



## New Insight into the Anticholinesterase Potential of *Cordia dichotoma* G. Forst. and *Cordia sebestena* L. Leaves, Phenolic Characterization of their Active Extracts by HPLC-DAD and Molecular Modeling

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### Abstract

Alzheimer's disease (AD) is the most prevalent form of dementia with cognitive impairment. Genus *Cordia* has been reported in the treatment of neurodegenerative diseases. We aimed to investigate the total phenolics (TPC), flavonoids (TFC) content and the phenolic composition of leaves of *Cordia dichotoma* G. Forst. (CDLME) and *Cordia sebestena* L. (CSLME) crude methanolic extracts. Subsequently, investigate the acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition activities of two plant leaves crude extracts and their subsequent fractions (*n*-hexane, dichloromethane, and ethyl acetate). The results demonstrate that CDLME had a higher TPC and TFC than CSLME. HPLC-DAD analysis of CDLME and CSLME demonstrated the existence of ten and five phenolic compounds, respectively. Rosmarinic acid (21595.123  $\mu\text{g g}^{-1}$ ), rutin (5258.113  $\mu\text{g g}^{-1}$ ) and dihydrokaempferol (3828.986  $\mu\text{g g}^{-1}$ ) showed high abundance in CDLME, while rosmarinic acid (22070.111  $\mu\text{g g}^{-1}$ ) showed high abundance in CSLME. *C. dichotoma* and *C. sebestena* ethyl acetate fraction displayed the highest inhibitory activity against AChE ( $\text{IC}_{50} = 15.12 \pm 1.05$  and  $25.52 \pm 0.15$   $\mu\text{g/mL}$ , respectively) and BChE ( $\text{IC}_{50} = 37.20 \pm 2.12$  and  $37.57 \pm 1.22$   $\mu\text{g/mL}$ , respectively). *C. dichotoma* and its subsequent fractions showed higher cholinesterase inhibition activity than *C. sebestena* and its subsequent fractions. The detected phenolic compounds achieved good binding scores towards AChE and BChE as reflected from molecular docking study. The obtained results stipulate that *C. dichotoma* and *C. sebestena* leaves contain highly valuable phenolic compounds, which may contribute to the determined anticholinesterase effects and can offer a scientific basis for additional research on anti-AD potential of those plants.

**Keywords:** *Cordia*; Alzheimer; neuroprotective; Phenolics; HPLC-DAD analysis; *in silico* study.

### 1. Introduction

Alzheimer's disease (AD) is currently among the biggest threats to world health. It ranks as the fifth main cause of mortality in America [1]. AD is a chronic, occult and fatal neurodegenerative disorder characterized by irreversible memory loss and

deteriorating cognitive abilities, that is relatively common among the elderly [2]. It impairs learning, memory, thinking, communication skills and judgment [3]. The deposition of amyloid beta ( $\text{A}\beta$ ) plaques, neurofibrillary tangles, phosphorylated tau proteins, mitochondrial abnormalities and ongoing brain deterioration are the major pathological findings associated with AD [4]. In addition,

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inflammation, lack of acetylcholine (ACh), oxidative stress and glutamate excitotoxicity are presumed to result in neurodegeneration in AD [5].

Cholinesterases (ChEs) belong to the carboxylic ester hydrolase group of enzymes, which control cholinergic nerves and break down esters of choline. ACh is engaged in the transmission of the signal at the synapse and its biological action is ended primarily by acetylcholinesterase (AChE) and secondly by butyrylcholinesterase (BChE) [6]. A decline in levels of ACh in the brain leads to pathophysiology of cognitive dysfunction occurring in AD [7]. The inhibitors of AChE and BChE will bring back the pharmacological action of ACh at the synapse and enhance memory and cognition in AD patients [2]. Although ChEs inhibitors such as tacrine, rivastigmine, donepezil and galantamine remain the most used medications for the treatment of AD, those drugs have limited therapeutic outcomes, many adverse effects and fail to stop disease completely [2] [8]. Although several AChE inhibitors that come from natural origins have been approved for the treatment of AD, the number of BChE inhibitory treatments from natural sources is considerably lower [9]. Safe and effective alternate treatments are urgently needed due to the devastating impacts of AD and/or drug-related side effects.

