Egypt. J. Chem. **Vol. 67**, No. 11 pp. 17 - 27 (2024)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Metabolic Profiling of *Millingtonia hortensis* L.f. Leaves Using UHPLC-MS/MS and Evaluation of its Antioxidant, Cytotoxic, Anticholinesterase Activities and Molecular Docking

Sohila M. Elnaggar[a]; Soumaya S. Zaghloul[a]; Hanaa A. Kassem[b]; Amira K. El Motayam[b]*

[a]*Department of Pharmacognosy, Faculty of Pharmacy, MSA University, Cairo, Egypt*

[b]*Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Cairo, Egypt*

Abstract

Background: *Millingtonia hortensis* L.f. known as Tree Jasmine or Indian Cork tree, stands as the only species within the genus *Millingtonia* and it is originated from South-East Asia, it belongs to family Bignoniaceae. The genus name, *Millingtonia*, is in honor of Sir Thomas Millington, the English botanist, while *hortensis* means "grown in gardens". The phytochemical screening of *M. hortensis* L.f. showed the presence of different chemical classes as carbohydrates, tannins, saponins, flavonoids, cyclohexylethanoid glycosides, and volatile oil. Different pharmacological actions were reported such as antiproliferative, antimutagenic, antibacterial, antifungal, and antioxidant activities. Aim: This study aims to investigate the phytochemical constituents of *M. hortensis* L.f. and evaluate its biological activities. Material and Methods: The total ethanolic extract *M. hortensis* L.f. was assessed by LC-MS/MS profiling followed by spectrophotometric determination of total phenolics and flavonoids. For biological evaluation, the antioxidant activity was assessed using (DPPH, ABTS, FRAP, and ORAC) assays. In-vitro cytotoxic study was performed using colorectal cancer (LS-513) and hepatocellular carcinoma (HepG2) cell lines and also anticholinesterase activity was determined. Furthermore, some of the identified compounds were analyzed by molecular docking studies to determine the binding affinity between the ligands and the enzyme (acetylcholinesterase). Results: The LC-MS/MS analysis of the total ethanolic extract resulted in identification of twenty eight compounds. The level of total phenolic contents was (34.137 ± 0.509 µg Gallic acid Eq/mg) in the total ethanolic extract while the level of total flavonoid content in total ethanolic extract was (10.256 ± 0.579 µg Rutin Eq/mg). The results of ABTs, ORAC and DPPH antioxidant assays demonstrated that dichloromethane fraction of *M. hortensis* L.f. leaves has the highest effect for free radical scavenging, while the n-hexane extract showed the highest effect for free radical scavenging in the FRAP assay, in-vitro cytotoxic activity showed that the total ethanolic extract has the least IC_{50} which indicates its highest activity compared to the other fractions, anticholinesterase activity obtained by Ellman's method indicates that dichloromethane fraction showed a significant activity against AChE causing 80.6 ± 0.007 % inhibition while ethyl acetate fraction showed least activity against AChE causing 31.8 ± 0.02 % inhibition at 0.1 mg/mL concentration. The molecular docking study revealed that apigenin was found to be more potent than hispidulin due to the strong interactions with amino acid residue SerA:200 , HisA:440 and those of the peripheral region are crucial for strong inhibitory activities against AChE. Conclusions: The results of this study revealed that the plant's identified phenolics and flavonoids, as detected in the LC-MS/MS analysis, may be correlated with the plant's notable antioxidant, cytotoxic, and anticholinesterase activities.

Keywords: *Millingtonia hortensis*, phytochemical profiling, LC-MS/MS, antioxidant, cytotoxic, anticholinesterase, molecular docking.

1. Introduction

Natural products from plants, animals or minerals are point of departure for treating human diseases. Medicinal plants are always available, and their acceptance is increasing progressively. There is no doubt that plants have an important role as it provides an essential assistance in ecosystems. Medicinal plants are a term that includes different types of plants used in herbalism and others possess medicinal activities. The presence of different active constituents in the medicinal plants give them the

*Corresponding author e-mail: **sohila.mahmoud105@gmail.com.;**(Sohila M. Elnaggar)
EJCHEM use only: Received date 09 July 2024; revised date 24 July 2024; accepted date 07 August 2024
DOI: 10.21608/ejchem.2024.302449.9974

chance to be used in drug development and synthesis [1].

The Bignoniaceae family includes trees, shrubs or lianas and rarely herbs. Bignoniaceae consists of about 82 genera and 827 species [2]**.** Though few of its species are present in temperate and sub-tropical regions, which makes an important part of the plant life [3].

M. hortensis L.f. belongs to family Bignoniaceae and it is the sole species in the genus *Millingtonia. M. hortensis* is an important medicinal plant found in southern Asia extending over India, Burma, Thailand and South China. It is known as Cork tree, Akash neem, Neem chameli or Tree Jasmine. It is named *Millingtonia* according to Thomas Millington the English botanist, while *hortensis* means "grown in gardens" [4].

M. hortensis L.f. is a tall tree that grows up to height 18 to 25 meters. Its wood is brittle and can be damaged by storms. It is characterized by having a corky bark and straight trunk with few branches. During the colder months, the tree blooms at the night, and by the next morning the ground is covered with fragrant blossoms [5].

In traditional medicines, *M. hortensis* L.f. leaves and roots are used as anti-asthmatic and antimicrobial. They are used also as antipyretic, cholagogue and tonic in folk medicine [6]. Dried flowers are utilized in cough conditions and are good lung tonic. Also the bark can be used to produce yellow dye [4].

