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Meryem Tourabi

Sidi Mohamed Ben Abdellah University

Amira Metouekel

Euromed University of Fes (UEMF) Route de Meknes

Asmae EL ghouizi

Sidi Mohamed Ben Abdellah University

Mohamed Jeddi

Sidi Mohamed Ben Abdellah University

Ghizalne Nouioura

Sidi Mohamed Ben Abdellah University

Hassan Laaroussi

Sidi Mohamed Ben Abdellah University

Md. eram hosen

University of Rajshahi

Kawtar Fikri Benbrahim

Sidi Mohamed Ben Abdellah University

Mohammed Bourhia

Ibn Zohr University

Ahmad Mohammad Salamatullah

King Saud University

Hiba-Allah Nafidi

Laval University

Gezahign Fentahun Wondmie (✉ resercherfent@gmail.com)

Bahir Dar University

Badiaa Lyoussi

Sidi Mohamed Ben Abdellah University

El Houssine Derwich

Sidi Mohamed Ben Abdellah University

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Efficacy of Various Extracting Solvents on Phytochemical Composition, Antioxidant Potential, Total Phenolic Content, and Antibacterial Properties, of Moroccan *Mentha longifolia* L. Leaf Extracts against Multidrug-Resistant Pathogenic Bacteria

Meryem Tourabi^{1*}, Amira Metouekel², Asmae EL ghouizi¹, Mohamed Jeddi³, Ghizal Nouioura¹, Hassan Laaroussi¹, Md. eram hosen⁴, Kawtar Fikri Benbrahim³, Mohammed Bourhia⁵, Ahmad Mohammad Salamatullah⁶, Hiba-Allah Nafidi⁷, Gezahign Fentahun Wondmie^{8*}, Badiaa Lyoussi¹, and El Houssine Derwich^{1,9}

¹Laboratory of Natural Substances, Pharmacology, Environment, Modeling, Health & Quality of Life, Faculty of Sciences, Sidi Mohamed Ben Abdellah University, Fez, Morocco.

²Euromed Research Center, Euromed Faculty of Pharmacy, Euromed University of Fes (UEMF) Route de Meknes, Fez 30000, Morocco

³ Laboratory of Biotechnology Microbial, Faculty of Science and Technology, Fez, Morocco

⁴Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi-6205, Bangladesh

⁵Department of Chemistry and Biochemistry, Faculty of Medicine and Pharmacy, Ibn Zohr University, Laayoune 70000, Morocco

⁶Department of Food Science & Nutrition, College of Food and Agricultural Sciences, King Saud University, 11 P.O. Box 2460, Riyadh 11451, Saudi Arabia

⁷Department of Food Science, Faculty of Agricultural and Food Sciences, Laval University, 2325 Quebec City, QC G1V 0A6, Canada

Department of Biology, Bahir Dar University, P.O.Box 79, Bahir Dar, Ethiopia

⁹Unity of GC/MS and GC-FID, City of Innovation, Sidi Mohamed Ben Abdellah University, Fez, Morocco.

Correspondances : meryem.tourabi@usmba.ac.ma (MT); resercherfent@gmail.com (GFW)

Abstract

The current work attempts to explore the influence of three extraction solvents on phytochemical composition, content of polyphenols, antioxidant potential, and antibacterial capacity of Moroccan *Mentha longifolia* L. leaves hydroethanolic, acetonic, and water extracts. To achieve this goal, the chemical composition was identified using an HPLC-DAD examination. The contents of polyphenols were assessed, while the total antioxidant capacity (TAC), the DPPH test, and the reducing power test (RP) were utilized to determine antioxidant capacity. To assess the antibacterial activity, the microdilution technique was carried out to calculate the minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) of extracts against four nosocomial bacteria (*Bacillus cereus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus*). Additionally, the antibacterial and antioxidant activities of all tested extracts were examined *in silico* against the proteins NADPH oxidase and *Bacillus cereus* phospholipase C. Study reveals *M. longifolia*

extracts contain high phenolic and flavonoids. Additionally, the hydroethanolic extract contained the highest amounts of phenolic and flavonoid content, with values of 23.52 ± 0.14 mg GAE/g DW and 17.62 ± 0.36 mg QE/g DW, respectively compared to the other two extracts. The same extract showed the best antioxidant capacity ($IC_{50} = 39 \mu\text{g/mL} \pm 0.00$), and the higher RP (EC_{50} of 0.261 ± 0.00 mg/mL), compared to the acetonetic and water extract regarding these tests. Furthermore, the hydroethanolic and acetonetic extracts expressed the highest TAC (74.40 ± 1.34 , and 52.40 ± 0.20 mg EAA/g DW respectively), compared with the water extract. Regarding antibacterial activity, the MIC value ranges between 1.17 and 12.50 mg/mL. The *in-silico* results showed that the antibacterial activity of all extracts is principally attributed to kaempferol and ferulic acid, while antioxidant capacity is attributed to ferulic acid.

Keywords: *Mentha longifolia* L., Phytochemical composition, Polyphenol content, Antioxidant, Antibiotic resistance, Antibacterial activity, Molecular docking.

1 Introduction

Aromatic medicinal plants (AMPs) are commonly employed in both folk medicine and gastronomy, primarily due to their great concentration of polyphenols, which offer several other advantageous properties including antioxidant and antimicrobial activity. [1]. Species of the *Mentha* genus are rich in antioxidants [2]. Because of their antioxidant capacity, these compounds have various therapeutic qualities, including antibacterial, anti-inflammatory, anti-diabetic, and cardioprotective effects, with low toxicity and high efficiency [3]. *Mentha longifolia* L., also known as "naanatouil" in Morocco, is a traditional medicinal plant that grows naturally and spontaneously in the Moroccan mountains. It is used for its therapeutic and medicinal characteristics. *M. longifolia*, like other mint species, is frequently used in the manufacture of herbal teas and spices. This plant is widely recognized in conventional medicine for its antimicrobial, antioxidant, analgesic, respiratory disorders, antiemetic, antimutagenic, anticancer, gastrointestinal disorders, infectious illnesses, inflammatory diseases, and menstrual disorders [4,5]. Nowadays, bacterial resistance to antibiotic drugs has been recognized as a problem since the beginning of the antibiotic era. Nevertheless, it is only in the last twenty years that the development of dangerous and resistant bacteria has become an alarming regularity [6]. Antibiotic resistance is globally spreading as a result of the misuse or overuse of antibiotics, as well as insufficient infection control and precautions. A growing list of microbial infections such as tuberculosis, blood poisoning, pneumonia, gonorrhea, and foodborne illnesses is becoming more difficult and nearly impossible to treat [7]. Because of the problem of antibiotic resistance, researchers are currently focusing on biologically active components derived from common herbal species used in phytotherapy, which may generate a new potential source of antibacterial activity [8]. In addition, medicinal plants are an enormous source of bioactive molecules (active plant substances), reduce the risk of several diseases like oxidative stress, neurodegenerative disorders, cerebral vascular accidents, cancer, and coronaropathies, in addition to some allergies or affections, repairing, toning, sedative, revitalizing or immunomodulating effects [9,10].

The current work attempts to examine the impact of various extraction solvents on the phytochemical composition, antioxidant potential, and *in vitro* antibacterial activity of *Mentha longifolia* L. leaf extracts from Morocco against resistant nosocomial bacteria. Furthermore, the *in-silico* study was also evaluated.

2 Materials and Methods

2.1 Plant material

The plant was collected in May 2021 in the town of Ifran in the Middle Atlas Mountains of Morocco, and it was identified by Professor Amina BARI, a botanist at the Sidi Mohamed Ben Abdellah University. The voucher specimen has been deposited at the faculty herbarium number: 001MLAV202162. Afterward, the collected plant was dried in the dark for two weeks, and then the aerial parts were removed and powdered using a professional herbs grinder. The obtained powder was packed in a glass flask and stored in an obscure and heat-protected locale until extraction.

