

Typhonium trilobatum* Demonstrates Both Antioxidant and Acetylcholinesterase Inhibitory Activities *In vitro

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(Received: November 12, 2018; Accepted: December 27, 2018; Published: January 17, 2019)

Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder of the elderly people. Cholinergic dysfunction and oxidative stress have been found to be related with the development of the disease. *Typhonium trilobatum* L. Scott has been used traditionally in folk medicine as a central nervous system stimulant. In this study, we have investigated the various extracts of *T. trilobatum* for acetylcholinesterase and antioxidant activities *in vitro*. All the tested extracts exhibited inhibition against acetylcholinesterase. Petroleum ether extract exhibited the highest inhibition with an IC₅₀ of 255.26 µg/ml. Likewise all the extracts were found to possess antioxidant activity. In reducing power assay, petroleum ether extract showed the highest reducing power with an absorbance of 0.874 at 0.1 mg/ml concentration and in DPPH radical scavenging assay, chloroform extract exhibited the highest scavenging activity with an IC₅₀ of 619.54 µg/ml. Phytochemical analyses of the four extracts revealed that the ethyl acetate extract contained the highest amount of flavonoids and petroleum ether had the highest amount of phenolics. These results suggest that *T. trilobatum* has both antioxidant and cholinesterase inhibitory activities, which may be useful in reducing the risk of AD.

Key words: Alzheimer's disease, *Typhonium trilobatum*, acetylcholinesterase inhibition, antioxidant activity.

Introduction

Alzheimer's disease (AD) is a degenerative disease of the central nervous system and most prevalent among the elderly people. AD patients present a progressive loss of the cholinergic synapses associated with reduction of acetylcholine levels in the hippocampus and cortex of the brain (Davies *et al.*, 1976; Terry *et al.*, 2003). This has been found to be linked to the development of dementia in AD (Bierer *et al.*, 1995). Therefore, inhibition of acetylcholinesterase, which enhances the concentration of acetylcholine at the synapse and improve the cholinergic deficit, is a therapeutic target for the development of drug for AD. Until now, out of the four drugs approved by the US Food and Drug Administration (FDA) to treat AD, three are cholinesterase inhibitors such as donepezil,

galantamine and rivastigmine and the rest one is memantine, a partial NMDA receptor antagonist (Graham *et al.*, 2017). These drugs are only effective in symptomatic relief but cannot completely cure the disease. Moreover, their use is limited due to their considerable side effects (Tanaka *et al.*, 2008). There are increasing evidences to suggest that oxidative stress is associated with the pathogenesis of AD (Lyras *et al.*, 1997; Galasko *et al.*, 2010). It has been shown that oxidative damage to the neurons presumably precedes neuropathology associated with AD (Nunomura *et al.*, 2001), suggesting that the intake of antioxidant might be beneficial in preventing or treating AD. As AD is a complex and multifactorial disease, the current strategy is to develop a compound with multiple targets, and plants are an important source of these compounds.

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Typhonium trilobatum, is a perennial herb belonging to the family Araceae, and widely distributed throughout Bangladesh. It is a popular leafy vegetable and being common, it is less pricey and so is consumed by poor people. It is an important source of vitamin such as thiamine, niacin, carotene and folic acid (Paul et al., 2011; WHO, 1990). Traditionally the plant is used in the treatment of different ailments. The whole plant is indicated for CNS-related conditions, as tonic and as a CNS stimulant in motor disorder. It is also used in snake bite, dermatitis, piles, boil, body ache, rheumatoid arthritis, edema, piles, helminthiasis, liver disease and spleen enlargement (Kirtikar et al., 1999; Vattayacharzo et al., 1989; Ghani, 2003). Medicinal uses of this plant have been described well in both Unani and Ayurvedic systems of medicine. Scientific investigations revealed that the plant has antimicrobial, antioxidant, anti-inflammatory, and nematocidal activities (Halder et al., 2011; Kandhasamy et al., 2008; Chattopadhyay et al., 1989; Ali et al., 2012). Given the ethnomedicinal uses of this plant, we have undertaken to evaluate the acetylcholinesterase inhibitory activity and antioxidant property of the extracts from *T. trilobatum*.