The phytochemical screening of *M. hortensis* L.f. showed the presence of carbohydrates, tannins, saponins, flavonoids, alkaloids, phenols, cyclohexylethanoid glycosides, coumarins and volatile oil [6].

Due to the presence of different phytochemical constituents in *M. hortensis* L.f., various pharmacological actions were reported such as antiproliferative, antimutagenic, antibacterial, antifungal, anticonvulsant and antioxidant activities. Also the flowers and leaves showed antiasthmatic and larvicidal activities, respectively [5].

This research aimed to identify various compounds present in the total ethanolic extract of *M. hortensis* through the LC-MS/MS. Furthermore, the analysis aimed to determine the total phenolic and flavonoid contents present in the plant's leaves. Additionally, investigation of the antioxidant, cytotoxic and anticholinesterase activities of *M. hortensis* L.f. was performed together with molecular docking study.

1. Material and methods

2.1. Plant material

Leaves of *M. hortensis* L.f. were collected on October 2020 from the Zoo, Giza, Egypt. Mrs. Therese Labib, a former director of El-Orman Botanic Garden and botanical Specialist and Consultant at Ministry of Agriculture, helpfully identified it. A voucher specimen was placed in the herbarium of "Pharmacognosy department, Faculty of Pharmacy, Cairo University, Cairo, Egypt" with code number 8-06-24-F.

2.2. Plant Extraction and fractionation

Leaves of *M. hortensis* L.f. were macerated in 70% ethanol for 24 hours, followed by filtration. This procedure was repeated three times till exhaustion. The resulting filtrates were collected and evaporated using rotary evaporator. A dark green sticky residue was obtained (320 g). 300 g of the residue was dissolved in ethanol:water (50:50 *v/v*), then it was successively partitioned with *n*-hexane, dichloromethane and ethyl acetate till complete exhaustion. The fractions were collected and evaporated yielding 146.38g, 25g and 12g, respectively.

2.3. LC-MS/MS

The UPLC analysis of secondary metabolites was conducted using a HSS T3 column (150 x 2.1 mm, particle size 2.5 μm; water) with a binary gradient at a flow rate of 300 μL min-1. The gradient consisted of 0 to 10 min, isocratic 90% A (ammonium formate/methanol, 99.0/1.0 *v/v*), 10% B (100% acetonitrile); 1 to 21 min, linear from 10 to 90% B; 21 to 25 min, isocratic 90% B; 25 to 28 min, isocratic 100% B. The injection volume was 10 μL (full loop injection). The eluted compounds were detected from *m/z* 50 to 1000 in negative ion mode using specific instrument settings. Detailed information on the separation methods, MS and MS/MS measurements, and data processing can be found in previous studies [7].

2.4. Estimation of total phenolic content

By using the Folin-Ciocalteau colorimetric technique, which is based on the development of a colored complex due to the interaction between the phenolic compounds present in the extract and

phosphomolibdic acid in Folin reagent, total phenolic content of the total ethanolic extract was estimated. Gallic acid (GAA) is used to calibrate the Folin-Ciocalteau assay [8].

2.5. Estimation of total flavonoids content

The development of acid stable complexes when aluminum chloride interacts with the C-4 keto group and either C-3 or C-5 hydroxyl group of flavones and flavonols is the basis for the colorimetric technique used to estimate the total flavonoid concentration. Additionally, it combines with ortho-dihydroxyl groups in flavonoid A- or B- ring to generate acid labile complexes [9].

2.6. Antioxidant activity

The antioxidant activity was assessed using the ABTS, ORAC, FRAP and DPPH assays.

2.6.1. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) antioxidant assay

Trolox stock solution was prepared in methanol, followed by 8 serial dilutions ranging from 800 to 100 μM. Samples were then prepared at the final concentration of 0.1 mg/ml in ethanol: DMSO (90:10 *v/v*), except for the *n*-hexane sample was prepared at final concentration 1 mg/ml. The assay was conducted using Arnao et al's method [10], with few modifications. In distilled water 192 mg of the ABTS was dissolved, then added to potassium persulphate. The final ABTS dilution was diluted with methanol. The reaction was incubated for 30 minutes, and the decrease in ABTS color intensity was measured at 734 *n*m.

2.6.2. Oxygen Radical Absorbance Capacity ORAC antioxidant assay

A series of serial dilutions of the trolox were prepared with varying concentrations, the samples were prepared in methanol:DMSO (90:10 *v/v*) at concentration of 0.05 mg/ml. The assay was performed according to Liang et al.'s method [11], incubating with fluorescein for 10 minutes at 37°C, followed by adding 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) and continuing for 60 minutes.

2.6.3. Ferric reducing antioxidant property (FRAP) antioxidant assay

After preparing a trolox stock solution in methanol, seven serial dilutions were prepared at concentrations of ranging from 800 to 25 μM. All samples were prepared as 0.2 mg/mL in ethanol with 0.1 DMSO, except for the ethyl acetate sample which was prepared as 1mg/mL in DMSO. The experiment was performed using Benzie et al technique [12], with a adjustments made in microplates. The newly synthesized tripyridyltriazine (TPTZ) reagents were mixed with the sample in a 96-well plate, and the resulting blue color was measured at 593 *n*m.

