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Phytochemical analysis of hydromethanolic extract and its fractions of myrtus communis leaves from Skikda, Algeria: LC-MS/MS analysis, antioxidant capacities, in-vitro and in silico assessment of anticholinesterase and antidiabetic activities

Jihane Bounaas[1]*, Abdelkader Basli[1]*, El Hassen Mokrani[2], Ismahan Ounissi[1], Samia Benzazia[1], Özen Tevfik[3], Marah Sarmad[3], Erenler Ramazan[4], Yildiz Ilyas[4] and Bensouici Chawki[5]

[1]Laboratory of Interaction Research, Biodiversity, Ecosystems and Biotechnology, Faculty of Sciences, University of August 20, 1955 Skikda, Skikda 21000, Algeria.

[2]Laboratory of Applied Biochemistry, Department of Biochemistry and Cellular and Molecular Biology, Faculty of Natural and Life Sciences, University Mentouri Brothers Constantine1 25017, Algeria.

[3]Department of Chemistry, Faculty of Science, Ondokuz Mayıs University, Samsun 55270, Turkey.

[4]Igdir University, Faculty of Health Sciences Nutrition and Dietetics Department, Igdir 76000, Turkey.

[5]Biotechnology Research Center, Ali Mendjli new city UV 03, BP E73, Constantine 25016, Algeria.

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*to whom all correspondence should be addressed: e-mail: a.basli@univ-skikda.dz

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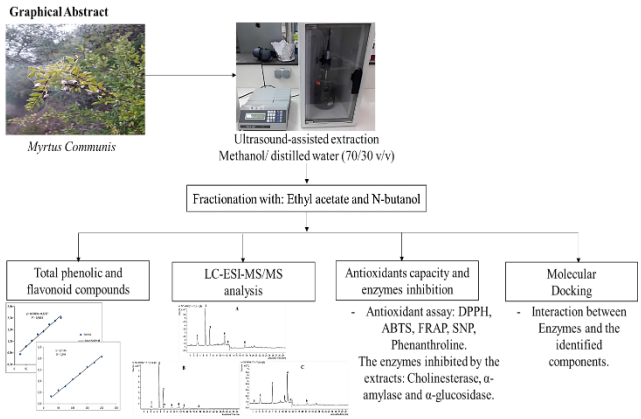
Graphical abstract



Abstract

The increasing prevalence of oxidative stress-related diseases, neurodegenerative and carbohydrate metabolism disorders necessitate the exploration of natural compounds with therapeutic potential. The main objective of this study was to explore in vitro the antioxidant, antidiabetic, and anticholinesterase activities of hydromethanolic extract, ethyl acetate, and N-butanol fractions from Algerian *Myrtus communis* leaves. This study further identified the biochemical composition of the extract and fractions through LC-ESI-MS/MS analysis. A molecular docking has been conducted to clarify the interaction mechanism between enzymes and the identified components. The antioxidant activity was carried out using ferric reducing power, $ABTS^{\bullet+}$, $DPPH^{\bullet}$, silver Nanoparticles and phenanthroline methods. The LC-ESI-MS/MS analyses of the hydromethanolic extract revealed high contents of gallic acid (278.150 µg L^{-1}) and luteolin (112.214 µg L^{-1}). The main constituents of ethyl acetate fraction were gallic acid (1502.228 µg L^{-1}), epigallocatechin (1204.629 µg L^{-1}) and catechin (410.925 µg L^{-1}). In the N-butanol fraction, shikimic acid (2425.644 µg L^{-1}), and gallic acid (220.417 µg L^{-1}) were the primary constituents. Based on the antioxidant results, the extract and fractions demonstrated remarkable antioxidant activity. The most effective was the ethyl acetate fraction, with an IC_{50} value inferior to 10 µg mL^{-1} against all tests used. Concerning the inhibition of cholinesterase, the hydromethanolic extract exhibited an interesting inhibitory effect against acetylcholinesterase (IC_{50}= 22.82 µg mL^{-1}) and butyrylcholinesterase (IC_{50}= 10.70 µg mL^{-1}). The extract and fractions showed significant inhibition against alpha amylase and alpha glucosidase, with IC_{50} ranging from 10.67 to 28.55 µg mL^{-1} and 3.45 to 5.05 µg mL^{-1}, respectively. The docking study showed that gallic acid exhibited the most favorable binding energy towards α-glucosidase. Conversely, Catechin demonstrated superior binding energies for AChE, BChE, and α-amylase. In conclusion, this species exhibits significant antioxidant capacity and enzymes inhibition, suggesting its potential applications in the prevention of many diseases linked to oxidative stress.

Keywords: *Myrtus communis*, antioxidant activity, enzymatic inhibition, LC-MS\MS, Molecular docking

1. Introduction

Civilizations of ancient times such as Egyptian, Indian, and Chinese used herbs to treat and prevent diseases. This knowledge was passed down from generation to generation and contributed to the development of modern phytotherapy that explores therapeutic uses of medicinal plants (Petrovska, 2012). According to the World Health Organization (WHO), more than 80% of the world population still uses medicinal plants as a natural alternative to synthetic medication (Jamshidi-Kia *et al*., 2017). Medicinal plants occupy an important place in research by means of various applications. They can be employed directly, modified to produce semi-synthetic compounds, or used as a basis for creating new synthetic substances with similar characteristics that can be more effective or easier to synthesize in larger quantities (Gurib-Fakim, 2006). Medicinal plant has its own unique mixture of compounds such as flavonoids, polyphenols, alkaloids, and many others that are responsible for various therapeutic properties, especially against oxidative stress and the diseases associated with it, such as Alzheimer, diabetes, and heart disease (Rudrapal *et al*., 2022).

The genus *Myrtus* is among the medicinal plants that have been widely used for centuries for their healing properties. It belongs to the family Myrtaceae, which contains 130 to 150 genus and more than 5600 species. This genus comprises two types of species: one is grown in the Mediterranean region, which is *Myrtus communis*, or true myrtle, and the other is grown in the central Sahara, which is *Myrtus nivellei*, or Saharan myrtle, and both are found in Algeria (Bouzabata *et al*., 2016). The available search result shows that *Myrtus communis* has a broader spectrum of pharmacological effects such as antioxidant, antidiabetic, antimutagenic, and analgesic activities. Additionally, it has antimicrobial activity due to the high concentration of bioactive compounds like terpineol, eucalyptol, α-pinene, linalool, and limonene. The latter showed activity against different bacterial species. Besides its antibacterial activity, *M. communis* has inhibitory effects on the growth of certain fungal species. Furthermore, *M. communis* has anti-inflammatory effects by reducing serum levels of pro-inflammatory cytokines, including IL6 and TNFα. Furthermore, studies showed that the extract of *M. communis* has a neuroprotective effect through AChE and BChE inhibition. Additionally, it has been found to inhibit the reduction of PUFAs (polyunsaturated fatty acids) and prevent the increase of their oxidative products. Moreover, myrtle exhibited cytotoxic effects on diverse cancer cell lines, inducing apoptosis (Alipour *et al*., 2014; Hennia *et al*., 2018).

