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**Experimental Biomedical Research** **Original article**

# Dual effect of epigallocatechine gallate on the pathology of Alzheimer's: Cholinesterases and amyloidogenesis

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## ABSTRACT

**Aim:** To evaluate the dual inhibitory effect of epigallocatechin gallate on Alzheimer's pathogenesis.
**Methods:** Cholinesterase inhibition studies were performed by kinetic Ellman methods and also the inhibitory effect of molecule on amyloid fibrillation is determined by dye binding thioflavin T method.
**Results**: Epigallocatechin gallate inhibited both types of cholinesterases and amyloid fibrillation. The inhibition of acetylcholinesterase indicated an uncompetitive inhibition whereas butyrylcholinesterase inhibition had an unique pattern. Compound inhibited butyrylcholinesterase with two types of inhibition in a dose related manner. Fibrillation destabilization is also observed by the compound.
**Conclusion**: Our results indicated that epigallocatechin gallate may be accepted as a candidate molecule as the dual effective drugs for neurodegenerative diseases.

**Key words:** Cholinesterase, amyloid, epigallocatechin gallate, Alzheimer's disease.

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## Introduction

Catechins are polyphenols that belong to plant-derived flavonoid family. Antioxidant effects are the well-known effects and their prooxidant behavior is also reported depending on the dose and route of administration [1,2]. Dietary sources, tea and especially green tea is a fundamental source of catechins for human. The major active component of the polyphenolic content of green tea is epigallocatechin gallate (EGCG). Besides antioxidant effects, some other beneficial roles of EGCG are reported as antiatherogenic, anticarcinogenic, antiapoptotic and its importance in neurodegenerative diseases is widely emphasized in experimental *in vivo* studies [3,4]. Many studies of human clinical trials showed to have an augmented healing effect on the cognitive dysfunction by the use of flavanol compounds.

Alzheimer's disease (AD) is a deteriorative neurological disorder characterized by loss of memory and mental functions, behavioral disorders and also impairment of cholinergic neurotransmission. Although the etiology of AD is not clarified definitely yet, there are some well accepted features about the basis of disease. Aggregation of amyloid peptides is reported as the most common reason in the onset and progression. Amyloid peptides are the proteolytic cleavage products of amyloid precursor protein (APP). Although this cleavage is a physiological process, the activity of specific enzymes results with the formation

of fragments (amyloid beta 1-40, amyloid beta 1-42) that form toxic aggregates (amyloid plaques) in the brain. The inhibition of aggregation is accepted as the main strategy for the treatment of the disease and there are several *in vitro* reports dealing with this approach [5-9].

Another well-known fact about the pathology and progress of the AD is the attenuation of cholinergic response. Whether it is caused by the apoptosis of cholinergic neurons or the decreased activity of acetylcholinesterase; it is mainly reported that the diminished acetylcholine levels has an important role in the severe symptoms of AD. Cholinesterase inhibitors are widely used medications to overcome this problem on the symptomatic treatment of the disease for an improved quality of lifetime. Cholinesterases are the serine hydrolases responsible for the cholinergic metabolism. Acetylcholinesterase (AChE; E.C.3.1.1.7) and butyrylcholinesterase (BChE; E.C.3.1.1.8) may use acetylcholine as the substrate with different affinities and both known to have roles in the maintanance of acetylcholine levels. Besides the enzymatic activity, many in vitro studies report another function of AChE in the molecular pathology of AD. Experiments show that, the peripheral anionic site (PAS) of the AChE interacts with amyloid peptides behaving as a core for promoting fibril aggregation [10].

Upon these observations experiments mainly focus on the generation and development of the dual effective cholinesterase inhibitors that target both the enzymatic activity and self-fibrillation [11,12].

There are several reports dealing with the inhibitory effects of flavonoids on the cholinesterases [13]. Our study is aimed to determine the inhibitory parameters of EGCG on cholinesterases and to evaluate the possible destabilization of amyloidogenesis within the determined pharmacological concentrations obtained by kinetic analysis. Based on our hypothesis EGCG may be nominated as a member of dual effective drugs family for Alzheimer's treatment.

