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Cholinesterase inhibitory effects of *Rhizophora lamarckii, Avicennia officinalis, Sesuvium portulacastrum and Suaeda monica:* Mangroves inhabiting an Indian coastal area (Vellar Estuary)

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Abstract

Alzheimer's disease is a progressive neurodegenerative illness accounting for approximately 50% of all types of dementia in elderly people. The only symptomatic treatment proven effective to date is the use of cholinesterase inhibitors to augment surviving cholinergic activity. The purpose of this study is to investigate cholinesterase inhibitory activity of mangroves as an alternative medicine for the treatment of Alzheimer's disease. About nine mangrove plants, which were used as folk medicine in tropical countries, were collected from Parangipettai, Vellar estuary, Tamilnadu, India. Nile Tilapia muscle homogenate was used as source of enzyme. Inhibitory effect of methanolic leaf extract was assessed under *in vitro* condition by incubating various concentration of the extract with total cholinesterase and butyryl cholinesterase and assessing their residual activities by Ellman's colorimetric method. The results showed that of the nine plants screened *Rhizophora lamarckii*, *Suaeda monica*, *Avicennia officinalis* and *Sesuvium portulacastrum* showed 50% inhibitory activity to both TChE and BChE at concentrations less than 2 mg/mL when compared to other plant extracts, which was comparable to the standard drug Donepezil. Phytochemical analysis showed the presence of alkaloids in high concentration which might be correlated to its cholinesterase inhibitory activity.

Keywords: *Alzheimer's disease, acetyl cholinesterase, butyryl cholinesterase, donepezil, mangrove plants, phytochemical analysis, inhibition*

Abbreviations: *TChE, (Total cholinesterase); BChE, (Butyryl cholinesterase); ChAT, (Choline Acetyltransferase); ChE, (Cholinesterase); AD, (Alzheimer's disease); ACh, (Acetyl choline); DTNB, (5, 5′-dithiobis [2-nitrobenzoic acid]); ATCI, (Acetylthiocholine iodide); BTCI, (Butyrylthiocholine iodide)*

Introduction

Mankind is afflicted by several neurological disorders of which one of the most insidious disorder is Alzheimer's disease a common dementia which occurs most often in people over the age of 60. About half million old people in United States suffer from this class of dementia. Alzheimer's disease (AD) is a chronic neurodegenerative disorder characterized by progressive deterioration of activities of daily living (ADL), behavioral disturbances and loss of cognitive functions [1]. This is due to loss of cholinergic activity in those areas of the brain responsible for higher mental functions, which partially accounts for the impairments in ADL, behaviour and cognition. Loss of cholinergic activity is due to substantial reduction in the activity of the enzyme choline acetyltransferase (ChAT) responsible for the synthesis of acetylcholine (ACh) [2,3]. Reduction in ChAT leads to a subsequent decline in levels of ACh necessary for neurotransmission in the brain. One of the most promising approaches for treating AD is to enhance acetylcholine (ACh) level in the brain. A variety of strategies have been envisaged to implement the replacement of acetylcholine, of which cholinesterases inhibition has shown consistent positive results in the treatment of Alzheimer's disease [4].

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Cholinesterase inhibitors act on the enzymes that hydrolyse ACh following synaptic release. In the healthy brain, acetylcholinesterase (AChE) predominates (80%) and butyrylcholinesterase (BChE) is considered to play a minor role in regulating brain ACh levels [5]. In the AD brain, BChE activity rises while AChE activity remains unchanged or declines. Therefore both enzymes are likely to have involvement in regulating ACh levels and represent legitimate therapeutic targets to ameliorate the cholinergic deficit. Epidemological data indicates an estimated doubling of dementia cases over the next 50 years [6]; hence the burden of care is set to increase substantially. Several cholinesterase inhibitors are being investigated for the treatment of AD. However only tacrine, rivastigmine, donepezil, galanthamine have been approved by food and drug administration in United States [7]. Because of bioavailability problems and side effects like hepatotoxicity and gastrointestinal disorder there is still a great interest in finding better cholinesterase inhibitors from natural sources [8,9]. Natural products are significant sources of synthetic and traditional herbal medicines. Alzheimer's disease is one area, where natural products have not been exploited to their potential. Some institutions are carrying out preclinical trials with isolated constituents like Huperzine, Bacosides, Hyperforin, Desoxy-peganine and ginko biloba (plant extract) on Alzheimer's disease [10]. Ocean, which is called 'mother of origin of life' posses nearly 3,00,000 of described species of plants and animals, which contain many novel product of pharmacological importance. Of which, Mangroves form a unique and dominant ecosystem comprised of intertidal marine plants, mostly halophytic trees, predominantly bordering margins of tropical coastlines around the world.