2.2 Preparation of extracts

In the current study, various solvents with different polarity were used to prepare the various extracts, and the extraction process was carried out, with minor modifications in agreement with El Ghouizi et al., [11]. Shortly, 10 mL of each selected solvent (ethyl acetate, chloroform, hexane, hydroethanolic 70% v/v, acetone, and distilled water) were added to 1 gram of plant powder, and the mixture was macerated and sustained under continuous agitation at room temperature for one week. After a previously performed test, which examined a variety of solvents, these conditions showed the most promising results (data not shown). Whatman N°1 filter was used to remove impurities from the extracts and thereafter condensed using a rotary vacuum evaporator at 40°C. The resulting crude extracts were collected and kept at -20°C before use.

2.3 Determination of dry matter content

Using a rotary evaporator under vacuum (BUCHI Rotavapor R-200) at 40°C, the solvents and water evaporated and the crude extracts obtained were weighed for yield determination. Furthermore, the yield of each extraction technique tested was calculated. The obtained dry matter content was expressed as a percentage and determined using the procedure below (1).

$$Y_{\text{extract}} \% = [(\text{Weight of dry extract (g)}) / (\text{Weight of dry plant (g)}) \times 100] \quad (1)$$

Y_{extract} is extraction yield expressed in % (w/w)

2.4 HPLC-DAD analysis

Mentha longifolia leaf extracts in aqueous, hydroethanolic, and acetone were examined utilizing reverse-phase high-performance liquid chromatography with a diode array detector (HPLC-DAD). For the analysis, a MOS-1 HYPERSIL 250 4.6 mm SS Exsil ODS 5 m analytical column with a Thermo Scientific HPLC system was used. The separation was achieved in gradient mode using two solvents, A (water) and C (acetonitrile), and the elution gradient was as follows: 80 percent A, 20 percent C for 1 minute, 60 percent A, 40 percent C for 2.5 minutes, and 80 percent A, 20 percent C for 4 minutes. The injection volume was 5 µL, and the flow rate was kept at 1 mL/min. Standard polyphenolic components gallic acid, ferulic acid, kaempferol, quercetin, vanillic acid, and naringenin were determined by correlating their retention time and UV spectra [12].

2.5 Evaluation of the antioxidant potential

2.5.1 Free radical scavenging activity (DPPH)

1, 1-diphenyl-2-picrylhydrazyl (DPPH) is a violet-colored free radical reduced in the presence of antiradical compounds. The DPPH radical scavenging activity of our samples was conducted using the procedure reported by Brand Williams et al with minor changes [13]. Briefly, 825 µL of DPPH ethanolic solution (65 µM), was added to 50 µL of each extract and standard solution. Absorbance readings were obtained at 517 nm after 60 minutes of incubation in the dark. The IC₅₀ values were determined to represent the 50% inhibition of DPPH radicals using the following formula (2).

$$\% \text{ inhibition} = [(Abs_{control} - Abs_{sample}/Abs_{control}) \times 100] \quad (2)$$

BHT was chosen as a standard and the IC₅₀ value was calculated from the percentage of the inhibition curve.

2.5.2 Reducing power assay

To determine the reducing power of our samples, we employed the method outlined by Shams Moattar *et al.* [14]. Briefly, 50 µL volume of plant extracts was added to 250 µL of phosphate buffer (0.2 M, pH 6.6) and 250 µL of potassium ferricyanide (1%). 250 µL of acetic trichloride acid (10%) was added to the mixture after 20 minutes of incubation in a water bath at 50 ° C, and the mixture was centrifuged at 3000 rpm for 10 minutes. 250 µL of the supernatant was then added to 250 µL of distilled water and 60 µL of ferric chloride (0.1%). The extract concentration that provides 0.5 of absorbance (EC₅₀) was calculated from the graph of absorbance, and ascorbic acid was employed as a positive control. The absorbance was measured at 700 nm.

2.5.3 Total antioxidant power test

Total antioxidant capacity (TAC) was conducted according to Mašković *et al.* [15]. The test for phosphomolybdate is based on the extract's transformation of Mo (VI) to Mo (V) and the formation of a green Mo (V) phosphate complex at an acidic pH. Shortly, 50 µL of sample extracts were combined with 1 mL of phosphomolybdenum reagent (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). After ninety minutes in a 95°C boiling water bath, absorbance readings were taken at 695 nm against a blank. Results were expressed as milligrams of ascorbic acid equivalent per gram of plant dry weight (mg AAE /g DW), and the standard curve was established using ascorbic acid as the standard.

2.6 Estimation of total phenolic content

The total phenolic content (TPC) of our samples was estimated according to El Ghouizi *et al* [11] utilizing the Folin Ciocalteu approach. 50 µL of each sample was combined with 500 µL of the Folin solution (0.2 N). 400 µL of a sodium carbonate solution (75 g/1L) was added after incubating for 5 minutes. The mixture was incubated for two hours in the dark, and the absorbance was read at 760 nm. The standard curve was created using the gallic acid solution, and the TPC was calculated as the quantity of gallic acid equivalent in milligrams per gram of plant dry weight (mg GAE/g DW).

2.7 Estimation of flavonoid content

The acetic trichloride (AlCl₃) test was carried out to assess the flavonoid content in the various samples [16]. 500 µL of AlCl₃ reagent (10%) were added to 500 µL of plant extracts and standard solution. After one hour of incubation in the darkness, absorbances were measured at 420 nm. The flavonoid concentration was represented as milligrams of quercetin equivalent per gram of plant dry weight (mg QE /g DW), and the standard curve was established using quercetin as the standard.

2.8 Evaluation of the antibacterial activity

2.8.1 Tested Microorganisms

Four strains were selected to investigate the antibacterial properties of our extracts, including two Gram-negative microbes, *Escherichia coli* (25922), *Pseudomonas aeruginosa* (27853), and two Gram-positive bacteria, *Staphylococcus aureus* (29213 (ATCC)), and *Bacillus cereus* (6633). Most microbes were clinically isolated from human specimens collected from the laboratory of microbiology, faculty of medicine, and pharmacy of Fez. The antibiotic resistance of the tested microbial strains was taken into consideration.

The selected strains were kept on nutritional agar (NG) and the sensitivity test was performed on Mueller-Hinton agar (MHB). On the selected bacterial strains, the different extracts were examined, firstly to calculate the minimum inhibitory concentration (MIC) using a liquid medium at 0.2% and secondly in the solid state to indicate the minimum bactericide concentration (MBC).

2.8.2 Preparation of bacterial suspension

The various bacteria were cultured in nine mL of MHB for 18–24 hours at 37°C. Besides fresh culture, the bacterial suspension was prepared using a sterile platinum loop where one or two colonies were collected and placed in a tube containing sterile physiological water (0.9%) and then the bacterial suspension was homogenized using a vortex. The optical density was calibrated at 0.5 Mc Farland at 625 nm. The spectroscopic density of the inoculum was updated to a concentration of 10^7 to 10^8 CFU/ ml.

2.8.3 Determination of the lowest inhibitory concentration (MIC)

The Lowest Inhibitory Concentration (MIC) represents the lower concentration capable of inhibiting any visible growth after an incubation time of 18 to 24 hours. The MIC of our different extracts (Hydroethanolic, Acetone, and water) was determined by microdilution technique in microplates under sterile conditions [17]. The examination of the minimum inhibitory concentration (MIC) was carried out by dilution of different extracts in dimethyl sulfoxide DMSO (2%) [18]. The first line wells were dispersed with 50 μ l (50 mg/ml) of each extract for inoculation, 50 μ l of MH was therefore added, and a series of dilutions were carried out. The MIC was calculated by adding 10 μ L of Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10- oxide) after the microplate had been incubated for one day at 37 °C (0.015 percent). Color is a strong indicator of the existence of bioactive microorganisms.

2.8.4 Determination of the minimal bactericidal concentration (MBC)

The MBC is the concentration of antibacterial that leaves no more than 0.01% of surviving germs [19]. After the MIC determination, samples are taken from every well free of bacterial growth placed on MH agar, and incubated at 37°C. After a 24-hour incubation time, the MBC is measured [20].

2.9 In silico assessment

2.9.1 Ligand Preparation

Phytochemicals present in extracts from the leaves of *Mentha longifolia* L. were identified using HPLC. These phytochemicals were selected for a docking study and their 3D structures in sdf format were obtained from PubChem [21]. The ligands were then prepared using Avogadro software (version: 1.2.0), where their structures were constructed and refined using the mmff94 force field. This process involved eliminating undesired groups, including hydrogen atoms, and optimizing the energy and geometry of the ligand structures [22].

