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Efficacy of Natural Extracts of Ginkgo Biloba and Berberry and a Synthetic Derivative of Genistein (ipriflavone), as Acetylcholinesterase Inhibitors, Comparative Study with Aricept® effect

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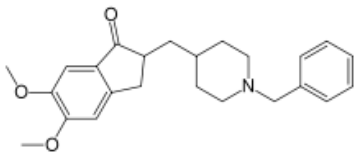
ABSTRACT: *Inhibition of acetylcholinesterase (AChE.3.1.1.7), the key enzyme in the breakdown of acetylcholine, is considered as a promising strategy for the treatment of neurological disorders such as Alzheimer's disease (AD). The brain AChE from female Egyptian Mediterranean buffalo (Bas Buballus) was purified by ammonium sulphate precipitation, Sephadex G-25, Sephadex G-100 and DEAE-cellulose. Finally, Polyacrylamide gel electrophoresis was carried out to clarify the enzyme purity. The effect of the natural extracts of Ginkgo biloba and berberry and a synthetic derivative of genistein; ipriflavone on the activity of pure AChE were carried out in an in vitro study.*

Ginkgo biloba ands berberry extracts inhibited AChE. The increase in the K_m-values with no differences in the V_{max}-values pointed toward competitive type of inhibition. On the other hand, ipriflavone could be accounted as non-competitive inhibitor to AChE, where it caused three fold decrease in V_{max} – value and did not alter the K_m-value. Moreover, AChE is inhibited in a mixed type of inhibition by Aricept ®. Interestingly, the inhibitory per cent of ipriflavone nearly equal to that of Aricept ® (70%). It is evident from the present study that ipriflavone is the strongest inhibitor.

Key words: *Acetylcholinesterase, Ginkgo biloba, berberry, ipriflavone, Aricept ®.*

1. INTRODUCTION

Alzheimer's disease (AD) is a progressive, neurodegenerative disease, which primarily affects the elderly population and is estimated to account for 50-60 per cent of dementia cases in those over 65 years of age (1). Cholinergic neurotransmission dysfunction in the brain contributes to the salient cognitive decline in AD. The loss of cholinergic cells, particularly in the basal forebrain, is accompanied by loss of the neurotransmitter, acetylcholine. The current mainstay treatments for AD are acetylcholinesterase [AChE.3.1.1.7] inhibitors, which increase the availability of acetylcholine at cholinergic synapse. AChE inhibitors are general chemical classes, such as rivastigmine, tacrine and donepezil (Aricept®), and have been tested for symptomatic treatment of AD (2). However, the non-selectivity of those drugs, and their limited efficacy due to their short half lives, poor bioavailability, nausea, vomiting, diarrhea and hepatotoxicity are some of the severe limitations to their therapeutic success (3). There have been a number of reports on the designing and development of synthetic AChE inhibitors (4-5), which were necessary for other studies on AChE inhibitors derived from medicinal plants (6-7).



Donepezil

A variety of plants have been reported to show AChE inhibitory activity and so may be relevant to the treatment of neurodegenerative disorders such as AD (8). Ginkgo biloba (GB) is an herbal medicine that has been used to treat a variety of ailments for thousands of years in China (6-8). An extract of GB has been found in several studies to improve the symptoms and slow the progression of AD (9). GB

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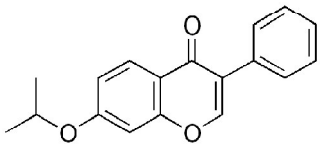
extract appears to be most effective in the early stages of AD. This could potentially mean that patients with early AD may be able to maintain a reasonably normal life (10). In addition, in traditional Eastern medicine, the extract of various *Berberidaceae (Berberis aquiflium, Berberis aristata and Berberis vulgaris)* is used for rheumatic complaints and other type of chronic inflammation (11). It has been reported that the functional components of the *Berberidaceae* extracts were alkaloid components such as berberine, oxyacanthine, berbamine and palmatine. *Berberidaceae* extract is used as anti-inflammatory and immunosuppressive medicine (11). Recent findings show that the extract of *Berberidaceae* prevents neuronal damage due to oxidative stress and it might act as a novel cholesterol-lowering compound (12). While berberry may have potential in the treatment of dementia disorders, its effect on the kinetic parameters of AChE has not been investigated. Furthermore, ipriflavone is a synthetic derivative of genistein; an isoflavone found in soy and other plant sources. Ipriflavone has been approved for the treatment of involutional osteoporosis in some European countries and in Japan (13). It has been indicated that genistein act as AChE inhibitor (14) while, there are no studies on the effect of ipriflavone on brain AChE activity.