For ages, Chinese and Ayurvedic cultures have utilized medicinal plants in the form of spices, vegetables, and crude medications to treat diminishing cognitive abilities [10] [9]. *Boraginaceae* (borage) is a family of blooming plants with about 150 genera and 2,700 species [11]. The genus *Cordia* includes over 300 species; most of them are shrubs or trees that are native to Asian, American and African countries [12]. Genus *Cordia* bears significance for its applications as food, medicine, and fuel [13]. *Cordia dichotoma* G. Forst., also known as clammy cherry and Indian cherry, can be searched out in Philippines, Southern China, tropical

Australia, Central South America, Cambodia, Thailand, Timor and India. The whole parts of the plant are edible, especially its fruit [14]. *Cordia dichotoma* has neuroprotective potential in Parkinson's disease caused by haloperidol in rats. So, *C. dichotoma* may have a potential application in treating AD [15]. *Cordia sebestena* L., known as orange Geiger tree, is native to Cuba, the Northern West Indies, the United States, and the Caribbean region. Both plants have been used since ancient times for treating several disorders such as urinary incontinence, inflammation, wounds, chest-related disorders, fever, diarrhea, influenza, tumors,

gastrointestinal disorders, and liver ailments [16] [17]. Both species have numerous pharmacological activities such as antioxidant, anticancer, antimicrobial, antidiabetic, anti-inflammatory and anti-ulcer activities. A chemical survey revealed many metabolites in the two species, such as coumarins, phenolic acids, flavonoids, sterols, terpenoids, carbohydrates, volatile oils, tannins, saponins, amino acids, pyrrolizidine alkaloids and fatty acids [18].

Phenolic metabolites are secondary metabolites of plant origin and can be used as treatments for numerous chronic ailments related to oxidation, such as neurodegenerative diseases, diabetes, and cardiovascular disorders [19][20]. Regarding the analytical techniques, the primary emphasis is directed to high-performance liquid chromatography (HPLC). It is usually the preferred method for the identification and quantification of polyphenols [21] because of its high reproducibility, high resolution, high efficiency, and comparatively rapid analysis without limitations on sample volatility [22]. In contrast, molecular docking studies are regarded as the most available and valuable tools of computational chemistry at the present time. It helps to investigate the possible mechanism of action and target interactions for a certain drug candidate and compare it to a reference drug [23][24][25].

Our ongoing program is to investigate the phenolic composition of the two plant leaves crude extracts using HPLC-DAD analysis. As it is the first phenolic profile of *C. sebestena* leaves. Aside, quantification of their total phenolics (TPC) and flavonoids content (TFC) by Folin-Ciocalteu and aluminum chloride methods, respectively. Enzymatic assays were conducted for the first time on the two plant leaves crude extracts and their different fractions to assess their inhibitory activities against AChE and BChE. So, the identified compounds in HPLC-DAD analysis of two species were further examined by molecular docking [26] to clarify the candidates with greater affinity for ChEs compared to the standard drug, donepezil.

## 2. Materials and methods

### 2.1 Plant material, extraction, and fractionation

*C. dichotoma* and *C. sebestena* leaves were gathered from Egypt (Orman Botanical Garden, Giza) during August 2021 [Figure 1]. The two plants were authenticated by Prof. Abd Haleem Abd El-Mogali, chief researcher, Flora and Phyto taxonomy Research Department, Agriculture Museum, Giza, Egypt. After identification, samples of dried leaves were placed at the herbarium of the Faculty of Pharmacy, Cairo University, under the identification [*C. dichotoma*. 7.9.2023- and *C. sebestena* L. 6.9.2023]. The two

plants leaves were dried in the shade. The dried leaves were thereafter crushed to a fine powder. The powdered leaves of *C. dichotoma* (6 Kg) and *C. sebestena* (4.5 kg) were soaked in 80% methanol of commercial grade (24 and 18 L, respectively) for two weeks, as every 1Kg crude plant will be extracted by 4 L of methanol [27]. The crude extracts of leaves underwent filtration by filter paper and this step was carried out three times. The filtrates from each species were then evaporated at a temperature not to exceed 50 °C using a rotatory evaporator operating at reduced pressure. Finally, we got about 430 g and 290 g of *C. dichotoma* (CDLME) and *C. sebestena* (CSLME) brown crude residue methanolic extracts, respectively.

The brownish CDLME (425 g) was dissolved in 1.5L distilled water and was successively fractionated with (500 mL of each solvent, 3 times each) *n*-hexane (CDLHF), dichloromethane (CDLDF) and ethyl acetate (CDLEF). Finally, we got the following fractions: CDLHF (30 g), CDLDF (23 g) and CDLEF (15 g) and the residual fraction (85 g). The brownish CSLME (250 g) was dissolved in 950 mL distilled water and was successively fractionated with (300 mL of each solvent, three times each) *n*-hexane (CSLHF), dichloromethane (CSLDF) and ethyl acetate (CSLEF). Lastly, we got four fractions, CSLHF (25 g), CSLDF (13 g) and CSLEF (11 g) and the residual fraction (37 g) [28]. These were refrigerated and kept in airtight containers until they were needed again.



**Figure 1.** Snapshot of *C. dichotoma* (A) and *C. sebestena* (B) leaves at collection point (Location: Orman Botanical Garden, Giza, Egypt).