2.9.2 Protein Preparation

Due to the notable antibacterial activity of the extract against *Bacillus cereus*, the docking study aimed to explore interactions with specific target proteins. These proteins included Phospholipase C (PDB ID: 2huc) from *Bacillus cereus* [23], and NADPH Oxidase from *Lactobacillus sanfranciscensis* (PDB ID: 2cdv) [24]. The 3D structures of these proteins, obtained from the Protein Data Bank (PDB), were in x-ray diffraction format. Employing Discovery Studio (version: 4.5) and Swiss pdb viewing software (version: 4.1), extraneous molecules like heteroatoms, water molecules, and non-target components were eliminated from the protein structures before initiating the docking process. To enhance stability, the

protein structures underwent energy minimization using the GROMACS96 force field. Following this, ligands were generated, and the modified protein structures were employed for conducting docking studies in pdb format.

2.9.3 Molecular docking

To explore the potential inhibitors derived from plant extracts for their antibacterial effects against *Bacillus cereus*, as well as their potential as antioxidants, a molecular docking study was conducted. The PyRx software tool (version: 0.8) was employed for this purpose [25]. The ligands were initially inputted using Open Babel and subsequently converted into PDBQT format. Additionally, the target protein was introduced into PyRx, with the protein being converted into macromolecules in PDBQT format. For the specific proteins, the center and grid box dimensions were set for optimal docking. For the protein with PDB ID 2huc, the coordinates were X: 88.3723 Å, Y: 68.9215 Å, Z: 14.0256 Å, and the grid box size was X: 43.3734 Å, Y: 47.8133 Å, Z: 57.55 Å. Similarly, for the protein with PDB ID 2cdu, the coordinates were X: 10.7221 Å, Y: 5.0086 Å, Z: 25.7258 Å, and the grid box size was X: 65.8874 Å, Y: 71.0272 Å, Z: 101.0575 Å. Docking calculations were executed using the PyRx program, while the analysis of binding interactions was carried out using Discovery Studio. The docking investigation involved utilizing the lowest binding scores, and the energy values were estimated in kcal/mole

3 Statistical Analysis

The different assays performed in this work were conducted in triplicate, and the findings have been expressed as mean $\pm$ SD. GraphPad Prism 9 software was used for the statistical analyses, along with one-way ANOVA and Tukey's multiple comparisons. One-way ANOVA and Tukey's multiple comparisons were used in the statistical studies, which were carried out using GraphPad Prism 9 software. Principal component analysis (PCA) was carried out with the use of the STATISTICA program, and Pearson's correlation coefficient (r) was utilized to determine relations between the examined variables.

4 Results and Discussion

4.1 Solvent screening

In phytochemical research, the most critical step in the extraction of phenolic and other bioactive constituents from vegetables, fruits, and plants is solvent selection. In summary, different factors, such as solvent polarity, temperature, and time, influence the extraction efficiency of phenolic components, and their effects can be independent or coupled [26]. In this work, results of the preliminary screening showed that three solvents (ethyl acetate, chloroform, and hexane) showed the most minimal effect in the antiradical scavenging activity, the reducing power, and the antioxidant activity. These three solvent extracts showed also a lower amount of total phenolic compounds. According to our preliminary results, the antioxidant power of *M. longifolia* was greatly impacted by the polarity of the extraction solvent which was confirmed by Aazza et al. [27]. The total phenolic and flavonoid content of the different assessed extracts varied between 7.130 ± 0.786 to 23.524 ± 0.139 mg GAE/g DW, and from 3.757 ± 0.255 to 17.622 ± 0.359 mg QE/g DW respectively. It should be noted that the hydroethanolic extract exhibited greater TPC and TFC levels. Considering the preliminary screening performed based on solvent polarity, we decided to select the stronger extracts (hydroethanolic, acetone, and water) to perform the phenolic composition and

antibacterial activities, and their results will be represented and discussed in the remaining sections of this paper.

4.2 Extraction Yields

Choosing the best extraction solvent mixture depending on material properties is crucial to attaining high yields. As solvents for extraction, ethanol 70%, acetone, and water were used in Table 1. The findings indicate that the high extraction yield was recorded during extraction with 70% EtOH with a rate of $10.5 \pm 0.074\%$, followed by water with a rate of $2.25 \pm 0.016\%$, however, acetone solvent achieved the lowest yield with a rate of $1.9 \pm 0.013\%$. According to researchers, the polarity of the solvent has a substantial effect on extract yield[28]. The extract yield in polar solvents (water and EtOH 70 %) was reported to be greater than in nonpolar solvents (acetone). Brahmi and coworkers reported that hydro-alcoholic solvents had the maximum extraction yield. Whereas ethanol (at 75 %) provided the best extraction rates for Algerine *Mentha spicata* (20.02 %), acetone gave the lowest rate (2.6%)[29].

	Water	EtOH 70%	Acetone
<i>M.longifolia</i> Leaves	2.25 ± 0.016	10.5 ± 0.74	1.9 ± 0.013

Table 1. Extractive yields of *M. longifolia* leaf extracts (% dry weight)

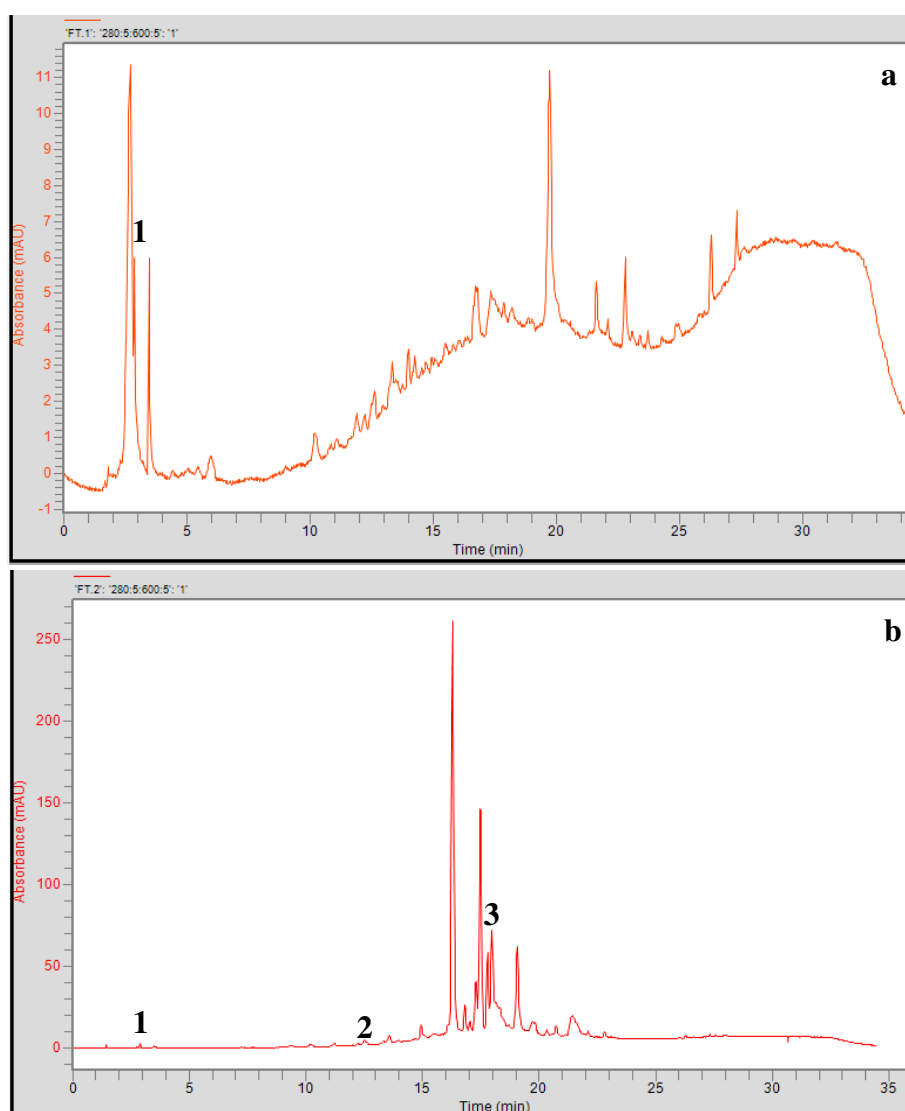
4.3 HPLC- DAD Examination of Phenolic Compounds

