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Antioxidant, Acetyl- and Butyrylcholinesterase Inhibitory Activities of a Memory Enhancer Formulation from Traditional Persian Medicine (TPM)

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Abstract

Alzheimer's (AD) is the most common type of dementia in humans. The cholinergic hypothesis is one of the most important theories behind the pathogenesis of AD. It claims that excessive activity of the enzyme acetylcholinesterase causes the degradation of acetylcholine, thereby reducing its concentration in the brain and leading to symptoms of dementia. AD ("Nesyan" in ancient Persian = forgetfulness) has been thoroughly investigated in Persian medicine. Various remedies have been recommended for the prevention, treatment, and symptom management of AD. One prescribed polyherbal formulation, *Safoof-e-Nesyan (SEN)*, includes *Cinnamomum verum* bark, *Zingiber officinale* rhizome, *Boswellia carterii* gum, *Acorus calamus* rhizome, *Syzygium aromaticum* flower, *Cinnamomum cassia* bark, and *Cyperus rotundus* rhizome. This study examines the impact of this formulation on acetylcholinesterase and butyrylcholinesterase activities, as well as its antioxidant properties. Methanol and dichloromethane extracts of each component and the whole formulation were prepared. DPPH free radical assessment revealed the lowest IC50 value (13.0±1.03 µg/ml) for *A. calamus* dichloromethane extract. The results of the enzyme inhibition assays showed that *SEN* could be considered an enzyme inhibitor. Overall, methanol extracts demonstrated greater effectiveness than dichloromethane extracts. Results indicated the highest inhibition of *S. aromaticum*, *B. carterii*, and *C. verum* against acetylcholinesterase. Contrary to this, the highest inhibition of butyrylcholinesterase enzyme activity was attributed to *C. rotundus*, *S. aromaticum*, and *A. calamus* extracts, which are responsible for the manifestation of anti-AD activity of the present formulation.

Keywords: Alzheimer's, acetylcholinesterase, butyrylcholinesterase, Persian Medicine

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1. Introduction

The German psychologist and neurologist Alois Alzheimer first discovered and described the disease in 1907 (1). The disease gradually causes dementia, which is also associated with impaired behavior, awareness, and function. Although medications may slow the disease and modulate the symptoms; the disease will eventually lead to death. Death occurs three to nine years after symptom on-

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set due to respiratory distress, whereas the onset of the disease is almost twenty years before the beginning of symptoms (1, 2). An estimated 35 million people worldwide have Alzheimer's (AD) or other forms of dementia, and it is estimated that this number will reach 65 million people in 2030 and 115 million in 2050 worldwide (3, 4). About 200 billion dollars a year is spent directly on treating dementia and the complications of living with AD, which will reach 2.54 trillion dollars in 2030 and 9.12 trillion dollars in 2050 (1, 5).

The clinical manifestations of AD include forgetfulness, speech disorders, and the inability to take care of oneself. Additionally, the disease can impair the psychomotor system, leading to movement problems such as stiffness and muscle tremors, while aggressive behaviors are observed in the advanced stages (6, 7).

About 90 to 95 percent of AD patients are over 65 years old. It is mainly seen in older people, while only about 5 to 10% of AD patients have an early onset, usually due to a genetic mutation (3, 4). Blood flow disturbance impairs the proper functioning of neurons. Therefore, cardiovascular disorders and high blood pressure are among the risk factors for AD. Studies have shown that people with high cholesterol are about three times more likely to develop AD (8, 9). Also, type 2 diabetes causes insulin resistance, and a decrease in the ability of neurons to respond to insulin and glucose metabolism can lead to neuronal dysfunction. Finally, the clearance of amyloid-betapeptides are reduced (10). Obesity is another condition that can also be considered a risk factor for the onset or progression of AD (11).

AD-approved drugs mainly control the signs and symptoms in patients and have an insufficient effect on preventing the progression of the disease. The mainstay of AD treatment is now brain neurotransmitters, which have been considered a treatment target (12, 13). Within the synapse, synthesized and stored acetylcholine (ACh) is broken down by two enzymes, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Cholinesterase inhibitors can increase the duration of acetylcholine (ACh) presence at the synapse (14). Concerned biochemical studies showed a severe deficiency of AC and the enzyme responsible for its production, cholinesterase, at the level of the neocortex. These studies led to the hypothesis of ACh deficiency in AD patients (15, 16). Several ACh supplements have been developed to manage AD. Second-generation AChE inhibitors, including rivastigmine, donepezil, and galantamine, are the first-line treatment for this disease in its early to moderate stages (17).

BChE, the second crucial enzyme, is distributed throughout the nervous system (18) and is directly made in the brain (19). This enzyme can play a more prominent role in inhibiting the enzyme AChE. Accordingly, BChE is responsible for continuing the cholinergic cycle. This enzyme converts AC to acetylthiocholine and impacts the treatment of AD (20).