This study contributes to existing literature by introducing ultrasound-assisted extraction as a viable method that opposes traditional simple maceration techniques employed in previous research and can enhance the yield and efficacy of bioactive compounds present in this species. Our study diverges from the approach taken by Bouaoudia-Madi *et al*. (2019) in the extraction conditions applied (amplitude, solvent, and time). Based on the search results, a few articles have investigated the chemical composition of Algerian *Myrtus communis* leaves (Babou *et al*., 2016; Dairi *et al*., 2015; Dellaoui and Berroukche, 2019). Unlike previous studies that primarily focused on hydroalcoholic extract and essential oil, there are also a limited studies investigating the inhibition of enzymes by Algerian *Myrtus communis* leaf extracts (Ouchemoukh *et al*., 2014). This work investigates the properties of both hydromethanolic extract and its fractions, ethyl acetate and N-butanol, suggesting a new approach for the use of *Myrtus communis* in therapeutic applications. Therefore, the aim of this study is to analyze the chemical composition of hydromethanolic extract, ethyl acetate, and N-butanol fractions of *Myrtus communis* leaves indigenous to northeastern Algeria using LC-ESI-MS/MS to evaluate in vitro its antioxidant activity, as well as the anticholinesterase and antidiabetic capacity, and to enhance our understanding of the molecular interaction involved by employing molecular docking.

2. Materials and methods

2.1. Plant materials

Several tests were conducted using *Myrtus communis* leaves that were collected in October 2021 in the Sidi Mezghiche area of Skikda province in northeastern Algeria (36°41'00"N 6°43'00"E).

2.2. Extraction and fractionation process

The powder of *M. communis* leaves was extracted with methanol and distilled water (70/30; v/v). For this, the ultrasound (vibra-cell™, USA) was used as extraction method, with extraction times of 30 min; and amplitude of 60%. The procedure is repeated three times with renewal of the solvents. The mixture then filtered using Whatman filter paper (pore size of 2 µm) and evaporated at 38°C. A small part of the resulting hydromethanolic extract was left to exhibit biological activity, and the rest was dissolved in distilled water and then extracted using organic solvents with increasing polarity: hexane, ethyl acetate, and N-butanol.

2.3. Measurement of total phenolic and flavonoid content

The total polyphenol content was determined using the method of Müller *et al*. (2010). In summary, 20 µL of samples were applied to 100 µL of diluted Folin Ciocalteu (1:10) and 80 µL of sodium carbonate (7.5%). After that, the 96-well microplate was incubated for 2 hours in the dark, then the absorbance was measured at 765 nm.

The method of Topçu *et al*. (2007) was used to determine the total flavonoids. The procedural steps are summarized as follows: 50 µL of samples were applied to 130 µL of MeOH, 10 µL of potassium acetate (CH_3COOK), and 10 µL of aluminum nitrate nonahydrate ($Al(NO_3)_2$, $9H_2O$). The microplate was incubated for 40 min, and the absorbance was examined at 415 nm.

2.4. LC-ESI-MS/MS Analysis

The 1260 infinity HPLC LC-MS/MS system coupled with an Agilent 6460 triple quadrupole mass spectrometer (USA) was used. The separation process utilized an Agilent Poroshell 120 SB-C18 column (3 × 100 mm, 2.7 µm), a type of reversed-phase column. The LC separation was performed using gradient elution with a mobile phase

consisting of water (A) and methanol (B), both containing 0.1% formic acid and 5 mM of ammonium formate. The gradient elution profile was as follows: at 3 min, A= 75%, B= 25%; at 12 min, A= 50%, B= 50%; at 16 min, A= 10%, B= 90%; at 21 min, A= 10%, B= 90%; and at 24 min, A= 97.5%, B= 2.5%. The injection volume was 5.12 µL, the flow rate was 0.4 mL/min, and the temperature was maintained at 40°C. The separated compounds were introduced into a mass spectrometer, which detects and identifies the target compounds based on their mass-to-charge ratios (m/z) and fragmentation patterns. LC-MS/MS systems often use electrospray ionization (ESI) as an ionization method, employing both positive and negative ionization techniques. The nebulizer gas N2 flow was 8 L min^{-1}, the source voltage was 4000V, and the capillary temperature was 300°C during the LC-MS/MS analysis (Atalar *et al*., 2023). The 30 standards used for this analysis are presented in Table 2. The multiple reactions monitoring (MRM) mode of the mass spectrometer was used to quantify the analytes.

Table 1. Total phenolic and flavonoid content, antioxidant activity of *Myrtus communis* extract and fractions

	IC_{50} (µg mL^{-1}) / $A_{0.5}$ (µg mL^{-1})						
Extract and fractions	**DPPH•**	**ABTS•+**	**FRAP**	**SNP**	**Phenanthroline**	**Total phenolic (mg GAE g^{-1} DW)**	**Flavonoid (mg QE g^{-1} DW)**
Hydromethanol	2.35 ± 0.28 ^{ab}	5.37 ± 0.50 ^{d}	8.72 ± 0.21 ^{d}	17.65 ± 0.2 ^{c}	2.57 ± 0.12 ^{d}	370.83 ± 8.30 ^{b}	79.05 ± 7.00 ^{b}
Ethyl acetate	1.23 ± 0.12 ^{a}	1.10 ± 0.10 ^{a}	5.25 ± 0.10 ^{a}	9.07 ± 0.12 ^{a}	1.28 ± 0.06 ^{ab}	400.75 ± 2.94 ^{a}	116.13 ± 7.20 ^{a}
N-butanol	2.08 ± 0.03 ^{ab}	2.28 ± 0.57 ^{b}	7.72 ± 0.16 ^{cd}	10.22 ± 0.08 ^{ab}	0.70 ± 0.02 ^{ab}	254.76 ± 3.37 ^{c}	62.16 ± 1.60 ^{c}
BHA	9.11 ± 0.89 ^{c}	1.55 ± 0.26 ^{ab}	5.60 ± 0.05 ^{ab}	73.47 ± 0.88 ^{d}	1.49 ± 0.08 ^{bc}	NT	NT
BHT	1.60 ± 0.36 ^{ab}	1.31 ± 0.06 ^{a}	14.48 ± 0.07 ^{e}	>200	2.20 ± 0.04 ^{cd}	NT	NT
Ascorbic acid	2.69 ± 0.22 ^{b}	4.04 ± 0.02 ^{c}	6.77 ± 1.15 ^{bc}	>200	8.30 ± 0.76 ^{e}	NT	NT
Quercetin	NT	NT	NT	11.25 ± 0.78 ^{b}	0.65 ± 0.04 ^{a}	NT	NT

NT: Not Tested, BHT: butylated hydroxytoluene, BHA: butylated hydroxyanisole, GAE: gallic acid equivalent, QE: quercetin equivalent, DW: dry weight. The results are reported as the mean ± SD. The values in the same column with varied superscripts (a, b, c, d, or e) are significantly different ($p < 0.05$).

