

A study on inhibitory effect of ethanolic extract of the *Pistacia lentiscus* on acetylcholinesterase activity

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Key Words:Alzheimer's disease
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Physostigmine**ABSTRACT****Background and Objective:** Changes of the enzyme acetylcholinesterase are involved in pathogenesis of different nervous disorders including Alzheimer's disease. This research work was conducted to evaluate the inhibitory effect of *Pistacia lentiscus* ethanolic extract on acetylcholinesterase activity.**Materials and Methods:** Activity of the enzyme acetylcholinesterase (AChE) was measured by Ellman method, using $\frac{\text{nmol}}{\text{min/mg}}$ scale. Then, Lineweaver Burk plot was used to calculate Km, Vmax and Ki. In all phases, the enzyme's concentration was constant and its activity was measured at six different concentrations of acetylthiocholine (5, 10, 15, 20, 25 and 30 mM) at room temperature (25 °C) and based on the optical absorption at 412 nm wavelength. Experiments were conducted in the presence of various concentrations of physostigmine and also *Pistacia lentiscus* (2, 4, 6, 8, and 10 ug/ml).**Results:** Ki of inhibitors were measured at different concentrations of acetylthiocholine (5, 10, 15, 20, 25 and 30 mM) and also in the presence of various concentrations of physostigmine and *Pistacia lentiscus* (2, 4, 6, 8, and 10 ug/ml). IC50 of physostigmine and *Pistacia lentiscus* were determined as 2.9 and 6.5 ug/ml, respectively.**Conclusion:** Since lower levels of Ki and IC50 indicate higher inhibitory effects on the enzyme, therefore, results show that physostigmine is a stronger inhibitor than *Pistacia lentiscus*.

1. Introduction

Alzheimer's disease (AD) is a type of dementia (dementia) and the problem is common in old age. With respect to increasing average age of the population, the incidence of this disease is increasing (1, 2). The incidence of AD is about 3 per thousand at age 65 and 70 per thousand in the age of 90 and its incidence is higher in women (3, 4). Oxidative stress, metal ions and the aberrant proteins are involved in the etiology of AD (5). In AD, memory loss, unusual behavior, personality changes and a decrease in the ability to interpret are seen (6). Acetylcholine

plays a key role in AD in such patients with its significant activity reduction. It is hydrolyzed by the enzyme acetylcholinesterase (AChE), therefore, the inhibition of the enzyme can lead to acetylcholine increase (7). Three drugs inhibit the enzyme AChE, i.e. anpzyll, ryvastgmyl and galantamine that have applications for mild to moderate stages of the disease used to improve cognitive function in the patients. In severe stages of the disease, memantine as a Damantyn derivative may be recommended. These drugs inhibit NMDA receptors and are neuroprotective (8, 9).

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Other drugs like physostigmine monomethyl are also as an AChE inhibitor that is a carbamate. It is easily ingested, has mucous membranes or subcutaneous absorption, and passes the blood-brain barrier. Physostigmine have adverse effects such as cramping, weakness, nausea, and vomiting, cramps bronchi, and stimulates the central nervous system. Thus, due to these complications, the need for a lower-risk drug is necessary (10). Pistacia lentiscus or Pistacia lentiscus tree, a tree species in the Mediterranean region and Southern USA can be considered in this respect. Compounds of this tree have diuretic and expectorant effects and could control bleeding (11). In warmer months, latex in the secreted droplets will enhance and improve the stomach, liver and appetite. It has been effective in obsessive and melancholy smell and its chewing could tighten the gums and teeth and strengthen memory. The main composition of Pistacia lentiscus family includes terpenes and resins (12, 13). It has also antioxidant properties to neutralize free radicals and inhibit oxidative stress with applications in AD and memory disorders (14). It is most likely that it causes the inhibition of the enzyme AChE that this potential was assessed in this study.