2.6.4. 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant assay

The study produced seven concentrations of Trolox were produced from a 100 μM stock solution in methanol. All samples were prepared with a final concentration of 0.05 mg/ml in ethanol: DMSO (90:10 *v/v*), except for the *n*-hexane sample which was prepared at a final concentration of 0.25 mg/ml. The DPPH (2, 2-diphenyl-1-picryl-hydrazyl) free radical assay was conducted according to Boly et al methodology [13], using 96 wells plate containing 100μL of freshly prepared DPPH reagent were added to 100 μL of the sample. The color intensity decrease was measured at 540 *n*m after 30 minutes incubation.

2.7. *In-vitro* cytotoxic activity

- Cell lines

Cytotoxicity test was determined in cell line Hepatocellular carcinoma (HepG2) and cell line colorectal cancer (LS-513) (Nawah-Scientific, Cairo, Egypt). In addition to 10% FBS, 100 units/mL of penicillin, and 100 mg/mL of streptomycin, the cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) media. Cultures were kept in a humidified environment with 5% CO_2 at 37 °C.

- MTT Assay

Cells were cultivated in 96 well-microtiter plates for a whole day, then incubated at 37 °C for 72 hours, with varying doses of the test samples. Next, 20 μL 5 mg/ml MTT was added to each well and incubated for 4 hours. The media was then removed, and 150 μl MTT solvent was added. Covered with tin foil, the cells were shaken on orbital shaker for 15 minutes, and optical density was measured at 570 *n*m using microplate reader.

2.8. Anticholinesterase activity

The dichloromethane and ethyl acetate fractions were dissolved in methanol with concentration of 3 mg/ml and diluted in phosphate buffer (0.1 M) with different concentrations (1-25 μg/Ml). Acetylcholinesterase was dissolved in one ml of 50 mM Tris Buffer that contain 0.1% Bovine serum albumin and then diluted in the buffer to 0.2 U/ml. Solution of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) (Sigma-Aldrich, Germany) was dissolved in Tris buffer (pH 7.5, 100 mM) to concentration 0.08 mM. The substrate (Acetylthiocholine iodide ATCI) 0.4 mM was prepared in distilled water. Tablets of

Donepzil 5 mg was used as a positive control. It was crushed and dissolved in methanol with a final concentration of 1 mg/ml.

For test assay, 25 μL of the enzyme solution was added to the cuvette followed by the plant sample 25 μL, 125 μL of DTNB and 25 μL substrate ATCI. A double beam spectrophotometer (Thermo electron corporation, USA) was used to measure the absorbance at 412 *n*m and it was measured every 2-3 minutes. Blank was performed the same as test, but the enzyme was replaced by buffer. The positive control was performed using the Donepzil 5 mg tablets instead of the plant extract.

The enzyme activity and the enzyme inhibition percentage of the control and tested samples were calculated from the rate of absorption with change in time (V = Abs/.t) as follow

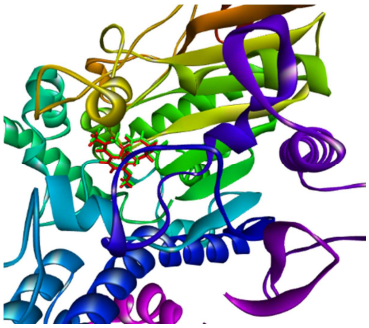
Enzyme activity % = 100 x V/V max

Enzyme inhibition % = 100 – percent enzyme activity

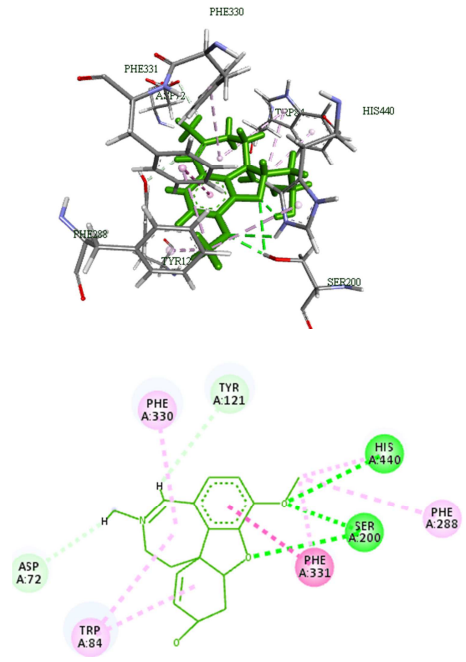
(Where V max is the enzyme activity in the absence of inhibitor drug).

2.9. Molecular docking study as anticholinesterase drug

Autodock 4.2 was used to perform molecular docking for Apigenin and Hispidulin in the active site of AChE (PDB code: 1W6R) [14]. Using Autodock tools, the crystal structure was produced in complex with the bound ligand galantamine, which was downloaded from the protein databank (https://www.rcsb.org/pdb) [14]. Following the removal of water molecules from the protein structure, the ligand and the protein structure were partially charged using Gasteiger charges. A grid box measuring 30 x 30 x 30 points spaced 0.375 Å and centered on the native ligand was used to define the active site. For every molecule, 100 docking trials with the default Autodock parameters were performed using the Lamarckian genetic algorithm [14]. Visualization was accomplished by using a discovery studio [15]. Re-docking the native ligand served as validation, yielding a rmsd value of 0.2896. The amino acids involved were identified, and as shown in Fig. 1, ligand interactions with the amino acids in the active site were investigated.



(a)



(b)

Figure 1: a) 3D overlay of the native (green) and re-docked ligand (red), b) 2D and 3D interactions of the native ligand.

