Searching for New Natural Inhibitors of Acetylcholinesterase Enzyme

Yasemin Camadan 1,a,⁎, Ebru Akkemik 2,b
1 Pharmacy Services, Vocational School of Health Services, Artvin Çoruh University, Artvin, Turkey.
2 Science and Technology Researchand Application Center, Siirt University, Siirt, Turkey.
⁎Corresponding author

ABSTRACT

Acetylcholinesterase enzyme (AChE) is the enzyme that catalyzes the hydrolysis of the neurotransmitter acetylcholine to choline. Inhibitors of this enzyme (AChE-i) are used to treat Alzheimer’s, a neurodegenerative disease. Due to the side effects of the drugs used, there has been an increased interest in investigating the inhibitory potentials of natural products which are presumed to have fewer side effects. For this purpose, the inhibitory effects of highland honey, chestnut honey, royal jelly and the seeds of peach, cherry, plum and apricot on human erythrocyte AChE enzyme was investigated in vitro in the present study. Extracts of the seeds and bee products were prepared in ethanol solvent. In order to determine the inhibitory effect of the extracts, the inhibition concentration (IC50) and K values which cause 50% inhibition of the enzyme were calculated using the Ellman method. It was found that among the natural product extracts studied, peach seed had the highest inhibitory effects against neurological disorder [10]. In order to investigate the inhibition effects of natural products (bee products such as highland honey, chestnut honey, royal jelly, donepezil, tacrine and galantamine, have been successfully used [7].

Since the drugs used as AChE inhibitors have various pitfalls such as poor bioavailability, undesired cholinergic side effects and hepatotoxicity, new compounds either synthetic or from plants are needed to be used as AChE inhibitors [8].

Today, many substances are being investigated as candidate drugs to help slow the rate of both cognitive and functional declines in neurodegenerative diseases such as AD [9]. Since phytochemicals found in plants and vegetables have pharmacological properties such as antioxidants, anti-allergics, anti-inflammatory and anticarcinogenic, they are used in the treatment of certain diseases, and their effects have also been investigated in neurological diseases. In previous studies, it was reported that tea, aged garlic extract and ginkgo exhibit protective effects against neurological disorder [10]. In order to investigate the inhibition effects of natural products (bee products such as highland honey, chestnut honey, royal jelly, peach, red cherry, plum and apricot fruits purchased from national companies, and the seeds of these fruits were used) on AChE enzyme associated with

Introduction

Alzheimer’s disease (AD) is the most common form of dementia, which consists of memory loss, cognitive impairment, and difficulty with problem solving and thinking. These symptoms deteriorate over time, becoming severe enough to interfere with daily activities and making the patient need help [1]. Although the etiology of AD is not fully known, according to the cholinergic hypothesis, cognitive and behavioral impairments affecting AD patients are related to a lack of cortical excitability, especially in cholinergic transmission. Acetylcholine levels in the brain can be increased by inhibition of acetylcholinesterase (AChE), thereby improving the cholinergic synapses of AD patients [2].

AD seriously affects the physical and mental health of older people. Aging is the biggest risk factor for the disease, and the incidence doubles every five years after the age of 65 [3]. Today, approximately 50 million people worldwide are affected by neurodegenerative diseases, and this number increases by 10 million each year. About 70% of these patients suffer from AD. This number is estimated to reach 152 million by 2050, and there is currently no treatment to cure neurodegenerative disorders [4].

Acetylcholinesterase (AChE, E.C.3.1.1.7), also called acetylcholine acetylhydrolase, is the carboxylic ester hydrolase which catalyzes the hydrolysis of choline esters [5]. The neurotransmitter hydrolyzes acetylcholine (ACh) and terminates synaptic transmission in cholinergic synapses. Many neurodegenerative diseases such as Alzheimer’s and Parkinson’s are linked with degeneration of the cholinergic system, which leads to a reduces neurotransmitters such as acetylcholine [6]. ACh concentration increases by the inhibition of AChE hydrolysis. Thus, inhibition of AChE hydrolysis constitutes an alternative treatment modality. For the treatment of neurodegenerative diseases, AChE inhibitors that can cross the blood-brain barrier, such as rivastigmine, donepezil, tacrine and galantamine, have been successfully used [7].

