Trop J Nat Prod Res, December 2024; 8(12): 9386 - 9398
ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Gas Chromatography-Mass Spectrometry Profiling, Antioxidant and Antibacterial Evaluations of Essential Oils Extracted from *Mentha piperita* and *Mentha pulegium*

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ARTICLE INFO

Article history:
Received 04 July 2024
Revised 08 August 3034
Accepted 25 November 2024
Published online 01 January 2025

ABSTRACT

Synthetic food preservatives protect against oxidation and pathogens but can harm consumer health. Plant-based bioactive components like phenolic acids, flavonoids, and essential oils (EOs) are crucial for safe alternatives. The study investigated the chemical composition and biological activities of EOs extracted from *Mentha piperita* and *Mentha pulegium*. Essential oils from both plant species were extracted using hydro-distillation method. Gas chromatography-mass spectrometry (GC-MS) profiling was performed to determine the EOs' constituents. The EOs were assessed for antioxidant properties using three assays, while their antibacterial activity was evaluated against *Escherichia coli* and *Staphylococcus aureus*. Molecular docking and dynamic simulation studies were performed to elucidate the interaction between EO constituents and target enzymes. The results revealed that the main components in *M. piperita* and *M. pulegium* EOs were isomintlactone (35.55%) and pulegone (74.04%), respectively. The EOs of *M. piperita* and *M. pulegium* exhibited remarkable antioxidant activity, with inhibitory concentration (IC_{50}) values of 3.24±0.22 and 3.51±0.22 mg/mL, respectively, in the 2,2-diphenyl-1-picrylhydrazyl radical scavenging (DPPH) assay. Strong antibacterial efficacy was observed with a minimum inhibitory concentration (MIC) of 4% for *M. pulegium* and moderate effects for *M. piperita* with MIC values of 0.5% against *Staphylococcus aureus* and its ATCC29213, and 1% against *Escherichia coli* and its ATCC25922. Molecular docking and dynamic molecular simulations showed that the identified chemical constituents in the test EOs have a good affinity to the target enzymes. These findings suggest that *M. piperita* and *M. pulegium* EOs are promising natural sources of antioxidant and antibacterial agents, potentially valuable in food preservation and health applications.

Keywords: *Mentha piperita*, *Mentha pulegium*, Essential oil, Chemical composition, Antibacterial, Antioxidant, Activity, *in silico*.

Introduction

Food preservatives, often synthetic chemical substances, have demonstrated protective effects against food oxidation and a wide range of pathogenic microorganisms. However, they may also have harmful effects on consumer health.[1]

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Citation: El maimouni MA, El amrani S, Lairini S, Bouslamti R, Er-rajy M, Mujwar S, Chalkha M, Chebbac K, El Akhal F, Elouali Lalami A. Gas Chromatography-Mass Spectrometry Profiling, Antioxidant and Antibacterial Evaluations of Essential Oils Extracted from *Mentha piperita* and *Mentha pulegium*. Trop J Nat Prod Res. 2024; 8(12): 9386 – 9398 https://doi.org/10.26538/tjnpr/v8i12.6

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

Therefore, replacing synthetic additives with safe and non-toxic natural products is crucial. Using plant-based bioactive components like phenolic acids, flavonoids, and terpenoid essential oils (EOs) has prompted significant attention due to their multifunctional properties.[2] These naturally occurring substances are easily accessible, less toxic, have fewer adverse effects (depending on the dosage), and provide several health benefits, such as antioxidant and antimicrobial effects.[3] They are used to protect food against pathogenic agents and bacteria that cause food spoilage, including rancidity, colour fading, or degradation related to auto-oxidation.[4] Essential oils derived from plants are composed of a few dozen to hundreds of substances, most of which are terpenes and terpenoids, including their oxygenated derivatives (aldehydes, ketones, alcohols, ethers, esters, and epoxides).[5] The well-known antioxidant characteristics can be identified directly by interacting with peroxyl radicals or indirectly by transcriptionally inducing antioxidant enzymes.[6] Conversely, the antibacterial effect might be observed as bacteriostatic and bactericidal effects on pathogenic bacterial strains, sometimes even at very low concentrations.[7]

Among the most economically significant genera with aromatic and medicinal properties are the *Mentha* species, which belong to the Lamiaceae family. These species are extensively utilized for health and medicinal purposes, primarily due to their large amounts of substances like thymol, menthol, carvacrol, and menthone.[8] These various bioactive compounds present in *M. piperita* and *M. pulegium* make them some of the most important spices and food preservatives, with interesting antimicrobial and antioxidant activities.[9] In investigative research, *in silico* studies using dynamic molecular simulations, molecular docking, and absorption, distribution, metabolism, excretion, and toxicity (ADME-Tox) analyses are usually conducted to validate and support the outcomes of the *in vitro* experiments. Molecular docking (M-docking) and dynamic molecular simulations may demonstrate the existence of a good affinity between the main chemical constituents identified in the EO studied and the target enzymes. The results of the *in vitro* and *silico* studies carried out for the first time in the present research, could increase the value of the EOs studied as a natural source of potential antioxidants and antibacterial agents. The present study was conducted to profile the chemical constituents of *M. piperita* and *M. pulegium* essential oils using the gas chromatography-mass spectrometry technique. The antioxidant and antibacterial activities of the essential oils using *in vitro* and *silico* approaches were also investigated.

Material and Methods

Plant Collection and Identification

The *M. piperita* and *M. pulegium* plants were cultivated and collected from Timezgana commune situated in Taounate Province, North Central Morocco (34°33′ 02.7″ N 4°40′49.3″ W) in April and May 2021. Professor Badr Satrani, a botanist at Morocco's Forestry Research Centre (FRC), Rabat, identified these species. Each plant's voucher specimen was deposited at the herbarium of Morocco's National Agency of Medicinal and Aromatic Plants.

Source of bacterial strains

Food-borne *Escherichia coli* and *Staphylococcus aureus*, as well as *E. coli* ATCC 25922 and *S. aureus* ATCC 29213, obtained from the Epidemiological Laboratory of the Regional Health Directorate, Fez-Meknes, were used to evaluate the antibacterial activity. Nutrient agar (Fluka, Seelze, Germany) was used to culture the bacterial species studies. The cultures were incubated at 37 °C for 24 hours.

Extraction of essential oils from Mentha piperita and Mentha pulegium

The entire aerial parts of the plants, including the branches, were shade-dried for 72 hours at room temperature (an average of 35±4 °C). EOs were extracted from dried plant material using a Clevenger-type apparatus through hydrodistillation. For this process, 250 g of the plants were boiled with 1 L of distilled water in a 2-liter flask for 2 hours and 30 minutes at a constant temperature of 85°C. The vapor containing the oils passed through a condenser, where it was condensed and collected in a burette. The final distillate comprised both hydrolate and essential oils. The EOs were collected by decantation, dried over anhydrous sodium sulfate, and then stored in amber-coloured vials at 4°C until usage.

