Dual-cholinesterase inhibition by an aqueous extract of *Emblica officinalis* fruit and its mode of inhibition by kinetic study

Chandra Shekhar and Suresh Kumar*

University School of Biotechnology, Guru Gobind Singh Indraprastha University, Sector 16C, Dwarka, New Delhi 110075, India

*Corresponding author; Dr. Suresh Kumar

E-mail ID: sk222ind@yahoo.com

Running Title: Anticholinesterase activity and inhibition kinetics of *Emblica officinalis* extract.

**Abstract**

*Emblica officinalis* (Family: Euphorbiaceae) commonly known as Indian Gooseberry or Amla, is widely used in Ayurveda for its memory enhancing, anti-aging, anti-stress and antioxidant properties. This study evaluated the anti-cholinesterase activity of an aqueous extract from the fruits of *Emblica officinalis* as these are the key enzymes involved in cholinergic transmission. Cholinesterase inhibitors are the class of compounds which inhibit cholinesterase enzyme and are used as drugs for symptomatic treatment of Alzheimer’s disease (AD). The assessment of cholinesterase inhibition was carried out using a colorimetric method based on Ellman’s reaction. An aqueous extracts of *Emblica officinalis* fruit showed concentration dependent acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibition with maximum inhibition of 71.96±0.005% and 61.22±0.001% at 100μg/ml final concentrations with IC$_{50}$ values of 26.84μg/mL and 33.11μg/mL respectively. The kinetic study using Lineweaver-Burk plots of an aqueous extract of *Emblica officinalis* fruit showed mixed mode of inhibition against both AChE and BuChE. This dual anti AChE and BuChE enzymes activity exhibited by an aqueous extracts of *Emblica officinalis* fruit showed potential in therapeutic application that might be beneficial in improving memory and other cognitive functions associated with the cholinergic system and might be used in future for symptomatic treatment of AD.

**Keywords:** *Emblica officinalis*, Acetylcholinesterase, Butyrylcholinesterase, Ellman’s method, Alzheimer’s disease.

**Introduction**

Cholinesterases (ChE) are the group of enzymes that plays very important role in cholinergic neurotransmission. There are two types of ChE enzyme, which are closely related in molecular structure but differ in distribution, substrate specificity, and function, acetylcholinesterase (AChE) (EC3.1.1.7) (true cholinesterase) and butyrylcholinesterase (BuChE) (EC 3.1.1.8) or plasma cholinesterase (pseudocholinesterase). AChE is present at all cholinergic junctions and bound to the basement membrane in the synaptic clefts where it hydrolyses acetylcholine (ACh). AChE is present in the cerebrospinal fluid and cholinergic nerve terminals, where it is thought to regulate free ACh concentration. BuChE is found in the liver, skin, gastrointestinal smooth muscle, kidneys, brain and plasma (plasma cholinesterase).

The physiological function of BuChE is not completely known. The circulating enzyme hydrolyses butyrylcholine (BCh) more rapidly than ACh [1-2]. According to cholinergic hypothesis, blocking the hydrolysis of ACh or BCh induced by ChE enzymes will result in increased concentration of ACh or BCh in the CNS leads to improved cognitive function [3]. ChE inhibitors are acting as drugs that prolong the existence of ACh after it is released from cholinergic nerve endings by inhibiting both AChE and BuChE. Therefore, enhancement of central cholinergic activity by inhibition of AChE or BuChE by using anticholinesterase is considered as a suitable strategy for symptomatic treatment of AD as well as other forms of dementia [4].
AD leads to neurodegeneration over the period of time and characterized by loss of memory, thinking ability, understanding, speech, behavioural changes, mutism, akinesia, ideational and ideomotor apraxia, visuospatial disorientation and sign language aphasial [5-8]. Currently, Federal Drug Administration approved limited drugs for symptomatic treatment of mild-to-moderate AD which includes ChE inhibitors such as tacrine, donepezil and rivastigmine. These drugs showed associated with side effects such as liver toxicity, aggression and depression, disturbances related to GI tract, cardiorespiratory, extrapyramidal, genitourinary and musculoskeletal. Weekly blood monitoring and expensiveness further adds limitations of these drugs [9]. All these limitations gives an urgent need to look for new lead compound from nature based products, including plants having potent ChEs inhibitory activities [10].