Since the drugs used as AChE inhibitors have various pitfalls such as poor bioavailability, undesired cholinergic side effects and hepatotoxicity, new compounds either synthetic or from plants are needed to be used as AChE inhibitors [8].
neurodegenerative diseases, were used in the present study. A literature survey revealed no study indicating the inhibition effect of extracts produced from the used material on AChE enzyme.

Honey and bee products have been used in folk medicine since the early years of human history. They were reported to be beneficial in the treatment of gastrointestinal disorders, wounds and burns, to provide protection against acute and chronic gastric lesions and to be effective as antimicrobial agents [11]. Honey, pollen and propolis are highly valuable bee products. The composition of these products is quite variable and depends on many bio-geographical factors such as plant species, climate, environmental conditions and beekeeper contribution [12]. Studies with honey indicate that it has antioxidant, antimicrobial, antiviral, anticancer and antidiabetic properties, and has protective activities on the nervous, cardiovascular, gastrointestinal and respiratory systems [13]. In addition to being a food source, royal jelly also increases the resistance of bees to diseases and pests. It is also an important food for human health. It was reported to have many pharmacological activities such as life-prolonging, antiallergic, anti-inflammatory, antihypercholesterolemic, antihypertensive and anti-inflammatory effects [14].

Various epidemiological studies showed that a diet rich in fruits and vegetables can reduce the incidence of noncommunicable diseases such as cardiovascular diseases, diabetes, cancer and stroke. These protective effects are in part attributed to phenolic secondary metabolites [15]. In addition to being an important source of antioxidants, fruits and vegetables have a significant antioxidant potential for the compounds in their seeds [16]. Anticholinergic, antidiabetic and antimicrobial effects were reported for fruit seed oils and different fruit seed extracts [17]. Thus, fruit seeds seem to have a very important potential in terms of health. However, the effects of peach (Prunuspersica L.), apricot (Prunusarmeniaca L.), plum (Prunuscerasiferaehrh) and cherry (Prunusavium L.) fruit seeds on AChE enzyme have not been investigated so far.

Peach (Prunuspersica L.) seeds extracts, one of the seed extracts used in the present study, contains many secondary metabolites such as phenolic compounds, carotenoids and tocopherols, which show biological activity and are considered to be disease-preventing [18]. Apricot (Prunusarmeniaca) fruit, on the other hand, has antioxidant and anti-inflammatory functions [19]. Plums (Prunuscerasifera Ehrh) and cherry (Prunusavium L.) fruits are rich in bioactive compounds such as phytochemicals, flavonoids, vitamins A, C, E, anthocyanins, carotenoids and phenolic compounds, which are also considered beneficial for the health [20,21]. Due to the undesirable side effects of the drugs used and the narrow range of therapeutic effects, new AChE inhibitors are needed. To this end, there has been an increasing interest in candidate natural compounds. Based on the effects of certain natural compounds on activities of neural neurotransmitters in the nervous system, the present study aimed to investigate the potential of various bee products and fruit seeds on AChE enzyme in vitro.

**Materials and Methods**

**Chemicals**

5,5’-dithio-bis(2-nitrobenzoic acid) (DTNB), AChE from human erythrocytes (SOUn), acetylcholine iodate (AChI), ethylenediaminetetraacetic acid (EDTA), sodium citrate and dimethyl sulfoxide (DMSO) were purchased from Sigma Chem. Co. Other chemicals were obtained from either Sigma-Aldrich (St. Louis, MO, USA) or Merck (Kenilworth, NJ, USA).

**Preparation of Honey Extracts**

About 5 g of samples were weighed and 100 ml ethanol was added as a solvent. Then, the samples were mixed with a shaker at room temperature for 24 hours. The solution was centrifuged at 10,000 g for 15 minutes to remove suspensions. Supernatant was removed with the help of an evaporator (Velp). The precipitate was dissolved at the minimum volume of the same solvent and kept at 4°C until used [9,12].