Materials and Methods

Chemicals: DPP (2, 2'-diphenyl-1-picrylhydrazyl), aluminum chloride, ammonium molybdate, potassium ferricyanide, catechin, Folin-cioaltea reagent, Tris-HCl and triton X-100 were obtained from Sigma-Aldrich, India. Gallic acid was obtained from Wako Pure Chemical Company Ltd., Japan. 5,5'-dithio-bis-(2-nitro) benzoic acid (DTNB), acetylthiocholine iodide, and donepezil were obtained from Sigma-Aldrich, Japan. Unless otherwise specified, all other chemicals were of analytical grade.

Plant materials: The stem and leaf of *T. trilobatum* were collected from University of Rajshahi campus, Rajshahi, Bangladesh, and identified by an expert taxonomist. A voucher specimen was submitted to the herbarium of the

Department of Botany, Rajshahi University.

Extraction and isolation: Dried powdered leaf and stem (300 gm) of *T. trilobatum* were extracted exhaustively with methanol by cold extraction method. The extract was then filtered and concentrated with a rotary evaporator under reduced pressure at 50°C temperature to afford the crude methanol extract (12.0 gm). An aliquot (10 gm) of the concentrated methanolic extract was partitioned by the method as described earlier and the resultant partitionates that is petroleum ether (PEF, 5.05 gm), chloroform (CLF, 1.28 gm), ethylacetate (EAF, 2.63 gm) and aqueous (AQF, 0.9 gm) extracts were obtained and used for the experiment purpose.

Phytochemical screening of the plant extract: Preliminary qualitative analysis of the extracts were carried out to determine the presence of various phytochemicals which include tannins, phenolics, flavonoids, alkaloids, saponins, steroids and glycosides in accordance with the methods as described earlier (Assaduzzaman et al., 2014).

Determination of total phenolic content: The total phenolic content of the various extracts of *T. trilobatum* was determined using the Folin-Ciocaltea reagent (Singleton et al., 1999). 0.5 ml of plant extract or solution of reference standard at different concentrations was added to 2.5 ml of Folin – ciocaltea (diluted 10 times with water) reagent and 2.5 ml of sodium carbonate (7.5%) solution. The reaction mixture was incubated for 20 minutes at 25°C and the absorbance of the final mixture was measured at 760 nm. Gallic acid was used as reference standard and the results were expressed as mg of gallic acid equivalent (GAE)/gm of dried extract.

Determination of total flavonoid content: Total flavonoid content of the various extracts of *T. trilobatum* was determined by aluminum chloride colorimetric method using catechin as a standard (Zhishen et al., 1999). The extract (1.0 ml) was added to 3.0 ml of methanol, 0.2 ml of 10% AlCl₃, 0.2 ml of 1M potassium acetate and 5.6 ml of distilled water. The resulting mixture was then incubated at room temperature for 30 minutes and the absorbance of the

mixture was measured at 420 nm. Catechin was used as standard. The results were expressed as mg of catechin equivalent (CE)/gm of dried extract.

Determination of ferric reducing antioxidant power (FRAP): The FRAP of the plant extracts of *T. trilobatum* was evaluated by the method as described earlier (Oyaizu 1986). The extract or standard solutions at different concentration (1 ml) were mixed with 2.5 ml each of potassium buffer (0.2 M) and potassium ferricyanide (1% w/v). The resulting mixture was incubated for 20 minutes at 50°C followed by addition of 2.5 ml of trichloro acetic acid (10% w/v) solution. The mixture was centrifuged at 3000 rpm for 10 minutes to collect the upper layer. 2.5 ml of solution from upper layer was mixed with 0.5 ml of ferric chloride (0.1% w/v) solution and 2.5 ml of distilled water and. The absorbance of the solution was then measured at 700 nm. Catechin was used as standard for comparison.

