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In-vitro cholinesterase inhibitory activity of dry fruit extract of *Phyllanthus emblica* relevant to the treatment of Alzheimer's disease

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Abstract

Alzheimer's disease (AD), a common type of progressive neurodegenerative disease, is characterized by low level of neurotransmitter (acetylcholine), oxidative stress and neuro-inflammation in brain stream. Effective treatment strategies rely mostly on either enhancing the cholinergic function of the brain by stimulating the cholinergic receptors, improve the level of acetylcholine from being a breakdown by cholinesterase enzymes or induce antioxidant therapy and anti-inflammatory agents. *Phyllanthus emblica* fruits are well known for its antioxidant activities with a rich source of vitamin C and polyphenols. A crude methyl extract (CME) of dry fruit of *P. emblica* evaluated for acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitory activity by Ellman's method and were found active in inhibiting AChE and BuChE with IC_{50} of 53.88 µg/ml and 65.12 µg/ml respectively, which strongly implies that that the CME of *P. emblica* is a rich source of AChE and BuChE inhibitors.

Keywords: Cholinesterase inhibitor, *Phyllanthus emblica*, Alzheimer's disease, Acetylcholinesterase inhibitor, Antioxidant, Butyrylcholinesterase inhibitor.

Introduction

The term neurodegeneration is applied in such conditions when the chronic breakdown of neuron occurs. [1] AD is a progressive neurodegenerative disease that primarily affects the older people with more than 65 years of age. [1] This disease is characterized by decline of memory and cognition. Some Pathological features are identified in AD like β amyloid plaques, neurofibrillary tangles (NFT), inflammation in the CNS and disturbances in the neurotransmitter distribution process. [3] Beside this, neurons located on the basal fore brain and hippocampi are mainly damaged in AD as a result huge cholinergic loss occurs. [4] This impairment cause irreversible damage in cognitive function, loss of memory, neurological and neuropsychiatric diseases. [5]

AD got a complex pathophysiology which involves different biochemical pathways. Acetylcholine (ACh) is the main neurotransmitter that is defected on AD. This ACh plays an important role in learning things and in memory function.[6] ACh is generally stored and released from the nerve terminals. In AD patients' large amount of ACHE and BuChE released in the system that makes the working half-life of ACh is too short. AChE enzyme hydrolyzes ester bond of the ACh molecule to make it choline and ester. BuChE enzyme is prominent in glial cells, which have almost same activity on ACh.[7, 8]

In AD patient's oxidative stress, increased enormously due to increased trace metal in the brain like Fe, Al or Hg or decreased polyunsaturated fatty acids in brain.[9, 10] This causes increased production of reactive oxygen species in brain tissue. Imbalance between production and removal of free radicals also found here. In this case antioxidants are suggested to the patients.

As ACh deficiency is the main hallmark in AD patients, enhancing ACh level is an essential strategy for treatment. Several strategies are applied to boost up ACh level in the brain, like, using the precursor of ACh, using agonist of ACh receptors and using cholinesterase inhibitors.[11-13] Prolonging the availability and activity of ACh is most effective among all. This is done by inhibiting AChE and BuChE. FDA approved several cholinesterase inhibitors (e.g. Donepezil, Galantamine, Rivastigmine) for the treatment of AD.[14, 15] But some of these drugs reported side effects like hepatotoxicity, narrow therapeutic index, short duration of action and low bioavailability.[16]

Phyllanthus emblica, commonly known as amla or amloki, belong to the family Phyllanthaceae, is a tree widely distributed throughout the Indian subcontinent.[17] In traditional Indian medicine dried fresh fruit are used for many disease. This fruit is reported to contain high amount of ascorbic acid (Vitamin C)

445mg/100g and other antioxidants like ellagitannins[18] such as emblicanin A, emblicanin B, punigluconin, pedunculagin. It's also rich on punicafolin and phyllanembin and other polyphenols like gallic acid, ellagic acid, flavonoids and kaempferol. [19, 20]

A preliminary study has established that, P. emblica shows antioxidant[20], immunomodulatory[21] and anticancer[22], anti-inflammatory[23], anti-bacterial activity and others. Although *P. emblica* has important medicinal values for the treatment of AD as it is a rich source of antioxidant and polyphenols, no studies have been yet examined its cholinergic activities. Therefore, objective of this study to evaluate the inhibitory activities of AChE and BuChE enzyme of *P. emblica* fruits in order to treat AD.

