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Cholinesterase inhibition and antioxidant activity of the stem bark of *Abroma augusta*: Correlation with phenolic and flavonoid content

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ABSTRACT

Abroma augusta is a popular folk medicine in Bangladesh used to treat a variety of diseases. The present study aimed to evaluate the anticholinesterase and antioxidant activity of the plant extract and its four fractions *in vitro* in order to explore new drugs or alternative treatment for AD. The cholinesterase inhibitory activity was performed by Ellman's method and the antioxidant activity by DPPH free radical scavenging assay. The methanol extract exhibited inhibition of both the acetyl- and butyryl-cholinesterase enzymes and showed antioxidant properties. When the extract was fractionated, the activities were mostly found in the chloroform fraction. The chloroform fraction showed the IC₅₀ values of 390.53±0.29 and 350.37±0.21 µg/ml for acetyl- and butyryl-cholinesterase, respectively. Importantly the chloroform fraction showed potential antioxidant activity with an IC₅₀ value of 10.25 ± 0.03 µg/ml for DPPH radical scavenging. The fraction was also found to be rich in phenolic content (97.8±0.07 mg GAE/gm of dried extract) and flavonoid content (108.60±0.49 mg CE/g dried extract). A linear correlation was found between phenolic and flavonoid content with acetyl- and butyryl-cholinesterase inhibition and antioxidant activity. The cholinesterase inhibitory and antioxidant activity of the chloroform fraction suggest that it may be useful in the treatment of AD. Further studies in animal model are required to confirm the anti-AD potential of the chloroform fraction.

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INTRODUCTION

Alzheimer's disease (AD) is a progressive neurological disorder accounting for majority of the dementia cases. The clinical features of AD include the impairment of memory and cognition, and psycho-behavioral disturbances (Rumiana *et al.*, 2024). The World Alzheimer's Report estimates over 55 million AD patients worldwide. If the number of AD cases increases in the same rate, the number is projected to double in twenty years (Alzheimer's Association, 2024). This will pose a serious threat and burden to the society. Therefore, development and discovery of drugs for treatment of AD is now a priority for the researchers.

Cholinergic system plays a vital role in the cognitive and retrieval of item in function in the brain. But in AD, there is a profound loss of cholinergic neurons and a gradual decline of the neurotransmitter acetylcholine. The gradual decline of acetylcholine is closely related with the degree of dementia in AD (Terry and Buccafusco, 2003). Acetylcholinesterase is a major enzyme involved in the metabolism of acetylcholine. Therefore, inhibition of acetylcholinesterase augments the concentration of acetylcholine at the synapse, elevates the neurotransmission and improves memory and cognition (Hampel *et al.*, 2018). Like acetylcholinesterase, butyrylcholinesterase also hydrolyzes acetylcholine. The currently used AD drugs inhibit both the AChE and BChE. Only three cholinesterase inhibitors donepezil, galantamine and rivastigmine are available for use (Pang *et al.*, 2025). Therefore, there is increased demand of cholinesterase inhibitors with more efficacy and safety.

Oxidative stress has been implicated in the pathogenesis of several neurodegenerative diseases including AD. The brains with AD showed an increased oxidation of lipids, proteins and DNA (Kao *et al.*, 2020). It has been suggested that oxidative stress plays a role in the formation and progression of AD (Bai *et al.*, 2022). Antioxidants are able to prevent the oxidative stress, thereby improve memory and

learning in mice (Pritam *et al.*, 2022). Therefore, an agent having cholinesterase inhibitory and antioxidant activities is desirable for mitigating AD.

