Assessment of in-vitro cholinesterase inhibitory and thrombolytic potential of bark and seed extracts of Tamarindus indica (L.) relevant to the treatment of Alzheimer’s disease and clotting disorders

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ABSTRACT

Background: Low level of acetylcholine (ACh) is an important hallmark of Alzheimer’s disease (AD), a common type of progressive neurodegenerative disorder. Effective treatment strategies rely mostly on either enhancing the cholinergic function of the brain by improving the level of ACh from being a breakdown by cholinesterase enzymes. Again atherothrombosis is major life-threatening cerebral diseases. Traditionally Tamarindus indica (L.) has widely known for its medicinal values. Our aim is to investigate the cholinesterase inhibitory activities as well as thrombolytic activities of the bark and seeds crude methanolic extracts (CMEs) in the treatment of AD and clotting disorder. Materials and Methods: The crude methanol extract was prepared by cold extraction method and was assessed for acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitory activities by the Ellman’s method. For thrombolytic activity clot lysis method was applied. Results: To compare both the fractions, extracts from the bark got more AChE inhibitory activity than the seed with the inhibitory concentration 50% IC₅₀ values of 268.09 and 287.15 µg/ml, respectively. The inhibitory activity of BuChE was quiet similar to that of AChE as IC₅₀ values of both the fractions were 201.25 and 254.71 µg/ml. Again in-vitro thrombolytic activity of bark was 30.17% and of seed it was 22.53%. Conclusion: The results revealed that the CME of bark and seed both have moderate cholinesterases inhibitory activities as well as thrombolytic activities, worth of further investigations to identify the promising molecule(s) potentially useful in the treatment of AD as well as in clotting disorders.

KEY WORDS: Alzheimer’s disease, cholinesterase inhibition, clotting disorder, Tamarindus indica, thrombolysis

INTRODUCTION

With increasing medical advancements, reduction of birth rate and increase of life expectancy reflect age associated diseases such as neurodegeneration, cardiovascular (CVS) diseases, and other nervous system related disorders [1]. Among the neuron degrading disease, Alzheimer’s disease (AD) is responsible for 60% of all dementia in the people having age more than 65 [2-4]. AD is a progressive neurodegenerative disorder characterize by impairment of learning, cognitive deficits, and behavioral disturbance [5-7].

Pathology of AD is complex and several pathogenic pathways are involved in the progression of this disease. Cholinergic deficit, oxidative stress, formation of neurofibrillary tangles, and deposition of senile plaque hypothesize are the main hallmark of AD [8,9]. Based on these information, several lines of pharmacological treatment have been developed such as cholinergic inhibitors, antioxidant therapy, neurotransmitter replacement, anti-inflammatory therapy, stimulation of muscarinic receptors, prevention of tau aggregation, prevention of tau hyperphosphorylation, increase of alpha-secretase activity, inhibition of beta and gamma secretase inhibitors, and others [10,11].

Among all the pathologic conditions of AD cholinergic deficits remains as one of the main reason of AD. Loss of acetylcholine (ACh) is considered to play a pivotal role in the learning and memory deterioration of AD patients [12-14].
Several strategies are available to improve cholinergic neurotransmission including stimulation of cholinergic receptors or increasing the ability of ACh released in the synaptic cleft by inhibiting acetylcholinesterase (AChE) enzyme [15-17].

Similar to AChE, butyrylcholinesterase (BuChE) enzyme singly or synergistically act on AChE to hydrolyze ACh as well as butyrylcholine (BuCh) in the nerve endings [18]. In the AD brains increased levels of BuChE correlate with the progression of AD. In healthy human brain, the function of BuChE is highly dominated by AChE. In low concentration, BuChE is less effective in hydrolyzing ACh, but the concentration of BuChE increases enormously in Alzheimer’s patient brain which is more capable of hydrolyzing ACh [19-21]. Therefore, inhibition of cholinesterase enzymes and preventing oxidative stress were found as a suitable strategy for AD treatment as well as for other types of neurodegenerative disorders including dementia.

Thrombosis is the process of blood clotting that obstructs the flow of blood through circulatory system. It is the first step of repairing process after injury [22]. However, in several cases, this clotted blood causes harm in the human body by blocking blood flow through an organ or part of the organ, which may lead to stroke, deep brain thrombosis, heart problems, and occlusion of peripheral artery. Beside this thrombosis is a critical stage for arterial disease associated with myocardial infarction and stroke which may be responsible for considerable morbidity and mortality of the patients. Moreover, for cancer patients, venous thrombosis can be the second leading cause of death [23]. To treat these this kind of diseased situation, thrombolytic agents such as tissue plasminogen activator, streptokinase (SK) or urokinase are frequently used [24]. However, these drugs display a huge side effect profile, including high risk of internal hemorrhage and severe anaphylactic reactions. Moreover, various treatments with SK are restricted due to immunogenicity [25].