The mangrove forest of Indo-Pacific area are rich in flora with 63 species of which *Rhizophora* and *Avicennia* are the predominant species with healing properties, making them popular in folk medicine. Root, leaf and stem extracts of *Rhizophora* trees have inhibitory properties against human pathogens like bacteria, virus and fungi [11]. Decoctions of bark of *Rhizophora* have been used as gargles for the treatment of throat cancer and as external rubefacients [12]. *Rhizophora* resin, which contains tannins with astringent properties, has been used for treating diarrhea and dysentery. Polysaccharides derived from leaf of *R. apiculata* (RAP) [13] are inhibitors of HIV-1 and SIV, so it can be used to treat early stage infection of AIDS. Bark of *R. mucronata* has been used to cure diabetes and bark of Prop roots has been used as mosquito repellent [14]. Young leaves of *A. marina* have been used for human consumption, cattle feed while its bark is used as astringent and minor fish stings. Seeds of *Avicennia officinalis* are used for rellieving ulcers. Fruits of *Bruguiera* are used as medicine for eye ailment. The medicinal properties of mangrove trees provide a wide domain for medical uses, which requires further study for possible drug development.

The scope of the present study is to evaluate the cholinesterase inhibitory activity of mangrove plant extract using Ellman's colorimetric method. For the inhibition studies, total cholinesterase (TChE) and BChE were isolated from fresh water fish Nile Tilapia, (*Oreochromis niloticus*) which occurs widely in tropical countries. It is an indicator species with richest sources of cholinesterase distributed widely in brain, liver and muscle [15]. Both Total and butyryl cholinesterase are uniformly distributed in muscle, so enzyme from muscle is used for study.

Materials and methods

Chemicals

Acetylthiocholine iodide (ATCI) was purchased from Sd.fine Chemicals Ltd, India. Butyrylthiocholine iodide (BTCI) and 5, 5′-dithiobis [2-nitrobenzoic acid] (DTNB) were purchased from Hi Media Laboratories, India. The organic solvents and Folin's-Ciocalteau's phenol reagent were purchased from SRL chemicals Ltd, India. All other chemicals were of highest purity grade commercially available.

Preparation of enzyme and determination of protein content

Nile tilapia fish were collected from fresh water pond in Rangium, located near by Thirumayam town, Pudukkottai District, Tamilnadu. The live fish were transported from a fresh water pond to the laboratory in an aerated tank and were maintained in fresh water under continuous aeration till analysis. Fish were killed by decapitation. Its muscle was excised and washed with homogenizing buffer (100 mM Phosphate buffer pH 7.4). Whole tissue was homogenized with ice-cold (1:4 w/v) 100 mM phosphate buffer pH 7.4 in Teflon–glass Potter-Elvejhem homogenizer (Remi Motors, India). The homogenate was centrifuged at 10,000 × g for 10 min and the supernatants were stored at −20°C till inhibition experiments. Protein content of the enzyme was determined using Folin's-Ciocalteau's phenol reagent and Bovine serum Albumin as standard by Lowry's method [16].

Preparation of plant extracts

Mangrove plants used for study are *Rhizophora annamalayana, Rhizophora apiculata, Rhizophora mucronata, Avicennia marina, Rhizophora lamarrckii, Avicennia officinalis, Bruguiera cylindrica, Suaeda monica and Sesuvium portulacastrum*, which was collected from Parangipettai, Vellar estuary, Tamilnadu. Professor Dr. K. Kathiresan, CAS in Marine Biology, Annamalai University, identified the species. Mangrove leaves were

washed, cut in to small pieces and air-dried. 1 gm of dried sample was suspended in methanol for 2 days and filtered. The filtrate was evaporated under reduced vacuum pressure in desiccators until dryness. The dried powder was dissolved in 0.5 M Tris- HCl pH 8.0 containing less than 10% of methanol.