Memantine is a non-competitive NMDA receptor antagonist. According to the findings, the over-activation of the NMDA receptor leads to the over-stimulation and toxicity of brain neurons, and is likely to result in neuronal death in AD. Therefore, memantine, by inhibiting this receptor, prevents over-stimulation and consequent neuronal death (21). The oxidation process has a significant role in the accumulation of amyloid beta peptides, resulting in neuronal destruction and the progression of AD. A decline in the oxidation-caused damage may prevent the progression of the disease. The effects of various antioxidants, including Vitamin E, *Ginkgo biloba* extract, and Coenzyme Q10 analog, on AD disease have been investigated in pre-clinical studies (22, 23). Vitamin E, selenium, unsaturated fatty acids, and certain herbal species are the most important antioxidants for preventing AD (24, 25). Diets such as the Mediterranean diet appear to be an effective option for preventing and controlling AD due to the abundance of their antioxidant principles (26). Several strategies have been proposed as complementary therapies for the prevention and treatment of AD, including recommendations for physical activity, mental stimulation, and a balanced diet (27).

In the world's traditional system of medicine, many herbs have been proposed to treat AD or impaired memory. A relevant study revealed the AChE inhibitory effects of a combination of herbs recommended in traditional Chinese medicine (28-30).

Persian medicine (PM) encompasses all the knowledge and methods for preventing, diagnosing, and treating diseases (31). Several single herbal remedies such as cinnamon, dandelion, mastic, borage, and lemon balm have been mentioned for the treatment of "Nesyan," or amnesia, in ancient TPM manuscripts such as *Al-Hawi* (Liber continens) and *Qanun-fitab*. Compound formulations are also mentioned in *Qarabadin* textbooks (32). Generally, treatment recommendations in traditional medicine are based on lifestyle, nutritional advice, and pharmacotherapy (33-36). Nutritional tips include eating small meals and avoiding "heavy" foods such as meat, eggplant, lentils, some fish, salty foods, spicy foods, milk, and jams, particularly those with hot temperaments like ginger and carrots (33-36).

Sweet flag rhizome, Lemongrass (aerial parts), Frankincense (resin), Mustard (seeds), Costus (rhizome), Jasmine (flower), Lavender (aerial parts), Chamomile (flowers), Valerian (root), Black seed (seeds), Black pepper (fruit), Tulip (aerial parts), and Ginger (rhizome) are among the most essential herbs for managing Alzheimer's disease in patients with Parkinson's (33-36).

To preliminarily assess a compound formulation in the field of antioxidant and enzyme inhibition, the current study has examined SEN, a multi-ingredient formulation extracted from "*Qarabadin-e-Azam*" (1922 AD), a formulary textbook of Persian Medicine (32, 37).

2. Materials and Methods

2.1. Traditional Medicine Manuscripts

The formulation, *SEN*, prepared in the present study, is mentioned as an effective preparation for treating amnesia in Traditional Persian Medicine (TPM) sources, such as *Qarabadin-e-Salehi*, *Kabir, Kaderi*, *Azam*, and *Makhzan-Al-Adwiya*.

2.2. Plant materials

SEN contains seven herbal ingredients, including *Cinnamomum verum* bark (PM905), *Zingiber officinale* rhizome (PM905), *Boswellia carterii* gum (PM961), *Acorus calamus* rhizome (PM963), *Syzygium aromaticum* flower (PM964), *Cinnamomum cassia* bark (PM966), and *Cyperus rotundus* rhizome (PM965).

The plants were purchased from an authorized medicinal plant shop and authenticated by a taxonomist from the Department of Phytopharmaceuticals (Traditional Pharmacy). The voucher specimens were deposited in the herbarium of the School of Pharmacy, Shiraz University of Medical Sciences.

2.3. Extraction

Plants were individually crushed and powdered using an electric grinder. A pair of 30 g of each plant was mixed with 300 ml of dichloromethane or methanol separately in different Erlenmeyer flasks and extracted in an ultrasonic bath for 15 minutes at 30 °C. The extracts obtained were filtered separately, concentrated on a rotary evaporator under reduced pressure, and dried. As performed with the plants, the formulated *SEN* was also extracted separately with methanol and dichloromethane. The prepared extracts were concentrated using a rotary evaporator, and the moisture and traces of solvents were removed in a speed vacuum and, ultimately, in a freeze dryer. All the concentrated extracts were weighed and stored at -18 °C before analysis.

2.4. DPPH Assay

To prepare the DPPH reagent, 1 mg of DPPH was added to methanol in a volumetric flask and brought to a volume of 25 mL. Concentrations ranging from 6.25 to 3200 mg/L were prepared from each extract and placed in a 96-well plate. Then, 200 µL of DPPH solution (100 mM) was added to all wells containing extracts. The plate was kept in the dark for 30 minutes, and the absorbance was read at 490 nm. Each test was performed in triplicate. Finally, the free radical scavenging percentage was calculated using the following formula, and the IC50 was subsequently calculated using Equation 1 (38).

$$DPPH\ Scavenging\ activity\ (\%) = 100 \times \left[100 - \frac{Absorbance\ Test - Absorbance\ Blank}{Absorbance\ Control}\right] \quad \text{(Eq. 1)}$$

2.5. Preparation of Phosphate Buffer

To prepare 1 liter of 1 M phosphate buffer solution, 174.18 g potassium dihydrogen phosphate and 136.09 g potassium hydrogen carbonate were used. Four parts of 1 M potassium hydrogen phosphate solution and six parts of 1 M potassium dihydrogen phosphate solution were used to adjust the pH to 8.9.