## Materials and methods

### *Chemicals*

Human recombinant AChE, Equine serum BChE, amyloid peptides 1-40/1-42, epigallocatechin-3-gallate (EGCG), acetylthiocholineiodide (ATCh), butyrylthiocholineiodide (BTCh) and Thioflavin T (ThT) were purchased from Sigma–Aldrich (MO, USA). All biochemical studies were performed in triplicate and data were expressed as mean ± SEM.

### *Enzymatic Analysis*

Enzyme activities (AChE and BChE) were assayed by the Ellman Method with/without various concentrations of inhibitor (EGCG). The tubes having zero concentrations of EGCG are used as the control. The activity medium was containing 100 mM MOPS pH 8.0, 0.05–0.5 mM ATCh or BTCh as the substrate, 0.125 mM dithiobisnitrobenzoic acid (DTNB) and inhibitor. Reactions were initiated by the addition of enzyme(s) (0.02 U/mL) and the absorbance change at 412 nm was monitored spectrophotometrically against the sample blank (Shimadzu-1601, Japan) at 25 °C [14].

### *Amyloid peptide fibrillation*

The inhibitory effect of EGCG on the amyloid aggregation is tested by Thioflavin T fluorescence method. ThT is a dye giving a characteristic fluorescence upon binding to peptides/polypeptides and also aggregates [15]. Due to characteristics of the binding, the

increase in the fluorescence with respect to control, is accepted as the increase of fibrillation (aggregation). Commercially available peptides were dissolved in 100 mM potassium phosphate buffer pH 7.2 and incubated with/without 50 µM of EGCG for 24 hours. The flouresence intensities were recorded and expressed as arbitrary unit (A.U.) at the time intervals given on the figure. ThT fluorescences were obtained using 8 µM ThT in 100 mM sodium phosphate buffer, pH 7.4, by spectrofluorometer (Shimadzu RF-5301, Japan) at wavelengths Ex: 442 and Em: 482. Rifampicin was used as the standard for fibril destabilization [16].

## Results

### *Kinetic Analysis*

In order to estimate the probable inhibitory effect of EGCG on cholinesterase activities and perform further kinetic evaluation, $IC_{50}$ values were determined as 55 ± 1,3 µM for AChE and 63 ± 1,1 µM for BChE. Inhibitor types and kinetic properties for both enzymes were determined by Lineweaver-Burk plots with secondary reciprocals. As shown in Figure 1 the inhibition of AChE by EGCG was uncompetitive (Figure 1).

EGCG inhibited BChE in a dose related manner. The inhibition type was uncompetitive at low inhibitor concentrations (25,50 µM)