Yield of *R. annamalayana, R. mucronata, R. apiculata, R. lamarrkii, A. marina, A. officinalis, Bruguiera cylindrica, Suaeda monica, Sesuvium portulacastrum* methanol leaf extracts were 12%, 11%, 13%, 16%, 10%, 13%, 16%, 10%, 16% respectively.

Total cholinesterase inhibition assay

The TChE activity was measured by the modified Ellman's colorimetric method [17,18]. 100 μL of tissue homogenate whose protein concentration is 519.4 μg was incubated with different concentration of plant extract for 45 min at RT. Enzyme control contains Tris-HCl buffer pH 8.0 instead of the extract. Methanol (10%) and Tris-HCl was used as solvent control. Reaction was arrested by the addition of 0.5 M Tris-HCl pH 8.0. Cholinesterase activity was assessed by the addition of 200 μL of 1 mM DTNB as chromogen and 200 μL of Acetylthiocholine iodide (ATCI) as substrate. Total reaction volume of Ellman's assay is 1 mL. The rate of hydrolysis of ATCI was observed as change in absorbance at 405 nm every 30 s for 3 min in UV- visible spectrophotometer (U-2800 model). Donepezil, a reversible inhibitor of cholinesterase, which is used as an effective drug for the treatment of Alzheimer's disease (AD) [19], was used as a standard drug.

Butyrylcholinesterase inhibition assay

The BChE activity was also assayed as above, using Butyrylthiocholine iodide (BTCI) as substrate instead of ATCI and inhibition studies was compared with standard drug donepezil. Enzyme control contains Tris-HCl buffer pH 8.0 instead of the extract. Methanol (10%) and Tris-HCl was used as solvent control. Enzyme activities were expressed as nmol/min/mg of protein. The experiments were done in triplicates and the values are presented as Mean ± SD.

Calculation of % of inhibition

Percentage inhibition of the mangrove plant extracts were calculated as

$$\frac{(\text{Enzyme activity of Control} - \text{Enzyme activity with the plant extract})}{(\text{Enzyme activity of Control})} \times 100$$

Calculation of IC_{50}

Various concentrations of plant extracts such as *R. annamalayana* (1.1–5.5 mg/mL), *R. mucronata* (0.2–1 mg/mL), *R. apiculata* (0.5–2.5 mg/mL), *R. lamarrkii* (1.6–8 mg/mL), *A. marina* (2.4–12 mg/mL), *A. officinalis* (2.4–12 mg/mL), *Bruguiera cylindrical* (1.2–6mg/mL), *Suaeda monica* (0.7–4.2 mg/mL), *Sesuvium portulacastrum* (1.6-8 mg/mL) were taken for the study and IC_{50} value (which shows 50% inhibition) was calculated by probit analysis method.

Statistical analysis

The assays were conducted in triplicate and the results were expressed as mean ± SD. Statistical differences between the control and treatment groups were evaluated by student t' test. $P < 0.05$ was considered to be significant.

Phytochemical screening methods

Mangroves were air dried and ground into uniform powder and aqueous extract of each sample was prepared by soaking 100 gm of dried powdered sample in 200 mL of distilled water for 72 h. The extracts were filtered using whatman filter paper No. 42. Chemical tests were carried out on the aqueous extract and on the powdered specimens using standard procedures to identify the constituents as described by Sofowara, Trease & Evans and Harborne [20–22].

Test for tannins. About 0.5 g of the dried powdered samples were boiled in 20 mL of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black coloration.

Test for saponin. About 2 g of the powdered sample was boiled in 20 mL of distilled water in a water bath and filtered. 10 mL of the filtrate was mixed with 5 mL of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion.

Test for flavonoids. 5 mL of dilute ammonia solution were added to a portion of the aqueous filtrate of each plant extract followed by addition of concentrated H_2SO_4. A yellow coloration observed in each extract indicated the presence of flavonoids. The yellow coloration disappeared on standing [20,21].

Test for terpenoids (*Salkowski test*). Five mL of each extract was mixed in 2 mL of chloroform, and concentrated H_2SO_4 (3 mL) was carefully added to form a layer. A reddish brown coloration at the inter face was formed to show positive results for the presence of terpenoids.