Plants are the wide source of bioactive principles and medicine and traditional medicine is one of the primary health-care system in many developing countries. *Tamarindus indica* is a rich source of bioactive molecules with numerous uses. Phytochemical investigation carried out on this plant revealed the presence of many active constituents, such as phenolic compounds, glycosides, malic acid, tartaric acid, mucilage, pectin, arabinose, xylose, galactose, glucose, and uronic acid [26-28]. The ethanolic extract of *T. indica* contains fatty acids and various essential electrolytes [28].

The seed and pericarp of *T. indica* are mainly composed of phenolic antioxidant compounds [29]. All extracts of *T. indica* exhibited good antioxidant activity [30]. Fruits of *T. indica* were well known in virtue of their effects on the lipid profile, systolic and diastolic blood pressure and other CVS effects, and the body weight of humans [31]. *T. indica* is often used for the treatment of various skin problems such as cuts, wounds, and abscesses. Bark and leaves are most commonly used either as a decoction or as a powder form in the external surface of the skin [32].

Although *T. indica* has important medicinal value, no studies have yet examined for its cholinesterase inhibitory activities as well as thrombolytic activities. This study discusses the investigation of dried bark and seed extracts of *T. indica* in AChE and BuChE inhibitory potential as well as thrombolytic point of view, relevant to the treatment of AD and blood clotting disorders.

**MATERIALS AND METHODS**

Chemicals that were used in this study are analytical grade. Acetylthiocholine iodide (ATCI), butyrylthiocholine iodide (BTCI), donepezil, galantamine, 5’dithio-bis-(2-nitro) benzoic acid, and SK were purchased from Sigma-Aldrich (Japan). Tris-HCl buffer, sodium chloride, magnesium chloride, and Triton X-100 were collected from Wako Pure Chemical Company Ltd. (Japan). Solvents (methanol, chloroform, etc.) were used in this experiment were analytical grade also.

**Plants Materials and Experiment**

Bark and seed of *T. indica* were collected from Mymensingh, Tangail and Dhaka, Bangladesh in January and February 2016. All plant parts were authenticated by an expert taxonomist. All tests are done in the Laboratory of Pharmacy Department, East West University.

**Preparation of the Sample**

After collecting barks and ripe fruits, these were dried into sun under shade. Seed was peeled out from the ripe fruit. The dried bark was cut into small pieces and ground into finer powder using a powerful grinder. The grinded sample was sieved to get uniform particle size and kept it into air-tight container to prevent it from light and moisture. Seeds were also ground by applying same method.

**Extraction**

Powdered bark (500 g) and grinded seed (200 g) were placed into an amber coated bottle and soaked into 1500 and 1000 ml of methanol, respectively. The contents were sealed into bottle for 10 days with occasionally stirred and shaken. After 10 days, the whole mixtures were filtered by Whitman No. 1 filter papers, and the filtrated solutions were concentrated under reduced pressure, heating below 50°C. Finally, 24.298 and 10.412 g of crude methanolic extracts (CMEs) of bark and of seed was obtained, respectively.

**Determination of AChE Inhibitory Activity [33-36]**

Modified Ellman’s colorimetric method was applied to run in-vitro AChE inhibitory assay and ATCI used as a substrate. For enzyme source, bovine brain was used. To run this method, bovine brain was homogenized in a homogenizer with 10 times of a homogenation buffer (10 Mm Tris-HCl buffer, pH 7.2), which contained 1 M NaCl, 50 mM MgCl, and 1% Triton X-100 and centrifuged for 30 min at 10,000 rpm. The supernatant that was form after centrifugation was treated then with super-saturated ammonium sulfate solution which results floating
precipitation. Collected precipitation was then solubilize into extraction buffer. Solution of this precipitation was used as an enzyme source. Cool temperatures were maintained (4°C) throughout the enzyme excretion procedure. AChE hydrolysis rate was monitored spectrophotometrically. Each extract or standard (various concentrations) was mixed with an enzyme solution (200 µL) (collected from bovine brain) and incubated at 37°C for 30 min. After that Ellman’s reaction mixture (400 µL, 0.35 mM ATCI, 200 µL, 0.7 mM 5, 5’-dithiobiocin (2-nitro benzoic acid)) (DTNB) in an extraction buffer (50 mM Tris.HCl buffer, 50 mM MgCl₂, 50 mM NaCl, 1% Triton X-100, pH 8.0) to adjust it 3 ml of final volume. Absorbance at 412 nm was taken after 30 min incubated this mixture at 37°C. The blank reaction was measured by substituting buffer saline for the enzyme. Donepezil was used as a positive control. AChE inhibitory activity by the extract was calculated using following formula:

\[
\frac{A_{\text{Absorbance of control}} - A_{\text{Absorbance of sample}}}{A_{\text{Absorbance of control}}} \times 100\%
\]