Determination of DPPH radical scavenging activity: DPPH radical scavenging activity of the extracts of *T. trilobatum* was determined according to the method as described earlier (Choi et al., 2000). 2 ml of methanol solution of the extract or standard at different concentration was mixed with 3 ml of methanol solution of DPPH (0.135 mM) into the test tube. The reaction mixture was incubated at room temperature for 30 minutes in dark place and the absorbance of the solution was measured spectrophotometrically at 517 nm. DPPH free radical scavenging ability (%) was calculated by using the formula:

$$[(A_{\text{absorbance of control}} - A_{\text{absorbance of sample}}) / A_{\text{absorbance of control}}] \times 100$$

Determination of acetylcholinesterase inhibitory activity: The acetylcholinesterase inhibitory assay was performed according to the colorimetric method of Ellman and associates (Ellman et al., 1961). Acetylcholinesterase enzyme was prepared from rat brain as described earlier (Asaduzzaman et al., 2014). The rates of hydrolysis by acetylcholinesterase were monitored spectrophotometrically. Each extract or standard (500 µl) was mixed with an enzyme solution (200 µl) and incubated at 37°C for 15 min.

Absorbance at 405 nm was read immediately after adding an Ellman's reaction mixture [3.5 ml; 0.5 mM acetylthiocholine, 1 mM DTNB] in a 50 mM sodium phosphate buffer (pH 8.0) to the above reaction mixture. Reading was repeated for 10 min at 2 min intervals to verify that the reaction occurred linearly. The blank reaction was measured by substituting saline for the enzyme. Donepezil was used as positive control. The percentage inhibition of acetylcholinesterase activity was calculated using the following formula:

$$[(A_{\text{absorbance of control}} - A_{\text{absorbance of sample}}) / A_{\text{absorbance of control}}] \times 100$$

Statistical Analysis: All analyses were carried out in triplicates. Data were presented as mean ± SD. Free R-software version 2.15.1 (<http://www.r-project.org/>) and Microsoft Excel 2007 (Roselle, IL, USA) were used for the statistical and graphical evaluations. Significant differences (p-value < 0.05) between the means were determined using the *t*-test.

Results and Discussion

AD is the major cause of dementia in the elderly people. It is a complex and multifactorial disease. Different mechanisms including amyloid and tau hypothesis, cholinergic and oxidative stress hypothesis have been proposed to explain the pathogenesis (Terry et al., 2003; Lyras et al., 1997; Masters et al., 2012). It is therefore suggested that an agent that can act on several targets would be an effective candidate as a potential drug to prevent the development of AD. In an epidemiological study, the incidence of AD in Indian subcontinent elderly people was found to be approximately 4.4 fold less when compared to a reference US population (Chandra et al., 2001). Environmental factors including dietary habits like intake of plant derived substances are thought to be responsible for this decreased incidence. In this context, curcumin, an ingredient of turmeric, has shown potential roles in ameliorating AD (Frautschy et al., 2010). *T. trilobatum* is a very popular vegetable consumed by

Bangladesh, India, and Pakistan, and has been used traditionally as tonic and central nervous system stimulant (Kirtikar *et al.*, 1999; Vattayacharzo *et al.*, 1989; Ghani, 2003). In this study, we have investigated the different extracts of *T. trilobatum* for acetylcholinesterase inhibitory and antioxidant activities using *in vitro* models.

Acetylcholinesterase inhibitory activity: The use of acetylcholinesterase inhibitors is currently accepted as the first line pharmacotherapy for symptomatic relief of AD. Inhibition of acetylcholinesterase can enhance cholinergic transmission by reducing the enzymatic hydrolysis of acetylcholine, leading to improvement of memory and cognition (Graham *et al.*, 2017). The effect of *T. trilobatum* on acetylcholinesterase was evaluated by

the Ellman's method (Ellman *et al.*, 1961) and the results have been shown in the Figure 1. All the tested extracts exhibited inhibition of acetylcholinesterase ranging from 23-65%. Petroleum ether extract showed the highest activity with IC₅₀ value of 255.26 µg/ml. The other extracts had comparatively less activity and failed to reduce 50% of the enzymatic activity. These results suggest that the *T. trilobatum* possesses considerable acetylcholinesterase inhibitory activity in comparing with the other medicinal plants *Bacopa moneira*, *Centella asiatica*, *Convolvulus pluricaulis*, *Glycyrrhiza glabra*, and *Aegle marmelos*, which are used in traditional Ayurvedic medicine for improving memory and cognitive function (Assaduzzaman *et al.*, 2014; Mathew *et al.*, 2014).