Test for cardiac glycosides (*Keller-Killani test*). Five mL of each extracts was treated with 2 mL of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 mL of concentrated sulphuric acid. A brown ring at the interface indicates a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer.

Test for alkaloids (Dragendorff's reagant). 1.5 mL of 10% HCl was added to about 5 mL of the extract and the mixture was heated for 20 min. It was cooled and filtered. 1 mL of Dragendorff's reagant was added. A reddish or orange colored precipitate is formed indicates the presence of alkaloids [23].

Test for phenols. 2 mL of extract was added to 2 mL of 5%ferric chloride solution ($FeCl_3$) a deep bluish green solution is formed with presence of phenols.

Results

Inhibitory effect of mangrove plant extract on TChE

TChE inhibitory activity was expressed as the mean of IC_{50} of triplicate determinations, the results of which are shown in Table I. Specific activity of TChE treated with mangrove leaf extract, in comparison with standard drug Donepezil at its IC_{50} concentration are illustrated in Table I. *Rhizophora lamarckii*, *Avicennia officinalis, Suaeda monica* and *Sesuvium portulacastrum* shows 50% inhibition at very low concentration (IC_{50} value are 0.9 mg/mL, 1.24 mg/mL, 1.42 mg/mL and 1.18 mg/mL respectively) indicating them as better inhibitors. While *Rhizophora mucronata, Rhizophora annamalayana, Avicennia marina, Brugueria cylindrica* & *Rhizophora apiculata* showed 50% inhibition at slightly higher concentrations, i.e., 1.9 mg/mL, 3.4 mg/mL, 3.2 mg/mL, 2.9 mg/mL and 2.6 mg/mL respectively. Inhibitory studies was compared with standard clinical drug Donepezil which showed 50% (IC_{50}) inhibition at concentration of 3.96 mg/mL and complete inhibition was observed at the concentration of 41.52 mg/mL. Methanol (10%) and Tris-HCl solvent control exhibited no inhibition and its activity was same as that of control.

Inhibitory effect of mangrove plant extract on BChE

BChE inhibitory activities are also expressed as above and the results are tabulated in Table II, which shows inhibitory effect of mangrove leaf extract on BChE, in comparison with standard drug Donepezil at its IC_{50} concentration. From the results it is clear that *R.lamarkii, A.officinalis, Suaeda monica* showed 50% inhibition at very low concentration of 1.26 mg/mL, 0.91 mg/mL and 0.52 mg/mL respectively when compared to *A. marina*, *S. portulagastrum*, *R. annamalayana, R. mucronata, R. apiculata B. cylindrica* which showed 50% inhibition at slightly higher concentrations. Donepezil, which is used as standard drug shows 50% inhibition at the concentration 8.87 mg/mL and complete inhibition, were observed at concentration 33.12 mg/mL.

Phytochemical analysis of mangrove plant extract

Phytochemical analysis of *Rhizophora lamarckii, Avicennia officinalis, Suaeda monica* and *Sesuvium portulagastrum* were carried out and the results are tabulated in Table III. Results revealed the presence of alkaloid, flavonoids, saponins, cardiac glycosides, terpenoids, phenolic contents and tannins. Highly positive results for alkaloids and positive results for flavonoids, cardiac glycosides, terpenoids, phenolic contents were observed in all the four species of mangrove plants. *R.Lamarkii, Suaeda monica,*

Table I. Anti – TChE activity of methanolic leaf extract of Mangrove plants.

S.No:	Plant	Family	Concentration exhibiting IC $_{50}$ (mg/mL)*
1	Donepezil	Standard drug	3.96 ± 0.13
2	*Rhizophora annamalayana*	*Rhizophoraceae*	3.41 ± 0.014
3	*Rhizophora mucronata*	*Rhizophoraceae*	1.97 ± 0.13
4	*Rhizophora lamarckii*	*Rhizophoraceae*	0.91 ± 0.007
5	*Rhizophora apiculata*	*Rhizophoraceae*	2.69 ± 0.02
6	*Avicennia officinalis*	*Avicenniaceae*	1.24 ± 0.011
7	*Avicennia marina*	*Avicenniaceae*	3.20 ± 0.022
8	*Suaeda monica*	*Chenopodiaceae*	1.42 ± 0.007
9	*Sesuvium portulacastrum*	*Aizoaceae*	1.18 ± 0.005
10	*Brugueria cylindrica*	*Rhizophoraceae*	2.92 ± 0.207

* Values are represented as Mean ± S.D

Table II. Anti – BChE activity of methanolic leaf extract of Mangrove plants.