## 2.2. The total phenolics and flavonoids contents

According to Folin-Ciocalteu colorimetric method, the TPC was determined [29]. Following the preparation of the gallic acid stock solution at a concentration of 1 mg/ml in methanol, the subsequent dilutions were prepared: 25, 50, 100, 200, 400, 600, 800 and 1000 µg/mL. The concentration at which

samples were prepared was 2 mg/mL in methanol. Briefly, the procedure involved mixing 10 µL of sample/standard with 100 µL of Folin-Ciocalteu reagent. After that, 80 µL of 1M Na<sub>2</sub>CO<sub>3</sub> was added, and the mixture was incubated for 25 minutes at room temperature (25 °C). The blue color that resulted was measured following the incubation at 630 nm using FluoStar Omega. Data are denoted as means ± SD. The TPC in samples was presented as µg gallic acid equivalent (GAE)/ mg extract. Gallic acid calibration curve was used to deduce the results.

To calculate the TFC, the aluminum chloride method was employed, with a few minor adjustments [30]. First, 2000 µg/mL of standard rutin was prepared as a stock solution in methanol, from stock solution the following dilutions were prepared: 1000, 500, 200, 125, 62.5, 31.4, 15.625 and 7.25 µg/mL. Samples were prepared at 2 mg/mL in methanol. In a 96-well microplate, 15 µL of sample/standard was placed, after that, 175 µL of methanol was added then 30 µL of 1.25 % AlCl<sub>3</sub>. Lastly, 30 µL of 0.125 M C<sub>2</sub>H<sub>3</sub>NaO<sub>2</sub> was added and the mixture was incubated for five minutes. After the incubation the color that resulted (yellow) was recorded at 420 nm using FluoStar Omega, a microplate reader. Data are signified as means ± SD. Rutin calibration curve was used to deduce the results. The TFC in samples were presented as µg rutin equivalent (RE)/ mg extract.

## 2.3. HPLC -DAD analysis

In order to prepare the sample, 100 mg of extract was dissolved in 10 mL of 100% methanol and shaken for one hour. Acrodisc syringe filter (Gelman Laboratory, MI) with a 0.45 µm pore size was used to filter all samples before injection in to HPLC vials (2 mL). The injection volume was 50 µL [31].

Agilent Technologies 1100 series liquid chromatograph was used for HPLC analysis. It is equipped with a degasser, autosampler, quaternary pump, and coupled with diode array detector (DAD) for quantification of phenolics. Compounds were separated using an Agilent rapid resolution Eclipse XDB-C18 (150 X 4.6 µm; 5 µm). The mobile phase consisted of acetonitrile (solvent A) and 2% acetic acid in water (v/v) (solvent B). The flow rate was kept constant at 0.8 mL/min during all run time (60 min) and the following was the gradient program.: 100% B to 85% B in 30 min, 85% B to 50% B in 20 min, 50% B to 0% B in 5 min and 0% B to 100% B in 5 min. Peaks were monitored simultaneously at 280 and 320 nm for the benzoic acid and cinnamic acid derivatives, respectively. Compounds were identified by comparing the retention time and the absorption spectra with readily available standards analyzed concurrently [32].

## 2.4. Anticholinesterase assays

The assays have been performed in accordance with the typical procedures as stated by [33]. First, 170  $\mu\text{L}$  of Tris-HCl buffer (200 mM, pH 7.5) was added; after that, 20  $\mu\text{L}$  of the crude extracts and fractions at different concentrations ranging from (250 to 3.906  $\mu\text{g mL}^{-1}$ ) and lastly, 20  $\mu\text{L}$  of the enzyme solution (0.1 U  $\text{mL}^{-1}$ ). Following a 10-minute incubation at room temperature (25  $^{\circ}\text{C}$ ) 40  $\mu\text{L}$  of DTNB (dithio-bis (2-nitrobenzoic acid)) and 20  $\mu\text{L}$  of the substrate (1.11 mM) were added. Acetylthiocholine and Butyrylthiocholine iodide were utilized as substrates in AChE and BChE assays. Methanol was used to dissolve the tested samples. The intensity of developed colors and control without the inhibitor was recorded at 405 nm using a microplate reader. In blank tests, buffer was used in place of the enzyme, and the absorbances were measured. The  $\text{IC}_{50}$  (50% inhibitory concentration) was determined via linear regression.

Data analysis was carried out using the Microsoft Excel program. Donepezil was used as a reference drug.

## 2.5. Drugs and chemicals

Methanol, *n*-hexane, dichloromethane, and ethyl acetate (analytical grade) were bought from El-Gomhuria Company (Egypt). Methanol (HPLC grade) was bought by Merck (Darmstadt, Germany). Donepezil was purchased from Pfizer (Egypt) and prepared into saline. HPLC-standards were bought from Sigma-Aldrich (Germany).