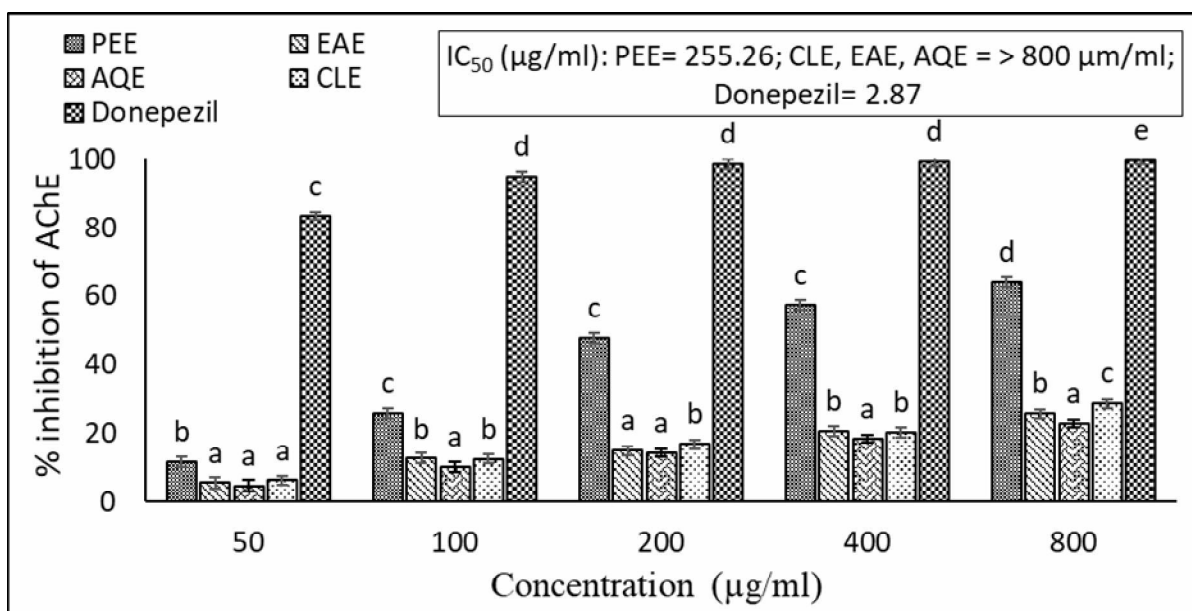


Figure 1. Acetylcholinesterase inhibitory activity of *T. trilobatum* extracts and standard donepezil. Results represent mean \pm SD (n=3). Different letters (a,b,c,d,e) indicate significant differences at $p < 0.05$. PEE, petroleum ether extract; CLE, chloroform extract; EAE, ethylacetate extract; AQE, aqueous extract.

Antioxidant activity: Free radical plays an important role in the pathogenesis of AD. Abeta protein is known to produce free radical and increased amount of oxidized protein, lipid and DNA has been observed in the brain of patients with AD (Lyras *et al.*, 1997; Galasko *et al.*, 2010). Naturally occurring antioxidants have been reported to play a

major role in the prevention of oxidative damage induced by free radicals (Commenges *et al.*, 2000; Darvesh *et al.*, 2010).