In the present study, Emblica officinalis fruits were selected to study ChE (AChE and BuChE) inhibition and to explore the mode of inhibition by kinetic studies. Emblica officinalis belongs to euphorbiaceae family. The rational for selecting this plant was that previous study demonstrated several CNS related activities by this plant like antidepressant activity in mice [11], learning and memory enhancing properties in rats [12], beneficial effects on memory deficit rats [13] and cholesterol lowering property [14]. Therefore, Emblica officinalis may prove to be a useful remedy for the management of AD on account of its multifarious beneficial effects.

Materials and Methods

Chemicals and Reagents: Acetylthiocholine iodide (ATChI), butryrythiocholine iodide (BTChI), acetylcholinesterase from electric eel (AChE), butyrylcholinesterase (BuChE) from equine serum, 5, 5-ditiobis [2-nitrobenzoic acid] (DTNB), sodium phosphate dibasic and sodium phosphate monobasic (Sigma Aldrich).

Plant Material: The fruit samples of plant Emblica officinalis (Voucher no. CS/USBT007) were collected from local shop in Delhi and authenticated by Botanist. The specimens of this plant sample are stored in a herbarium at University School of Biotechnology, Guru Gobind Singh Indraprastha University, Delhi, India.

Equipments and instruments: 96-well plate (Corning Inc. NY), eppendorf tubes, centrifuge tubes, tips (Tarsons products Ltd. India), eppendorf tube stand, pipettes (Biomate), weighing balance, aluminium foil, tissue paper, ice box, blotting paper, vortex machine (REMI), magnetic stirrer (REMI), spatula, muslin cloth, spectrofluorometer (Spectra Max), centrifuge machine (Sigma) and lyophilizer (Thermo scientific Heto Freeze Dryer).

Preparation of plant extract: The fresh sample of plant material was air dried at room temperature and powdered using electric grinder. 2 gm of sample was weighed and extracted with 40 ml of distilled water (1:20 w/v). The sample was filtered using muslin cloth, which was then freeze dried in lyophilizer. Finally, the sample was collected and kept in -20°C. Percentage yield of sample extract was 11.6% (this value is based on one batch extraction).

The percentage yield of the extract was calculated using the formula below:

\[
\text{% yield} = \frac{\text{weight of the extract}}{\text{weight of plant material}} \times 100
\]

Cholinesterase inhibitory assay: Dual anticholinesterase activity (AChE and BuChE inhibition) was determined by the spectrophotometer using the Ellman’s method with slight modifications given in other papers [15-16]. The assessments of ChE inhibition were carried out in flat-bottom 96- well microtitre plates using the colorimetric method. A typical run consisted of 5μl of AChE/BuChE solution, at final assay concentration of 0.08 U/ml; 200μl of 0.1 M phosphate buffer pH 8; 5μl of DTNB at a final concentration of 0.5mM prepared in 0.1 M phosphate buffer pH 7 containing 0.12 M of sodium bicarbonate; and 5μl of the test extract (Emblica officinalis fruit). The final assay concentration used for an aqueous extract of the plant material was 100μg/ml. The reactants were mixed and pre-incubated for 15 min at 30°C. The reaction was initiated by adding 5μl of ATChI/BTChI at a final concentration of 0.5mM. As a control the inhibitor solution was replaced with buffer. The control was assayed in triplicate. To monitor any non- enzymatic hydrolysis in the reaction mixture two blanks for each run were prepared in triplicate. One blank consisted of buffer replacing enzyme and a second blank had buffer replacing substrate. Change in absorbance at 412 nm was measured on spectrophotometer, 96 well plate reader for a period of 2 min at 25°C. The reaction involved in this is enzyme hydrolyses the substrate ATChI/BTChI resulting in the product thiocholine which reacts with Ellman’s reagent (DTNB) to produce 2-nitrobenzoate-5-mercaptotiocholine and 5-thio-2-nitrobenzoate which can be detected. Mean absorbance per minute values were calculated and blank well values were subtracted from the corresponding sample mean. Percentage inhibition was calculated using the equation:

\[
\text{Percentage inhibition} = \frac{(\text{Control} - \text{Extract}) \times 100}{\text{Control}}
\]
Dose response curve and inhibition kinetic studies: The values of percentage inhibition of enzymes from Ellman's assay were plotted against the concentration range (6.25 to 100μg/mL, final assay concentration) of an aqueous extract of *Emblica officinalis*. The concentration of extract that inhibited the hydrolysis of substrates by 50% (i.e. IC\textsubscript{50} value) was determined by monitoring the effect of various concentrations. Each concentration was run equivalent to n = 3. The concentration-response curves were fitted to the data points using Microsoft Excel software and IC\textsubscript{50} value was calculated from the standard curve equations. For kinetic studies, the enzyme was pre-incubated with different substrate concentrations (0.0625 to 0.5mM ATChI/BTChI). Lineweaver-Burk (LB) plots [17] were then plotted reciprocal of initial enzyme velocity against reciprocal of substrate (ATChI/BTChI) concentrations in the presence and absence (control) of different concentrations of an aqueous extract of *Emblica officinalis* fruit.

Statistical analyses: The results are expressed as the mean ± SEM. Microsoft Excel software was used to plot and calculate the equation of the curve.

Results

The results showed that an aqueous extract of *Emblica officinalis* fruit showed concentration dependent inhibition against both AChE and BuChE enzymes. The maximum inhibition of 71.96%±0.005 SEM and 61.22%±0.001SEM was observed for AChE and BuChE respectively at final assay concentration of 100μg/mL. The IC\textsubscript{50} values calculated from the equation obtained from the concentration versus percentage inhibition curve were 26.84μg/mL and 33.11μg/mL for AChE and BuChE respectively (Figure 1A and 1B). The potency of inhibition by an aqueous extract of *Emblica officinalis* is more for AChE compared with BuChE.

The modes of enzyme inhibition were derived from the LB plots between the reciprocal of substrate concentration on x-axis and reciprocal of velocity on y-axis [17-19]. The LB plot of an aqueous extract of *Emblica officinalis* fruit showed mixed inhibition kinetics against both AChE and BuChE as the intersection of lines occurred neither on x-axis or y-axis but in between the axis in third and second quadrants in cases of AChE and BuChE respectively (Figure 2A and 2B). This mechanism of inhibition observed might be because of presence of different phytocattributes present in an aqueous extract of *Emblica officinalis*.

![Figure 1A](image1.png)  
Fig 1A: Percentage inhibition of AChE activity of different concentration of an aqueous extract of *Emblica officinalis* fruit. [The equation of the line is $y = 18.537 \ln(x) - 11.077$; $R^2 = 0.985$]. Values are expressed as mean ±SEM (n=3).

![Figure 1B](image2.png)  
Fig 1B: Percentage inhibition of BuChE activity of different concentration of an aqueous extract of *Emblica officinalis* fruit. [The equation of the line is $y = 10.462 \ln(x) + 13.523$; $R^2 = 0.9653$]. Values are expressed as mean ±SEM (n=3).
Fig 2A: Lineweaver-Burk plot representing the reciprocal of initial enzyme velocity versus the reciprocal of acetylthiocholine iodide (ATChI) concentration in the presence and absence (control) of different concentrations of an aqueous extract of *Emblica officinalis* fruit.

Fig 2B: Lineweaver-Burk plot representing the reciprocal of initial enzyme velocity versus the reciprocal of butyrylthiocholine iodide (BTChI) concentration in the presence and absence (control) of different concentrations of an aqueous extract of *Emblica officinalis* fruit.