2. Results and discussion:

3.1. LC-MS/MS profiling for the secondary metabolites of *M. hortensis* L.f. total ethanolic extract

UHPLC-MS analysis was used for the first time for performing a comprehensive chemical profiling of

Millingtonia hortensis L.f. leaves. Twenty-eight compounds were identified depending on molecular formulae, characteristic fragmentation patterns and comparing to published data and compound libraries, e.g. dictionary of natural products and pubchem. Three main classes were identified; flavonoids, organic acids and fatty acids. The identified compounds with their data are compiled in Table (1). Figures. (2 and 3).

Table (1): Metabolites identified in the total ethanolic extract of *M. hortensis* L.f. leaves analyzed using UHPLC/MS-MS technique in negative mode

Peak no.	Rt (min.)	Identified Compounds	[M-H]-	MS/MS fragments	Error (ppm)	Molecular formula	Chemical class
1	1.05	α-Hydroxyglutaric acid	147.0303	129, 85	2.9	C5H8O5	Organic acids
2	1.06	Citric acid	191.0206	173, 129, 111, 67	4.7	C6H8O7	
3	1.07	Malic acid	133.0146	115, 89, ,71	2.8	C4H6O5	
4	1.12	Shikimic acid	173.0463	155, 111, 93	4.5	C7H10O5	
5	1.14	Monosaccharide	209.0674	191, 173, 129	3.6	C7H14O7	Carbohydrates
6	1.16	Gluconic acid	195.0518	177, 159, 141	4.1	C6H12O7	Organic acids
7	1.18	Ribonic acid	165.0410	147, 129, 121	3.4	$C_5H_{10}O_6$	
8	1.31	Hexose	179.0567	161, 117, 99	3.4	$C_6H_{12}O_6$	Carbohydrates
9	1.36	Rengyoside B	319.1409	157, 113	3.4	$C_{14}H_{24}O_8$	Aliphatic C6-C2 alcohols glycosides
10	1.36	Rengyoside A	321.1566	179, 159	3.5	$C_{14}H_{26}O_8$	
11	4.14	Salidroside	299.1147	179, 119	3.7	$C_{14}H_{20}H_7$	Phenylethanoid glycosides
12	6.56	Hispidulin 7 glucuronide	475.0895	299, 284,	2.8	$C_{22}H_{20}O_{12}$	Flavonoids
13	6.88	Phenethyl rutinoside	429.1770	352, 265	0.9	$C_{20}H_{30}O_{10}$	Phenylethanoid glycosides
14	7.3	Acetoside	623.1965	461	-2.6	$C_{29}H_{36}O_{15}$	
15	7.74	Luteolin-O-Rutinoside	593.15	501, 473, 383, 353	2.1	$C_{27}H_{30}O_{15}$	Flavonoids
16	7.85	Isorhamnetin 3-O-hexoside	477.10	315, 300	0.8	$C_{22}H_{22}O_{12}$	
17	8.13	Apigenin-6-C- hexose (Isovitexin)	431.0995	341, 311, 269	2.7	$C_{21}H_{20}O_{10}$	
18	8.26	Hispidulin-7-glucoside	461.11	299, 284	3	$C_{22}H_{22}O_{11}$	
19	8.42	Trihydroxy-11-octadecanoic acid	329.2344	311, 293	3.3	$C_{18}H_{34}O_5$	Fatty acids
20	10.4	Scutellarein	285.0414	267, 241, 223	3.4	$C_{15}H_{10}O_6$	Flavonoids
21	10.45	Hispidulin	299.0571	284, 268, 175	3.4	$C_{16}H_{12}O_6$	
22	10.53	Isorhamnetin	315.0523	300, 271	4.1	$C_{16}H_{12}O_7$	
23	10.87	Apigenin derivative	539.0992	269	1.6	$C_{30}H_{20}O_{10}$	
24	10.89	Apigenin	269.0466	151, 117, 149	4	$C_{15}H_{10}O_5$	
25	19.57	Alpha-Linolenic acid	277.2188	233, 325	5.5	$C_{18}H_{30}O_2$	Fatty acids
26	21.99	Linoleic acid	279.2346	-	6	$C_{18}H_{32}O_2$	
27	23.64	Palmitic acid	255.2346	211	6.5	$C_{16}H_{32}O_2$	
28	24.46	Oleic acid	281.2503	112	6.1	$C_{18}H_{34}O_2$	

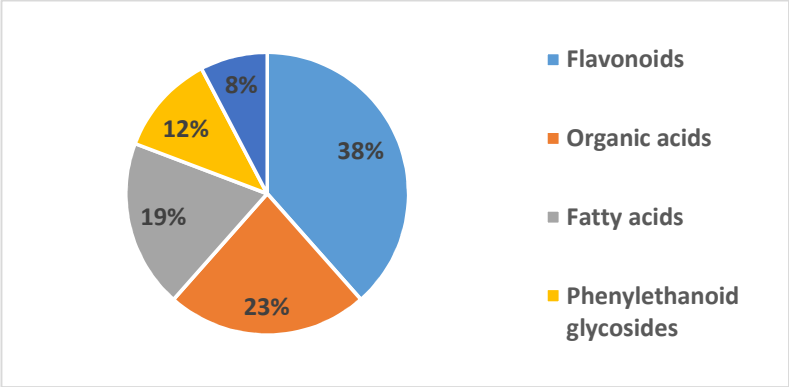


Figure (2): Percentage of the different classes of identified compounds by LC-MS/MS present in *M. hortensis* L.f. leaves