2.6. Preparation of Glycerin Buffer

To prepare 100 mL of glycerin buffer, 25 mL of glycerin with phosphate buffer (pH 8) was used to stabilize the enzyme.

2.7. Preparation of DTNB Solution

To prepare 10 mL of 5,5-dithiobis-2-nitrobenzoic acid (DTNB) solution, 39.6 mg of DTNB powder was dissolved in distilled water, and the volume was brought up to 10 mL with distilled water.

2.8. Enzyme Assay

Solutions of four serial concentrations, including 10, 5.0, 2.5, and 1.25 mg/ml of the extracts, were prepared using DMSO and 6 ml of phosphate buffer. Ellman's spectrophotometric method was used to measure the AChE and BChE activities of the solutions, using DTNB reagent, which records the level of cholinesterase activity as an increase in absorbance (39).

2.9. Preparation of Enzymes and Substrates

In this study, enzymes were used at concentrations of 0.075 mM. To prepare the enzyme solutions, AChE and BChE, 237.9 mg of Butyrylthiocholine iodide (BTCI) and 216.8 mg of acetylthiocholine iodide (ATCI) were separately added to 10 mL of distilled water. Then, 2 mL of glycerin buffer was added to the vials containing the enzyme solutions. The solution was then divided into four 500 µl portions in Eppendorf tubes. Each part was individually prepared up to 15 ml using glycerin buffer and further divided into 15×1 ml volumes in Eppendorf tubes, stored at -18 °C to maintain enzyme stability (40).

To assess enzyme activity, a 24-well plate was used, with the first column of each plate loaded with the enzyme. Columns 2 to 6 were loaded with plant extracts (each column belongs to an extract) and enzymes. Solutions of five different plant extracts with identical ranges of concentrations (1.5, 2.5, 5, and 10 mg/ml) were transferred into each plate. Two ml of phosphate buffer, 50 µl of Ellman's reagent, and 20 µl of substrate were added to each plate. In rows A-D, 200 µl of plant extracts in DMSO and buffer were added to columns 2-6. Since DMSO may have an enzyme-inhibitory effect, in the first column, 200 µl of DMSO blank (i.e., the same ratio 2:3 of DMSO /phosphate buffer as the extracting solvent) as a blank was added into cells A-D. Then, 50 µL of the enzyme was added to all rows A, B, and C. Cell D of the first column was designated as the enzyme blank, and the other D-cells were designated as the blanks for the extracts. The absorbance of each well was measured at six time durations: 0, 1, 2, 3, 4, and 5 minutes, using a spectrophotometer. Higher absorption intensity is considered an indication of higher enzyme activity.

2.10. Standard Enzymatic Assay

In the assessment of the enzymatic activity of plant extracts, tacrine, a standard enzyme-inhibiting drug, was used as a positive control. Tacrine tests were performed in a 24-well plate similar to those used for plant extracts. A 2 mg/mL tacrine solution in DMSO and phosphate buffer (pH 8) was prepared using the same ratio as the extracts. This solution was used to prepare concentrations of 0.2 mg/mL, 0.02 mg/mL, 0.002 mg/mL, and 0.0002

Table 1. Extraction yield and DPPH assay of SEN extracts and their plant components.

Drug / Herbs	Methanol extract yield (w/w %)	Dichloromethane extract yield (w/w %)	Methanol extract IC50 (µg/ml) DPPH assay	Dichloromethane extract IC50 (µg/ml) DPPH assay
SEN	12.4	25.2	151.0±5.11	669.0±3.38
C. verum	7.0	5.1	51.4±1.4	481.3±6.0
Z. officinale	5.6	3.0	998.0±3.0	50.0±8.3
B. carterii	2	23	2520±2.10	2279.7±5.0
A. calamus	8.0	4.8	63.0±3.2	13.0±1.03
S. aromaticum	7.7	7.0	4163±11	1518.4±4.0
C. cassia	3.0	17	154.0±9.3	21.0±2.11
C. rotundus	4.6	5.5	252.2±5.1	94.0±1.6

mg/mL for enzymatic testing. The remaining steps were the same as those conducted with the extracts (40).

3. Results

3.1. Extraction

The percentage yields of methanol and dichloromethane extracts of *SEN* and its plant drug components are given in Table 1.

3.2. DPPH Assay

The results of the DPPH radical scavenging activity of methanol and dichloromethane extracts of *SEN* and its herbal components, along with their respective IC50 values, are represented in Table 1.