Determination of the chemical composition of the essential oils

The constituents of *M. piperita* and *M. pulegium* EOs were determined employing a Shimadzu GC (Trace GC Ultra, Hewlett-Packard, HP 6890), coupled with an MS (MS, HP 5973) (Tokyo, Japan), supplied with HP-5MS column (60 m × 0.32 mm × 0.25 µm). The GC-MS analysis was conducted following the standard procedures. Helium (≥99.995%; Sigma-Aldrich, USA) was used as the carrier gas at a flow rate of 1.0 mL/min; a temperature rise of 5°C/min was planned for the column, from 40 to 280°C; and at 220 °C, a split mode injection of 1 µL of sample volume was performed. The samples of EOs were diluted (1/20 v/v) in hexane (≥99%; Sigma-Aldrich, USA) before injecting the sampling port. Quantitative analysis was made via HP ChemStation software, and the components were identified using their Kovats retention index (KI), which was computed against the n-alkanes (C8-C23) series. Besides that, the identification was validated by comparing its constituents' mass spectral fractionation with the NIST MS Search database 2012 and the Adams terpenes library.[10]

Measurement of the antioxidant activity of essential oils using the DPPH radical assay

The DPPH test was conducted accurately as previously described in the literature, with a few modifications.[11] The DPPH solution was obtained by solubilizing 0.005 g of DPPH in 200 ml of absolute ethanol (≥99.8%; Sigma-Aldrich, USA). Then, 25 µL of the sample at various concentrations was mixed with 825 µL of DPPH. The tubes were agitated and kept at ambient temperature in the dark for an hour. The absorbance readings were obtained at 517 nm using a UV spectrophotometer (BK-D560; BIOBASE, China). The standard used was butylated hydroxytoluene (BHT). Equation 1 was employed to estimate the antiradical activity.[11] The IC_{50} values were determined graphically from a plot of I % against the concentrations. Every test was performed three times.

$$\text{I \% of DPPH} = ([\text{Abs Ctrl} - \text{Abs spl}]/\text{Abs ctrl}) \times 100$$

…… (Equation 1)

Where Abs ctrl is the absorbance of the employed control (consisting of all reagents, except the test EOs), and Abs spl is the absorbance of test EOs.

Evaluation of the antioxidant activity of essential oils using the cation radical ABTS assay

Method to evaluate the antioxidant activity of essential oils (EOs) involves assessing their ability to inhibit the ABTS radical cation (2,2'-azino-bis [3-ethylbenzothiazoline-6-sulfonic acid]). This procedure, adapted from the method described by De León-Zapata *et al*.,[12] is based on the principle of converting the stable ABTS+ radical cation back to ABTS in the presence of antioxidants. To regenerate the ABTS+ radical cation, a solution of ABTS (7 mM) was mixed with potassium persulfate (2 mM) and then stored for 16 hours at room temperature in the dark before use. The spectrophotometric absorbance of the solution was adjusted to 0.70 at 734 nm by dilution with ethanol. Then, 50 µL of the sample at different concentrations was combined with 825 µL of ABTS+. After 6 minutes, the absorbance was measured at 734 nm. The antioxidant activity of each test EO was compared to that of gallic acid, and the antiradical activity was determined accordingly to Equation 2. The IC_{50} values were computed graphically with the use of linear regression. Every test was conducted three times.

$$\text{I \% of ABTS} = ([\text{Abs Ctrl} - \text{Abs spl}]/\text{Abs ctrl}) \times 100$$

…… (Equation 2)

Where Abs ctrl is the absorbance of the positive control (which included all reagents, except the EOs for testing), and Abs spl is the absorbance of test EOs.

Evaluation of the total antioxidant capacity (TAC) of the essential oils

The phosphomolybdate test was performed using the technique described by Prieto *et al*.,[11] with slight modifications. The phosphomolybdate reagent was prepared by combining sulfuric acid (99.999%; Sigma-Aldrich, USA; 25 mL in 225 mL of distillate water), sodium phosphate (96%; Sigma-Aldrich, USA; 3.28 g in 250 mL of distilled water), and ammonium molybdate (99.999%; Sigma-Aldrich, USA; 3.7 g in 250 mL of distilled water). Briefly, 100 µL of sample and standard were mixed with 1 mL of phosphomolybdate reagent. The absorbance measurement was made at 700 nm after incubation for 1 hour and 30 minutes at 96°C. A standard blank solution, which included 1 mL of reagent solution and an appropriate quantity of the solvent used to prepare the sample, was incubated with the other samples under the same conditions. The antioxidant efficacy was computed employing an established calibration range with ascorbic acid (99%; Sigma-Aldrich, USA) and recorded in milligrams of ascorbic acid equivalent per gram of EO (mg AAE/g EO). Every test was conducted three times.

Evaluation of the antibacterial activity of the essential oils using the disc diffusion method

The antibacterial effect of the two EOs was first screened against bacterial strains using the disc diffusion method.[13] Muller Hinton (Fluka, Seelze, Germany) plates were seeded with a bacterial inoculum adjusted to 0.5 Mc Farland standard. Subsequently, the inoculated agar

was covered with a sterile Whatman paper disc (6 mm in diameter) that had been impregnated with 10 µL of each EO. After incubation at 37°C for one day, inhibition diameters, including disc diameter, were determined. Novobiocin and chloramphenicol antibiotics (OXFOR, England) were chosen as positive controls. Sterile distilled water was applied as a negative control. Antibacterial activity was considered highest when the zone of inhibition exceeded 15 mm, moderate between 15 and 8 mm, and low below 8 mm.[14] The analyses were repeated in triplicates.

Determination of the minimum inhibitory concentration of the essential oils

The minimum inhibitory concentration (MIC) of the two EOs was established using the broth microdilution technique.[15] A 96-well polypropylene microtiter plate was loaded with 50 µL of MH broth (Fluka, Seelze, Germany) supplemented with 0.15% (w/v) agar (Fluka, Seelze, Germany). An aliquot of 50 µL of EO at a terminal concentration of 4% (v/v) was poured into the first well. Then, 50 µL was pipetted from the first well and transferred to the subsequent well to obtain a ½ serial dilution. This procedure was repeated up to the twelfth well, and the last 50 µL of the combination was thrown away. Lastly, each well was filled with 50 µL of bacterial culture, resulting in a total concentration of around 10^6 CFU/mL. The concentrations of the EO in the wells varied between 4 and 0.00195% (v/v). Next, the 96-well plate was sealed with parafilm and incubated for a full day at 37°C. Then, 5µL of resazurin was added to each well, and the plate was incubated at 37°C for 2 hours. The MIC was defined as the lowest EO concentration that inhibited bacterial growth, as observed by the resazurin's colour being unchanged. Positive growth was measured by reducing the blue resazurin dye to pink resorufin[16]. The analyses were repeated in triplicates.

Molecular docking analysis

Before the docking study, the main chemical components identified in EOs derived from *M. piperita* and *M. pulegium* species were drawn in ChemDraw 16.0, and then the geometry was MM2 optimized.[17] The Discovery 2021 software was used to explore the ligand-protein interaction,[18] eliminate water molecules, curate missing side-chain residues, and merge non-polar hydrogens. For the antioxidant analysis, the Nicotinamide Adenine Dinucleotide Phosphate Hydrogen (NADPH) oxidase receptor extracted from the protein data bank (PDB ID: 2CDU with a resolution of 1.80 Å) was utilized.[19] A grid was created with parameters X = 10.172, Y = 0.704, and Z = 6.106 Å. The protein-ligand complex was docked using Lamarck's genetic algorithm (LGA) to get the lowest binding free energy (ΔG). The 3D structure of enzymes obtained from the Research Collaboratory for Structural Bioinformatics (RCSB) database (PDB codes: 1KZN for *E. coli* with a resolution of 2.30 Å) was used for the antibacterial study.[20] A grid was created with parameters X = 19.500, Y = 30.390, and Z = 34.740 Å. Following the algorithm LGA, docking investigations of the protein-ligand complex were executed to find the lowest ΔG. For M-docking studies, a grid of 60*60*60 points in the x, y, and z directions, as well as a total number of 100 solutions calculated in each case, with a population size of 300, were employed.