## 2.6. Molecular docking studies

The phenolic compounds identified in HPLC-DAD analysis of CDLME and CSLME were examined for their AChE and BChE inhibition activity using the molecular docking technique [34]. This was done to investigate the affinity of the examined candidates towards AChE and BChE compared to the co-crystallized inhibitors of both. In the beginning, the identified chemical structures of the 10 phenolic compounds were sketched in ChemDraw and introduced individually for preparation in the working window [35]. While the target AChE and BChE protein structures were downloaded from the Protein Data Bank with PDB IDs of 7E3H and 4BDS, respectively. Each receptor was prepared for the docking step by correction of its missed parts, 3D hydrogenation of its atoms, and energy minimization as well [36]. Finally, a database of the prepared 10 phenolic compounds combined with the co-crystallized inhibitor in each case was docked against

the appropriate protein [37]. The candidates that achieved the superior binding scores on both targets were selected for further investigation. Moreover, it is important to note that a validation step for each applied docking process was performed by redocking the co-crystal of each receptor inside its binding pocket. Where the validity of the software was confirmed by low root mean square deviation (RMSD) values ( $< 2 \text{ \AA}$ ) [38].

## 3. Results

### 3.1. The total phenolic and flavonoid contents

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The phenolics ( $\mu\text{g GAE/1 mg}$  of sample) and flavonoids ( $\mu\text{g RE/1mg}$  of sample) contents in CDLME and CSLME are presented in Table 1.

**Table 1** Total phenolics and flavonoids contents of CDLME and CSLME.

Sample	TPC ( $\mu\text{g GAE/1 mg of sample}$ )	TFC ( $\mu\text{g RE/1mg of sample}$ )
CDLME	137.9 $\pm$ 6.76	22.44 $\pm$ 1.21
CSLME	87.94 $\pm$ 4.41	18.25 $\pm$ 1.75

CDLME: *Cordia dichotoma* leaves crude methanolic extract; CSLME: *Cordia sebestena* leaves crude methanolic extract; GAE: Gallic acid equivalent; RE: Rutin equivalent.

### 3.2. HPLC-DAD phenolic-profiling

The CDLME chromatogram (**Figure 2**) permitted the identification and quantification of ten phenolic compounds, namely, ferulic acid, gallic acid, rosmarinic acid, chlorogenic acid, *p*-coumaric acid, cinnamic acid, rutin, dihydrokaempferol, kaempferol and chrysin. Rutin, rosmarinic acid and dihydrokaempferol were the highly abundant phenolics and their concentrations were 5258.113, 21595.123 and 3828.986  $\mu\text{g/g}$ , respectively. Furthermore, the CSLME chromatogram (**Figure 3**) permitted the identification and quantification of five phenolic compounds, namely, gallic acid, chlorogenic acid, dihydrokaempferol, rosmarinic acid and cinnamic acid. Among them, dihydrokaempferol and rosmarinic acid displayed the highest concentration (708.694 and 22070.111  $\mu\text{g/g}$ , respectively). HPLC-DAD phenolic-profiling of CDLME and CSLME was presented in **Table 2**.

### 3.3. Anticholinesterase assays

CDLME and CSLME and their respective fractions were investigated for inhibition of AChE and BChE at different concentrations using Ellman method [33]. Generally, the tested crude extracts and their subsequent fractions displayed concentration -

dependent AChE and BChE inhibitions. In AChE assay, donepezil was used as a reference drug that displayed an  $IC_{50}$  of  $0.22 \pm 0.03 \mu\text{g/mL}$ . The tested samples' AChE inhibitory activity was arranged in ascending order as follows:

CDLEF>CSLEF>CDLME>CSLME>CDLDF>CSLDF>CDLHF>CSLHF. Similarly, in BChE assay, donepezil was used as a reference drug that displayed an  $IC_{50}$  of  $0.77 \pm 0.01 \mu\text{g/mL}$ . The examined samples' BChE inhibitory activity was arranged in ascending order as follows: CDLEF> CSLEF> CDLME> CSLME> CDLDF>CSLDF> CDLHF> CSLHF.

It was obvious that *C. dichotoma* and *C. sebestena* ethyl acetate fractions exhibited the highest enzyme inhibitory potencies against AChE ( $IC_{50}$ =  $15.12 \pm 1.05$  and  $25.52 \pm 0.15 \mu\text{g/mL}$ , respectively) and BChE ( $IC_{50}$ =  $37.20 \pm 2.12$  and  $37.57 \pm 1.22 \mu\text{g/mL}$ , respectively). Results of cholinesterase inhibitory activity of *C. dichotoma* and *C. sebestena* are shown in table 3.