Traditional medicine has now drawn interest as an alternative medicine for their potency, efficacy and safety. A good number of medicinal plants used as traditional medicine in Bangladesh have already been evaluated for inhibitory potential against cholinesterase with positive outcome (Uddin and Zidorn, 2020). But still there are a large number of plants which remained unexplored. *Abroma augusta* Linn., commonly known as Ulatkambal in Bengali and devil's cotton in English, is an evergreen tree that belong to the family Malvaceae. It is widely distributed (native or cultivated) throughout the country. The plant is found in the warm area of India such as Uttar Pradesh, Sikkim, Khasi Hills, and Assam. It is also found in Java, Philippines and China (Uddin and Zidorn, 2020). The plant can grow up to 2-4 m tall, normally branching at the height of 1-2 m and is often multi-stemmed from the base. Traditionally, the plant is used in the management of headache with sinusitis. It is also used in dysmenorrhea, amenorrhoea, diabetes, anti-scabies, anti-dermatitis, anti-inflammatory, uterine disorder, diabetes, and rheumatic pains (Ghani, 2003 and Rakesh *et al.*, 2023). Experimental evidences revealed that the plant has a good deal of biological and pharmacological activities, including antioxidant, anticancer, antifertility, anti-inflammatory, antidiabetic, antinociceptive, skin damage repairing, antilipidemic activity, antifungal, antibacterial, insecticidal, and cytotoxic effects (Khan *et al.*, 2003, Khanra *et al.*, 2015 and Miah *et al.*, 2020). Phytochemical studies reported the isolation and identification of a number of bioactive compounds such as terpenoids, alkaloids from various parts of the plant *A. augusta* (Ahmad *et al.*, 2003, Chowdhury *et al.*, 2019 and Rahman *et al.*, 2016). Although the plant has shown potential bioactivities, no study has been done on its neuroprotective properties. In this study, we have evaluated the anticholinesterase and antioxidant properties of the stem bark of *A. augusta* with a view to seek a remedy for the recovery of AD.

MATERIALS AND METHODS

General

Extracting, eluting and other solvents including methyl alcohol, n-hexane, cyclohexane, chloroform, benzene, ethylacetate, acetonitrile, acetone, dichloromethane, are obtained from Duksan pure chemicals Ltd., Korea Republic. Reagent 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was bought from Sigma (Merck KGaA, Darmstadt, Germany). Acetylcholinesterase (AChE) from electric eel and butyrylcholinesterase enzyme from equine serum, acetylthiocholine iodide (ATCI) and S-butyrylthiocholine iodide were purchased from Sigma-Aldrich, Germany. A Shimadzu UV-1800 spectrophotometer was used to record the absorbance of test samples.

Plant collection, extraction and fractionation

The plant material (stem bark of *Abroma augusta*) was collected from Rajshahi district, Bangladesh and was identified by taxonomist Mr. A.H.M. Mahbubur Rahman, Dept. of Botany, University of Rajshahi and the voucher specimen was kept at the herbarium of same department (RR-134). After collection, the stem bark was washed, sun-dried and crushed into coarse powder using a grinder and was subjected to further analysis.

The coarse powder was overwhelmed with adequate quantity of methanol following cold extraction method. The filtrate was then taken, concentrated in vacuo for collecting the crude methanol extract (MAA). The extract was kept at 4 degree centigrade in the refrigerator awaiting use. The crude extract was then fractionated with four solvents such as n-hexane, chloroform, ethyl-acetate and water according to the modified Kupchan method (Kupchan, 1970) to obtain corresponding n-Hexane fraction (AAH), chloroform fraction (AAC), ethylacetate fraction (AAE) and aqueous fraction (AAA).

Estimation of total phenolic content (TPC)

The TPC of the crude methanol extract and its fractions was determined by spectrophotometric method (Majhenic *et al.*, 2007). In brief 0.5 ml of sample at concentration of 0.5 mg/ml was mixed with

2.5 ml of Folin-Ciocalteu (diluted 10 times with water) reagent and 2.5 ml of sodium carbonate (7.5%) solution. Following incubation for 20 minutes at 25°C, the absorbance of the mixture was measured at 760 nm. The phenolic content was determined using the standard curve obtained for gallic acid and the results were expressed as mg of gallic acid equivalent (GAE) per gm of dried extract. Each of the sample was tested for three times and mean value had taken as the total content.

Estimation of total flavonoid content (TFC)

The TFC of plant extract/fractions was measured by the aluminum chloride colorimetric method as described by Dewanto method (Dewanto *et al.*, 2002) using catechin as a standard. 1 ml plant extract with a concentration of 500 µg/ml was taken in a 10 ml of volumetric flask, and then 5 ml of distilled water followed by 0.3 ml of 5% sodium nitrite were added to it. About 0.6 ml of 10% aluminum chloride was added after 5 minutes. Again after 5 minutes 2 ml of 1M sodium hydroxide was added and volume was made up to the mark with distilled water. The solution was under through mixing and absorbance was taken as 510 nm wavelength. The flavonoid content was determined using the standard curve obtained for catechin and the results were expressed as mg of catechin equivalent (CE) per gm of dried extract. Each of the samples was tested for three times and readings so obtained were averaged.