**Preparation of Seed Extracts**

The method described by Velioglu (2007) was used with small modifications depending on laboratory and sample characteristics. A 5 g seed sample was homogenized in 50 ml ethanol using a homogenizer (OV-VELP Scientific). Then, the solution was extracted at room temperature in the dark with an orbital shaker for two hours. After filtering with Whatman No.1 filter paper, the mixture was vacuum dried at 40°C using a clear filtrate rotary evaporator (Heldolph-Heizbad-Hei-VAP-517-61000-00-0). The resulting dry crude extract was weighed to calculate extraction efficiency. Then the extract was dissolved in 1 mg/1mL of DMSO and stock solution was prepared. In inhibition studies, extracts were used after dilution with distilled water [22].

**Determination of Esterase Activity**

Spectrophotometric method described by Ellman et al. (1961) was used with minor modifications to determine the effect of bee products on AChE activity. Hydrolysis of the enzyme which uses acetylcholine iodine (AChI) as substrate produces thiocyanate and iodate. The absorption of yellow colored 5-thio-2 nitrobenzoic acid formed by the reaction of thiocyanate with 5,5’-dithio-bis(2-nitrobenzoic acid) (DTNB) was measured spectrophotometrically at 412 nm. For the activity measurement, Tris/HCl buffer (1 M, pH: 8.0), sample extracts at different concentrations and human erythrocyte AChE (500 units /mg protein) were added to the reaction. The reaction was allowed for 10 minutes at 25°C. Finally, acetylcholine iodate (AChI) was added to the reaction and activity measurements were performed [23].
Kinetic Studies

In order to determine the effect of highland honey, chestnut honey, royal jelly and fruit seeds on my AChE enzyme, the activity of the enzyme was measured in five different concentrations of samples (highland honey 0.1315-0.9205 mg/mL, chestnut honey 0.0313-2.191mg/mL, royal jelly 0.0325-0.3900 mg/mL, cherry seed 0.05-0.795 mg/mL, apricot seed 0.05-0.795 mg/mL, plum seed 0.1-1.59 mg/mL, peach seed 0.0112-0.112 mg/mL) mg/mL, in a fixed substrate concentration. The activity of the tube that did not contain sample was used as blank, and its activity was considered 100%. Each experiment was repeated three times. Activity (%)-[I] charts were drawn for each sample to find the IC₅₀ values. IC₅₀ values of the samples were calculated from these charts [24]. Activity measurements were made at five different substrate concentrations to determine the Vₘₐₓ and Kᵣ parameters of the enzyme. Kᵣ (0.07272 mM) and Vₘₐₓ (181.81 EU) values were calculated by drawing Lineweaver-Burk charts [25].

Using these parameters, the Kᵢ values of the substances were calculated with the following formula [26]:

\[ \frac{V}{K_m (1 + \frac{I}{K_i}) + S} \]

In the equation, Vₘₐₓ is the maximal speed, Kᵣ is the substrate concentration at which half of the maximum speed is reached, I is the compound concentration, Kᵢ is the inhibition constant, S is the substrate concentration of the substrate. Nonlinear regression was used to calculate the inhibition constant values (Kᵢ) using the equation above.

Results and Discussion

The key role that acetylcholinesterase (AChE) plays on the central nervous system in Alzheimer’s disease (AD) encourages research to identify more effective inhibitors and safer drug candidates for the enzyme. However, adverse side effects of AChE inhibitor drugs used in the pharmacological treatment of AD limits their use [27]. Therefore, currently there has been much interest in development of more effective and useful drug candidates.

In the present study, the effect of extracts obtained from natural products on human erythrocyte AChE was investigated in vitro conditions. Extracts of highland honey, chestnut honey, royal jelly and fruit seeds were prepared with ethanol solvent, and their inhibition effects were investigated. IC₅₀ and Kᵢ values of the samples which exerted inhibition effect were calculated (Table 1).