Ipriflavone

We undertook the present study to test the efficacy of extracts of GB and berberry in addition to ipriflavone on purified AChE through *in vitro* study compared to the effect of Aricept ® as a commercial drug.

2. MATERIAL AND METHOD

2.1. Chemicals

Acetylthiocholine iodide (ACTI), 5, 5′- dithiobis 2-nitrobenzoic acid (DTNB), Sephadex G-25, Sephadex G-100, and diethyl aminoethyl-cellulose (DEAE-cellulose) were purchased from Sigma Chemical Company (St. Louis, Mo., USA). Polyacrylamide gel was obtained from Merck (Germany). Ipriflavone (fine grade, the pure active material) and Aricept® (donepezil as commercial drug mixed with tablet pharmaceutical ingredients) (were obtained from Inspired Living, Italy and Pfizer, Alexandria, Egypt, respectively. Plants extracting solvents (HPLC grade) were obtained from Merck (Germany). Al1 the other reagents were of analytical grade and highest purity available commercially.

2.2. Herbal Extraction

GB (green leaves) and berberry (green leaves) (Berberis vulgaris) were collected in the spring and authenticated by Prof. Dr. Eldareir, S. Prof. of plant ecology, Faculty of Science, University of Alexandria Egypt. These plants were washed, dried at 37 °C for 24 hours and milled well to fine powders separately in a Braun homogenizer. The GB powder (20g) was extracted by shaking with 100 ml ether for 30 minutes. This extraction procedure was repeated two times and the ether was evaporated. The residue was successively partitioned three times for each of the following solvents; chloroform, acetone and finally ethanol (all solvents were HPLC grade) (100 ml per each time). Each fraction was evaporated and all the extracted compounds were collected as GB extract (2.5g). According to extraction procedure the extracts could contain alkaloids, flavanoides and saponin (10). In addition, the fine powder of berberry (100g) was dispersed in five volumes ethanol (99.8%) and refluxed for one hour at 70 °C. The extraction procedure was repeated three times. The ethanol extract was concentrated and dried under vacuum to get a residue (9.8g); this residue contains mainly berberine according to the extraction procedure of Yoo *et. al*. (11).

2.3. Preparation of Crude Enzyme Extract

Fresh brain tissue was obtained from male *Egyptian Mediterranean buffalo* (*Bos Buballus*) immediately after slaughter and washed with ice-cold phosphate buffer (pH 7.6, 0.1 M), containing NaCl (0.2M) and Na_2EDTA (0.001M). Then, the brain tissue was stored under ice-cold toluene for three days in a refrigerator. The brain tissue was washed again with ice-cold phosphate buffer and distilled water three times. Then, the brain tissue (300g) was homogenized with four volumes cold 0.1 M phosphate buffer (pH7.6) containing NaCl (0.2M), Na_2 EDTA (0.001M), 5mM protease inhibitor cocktail and 0.5% Triton X-100 using a Polytron

(Tekmar model, TR 10, Germany). The homogenate (F1) was centrifuged at 6000 rpm for 30 minutes using cooling centrifuge at 4 °C (Hitachi, Germany). The clear supernatant was collected and used as a crude preparation of AChE (F2) (15). The total volume of this supernatant was measured and the protein content was determined according to Lowry's method (1951) (16).