Molecular dynamic simulation

A molecular dynamic (M-dynamic) simulation was conducted to validate the thermodynamic stability of ligand-protein complexes, including the ligands pulegone and isomintlactone with the Deoxyribonucleic Acid (DNA) gyrase enzyme of *E. coli*, as well as the ligands menthone and isomintlactone with the NADPH oxidase enzyme. These complexes were shortlisted based on their docking score, physicochemical analysis, and detected chemical contacts with the target receptors. The M-dynamic simulations were executed for 100 ns using the Desmond module of Schrodinger's Maestro software.[21] The process involved the addition of explicit solvent molecules and their neutralization by adding the corresponding ions. The steepest-descent algorithm was used to relax the system and eliminate any steric clashes or poor contacts within atoms to minimize the system's energy. The system was brought to equilibrium through short series simulations with low temperature and constant pressure (NPT). Positional constraints were applied to the system in addition to a progressive increase in temperature.[22] The system's stability and balance were enhanced before the simulation to ensure the desired outcomes. The simulation was performed for 100 ns, considering the system's energies, atomic positions, and RMSD (Root Mean Square Deviation) values. This aids in comprehending the system's dynamic behaviour and provides long-term intuitions on the complex's structure and functional stability.[23]

Determination of ADME-Tox properties

A comprehensive grasp of absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties is indispensable in evaluating the feasibility of potential therapeutics within the swiftly evolving realm of drug discovery.[24,25] A compound's "drug-likeness" is crucial for its potential for successful development as an orally administered medication. In this regard, a thorough *in silico* assessment of these features was performed for the primary chemical compounds found in the test EOs of *M. piperita* and *M. pulegium* using the SwissADME and pkCSM web servers.[26,27] The key parameters to determine a drug's bioavailability were considered in the analysis, including lipophilicity, water solubility, saturation, flexibility, and different pharmacokinetic characteristics. Furthermore, a drug similarity assessment was conducted based on adopted pharmaceutical norms, including Lipinski's five rules.[28]

Statistical analysis

Three replicates of each test were conducted. The data were analyzed using three measurements obtained at each data point in each repetition to compute medians and standard deviations. GraphPad Prism 10, Statistical Package for the Social Sciences (SPSS; version 2022), and Excel 2010 were applied to analyze the data and presented as mean ± standard deviation.

Results and Discussion

The chemical composition of the essential oils extracted from Mentha species

The results of the GC-MS analysis of *M. piperita* and *M. pulegium* EOs are presented in Table 1 and Figure 1. The obtained results revealed that *M. piperita* EO contained 82.44% of four volatile substances, and three major constituents were detected in *M. pulegium* EO, accounting for 86.07% of the total EO.

The analysis of the chemical composition of *M. piperita* EO indicated that its main components were pulegone (10.37%), menthone (18.48%), eucalyptol (18.04%), and isomintlactone (35.55%). Extensive research on the composition of *M. piperita* EO has previously been published. Guedes *et al.*,[29] found that one species of a Brazilian EO contained menthol (59.73%), isomenthone (18.45%) and methyl acetate (6.02%). In the same country but in a different region, another study,[30] identified carvone D (49.27%) and limonene (37.18%) as major components in *M. piperita* EO. Algerian oil is characterized by the following major components: menthol (49.89%), menthone (20.84%), isomenthone (7.25%), 1,8-cineole (6.73%), and ciscarane (4.99%).[31] In India, a group of researchers,[32] obtained menthol (34.82%), carvone (19.54%), and menthone (9.10%). Furthermore, the major components of Korean *M. piperita* EO are menthol (4.30%), caryophyllene (5.50%), and 1,8-cineole (62.16%), as reported in the literature.[33] Iranian and Saudi Arabian *M. piperita* EOs contain similar components. The Iranian EO,[34] contains menthol (36.9%), menthone (28.8%), menthyl acetate (4.54%), and 1,8-cineole (3.75%), and the Saudi Arabian EO,[9] contains menthol (36.02%), menthone (24.56%), menthyl acetate (8.95%), and menthofuran (6.88%).

On the other hand, the EO derived from *M. pulegium* was composed of 22 components. Pulegone (74.04%), piperitone (7.94%), and menthone (4.09%) make up their most significant compositions. It has been observed that the presence of pulegone as a major constituent of this oil suggests that it belongs to the pulegone chemotype. These findings are consistent with most previous studies on *M. pulegium* essential oil, although the percentages differ. Other researchers have found other major components of *M. pulegium* EO to include pulegone (75.48%), carvone (6.66%), and dihydrocarvone (4.64%) as reported by Chraibi

et al.,[35] isomenthone (13.4%) by El Asbahani *et al*.,[36] menthone (21.16%), and pulegone (40.98%) by Bouyahya *et al*,[7]

Table 1: The chemical composition of *Mentha piperita* and *Mentha pulegium* essential oils

Compound	RI Obs	RI Lit	(%)	
			M. piperita	*M. pulegium*
Sabinene	897	920	1.13	-
β-Pinene	943	949	2.53	-
α-Pinene	948	949	1.71	0.39
3-Methylcyclohexanone	952	937	-	0.48
3-Octanone	979	985	-	0.84
D-Limonene	1018	1029	2.90	0.94
Eucalyptol	1059	1031	18.04	-
Linalool	1082	1085	0.78	-
α-Fenchol	1138	1100	2.15	-
Borneol	1138	1169	1.08	-
Isomintlactone	1142	1219	35.55	-
p-Menth-3-en-8-ol	1143	1149	-	1.17
Menthone	1148	1157	18.48	4.09
α-Elemene	1170	1177	-	0.90
Piperitone oxide	1171	1230	-	0.30
Piperitone	1190	1232	-	7.61
Pulegone	1212	1237	10.37	74.04
1,6,6-Trimethyl-8-Oxabicyclo(3.2.1)octan-2-one	1230	1229	-	0.33
Acetaldehyde	1310	1309	-	2.68
α –Terpinyl-Acetate	1350	1352	-	0.37
Menthofuran	1368	1364	0.99	0.32
(+)-Mintlactone	1368	1368	0.75	0.31
Caryophylene oxide	1368	1583	-	0.57
Acid methyl ester	1372	1378	-	0.75
α-Humulene	1494	1446	-	1.56
Caryophyllene	1494	1466	1.65	0.76
Humulene epoxide II	1518	1593	-	0.86
p-Cresol	1565	1071	0.68	-
Benzofuranone	1579	1539	-	0.28
β-Resorcylaldehyde	1650	1450	1.23	0.45
Monoterpene hydrocarbons			8.27	1.33
Oxygenated monoterpenes			88.19	88.17
Sesquiterpene hydrocarbons			1.65	3.79
Oxygenated sesquiterpene			0.00	0,86
Others			1.91	5.85
Total			**100**	**100**

RI Obs: Retention index obtained; RI Lit: Retention index of literature

pulegone (69.8%) and piperitenone (3.1%) by Ait-Ouazzou and its colleagues [37] and piperitone (38.00%), piperitenone (33.0%), and α-terpineol (4.7%) by Mahboubi and Haghi.[38]

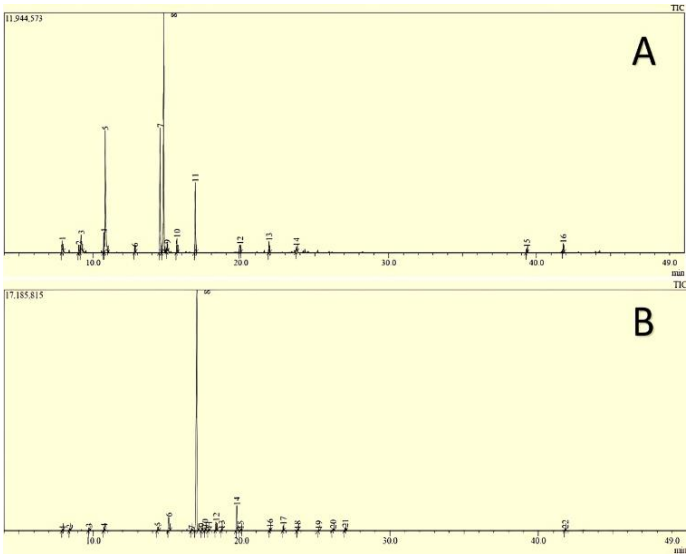


Figure 2: Gas chromatogram profiles of essential oils obtained by gas chromatography/mass spectrometry (GC/MS). A: *Mentha piperita*; B: *Mentha pulegium*.