Antioxidant activity of the *T. trilobatum* extract was evaluated by two different assays, DPPH radical scavenging assay and reducing power assay. DPPH is a stable free radical whose level decreases following

exposure to a proton radical scavenger. The results of DPPH radical scavenging assays of the different extracts of *T. trilobatum* have been shown in Figure 2A. Our results demonstrated the DPPH scavenging activity of the tested extracts. Similar to acetylcholinesterase inhibitory activity, petroleum

ether extract showed the highest activity followed by chloroform extract and aqueous extract with IC_{50} values of 529.47, 619.54, and 625.32 $\mu\text{g/ml}$, respectively. The ethylacetate extract had comparatively less activity and failed to reduce 50% of DPPH scavenging activity.

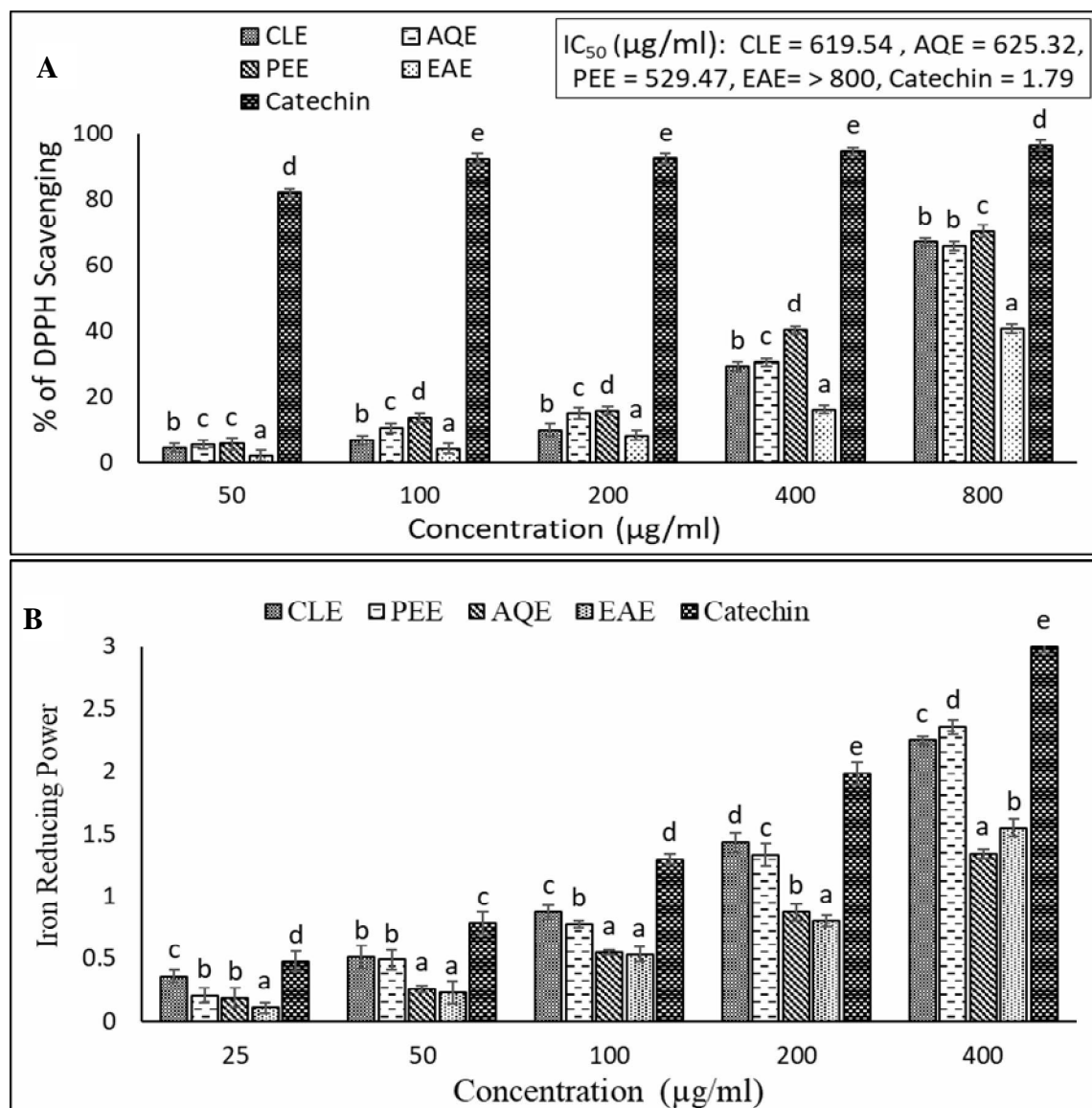


Figure 2. Antioxidant potential of *T. trilobatum* extracts. (A) DPPH radical scavenging activities of *T. trilobatum* extracts and standard catechin. (B) Reducing power of *T. trilobatum* extracts and standard catechin. Results represent mean \pm SD ($n=3$). Different letters (a,b,c,d,e) indicate significant differences at $P<0.05$. PEE, petroleum ether extract; CLE, chloroform extract; EAE, ethylacetate extract; AQE, aqueous extract; CA, catechin.

The reducing power represents the ability of an antioxidant to donate electrons. We have assessed the

reducing ability of *T. trilobatum* extract by ferric reducing antioxidant power (FRAP) method based on

the reduction of the Fe³⁺-ferricyanide complex to the ferrous form. The results of the reducing power of the different extracts have been shown in Figure 2B. All the extracts increased the absorbance significantly with the increase of concentration of the extract, demonstrating the antioxidant potential of *T. trilobatum*. Chloroform extract showed the highest activity with an absorbance of 0.874 at 100 µg/ml

concentration. Whereas the absorbance of ethylacetate, petroleum ether and aqueous extracts were 0.776, 0.534 and 0.548, respectively at the same concentration. Ascorbic acid was used as a standard which gave an absorbance of 1.301. Taken together, our results demonstrated the appreciable antioxidant activity of the extract of *T. trilobatum*.