Materials and Methods

Chemicals

Tris-HCl, Triton-X, Acetylthiocholine Iodide (ATCI), Butyrylthiocholine Iodide (BTCI), Donepezil, Galantamine and 5,5'-dithio-(2-bisnitro)-Benzoic Acid (DTNB) were obtained from Osaka, Japan. Chemicals are used for experiment were highly specified and were analytical grade.

Plant materials

Preparation of fruit extract

Fruits of *P. emblica* are collected from the city of Rajshahi, Bangladesh and identified by expert taxonomist. Fresh fruits are washed thoroughly to remove foreign materials like dust and others. The cleaned fruits are cut into small pieces and dried under shade at room temperature. Dried fruits are then grinded to powder (200 g). Powder was placed into an amber coated bottle soaked with 500 ml of methanol for 7 days. The whole mixture was filtered through cotton, then with Whatman No. 1 filter paper. Finally filtrate was concentrated through a rotary evaporator under reduced pressure at 50^0C to obtain the crude methanol extract (CME) (5.08 g). This CME undergoes the experiment.

Determination of AChE Inhibitory activities

The AChE inhibitory activity was preformed according to the colorimetric method of Ellman's method using acetylcholine iodide as a substrate.[24] For enzyme source, bovine brain were homonized with 10 volumes of extraction buffer [50mM Tris-HCl (pH 7.4) which contain 50M NaCl, 50mM $MgCl_2$ and 1 % Triton X-100] and centrifuged at 10000 rpm for 30 minute. The resulting supernatant collected and treated with super saturated ammonium sulfate solution, left for few hours at 4^oC to complete precipitation of proteins and again centrifuged at 12000 rpm for 20 minutes. The resulting precipitation was solubilized with extraction buffer. This solution used as an enzyme source.

AChE inhibitory assay was carried out by modified Ellman's method. For positive control 200µl enzyme solution, fruit extract and extraction buffer incubated for 2 hours at room temperature. 200µl DTNB and 400µl ATCI added to the mixture, incubated for 37^0C for 30 min. Absorbance taken at 405nm. For negative control almost the same procedure applied. Fruit extract, enzyme 200µl and hominization buffer incubated at room temperature for 2 hours. After that, only 400µl ATCI and 200µl extraction buffer, incubated it at 37^0C for 30 min. Absorbance was taken at 412nm. The rates of hydrolyzing AChE were monitored spectrophotometrically. The blank reaction was measured by substituting saline without Ellmans reaction mixture. Donepezil used as a standard. Percentage of inhibition was calculated by,

$$\%\ of\ inhibition\ of\ AChE = \frac{Positive\ Control - Negative\ sample}{Positive\ control} \times 100$$

Determination of BuChE inhibitory activity

The Ellmans reaction for determining BuChE was performed here. Human blood collected and centrifuged at 4000 rpm for 5 minutes, resulting supernatants was used as an enzyme source. Extraction steps are carried out at 4^0C. For positive control about 50 ml of enzyme solution, plant extract/standard and extraction buffer was incubated at 37^0C for 2 hours. 200µl DTNB and 400µl BTCI added to the mixture, incubated for 37^0C for 30 min and absorbance taken. For negative control BTCI is replaced with extraction buffer. The rate of hydrolysis of BuChE was monitored spectrophotometrically. Absorbance was taken at 412nm.From the difference from the positive data and Butyrylcholine iodide negative data activity of extract measured with the use of the same formula. Galantamine was used as a standard.

$$\%\ of\ inhibition\ of\ BuChE = \frac{Positive\ Control - Negative\ sample}{Positive\ control} \times 100$$

Statistical analysis

All analyses were performed at least 3 times. Data were given as Mean ±SD. Microsoft excel 2007 (Roselle, II, USA) were used for the statistical and graphical evaluation.

Results

Extract (CME) of *P. emblica* shows AChE inhibitory activity. Reduction of ACh in the CNS is a major characteristic of AD. Therefore, inhibition of AChE and BuChE level is an important strategy to treat AD. The inhibitory activity of *P. emblica* was determined by Ellman's method. This method based on the reaction between acetylthiocholine iodide or butyrylthiocholine iodide, DINB, fruit extract and the enzyme solution. The enzymatic activity measured spectrophotomatrically, when yellow color complex produced by the decline by reaction of DINB ion. The result was given in Table 1, Figure 1 and in Figure 2. CME was found to inhibit 62.18% of AChE and 59.17% BUChE at a concentration of 100 ug/mL, which indicated it was a potential source of cholinergic inhibitor.