3.3. Enzyme Inhibition

The results of the enzyme test were based on finding a percentage of inhibition. To this end, the absorbance of plates containing extract and enzyme, as well as those containing tacrine and enzyme, was measured at six different time intervals (in minutes) using a spectrophotometer; the results of which helped us determine a percentage of inhibition. To determine the percentage of enzyme inhibition, the activity of the enzyme in the presence of the extracts (as a possible inhibitor) is compared to its activity in the absence of the inhibitor (control). The percentage of enzyme inhibition of the extracts was calculated using the following equation, which allowed for the quantification of the extent to which the extract inhibited the enzyme's activity.

$$\text{Inhibitation of Extract (\%)} = \left[1 - \frac{\text{the slope of first diagram}}{\text{the slope of second diagram}}\right] \times 100 \quad \text{(Eq. 2)}$$

The results of the AChE enzyme inhibitory activity of the methanol extract of SEN showed no inhibition against AChE (0.0798x + 0.0191, $R^2 = 0.9652$) at the concentration of 2.5 mg/ml (Table 2).

The results of AChE inhibitory activity assays were recorded as 0.1979x+0.3335 (R^2=0.9905) in the absence of an inhibitory

Table 2. AChE activity in the presence of 2.5 mg/ml of methanol extract.

Time (Min)	Average±SD*	Blank	Average-Blank
T1	0.63±0.02	0.54	0.10
T2	0.69± 0.05	0.55	0.14
T3	0.77±0.09	0.53	0.24
T4	0.86±0.13	0.52	0.33
T5	0.98± 0.16	0.53	0.44

Table 3. AChE enzyme activity in the absence of an inhibitor.

Time (Min)	Average Absorbance	Blank	Average-Blank
T1	0.64±0.18	0.10	0.54
T2	0.87±0.24	0.10	0.77
T3	1.07±0.26	0.11	0.95
T4	1.25±0.29	0.13	1.12
T5	1.42±0.34	0.13	1.28

compound (Table 3).

Similarly, the percentage inhibition of AChE and BChE enzymes was obtained by tacrine (Table 4, Figure 1.2).

The percentage inhibition of both enzymes, as declared by different plant extracts, is shown in Figures 3-6.

4. Discussion

AD disease is the most common form of amnesia in humans (41). Although the neuropathophysiology of this disease is not entirely known, one of the most plausible hypotheses for AD is the cholinergic theory. According to this hypothesis, dysfunction of the brain's cholinergic system reduces a person's ability to perceive and recognize. Cholinesterase inhibitors are the key therapeutic classes of medications to fight AD symptoms, and in this category, FDA-approved anti-AD drugs are rivastigmine, donepezil, and galantamine (17, 42).

BChE is the second most crucial enzyme in the breakdown of AC. This enzyme is distributed throughout the body's nervous system (18). This enzyme can play a more prominent role in inhibiting the enzyme AChE. If the enzyme AChE is inhibited for any reason, the enzyme BChE is responsible for continuing the cholinergic cycle and converting acetylcholine to acetylthiocholine. This enzyme can play a vital role in treatments related to the cholinergic system, especially AD (20).

In addition to conventional medicine, complementary medicine offers strategies to help treat or manage AD, including physi-

Table 4. Percentage of enzyme inhibition by tacrine at different concentrations.

Percentage Inhibition of AChE enzyme	Tacrine concentration (mg/ml)	Percentage Inhibition of BchE enzyme	Tacrine concentration(mg/ml)
62	0.2	75	0.02
41	0.02	55	0.002
21	0.002	20	0.0002

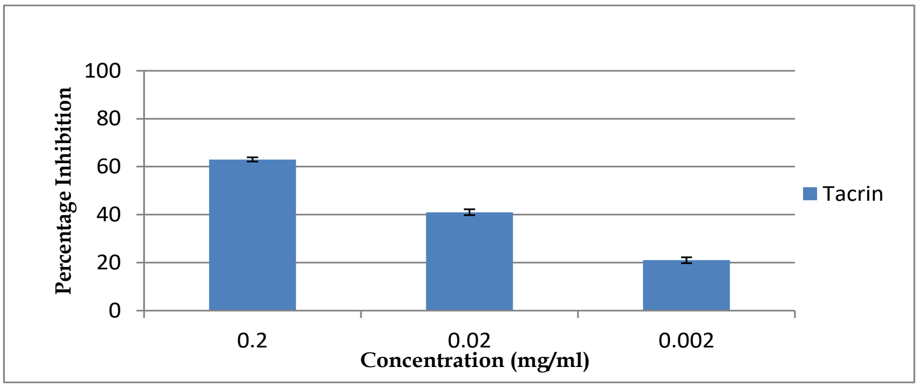

Figure 1. AChE enzyme inhibition (%) of tacrine at different concentrations.