HPLC-DAD chromatograms of the three extracts, water, hydroethanolic, and acetone, are shown in Table 2 and Figures 1 (a, b, and c), respectively. The chemical profiles of the three extracts vary considerably, indicating the distinct efficacy of each extraction method. The aqueous extract contains mainly compounds with a retention time of around 19.732 minutes, as shown by the highest peak in Figure 1. These are rather polar molecules, which is quite normal in a water extract. This extract contains gallic acid, known for its antioxidant properties. Gallic acid has been associated with a variety of health advantages, including potential antioxidant effects[4]. The hydroethanolic extract reveals a diverse range of compounds, with significant peaks around 16.281 minutes (non-identified) and 17.975 minutes (kaempferol). A range of moderately polar to apolar molecules, demonstrating the efficiency of the mixed ethanol/water solvent. Kaempferol, present in hydroethanolic extract, is a flavonoid known for its antioxidant and anti-inflammatory potential. The acetone extract shows a distinct chemical profile, with significant peaks at around 16.258 minutes (non-identified) and 17.988 minutes (kaempferol). This is an extract containing molecules that are rather medium in polarity. The presence of chlorobenzoic acid and kaempferol in the acetone extract shows that the acetone extract may contain compounds with antimicrobial, and antioxidant properties[30], similar to those observed in the hydroethanolic extract. Our data are in line with studies[4,31], of other plant extracts. Our findings highlight the distinct chemical profiles of the three extracts, suggesting the effectiveness of each extraction method. These results confirm the value of *Mentha longifolia* extracts as potential sources of bioactive compounds with diversified therapeutic potential, mainly antioxidant and possibly anti-bacterial activity.

Table 2.HPLC-DAD chromatographic study of individual phenolic composition identified in the various extracts of *Mentha longifolia* leaves.

Peak	Standards	RT(min)	Area %		
			Water	EtOH 70%	Acetone
1	Gallic acid	2.886	6.22	0.14	0.01
2	Ferulic acid	12.236	ND	0.14	ND
3	Kaempherol	17.975	ND	6.05	2.80

ND: Non detected



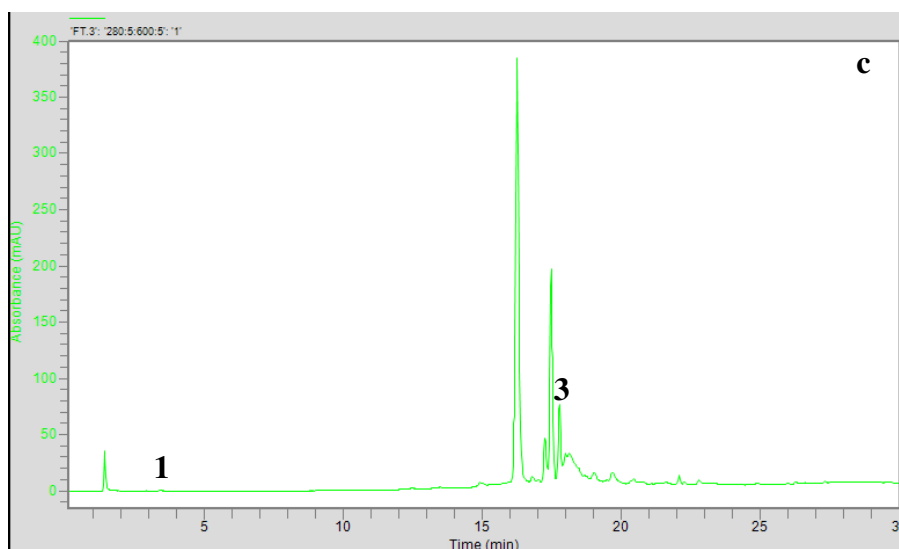


Figure 1. HPLC-DAD chromatogram of *M. longifolia* leaves extracts (a) aqueous (b) ETOH70% (c) acetone.

4.4 Phenolic and flavonoid content of the different *Mentha longifolia* L. leaf extracts

Phenolic compounds are the most common type of phytochemical found in a wide range of plant-based products, including phenolic acids (hydroxycinnamic acids, and hydroxybenzoic), polyphenols (condensed tannins, and hydrolysable), flavonoids (rutin, naringenin, quercetin, apigenin, kaempferol) [32], because of its importance in the pharmaceutical, and food industries, these compounds have been extensively researched [33]. In this context, *M. longifolia* (Horsement) is considered a potential source of bioactive compounds as well as micronutrients, which can be explored as a promising alternative for the formulation of nutraceutical products [34]. As previously confirmed by our results, the phenolic content strongly depends on the selected extraction solvent.

Figure 2 (a) demonstrates the data of the total phenolic content of the tested *M. longifolia* extracts, and found that water extracts had the lowest value (17.90 ± 0.49 mg AGE/g DW), whereas acetone extracts had the greatest value (23.52 ± 0.14 mg AGE/g DW). This study suggests that hydroethanolic was the most efficient extraction solvent for extracting polyphenols, followed by acetone and water, suggesting that polar solvents extract more phenolic content than apolar solvents. Our results are higher than that reported by [35,36]. In contrast, Ertaş et al. found that the phenolic content of methanol, acetone, and petroleum ether extracts ranged from 217.10 ± 1.82 to 225.65 ± 3.42 µg GAE/mg and were higher than our extracts [37].

Regarding flavonoids content results are summarized in Figure 2 (b) and suggested that the hydroethanolic extract showed the highest amount of flavonoids (17.622 ± 0.359 mg QE/g DW), followed by acetone extract (11.174 ± 0.330 mg QE/g DW), whereas water extract showed the lowest amounts with (4.114 ± 0.497 mg QE/g DW), these findings were inferior to those found by Hajlaoui et al and Ertaş et al [37,38].

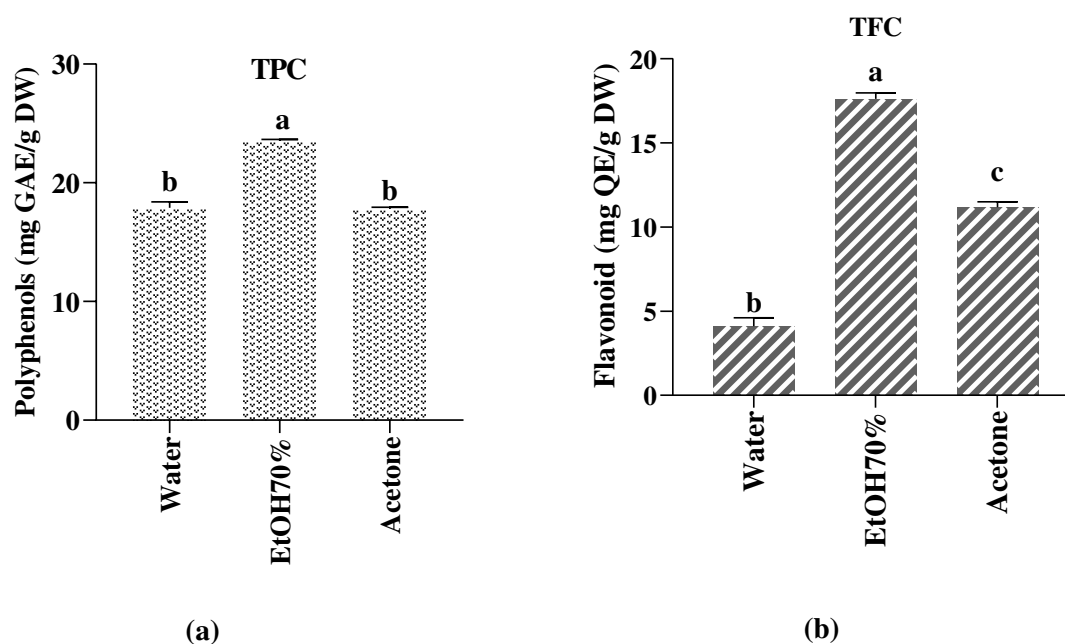


Figure2: (a) Total phenolic content (TPC) of *M. longifolia* extracts. (b) Total flavonoid content (TFC) of *M. longifolia* extracts. Tukey's multiple range test showed that results with the same letter in the same test are not statistically different ($p < 0.05$).