Menthol, a primary component of *M. piperita* EO, is commonly employed in pharmaceuticals, cosmetics, and food products due to its cooling and analgesic properties. Menthone, another compound found in these species, is used in the production of perfumes and flavourings. Thymol and carvacrol, which are present in high amounts in plants like *M. pulegium*, are known for their antimicrobial and antioxidant properties. The quantitative and qualitative differences in the EOs' composition could be linked to several factors, including the plant's origin, climate, geographical locations, and genetic diversity.[9,39] This variation may also be due to environmental variables, agronomic characteristics, the drying process, oil extraction technology, and the harvesting period.[31,40,8]

The antioxidant activity of the essential oils extracted from Mentha species

The antioxidant capacity of the resulting EOs was assessed via three previously established techniques (DPPH, ABTS, and TAC). A single technique will offer basic insights regarding antiradical characteristics, but several approaches will characterize the sample's antioxidant qualities in greater depth.[41] Scavenging free radical DPPH was an approach to investigate the antioxidant action of the EOs from the two *Mentha* species. According to the results outlined in Figure 2, *M. piperita* EO exhibited the highest antiradical action with an IC_{50} value of 2.99±0.20 mg/mL, followed by *M. pulegium* EO, which had an IC_{50} value of 3.24±0.20 mg/mL, compared to standard BHT (IC_{50} = 0.039±0.023 mg/mL). The difference in antioxidant capacity of the two EOs might be attributed to their different chemical profiles, involving various functional groups, polarity, and chemical behaviour.[42] The obtained IC_{50} value for *M. piperita* (IC_{50} = 2.99±0.20 mg/ml) was lower than that reported by other authors, such as Raeisi *et al*.,[43] who found an IC_{50} value of 7.81±0.23 mg/mL. Meanwhile, the EO of Algerian *M. piperita*,[44] showed a higher activity of radical scavenging than that obtained in the present study, which had a value of 17.0 µg/mL. The variations in the antiradical capacity of peppermint oil can be attributed to the complex and changeable chemistry of this EO, which is affected by diverse elements like the climate, cultivar, and geographical position.[45] For *M. pulegium* EO, the result of the antioxidant potency (IC_{50} of 3.24±0.20 mg/mL) was lower than those found in the studies carried out on the same plant by Bouyahya *et al*.,[7] and Benabdallah *et al*.,[44] which showed higher radical scavenging activities (IC_{50} values of 0.32±0.002 and 0.025 mg/mL, respectively).

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

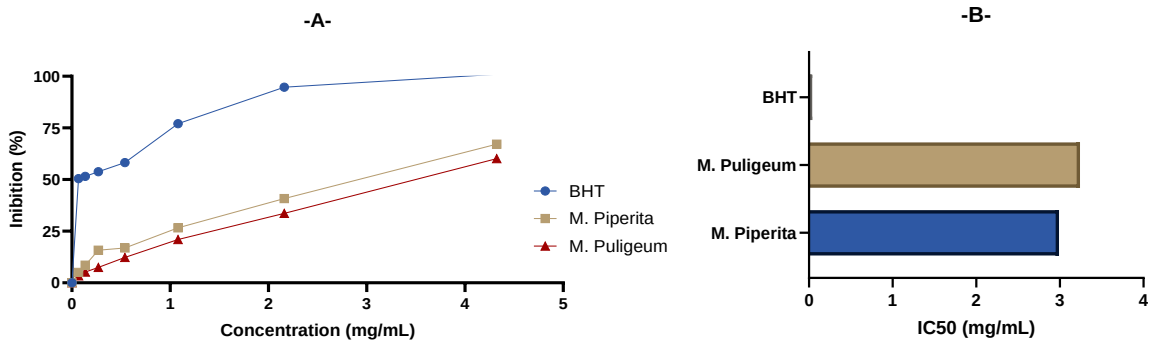


Figure 3: Antioxidant activity of essential oils extracted from *Mentha* species using butylated hydroxytoluene as a reference.
A: Anti-radical activity of *Mentha piperita* EO, *M. pulegium* EO and BHT by DPPH; B: IC_{50} values of anti-radical activity of *M. piperita* EO, *M. pulegium* EO and BHT; BHT: butylated hydroxytoluene; DPPH: 2,2-diphenyl-1-picrylhydrazyl; EO: Essential oil.

In contrast, several researchers have reported that this EO has lower antioxidant activity (IC_{50} values of 14.73±0.15 mg/mL,[46] and 6.2±0.2 mg/mL,[47]) than the values obtained in the present study. This suggests that the chemical composition of *M. pulegium* EO could be the source of its antioxidant activity.

The results of the ABTS test support the findings of the DPPH assay by classifying *M. piperita* EO as the most active, with an IC_{50} value of 0.96±0.29 mg/mL, while *M. pulegium* EO had the lowest antioxidative activity, with an IC_{50} value of 1.02±0.21 mg/mL (Figure 3). However, the gallic acid used as an antioxidant standard showed a higher potency than both test EOs, with an IC_{50} value of 0.102±0.05 mg/mL. Similar previous research has revealed that the EOs of both *Mentha* species have varied antioxidant activities, with IC_{50} values ranging from 1.41±0.01 to 6.59±0.02 µg/mL for *M. piperita*,[48] 155 µg/mL,[49] and 5.72±0.060 mg/mL,[42] for *M. pulegium*. These findings do not corroborate with our results. The variations in the outcomes may be due to several factors, such as the plant material's place of origin, the plant part utilized, the harvest season, the stage of phenology, and the conditions of extraction, and storage. The analysis of the total antioxidant capacity of the essential oils showed that the phosphomolybdate reduction capacity of *M. piperita* EO was 10.42±0.03 mg AAE/g EO. The value appeared to be higher than the findings for the same species from Corsica,[42]. Furthermore, the phosphomolybdate test on *M. pulegium* EO confirms its potent antioxidative ability (9.94±0.21 mg AAE/g EO). Therefore, the EO in the present study has a much higher antioxidative activity than those of the same species found in Algeria (0.159 mg AAE/g EO).[50] Similarly, the research performed by Baali *et al.*,[42] on the chemical composition and biological activity of *M. pulegium* EO yielded a value (0.109 mg AAE/g EO) that was significantly lower than that obtained in the present study.

Antibacterial activity of the essential oils extracted from Mentha species

The disc diffusion method was used to investigate the inhibitory power of EOs on bacterial strains, with results shown in Table 2 and Figure 4. The antibacterial activity of *M. pulegium* EO was low against foodborne *S. aureus* and *E. coli*, with a value of 9.00±0.00 mm as the inhibition diameter for both strains. *Mentha piperita* EO produced zones of inhibition ranging from 9.33±0.89 mm for *E. coli* isolated from food to 19.00±1.33 mm for *E. coli* ATCC. The order of efficacy of the antibacterial activity of EOs against the study bacteria is *M. pulegium* > *M. piperita*. For *M. pulegium* EO antibacterial activity, a previous Moroccan study ,[40] discovered 15 mm as the inhibition diameter against *S. aureus* MBLA and *S. aureus* 994. The results of Benahmed *et al.*,[51] were higher for *S. aureus* (20.33±5.69 mm). Several more investigations have demonstrated the positive activity of *M. pulegium* EO.[52,53,54] The inhibitory properties of this oil may be linked to the high proportions of menthone, piperitenone, and pulegone.[37] Also, pulegone, a ketone recognized for its inhibitory properties, has been implicated. Several biological features of pulegone have been identified, including antibacterial activity.[55] Benahmed *et al.*,[44] argued that it is challenging to identify the specific chemicals responsible for the antibacterial activity. Thus, synergistic effects between the different constituents may also explain the antimicrobial activity of this EO.[51]

Table 2: Effect of essential oils and antibiotics on inhibition zone diameters of test bacterial strains

Compound	Inhibition zone diameter (mm)			
	S. aureus	***E. coli***	***S. aureus* ATCC 29213**	***E. coli* ATCC 25922**
M. pulegium EO	9.00±0.00a	9.00±0.00a	19.00±1.33b	10.33±0.44 a
M. piperita EO	12.66±0.44b	9.33±0.89 a	14.33±0.44b	19.00±1.33c
Novobiocin	30.00±0.60c	9.66±0.40 a	37.66±0.40d	16.33±1.10b
Chloramphenicol	23.33±0.40b	25.00±0.60c	27.33±1.10d	17.33±0.80 a

EO: Essential oil; Means denoted with different alphabets are significantly different ($p < 0.05$).