**Table 2.** HPLC-DAD phenolic-profiling of CDLME and CSLME

Compound	$R_t$ (min)	Sample concentration (ug/g)	
		CDLME	CSLME
Gallic acid	4.1	375.840	476.186
Chlorogenic acid	13.2	613.958	272.083
Ferulic acid	21.6	101.462	
<i>P</i> -Coumaric acid	24.7	483.652	-
Rutin	26.1	5258.113	-
Dihydrokaempferol	28.2	3828.986	708.694
Rosmarinic acid	30.1	21595.123	22070.111
Cinnamic acid	35.4	75.739	78.092
Kaempferol	40.8	225.441	-
Chrysin	53	52.0591	-

**Table 3.** Results of cholinesterase inhibitory activities of *C. dichotoma* and *C. sebestena*.

Sample	Acetylcholine esterase $IC_{50}$ (AChE, $\mu\text{g/mL}$ )	Butyrylcholine esterase $IC_{50}$ (BChE, $\mu\text{g/mL}$ )
CDLME	$38.76 \pm 0.44$	$50.79 \pm 4.18$
CDLEF	$15.12 \pm 1.05$	$37.20 \pm 2.12$
CDLDF	$56.79 \pm 1.16$	$67.05 \pm 3.22$

CDLHF	$125.18 \pm 0.57$	$150.63 \pm 1.59$
CSLME	$51.79 \pm 2.4$	$52.71 \pm 0.48$
CSLEF	$25.52 \pm 0.15$	$37.57 \pm 1.22$
CSLDF	$67.53 \pm 0.8$	$70.48 \pm 1.69$
CSLHF	$131.50 \pm 1.02$	$163.56 \pm 10.71$
Donepezil	$0.22 \pm 0.03$	$0.77 \pm 0.01$

### 3.4. Molecular docking studies

The phenolic compounds identified in HPLC-DAD analysis of CDLME and CSLME were examined for their inhibitory effect on AChE and BChE via molecular docking. The docked co-crystallized inhibitors of AChE (donepezil) and BChE (tacrine) obtained binding scores of  $-8.78$  and  $-6.22$  kcal/mol, respectively. The native donepezil and tacrine binding modes were studied to clarify the crucial amino acids responsible for the inhibitory activity in each. For donepezil, it was observed that Asp74, Tyr337, Trp286, and Phe295 were the amino acids involved in its interactions within the AChE binding site. However, tacrine bound both His438 and Trp82 amino acids of the BChE active pocket.

Phenolic compounds, rutin, chlorogenic acid, and rosmarinic acid were the most promising ones for both AChE and BChE targets. They achieved binding scores of  $(-8.91$  and  $-22.37)$ ,  $(-7.66$  and  $-11.51)$ , and  $(-7.89$  and  $-8.99)$  kcal/mol towards AChE and BChE receptors, respectively. The binding scores of the studied 10 phenolic candidates are given in the supplementary data (**Table S1**)

**Figure 4** clarifies that rutin was able to bind Asp74 with an H-bond and Trp286 with an H-pi bond inside the AChE binding site. However, it bound His438 and Gly117 with two H-bonds, Gly116 with a pi-H bond, Trp82 with two pi-pi interactions, and an unknown metal ion with an ionic bond within the binding site of the BChE receptor. Chlorogenic acid formed two H-bonds with Asp74 and Tyr124 amino acids of AChE, and three H-bonds with His438, Ser79, and Gln67 amino acids of BChE. Furthermore, rosmarinic acid bound both Asp74 and Tyr341 of AChE with two H-bonds and one pi-pi bond, respectively. Regarding the BChE binding site, rosmarinic acid demonstrated the formation of one H-bond with His438 amino acid of BChE.

Collectively, the aforementioned findings propose the superior inhibitory activities of chlorogenic acid, rosmarinic acid and rutin towards the AChE and BChE receptors.



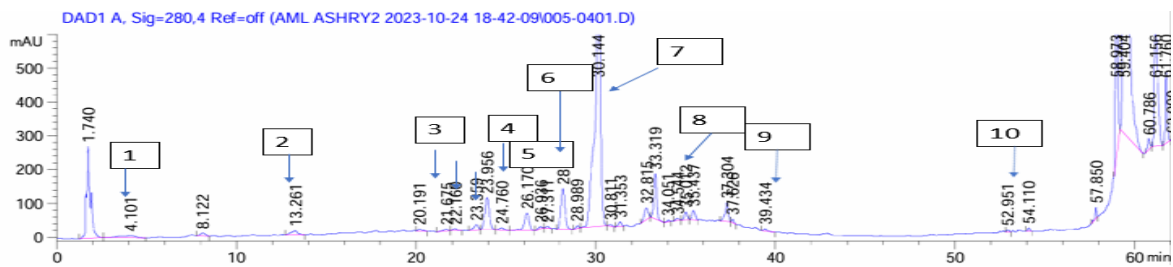


Figure 2. Chromatogram of the HPLC-DAD analysis of CDLME.