Assessment of the cholinesterase inhibitory activity

The anti-cholinesterase (AChE and BuChE) activity of the extractives was assessed according to Ellman's method (Ellman *et al.*, 1961) using substrates such as acetylthiocholine iodide (ATCI) and S-butyrylthiocholine iodide (BTCl). A mixture containing about 200 µl of extract/fractions of different concentration, 200 µl of enzyme extract and 2.00 ml of 50 mM Tris-HCl buffer, pH 7.4, was taken in test tubes and kept for sixty minutes at room temperature. Then about 200 µL of 0.7 mM DTNB and 400 µl of 0.35 mM ATCI/BTCl were added to it and the absorbances of the mixture were recorded at a wavelength of 412 nm, every 30 sec for 3 minutes

against a blank solution. Donepezil and galantamine were used as working references for the determination of anti-AChE and anti-BuChE activity of extracts, fractions and compounds respectively. The percent (%) of inhibition, I%, was calculated by using the following equation.

$$I\% = \{(A_0 - A_1)/A_0\} \times 100$$

Where, A_0 is rate of the change of absorbance of the control solution per minute and A_1 is the rate of the change of absorbance of the test sample per minute. IC_{50} value of extract and fractions were attained by plotting the percentage of inhibition value against the respective concentrations. The pertinent analyses were executed thrice.

Assessment of antioxidant activity *in vitro*

The possible antioxidant activity of the extracts, fractions and compounds were assessed by using DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging method according to Braca method (Braca *et al.*, 2001). In brief, test tubes containing 2 mL of methanol solution of test samples at varying concentration were taken. 2 ml 0.004% methanol solution of DPPH was added to each of the test tube and kept in dark place for one hour to complete the reaction. Then the absorbances of the samples were taken at 517 nm using a UV-spectrophotometer. Catechin was used as standard substance to make a comparison. The every test was executed three times and the results were converted to mean. The percent of free radical scavenging (I %), was estimated by using the following equation.

$$I\% = \{(A_0 - A_1)/A_0\} \times 100$$

Where, A_0 represents the absorbance of the control solution and A_1 represents the absorbance of the samples. By plotting the percentage of free radical scavenging against the corresponding concentrations, IC_{50} value of extract, fractions and compounds were obtained.

Statistical analysis

In this investigation, each of the respective experiment was run thrice. The data are tabulated as

mean $\pm$ SD. The statistical and graphical analyses were accomplished with Graph Pad Prism (10.0.1) and Microsoft Excel 2013. In order to estimate the statistical significance (p value < 0.05) between mean values, T-test was presumed. Pearson correlation analysis was completed for correlation analysis.

RESULTS AND DISCUSSION

Medicinal plants are long being used as traditional medicine in the treatment different diseases including AD. Such plants have provided alternative medicines to the existing therapies. A number of phytochemicals have been isolated from plants that inhibited cholinesterase enzymes and improved memory and cognition (Koul *et al.*, 2023). The natural compounds or alternative medicines are now being preferred to the synthetic compounds due to toxicities. In continuation of our efforts to explore cholinesterase activity from Bangladeshi medicinal plants, we carried out an investigation on the folk medicine *Abroma augusta* used in the management of headache and other ailments (Ghani, 2003 and Rakesh *et al.*, 2023). We report for the first time the anticholinesterase and antioxidant activity of the extract *in vitro*.

Phytochemical analysis

Quantitative analysis was carried on the phenolic and flavonoid content of the crude methanol extract and its fractions and the result has been presented in the Table 1. Among the tested extractives, the chloroform fraction was found to possess the highest amount of phenolics and flavonoids. The total phenolic content was 92.88 ± 0.48 , 59.83 ± 1.13 , 97.80 ± 0.07 , 46.69 ± 0.67 , 46.69 ± 0.67 , and 35.61 ± 0.36 mg GAE/g of dry extract for methanol extract and its *n*-hexane, chloroform, ethylacetate and aqueous fractions. While the flavonoid content was 107.80 ± 1.36 , 5.43 ± 0.45 , 108.60 ± 0.49 , 67.75 ± 0.34 , and 40.38 ± 0.52 mg CAE/g of dry extract for the same, respectively.