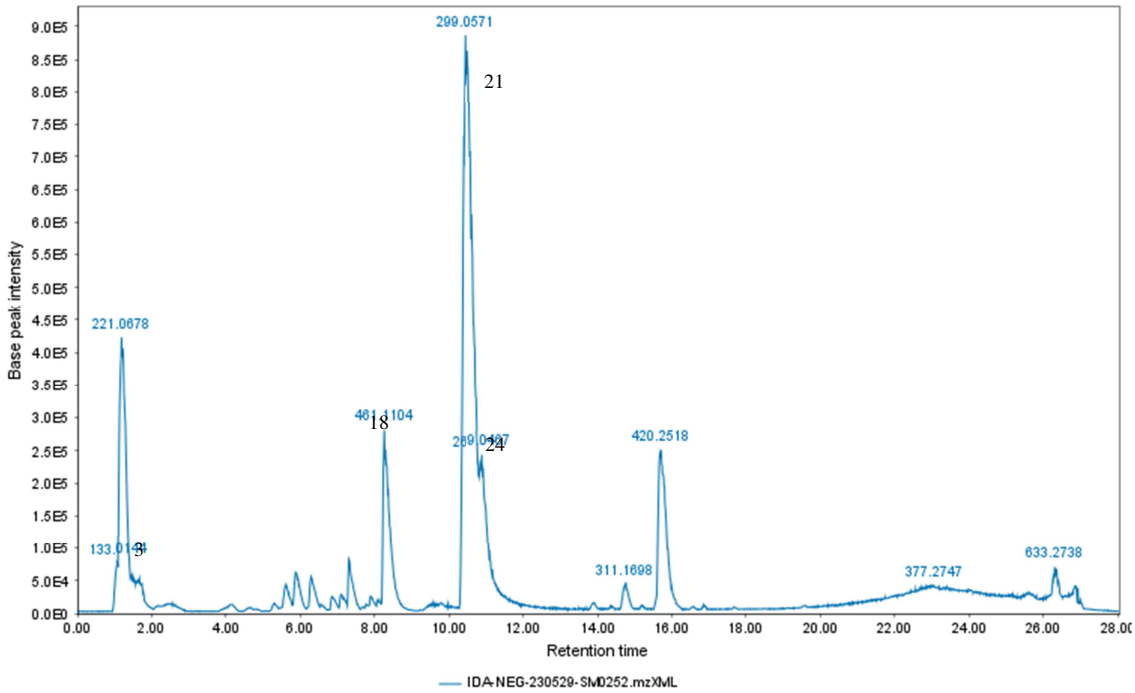


Figure (3): Total ion chromatogram in the negative mode for the total ethanolic extract of *M. hortensis* L.f. leaves

I-Flavonoids:

Ten flavonoids were identified in *M. hortensis* L.f. leaves and their identification is discussed here in details including hispidulin and scutellarein which were detected previously.

Hispidulin was identified with $[M-H]^-$ at *m/z* 299.05 ($C_{16}H_{12}O_6$) that yields fragments masses at *m/z* 284 [M-H-CH_3], *m/z* 268 [M-H-284-O], *m/z* 175 [M-H-268-phenol] and *m/z* 137 [M-H-Glu] as shown in fig. (4) (supplementary file) [16]. Other hispidulin derivatives were also detected at **peaks; 12 and 18** with $[M-H]^-$ *m/z* 475.08 ($C_{22}H_{20}O_{12}$) and $[M-H]^-$ at *m/z* 461.11 ($C_{22}H_{22}O_{11}$) respectively. Peak 12 was identified as Hispidulin 7 glucuronide which upon fragmentation gives a product ion peak at *m/z* 299 corresponding to hispidulin aglycone after the loss of a glucuronide moiety [16]. On the other hand peak

18 was identified as Hispidulin-7-glucoside which upon the loss of glucose it showed fragments masses at *m/z* 299 [17].

Another flavonoid that was detected in *M. hortensis* L.f. leaves was apigenin at **peak 24** with $[M-H]^-$ at *m/z* 269.04 ($C_{15}H_{10}O_5$) shown in fig. (5) (supplementary file) [18]. In addition two apigenin derivatives were identified at peaks 17 and 23 with $[M-H]^-$ at *m/z* 431.099 ($C_{21}H_{20}O_{10}$) and $[M-H]^-$ at *m/z* 539.099 ($C_{30}H_{20}O_{10}$) respectively [19].

Isorhamnetin which is a flavonoid aglycone with $[M-H]^-$ at *m/z* 315.05 ($C_{16}H_{12}O_7$) detected at **peak 22** while its glycoside Isorhamnetin 3-O-hexoside was identified at peak 16 with $[M-H]^-$ at *m/z* 477.10 ($C_{22}H_{22}O_{12}$) yielding mass the aglycone fragment at *m/z* 315.05 after the loss of 162 amu [20].

Peak 20 was identified as scutellarein showing a molecular ion peak at *m/z* 285.04 ($C_{15}H_{10}O_6$) and yields fragments masses at *m/z* 267 [M-H-H_2O], *m/z* 241 [M-H-COO^-] and *m/z* 223 [M-H-H_2O and COO^-] [21].