2. Materials and Methods

AChE activity was studied using DTNB. The assay of human serum cholinesterase with the Ellman reaction involves reaction of 5, 5-dithio-bis (2- nitrobenzoic acid) (DTNB) with thiocholine liberated from its esters by enzymatic hydrolysis. The yellow 5-thio-2-nitrobenzoate (TNB) is formed that is detected by colorimetric substrates for the assay. The absorbance at 412 nm is measured immediately (16). In the first part of experiments, activity of AChE was assessed for different concentrations of substrates. The concentrations ranged from 5,10,15, 20, 25, and 30 mM. The composition of each tube contained 2.6 ml of Tris buffer. 0.2 ml of the enzyme, 0.1 ml of DTNB and 0.04 ml of the substrate acetylthiocholine. Tested enzyme inhibitors were physostigmine and Pistacia lentiscus. To compare and calculate the impact of inhibitor, Ki was calculated. The composition of each tube contained 2.7 ml of Tris buffer, 0.2 ml of the enzyme, 0.1 ml of DTNB and 0.04 ml of the substrate (at a concentration of 5, 10, 15, 20, 25, and 30 mM) and 0.06 ml of the inhibitor (physostigmine at concentrations of 2.5 and 7.5

and Pistacia lentiscus at concentrations of 5 and 7.5 ug/ml). To calculate, the physostigmine and Pistacia lentiscus at concentrations of 2, 4, 6, 8, 10ug/ml and fixed substrate concentration of 20 mM were used and also in various stages of testing, the same time concentrations were measured by enzyme activity. The tests were used to calculate the speed of the enzyme from the following equation:

$$\text{Rate} = \frac{\Delta A \times V}{1.36 \times 10^{-2}} \times \frac{1}{C \times Y}$$

Delta A: differences in light absorption, V: Covet volume equivalent to 3.1 ml, Y: amount equal to 0.2 ml enzyme and C: enzyme concentration of 1 mg / ml.

After calculating the rate of the enzyme in different situations, with and without inhibitor, and in the presence of different concentrations of different inhibitors, to obtain percentage and percentage inhibitory activity of the enzyme reaction, the following formula was used:

$$\text{Inhibitory Percent} = \frac{\text{Speed in the presence of inhibitor} - \text{Speed in the absence of inhibitor}}{\text{Speed in the absence of inhibitor}}$$

$$\text{ACT \%} = 100 - \text{Inhibitory Percent}$$

The amount and the reaction was calculated using Lineweaver Burk chart. In order to calculate the inhibitor, the Dixon equation was used.

2.1. Preparation of *Pistacia lentiscus* ethanolic extract

One hundred g of plant powder was added to 1 l of ethanol and incubated for 3 days in a cool and dry place away from light with daily agitation. Thereafter, the extract passes through appropriate filters till a transparent and clear solution was achieved. The resulting solution was condensed using rotatory evaporator till dried. Then, the final extract was kept in a closed container in the refrigerator.

3. Results

For control experiments, the concentrations of 5, 10, 15, 20, 25 and 30 mM of iodized acetylcholine (substrate) in the presence of 1 mg/ml of the enzyme AChE was used in the test, with K_m and V_{max} of 150 and 5 mM (1) (Figure 1). The concentration physostigmine 7.5 ug/ml in the presence of 5 mM, 10, 15, 20, 25 and 30 mM of substrate concentration 1 mg / ml of the enzyme AChE was used the V_{max} of control (150) and the apparent K_m of 40 mM to lose (Figure 2), which indicates the competitiveness of the inhibitory effect of the cortex (Diagram 2). When the concentration of the Pistacia lentiscus 7.5 ug/ml was used, the V_{max} such as state control and the apparent K_m 6.66 mM was obtained. This plant has also inhibitory effects on