2.5. Determination of antioxidant activity

The antioxidant activity of the hydromethanolic extract and its different fractions of *Myrtus communis* leaves were determined using DPPH• (2,2-diphenyl-1-picrylhydrazyl), SNP (Silver nanoparticle), ABTS•+ (2,2′ -Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid), diammonium salt), FRAP (Ferric reducing antioxidant power), and Phenanthroline. Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), quercetin and ascorbic acid were used as standards.

2.5.1. DPPH• scavenging activity

The radical scavenging activity was evaluated using Blois's, (1958) method. 40 µL of samples were mixed with 160 µL of DPPH•. After that, the microplate was incubated for 30 min in the dark then the absorbance was measured at 517 nm.

2.5.2. ABTS•+ scavenging activity

The ABTS•+ scavenging activity of samples were assessed according to the method developed by Re *et al*. (1999). In summary, 40 µL of each sample was applied to 160 µL of ABTS•+. After that, the microplate was incubated for 10 min in the dark then the absorbance was measured at 734 nm.

2.5.3. FRAP assay

The assay were determined using the method reported by Oyaizu, (1986) with slight modification. 10 µL of samples was mixed with 40 µL of phosphate buffer (pH 6.6) and 50 µL of potassium ferricyanide (1%), the microplate was incubated at 50°C for 20 min. After that, 50 µL of tricarboxylic acid (10%), 40 µL of H_2O and 10 µL of ferric chloride (0.1%) were added. The absorbance was measured at 700 nm.

2.5.4. SNP assay

The SNP assay was determined by Özyürek's *et al*. (2012) method. 20 µL of samples were added to 130 µL of SNP solution (silver nitrate (1 mM) + trisodium citrate (1%)) and 50 µL of H_2O. After 30 min of incubation at 25°C, the absorbance was measured at 423 nm.

2.5.5. Phenanthroline assay

The phenanthroline assay was measured following the method of Szydłowska-Czerniak *et al*. (2008). In a 96-well microplate, 10 µL of samples were mixed with 50 µL of ferric chloride (0.2%), 30 µL of Phenanthroline (0.5%) and 110 µL of methanol. The plate was incubated at 30°C for 20 min, and the absorbance was measured at 510 nm.

The percentage inhibition was determined by the following equation:

$$\%\ \text{inhibition} = 100 \times (\text{Abs of control} - \text{Abs of sample}) / \text{Abs of control} \quad (1)$$

For DPPH• and ABTS•+, the results were expressed as IC_{50} values (µg mL^{-1}). For FRAP, SNP and phenanthroline tests, the results were expressed as $A_{0.50}$ values (µg mL^{-1}).

2.6. Inhibition assay of enzymatic activity

2.6.1. Anticholinesterase activity

The tested sample were evaluated using the method established by Ellman *et al*. (1961). 150 µL of sodium phosphate buffer (100 mM, pH 8.0), 10 µL of the test sample and 20 µL of Acetylcholinesterase (AChE) or Butyrylcholinesterase (BChE), were added. The mixture was incubated at 25°C for 15 min. 10 µL of DTNB (5, 5'-Dithiobis (2-nitro-benzoic acid) and 10 µL of acetylthiocholine iodide (0.71 mM, AChE's substrate) or butyrylthiocholine iodide (0.2 mM, BChE's substrate) were

added. The absorbance was measured at 412 nm after 0 min and 15 min. Galantamine was used as standard.

2.6.2. Alpha-amylase inhibitor activity

The method of Yang *et al*. (2012) was used to determine α-amylase inhibitor activity. The procedural steps are summarized as follows: 82 µL of extracts were added to 10 µL of α-amylase (1U). After 10 min of incubation at 37°C, 8 µL of starch (1%) was added. The microplate was incubated again at 37°C for 10 min. After that, 50 µL of HCl (10%), 15 µL of IKI and 50 µL of H_2O were added. The absorbance was read at 630 nm. Acarbose was used as standard.

2.6.3. Alpha glucosidase inhibitor activity

The procedural steps are summarized as follows: 10 µL of samples were added to 25 µL of α-glucosidase (0.2 U/mL), 25 µL of 4-Nitrophenyl β-D-glucopyranoside (PNPG, 0.5 Mm) and 50 µL of phosphate buffer (20 mM, Ph 6.9). The microplate was incubated at 37°C for 10 min. After that, 100 µL of $NaCO_3$ (0.2 M) was added (Mayur *et al*., 2010). The absorbance was read at 410 nm. Acarbose was used as standard.

3. Statistical analysis

The statistical analysis was performed using IBM SPSS statistics version 22. One-way analyses of variance (ANOVA) were conducted to compare the means between different treatments. Tukey's multiple range tests were used for post-hoc analysis when the results were statistically significant ($p < 0.05$). Each sample was tested three times. The results are reported as the mean ± standard deviation. The Pearson correlation coefficient (r) was chosen to determine the relationship between antioxidant activity, flavonoid, and total phenolic compounds.

4. Molecular docking study

A molecular docking study was performed on 6 key compounds from *Myrtus communis* extract and fractions to evaluate their binding affinities and investigate their interaction modes within the active sites of AChE, BChE, α-amylase, and α-glucosidase. The crystal structures of these enzymes were obtained from the Protein Data Bank, using the following PDB IDs: 4M0E for AChE, 2XQF for BChE, 4GQR for α-amylase, and 3L4X for α-glucosidase. The docking process was set up using LeadIT 2.1.8 software (available at www.biosolveit.com). This preparation involved removing cofactors, heteroatoms, and water molecules, except for those within the active pockets (Boulhissa *et al*., 2021). Subsequently, missing atoms were added, formal charges were calculated, and the active sites were defined by selecting residues within a 6.5 Å radius around the inhibitor in the crystal structure (Djehiche *et al*., 2024). This selection was further refined by including residues beyond 6.5 Å that were critical for maintaining the continuity of the cavity (Ikhlef *et al*., 2024). The protonation states and orientations of side chains for each amino acid in the active sites were then reviewed, and the resulting structures were fully minimized and exported as mol2 files (Srief *et al*., 2023).