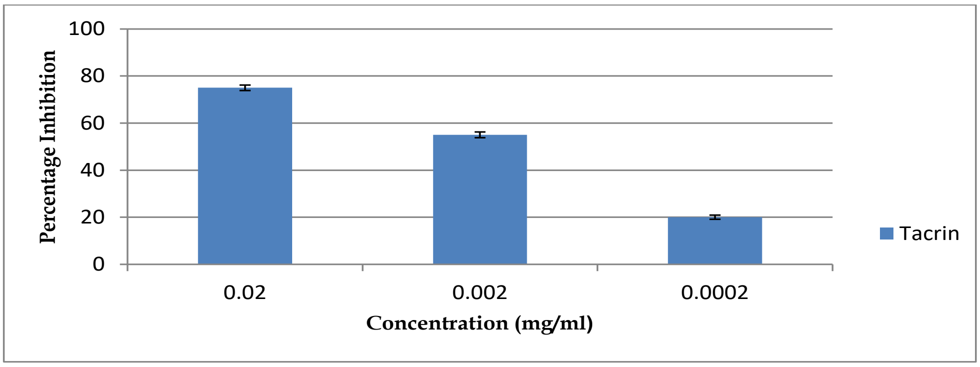

Figure 2. AChE enzyme inhibition (%) of tacrine at different concentrations.

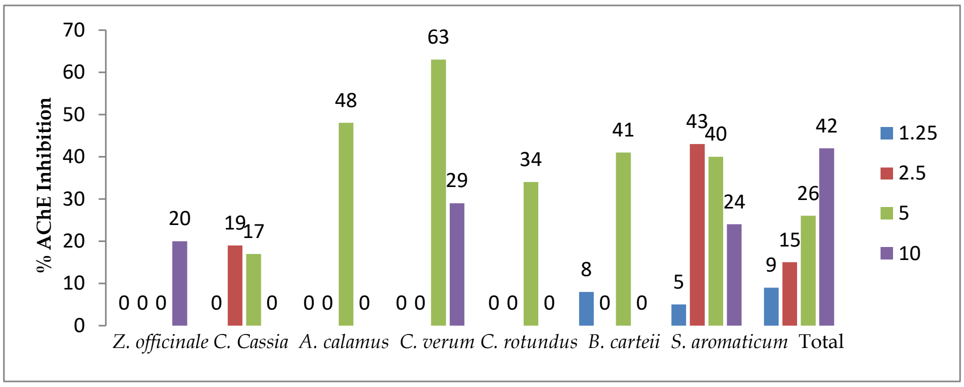

Figure 3. AChE Inhibition of dichloromethane extracts of *SEN* and its plant components.

cal exercise, mental activity, and a balanced diet (27). There have been several studies on the effects of antioxidants in preventing AD. Due to the oxidative effects of amyloid-beta, the role of antioxidants in protecting the body against AD has received increasing attention.

The most important antioxidants used to prevent or slow the progression of AD are vitamin E, selenium, and unsaturated fatty acids (22, 27). Additionally, foods that follow the Mediterranean diet appear suitable for preventing and managing AD due to their increased abundance of antioxidants (26). In addition, various parts of Ginkgo biloba, a distinctive plant species of the family Ginkgoaceae with a long history of use, have also been suggested as a complementary treatment for cognitive disorders such as AD. The leaf extract of this plant, known as EGb 761, is available as a supplement in the pharmaceutical market (24). In the traditional medicine of various world regions, such as China and Iran, multiple treatments have been proposed for AD or memory enhancement. Earlier stud-

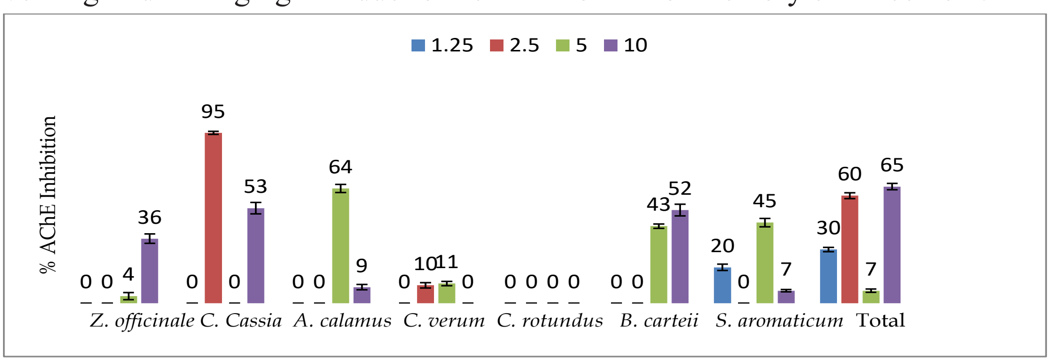

Figure 4. AChE inhibition of *SEN* methanol extract and its components.

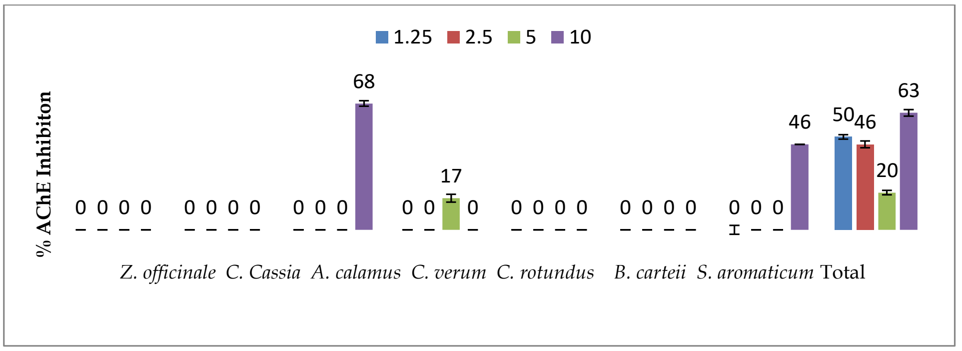

Figure 5. BChE Inhibition (%) of SEN dichloromethane extract and its components.