2.4. Partial Purification Procedure of Brain Acetylcholinesterase

Unless otherwise stated, all the procedures of purification were carried out at 2-4 °C in a cold room. The collected crude extract of AChE was submitted to four steps of purification; ammonium sulphate fractionation (30-70%), two steps of Sephadex gel chromatography (G-25 and G-100) and a step of DEAE-cellulose chromatography according to the method of Rao and Dasgupta (1991) (15).

2.5. Electrophoresis

Polyacrylamide gel electrophoresis was carried out according to the procedure of Davis (1964) (17) to estimate the enzyme purity. The gel concentration for the running gel was 8% polyacrylamide net work and the running solution was Tris-glycine buffer (pH 8.6, upper buffer 0.054 M; lower buffer 0.108 M). A stacking gel was 4% polyacrylamide net work (Stacking gel is a less concentrated gel which permit a rapid migration of proteins through it causes them to accumulate and stack as a very thin zone at the stacking gel/running gel boundary, and most importantly, since the 4% stacking gel affects the mobility of the large components only slightly, the stack is arranged in order of mobility of the proteins in the mixture. This stacking effect results in superior resolution within the running gel, where polypeptides enter and migrate much more slowly, according to their size and shape). The applied current was 50 mA Protein was stained with Coomassie Blue according to the method of Chambach *et al.* (1967) (18).

2.6. Acetylcholinesterase Activity Assay

The AChE activity assay was carried out using an acetylcholine iodide substrate, based on colorimetric method, as described previously (19). The residues from GB and berberry were initially dissolved in 0.2% DMSO, and immediately diluted to a concentration (1 mg/ml) in buffer A (100mM sodium phosphate buffer, pH8.0) also Aricept® and ipriflavone dissolved to the same concentration before use. In ELISA plate (Bio Tec. USA), 151µl of phosphate buffer (pH 8.0) was directly put in ELISA blank well and 131µl of phosphate buffer (pH 8.0) was directly put in ELISA activity wells. Then to the blank and activity wells, 5ul of substrate ACTI (75 mM) was added and, then 20µl of enzyme was added in Activity ELISA wells only. The plate was preincubated for 15 min at 37 °C before the addition of the second substrate, 0.32 mM DTNB. DTNB (60µl) was added in both the blank and activity wells. Absorbance was measured at 405 nm every two minutes. Values obtained were analyzed; blank reading were subtracted from sample readings. Absorbance vs Time graph was plotted.

2.7. Specific Activity

[A] x [Total Volume in cuvette (µl)] / ([Molar extinction coefficient of DTNB] x [Volume of Brain extract (µl)] x [Protein Concentration (mg/ml)])

Where: -

Specific activity = moles of substrate hydrolyzed / minute / mg of protein

A = change in O. D. per minute = Slope

Molar extinction coefficient of DTNB = 1.36×10^4

Volume of Brain extract = 20µl

Volume in Cuvette = 20µl [vol. of brain extract] + 131µl [vol. of phosphate buffer, pH 8.0] + 60 µl of DTNB + 5µl of Acetylthiocholine Iodide = 216 µl.

2.8. Determination of the Kinetic Parameters of AChE

In the activity assay system, we changed the substrate's concentrations (ACTI, 7.5-75 mM). Lineweaver-Burk plots were used to determine the kinetic parameters of AChE (20).

The per cent inhibition was calculated by using the following equation: % inhibition= {[Activity of control – Activity in presences of 1 mg/ml inhibitor] / activity of control}X 100

3. RESULTS

3.1. Partial Purification of AChE

The key steps in the purification of brain AChE were summarized in table (1). AChE was partially

purified about 202-fold starting from the crude homogenate to the final step. The DEAE-cellulose fraction retained approximately 11% of the original total activity (yield). The electrophoretic behavior of AChE during purification steps was shown in Fig. 1. The fraction of DEAE-cellulose column resolved as less number bands of protein (Fig. 1, lane 4). The first band on the PAGE corresponds to AChE according to the protein marker which we used.