The three-dimensional coordinates of the studied compounds were retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and subsequently prepared for docking using the LigPrep tool within Schrodinger's Maestro version 11.3 (Maestro, 2015). During the preparation phase, we generated all possible tautomer, protonation states (at a physiological pH of 7.4±1), and enantiomers for each compound, ensuring a comprehensive exploration of their chemical diversity (Mokranı *et al*., 2022). Following this, molecular docking simulations were performed using FlexX 2.1.8 (Rarey *et al*., 1996), which applies an incremental construction strategy to accurately model ligand binding. The docking process was guided by the FlexX scoring function, which ranks the compounds based on their binding free energy (ΔG) expressed in kJ/mol. To validate the docking protocol, the co-crystallized ligand of each enzyme was re-docked into its active site, achieving a Root Mean Square Deviation (RMSD) of less than 2Å, which confirmed the reliability of the docking setup. Once validated, the same docking parameters were employed to assess the binding affinities of the studied molecules (Hioual *et al*., 2014).

5. Results and Discussion

5.1. Total phenolic and flavonoid content

The results obtained are presented in Table 1. The total polyphenol was presented as mg GAE g^{-1} DW using the gallic acid calibration curve (y = 0.0089x + 0.3217) and the total flavonoid content was presented as mg QE g^{-1} DW using a calibration plot of quercetin (y = 0.0106x). According to the findings, the ethyl acetate fraction had the highest phenolic and flavonoid content, followed by hydromethanolic extract and then the N-butanol fraction. It is possible that the selection of extraction solvent might have an impact on this result (Lefebvre *et al*., 2021). The same observation was found in a study by Bouaziz *et al*. (2015). In Şafak's *et al*. (2023) study on different extracts of *M. communis* leaves gathered from Silifke, Turkey, it was observed that the ethyl acetate extract demonstrated high phenolic content, followed by butanol, methanol, water, and then dichloromethane extracts, while the flavonoid content presents in large quantities in the methanol extract, followed by ethyl acetate, butanol, dichloromethane, and then water. In other studies carried out by Amensour *et al*. (2010) and Bouyahya *et al*. (2016), the ethyl acetate extract gave the least yield of phenolic and flavonoid content. In the study conducted by Bouaoudia-Madi *et al*. (2019), ultrasound-assisted extraction was performed using ethanol (70%; v/v) at an amplitude of 30% for 7.5 min. A total phenolic content of 241.66 ± 12.77 mg GAE g^{-1} DW was obtained, which was slightly lower than our hydromethanolic extract, whereas, their flavonoid content (18.99 ± 1.3 mg QE g^{-1}) was relatively low.

Therefore, all of our myrtle leaf extracts have an important source of phenolic and flavonoid compounds. However, we observed differences in the value and order of the extracts. This difference in our results may be due to various factors, such as geographical, climatic, and genetic factors, the degree of plant maturation, extraction method, and extraction conditions (Ksouri *et al*., 2008).

Table 2. Phytochemical profile of hydromethanolic extract, ethyl acetate and N-butanolic fractions of *Myrtus communis*

No	Analyte	RT (min)	Concentration µg L^{-1}			Ion Source	Ion Transitions	Ion Mode	R^2	LOQ (µg L^{-1})	LOD (µg L^{-1})	Linearity Range (µg L^{-1})
			Hydro methanolic	Ethyl acetate	N-butanol							
1	Shikimic acid	1.297	ND	ND	2425.644	ESI	173.0 -> 93.1	-	0.9992	78.7970	20.3983	75-4800
2	Gallic acid	3.182	278.150	1502.228	220.417	ESI	169.0 -> 125.1	-	0.9986	18.5862	7.1674	31.25-500
3	Protocatechuic acid	5.451	19.633	52.849	ND	ESI	153.0 -> 109.0	-	0.9969	13.1729	3.1564	15.625-250
4	Epigallocatechin	6.792	85.493	1204.629	114.458	ESI	307.0 -> 139.0	+	0.9995	3.8750	2.0903	12.5-200
5	Catechin	6.896	ND	410.925	ND	ESI	288.9 -> 245.1	-	0.9946	7.5013	1.7055	343.750-5500
6	Chlorogenic acid	7.333	ND	ND	ND	ESI	353.0 -> 191.0	-	0.9981	25.9023	11.5890	31.25-500
7	Hydroxybenzaldehyde	7.618	ND	ND	ND	ESI	121.0 -> 92.0	-	0.9993	12.8651	4.9742	15.625-250
8	Vanillic acid	7.782	ND	ND	ND	ESI	167.0 -> 151.8	-	0.9958	1424.2132	219.0421	1250-20000
9	Caffeic acid	7.820	ND	ND	ND	ESI	178.9 -> 135.1	-	0.9994	24.1620	6.9205	31.25-500
10	Syringic acid	8.375	ND	ND	ND	ESI	197.1 -> 181.8	-	0.9990	857.3388	358.5000	1250-20000
11	Caffeine	8.412	ND	ND	ND	ESI	195.0 -> 137.9	+	0.9986	15.4959	6.8099	18.75-300
12	Vanillin	8.560	ND	ND	ND	ESI	153.0 -> 125.0	+	0.9949	40.5411	14.5885	62.5-1000
13	o-coumaric acid	9.307	ND	40.832	ND	ESI	163.0 -> 119.1	-	0.9996	7.9973	4.0164	15.625-500
14	Salicylic acid	9.527	ND	ND	ND	ESI	137.0 -> 93.1	-	0.9981	82.9646	47.6695	112.5-1800
15	Naringenin	14.746	ND	ND	ND	ESI	270.9 -> 119.1	-	0.9960	0.4575	1.3694	31.25-500
16	Rutin	12.266	ND	ND	ND	ESI	611.0 -> 302.8	+	0.9980	240.6720	59.5597	125-2000
17	Polydatine	9.615	ND	ND	ND	ESI	390.9 -> 228.9	+	0.9987	1.8411	1.1471	7.8125-125
18	trans-ferulic acid	10.080	ND	ND	ND	ESI	193.1 -> 133.9	-	0.9950	11.5276	6.1184	31.25-1000
19	Sinapic acid	10.385	ND	ND	ND	ESI	223.1 -> 208.0	-	0.9972	4.9652	1.9437	125-2000
20	p-coumaric acid	11.482	ND	ND	ND	ESI	163.0 -> 119.0	-	0.9987	17.5416	3.5348	31.25-500
21	Protocatechuic ethyl ester	11.739	ND	ND	ND	ESI	181.0 -> 107.9	-	0.9996	24.9201	14.5610	15.625-1000
22	Hesperidin	11.812	ND	ND	ND	ESI	611.0 -> 302.9	+	0.9957	17.6753	4.1396	31.25-500
23	Hesperetin	15.950	ND	ND	ND	ESI	300.9 -> 164.0	-	0.9966	0.649	0.3008	31.25-500
24	Isoquercitrin	11.414	48.814	185.706	157.823	ESI	464.9 -> 302.8	+	0.9982	11.2680	9.9382	18.75-300
25	Quarcetin-3-xyloside	12.441	ND	ND	ND	ESI	432.7 -> 299.5	-	0.9900	69.4059	18.7126	125-2000
26	Kaempferol-3-glucoside	12.878	5.639	20.193	12.919	ESI	448.8 -> 286.9	+	0.9997	4.5238	1.1609	7.8125-125
27	Chrysin	14.162	ND	ND	ND	ESI	254.9 -> 153.0	+	0.9989	0.1338	0.0737	1.5625-25