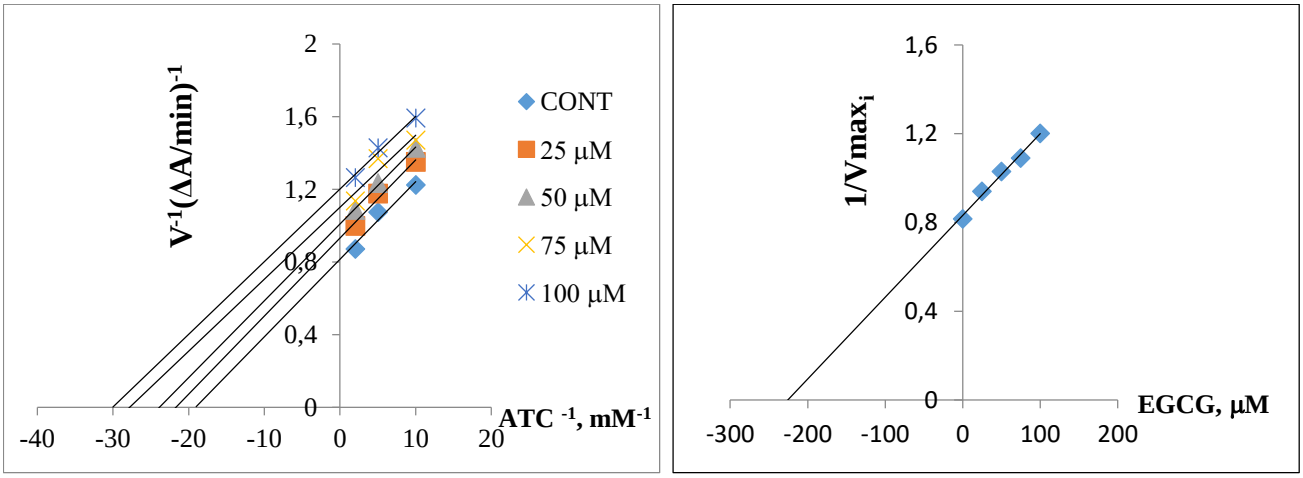


**Figure 1**. The Lineweaver-Burke (A) and reciprocal (B) plot of AChE inhibition by EGCG. Each point is the average of three determinations.

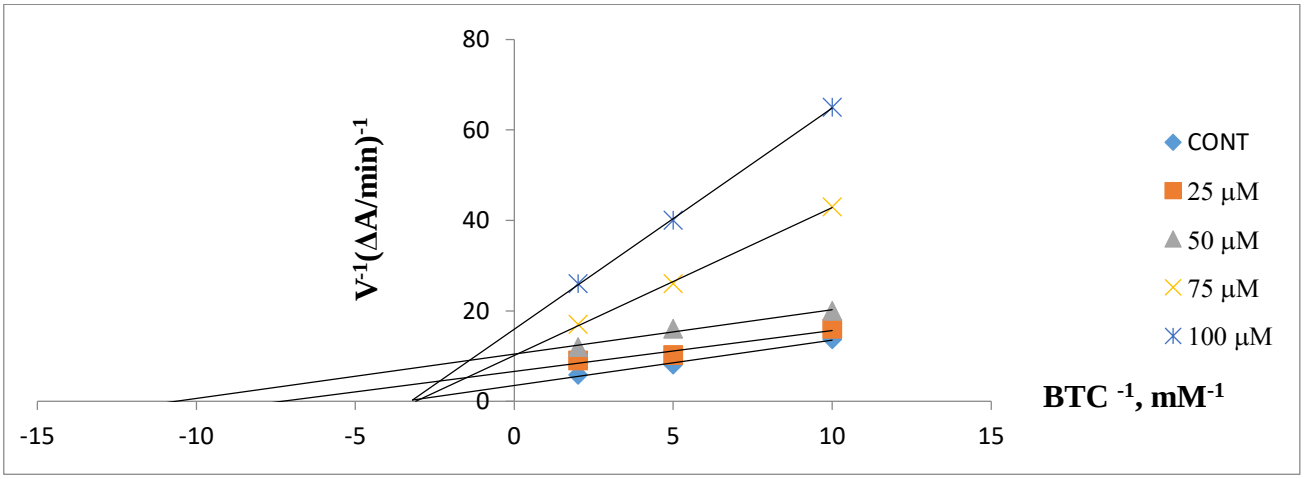


**Figure 2**. The Lineweaver-Burke plot of BChE inhibition by EGCG. Each point is the average of three determinations.

whereas at high inhibitor concentrations (75,100 µM) inhibition was noncompetitive (Figure 2). The reciprocal plots for each are given respectively in Figure 3 A and B.

The types of inhibitions and inhibitor-enzyme dissociation constant ($K_i$) values were given in Table 1.

***Amyloid Destabilization***

The inhibitory effect of EGCG on three forms of fibrillation (1-40-140, 1-42-1-42 and 1-40-42) was tested with the Tht fluorescence method. As shown in Figure 4 EGCG inhibited the formation of all types of fibril aggregation from the fourth hour of experiment. Rifampicin which is a well-known standard destabilizing agent was used in our experiment for the same purpose [16].