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

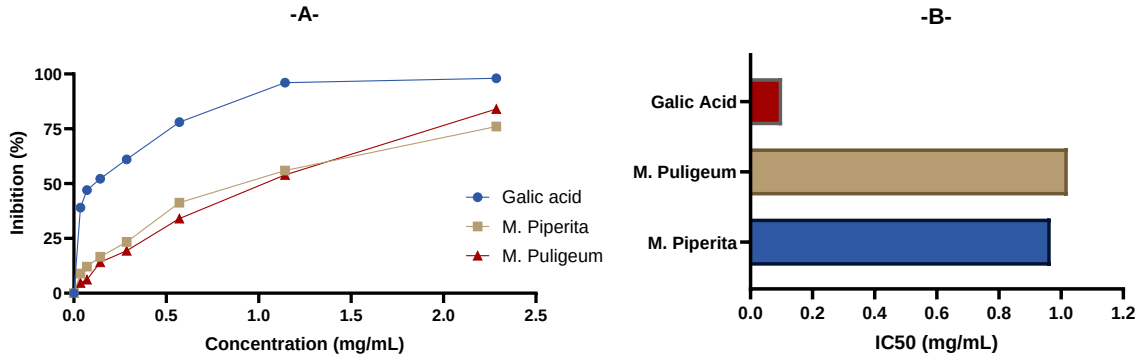


Figure 4: Antioxidant activity of essential oils extracted from *Mentha* species using galic acid as a reference.
A: Anti-radical activity of *M. piperita* EO, *M. pulegium* EO and gallic acid by DPPH; B: IC_{50} values of anti-radical activity of *M. piperita* EO, *M. pulegium* EO and gallic acid; DPPH: 2,2-diphenyl-1-picrylhydrazyl; EO: Essential oil.

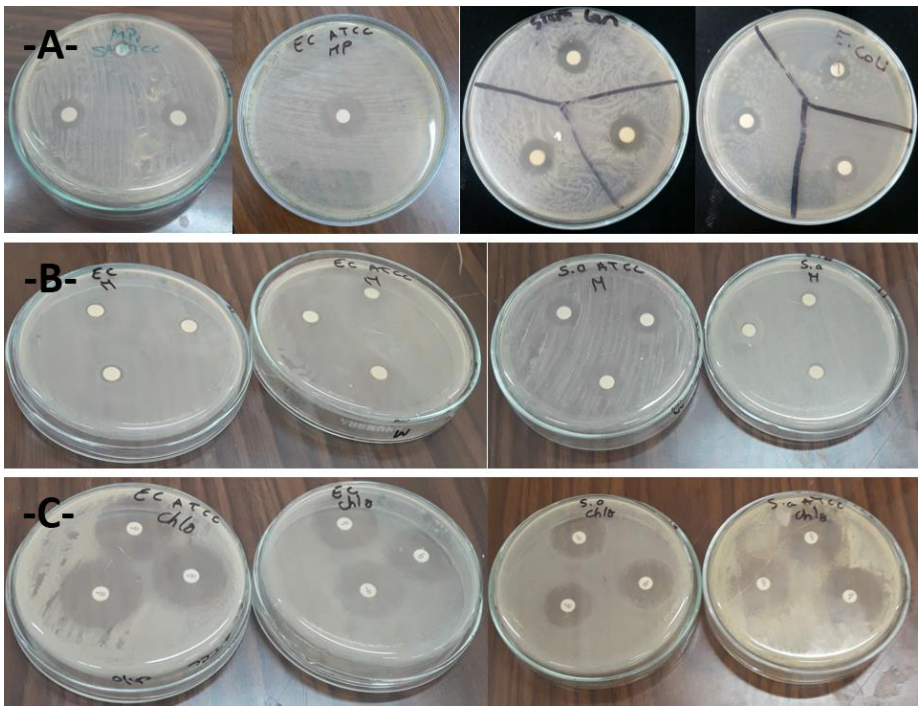


Figure 5: The inhibition zone diameters of essential oil against *S. aureus, S. aureus* ATCC 6538, *E. coli*, and *E. coli* ATCC 25922.
A: *Mentha piperita* essential oil; B: *M. pulegium* essential oil; C: Chloramphenicol

Concerning *M. piperita* EO, the results remain moderate compared to other studies conducted in other countries.[9,45] According to a previous stud,[56] the antimicrobial activity of the EOs of three mint species (*M. piperita*, *M. aquatica*, and *M. longifolia*) showed that *M. piperita* had the highest inhibitory power against pathogenic strains, including *E. coli* ATCC 25922 with an inhibition diameter of 22.6±1.67 mm and *S. aureus* ATCC 6538 with a diameter of 39.8±0.44 mm. The lower antibacterial effect of *M. piperita* observed in the present study could be attributed to menthol, one of its major compounds known for its low antibacterial activity.[57] To explain these results, EOs' antibacterial effects are hard to trace to a single molecule because of their complexity and variety. However, several studies have found a correlation between the chemical composition of the most abundant constituents in EOs and their antibacterial properties.[37,58] Essential oils have complicated combinations consisting of several parts, their structures, and interactions are connected to their antibacterial activity. There are three possible kinds of interactions, which include antagonistic, synergistic, and additive.[59]

The minimum inhibitory concentration of the essential oils extracted from Mentha species

The MIC results (Table 3) confirm the disc diffusion test. The four bacteria were sensitive to the action of the test EOs. The EO of *M. pulegium* showed a MIC value of 4.0±0.0% against the four test bacteria. *Mentha piperita* EO displayed a moderate antibacterial effect with MIC values of 0.5± 0.0% (v/v) against *S. aureus* and *S. aureus* ATCC 29213, and 1.0±0.0% (v/v) against *E. coli* and *E. coli* ATCC 25922. The MIC value obtained for *M. pulegium* was higher than that of Chraibi *et al*.,[55] who found a MIC of 0.1% (v/v) for *E. coli* and 0.05% (v/v) for *S. aureus*. Similarly, other studies,[54] revealed that *M. pulegium* EO had an inhibitory action on these strains with MIC values of 1.40 and 2.80% against *E. coli* and *S. aureus*, respectively. A similar study,[47] demonstrated that the different test bacterial suspensions were sensitive to *M. pulegium* EO, including *E. coli*, with a MIC of 3.2% (v/v). The same same species from Taounate,[60] showed inhibitory action for the two test strains, with MIC values of 0.5% (v/v) and 1% (v/v) for *S. aureus* and *E. coli*, respectively.