3.2. Inhibitory Activity of the Tested Compounds against AChE

The concentration of inhibitors was chosen similar to the recommended dose of Aricept® for this reason we did not perform the dose-response curve for our inhibitors in order to compare the efficiency of inhibitory effects of natural product with synthetic drug at its recommended dose. The inhibitory effects of the extracts of GB and berberry in addition to ipriflavone compared to a positive control (Aricept®) on the AChE activity are described in table (2) and figure (2). The Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) of AChE were determined by Lineweaver-Burk plots (1/V versus 1/[S]; reciprocal plots). The reciprocal plots of either GB extract or berberry extract can be seen to be intersected on the 1/V axis; both extracts were found to inhibit AChE activity in a competitive manner. The Per cent inhibition values of AChE activity were 35% and 50%, respectively (Fig. 3) compared to the control value.

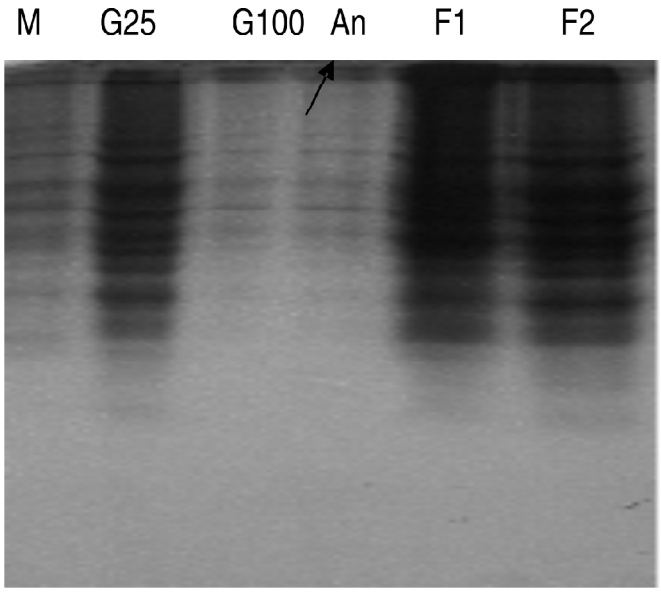


Figure 1: **No denaturing PAGE analysis of the partially purified AChE. Each purification step of AChE was loaded to each lane of an 8% Polyacrylamide gel after being concentrated. The gel was run for 1.5 h at 150 V. After electrophoresis, the gel was stained for total protein with Coomassie Blue. F1: Homogenate, F2: Crude preparation of AChE, G25: fraction obtained from gel filtration with Sephadex G25, G100: fraction obtained from gel filtration with Sephadex G 100 and An: fraction obtained from anion exchanger DEAE – cellulose.**

Protein markers are; B–galactosidase, 116 KDa, Bovine serum albumin, 66.2KDa, Oval albumin, 45KDa, Lactate dehydrogenase, 35KDa, Restriction endonuclease (BSP 981), 25.0kDa, B. Lactoglobulin, 18.0KDa and Lysozyme, 14.0kDa.

The black arrow refers to AChE band.

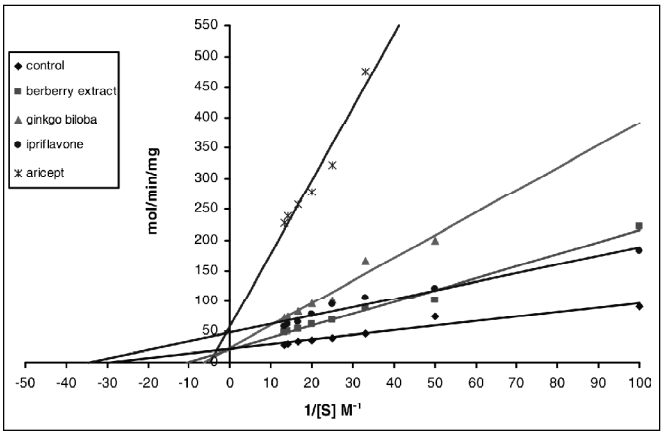


Figure 2: **Double reciprocal 1/V versus 1/[S] lineweaver Burk plot for the partially purified AChE in absence and presence of GB extract, berberry extract, Ipriflavone and Aricept®, at concentration of 1 mg/ml. The data were analyzed by Lineweaver-Burk plot and the values of Km and Vmax were averaged**