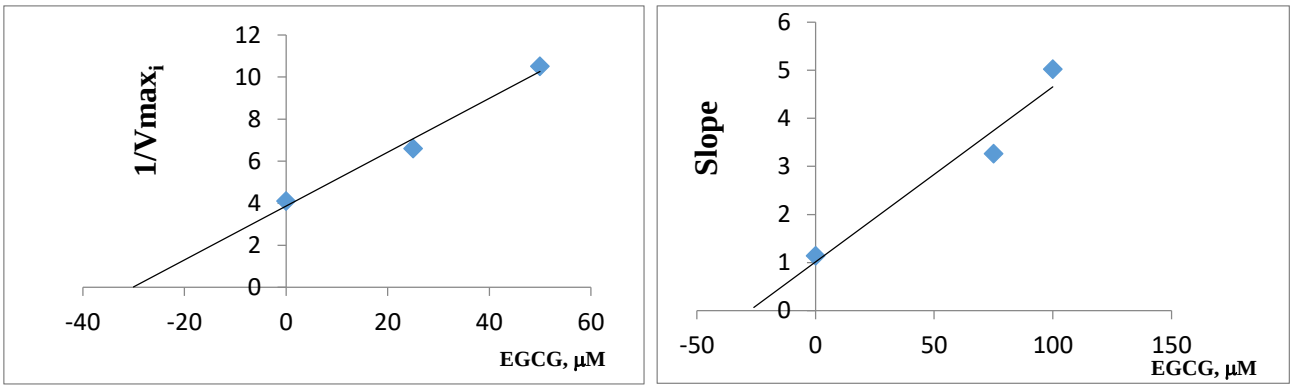


**Figure 3**. The reciprocal plots for (A) uncompetetive (B) noncompetetive inhbition of BChE inhibition by EGCG. Each point is the average of three determinations.

**Table 1.** The kinetic data of inhibitory parameters of EGCG on cholinesterases. Values are expressed as mean ± SEM.

| Enzyme | Inhibition type | Ki (µM) |
|---|---|---|
| **AChE** | Uncompetitive | 222.3 ±1.25 |
| **BChE**<br>**25-50 µM EGCG**<br>**75-100 µM EGCG** | <br>Uncompetitive<br>Noncompetitive | <br>28.1 ±0.33<br>32.3 ± 0.46 |

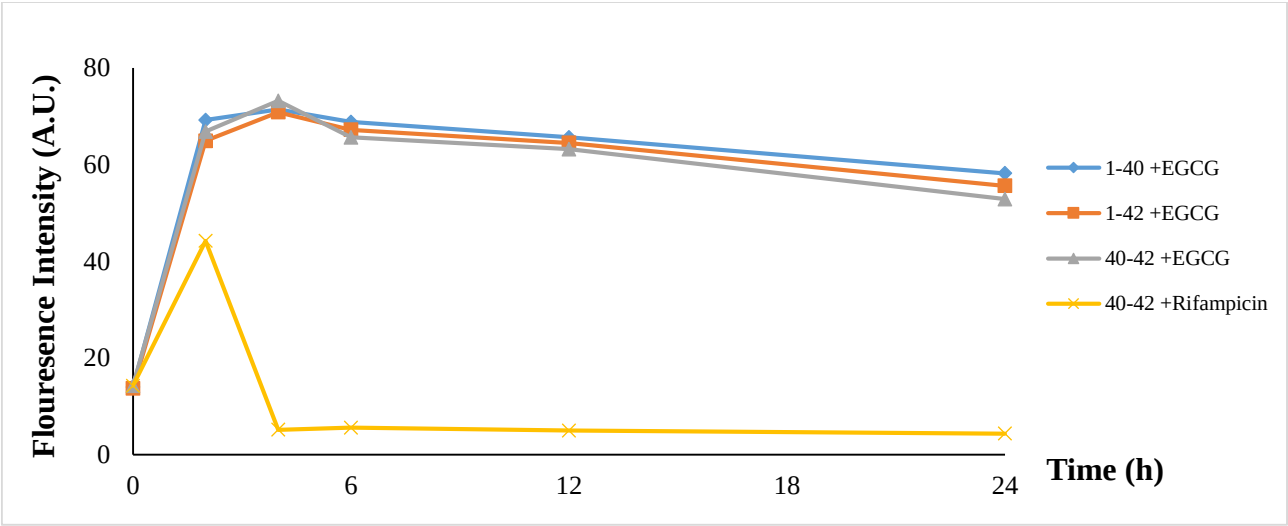


**Figure 4**. Inhibitory effect of EGCG on amyloid peptide aggregation. Each point is the average of three determinations.