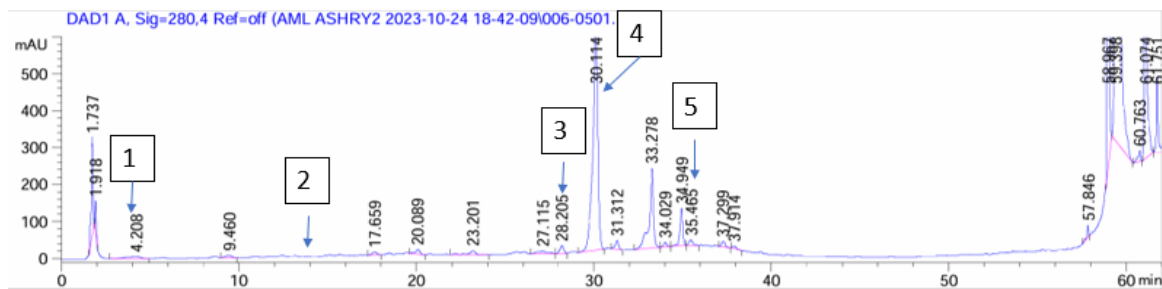


Figure 3. Chromatogram of the HPLC-DAD analysis of CSLME.

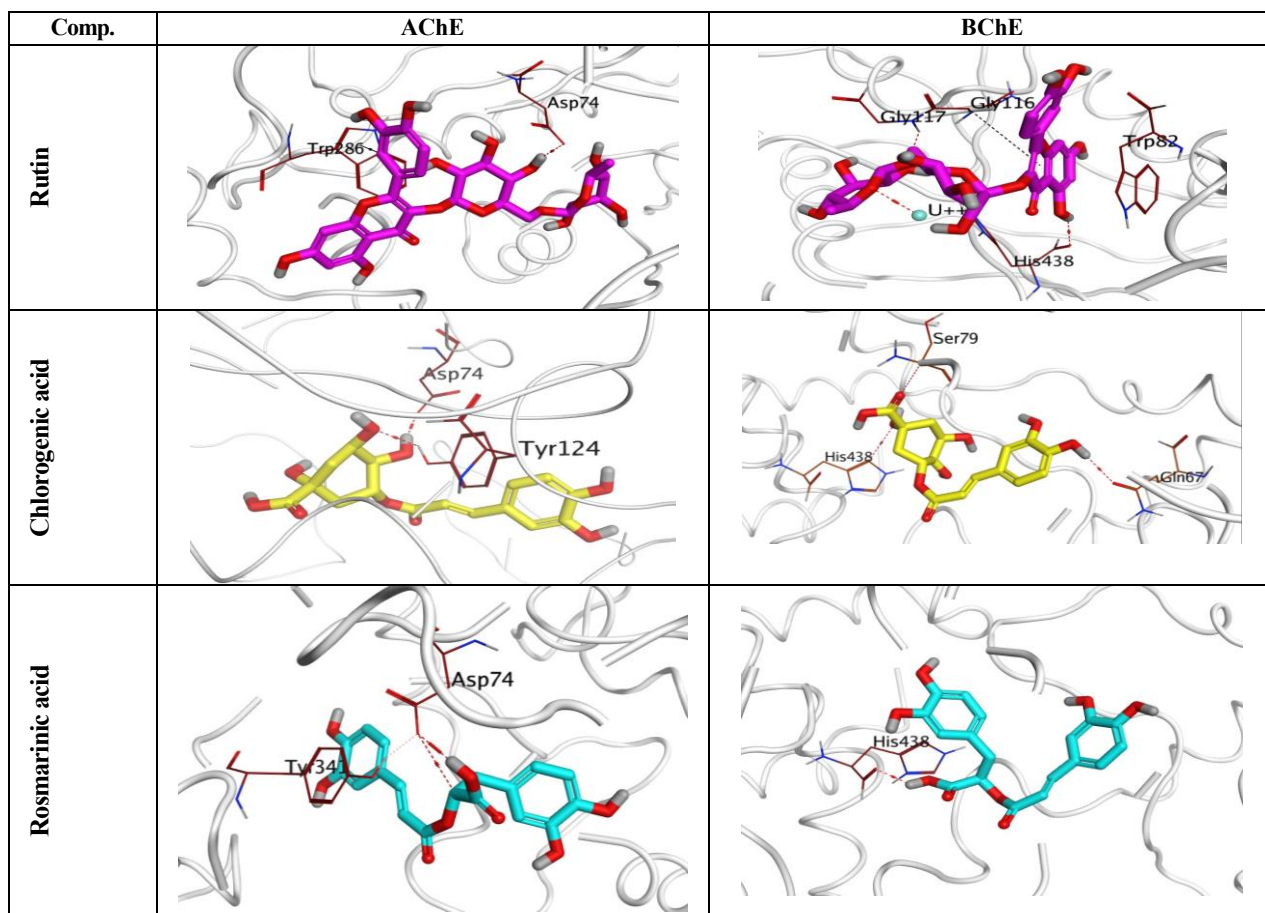


Figure 4. 3D interactions of rutin, chlorogenic acid, and rosmarinic acid towards the AChE and BChE target receptors.