28	trans-cinnamic acid	14.331	ND	ND	ND	ESI	149.0 -> 131.1	+	0.9999	22.0279	11.1853	31.25-500
29	Kaempferol	16.456	ND	ND	ND	ESI	284.9 -> 116.9	-	0.9997	5.4004	1.8683	312.5-10000
30	Luteolin	17.328	112.214	128.384	167.736	ESI	285.0 -> 133.1	+	0.9962	21.4535	20.0000	31.25-500

ND: Not detected, RT: retention time, LOQ\LOD: limit of quantification\ limit of detection, R2: coefficient of determination.

5.2. LC-ESI-MS/MS Analysis

The results of LC-MS/MS analysis are summarized in Table 2. The contents of gallic acid (278.150 µg L^{-1}) and luteolin (112.214 µg L^{-1}) were found in high concentration in hydromethanolic extract. The ethyl acetate fraction showed a high concentration of gallic acid (1502.228 µg L^{-1}), epigallocatechin (1204.629 µg L^{-1}), and catechin (410.925 µg L^{-1}). The compounds that ranked second in abundance were: isoquercitrin (185.706 µg L^{-1}) and luteolin (128.384 µg L^{-1}). In the N-butanol fraction, shikimic acid (2425.644 µg L^{-1}), and gallic acid (220.417 µg L^{-1}) were the primary constituents, while luteolin (167.736 µg L^{-1}), isoquercitrin (157.823 µg L^{-1}) epigallocatechin (114.458 µg L^{-1}) were the second abundant compound. The hydromethanolic extract and fractions share some common components. However, their concentrations varied among the fractions, possibly due to the characteristics of the solvent employed (Lefebvre *et al*., 2021).

Table 3. Pearson correlation coefficient (r) between phenolic, flavonoid compounds and antioxidant activities

	Total phenolic	Flavonoid	DPPH	ABTS	FRAP	Phenanthroline	SNP
Total phenolic	1	0.849 [a]	- 0.453	0.105	- 0.421	0.594	0.204
Flavonoid	0.849 [a]	1	- 0.812[a]	- 0.416	- 0.810	0.098	- 0.321

[a]*the correlation is significative at 0.01.*

In a study by Aidi Wannes *et al*. (2010) on methanolic extract of *Myrtus communis* leaves from Nabeul, Tunisia, demonstrated the presence of caffeic acid, vanillic acid, and syringic acid, which were not detected in our extract and fractions. In the same study, catechin was present in trace amounts. In Iran, Vafadar Shoshtari *et al*. (2017) identified rutin, chlorogenic acid and p-coumaric acid that were not detected in our extract and fractions. Another study on ethanolic extract from Blida, Algeria (Dellaoui and Berroukche, 2019) revealed the presence of chrysin, naringenin and kaempferol, which were absent in our hydromethanolic extract and fractions. The difference in our finding is due to the geographical, climatic, and genetic factors, the degree of plant maturation, extraction solvent, extraction methods, and extraction condition (Ksouri *et al*., 2008).

Table 4. AChE, BChE, alpha amylase and alpha glucosidase inhibition by hydromethanolic extract and fractions

	IC_{50} (µg mL^{-1})			
Extract and fractions	**AChE**	**BChE**	**Alpha amylase**	**Alpha glucosidase**
Hydromethanol	22.82 ± 0.05 [b]	10.70 ± 0.22 [a]	28.55 ± 0.22 [c]	5.05 ± 0.20 [b]
Ethyl acetate	35.71 ± 0.30 [c]	24.58 ± 1.30 [b]	18.66 ± 0.49 [b]	3.45 ± 0.36 [a]
N-butanol	41.71 ± 0.07 [d]	22.07 ± 0.61 [b]	10.67 ± 0.58 [a]	4.86 ± 0.09 [b]
Galantamine	4.66 ± 0.42 [a]	34.75 ± 1.99 [c]	NT	NT
Acarbose	NT	NT	10.52 ± 0.02 [a]	5.24 ± 0.06 [b]

NT: Not Tested. The results are reported as the mean ± SD. The values in the same column with varied superscripts (a, b, c, d) are significantly different ($p < 0.05$).

5.3. Antioxidant activities

The antioxidants can trap free radicals and stabilize them, thereby decreasing their potential to cause damage. This is done either by the transfer of a hydrogen atom, the transfer of an electron, or by chelating pro-oxidant metal ions (Lü *et al*., 2010). Table 1 provides a summary of the study's findings. According to the data, the extract and fractions of *M. communis* leaves exhibited the highest activity against all tests used. Regarding the $DPPH^{\bullet}$ activity, the hydromethanolic extract and fractions were ranked based on their level of effectiveness, as follows: ethyl acetate fraction, N-butanol fraction, and then hydromethanolic extract. Our findings are in line with those obtained by Bouaziz *et al*. (2015) and Şafak *et al*. (2023). Our ethyl acetate fraction demonstrated superior effectiveness compared to the standards used and the values reported by Al-Maharik *et al*. (2023) and Moein *et al*. (2015).

Concerning the $ABTS^{\bullet+}$ assay, the hydromethanolic extract and fractions showed strong free radical scavenging activity, particularly the ethyl acetate fraction, which exhibited better activity than the standards, while the hydromethanolic extract was a bit off lower than the standards utilized. This observation is consistent with the findings of a study by Bouaziz *et al*. (2015). On the other hand, Amensour *et al*. (2010) found that the ethyl acetate

extract exhibited weak activity against $ABTS^{\bullet+}$, with a maximum inhibition rate of 80% at a concentration of 1000 µg mL^{-1}.

After conducting the FRAP assay, the extract and fractions had an important capacity to reduce the iron Fe^{3+} to Fe^{2+}; the most effective one was the ethyl acetate fraction with $A_{0.5}$ of 5.25 ± 0.10 µg mL^{-1}, almost the same value as BHA (5.60±0.05 µg mL^{-1}), and more effective than ascorbic acid and BHT. The hydromethanolic extract and N-butanol fraction were slightly lower than BHA and ascorbic acid. Our findings regarding the FRAP activity are consistent with those reported by Bouaziz *et al*. (2015) and were superior to those reported by Moein *et al*. (2015) and Yangui *et al*. (2021).