Table 1. Total phenolic and flavonoid contents of different extracts from *T. trilobatum*.

Sample	TPC (mg GAE/g dried extract)	TFC (mg CE/g dried extract)
PEE	2.94 ± 0.16 ^(a)	46.53 ± 0.21 ^(a)
CLE	4.53 ± 0.18 ^(b)	20.94 ± 0.35 ^(b)
EAE	6.14 ± 0.45 ^(d)	32.87 ± 0.57 ^(c)
AQE	1.24 ± 0.22 ^(c)	1.88 ± 0.19 ^(d)

PEE, petroleum ether extract; CLE, chloroform extract; EAE, ethylacetate extract; AQE, aqueous extract. TPC: Total phenolic content, TFC: Total flavonoid content. Means in each column with different subscript letters (a, b, c, d, e, f) differ significantly (p < 0.05).

Phytochemical Analyses: A preliminary phytochemical analyses was conducted first to understand the presence of different classes of phytochemicals in the extract of *T. trilobatum*. The extracts showed the presence of phenolics and flavonoids, phytosterols, alkaloids, tannins, and saponins which is consistent with the previous study (Kandhasamy *et al.*, 2008). Phenolics and flavonoids are important classes of phytochemicals that are reported to have antioxidant activity due to their redox properties. Quantitative analyses of the four extracts (Table 1) revealed that the ethylacetate extract contained the highest amount of phenolic (6.14 ± 0.45 mg GAE/gm dried extract) followed by chloroform extract (4.53 ± 0.18 mg GAE/gm dried extract), petroleum ether extract (2.94 ± 0.16 mg GAE/gm dried extract) and aqueous extract (1.24 ± 0.22 mg GAE/gm dried extract). In contrast, petroleum ether contained the highest amounts of flavonoids (46.53 ± 0.21 mg CE/gm dried extract) followed by chloroform extract (20.94 ± 0.35 mg CE/gm dried extract), ethyl acetate (32.87 ± 0.57 mg CE/gm dried extract), and aqueous extract (1.88 ± 0.19 mg CE/gm dried extract).

Conclusion

Results of the present study clearly demonstrated that the extract of *T. trilobatum*, has considerable amount of phenolics and flavonoids and possesses both cholinesterase inhibitory activities and antioxidant properties. These findings suggest that increasing the intake of this vegetable may be helpful in preventing or reducing the risk of AD.

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