| Table 1. Inhibition effects of various natural extracts on AChE enzyme |
|-----------------------------|-----------------|-----------------|-----------------|
| Sample          | Solvent | IC₅₀ (mg/mL) | Kᵢ (Avg.) (mg/mL) |
| Highland honey   | Ethanol  | 1.23±0.0077   | 0.0128±0.0070     |
| Chestnut honey   | Ethanol  | -              | -                |
| Royal jelly      | Ethanol  | 0.397±0.1357  | 4.22E-06±4.97E-07 |
| Cherry seed      | Ethanol  | -              | -                |
| Plum seed        | Ethanol  | 0.687±0.137   | 0.095±0.039       |
| Apricot seed     | Ethanol  | 0.460±0.041   | 0.101±0.033       |
| Peach seed       | Ethanol  | 0.056±0.0037  | 0.0176±0.0088     |

IC₅₀ values were 0.2555 mg/mL and 0.3006 mg/mL for ethanol extracts of highland honey and royal jelly, respectively. For chestnut honey, an inhibition effect on the enzyme was observed, but the IC₅₀ value could not be calculated (Table 1, Figure 1). To date, the effects of honey and other bee products were detected only for physiological diseases, while their role in psychological or neurodegenerative diseases is still unknown. Therefore, the effect of bee products on the activity of enzymes attracts the attention of researchers. A study found that the IC₅₀ value of chestnut honey was 41.60 ±6.05 μg/mL (Galantamine was used as a standard inhibitor for AChE and IC₅₀ value was determined 2.727± 0.08 (μg/mL) and it was suggested that bioactive compounds in plants may have inhibitory effects [28]. In different studies found that the extracts of chestnut honey and mad honey had IC₅₀ values ranging from 76 to 129 mg/mL on hyaluronidase enzyme, from 12 to 34 mg/mL on urease enzyme, 1.705 (mg/mL) on hCA I isoenzyme and 2.830 (mg/mL) on hCA II isoenzyme [12, 29]. Akbulut and Akkemik (2018) found that ethanol extracts of highland honey and chestnut had IC₅₀ values ranging from 0.060-2.768 mg/mL on the cancer-related thioredoxin reductase enzyme [9]. It was seen that the results were compatible with our study. Studies of bee products on AChE, a neurotransmitter enzyme, are limited..

The IC₅₀ values of plum, apricot and peach seeds were calculated as 0.7049, 0.4544 and 0.05708 mg/mL, respectively (Table 1). Kᵢ values were in the range of 0.01761-0.1601 (mg/mL). However, IC₅₀ value could not be determined for cherry seed extract because it did not inhibit AChE enzyme activity sufficiently.
Although various studies were conducted on the antioxidant, anticancer, antimicrobial, anti-inflammatory effects of fruit seed extracts, studies investigating their effects on enzyme inhibition are limited. Acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE,pseudocholinesterase) enzymes were inhibited by methanolic extracts of grape (Vitisvinifera L.) seeds [30]. In studies with Acacia catechu willd, Cola acuminata, Cuminum cuminum and Lawsonıa Inermıs (Henna) seeds, IC50 values of seed extracts were found as 204.38 ± 2.54 (µg/mL) [31], 14.6 ± 1.04 (µg/mL) [32], 0.437µg/mL [33] and 66.6 (mg/L) [34] respectively. Similar to the literature, it was observed that selected seed extracts had inhibitory effects on the enzyme activity (Table 1, Figure 2).

As a result of the study, it was observed that natural products showed inhibition against the enzyme at certain ranges, but it was found that the extract obtained from the peach seed exerted the highest inhibition activity (Table 1, Figure 2).

**Figure 1.** In vitro effects of highland honey and royal jelly on AChE enzyme activity

**Figure 2.** In vitro effects of apricot, peach and plum seed on AChE enzyme activity

**Conclusion**

While dietary intake of some fruits plays a role in delaying the onset of Alzheimer’s disease, natural products are also used in the treatment of neurological diseases by altering the activities of neural neurotransmitters in the central nervous system. In order to reduce the side effects of the drugs used today and to obtain more effective and inexpensive drugs, the interest of scientists has shifted towards the extracts and molecules obtained from natural products. For this purpose, the inhibition effect of extracts from natural products on AChE is valuable to guide the future studies. Considering the results of the present study, it could be stated that honey and fruit seeds may have beneficial a role in the treatment for human health. However, more studies are needed to reach a definite conclusion.
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Conflicts of Interest

The authors declared that they have no conflict of interest.

References