I- Organic acids:

In addition to the identified flavonoids, six organic acids were detected in the LC-MS/MS profiling of *M. hortensis* L.f. leaves. Peak 1 showed $[M-H]^-$ at *m/z* 147.03 ($C_5H_8O_5$) yielding product ion peaks at *m/z* 129 [M-H- H_2O] and *m/z* 85 [M-H-129.02-COO^-] and it was identified as α-Hydroxyglutaric acid. Peak 2 was identified as Citric acid showing $[M-H]^-$ at *m/z* 191.02 ($C_6H_8O_7$) yielding fragments masses at *m/z* 173 [M-H-H_2O], *m/z* 129 [M-H-173.01-COO^-], *m/z* 111 [M-H-129-H_2O] and *m/z* 67 [M-H-111-COO^-] [22]. Peak 3 and 4 with $[M-H]^-$ at *m/z* 133.01 ($C_4H_6O_5$) and $[M-H]^-$ at *m/z* 173.04 ($C_7H_{10}O_5$) were identified as malic acid and shikimic acid, respectively. Both showed the loss of water (18 amu) and COO^- group (44 amu) yielding fragment masses at *m/z* 115 [M-H-H_2O], and *m/z* 89 [M-H-COO^-] for malic acid as shown in fig. (6) (supplementary file) [20] and fragments masses at *m/z* 155 [M-H-H_2O], and *m/z* 111 [M-H-155.00-COO^-] for shikimic acid [23].

Also, gluconic acid and ribonic acid were identifies as peaks 6 and 7 respectively. Peak 6 showed $[M-H]^-$ at *m/z* 195.05 ($C_6H_{12}O_7$) yielding product ions at *m/z* 177 [M-H-H_2O], *m/z* 159 [M-H-177.04- H_2O], and *m/z* 141 [M-H-159.03-H_2O] [24]. Peak 7 showed $[M-H]^-$ at *m/z* 165.04 ($C_5H_{10}O_6$) yields fragments masses at *m/z* 147 [M-H-H_2O], *m/z* 129 [M-H-147- H_2O], and *m/z* 121 [M-H-COO^-] [24].

II- Fatty acids:

Five fatty acids were detected including trihydroxy-11-octadecanoic acid **(peak 19)** alpha-linolenic acid **(peak 25)**, linoleic acid **(peak 26)**, palmitic acid **(peak 27)** and oleic acid **(peak 28)** with M-H *m/z* at 329.23, 277.21, 279.23, 255.23, and 281.25 with predicted molecular formula $C_{18}H_{34}O_5$, $C_{18}H_{30}O_2$, $C_{18}H_{32}O_2$, $C_{16}H_{32}O_2$, and $C_{18}H_{34}O_2$ respectively. Alpha-linolenic acid and palmitic acid showed the loss of COO^- group yielding fragments masses at *m/z* 233 fig. (7) (supplementary file) [20] and at *m/z* 255, respectively [22].

III- Phenylethanoid and aliphatic C6-C2 alcohols glycosides:

Two aliphatic C6-C2 alcohols which were reported previously, rengyoside A and B were identified in which rengyoside A (peak 10) was assigned the molecular formula $C_{14}H_{26}O_8$ showed at $[M-H]^-$ *m/z* 321.1566 yielding fragment mass *m/z* 159 [M-H-Glu] as shown in fig. (8) (Supplementary file). While rengyoside B which was identified at $[M-H]^-$ *m/z* 319.1409 ($C_{14}H_{24}O_8$) yields fragment mass at *m/z* 157 [M-H-Glu] [25].

Three phenylethanoid glycosides; salidroside, acetoside and phenethyl rutinoside at *m/z* 299.1147 ($C_{14}H_{20}H_7$), *m/z* 623.1965 ($C_{29}H_{36}O_{15}$) and *m/z* 429.1770 ($C_{20}H_{30}O_{10}$), respectively. Salidroside gave rise to fragments at *m/z* 179 and 119. These two fragments were due to the cleavage of the glycosidic linkage leading to the formation of *m/z* 179 and 119 [26].

3.2 Spectrophotometric investigation of total phenolic and flavonoids contents and antioxidant activity:

The amount of the total phenolic and flavonoids contents in the ethanolic extract of *M. hortensis* L.f. was 34.137 ± 0.509 μg Gallic acid Eq/mg and 10.256 ± 0.579 μg Rutin Eq/mg respectively.

The antioxidant activity was determined by ABTS, DPPH, FRAP, and ORAC assays as shown in Table (2) and Fig. (9).

The ABTS, ORAC and DPPH assays showed that dichloromethane fraction of *M. hortensis* L.f. leaves has the highest capacity for free radical scavenging followed by the ethyl acetate fraction, while the *n*-hexane fraction showed the highest capacity for free radical scavenging in the FRAP assay followed by the ethyl acetate fraction.The significant antioxidant activity of *M. hortensis* L.f. may be explained by the mount of phenolic and flavonoid present in its leaves.

Table (2): Antioxidant activity of *M. hortensis* L.f. leaves using different assays

Samples	ABTS Assay	ORAC Assay	DPPH Assay	FRAP Assay
Total ethanolic extract	1251.927 ± 98.045	8101.25 ± 769.54	118.090 ± 5.895	606.388 ± 88.388
***n*-hexane fraction**	103.565 ± 3.538	2349.55 ± 288.79	34.612 ± 2.440	1892.5 ± 76.014
Dichloromethane fraction	6992.749 ± 47.578	39657.96 ± 1302.88	760.002 ± 11.894	736.019 ± 74.604
Ethyl acetate fraction	3303.228 ± 101.903	36896.14 ± 2117.09	507.225 ± 23.671	1155.907 ± 57.469

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
ORAC: Oxygen radical absorbance capacity
DPPH: 2,2-diphenyl-1-picrylhydrazyl
FRAP: Ferric reducing antioxidant power