S.No:	Plants	Family	Concentration exhibiting IC_{50} (mg/mL)*
1	Donepezil	Standard drug	8.87 ± 0.43
2	*Rhizophora annamalayana*	*Rhizophoraceae*	2.72 ± 0.06
3	*Rhizophora mucronata*	*Rhizophoraceae*	3.05 ± 0.075
4	*Rhizophora lamarckii*	*Rhizophoraceae*	1.26 ± 0.01
5	*Rhizophora apiculata*	*Rhizophoraceae*	5.39 ± 0.050
6	*Avicennia officinalis*	*Avicenniaceae*	0.911 ± 0.007
7	*Avicennia marina*	*Avicenniaceae*	1.96 ± 0.02
8	*Suaeda monica*	*Chenopodiaceae*	0.52 ± 0.018
9	*Sesuvium portulacastrum*	*Aizoaceae*	1.0 ± 0.017
10	*Brugeria cylindrica*	*Rhizophoraceae*	1.89 ± 0.038

* Values are represented as Mean ± S.D

Table III. Phytochemical screening of Mangrove leaf extract.

S.No:	Alkaloids	Flavonoids	Tannins	Saponins	Cardiac glycosides	Terpenoids	Phenols
R.Lamarkii	+++	++	+++	++	+	+	+
Suaeda monica	+++	++	+++	−	+	+	+
Sesuvium Portulagastrum	+++	++	−	+	−	+	+
Avicennia Officinalis	+++	++	+++	++	+	+	+

− No response; + low content; ++ moderate content: +++ high content

Avicennia officinalis showed highly positive result for tannins, while *Sesuvium portulagastrum* showed negative result. *Suaeda monica* showed negative result for the presence of saponins which is observed as positive for other mangrove plants.

Discussion

The main finding of present study was that certain species of mangrove plants, which possess antimicrobial and antidiabetic activity, showed anticholinesterase activity. *Rhizophora lamarckii, Avicennia officinalis, Suaeda monica, Sesuvium portulacastrum* showed effective inhibition at lesser concentrations (less than 2 mg/mL) for both TChE and BChE. From the results it is clear that methanolic extract of *Rhizophora lamarckii, Avicennia officinalis, Suaeda monica* and *Sesuvium portulacastrum* shows dual cholinergic activity i.e., they are active against both TChE & BChE. Plant extracts, which have dual anti-ChE activity, may be appropriate to patients with those forms of the disease, where the level of TChE has not yet significantly declined [24], but a possibility that BChE could hydrolyse ACh exists [4], i.e. at a moderate stage of AD.

Phytochemical analysis of mangrove leaf extracts showed the presence of alkaloids in higher concentration, moderate level of flavonoids and trace amount of terpenoids and cardiac glycosides. Presence of high concentration alkaloids, tannins and moderate amount of flavonoids and saponins in Rhizophoraceae and Avicenniacea species has been widely reported by researchers [25]. Since most of the alkaloids isolated from natural source-like Huperzine A and B from *Huperzia serrata* [26], Galanthamine from *Galanthus woronowii* [27] and lycoperine A [28] exhibited cholinesterase inhibitory activity, the anticholinesterase activity of *Rhizophora lamarckii, Avicennia officinalis, Suaeda monica* and *Sesuvium portulagastrum* might be due to presence of alkaloids in high concentration.

Conclusion

To conclude, the present study indicates that, of the nine mangrove plants screened for anti-cholinesterase activity, methanolic extracts of *Rhizophora lamarckii, Avicennia officinalis, Sesvium portulacastrum and Suaeda monica* showed the highest inhibitory activity to both ChEs. *Rhizophora lamarckii* acts as potent inhibitor of both TChE and BChE. Further work is in progress to identify the compounds responsible for inhibition of cholinesterases from *Rhizophora lamarckii*.

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