## Discussion

In our study EGCG is found to inhibit both types of cholinesterases and also amyloid aggregation. Although the calculated $IC_{50}$'s were not promising when compared to the values of donepezil (23.1 nM for human recombinant AChE and and 7.4 nM for BChE) the discovery of dual inhibiory effect was satisfactory [17].

The plots indicated an uncompetetive inhibition of AChE and a dose related inhibition pattern for BChE.

Due to its physiological importance, AChE is one of the most investigated enzymes for the *in vivo* and *in vitro* effects of herbal medicines. There are several reports indicating the roles of ginsenosides [18], flavanoids [19], and other types of plant-based sources on the activity of cholinesterases.

The active site of cholinesterases (active gorge) has five different regions for proper catalysis. The amino acid content of these sites differ for AChE and BChE and contribute to the differences in catalytic activites and their behavior against the inhibitors. It is mainly known that the gorge of AChE is smaller than the active gorge of BChE by the replacement of 14 aromatic amino acids with the aliphatic ones. The common catalytic site is a catalytic triad having Ser-His-Glu residues [20].

In our model, the uncompetitive inhibition of AChE indicates an interaction of EGCG with a site other than the catalytic triad of the enzyme. The smaller gorge of AChE may block the entrance of EGCG to active site and EGCG is captured by the PAS. The peripheral anionic site (PAS) which is placed on the entry of active gorge has Asp and Tyr amino acids. EGCG is a polyphenolic substance having free hydroxyl residues in its structure. Due to the probable hydrogen bond formation between Asp/Tyr amino acids with these polyhydroxyls an uncompetitive inhibition is caused by EGCG [21].

The inhibition of BChE with EGCG showed an interesting pattern. At concentrations below $IC_{50}$ values the inhibition was uncompetitive whereas noncompetitive inhibition was observed at concentrations greater than 50 µM. These results implied a multi-site interaction of molecule with the enzyme. In order to prove our assumption Hill plot was constructed [22]. By the plot, inhibitor binding sites were calculated as 1.9 and 2.4 at low and high concentrations respectively having an average 2.15. The n values greater than 1 obtained by Hill plot indicates more than one binding sites for inhibitor – enzyme relationship and this data clarified dose related inhibition pattern. Also findings were in accordance with the previous studies dealing with this type of inhibitions [17,23]. This observation is generally reported due to conformational change at the active site of BChE by increasing concentrations of inhibitor. Several reports mention the importance of Leu and Val residues of acyl bonding pocket of BChE for the binding of large molecules [24,25]. We postulate that this change of inhibition type is caused by the binding of EGCG to PAS at low concentrations and the increased concentrations predominating interaction with the second site, acyl pocket.

EGCG also inhibited the amyloid fibril aggregation in a time course manner. Previous studies report [26] the destabilizing effect of EGCG on Ab42 fibrillation but we investigated all types of fibrillation processes. All types of fibril formations was observed to increase by the two hours of experiment and EGCG destabilized the fibrillation from fourth hours. Rifampicin which is a well- accepted fibrillation destabilizer inhibited fibril formation by 90.9% in 24 h and EGCG is inhibited only by 7%. The selected

concentration of EGCG was only 50 µM in order to sustain and mimic $IC_{50}$ levels and we conclude that the fibrillation inhibitions may be repeated in dose dependent manner.

## Conclusion

The dual inhibitory effect on both cholinesterases and amyloidogenesis is a well investigated effect of molecules for the treatment of AD and depending on this approach our findings add a new insight to known beneficial effects of EGCG on neurodegenerative diseases.

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***Conflict of Interest:*** *The authors declare that they have no conflict of interest.*

## Open Access Statement



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