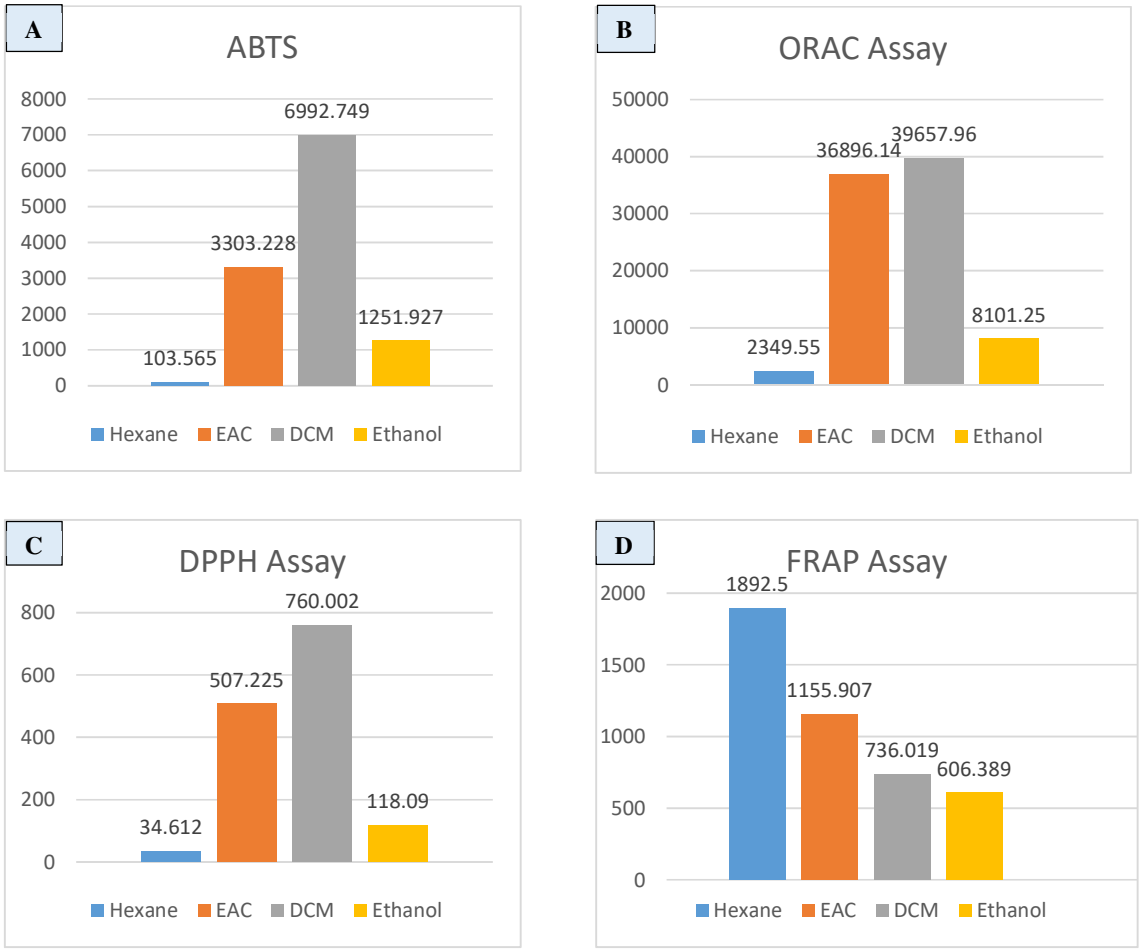


Figure (9): Micro molar Trolox equivalent per mg extract (μMTE/mg extract) (A) 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), (B) Oxygen radical absorbance capacity (ORAC), (C) 2,2-diphenyl-1-picrylhydrazyl (DPPH) and (D) Ferric reducing antioxidant power (FRAP) of *M. hortensis* L.f. extracts/fractions. EAC: ethyl acetate; DCM: dichloromethane.

3.3 Cytotoxicity of the extract and fractions of *Millingtonia hortensis* L.f. leaves on colorectal cancer (LS-513) and hepatocellular carcinoma (HepG2) cell lines:

Total ethanolic extract and *n*-hexane fraction in addition to other fractions (dichloromethane and ethyl acetate) were tested on both cell lines colorectal cancer (LS-513) and hepatocellular carcinoma (HepG2) by MTT colorimetric assay to detect their IC_{50}. According to the results shown in Table (3) the total ethanolic extract showed the least IC_{50} on both cell lines (**LS-513 and HepG2)** indicating its superior activity on the other tested fractions.

Table (3): MTT assay for cytotoxic activity on colorectal cancer cell line (LS-513) and hepatocellular carcinoma (HepG2):

Extract	IC_{50} (μg/ml)	
	Colorectal cancer cell line (LS-513)	Hepatocellular carcinoma (HepG2)
Total ethanolic extract	23.848 ± 0.5356	14.040 ± 0.2450
n-hexane fraction	45.684 ± 0.8372	37.463 ± 0.2516
Dichloromethane fraction	39.389 ± 0.9120	64.495 ± 0.4160
Ethyl acetate fraction	35.996 ± 0.6680	30.878 ± 0.4335
Doxorubicin (Control)	0.44 ± 0.466	4.79 ± 0.929

The significant cytotoxic activity of the total ethanolic extract may be explained by the presence of several compounds with cytotoxic activity, such as apigenin and hispidulin, as revealed by the LC-MS/MS results obtained from the extract. Hispidulin has been reported to have antiproliferative effect against hepatoma cell line HepG2 [27]. While apigenin inhibited the cell growth and induced apoptosis in various cancer types, including lung, liver and breast cancer [28].