4.5 Antioxidant activity of the different *Mentha longifolia* L. leaf extracts

Results of the antiradical property (DPPH), reducing power, (PR), and the total antioxidant capacity (TAC) of the selected *Mentha longifolia* L. leaf extracts were summarized in Figure 3 and showed a significant variation between the different extracts. Our findings showed that hydroethanolic extract exhibited the highest total antioxidant capacity (74.40 ± 1.34 mg AAE/g) followed by acetone extract (52.40 ± 0.20 mg AAE/g), and water extract which showed the lowest value (20.98 ± 0.08 mg AAE/g) (Figure 3 (c)). In the same way, the aqueous extract presented the lowest radical scavenging inhibition with the highest $IC_{50} = 306 \pm 0.1$ μ g/mL for the DPPH test and the highest $EC_{50} = 0.80 \pm 0.03$ mg/mL for the reducing power (RP) compared to standards. However, the hydroethanolic extract showed higher free radical scavenging activity (DPPH) and reducing power (RP) ($IC_{50} = 39.00 \pm 0.00$ μ g/mL and $EC_{50} = 0.261 \pm 0.00$ mg/mL respectively) followed by the acetone extract ($IC_{50} = 43.00 \pm 0.00$ μ g/mL and $EC_{50} = 0.324 \pm 0.00$ mg/mL respectively) showing no statistical significance with BHT and ascorbic acid used as standards (Figure 3 (a, b)). These outcomes were stronger than those reported by Bahadoi *et al* for the Iranian *M. longifolia* ethanolic and aqueous extracts in which they reported DPPH IC_{50} values ranged between 195.96 ± 0.94 , and 162.08 ± 3.90 mg TEs/g extract, and RP EC_{50} values ranged between 239.87 ± 3.95 , and 346.20 ± 0.17 mg TEs/g sample [5]. The findings of this study showed that hydroethanolic is the most effective extraction solvent and it had the highest levels of phenolic and flavonoid content, which were closely related to the strong antioxidant activities (DPPH, TAC, and RP) that were noticed. It was followed by acetone and water. Our findings are consistent with those previously published, which reported that high-polarity solvents are extensively used to extract antioxidant compounds such as hydroethanolic, water, acetone, and methanol. It is well known that ethanol, methanol, and water are polar solvents commonly used to extract polar molecules like phenolic and flavonoid components [39].

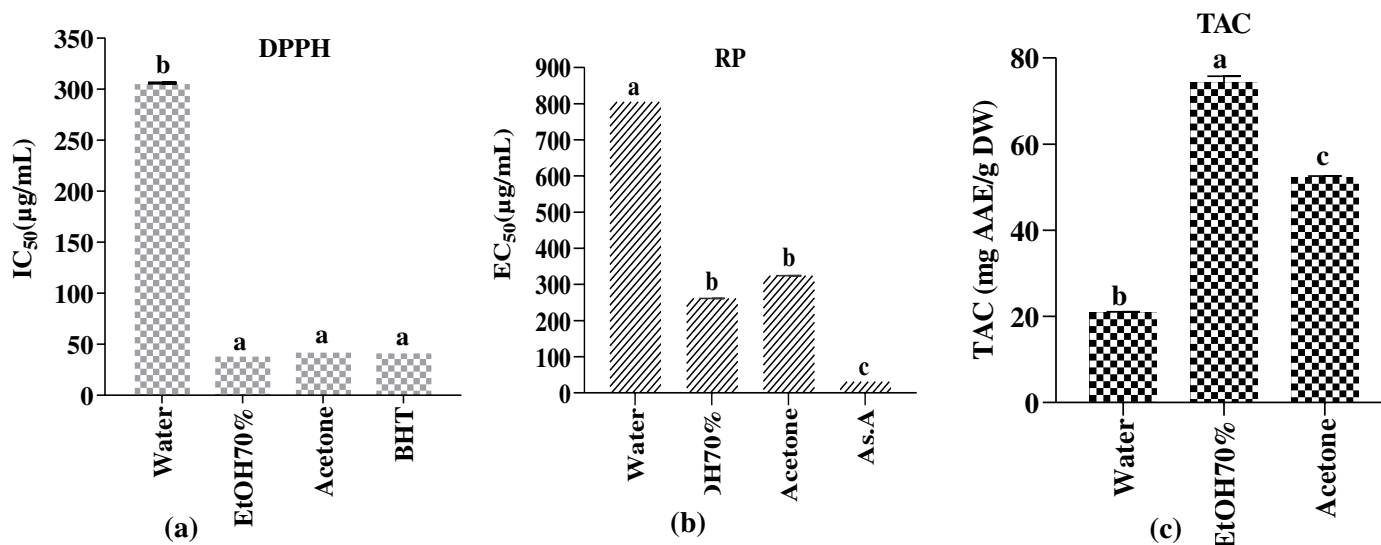


Figure 3: (a) radical scavenging activity of *M. longifolia* leaf extracts (DPPH assay), (b); reducing power of *M. longifolia* extracts; (c) Total antioxidant activity (TAC) of *M. longifolia* extracts. Tukey's multiple range test showed that results with the same letter in the same test are not statistically different ($p < 0.05$).

Previous studies have been conducted to identify the phenolic and flavonoid profiles of *M. longifolia* and revealed a variety of natural antioxidant molecules such as phenolic acids (gallic acid, vanillic acid, rosmarinic acid, caffeic acid, syringic acid, p-coumaric acid, o-coumaric acid, ferulic acid, trans-cinnamic acid, and chlorogenic acid) and flavonoids (naringenin, rutin, quercetin, apigenin, kaempferol)[35,40,41]. The authors of an Iranian study stated that rosmarinic acid and cinnamic acid were principally identified in their *M. longifolia* areal part extracts [5]. Furthermore, flavone glycosides such as apigenin and luteolin were the major flavonoids detected in the Moroccan *M. longifolia* areal part extracts [42]. The findings of the present work suggest that the strong antioxidant activity of *M. longifolia* extracts is mostly attributed to its phenolic profile[5,43].

4.6 Evaluation of the antibacterial activity

The outcomes of the antibacterial effect of *M. longifolia* leaf extracts are represented in Tables 3, and 4. In this study, a total of 4 nosocomial pathogenic microbial strains (2 Gram-positive and 2 Gram-negative) were used to investigate the antibiotic potential of our samples. Results showed that all extracts revealed strong bacterial growth inhibition against all tested pathogenic bacteria. In particular, acetone extract showed the highest inhibitory effect against *Bacillus cereus* with a MIC = 1.17 ± 0.05 mg/mL, and MBC = 1.50 ± 0.05 mg/mL, and *Staphylococcus aureus*, (MIC = 2.34 ± 1.10 mg/mL, and MBC = 6.25 ± 0.43 mg/mL). Additionally, acetone extract exhibited higher activity against *Escherichia coli* with a MIC = 6.25 ± 0.00 mg/mL. It is crucial to record that acetone extract has exhibited a powerful antibacterial effect against Gram-positive bacteria, more than Gram-negative bacteria except *Pseudomonas aeruginosa*. Acetone plant extract tends to be more effective in terms of antibacterial activity; indeed, Felhi *et al.* reported that the acetone extract of fruit bark and seeds of *E. elaterium* signaled the highest activity against *Staphylococcus aureus* and *Bacillus subtilis*[44]. However, Zhang *et al.* have demonstrated in their research that the ethanolic extract of *Mentha arvensis* exhibited strong antibacterial activity against *Acinetobacter baumannii* [45]. Another study on methanolic extract of the aerial parts of *M. longifolia* ssp.

had no antibacterial effect [46]. Preeti *et al.* evaluated the antibacterial efficacy of several *Mentha piperita* leaf solvent extracts against pathogenic bacteria notably *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Escherichia coli*, *Proteus vulgaris*, and results revealed that water and ethyl acetate extracts exhibited the strongest antibacterial activity [47].