the activity of the enzyme AChE that is competitive (Figure 3). IC_{50} for physostigmine and Pistacia lentiscus, according to the charts of activity of the enzyme AChE was calculated at concentrations of 2, 4, 6, 8, 10 ug/ml of these two inhibitors in order for physostigmine and Pistacia lentiscus 2.9 and 6.5 ug/ml, respectively (Figures 4 and 5). K_i reaction in the presence of the substrate with concentrations of 5, 10, 15, 20, 25 and 30 mM Venice concentration of 1 mg / ml of the enzyme AChE, the physostigmine and the concentration of Pistacia lentiscus 7.5 ug/ml respectively 1.07 and 22.7 ug/ml was the apparent K_m using the formula results and charts help Lineweaver Burk, respectively (Figure 6). All results related to K_m , V_{max} , K_i , IC_{50} of the enzyme inhibitors physostigmine and Pistacia lentiscus are presented in Table 1.

Table 1. Kinetic factors with and without inhibitors of acetylcholinesterase (AChE)

K_m (mM)	V_{max} (nmole/min/mg)	K_i (ug/ml)	IC_{50} (ug/ml)	Inhibitor Type	Inhibitory concentration (ug/ml)	Component name
5	150	-	-	-	.	normal
40	150	1.07	2.9	competitive	7.5	Physostigmine
6.66	150	22.7	6.5	competitive	7.5	<i>Pistacia lentiscus</i>

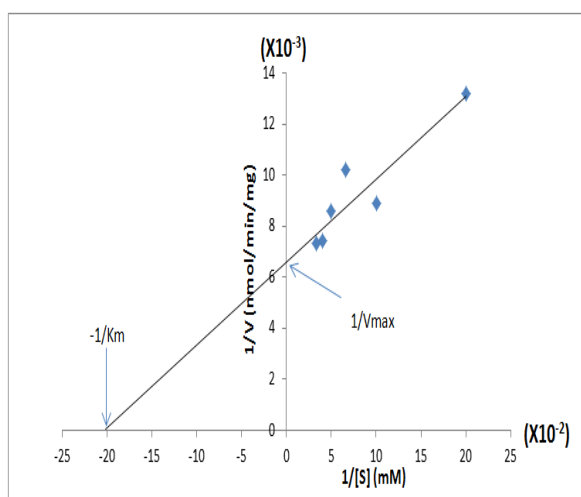


Fig. 1. Lange Lineweaver Burk chart: The maximum and Michaelis-Menten constant velocity of acetylcholinesterase without inhibitor.

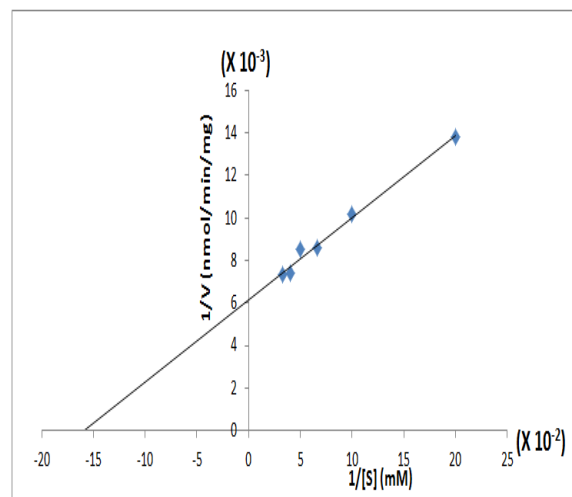


Fig. 2. Lange Lineweaver Burk curve: Calculate the maximum speed and Michaelis-Menten constant for acetylcholinesterase inhibitors in the presence of 7.5 ug/ml of physostigmine and substrate concentrations of acetylcholine 5, 10, 15, 20, 25 and 30 mM