3.4 Anticholinesterase activity of *M. hortensis* L.f. leaves:

Acetylcholine is one of the most important neurotransmitters in the human nervous system as it works on activating the skeletal muscles through the peripheral nervous system, also it has different effects on the autonomic nerve system (ANS), specifically by sympathetic and parasympathetic neurons. Acetylcholine (ACh) is synthesized from choline and the acetyl coenzyme in the presynaptic neuron. Acetylcholine is hydrolyzed by acetylcholinesterase after binding with its receptors to terminate synaptic transmission. The hydrolysis of acetylcholine by cholinesterase enzyme is inhibited by acetylcholinesterase inhibitors (AChEIs) leading to enhancing the level of the neurotransmitter. Many medicinal plants are acting as AChEIs due to their effects on the muscarinic receptors, they may also show some activity on nicotinic receptors at high concentrations. This could potentially help in managing dementia in individuals diagnosed with Alzheimer's disease as they experience decreased acetylcholine levels in the brain. Studies have indicated that anticholinesterase may offer some improvement in dementia related symptoms as cognition [29].

Based on the superior antioxidant activity exhibited by both dichloromethane and ethyl acetate fractions, they were chosen for evaluation of their anticholinesterase properties. The results obtained by Ellman's method showed that dichloromethane fraction had a significant activity against AChE causing 80.6 ± 0.007 % inhibition, while ethyl acetate fraction showed least activity against AChE causing 31.8 ± 0.02 % inhibition at 0.1 mg/mL concentration. AChE inhibition by positive control donepzil was 99.22 ± 0.001 % at 5 mg/mL. This study demonstrated that the dichloromethane fraction displayed a significant inhibitory effect against the enzyme AChE.

3.5 Molecular docking study:

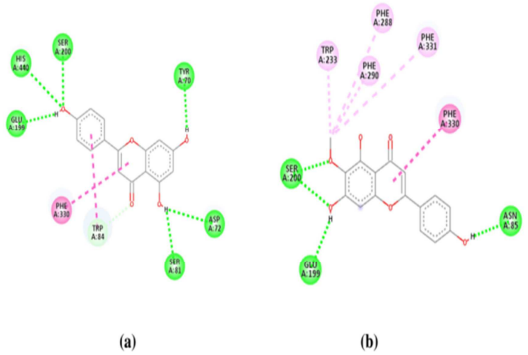
To explore the molecular basis behind the AChE inhibitory activity of the extracted compounds, Apigenin and Hispidulin were docked in the active site of AChE obtained from the protein databank (PDB ID: 1W6R) using Autodock 4.2. The docking scores in (kcal/mol) and major interactions are provided in Table (4) and Fig. (10) along with the score and interactions of Galantamine. In this context, it is clear that apigenin and hispidiulin displayed similar interactions as co-crystallized ligand galantamine.

Apigenin and hispidulin exhibited hydrogen bonds with SerA:200 in a similar way as Galantamine. Apigenin forms hydrogen bond with HisA 440; an amino acid of the catalytic triad of AchE . This may explain the higher AChE inhibitory activity of apigenin. Additionally, apigenin displayed interaction

with TryA: 70; an amino acid residue in the peripheral region that contributes to the enzyme inhibitory activity. Furthermore, the van der Waals interactions with PheA:330,TrpA:84 in Apigenin and with PheA:330,PheA:331, PheA:288,PheA:290, TrpA:233 in Hispidulin played important role in peripheral interaction for the enzyme inhibitory activities of the compounds.

Table (4): The amino acid with which the ligand with the relevant interactions

Ligand		Galantamine	Apigenin	Hispidulin
Interactions	Hydrogen bonds with SerA:200 and HisA:440 Vander waals interactions with PheA:330,PheA:331, TrpA:84, AspA:72, TyrA:121, PheA:288	Hydrogen bonds with SerA:200, HisA:440. GluA:199, AspA:72, SerA:81,TyrA:70 Vander waals interactions with PheA:330,TrpA:84	Hydrogen bonds with SerA:200, GluA:199 and AsnA:85 Vander waals interactions with PheA:330,PheA:331, PheA:288,PheA:290 TrpA:233	
Docking scores (Kcal/m)	-8.43939018	-6.81736469	-5.516819	



(a) (b)

Fig. (10) 2D interactions of Apigenin (a) and Hispidulin (b) with AChE

Finally, molecular docking was performed with acetylcholinesterase to analyze and potentially understand the possible binding interactions underlying the relationship between our target compounds and acetylcholinesterase essential amino acids.

The results of the molecular docking study demonstrated the strong interactions with amino acid residue SerA:200 , HisA:440 and peripheral region are essential for potent inhibitory activities against AChE. Apigenin was found to be more potent than hispidulin in this study.

3. Conclusion:

This study investigates the phytochemical and biological activities of *M. hortensis* L.f. leaves. It demonstrated that the plant's high concentration of phenolics and flavonoids contents identified in the LC-MS/MS study was reflected on the plant's considerable antioxidant, cytotoxic and anticholinesterase activities.

Further analysis is necessary to isolate and identify the active compounds found in various extracts of *M. hortensis* L.f. to understand how they work. Moreover, it can be said that extracts from *M. hortensis* L.f. may be used as a valuable resource for natural products that may help in the prevention or delaying of Alzheimer's disease, as well as providing antioxidant and cytotoxic properties.

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