The phenanthroline method is used to assess the antioxidant capacity of a compound to reduce Fe^{3+} to Fe^{2+} by reacting with orthophenanthroline, which results in the formation of an orange-colored complex (Szydłowska-Czerniak *et al*., 2008). According to the result, the N-butanol fraction with an $A_{0.5}$ of 0.70±0.02 µg mL^{-1} was nearly the same as quercetin's value of 0.65±0.04 µg mL^{-1} followed by the ethyl acetate fraction, BHA, BHT, hydromethanolic extract, and then ascorbic acid.

For the SNP assay, the hydromethanolic extract and fractions exhibited strong activity similar to that of quercetin, with the ethyl acetate fraction found to hold an advantage. Based on the provided search results, there is no specific mention of SNP assay being used to test the antioxidant capacity of *Myrtus communis* extracts.

The high antioxidant activity recorded in our extract and fractions is attributed to their richness in functional groups such as hydroxyl groups (-OH), carboxylic groups (-COOH), methoxy groups ($-OCH_3$), and phenolic groups (C_6H_4OH), as well as the specific position and the presence of multiple -OH groups and other substituents on the aromatic ring, which give a complementary mechanism of action to neutralize free radicals (J. Chen *et al*., 2020; Gulcin, 2020).

Several factors may explain the difference observed between our findings and the findings mentioned above. Besides the region, seasons, and climatic factors, the extraction method, the methodologies used to test the antioxidant capacity, and the experimental conditions such as PH, reagent and sample concentration, etc. have the potential to influence the efficiency of the extracts (Di Majo *et al*., 2011; Munteanu and Apetrei, 2021). Although these methods used to test the capacity of antioxidants have allowed us to draw conclusions about the potency of extracts, it is important to note that some of these tests may be artificial and do not show the complexity of oxidative stress in food or living organisms (Munteanu and Apetrei, 2021).These extracts may then be potent in a laboratory test, but they might also be less effective or inactive under biological conditions. Additional tests, using other methods, and ex vivo and in vivo studies are required to confirm the efficacy of extracts.

5.4. Relationship between antioxidant activity, total phenolic and flavonoid contents

The total phenolic, flavonoid, and antioxidant assays were subjected to a correlation analysis. The findings are shown in Table 3. The total phenolic and flavonoid compounds showed a very strong positive relationship (r = 0.849). A strong negative correlation was noted between flavonoid, DPPH, and FRAP with values of -0.812 and -0.810, respectively. There was a weak positive relationship between total phenolic, $ABTS^{\bullet+}$, and SNP with r below 0.25. A weak negative relationship was observed between flavonoid and SNP, indicating that there is an opposite relationship between them but in a very feeble manner. The total phenolic and phenanthroline showed a moderate positive correlation. A very weak positive relationship and not statistically significant was observed between flavonoid and phenanthroline with r equal to 0.098. A moderate negative relationship was observed between total phenolic, $DPPH^{\bullet}$, and FRAP and between flavonoid and $ABTS^{\bullet+}$, meaning that the increase in total phenolic and flavonoid was accompanied by the decrease in $DPPH^{\bullet}$, FRAP, and $ABTS^{\bullet+}$ activity, but in a moderate manner. Several studies indicate a strong correlation between the antioxidant activity of plant extracts and their phenolic content, as determined by $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and FRAP tests (Asem *et al*., 2020; Dudonné *et al*., 2009). As observed in our case, a strong correlation was only noted between flavonoid, DPPH, and FRAP assays, while the others showed either a moderate or weak correlation. Therefore, while the amount of total phenolic and flavonoid compounds is important, it is not the only determinant of the antioxidant activity of plant extracts but also the structure of phenolic compounds, the presence of a specific compound and its concentration, and also the synergy between all compounds present in the extract (Munteanu and Apetrei, 2021).

5.5. Enzymatic inhibition

The Table 4 summarizes the BChE, AChE, alpha amylase and alpha glucosidase inhibition by extract and fractions.

5.5.1. Cholinesterase inhibition

Cholinesterase occurs in two forms: butyrylcholinesterase and acetylcholinesterase. Both of them are extremely important as they contribute to the regulation of acetylcholine (ACh) levels, which is vital for cholinergic neurotransmission. On the other hand, during high cholinesterase activity, the levels of ACh became low. This decrease in ACh levels causes memory and concentration problems that are symptoms of Alzheimer's disease (AD) (Z.-R. Chen et al., 2022). Cholinesterase inhibition is one of the strategies that could help delay or prevent the progression of AD. In our study, the hydromethanolic extract and fractions exhibited an interesting inhibitory effect against AChE; however, they were less effective than galantamine (Table 4). The hydromethanolic extract and the fractions showed the highest BChE inhibition, ranging from 10.70 to 24.58 µg mL^{-1} and were more effective than galantamine (IC_{50}= 34.75±1.99 µg mL^{-1}). Our study is consistent with the findings of Begum *et al*. (2012) and Ouchemoukh *et al*. (2014). This similarity in our results suggests that the extract and fractions have potential therapeutic applications for treating neurodegenerative diseases like Alzheimer's. However, in another study conducted by Tumen *et al*. (2012) on *M. communis* leaves

collected from Mersin, Turkey, all extracts (dichloromethane, acetone, ethyl acetate and methanol) did not exhibit significant inhibition against AChE. The difference in our results could be attributed to geographical origin, environmental factors, and extraction method, which may influence the production of secondary metabolites that exhibit cholinesterase inhibition. Many studies highlight alkaloids as key compounds responsible for cholinesterase inhibition (Tamfu *et al*., 2021). According to the literature, the leaf of *Myrtus communis* does not contain significant amounts of alkaloids (Dellaoui and Berroukche, 2019; Mahmoudvand *et al*., 2015; Sisay *et al*., 2017). However, the extract and fractions still exhibited significant cholinesterase inhibition. This suggests that other bioactive compounds present in the extract may play an essential role in this activity. Phenolic compounds can inhibit cholinesterase, and their effectiveness depends on the number and position of methoxy (-OCH_3) and free hydroxyl (-OH) groups located on the phenol ring as well as the presence of -OCH_3 substitutions. On the other hand, flavonoids can alter the structure of cholinesterase, preventing access to the active site (Tamfu *et al*., 2021).

