg/mL (Table 8 and Figure 12).

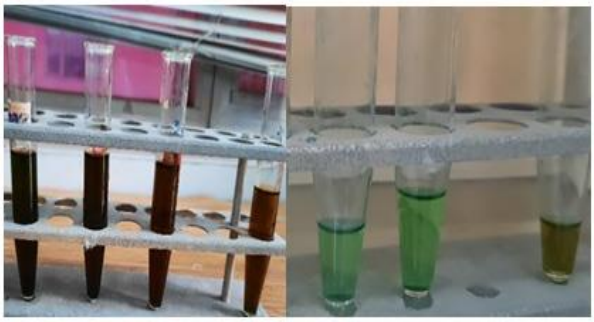

Figure 11. Color changes induced by rosmarinic acid

Table 8. Absorbance at 700 nm

Concentration (µg/ml)	Absorbance
50	3
25	3
10	1.7
5	1.657
2.5	1.113
1.25	0.46
0.625	0.44

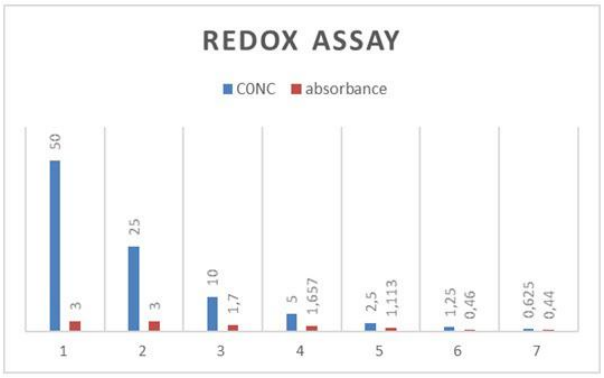

Figure 12. Absorbance at 700 nm

For the rosmarinic acid that was purified from methanolic peppermint extract obtained from reflux condenser and analyzed, the DPPH technique revealed the highest proportions of antioxidant activity of rosmarinic, which were 95.58% (at 100 µg/mL of RA) and 87.83% (at 100 µg/ml of RA) by H_2O_2 scavenging assay. The HPLC analysis at 270 nm indicated that the reflux acidify methanolic extracts with a higher concentration of rosmarinic acid whose concentration

was 1.9 mg/ml corresponded to (w/w) the rosmarinic acid content. This experiment connects technology and science to daily life while also demonstrating that commonly utilized plants have large levels of antioxidant-like compounds.

Several biotechnological and biochemical techniques have been developed to facilitate the extraction and production of bioactive compounds from medicinal plants, such as *M. peppermint*. Thanks to the identification and development of these techniques, numerous herbal plants were used for the production of various secondary metabolites, such as rosmarinic acid, cryptotanshinone, camphor, ferruginol, and sclareol (19, 20).

Rosmarinic acid is a natural phenolic compound extracted from *Rosemarinus officinalis* L. Rosmarinic acid contains two phenolic rings, both of which have the ortho position hydroxyl groups. There is a carbonyl, an unsaturated double bond, and a carboxylic acid between the two phenolic rings. The structures of both phenolic rings are different from the flavonoids (20).

The rosmarinic acid has many biological activities, such as inhibiting the HIV-1, antitumor, and anti-hepatitis as well as protection of the liver, inhibition of the blood clots, and anti-inflammation (19).

Based on related literature, there are several reports concerning the phytochemical compositions of several herbal plants. To the best of our knowledge, there is no detailed study about the Rosmatitic acid extracted from *M. piperita*.

The results are obvious and consistent, which makes it much easier to replicate, contrast, and assess them. The adoption of an HPLC technique to assess rosmarinic acid content transforms this experiment into a fascinating and versatile chromatographic approach with a wide range of applications.

Authors' Contribution

Study concept and design: F. E. H. A.
Acquisition of data: U. M. N. A.
Analysis and interpretation of data: U. M. N. A.
Drafting of the manuscript: F. H. S.
Critical revision of the manuscript for important intellectual content: F. E. H. A., U. M. N. A. and F. H. S.
Statistical analysis: F. E. H. A.
Administrative, technical, and material support: F. E. H. A., U. M. N. A. and F. H. S.

Conflict of Interest

The authors declare that they have no conflict of interest.

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