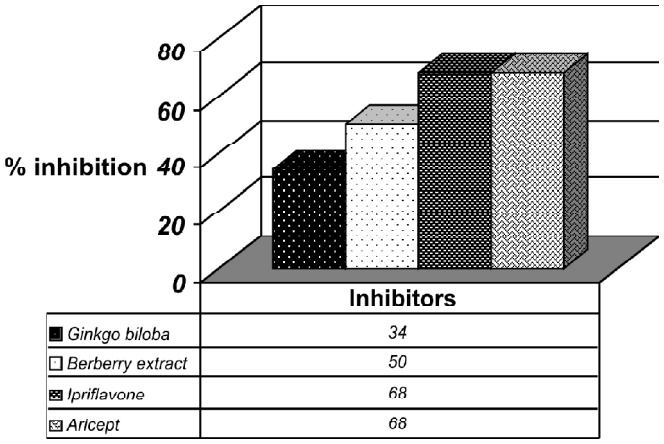


Figure 3: **Partially purified AChE from brain prepared by the procedure of Rao and Dasgupta, 1991. Per cent inhibition of AChE activity in the presences of berberine, Aricept, Ginkgo biloba and Ipriflavone, at a concentration of 1 mg/ml**

Furthermore, AChE activity was also inhibited by ipriflavone (Fig. 2). The reciprocal plot of ipriflavone was intersected on the left side of 1/[S] axis; suggesting that the inhibition was non-competitive. In addition, the results show that the per cent inhibition value of ipriflavone was 69% and the Ki- value was 1.68M. Finally, the Lineweaver-Burk plot shows that Aricept ® acts as a mixed type inhibitor for AChE resulting in 70 per cent inhibition with a Ki value 0.84M.

4. DISCUSSION

Acetylcholinesterase activity tends to increase with aging. Increased activity and stress cause an excessive breakdown and removal of the neurotransmitter, acetylcholine. Consequently, this enzyme reduces message-delivery activity to the muscle and over ambitiously "cleans house" of acetylcholine in human brain (1). The resultant decrease in ACh- dependent neurotransmission is thought to lead to the functional deficits of AD. Clinical drug trials in patients with AD have focused on drugs that augment levels of ACh in the brain to compensate for losses of cholinergic function in the brain. These drugs have included ACh precursors and cholinesterase inhibitors (10). AChE inhibitors as tacrine, rivastigmine and donepezil (Aricept ®) are the only drugs currently approved for the treatment of AD; however, these drugs are known to have limitations for clinical use (21). Following the identification of Aricept ® as a cholinesterase inhibitor, there have been many detailed studies on its *in vitro* and *in vivo* pharmacological studies (22). However, there is only limited published informations on the inhibitory effect of the extracts of GB and berberry on AChE activity (8, 23). In addition, there is no any published informations on the effect of ipriflavone on the activity of the same enzyme.

To study the effect of a compound on enzyme kinetics, it must be examined on the purified form of the enzyme; the purified form has higher quantity and quality (24). In our study, AChE from brain tissue was purified according to the method of Roa and Dasgupta (1991) (15). After the last step of purification, the specific activity of brain AChE has increased about 202-fold (Table 1).