## 4. Discussion

The strong antioxidant potential of phenolic compounds can be extremely beneficial to health [39]. Modern research has shown that flavonoids, phenolic acids and their derivatives significantly improve cognitive function and inhibit or delay neurodegenerative disorders. Their mechanisms involved inhibition of ChEs, formation of A $\beta$  and modulation of signaling pathways [40] [41]. Folin-Ciocalteu assay is the most popular and convenient method to determine the TPC in plants because of its good reproducibility. Plant extracts contain polyphenols that interact with Folin-Ciocalteu reagents (specific redox reagents) to form a blue color complex that can be measured by visible-light spectrophotometry [42]. The determined TPC in this study in CDLME and CSLME were  $137.9 \pm 6.76$  and  $87.94 \pm 4.41$   $\mu\text{g GAE}/1$  mg sample, respectively.

The TFC in samples of medicinal plants is determined using widely used spectrophotometric techniques that rely on the aluminum complexes formation [43]. The TFC in CDLME and CSLME was found to be  $22.44 \pm 1.21$  and  $18.25 \pm 1.75$   $\mu\text{g RE}/1$  mg sample, respectively. The previously reported TFC in *C. dichotoma* leaves was 160 mg quercetin E/g extract [44]. From the obtained results, it was observed that flavonoids in both plants constitute a minor proportion of the total phenolics. Aside, CDLME has more TPC and TFC than CSLME.

As fingerprint analysis is a simple and reliable procedure, it is widely used to determine the chemical composition and relative amounts of phenolic compounds in a variety of plant extracts [45]. Among them, HPLC has been the most frequently used approach to quickly analyzing the bioactive components of medicinal plant extracts [46], as it allows comprehensive profiling of complex plant samples [47]. Thus, HPLC-fingerprint analysis was used to do phytochemical research on the aforementioned crude extracts, seeking to identify their phytoconstituents and establish correlations between the ChEs inhibitory potency with and chemical constituents. HPLC-fingerprint analysis of CDLME revealed the presence of ten phenolic compounds (Table 2). In this context, rutin, kaempferol, rosmarinic acid, chlorogenic acid, and gallic acid were previously reported in *C. dichotoma* leaves [18]. Meanwhile, *p*-coumaric acid and ferulic acid were reported in other parts of the plant rather than the leaves [48]. For the best of our knowledge, dihydrokaempferol and chrysin were reported here in *C. dichotoma* leaves for the first time.

HPLC-fingerprint analysis of CSLME revealed only five phenolic compounds (Table 2). Chlorogenic acid, rosmarinic acid and dihydrokaempferol were reported in *C. sebestena* leaves for the first time in our investigation. However, rosmarinic acid and its methyl ester were previously isolated from *C. sebestena* fruit [49]. Rosmarinic was detected here in the two species at very high concentrations (Table 2) and this aligns with the previous reports that rosmarinic acid is abundant in species of the family *Boraginaceae* [50].

AD currently affects about 50 million individuals globally, and by 2050 that number will be tripled [51]. Drugs approved for AD, including, ChEs inhibitors, have a lot of negative side effects (hepatotoxicity) and they are only useful for moderate cases of AD [52]. There is much research being done on plants to find possible medicinal ingredients that could be used to treat neurological conditions [53]. *In vivo* and *in vitro* ChEs inhibitory effects of traditionally used herbal remedies have been shown to improve cholinergic and cognitive functions [54]. The inhibition of AChE and BChE, the main enzymes in the breakdown of ACh and butyrylcholine, is of prime importance [55]. ChEs inhibitors have been shown in several trials to have a multitude of medicinal effects, such as antioxidant action, amyloid precursor protein processing regulation, and the prevention of A $\beta$  formation. AChE inhibitors raise the amount of ACh at the synapse, enabling the signal's potentiation, and improve cognition as well as functional outcomes in daily living [56].

In the current study, the main concern is the *in vitro* ChEs inhibitory activities of *C. dichotoma* and *C. sebestena* crude extracts and their subsequent fractions. In AChE inhibitory assays, the IC<sub>50</sub> values for CDLME and CSLME were found to be  $38.76 \pm 0.44$  and  $50.79 \pm 4.18$   $\mu\text{g}/\text{ml}$ , respectively. Numerous herbs used in traditional medicine have been studied for their potential to improve memory; however, few of them have been found to show an acceptable level of AChE inhibitory potency [57]. Compared to those plants, *C. dichotoma* crude extract seemed to be a more effective AChE inhibitor. CDLEF and CSLME had the lowest IC<sub>50</sub> values (IC<sub>50</sub> =  $15.12 \pm 1.05$  and  $25.52 \pm 0.15$   $\mu\text{g}/\text{mL}$ , respectively) (Table 3), demonstrating that they displayed the best inhibition of the enzyme and the polarity of the active compounds.