The study by Chraibi *et al*.,[60] on *M. piperita's* EO showed its significant inhibitory action against *S. aureus* and *E. coli*, with MIC values of 0.062

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

and 1%, respectively. Also, another study conducted for Moroccan EO,[61] showed high activity for all test bacteria (*S. aureus* 994 with a MIC value of 0.25%, *E. coli* K12 with a MIC value of 0.0625%). In another study,[56] the species of peppermint collected in June 2000 in Serbia showed moderate MIC values against *E. coli*. Some researchers have observed a link between the chemical composition of the most prevalent components in EOs and antibacterial activity.[62] Meanwhile, some hydrocarbons also have antibacterial activity. Oxygenated terpenoids (such as alcohols and phenolic terpenes) are often responsible for the antimicrobial effects of EOs.[63]

Molecular interactions between chemical constituents and target enzymes according to the molecular docking analysis

The M-docking study offered a promising explanation for the major chemical constituents identified in EOs derived from *M. piperita* (pulegone, menthone, isomintlactone, and eucalyptol), and *M. pulegium* (pulegone, piperitone, and menthone) for their antibacterial and antioxidant activities. The *in silico* results obtained are summarized in Table 4. For the antibacterial study, the chemical constituents displayed docking scores varying within the range of 4.95 to -5.70 kcal/mol, indicating a strong binding affinity to the target enzyme of *E. coli* strain (1KZN), compared to the reference antibiotic, chloramphenicol, which scored -5.65 kcal/mol. According to the outcomes listed in Table 4, the two main components, isomintlactone identified in *M. piperita*, and pulegone identified in *M. pulegium*, showed important binding affinity to the target receptor of *E. coli* with docking scores of -5.70 and -5.52 kcal/mol, respectively. For the complexes, NADPH oxidase receptor (ID: 2CDU) with different chemical substances, the energy scores were between -5.11 and -6.39 kcal/mol, demonstrating strong binding potential compared to the standard antioxidant gallic acid (-4.02 kcal/mol). Among the identified constituents, isomintlactone and menthone, exhibited notable binding affinities against the NADPH oxidase receptor, as evidenced by their docking scores of -6.39 and -5.93 kcal/mol, respectively.

Table 3: Binding energy scores (Kcal/mol) of essential oils

Compound	Chemical constituent	Binding energy	
		Antibacterial activity	Antioxidant activity
M. pulegium essential oil	Pulegone	-5.52	-5.66
	Piperitone	-5.23	-5.11
	Menthone	-5.16	-5.93
M. piperita essential oil	Menthone	-5.16	-5.93
	Pulegone	-5.52	-5.66
	Isomintlactone	-5.70	-6.39
	Eucalyptol	-4.95	-5.74
Chloramphenicol		-5.65	-
Gallic acid		-	-4.02

Following the binding energy results of molecular docking, the two compounds, pulegone, and isomintlactone, emerged as promising multi-target agents against the *E. coli* receptor (1KZN) (Figure 5). After visual observation of the results for complex isomintlactone-1KZN, three hydrogen bonds with residues ARG 76, ARG 136, and GLY 77 were observed, with distances equal to 2.73, 2.98, and 2.02 Å, respectively. Also, there are three alkyl bonds with residues ILE 78, PRO 79, and ALA 47. For complex pulegone-1KZN, there are two hydrogen bonds with residues ARG 76 and GLY 77 with distances equal to 3.02 and 1.67 Å, respectively. Also, there are three alkyl bonds with residues PRO 79, ILE 78, and ARG 76. The study indicated that the identified components can effectively inhibit the studied receptors. On the other hand, based on M-docking binding energy results, the two compounds, menthone, and isomintlactone, emerged as promising antioxidant agents against the 2CDU receptor (Figure 5). After visual observation of the results, for the isomintlactone-2CDU complex, three hydrogen bonds with residues GLY 161, GLY 158, and ILE 160 were observed, with distances equal to 1.99, 2.38, and 1.91 Å, respectively. Also, there are two alkyl bonds with residues PHE 245, and PRO 298. For the menthone-2CDU complex, there is one hydrogen bond with residue TYR 159, with a distance equal to 1.61 Å. There are three alkyl bonds with residues PHE 245, ILE 160, and PRO 298. These results suggest that the two identified components can be good inhibitors against the receptors studied.

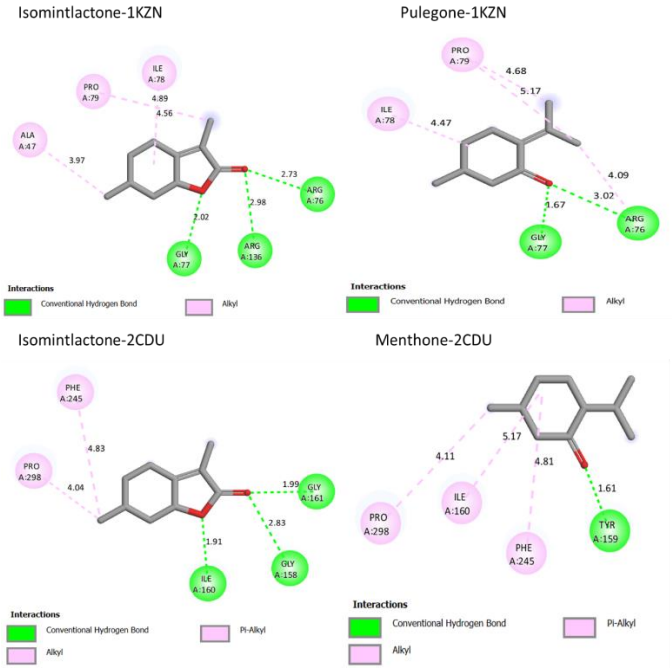


Figure 6: 2D interactions occur between isomintlactone and pulegone with the 1KZN receptor, as well as between isomintlactone and menthone with the 2CDU receptor.

Molecular dynamic simulation outcome

A drug-receptor complex has to be sufficiently stable over a nano-scale time range to execute a therapeutic response. As a result, the macromolecular complex underwent a 100 ns M-dynamic simulation using Schrodinger's Desmond software (version 2022.4). The bacterial target DNA gyrase enzyme's monomeric chain had 186 amino acids, consisting of 1,446 heavy atoms out of 2,884. Structural alterations and RMSD analysis of the macromolecular backbone were executed during the 100 ns simulation to evaluate their thermodynamic stability. The complexed ligand pulegone, consisting of eleven heavy atoms of twenty-seven, lacks any rotatable bond. The RMSD value of the receptor's backbone was found to fluctuate between 1.25 and 2.25 Å, while the bound ligand emodin exhibited stable conformation until 85 ns of the simulation within the macromolecular cavity, with an RMSD value in the range of 4-6 Å, followed by a sharp conformational change within the target cavity.

Throughout the M-dynamic simulation, ligand pulegone was found to be interacting with pathogenic bacterial enzymes via the formation of hydrophobic bonds with the amino acids Val43, Ala47, Val71, Ile78, Ala87, Ile90, Met91, Val120, Val123, Leu132, and Val167, while amino acids Asn46, Glu50, Asp73, Gly77, and Thr165 were found to be interacting via water bridges and ionic bonds. The bacterial enzyme-isomintlactone complex displayed a high degree of stability throughout the simulation. The RMSD value of the receptor's backbone fluctuated between 1.0 and 2.0 Å. Meanwhile, the bound ligand isomintlactone exhibited stable conformation throughout the simulation within the macromolecular cavity, with an RMSD value of 3.8-4.8 Å within the target cavity. The NADPH oxidase enzyme's monomeric chain consists of 450 amino acids, with 3,453 heavy atoms out of 6,905 total atoms.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

The complexed ligand isomintlactone, composed of twelve heavy atoms of twenty-six atoms, did not have any rotatable bond. The target enzyme NADPH oxidase-isomintlactone complex displayed stability throughout the simulation. The RMSD value of the NADPH oxidase enzyme's backbone was found to fluctuate between 1.5 and 2.2 Å, whereas the bound ligand isomintlactone exhibited an RMSD value of 7.5-11.5 Å with a couple of fluctuations within the target cavity.