Our study showed that the extract of GB and berberry can manifest inhibitory AChE activity, although they are less effective than Aricept® at the same concentrations. Moreover, according to the characters of competitive inhibition; GB and berberry extracts are considered as competitive inhibitors to AChE (K_m values are increased by 3 and 6 fold, respectively with no change in V_{max} values). GB extract and berberry extract are known to be reversible inhibitors to AChE (25, 26). The action mechanisms of these herbs are not well known. It has been suggested that the chemical composition of GB extract are flavonoids, terpenoids and Ginkgolic acids (27). While, the main functional components found in berberry extract are alkaloids such as berberine (12). It has been reported that the extracts of GB and the berberine have a dose dependent- inhibitory activity on AChE (28). Although, it has been reported that the effect of GB is comparable to that of donepezil in the treatment of dementia (29) and berberry extract reduces neuronal damage (11). This requires confirmation in an *in vivo* study. A very useful study would be four –armed comparing the effect of placebo, donepezil, GB and combined donepezil/ GB therapy. In addition, berberry extract and ipriflavone would be tested in the same manner.

However, we can conclude that the water extracts of ginkgo biloba (GB) and berberry can be used by human to alleviate the increased activity of AChE during the suffering from dementia because they already used as nutriceutics from thousands of years in traditional Eastern medicine and in China, in addition there are many drugs in the Egyptian market contains GB and berberry as a powder or as an extract such as PURGB21 (GRANDMAS REMEDIES, Co. USA) and Berberis Formula (Innovative medicine, Co. USA) (6-8, 11).

On the other hand, ipriflavone could be accounted as non-competitive inhibitor to AChE, where it caused three fold decrease in the V_{max} value and did not alter the K_m one. The inhibitory effect of ipriflavone on AChE activity may be due to its similarity in structure to Aricept®. Interestingly, the inhibitory per cent of ipriflavone nearly equal to that of Aricept® where each of them inhibited AChE activity by about 70% (Fig. 3). It is evident from the present results that the strongest inhibitory activities were exhibited by ipriflavone. Moreover,

Table 1
Summary of Purification of Acetylcholinesterase from the Brain of *Egyptian Mediterranean buffalo* (*Bos Buballus*)

Steps	*Activity (mol/min)*	*Protein (mg/ml)*	*Volume (ml)*	*Total activity mol/min*	*Total protein concentration (mg)*	*Specific activity mol/min/mg*	*Yield %*	*Purification Fold*
Crude	0.053	0.59	650	34.45	389	0.09	100	1
$(NH_4)_2SO_3$ Precipitation	0.65	0.826	20	13	16.52	0.79	38	8.7
Sephadex G25	2.02	0.529	5	10.1	2.65	3.81	29	42.3
Sephadex G100	0.834	0.09	5	4.17	0.45	9.3	12	103.3
DEAE- cellulose	0.308	0.017	12	3.7	0.204	18.14	11	201.6

Results represented as an average of two independent runs.
Yield= total activity of each step/ total activity of the first step (crude) X100.
Fold= specific activity of each step/ specific activity of the first step.
Specific activity= total activity / total protein at each step.

Table 2
Kinetics Parameters in Absence and Presences of Inhibitors

Inhibitors	$K_m * 10^{-3}$ *(M)*	$V_{max} * 10^{-3}$ *(mol/min/mg protein)*	$Ki * 10^{-3}$ *(mM)*
Control	33 ± 1.2	50 ± 0.0	–
Ginkgo biloba	200 ± 3.2	50 ± 0.3	
Berberry extract	100 ± 1.5	50 ± 0.1	
Ipriflavone	33 ± 1.7	16 ± 0.4	1.68
Aricept	227 ± 10.1	16 ± 0.5	0.84

Partially purified AChE from brain tissue prepared by the procedure of Rao and Dasgupta, 1991. Kinetic measurements were carried out on purified form in absences and presences of individual (Ginkgo biloba, berberine, ipriflavone and Aricept) at a concentration of 1 mg/ml. The data were analyzed by Lineweaver-Burk plot and the values of Km and Vmax were averaged. The results are given as means ± SEM.

potential applications in human treatment have must be applied to evaluate its effects.

However, the precise compounds for the AChE inhibitory activity are still of interest. As these herbal extracts are considered to be more safe than Aricept® and other drugs that increase cholinergic activity. Therefore, it is of paramount importance to find novel and more potent AChE inhibitors from plant extracts or synthetic derivatives.

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