5.5.2. Alpha amylase and alpha glucosidase inhibition

The inhibition of alpha amylase and alpha glucosidase affords the control of glucose level (Tiwari and Rao, 2002). Concerning alpha amylase inhibition, the N-butanolic fraction (10.67±0.58 µg mL^{-1}) was the most effective compared to the other and was slightly lower than acarbose (10.52±0.02 µg mL^{-1}) (Table 4). For alpha glucosidase inhibition, all samples demonstrated interest activity with IC_{50} values ranging from 3.45 to 5.05 µg mL^{-1} (Table 4). In a study by Şafak *et al*. (2023), the ethyl acetate showed also high activity against the α-glucosidase, with 96% inhibition at a concentration of 0.5 mg mL^{-1}, whereas, contrary to our findings, their N-butanol extract revealed low activity against the same enzyme (35.52% at 1 mg L^{-1}). Another study by GHOLAM *et al*. (2008) on the methanolic and aqueous extracts from Kerman, Iran, the extracts showed the highest alpha glucosidase inhibitory activity, with values of 97±3% and 99±3%, respectively. This indicated that the plant extracts have the potential to enhance glycemic control, suggesting the need for further research into their application in diabetes care. However, in vivo results may confirm or invalidate these in vitro results, showing the need for extensive studies that link laboratory results with real life biological responses.

Table 5. Binging energies values of 6 major compounds derived from *Myrtus communis* extract and fractions with AChE, BChE, α-amylase and α-glucosidase enzymes, compared to Galantamine and Acarbose

Compound	Binding energy (KJ/mol)			
	AChE	**BChE**	**α-amylase**	**α-glucosidase**
Shikimic acid	-16.75	-16.05	-20.12	-25.45
Gallic acid	-22.97	-23.95	-20.83	-28.26
Epigallocatechin	-35.17	-32.83	-23.69	-20.91
Catechin	-36.48	-35.58	-25.24	-23.43
Isoquercitrin	-28.59	-31.89	-16.09	-23.07
Luteolin	-27.57	-30.48	-24.61	-23.90
Galantamine	-21.03	-23.66	NT	NT
Acarbose	NT	NT	-20.46	-13.30

NT: Not tested.

5.6. Molecular docking

Molecular docking studies were performed to estimate the binding affinities and the interaction mode of 6 major compounds from *Myrtus communis* extract and fractions against the AChE, BChE, α-amylase, and α-glucosidase active sites. Galantamine served as the reference standard for AChE and BChE, while acarbose was employed for α-amylase and α-glucosidase.

As shown in Table 5, gallic acid exhibited the most favorable binding energy towards α-glucosidase, highlighting its significant potential as a potent inhibitor of this enzyme. Conversely, catechin demonstrated superior binding energies for AChE, BChE, and α-amylase, underscoring its broader inhibitory effectiveness across multiple enzymes. Based on these promising results, both compounds were selected for a more detailed examination of their interaction mechanisms within the active sites of the respective enzymes.

As depicted in Figure 1, molecular docking analysis reveals that Catechin binds to both the Catalytic Anionic Site (CAS) and the Peripheral Anionic Site (PAS) of AChE, in contrast to galantamine, which only interacts with the CAS. This dual-site binding is significant, as the most effective AChE inhibitors developed recently target both the CAS and PAS simultaneously, similar to Catechin (Mokrani *et al*., 2019). Additionally, catechin forms seven hydrogen bonds with key residues, including Trp86, Gly121, Gly112, Tyr133, Glu202, and Ser203. Notably, Ser203 is part of the AChE catalytic triad, essential for the enzyme's function (Cheung *et al*., 2013). The extensive hydrogen bonding network formed by Catechin may contribute to its superior inhibitory potency against AChE compared to galantamine, which establishes only three hydrogen bonds with Tyr133, Glu202, and Tyr337 (Figure 2). The BChE-Catechin docked complex showed a binding energy of -35.58 kJ/mol and formed six hydrogen bonds with key residues, including Trp82, Gly116, Gly117, Glu197, and Ser198, the latter being part of the BChE catalytic triad (Wandhammer *et al*., 2011). The difference in BChE inhibitory potency between catechin and galantamine may be attributed to the disparity in the number of hydrogens bonds each

compound forms with the enzyme (Figure 3). Indeed, catechin establishes six hydrogen bonds, whereas galantamine forms only three with Trp82, Trp430, and Tyr440. Moreover, catechin binds to both the CAS and PAS of the BChE active site, while galantamine interacts only with the CAS (Figure 4).

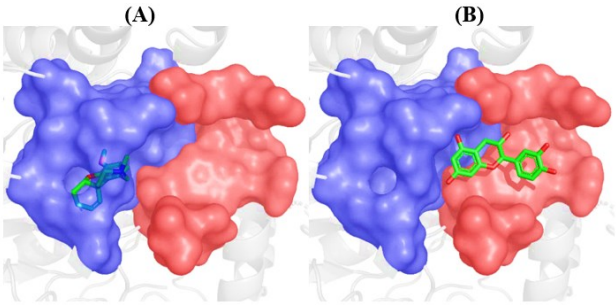


Figure 1. Positioning of Galantamine (A) and Catechin (B) into the AChE-active site. The CAS region of the cavity is represented in blue and the PAS in red. The color code of the ligand atoms is the following: carbon in green, oxygen in red and nitrogen in blue

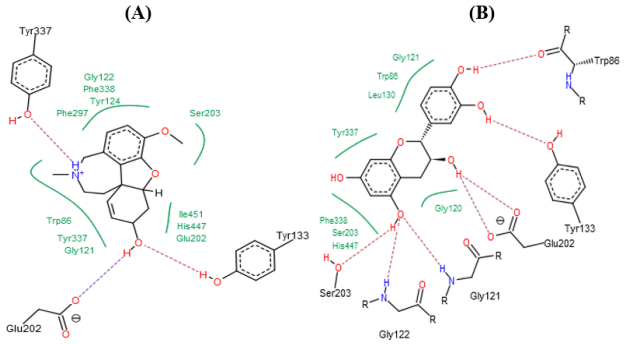


Figure 2. Binding mode interaction of Galantamine (A) and Catechin (B) into the AChE-active site. Purple broken lines show the hydrogen bonds and green area the hydrophobic interactions

The docking study showed that Catechin is well-suited to the active site of α-amylase (Figure 5), forming seven hydrogen bonds with critical residues like His101, Asp197, Trp59, Gln63, and with two water molecules within the active site. This extensive network of hydrogen bonds likely plays a key role in Catechin's strong inhibitory effect. In comparison, although Acarbose also forms seven hydrogen bonds, it interacts with a different set of residues (Asp300, Arg195, Asn298, and Thr163), highlighting the distinct inhibitory strategies employed by Catechin and Acarbose (Figure 6).

On the other hand, Gallic acid demonstrated a significantly favorable binding energy of -28.26 kJ/mol, substantially surpassing Acarbose, the standard inhibitor, which recorded a binding energy of -13.30 kJ/mol. This strong affinity for the α-glucosidase active site, coupled with its ability to form eight hydrogen bonds with key residues such as Asp203, Arg526, and Asp542, as well as with two water molecules in the active site, highlights Gallic acid's potential as a promising lead compound for therapeutic development (Figure 7 and 8). The higher inhibitory potency of Gallic acid compared to Acarbose may be due to its ability to form more hydrogen bonds with the enzyme.