The M-dynamic evaluation of the NADPH oxidase enzyme complexed with isomintlactone revealed that the root mean square fluctuation (RMSF) for Cα backbone ranges from 0.5-2.5 Å, while for the ligand isomintlactone, it ranges from 4-5 Å. Throughout the simulation, ligand isomintlactone was found to be interacting with NADPH oxidase enzyme via formation of hydrophobic bonds with the amino acids Tyr159, Ile160, Tyr188, Phe245, Tyr288, Ile297, Pro298, Leu299, and Leu346, whereas Lys187, Tyr188, Ala300, and Gly329 were interacting via hydrogen bonds. Also, amino acids Ser41, Tyr159, Lys187, Tyr188, Asp282, Tyr296, Pro298, Ala300, Thr301, Ser326, Ser328, and Gly329 were observed to be interacting via water bridges and ionic bonds. The complexed ligand menthone, consisting of eleven heavy atoms of twenty-nine atoms in total, lacks any rotatable bond. The RMSD value of the NADPH oxidase enzyme's backbone fluctuated between 1.4 and 2.3 Å, while the bound ligand menthone had a conformational change at 40 ns of the simulation to achieve the most stabilized conformation and remained stabilized till the end of the simulation with an RMSD value ranging between15-18 Å. Figure 6 demonstrates the revealed RMSD for macromolecular complexes of (a) DNA gyrase of *E. coli* complexed with pulegone; (b) DNA gyrase of *E. coli* complexed with isomintlactone; (c) NADPH oxidase complexed with isomintlactone; and (d) NADPH oxidase complexed with menthone.

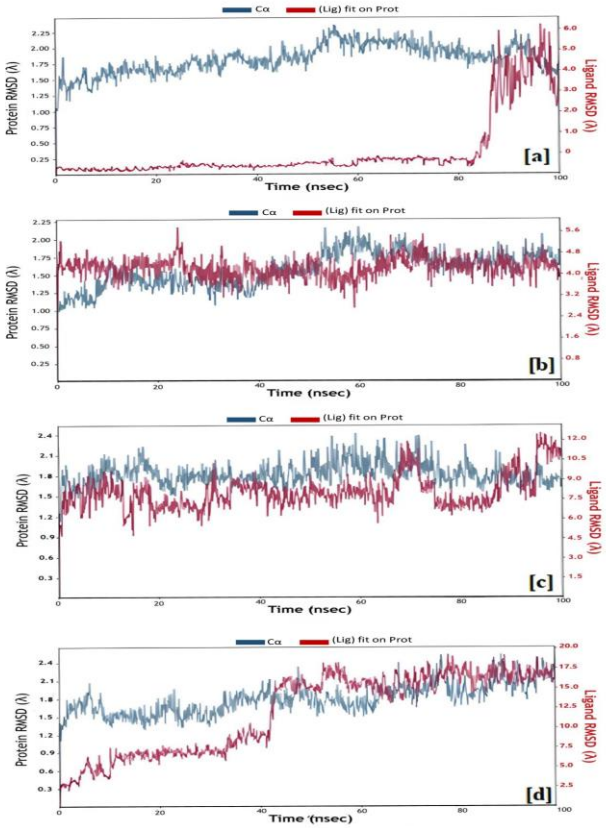


Figure 6: Root mean square deviation for Cα chain backbone and complexed ligand for (a) DNA gyrase of *E. coli* complexed with pulgone; (b) DNA gyrase of *E. coli* complexed with isomintlactone; (c) NADPH oxidase complexed with isomintlactone; and (d) NADPH oxidase complexed with menthone detected while executing 100 ns M-dynamic simulation.

The M-dynamic-based evaluation of the NADPH oxidase enzyme complexed with menthone revealed that the RMSF for the Cα backbone ranges from 0.8-2.4 Å, while for the ligand menthone, it ranges from 7-8 Å. Root mean square fluctuation for macromolecular complexes of (a) DNA gyrase of *E. coli* complexed with pulegone; (b) DNA gyrase of *E. coli* complexed with isomintlactone; (c) NADPH oxidase complexed with isomintlactone; and (d) NADPH oxidase complexed with menthone are depicted in Figure 7. Throughout the simulation, ligand menthone was found to be interacting with the NADPH oxidase enzyme via the formation of hydrophobic bonds with the amino acids Phe39, Leu40, Leu46, Tyr159, Ile160, Tyr188, Phe245, Ile297, Leu299, Val338, and Ala402, whereas Gly43, Ile44, Ala45, Tyr159, Ala300, and Asn403 via hydrogen bonds, while amino acid Ser41, Tyr159, Lys187, Tyr296, Ala300, Thr301, Arg305, Ser327, Gly329, Ser399, and Asn403 were found to be interacting via water bridges and ionic bonds. Figure 8 illustrates the interacting residues of (a) DNA gyrase of *E. coli* complexed with pulegone; (b) DNA gyrase of *E. coli* complexed with isomintlactone; (c) NADPH oxidase complexed with isomintlactone; and (d) NADPH oxidase complexed with menthone.

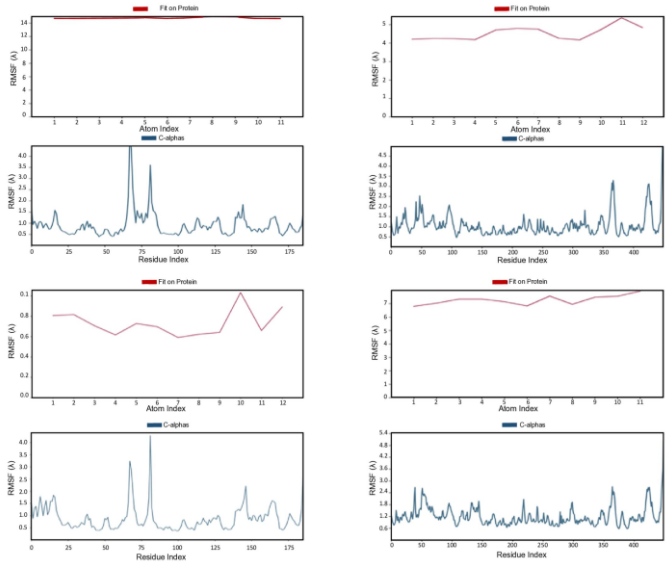


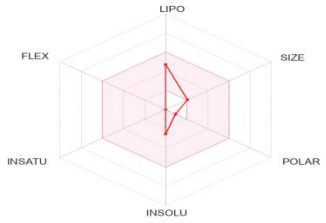
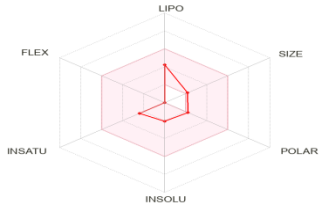
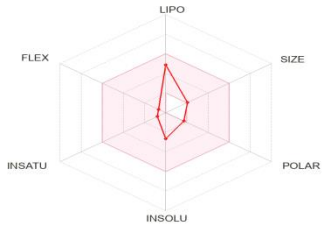
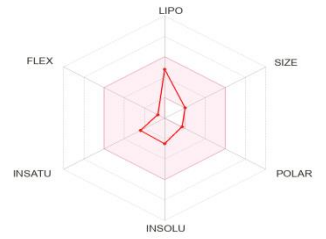
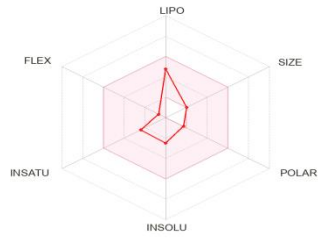
Figure 7: Root mean square fluctuation for (a) DNA gyrase of *E. coli* complexed with pulgone; (b) DNA gyrase of *E. coli* complexed with isomintlactone; (c) NADPH oxidase complexed with isomintlactone; and (d) NADPH oxidase complexed with menthone detected while executing 100 ns M-dynamic simulation.