Table 1: AChE and BuChE inhibitory activity of CME fractions of *P. emblica* fruits

Concentration (µg/mL)	% AChE Inhibition		% BuChE inhibition	
	P. emblica	Donepezil (Std.)	*P. emblica*	Galantamine
25	32.17 ± 1.08	84.02 ± 1.19	29.29 ± 2.02	80.34 ± 2.07
50	48.43 ± 2.43	91.29 ± 1.72	44.18 ± 1.76	88.06 ± 1.73
100	62.18 ± 1.61	92.57 ± 1.56	59.17 ± 1.92	90.33 ± 1.08
200	71.52 ± 1.39	94.73 ± 1.82	70.20 ± 2.21	92.47 ± 1.62

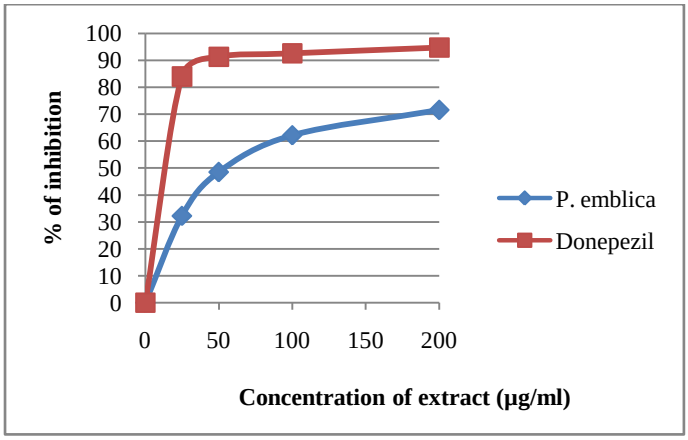

Figure 1: AChE inhibitory activity of CME of *P. Emblica* fruit extract compared with the standard

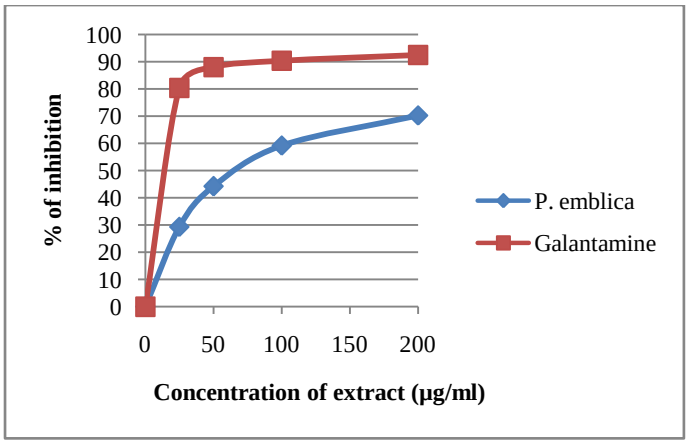

Figure 2: BuChE inhibitory activity of CME of *P. Emblica* fruit extract compared with the standard

Discussion

Inhibition of AChE and BUChE is considered one of the most important strategies to treat AD patients. Therefore, it has been suggested that either plant or plant derived compound that inhibits AChE and BUChE would have been effective for developing newer drugs. CME of *P. emblica* fruit shows its potency in inhibiting both AChE and BuChE with with IC_{50} of 53.88 µg/ml and 65.12 µg/ml respectively.

P. emblica has been reported to possess antioxidant and antiinflammatory properties.[22, 23] Our finding indicates that it also provides AChE inhibitory and BuChE inhibitory properties. In AD treatment strategy, inhibition of AChE and BuChE is well accepted. In vitro study indicated that CME of *P. emblica* inhibit bovine brain AChE and human blood plasma BuChE.

Conclusion

The result from the total experiment suggests that fruit extract of CME of *P. emblica* potentially inhibit both AChE and BUChE. As *P. emblica* also provides antioxidant and anti-inflammatory activities, this fruit can be used for the treatment of AD.

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