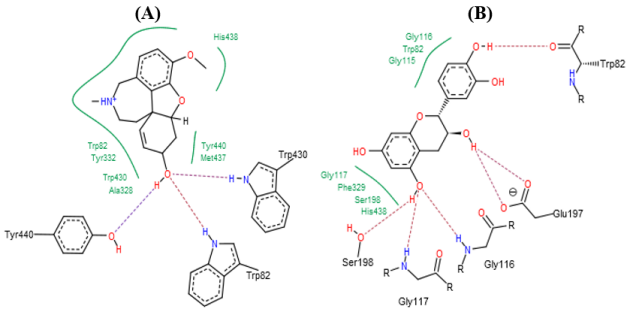


Figure 3. Binding mode interaction of Galantamine (A) and Catechin (B) into the BChE-active site. Purple broken lines show the hydrogen bonds and green area the hydrophobic interactions

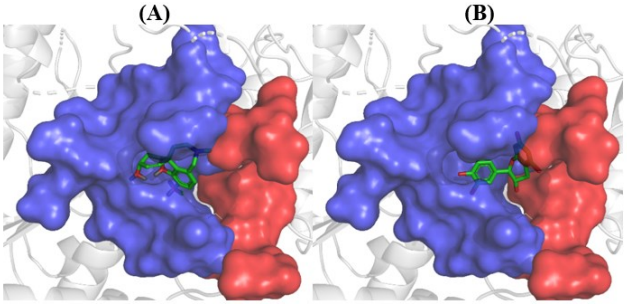


Figure 4. Positioning of Galantamine (A) and Catechin (B) into the BChE-active pocket. The color code of the ligand atoms is the following: carbon in green, oxygen in red and nitrogen in blue

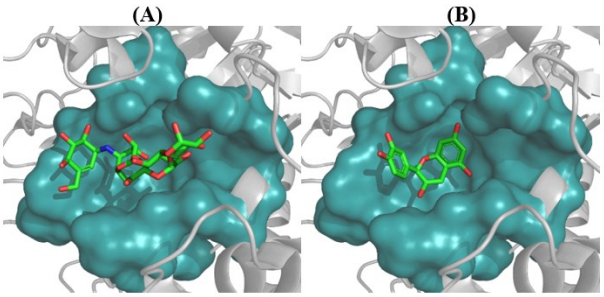


Figure 5. Positioning of Acarbose (A) and Catechin (B) into the α-amylase active site. The active pocket is represented in cyan "surface" whereas the ligand atoms are color-coded as follows: carbon in green, oxygen in red and nitrogen in blue

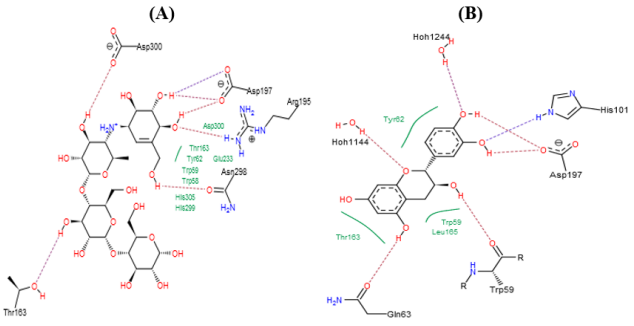


Figure 6. Binding mode interaction of Acarbose (A) and Catechin (B) into the α-amylase active site. Purple broken lines show the hydrogen bonds and green area the hydrophobic interactions

The presence of catechin could explain the significant effect of the ethyl acetate fraction against cholinesterase and α-amylase. Even in the absence of catechin, the hydromethanolic extract and N-butanol fraction still demonstrated a notable effect. This suggests that the effect could be attributed to the synergy between all

compounds or possibly that another bioactive compound is responsible for these effects.

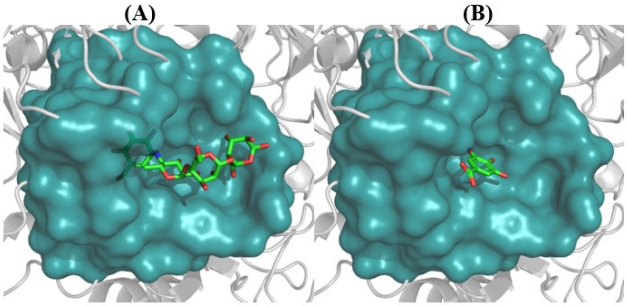


Figure 7. Positioning of Acarbose (A) and Gallic acid (B) into the α-glucosidase active site. The color-coded is as follows: carbon in green, oxygen in red and nitrogen in blue

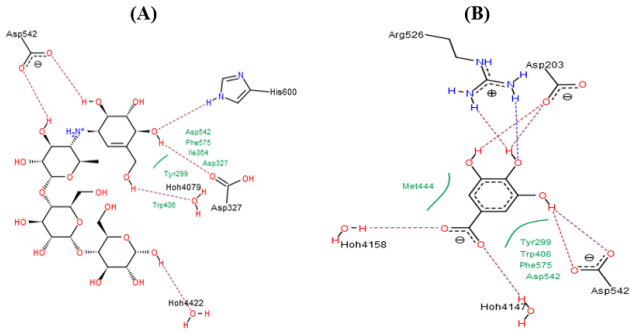


Figure 8. Binding mode interaction of Acarbose (A) and Gallic acid (B) into the α-glucosidase active site. Purple broken lines show the hydrogen bonds and green area the hydrophobic interactions

6. Conclusion

In conclusion, extraction conditions such as method, time, solvent, etc. influence the yields of polyphenols and flavonoids as well as the biological activities of plant extracts. This emphasizes the importance of optimizing extraction techniques to ensure consistent quality and efficacy of plant extracts, facilitating comparisons and applications in research and industry.

Based on our findings, *Myrtus communis* leaves showed potential antioxidant and anticholinesterase activities and highlighted the ability to significantly inhibit key enzymes involved in carbohydrate digestion, namely alpha amylase and alpha glucosidase. This indicates that this species is considered a natural antioxidant source and has the potential to be used as a dietary supplement and in preventing or reducing diseases associated with oxidative damage and metabolic disorders.

The LC-MS/MS analysis of the samples showed their main chemical components. The results of the molecular docking experiment, indicated that gallic acid exhibited the most favorable binding energy towards α-glucosidase and catechin demonstrated superior binding energies and inhibitory activity on AChE, BChE, and α-amylase, suggesting that both compounds could be used as a natural inhibitor.

While these in vitro findings are promising, it is essential to use different techniques (electrochemistry and chromatography) to determine the antioxidant activity and validate the effects in an in vivo study.

Conflict of interest

The authors report no conflicts of interest.

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