ADME-Tox properties of the essential oils

The primary aim of this investigation was to assess the medicinal properties of the main chemical components identified in EOs and subsequently evaluate their toxicity when utilized in pharmaceutical applications. The results (Table 5) of the oral bioavailability of all the main constituents identified in the EOs indicated that all the constituents exhibited properties within the coloured zone. The coloured zone indicates the optimal range for each characteristic. The outcomes of ADMET prediction are presented in Table 6. ADMET prediction results for the main compounds identified in EOs, included water solubility, gastrointestinal absorption, blood-brain barrier (BBB) permeability, P-glycoprotein substrate inhibition capacity, cytochrome P450 enzyme substrate, and toxicity (Table 6). All water solubility values were negative, implying that the major constituents identified in the EOs are highly soluble in water.[8] Concerning intestinal absorption, the five main chemical constituents identified in the EOs exhibited high intestinal absorption rates in humans, surpassing 96%, thus suggesting promising potential bioavailability. In terms of distribution, quantified by steady-state volume of distribution (VDss), a log (VDss) value less than -0.15 indicates a relatively low volume of distribution, while a value greater than 0.45 suggests a relatively high volume of distribution.[64]

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Table 4: Lipinski's rule and the oral bioavailability properties of the primary chemical components identified in essential oils

Structural name	Oral bioavailability	Rule of Lipinski					
		MW	LogP	n-Rot-bond	TPSA	n-HBA	n-HBD
Eucalyptol		154.25	2.74	0	9.23	0	1
Isomintlactone		166.22	2.04	0	26.30	2	0
Menthone		154.25	2.64	1	17.07	1	0
Piperitone		152.23	2.56	1	17.07	1	0
Pulegone		152.23	2.71	0	17.07	1	0

LIPO: lipophilic; SIZE: molecular weight; POLAR: polarity; INSOLU: solubility; INSATU: saturation; FLEX: flexibility; MW: molecular weights; n-Rot-bonds: number of rotatable bonds; n-HBA and n-HBA: number of hydrogens bond acceptors and donors; TPSA: topological polar surface area; Log P: partition coefficient.

The results revealed that only eucalyptol exhibited a significant VDss value, exceeding 0.45, indicating a relatively low volume of distribution for this compound. For the remaining compounds, with values greater than zero, their volume of distribution is categorized as relatively medium.

The benchmark for BBB permeability categorizes it as good if its value exceeds 0.3, while it is considered poor if it falls below -1. The BBB report indicates that all the primary chemical components in essential oils have an average BBB permeability. However, it is notable that the BBB values for these components exceed -1. This indicates that, while they may not exhibit exceptionally high permeability, they still have sufficient potential to cross the BBB. According to the criteria outlined for the central nervous system (CNS) permeability index, compounds with LogPS values above -2 are classified as capable of penetrating the CNS, while those with LogPS values below -3 are classified as unable to penetrate the CNS.[65] As shown in Table 6, all the main chemical components identified in EOs have LogPS values falling between -2 and -3. Therefore, it can be inferred that all these compounds have the potential to penetrate the CNS. Cytochrome P450 isoenzymes play a crucial role in drug metabolism within the liver.[66] CYP2D6 and CYP3A4 are crucial for the human body's detoxification processes and drug pharmacokinetic modulation. The primary chemical constituents identified in the test EOs did not function as inhibitors or substrates for CYP2D6, CYP2C9, CYP3A4, CYP1A2, and CYP2C19. The total clearance of a drug provides insights into its half-life, with a low clearance value indicating a longer half-life for the compound.[67]

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Table 5: ADMET prediction results for the main chemical components identified in essential oils

Compound	Absorptions		Distributions			Metabolism					Excretion	Toxicity
	Water solubility	Intestinal absorption	VDss	BBB	CNS	Substrate / Inhibitor CYP450					Total clearance	AMES toxicity
						2D6	3A4	1A2	2C19	2C9		
	Numeric (log mol/L)	Numeric (%Absorption)	Numeric (Log L/kg)	Numeric (Log BB)	Numeric (Log PS)	Category (Yes/No)					Numeric (Log ml/ min/kg)	Category (Yes/No)
Eucalyptol	-2.63	96.50	0.491	0.368	-2.97	No	No	No	No	No	1.009	No
Isomintlactone	-2.12	97.67	0.19	0.56	-2.59	No	No	No	No	No	0.65	No
Menthone	-2.87	96.73	0.20	0.59	-2.11	No	No	No	No	No	0.24	No
Piperitone	-2.80	96.88	0.19	0.59	-2.11	No	No	No	No	No	0.19	No
Pulegone	-2.58	96.7	0.18	0.55	-2.41	No	No	No	No	No	0.19	No

ADMET: absorption, distribution, metabolism, excretion, and toxicity; VDss: volume of distribution at steady state; BBB: blood-brain barrier; CNS: central nervous system.

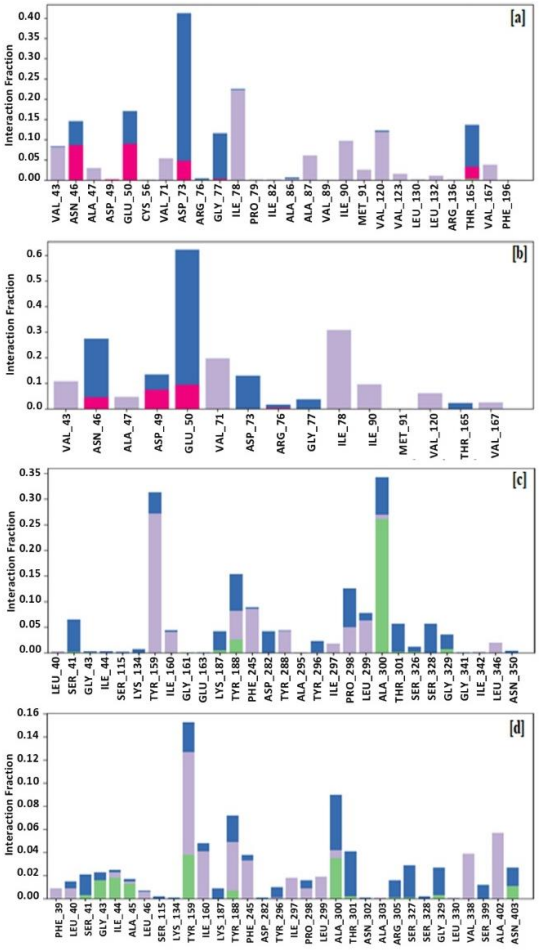


Figure 8: Protein-ligand contacts (P-LCs) identified between (a) DNA gyrase of *E. coli* complexed with pulgone; (b) DNA gyrase of *E. coli* complexed with isomintlactone; (c) NADPH oxidase complexed with isomintlactone; and (d) NADPH oxidase complexed with menthone.
Contacts were shown with colour-coded bars: green for hydrogen bonds, blue for water bridges, and purple for hydrophobic intercontact.

The main chemical components identified in EOs exhibit low clearance values, suggesting that these compounds have extended half-lives for their pharmacological properties. The Ames toxicity and skin sensitization tests are widely utilized methods for assessing the toxicity of compounds. The main chemical components identified in EOs in the present study demonstrated non-toxic properties, indicating their safety.

Conclusion

The present study found that the chemical composition of EOs from *M. piperita* and *M. pulegium* included pulegone and menthone. The test EOs exhibited remarkable antioxidant and antibacterial effects. Pulegone and menthone exhibited significant binding affinities to the target enzymes (1KZN and 2CDU) with high docking scores compared to the standard drugs used. Also, they exhibited favourable absorption, distribution, and metabolic properties, coupled with low total clearance rates and a demonstration of a lack of toxicity. The study's findings suggest that the selected native species of *M. piperita* and *M. pulegium* are significant natural sources of compounds with extraordinary antioxidant and antibacterial activity, and their use in food applications is strongly recommended. Future research on formulation studies and the potential toxicity of examined EOs is needed for safety concerns, including *in vitro* and *silico* studies using bioactive molecules.

Conflicts of interest

The authors claim to have no conflicting interests.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgements

The authors wish to express their special gratitude to everyone who contributed to this study. They are also highly grateful to the Centre of Excellence, Drug Design and Molecular Modelling Centre, Chitkara College of Pharmacy, Chitkara University, Punjab, India for providing resources for completing this research article.

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ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

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