BChE is yet another medication target for AD, since its concentrations and activity noticeably rise in the latter stages of AD [58]. While AChE was the main focus of early research, dual inhibitors of AChE and BChE such as rivastigmine are now believed to have greater sustained efficacy throughout AD and may aid in slowing the disease's progression [59]. In these investigations, CDLME and CSLME were seen to display inhibition of BChE with an  $IC_{50}$  values of  $50.79 \pm 4.18$  and  $52.71 \pm 0.48$   $\mu\text{g/mL}$ , respectively. This result suggests that the two plant crude extracts have good BChE inhibitory activity. CDLEF and CSLEF showed the lowest  $IC_{50}$  values of ( $IC_{50}=37.20 \pm 2.12$  and  $37.57 \pm 1.22/$   $\mu\text{g/mL}$ , respectively), confirming the polarity of the active compounds. The hexane fraction of both plants in two enzyme assays (AChE and BChE assays) had the highest  $IC_{50}$  values (**Table 3**), indicating that it displayed the worst inhibition of both enzymes. From these findings, it was clear that the crude extract of *C. dichotoma* leaves crude extract presented higher AChE and BChE inhibitory activity than *C. sebestena* leaves crude extract and this may relate to the higher TFC and TPC in it. Additionally, all tested fractions of *C. dichotoma* presented higher AChE and BChE inhibitory activity than fractions of *C. sebestena*.

HPLC analysis of the two plants indicated the existence of diverse phenolic compounds, which were reported to have potent anti-AD activity by different mechanisms. In details, *p*-coumaric acid alleviated memory deficits and improved hippocampal synaptic plasticity impairment in aluminum chloride AD model in rats [60]. Rosmarinic acid [61], and chlorogenic acid [62] enhanced the deficit of short-term memory, increased the amount of ACh in the brain and improved cholinergic signal transduction in scopolamine-induced AD. Rutin, according to a study, protected neuronal morphology from harmful tau oligomers and reduced their cytotoxic effects in an AD model in mice [63]. Chrysin [64], ferulic acid [65], and kaempferol [66] have neuroprotective effects *via* reducing amyloidosis, oxidative stress, inflammation and apoptosis in different models in rats. In a model of AD mice, cinnamic acid activates PPAR $\alpha$  to promote lysosomal biogenesis and decrease A $\beta$  [67]. Consistent with our current investigation, prior investigations have shown that phenolic compounds have ChEs activity. *P*-coumaric acid, ferulic acid, rosmarinic acid, chrysin [68] [69], rutin [70] and kaempferol [71] are efficient *in vitro* AChE and BChE inhibitors. Chlorogenic acid was found to inhibit AChE ( $IC_{50}=98.17$   $\mu\text{g/ml}$ ) . [62].

From the previous results, we conclude that crude extracts' anti-ChEs may have contributed to the

identified phytochemicals' neuroprotective properties. This research offers an evidence-based framework for further investigation and isolation of bioactive phytoconstituents for clinical trials.

## 5. Conclusion

Phytochemical analysis of CDLME and CSLME demonstrated the existence of valuable phenolic compounds that possess neuroprotective properties. HPLC analysis revealed the identification for the first time of dihydrokaempferol and chrysin in *C. dichotoma* leaves, while rosmarinic acid, chlorogenic acid, and dihydrokaempferol in *C. sebestena* leaves. Our study demonstrated that *C. dichotoma* and *C. sebestena* crude methanolic extracts and their polar fractions exert substantial ChEs inhibition activity, which may prove beneficial in the management of AD. *C. dichotoma* showed higher ChEs inhibition activity than *C. sebestena*. The results were confirmed by molecular simulation studies performed on the identified compounds containing these enzymes. Rutin, chlorogenic acid, and rosmarinic acid were the most promising ones for both AChE and BChE targets with binding scores of (-8.91 and -22.37), (-7.66 and -11.51), and (-7.89 and -8.99) kcal/mol, respectively. As far as we are aware, our research is the first to investigate the inhibition of cholinesterase by *C. dichotoma* and *C. sebestena* as well as phenolic profile of *C. sebestena*.

## Lists of abbreviations:

A $\beta$ , Amyloid beta; ACh, Acetylcholine; AChE, Acetylcholinesterase; AD, Alzheimer's disease ;BChE, Butyrylcholinesterase; CDLMF, *Cordia dichotoma* leaves dichloromethane fraction; CDLEF, *Cordia dichotoma* leaves ethyl acetate fraction; CDLHF, *Cordia dichotoma* leaves hexane fraction; CDLME, *Cordia dichotoma* leaves crude methanolic extract; ChEs, Cholinesterases; CSLDF, *Cordia sebestena* leaves dichloromethane fraction ;CSLEF, *Cordia sebestena* leaves ethyl acetate fraction ;CSLHF, *Cordia sebestena* leaves hexane fraction; CSLME, *Cordia sebestena* leaves crude methanolic extract; GAE, Gallic acid equivalent; HPLC, High-performance liquid chromatography; HPLC-DAD ,High-performance liquid chromatography with diode-array detection ;RE, Rutin equivalent; TFC, Total flavonoids content; TPC, Total phenolics content.

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