**Determination of BuChE Inhibitory Activity [33-36]**

BuChE inhibitory assay was also performed by modified Ellman’s colorimetric method, where BTCI acts as a substrate. Human blood plasma was used as source of BuChE enzyme. BuChE hydrolysis rate was examined spectrophotometrically. Each extract or standard (various concentrations) was mixed with an enzyme solution (50 µL) and incubated at 37°C for 30 min. After adding Ellman’s reaction mixture (400 µL, 0.35 mM butyrylthiocholine, 200 µL, 0.7 mM DTNB in an extraction buffer (50 mM Tris.HCl buffer, 50 mM MgCl₂, 50 mM NaCl, 1% Triton X-100, pH 8.0) to the above reaction mixture to adjust final volume 3 ml. To verify the result, all reading were repeated 3 times. The blank reaction was measured by substituting extraction buffer for the enzyme. Galantamine was used as a reference standard. BuChE inhibitory activity was calculated using the following formula:

\[
\frac{A_{\text{Absorbance of control}} - A_{\text{Absorbance of sample}}}{A_{\text{Absorbance of control}}} \times 100\%
\]

**Thrombolytic Activity Test [37]**

For thrombolytic activity test human blood was used. Blood was withdrawn from healthy human volunteers having no history of taking oral contraceptive pills or undergoing anticoagulant therapy. 1.0 ml of venous blood from each volunteer was transferred to the previously weighed eppendorf tubes (volume 1.5 ml) and incubated for 45 min at 37°C and was allowed to form clot. Bark and seed extracts (100 mg) were suspended into 10 ml of distilled water. After clot formation, the serum was completely removed from eppendorf tube. Clot was again weighed to determine the clot weight. For each eppendorf tube with the preweighed clot, 100 µl aqueous solution of the crude extract was added separately. 100 µl of SK (30,000 IU) were added to the positive and 100 µl distilled water were added to negative control tubes, respectively. All tubes were then again incubated for 90 min at 37°C to observe clot lysis. After 90 min, the released fluid was removed again and tubes were again weighed to observe the difference in weight. Difference obtained in weight taken before and after clot lysis by the extract, positive control and negative control, was expressed as percentage of clot lysis and the equation is shown below:

\[
\frac{\text{Weight of clot after release of fluid}}{\text{Weight of clot before release of fluid}} \times 100\%
\]

**RESULTS**

**AChE Inhibitory Activity**

Ellman’s colorimetric method was applied to determine the AChE inhibitory activity of the plant extract. This method is based on determining the amount of thiocholine releases when ACh is hydrolyzed by AChE enzyme. DTNB is a coloring agent which binds with the ACh and forms color. In this test, the color indicates the presence of free ACh which was not hydrolyzed by AChE in the reaction mixture. As reduction of AChE in hippocampus and cortex is a remarkable hallmark of AD, inhibition of AChE ensures more ACh in the brain. The inhibitory activities of the different fractions are shown in Table 1. It can be observed that AChE inhibitory activity T. indica was concentration-dependent, because with the increase of the concentration, activity increased. To compare both the fractions, extract from the bark got more enzyme inhibitory activity than the seed sharing inhibitory concentration 50% (IC₅₀) values of 268.09 and 287.15 µg/ml, respectively. Donepezil was used as reference standard.

**BuChE Inhibitory Activity**

BuChE possess the capability of hydrolyzing ACh, as it can act both itself and synergistically with AChE. Modified Ellman’s method was also applied to quantify the BuChE activity of the plants extract. BuChE inhibitory activity of different fractions of T. indica is reported in Table 2. The result truly indicates that both bark and the seed extracts have the ability to inhibit
BuChE. IC_{50} values of both the fractions were 201.25 and 254.71 µg/ml, respectively. Galantamine was used as reference standard.