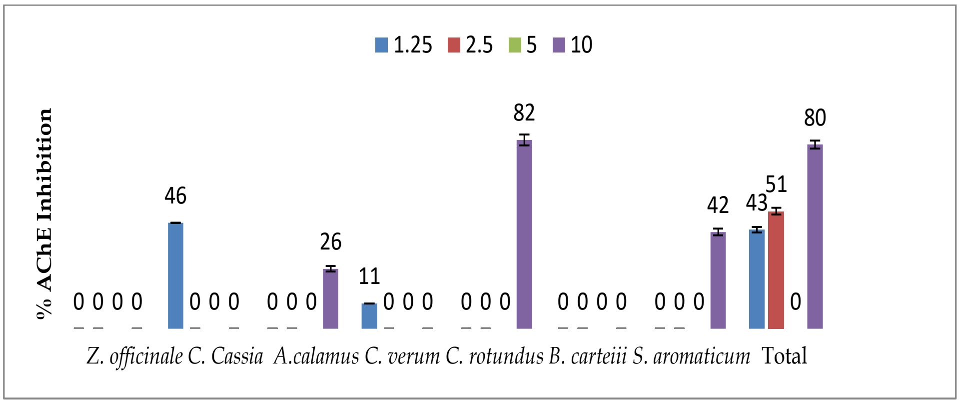

Figure 6. BChE Inhibition (%) of SEN methanol extract and its components.

ies have documented the inhibitory effects of a polyherbal product recommended in traditional Chinese medicine against the enzyme AChE (28, 29).

Various chemical components of the ingredient plant extracts in SEN may act as inhibitors, capable of binding to cholinesterase enzymes and reducing their activity, thereby indicating the extent to which the enzyme's activity is reduced compared to a control.

SEN is one of the effective compound medications used for the treatment of AD in TPM (33-36). Therefore, this study was designed to screen the anticholinesterase effect of methanol and dichloromethane extracts of *SEN* and its plant ingredients. The present study found that the dichloromethane extract specifically inhibited the enzyme BchE. In contrast, the methanol extracts showed higher effectiveness, which may be attributed to the fact that the methanol extracts contain a significantly wider range of bioactive compounds than the dichloromethane extract. Due to the polar nature of methanol, the methanolic extract contains highly polar components, such as polyphenols, terpenoids, and flavonoids —a group of compounds rich in oxygen functions. These compounds demonstrate prominent antioxidant properties and AChE enzyme inhibition, thereby effectively contributing to anticholinesterase activity and aiding in the treatment of AD (43, 44).

As shown in Figure 3, the dichloromethane extracts of *C. verum* exhibited the highest percentage of inhibition against the enzyme AChE at a concentration of 5 mg/mL. However, other individual plant components of the formulation have shown a lower degree of effectiveness. *C. verum* declared 63% inhibition at this concentration, which can be considered the main effective anticholinesterase component. However, the low percentage of inhibition of the AChE enzyme by *SEN* dichloromethane extract at 5 mg/ml can be attributed to its low proportion among the SEN components. Based on the results obtained, the targeted changes in the proportion of *SEN* components are likely to increase enzyme inhibition. *C. verum* enzyme inhibiting activity significantly differs from *SEN* at 5mg/ml (p-value <0.001). It may be argued that cinnamon demonstrated anti-acetylcholinesterase activity due to the presence of cinnamic aldehyde, a methylpropanoid in *C. verum*. This plant contains large amounts of phenols, flavonoids, and tannins in addition to alkaloids and saponins (45).

The marker chemical components of *C. verum* essential oil are (E)-cinnamaldehyde, trans-cinnamic acid, and cinnamyl acetate (46). The methanol extract and essential oil of *C. zeylanicum*, containing cinnamaldehyde as the marker component (66.74%), have demonstrated AChE and BChE inhibitory properties with IC50 values of 77.78% and 88.62%, respectively (47).

Interestingly, all plant extracts showed a significant degree of inhibition of the enzymes at 5 mg/ml than at 10 mg/ml. It has been demonstrated that the enzyme inhibitory activity of the extracts is not dose-dependent and cannot be predicted. A reason behind this characteristic performance may be the presence of polyphenols in the plant, which act as chelating agents and enzyme inhibitors. A study on the effect of tea polyphenols on the enzyme lipase found that these compounds are highly capable of binding and inactivating proteins. This characteristic behavior of polyphenols acts as a polydentate ligand, capable of simultaneously attaching to the surfaces of multiple proteins, thereby causing protein deposition. Tannins, widespread and vital polyphenolic compounds found in many plant species, have a chelating effect on various enzymes (48).