In our current study, we noticed that Gram-positive strains were more responsive to our extracts compared to Gram-negative strains. The reason for the lower sensitivity of Gram-negative bacteria could be attributed to the complexity of their double membrane, which includes a cell envelope made up of a lipoprotein and a lipopolysaccharide layer (LPS). Unlike Gram-positive bacteria's single membrane, this structure works as a biological barrier to antibacterial drugs [48]. The demonstrated antibacterial activity of our *Mentha longifolia* extracts can be explained by its rich phytochemical profile, which includes caftaric acid, rosmarinic acid, cryptochlorogenic acid, p-coumaric acid, m-coumaric acid, chlorogenic acid, caffeic acid, gallic acid, luteolin, apigenin, quercetin, rutin, coumarins, and isocoumarins [49], [50]. Indeed, previous studies have reported greater antimicrobial potency for caffeic, cryptochlorogenic, as well as chlorogenic acids [49,51,52]. Other phenolic compounds found in various species of the genus *Mentha* such as ellagic acid, ferulic acid, galocatechin, epigallocatechin gallate, and catechins were reported to exhibit antibacterial or bacteriostatic effects against multiple bacterial strains including *Escherichia coli*, *Staphylococcus aureus*, *Bacillus aureus*, *Bacillus pumilis*, *Bacillus subtilis*, and *Pseudomonas aeruginosa*. This effect is associated with their capability to penetrate the bacterial wall and reach the bacterial cytoplasm [52].

As stated previously, several solvent combinations have been applied to effectively extract phenolic components from plant material. Water, ethanol, methanol, acetone, and their aqueous mixes are the most commonly used solvents [53]. The significant antibacterial impact found in our investigation can be attributed to acetone's ability to extract antimicrobial compounds due to its power to dissolve hydrophilic and lipophilic components [54]. Furthermore, acetone is the most effective extractor of plant material since it can extract molecules with a wide range of polarities and has low toxicity in biological assays, making it a highly valuable extraction solvent [55]. Interestingly, acetone was shown to be the most effective solvent for extracting polyphenols, such as hydroxycinnamic acids (ferulic acid, sinapic acid, caffeic acid, p-coumaric acid, chlorogenic acid, luteolin, quercetin, vanillic acid, and catechin), and hydroxybenzoic acids (gallic acid, and protocatechuic acid) [56]. These molecules have been mentioned to have a powerful antibacterial effect through several mechanisms, notably membrane instability, membrane hyper-permeabilization, hyper-acidification, enzyme inhibition, and the generation of reactive quinones [57]. According to Campos *et al.*, these acids might also inhibit the synthesis of nucleic acids by Gram-negative and Gram-positive bacteria [58]. Because of their propenoid side chain, hydroxycinnamic acids are a little more polar than corresponding hydroxybenzoic acids, which may assist their transit through the cell membrane increasing their toxic effect into the bacterial cell [59,60]. In the same context, gallic and ferulic acids have been shown to cause bacterial cell death by altering bacterial hydrophobicity (interacting with the surface of Gram-negative and Gram-positive bacteria), potentially acidifying their cytoplasm by increasing K⁺ release and inducing protein denaturation, which can change the cytoplasmic membrane permeability, induce intracellular material release, and cause membrane damage. [60]. Moreover caffeic acid, and p-coumaric acid impact cell membrane structure through inflexibility and alterations in the phospholipid chains' stability [61].

Table 3. Minimum Inhibitory Concentration (MIC) of *M. longifolia* extracts in gram-

Extracts	Gram-negative Bacteria		Gram-positive Bacteria	
	<i>P. aeruginosa</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>B. cereus</i>
Etoh70%	6.25 ± 0.00 ^a	12.50 ± 0.00 ^a	4.68 ± 0.21 ^a	3.12 ± 0.00 ^a
Acetone	12.50 ± 0.00 ^b	6.25 ± 0.00 ^b	2.34 ± 0.11 ^b	1.17 ± 0.05 ^{ab}
Water	9.37 ± 0.41 ^a	12.50 ± 0.00 ^a	4.68 ± 0.21 ^a	4.61 ± 0.76 ^a

negative and gram-positive bacteria (mg/mL)

Tukey's multiple range test showed that results with the same letter in the same test are not statistically different ($p < 0.05$).

Table 4. Minimal bactericidal concentration (MBC) *M. longifolia* extracts in gram-negative and gram-positive bacteria (mg/mL)

Extracts	Gram-negative Bacteria		Game-positive Bacteria	
	<i>P. aeruginosa</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>B. cereus</i>
Etoh70%	25 ± 1.28	25 ± 3.78	25 ± 2.26 ^a	1.56 ± 0.07
Acetone	-	-	6.25 ± 0.43 ^b	1.50 ± 0.05
Water	-	-	-	-

Tukey's multiple range test showed that results with the same alphabet in asimilar test are not statistically distinct ($p < 0.05$).

4.7 *In silico* assessment

4.7.1 Molecular docking against Phospholipase C *Bacillus cereus*

Three compounds identified from *Mentha longifolia* L. leaf extracts through HPLC were subjected to molecular docking studies, and the findings indicated that all three compounds bind to the same region within the active site (Figure 4). Among these compounds, Kaempferol displayed the highest binding energy at -9.67 kcal/mol, followed by Ferulic acid at -9.5 kcal/mol and Gallic acid at -8.75 kcal/mol (Table 5).

The interaction analysis between Phospholipase C (PDB ID: 2huc) and the top-performing compound Kaempferol revealed the formation of five hydrogen bonds at specific amino acid residues: Cys8 (2.83 Å), Thr9 (2.00 Å), Ala11 (2.30 Å), Thr112 (2.95 Å), and Lys134 (3.00 Å) (Figure 4a). In the case of 2cdu and Ferulic acid, there were four hydrogen bonds formed with Thr9 (2.43 Å), Ala11 (2.40 Å), Gly12 (2.17 Å), and Thr112 (2.27 Å) (Figure 4b). Notably, both Kaempferol and Ferulic acid shared a hydrogen bond at the exact same position, suggesting a similar interaction pattern with the target protein.

Additionally, the third compound, Gallic acid, exhibited interactions with the target protein 2huc, forming three hydrogen bonds at Thr9 (2.27 Å), Thr112 (2.78 Å), and Asp282 (2.12 Å) (Figure 4c).

Table 5. Binding energy, interacting amino acid residues, bond types, and their distance between Phospholipase C (PDB ID: 2huc) of *Bacillus cereus* and the three docked compounds.

Complex	Binding energy (kcal/mole)	Amino acid residues	Bond types	Distance (Å)
2cdu +Kaempherol	-9.67	Cys8	H	2.83
		Thr9	H	2.00
		Ala11	H	2.30
		Thr112	H	2.95
		Lys134	H	3.00
2cdu +Ferulic acid	-9.5	Thr9	H	2.43
		Ala11	H	2.40
		Gly12	H	2.17
		Thr112	H	2.27
		Lys134	A	4.97
		Ala300	A	5.35
		Ala303	A	4.76
2cdu +Gallic acid	-8.75	Asp282	PIA	3.45
		Thr9	H	2.27
		Thr112	H	2.78
		Asp282	H	2.12
		Ala11	PA	5.12
		Thr113	AS	4.75