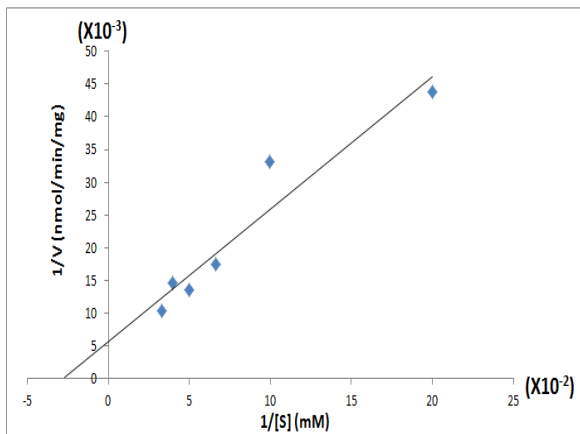


Fig. 3. Lange Lineweaver Burk curve: Calculate the maximum speed and Michaelis-Menten constant for acetylcholinesterase inhibitors in the presence of 7.5 ug/ml of *Pistacia lentiscus* and substrate concentrations of acetylcholine 5, 10, 15, 20, 25 and 30 mM

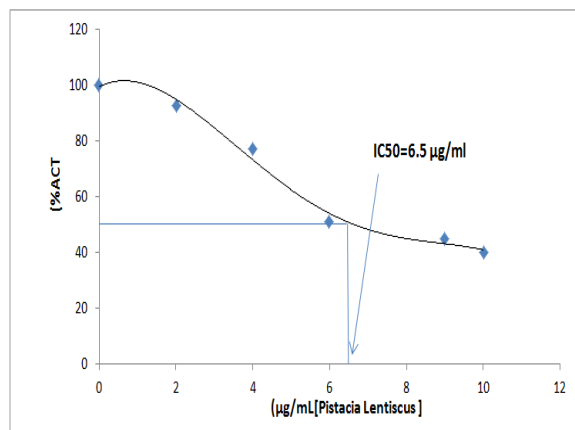


Fig. 6. The effect of different inhibitory concentrations of *Pistacia lentiscus* extract on the acetylcholinesterase enzyme (IC50 calculation inhibitors).

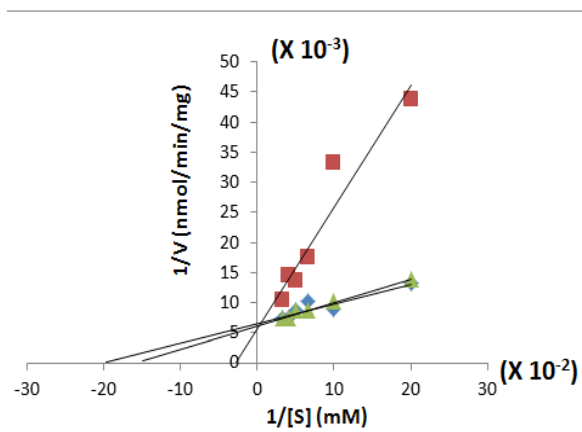


Fig. 4. Lange Lineweaver Burk curve: Calculate the maximum speed and Michaelis-Menten constant for acetylcholinesterase inhibitors in the presence of 7.5 ug/ml of physostigmine and *Pistacia lentiscus* and substrate concentrations 5, 10, 15, 20, 25 and 30 mM)

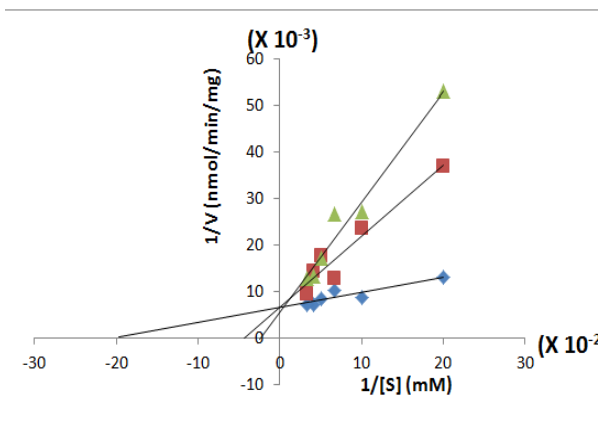


Fig. 7. Lange Lineweaver Burk curve: Calculate the maximum speed and Michaelis-Menten constant for acetylcholinesterase inhibitors in the presence of 2.5 and 5 ug/ml of physostigmine, *Pistacia lentiscus*, respectively, and substrate concentrations of 5, 10, 15, 20, 25 and 30 mM