As mentioned earlier, the *SEN* methanol extract at concentrations of 2.5 and 10 mg/mL showed 60% and 65% inhibition of the AChE enzyme, respectively. Since all components of *SEN* except *C. cassia* did not have significant inhibition at a concentration of 2.5 mg/ml, it can be concluded that *C. cassia* is considered the active pharmaceutical ingredient of *SEN*. Also, its effect at a concentration of 10 mg/ml supports this claim. The methanol extract of *C. cassia* at 2.5 mg/mL showed a significant difference from *SEN* (p-value < 0.0001). *B. carterii*, *S. aromaticum*, and *A. calamus* exhibited anticholinesterase at 5 mg/ml, while *SEN* lacked a significant inhibition at this concentration. *C. cassia* contains tannin, an oligomeric proanthocyanidin compound called cinnamtannin, cinnamaldehyde, polyphenolic compounds, eugenol, and flavonoids (49). Therefore, the favorable results of the antioxidant assay of *C. cassia* methanol extract can be attributed to the presence of these bioactive compounds. Among the marker chemical constituents, the ethanolic extract of this plant, with (E)-cinnamaldehyde being the most prominent active constituent, has been reported to exhibit 85.11% inhibition of BChE and 63.02% inhibition of AChE at 200 µg/mL (50). Other constituents, such as cinnamic acid, cinnamyl acetate, and eugenol, may also contribute to anticholinesterase activity.

B. carterii, commonly known as frankincense, also shows anti-inflammatory effects due to the presence of boswellic acid and its analog compounds and triterpenes among its components, which can contribute to the improvement of AD symptoms. The difference in extraction efficiencies between dichloromethane and methanol can be attributed to their oleo-gum resin content, which has high solubility in methanol and can act as an interfering agent in critical assays (51). The *B. carterii* constituents, particularly two specific boswellic acids, 11α-hydroxy-β-boswellic acid and 11-keto-β-boswellic acid, isolated from this plant, have shown notable inhibitory effects on acetylcholinesterase (52). Moreover, 3-O-acetyl-11-keto-β-boswellic acid has exhibited significant anti-inflammatory and neuroprotective effects in Alzheimer's disease. It also plays a role in modulating the cholinergic system by inhibiting acetylcholinesterase activity, thereby enhancing choline concentrations and its interaction with nicotinic receptors, which ultimately contributes to its anti-inflammatory properties (53).

S. aromaticum, commonly known as cloves, is from the Myrtaceae family. The main organs used are unopened buds. As illustrated in Figure 5, *S. aromaticum* extract at a concentration of 10 mg/ml demonstrated good enzyme inhibition, consistent with earlier studies. Therefore, the methanol extract of *SEN* is expected to have a significant inhibitory effect on AChE enzyme activity.

S. aromaticum can be considered a suitable choice in the treatment of AD due to the favorable DPPH radical scavenging properties of its dichloromethane and methanol extracts and the percentage inhibition of AChE declared by both the extracts at a concentration of 5 mg/ml (54). *S. aromaticum* contains eugenol, thymol, monoterpenes, cinnamaldehyde, and flavonoids (55). According to the results of an earlier study, clove extract could inhibit both AChE and BChE enzymes (56). The alcoholic extract of the plant demonstrated a higher degree of efficacy than the aqueous extract against both enzymes (57). *S. aromaticum* aqueous extract can mitigate iron-mediated oxidative brain damage by preventing oxidative stress and controlling gluconeogenesis (58). Eugenol, the most abundant component of clove oil, has been identified as a potent inhibitor of both AChE and BChE. Additionally, cholinesterase inhibition plays a role in the insecticidal properties of clove oil, as it can disrupt the insect's nervous system by inhibiting AChE (59).

In Figure 3, the *SEN* dichloromethane extract at a concentration of 10 mg/ml inhibited BChE by 63%. *A. calamus* has demonstrated effective inhibition against the BChE enzyme. This plant contains terpenes, flavonoids, proanthocyanidins, phytosterols, and the bioactive compounds α- and β-asarones, which exhibit significant enzyme inhibitory properties (60). α- and β-Asarone have been shown to have the capacity to penetrate the blood-brain barrier, suggesting their potential utility in the treatment of neurological disorders. These compounds simultaneously mitigate stress-induced neuronal damage and enhance hippocampal structure in rodent models. Moreover, they exhibit efficacy in modulating oxidative stress and inflammatory responses, thereby preventing cognitive impairments in neuroinflammatory conditions (61). The documented anti-inflammatory and neuroprotective effects of *Acorus calamus* further suggest its therapeutic potential in ameliorating AD pathology in animal models (62).

The interesting characteristic of SEN methanol extract is its enzyme-inhibiting activity at low concentrations. Among the principal phytochemicals identified in *C. rotundus* are α-cyperone, cyperene, α-selinene, and cyperotundone. These compounds are associated with the plant's antioxidant, antibacterial, DNA-protective, and anti-inflammatory activities (63). Figure 4 shows the degree of inhibition of the butyrylcholinesterase enzyme by methanol extracts of the *SEN* plant ingredients. *C. rotundus*, the main plant component of the formulated *SEN*, inhibited this enzyme by 82% at a concentration of 10 mg/ml (Figure 4).