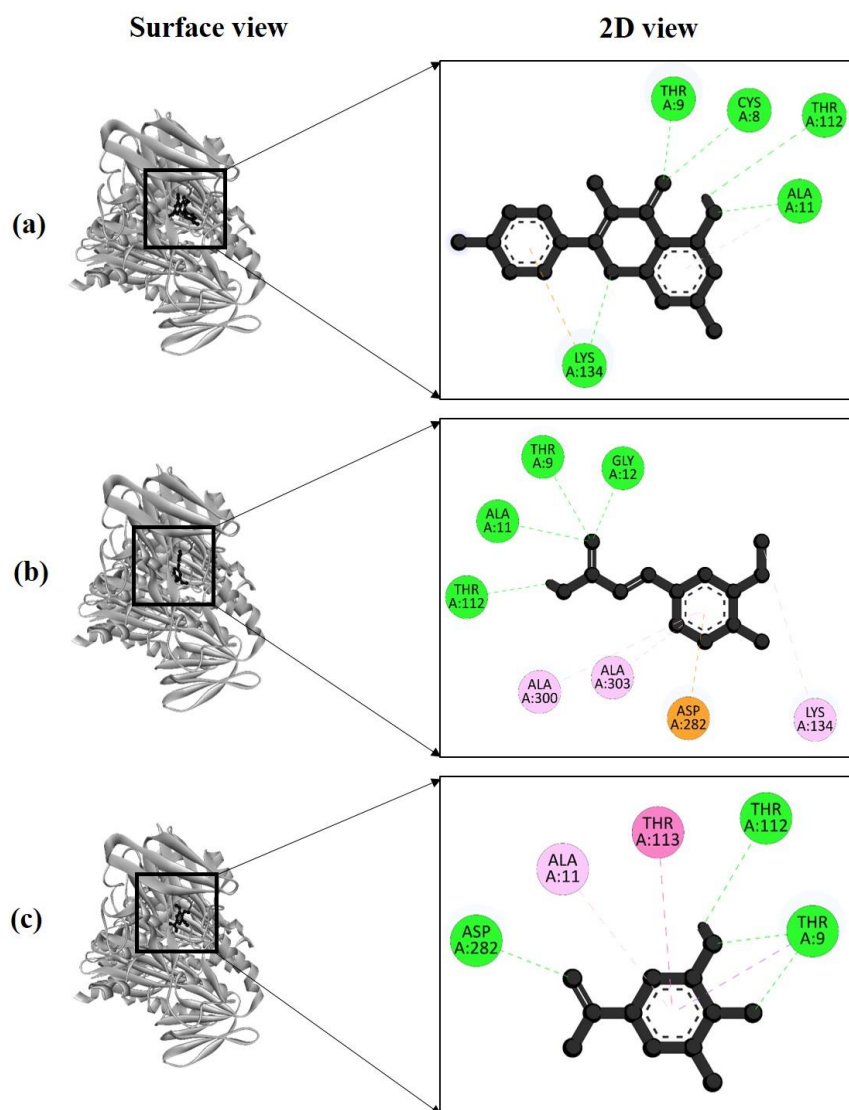


Figure 4. The molecular docking study by Phospholipase C (PDB ID: 2huc) of *Bacillus cereus* and the active compounds from extract; surface and 2 D view of compounds (a) Kaempferol, (b) Ferulic acid and (c) Gallic acid respectively.

4.7.2 Molecular docking against NADPH oxidase

In terms of binding affinity to the target enzyme NADPH oxidase, Ferulic acid exhibited the strongest interaction with a binding energy of -8.4 kcal/mol, followed by Kaempferol and Gallic acid with binding energies of -8.0 and -7.9 kcal/mol, respectively (Table 6). Ferulic acid formed interactions with NADPH oxidase (PDB ID: 2cdu) through four hydrogen bonds, which significantly contributed to its binding stability. These hydrogen bonds were observed with specific amino acid residues: Trp1 (2.05 Å), Asp122 (2.72 Å), His128 (2.83 Å), and Thr65 (3.63 Å) (Figure 5a).

On the other hand, both Kaempferol and Gallic acid exhibited an equivalent number of hydrogen bonds when interacting with the target protein. Specifically, they formed hydrogen bonds with Trp1 (at 2.28 Å) and His228 (at 2.67 Å) for Kaempferol (Figure 5b), and with Trp1 (at 2.46 Å) and Asp55 (at 1.85 Å) for Gallic acid (Figure 5c), respectively.

Table 6. Binding energy, interacting amino acid residues, bond types, and their distance between NADPH oxidase (PDB ID: 2cdu) and the three docked compounds.

Complex	Binding energy (kcal/mole)	Amino acid residues	Bond types	Distance (Å)
2huc +Ferulic acid	-8.4	Trp1	H	2.05
		Asp122	H	2.72
		His128	H	2.83
		Thr65	H	3.63
		Phe70	A	4.86
		Tyr79	A	4.56
		Phe66	A	3.96
		His69	PIA	1.42
2huc +Kaempherol	-8.0	Trp1	H	2.28
		His228	H	2.67
		Phe66	PPS	4.11
2huc +Gallic acid	-7.9	Trp1	H	2.46
		Asp55	H	1.85
		Phe66	PPS	4.43

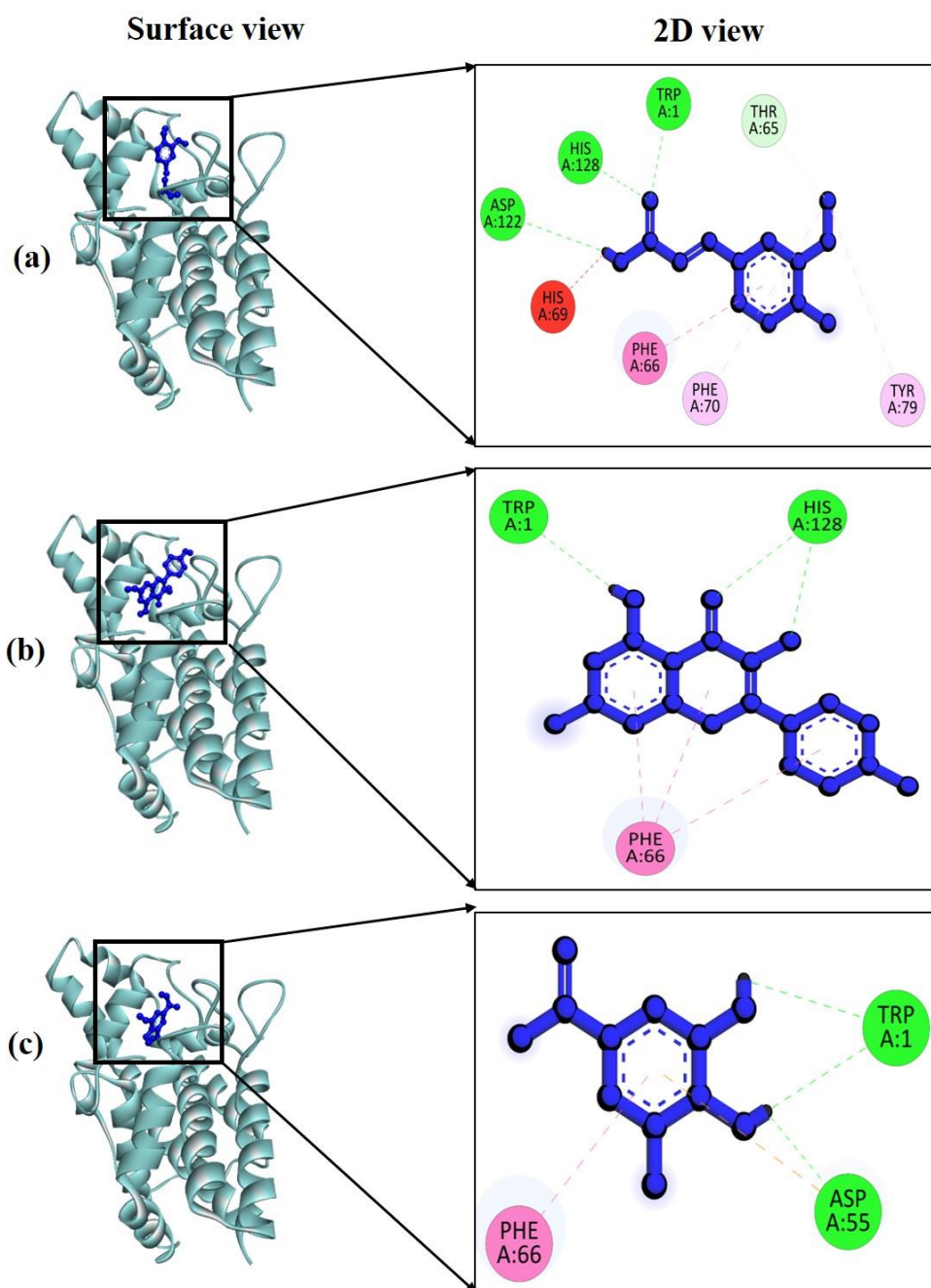


Figure5. The molecular docking study by targeting NADPH oxidase (PDB ID: 2cdu) and the HPLC detected active compounds from extract; surface and 2 D view of compounds (a) Ferulic acid, (b) Kaempferol and (c) Gallic acid

5 Statistical analysis

5.1 Correlation test

The correlation test is well-known for showing any causal relationships between the various factors studied in the research. The results are summarized in Table 7 and exhibited a strong excellent correlation between phenolic, and flavonoid contents ($r = 0.845$), and total antioxidant capacity (TAC) ($r = 0.802$ and 0.997 respectively). Adversely, phenolic content and the antioxidant activity measured by DPPH and RP showed a considerable negative correlation ($r = -0.498$. and -0.571 respectively), and between the antioxidant tests

themselves, DPPH/TAC ($r = -0.917$), and TAC/ RP ($r = -0.948$). Our results demonstrated also a strong positive correlation between the polyphenolic content and the antibacterial activity of the studied *M. longifolia* extracts against all microbiological strains. Our data are consistent with those previously published which reported a positive relationship between the antioxidant capacity, phenolic content, and the antibacterial efficiency [62,63].