Anticholinesterase activity

It is increasingly evident that the inhibitors of cholinesterase enzymes augment cholinergic function by increasing the levels of ACh in the brain and improve

memory and cognition (Hampel *et al.*, 2018). Acetylcholinesterase is a major therapeutic target for improvement of AD due to its catalytic role in the breakdown of the acetylcholine (ACh) into acetic acid and choline. In the present study, the cholinesterase inhibitory activity of the extract and fractions of *A. augusta* was tested by Ellman's method *in vitro* (Ellman *et al.*, 1961). The results (Fig. 1 and Table 2) showed that the methanol extract of the plant inhibited the AChE in a dose dependent manner with IC₅₀ 355.63 ± 0.21 µg/mL.

When the extract was fractionated into four solvent fractions having different polarity, the activity was found to be the highest in the chloroform fraction with IC₅₀ value of 390.53 ± 0.29 µg/mL. The remaining n-hexane, ethylacetate and aqueous fractions showed activity, but failed to inhibit to 50% of enzyme activity at the same concentration. Strong correlation has been found between phenolic ($r=0.952$) and flavonoid ($r=0.853$) content with acetylcholinesterase inhibitory activity (Table 3).

Table 1. TPC and TFC of *A. augusta* methanol extract and its four solvent fractions

Samples	TPC mg GAE/g dried extract	TFC mg CE/g dried extract
MAA	92.88 ± 0.48 ^(a)	107.80 ± 1.36 ^(a)
AAH	59.83 ± 1.13 ^(b)	5.43 ± 0.45 ^(d)
AAC	97.80 ± 0.07 ^(a)	108.60 ± 0.49 ^(a)
AAE	46.69 ± 0.67 ^(c)	67.75 ± 0.34 ^(b)
AAA	35.61 ± 0.36 ^(d)	40.38 ± 0.52 ^(c)

Values are mean ± SD; different superscripts in a column (a–d) indicate significant differences ($p < 0.05$). TPC: total phenolic content, TFC: total flavonoid content, MAA: Methanol extract of *A. augusta*, AAH: n-hexane fraction, AAC: chloroform fraction, AAE: ethyl acetate fraction, AAA: aqueous fraction.

Table 2. IC₅₀ values of extract and fractions from *A. augusta* in anticholinesterase activity assay and DPPH free radical scavenging assay

Samples	IC ₅₀ values (µg/ml)		
	AChE	BuChE	DPPH radical scavenging
DON	2.07 ± 0.02 ^a	-	-
GAL	-	1.29 ± 0.01	-
CAT	-	-	4.62 ± 0.03
MAA	355.63 ± 0.21	>500	28.27 ± 0.03
AAH	>500	>500	46.50 ± 0.05
AAC	390.53 ± 0.29	350.37 ± 0.21	0.25 ± 0.03
AAE	>500	>500	15.74 ± 0.08
AAA	>500	>500	138.70 ± 0.20

^a Each value is represented as average ± SD.

Table 3. Pearson correlation coefficients of total phenolic and flavonoid contents with acetyl- and butyrylcholinesterase inhibition, and DPPH radical scavenging activities

Activities	TPC	TFC
AChEI	0.952	0.853
BuChEI	0.539	0.536
DRS	0.633	0.502

AChEI: Acetylcholinesterase inhibition, BuChEI: Butyrylcholinesterase inhibition, DRS: DPPH radical scavenging.

Similar to acetylcholinesterase, butyrylcholinesterase is another therapeutic target due its ability to hydrolyze acetylcholine. It has been found that inhibition of BuChE elevates the level of acetylcholine in the synaptic cleft and improves the cholinergic

neurotransmission (Li *et al.*, 2000). The inhibitory activity of the methanol extract and its fractions were similarly evaluated and the result has been presented in Fig 2 and Table 2. The methanol extract showed inhibition against butyrylcholinesterase enzyme.

At a concentration of 500 $\mu\text{g/mL}$, the percent inhibition was $50.40 \pm 0.26\%$. Among the fractions, chloroform fraction displayed the highest inhibition with IC_{50} value of $350.37 \pm 0.21 \mu\text{g/mL}$. The other fractions showed less activity. These results suggest that the chloroform fraction is a dual cholinesterase inhibitor. A moderate correlation was observed between phenolic ($r=0.539$) and flavonoid ($r=0.536$) content with butyrylcholinesterase inhibitory activity (Table 3).