C. cassia is a plant of the Lauraceae family; the consumable part of the plant is the inner bark of the tree. This plant has been reported to have antioxidant and anti-inflammatory effects (64,65). In a study by Yu *et al*., the plant was also found to provide anti-anxiety effects (65). The anti-AD effect of its extract is performed by inhibiting the formation of toxic Aβ oligomers, as shown in previous studies (66). In a 2012 study by Kumar et al., cinnamon was found to have significant inhibitory effects on AChE and BChE enzymes. The plant exhibited a slightly more potent inhibition of BChE than AChE (57). Chinese cinnamon shares many characteristics with Ceylon cinnamon, making it a potential inhibitor of the cholinesterase enzymes.

Ginger, known as *Z. officinale*, is a plant in the Zingiberaceae family; the rhizomes are used for medicinal purposes. In addition to the antioxidant effects, this plant shows anticholinesterase activity, which justifies the plant's ability to fight AD. In a study, Oboh *et al*. reported the dose-dependent inhibitory effects of ginger on the AChE enzyme (67).

Alongside volatile compounds like β-sesquiphellandrene, zingiberene, ar-curcumene, α-farnesene, and β-bisabolene, ginger contains non-volatile markers such as 6-, 8-, and 10-gingerols and 6-shogaol, noted for their significant pharmacological activities. 6-Shogaol exhibits potent antioxidant and anti-inflammatory effects, primarily attributed to its α,β-unsaturated ketone group in its molecular structure. Moreover, carbon chain length notably affects bioactivity, with 10-gingerol showing the greatest efficacy among gingerols (68). 6-Gingerol primarily inhibits BChE at 1 mM concentration, though it has also been reported to reduce whole-brain acetylcholinesterase activity in mice. White ginger aqueous extract demonstrated greater AChE inhibition than red ginger (69). Additionally, 6-gingerol shows strong binding to AChE active sites, indicating its potential to modulate Alzheimer's disease-related cholinesterase activity, apoptosis, and associated regulatory pathways (69).

B. carterii and *B. serrata* from the Burseraceae family are trees that produce gum exudate when the trunk is cut off at the age of 8 to 10 years. It has been used as a memory enhancer in Persian and ethnomedicine. It is also recommended for pregnant mothers to increase their children's intelligence (70). The results of the present study thus corroborate earlier studies; various boswellic acid and its analogues structures, as the main components of *B. serrata*, demonstrate inhibitory effects on the AChE enzyme (71, 72).

A. calamus, known as the sweet flag, is a plant in the Acoraceae family. The rhizome of the plant is used, which is collected late in the summer. The protective effect of this plant on the nervous system, as well as its ability to reduce oxidative stress and inflammation, has been demonstrated in several studies (73, 62). The study by Mukherjee *et al*. shows the sweet flag's inhibitory effects on AChE, particularly due to the presence of β-asarone, which has been identified as the principal chemical constituent in its rhizomes (74). *A. calamus* is considered one of the most efficient herbal treatments for AD in Persian Medicine.

C. rotundus belongs to the Cyperaceae family. The plant's rhizomes are believed to possess medicinal properties. A study conducted earlier showed that *C. rotundus* could inhibit the plant-derived AChE enzyme (63). In conclusion, it is worth noting that the targeted combination of herbs used in Traditional Persian Medicine formulations may serve different purposes, such as enhancing efficacy, minimizing side effects, and ultimately increasing patient interest, thereby contributing to their overall effectiveness (75).

5. Conclusion

According to the results of this study, the observed significant enzyme inhibitory activity of *Safoof-e-Nesyan*, the prepared anti-Alzheimer formula, towards AChE and BChE, as well as its free radical scavenging effect, make this formula a suitable candidate for further investigation in various biological evaluations for the prevention or treatment of AD diseases. A further focusing on the mechanism of action of the active principles of this formulation, such as performing comprehensive enzyme assay, modifying the proportion of bioactive components of the formulation, and conducting in vivo human studies, are some of the proposed guidelines that help to achieve a standard traditional formulation as natural adjunctive therapy for improving memory impairment and/or symptomatic treatment of AD diseases.

Statistical Analysis

Statistical data analysis was performed using GraphPad Prism and ANOVA test. Significant differences were observed between the extracts of different plants at identical concentrations. Values expressed as Mean±SD.

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Authors contributions

Concept – M. M. Zarshenas, M. A. Farboodniay Jahromi; Literature Search – S. Sanei; Supervision – M. R. Moein; M. M. Zarshenas, M. A. Farboodniay Jahromi; Resources – M. A. Farboodniay Jahromi, S. Sanei; Materials – S. Sanei, F. Farmani; Data Collection and/or Processing – S. Sanei, F. Farmani; Analysis and/or Interpretation – M. M. Zarshenas, M. A. Farboodniay Jahromi; Writing – M. M. Zarshenas, M. A. Farboodniay Jahromi and Ehsan Amiri-Ardekani; Critical Reviews – M.A. Farboodniay Jahromi, M. M. Zarshenas.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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