Table7. Pearson correlation coefficients

	Polyphenols	Flavonoids	TAC	DPPH	RP
Polyphenols	-	0.845	0.802	-0.498	-0.571
Flavonoids	0.845	-	0.997*	-0.885	-0.922
TAC	0.802	0.997*	-	-0.917	-0.948
DPPH	-0.498	-0.885	-0.917	-	0.996*
RP	-0.571	-0.922	-0.948	0.996*	-
<i>P. aeruginosa</i>	-0.874	-0.477	-0.410	0.013	0.099
<i>E. coli</i>	0.513	-0.026	-0.101	0.489	0.411
<i>S. aureus</i>	0.511	-0.029	-0.104	0.491	0.414
<i>B. cereus</i>	0.078	-0.468	-0.534	0.826	0.774

Statistical significance. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.2 Principal component analysis

The previously acquired results were treated using the principal component analysis (PCA), and the key outputs are displayed in Figures 6(a, b). The principal component analysis (PCA) for total phenolic, flavonoid contents, and antioxidant activity of the studied extracts is shown in Figure 6 (a). Most importantly, PC1 and PC2 explained 100% of the variation, which was sufficient to represent all of the selected variables. The PC1 against PC2 score plot indicates a significantly positive relationship between TPC, TFC, and TAC, showing that the extracts with the highest potential for antioxidants total likewise had the most TPC and TFC content. These three parameters, particularly TPC, were shown to be positively correlated to the hydroethanolic solvent used in the extraction and negatively correlated to water. Moreover, DPPH and RP assays are regrouped negatively with the total phenolic and flavonoid contents, which supports the contribution of phenolic compounds in the DPPH scavenging ability and reducing the power of the different tested extracts, and the observed anti-*Bacillus cereus*, *Staphylococcus aureus* effects. While it's negative part represents RP and DPPH.

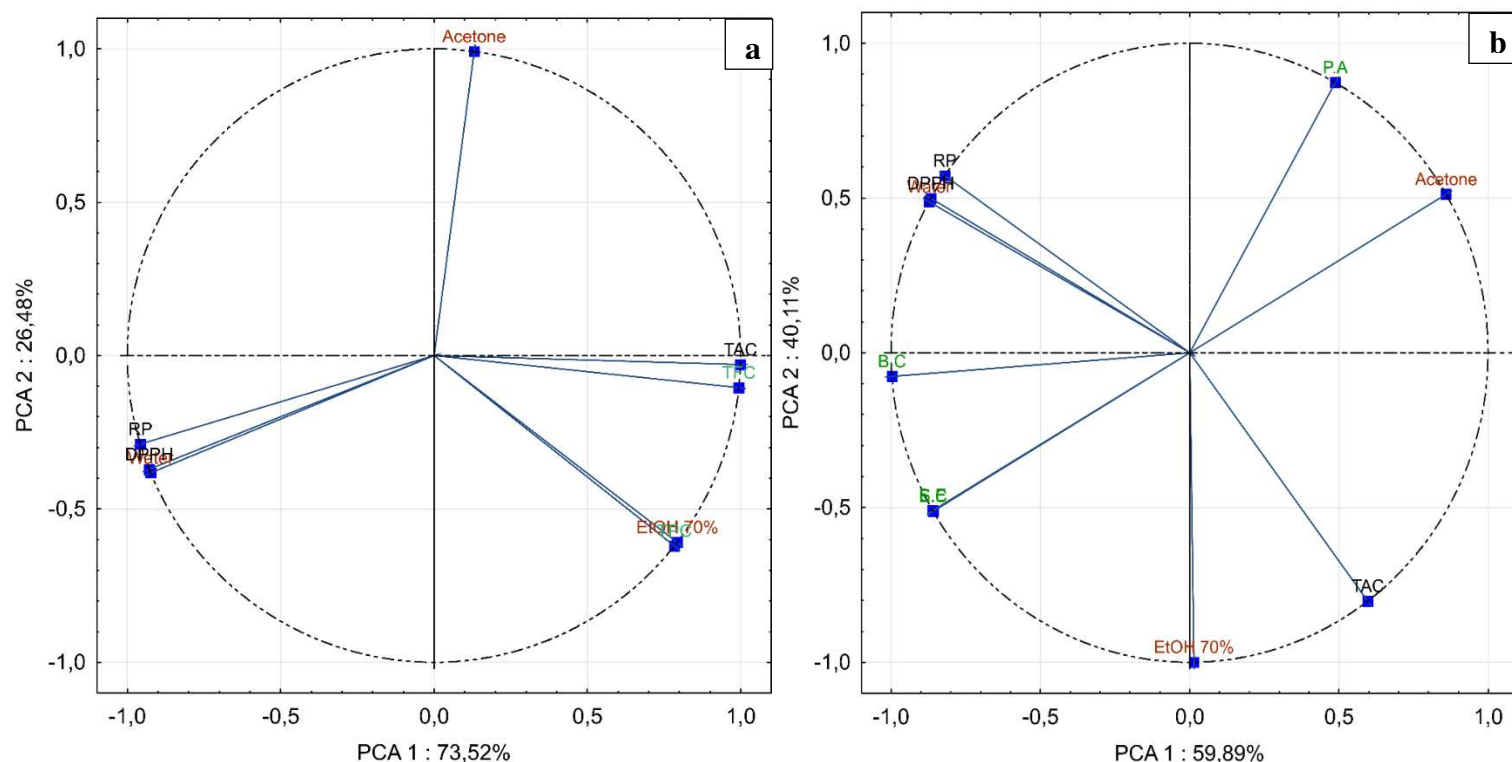


Figure 6: Principal component analysis (PCA). (a) Bbiplots for phenolic compounds and antioxidant activities of studied solvent extracts, (b) Bbiplots for antioxidant, and antibacterial activities of studied solvent extracts.

Figure 6 (b) illustrates the PCA for antibacterial, and antioxidant activities of investigated extracts, with the two main components (PC1 and PC2) explaining 100% of the variation. The first component explained 59.81% of the variation and included TAC and *Pseudomonas aeruginosa* in its positive part, while the negative part included RP, DPPH, *Bacillus cereus*, and *Staphylococcus aureus*. Although the second component explained 40.11 % and included water, RP, and DPPH in its positive part.

6 Conclusion

The outcomes of this investigation demonstrated that the different *Mentha longifolia* L leaf extracts contain a significant concentration of polyphenols and flavonoids. In addition, the phenolic composition of various polarities extracts indicated that the best yields of extracts with an ETOH of 70% were also the ones with the greatest variety of phenolic components. The investigated extracts demonstrated powerful antioxidant potential, with the highest level of activity shown by the hydroethanolic extract, followed by the acetone and water extracts. The antibacterial assays showed that all tested extracts demonstrated a powerful toxic effect against all tested bacterial strains, with a better effect of the acetone extract against Gram-positive bacterial strains. Our findings suggest that the demonstrated properties of *Mentha longifolia* L extracts can be used in various applications, including the development of a new antibiotic in the pharmaceutical sector and the preservation of fresh nutrition in the food industry. However, further *in vivo* experimental investigations are needed to evaluate the safety profile and eventual toxicity of Moroccan *Mentha longifolia* L. leaf extracts.

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M.B, and A.E.; A MS, HAN, and GFW writing—review and editing . Supervision, B.L, K.B, and E.D. All authors have read and agreed to the published version of the manuscript.

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Plant collection approval: No approval is needed to collect *Mentha longifolia* L. in Morocco for research purposes.

IUCN Policy statement. The collection of plant material complies with relevant institutional, national, and international guidelines and legislation.

Data Availability Statement: All data generated or analyzed during this study are included in this published article.

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