Thrombolytic Activity Test

100 µl SK as a positive control (30,000 IU) was added to the clots along with 90 min of incubation at 37°C, showed 72.95% clot lysis. Clots when treated with 100 µl distilled water (negative control) showed only negligible clot lysis (3.60%). The in-vitro thrombolytic activity study with T. indica revealed 30.17% clot lysis with bark and 22.53% with the seed. The percentage of weight loss of clot after application of extract solution was the functional indication of thrombolytic activity of the extract. % of clot lysis obtained after treating clots with different concentration of the sample is given in Table 3.

DISCUSSION

Due to medical advancement reduction of birth rate is increasing life expectancy which results an increase of problem in this century [38]. AD is one of the most common among them having no cure at all. However, there are several hypotheses that have been established to suppress the severity of this disease. A number of factors have been identified; among them oxidative stress and cholinergic dysfunctions were found as the major contributor factors of AD [38,39]. In AD patients, the number of degrades time to time, so a neuroprotective approach also might be beneficial to this condition [40-42].

T. indica was used from the very past for various disease condition. It has been renowned for its several medicinal importance. It is a rich source of antioxidant that was previously established. Extract of the T. indica possesses lipid peroxidation, antioxidant enzyme activities, H_{2}O_{2}-induced ROS production, and gene expression patterns in liver HepG2 cells [43,44]. In this study, we evaluate its anti-Alzheimer’s activity along with its thrombolytic activity. Our finding suggested that T. indica not only possesses anti-Alzheimer’s activity but also thrombolytic activity. It is well recognized that inhibition of AChE is one of the most effective strategy.

In this study, we found that inhibition of AChE by both CME was occurred by dose-dependent manner. From Table 1, it is found that AChE inhibitory activity T. indica was concentration-dependent. To compare both the fractions, extract from the bark got more enzyme inhibitory activity than the seed with the IC_{50} of 268.09 and 287.15 µg/ml, respectively. Donepezil was used as positive control, and at the same concentration, it inhibits near about 94% of the enzyme. All the tests were run for at least three times to get linear and reliable results.

Like AChE, BuChE is also important in the breakdown of ACh. It can cleave ACh alone with synergistically with AChE. So preventing this BuChE is also important strategy in the treatment of AD [45]. Our study revealed that T. indica has anti-BuChE activity [Table 2]. CME of both bark and seed indicates that these are capable of inhibiting this enzyme too. IC_{50} values of bark and seed the fractions were 201.25 and 254.71 µg/ml, where at the same concentration galantamine inhibits almost 95% of the enzyme.

Plants are always been an excellent source for treating clotting disorder and stroke [46,47]. There are several thrombolytic drugs obtained from various plant sources. In our study, two different extracts from T. indica showed the thrombolytic activity in compare to the standard drug. The maximum clot lysis activity was mostly observed in CME of the bark with 30.17% than the seed 22.53%, which means bark can lysis more blood clot than the seeds. At the given concentration SK solubilize 72.95% clot.

| Table 2: BuChE inhibitory activity of CME fractions of T. indica |
| --- | --- | --- |
| Concentration (µg/mL) | Bark | Seed | Galantamine (standard) |
| 25 | 15.64±1.09 | 10.33±1.81 | 76.12±2.19 |
| 50 | 28.03±1.21 | 22.19±1.10 | 87.29±1.77 |
| 100 | 37.66±1.57 | 30.07±1.75 | 92.57±2.56 |
| 200 | 49.54±1.74 | 41.52±1.68 | 93.73±1.23 |
| 300 | 65.64±1.07 | 57.41±1.55 | 94.62±1.79 |

BuChE: Butyrylcholinesterase, CME: Crude methanolic extracts, 
T. indica: Tamarindus indica

| Table 3: % lysis of clot by SK, methanolic extract of T. indica bark, seed and control |
| --- | --- | --- | --- |
| Sample | Concentration (µg/ml) | Blood clot weight before treatment (ava.) | Blood clot weight after treatment (ava.) | % of clot lysis |
| Control (distilled water) | 100 | 0.648±0.038 | 0.611±0.046 | 3.60 |
| Bark extract | 100 | 0.748±0.027 | 0.447±0.039 | 30.17 |
| Seed extract | 100 | 0.792±0.035 | 0.569±0.022 | 22.53 |
| Standard (SK) | 100 | 0.825±0.0411 | 0.0955±0.0478 | 72.95 |

SK: Streptokinase, T. indica: Tamarindus indica
CONCLUSION

The result suggested that CMEs of both bark and seed from *T. indica* moderately inhibit both AChE and BuChE. These fractions also possess thrombolytic activity. Further study is needed to identify the specific molecule (or molecules) that are mainly responsible for those effects which might be a potential source for cholinesterase inhibitors in AD treatment and in neuroprotection, in general.

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