**Discussion and Conclusion**

Anticholinesterases are the drugs that prolong the action of ACh after it is released from cholinergic nerve endings by inhibiting both AChE and BuChE enzymes or by enhancing the concentration of ACh or by increasing residence time in the synapse and can slow the degenerative process. The current therapeutic approach for treatment of mild to moderate AD includes drugs such as donepezil, tacrine and rivastigmine to improve cognitive function. The limitations associated with the use of above mentioned drugs are their side effects such as diarrhoea, nausea, vomiting, fatigue, insomnia, muscles cramps, loss of appetite and hepatotoxicity. In view of these drawbacks, a number of plants or plant derived compounds showing anticholinesterase properties are explored which can used as new therapeutic option in symptomatic treatment of AD [20].

The current study demonstrated that aqueous extracts of *Emblica officinalis* fruits inhibited AChE and BuChE in a concentration dependent manner. As per the previous study, methanolic extract of *Emblica officinalis* has already demonstrated anticholinesterase properties [21]. The present study is complementary to the previous results, however an aqueous extract of *Emblica officinalis* fruits, used in this study, found potent enough in AChE inhibitory activity by 71.96±0.005%, (IC₅₀ 26.84μg/ml). The phytoconstituents present in the aqueous extract might be different from that of methanolic extract which needs further study. The present study for the first time demonstrated that an aqueous extract of *Emblica officinalis* possess anti-BuChE properties with 61.22±0.001% inhibition. The IC₅₀ value calculated from the inhibition curve was 33.11μg/ml. Inhibition of AChE appears to be much more potent than BuChE by an aqueous extract of *Emblica officinalis*.

The present results also demonstrated the mechanism of ChE inhibition by kinetic study using LB plot for the first time. The results demonstrated mixed type inhibition kinetics for both the enzymes. This mixed mode of inhibition revealed that an aqueous extract of *Emblica officinalis* (inhibitor) might bind to the enzyme AChE/BuChE whether or not the enzyme has already bound to the substrate ATChI/BTChI but has greater affinity for one state or other. This mixed type inhibition kinetics is typical of some medicinal plants, due to the great variety of compounds they contain all acting in different ways [22].

Previous reports suggests that an enzyme AChE role in the development of neuro-toxic senile plaques by accelerating Aβ deposition and aggregation. It has been shown that AChE forms a stable complex with senile plaque components through its peripheral anionic site which might be involved in accelerating Aβ fibril formation [23]. It has also been suggested that mixed or non-competitive type inhibitors have been put forward as model candidates for inhibiting AChE-induced Aβ aggregation due to their ability to bind to the peripheral anionic site [24]. Other studies also suggested that the Aβ aggregating property of AChE during the early stages of AD can be inhibited by mixed or non-competitive type of inhibitors [25]. The AChE inhibition kinetics in the present study indicates a putative mechanism by which the aqueous extract may have a novel therapeutic
potential for AD, as these compounds might be able to ameliorate cognitive deficiency by inhibiting Aβ aggregation. One of the main benefits of using natural product such as *Emblica officinalis* is the wide range of medicinal properties reported for central nervous system related activities. For example, hydroalcoholic extract of *Emblica officinalis* is known to protect against kainic acid induced status epilepticus (seizures), cognitive decline and oxidative stress in rats which shows anti-inflammatory, antioxidant and neuroprotective activities of this plant [26].

Hydroalcoholic extract of *Emblica officinalis* Gaertn is known to protect against PTZ-induced seizures in rats [27]. *Emblica officinalis* also showed their effects on cholinergic functions in scopolamine induced amnesia in mice [28]. It can therefore be concluded that *Emblica officinalis*, in addition to possessing memory enhancing with antiaging, antistress and antioxidant agents, also exhibit anti-AChE/BuChE properties and thus could potentially provide novel leads for alleviating the symptoms associated with AD.

**Conflict of interest**

The authors declare that there is no conflict of interest to reveal.

**References**