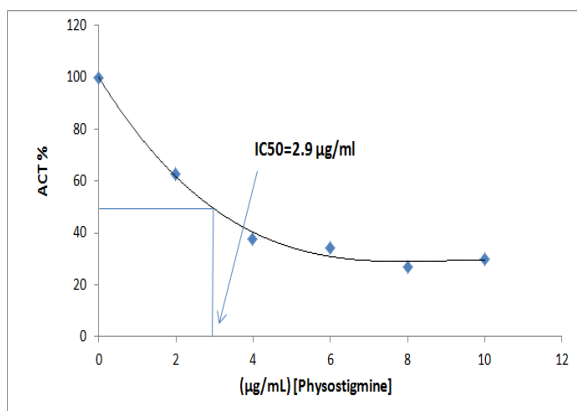


Fig. 5. The effect of different inhibitory concentrations of physostigmine on the acetylcholinesterase (AChE) enzyme (calculated inhibitors IC50).

4. Discussion

AChE (EC 3.1.1.7) is an enzyme essential to nervous system. Cholinergic synapses play important physiological roles (17). Some of the chemical compounds in the active site of the enzyme or chemical agents capable of coenzyme or enzyme activator ions are combined and thus the catalytic activity of enzymes to create a barrier, These compounds are mentioned as inhibitors. Many inhibitor compounds as drugs or insecticides (organophosphates) are used If a chemical can act as a barrier to the catalytic activity of the enzyme AChE is effective in the treatment of AD. Therefore, in our study, the effect of ethanolic extract of *Pistacia lentiscus*

and the drug physostigmine was examined on the activity of the enzyme in vitro. Km of enzyme response in control and in the presence of 5 mM of 7.5 ug/ml of Pistacia lentiscus to 6.66 mM was obtained, which concluded that ethanolic extract of Pistacia lentiscus has its impact on AD by inhibiting the activity of the enzyme. The apparent Km of the enzyme reaction in the presence of 7.5 ug/ml of physostigmine is 40. The apparent Km reaction than the presence of Pistacia Lentiscus, Pistacia lentiscus has more effect than the physostigmine. The IC50 as compared to Dygrjht Physostigmines with Pistacia Lentiscus, when the concentration of 2, 4, 6, 8, 10 ug/ml of the inhibitor and the substrate with a constant concentration of 20 mM used is another confirmation of the greater inhibitory effect than Pistacia lentiscus obtained physostigmines (IC50 for physostigmines for on the Pistacia lentiscus to the 2.9 for a 6.5 ug/ml is) (Approximately 2-fold). Km apparent reaction when the other concentrations of 2.5 physostigmine or 5 ug/ml of Pistacia lentiscus of substrate and enzyme used in the presence of the same, respectively, 20 and 40 mM and Vmax in both cases was 143 and the control condition. The findings also show that Pistacia lentiscus and physostigmine that are both competitively inhibit enzyme activity Ki reaction in the presence of different concentrations of the substrate and the concentration of 1 mg / ml of the enzyme, the physostigmine with a concentration of 7.5 ug/ml concentration of 7.5 to 1.07 and Pistacia lentiscus with the 22.7 ug/ml, respectively. Thus, physostigmine has more inhibitory power versus Pistacia Lentiscus.

Conclusion

The findings of this study showed that Pistacia lentiscus could improve AD through inhibition of AChE. However, regarding side effects such as dizziness and nausea, its impact has been equivalent to half of the affected physostigmine. Because certainly different types of tannins and resins in the Pistacia lentiscus extract exist, therefore, we need to investigate further purified active substance of this extract. The other ingredients in this herb can have a positive or negative impact on the ability of inhibitory active substance.

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Conflict of interest: We have no conflicts of interest to disclose for this research study.

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