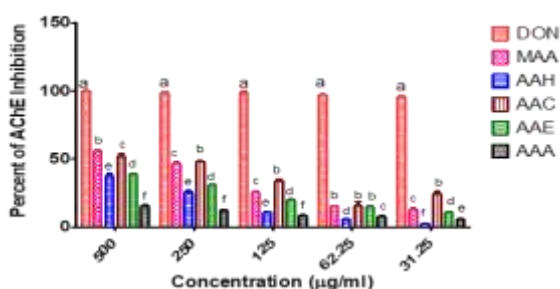


Fig. 1. Acetylcholinesterase inhibitory activity of the extracts and solvent fractions from *A. auugusta*. Mean with different letters (a-f) differ significantly ($p < 0.05$).

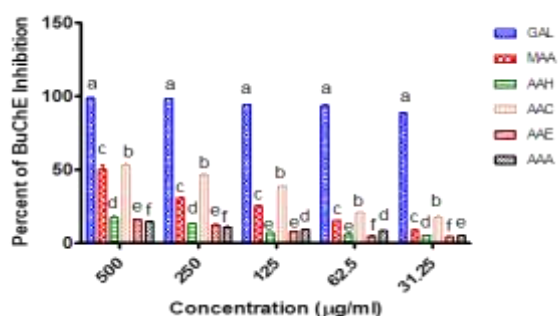


Fig. 2. Butyrylcholinesterase inhibitory activity of the extracts and solvent fractions from *A. auugusta*. Mean with different letters (a-f) differ significantly ($p < 0.05$).

Antioxidant activity

The DPPH free radical scavenging assay is a simple and quick method used to preliminarily test the antioxidant activity of plant extracts/fractions (Braca et al., 2001). The extract or fractions that show good results in this assay constitute a reliable basis for further studies. The methanol extract of *A. auugusta*

and its fractions were assessed by DPPH assay and the results have been shown in the Fig. 3 and Table 2. The methanol extract demonstrated considerable scavenging activity with IC_{50} value of $28.27 \pm 0.03 \mu\text{g/mL}$. After fractionation, the highest activity was found in the chloroform fraction followed by the ethylacetate and n-hexane fraction with IC_{50} values of 0.25 ± 0.03 , 15.74 ± 0.08 and $46.50 \pm 0.05 \mu\text{g/mL}$, respectively. The aqueous fraction showed mild activity. These results revealed that the chloroform fraction has potential antioxidant activity, suggesting a source of antioxidants that may be effective in ameliorating the oxidative stress in oxidative stress induced diseases. Pearson's correlation study showed moderate correlation of phenolic and flavonoid content with DPPH scavenging activity with r values of $0.502 \sim 0.633$, respectively (Table 3).

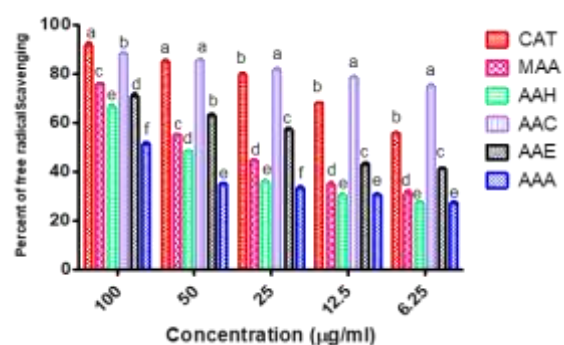


Fig. 3. DPPH free radical scavenging activity of extracts and solvent fractions from *A. auugusta*. Results are set as mean $\pm$ SD ($n=3$) IC_{50} value ($\mu\text{g/mL}$). Mean with different letters (a-f) differ significantly ($P < 0.05$).

CONCLUSION

The findings of this study clearly demonstrate the acetyl- and butyryl-cholinesterase inhibitory and antioxidant activity of the methanol extract of *A. auugusta*. Among the fractions, chloroform fraction was rich in phenolics and flavonoids and showed the most potent activity. A linear correlation was found between phenolic and flavonoid content with acetyl- and butyryl-cholinesterase inhibition and antioxidant activity. Further studies in animal model are required to confirm the anti-AD potential of the chloroform fraction.

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