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ORIGINAL ARTICLE

In vitro antioxidant and anti-cholinesterase activities of *Rhizophora mucronata*

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Abstract

Context: *Rhizophora mucronata* Lam. (Rhizophoraceae), commonly known as Asiatic mangrove, has been used traditionally among Asian countries as folk medicine.
Objective: This study investigates the cholinesterase inhibitory potential and antioxidant activities of *R. mucronata*.
Materials and method: *Rhizophora mucronata* leaves were successively extracted using solvents of varying polarity and a dosage of 100–500 μg/ml were used for each assay. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities were assessed according to the method of Ellman. *In vitro* antioxidant activity was assessed using free radical scavenging, reducing power, and metal-chelating activity (duration – 3 months). Total phenolic and flavonoid content were quantified spectrophotometrically. Compound characterization was done using column chromatography, NMR, FTIR, and LC-MS analysis.
Results: Methanolic leaf extract (500 μg/ml) exhibited the highest inhibitory activity against AChE (92.73 ± 0.54%) and BuChE (98.98 ± 0.17%), with an IC_{50} value of 59.31 ± 0.35 and 51.72 ± 0.33 μg/ml, respectively. Among the different solvent extracts, methanolic extract exhibited the highest antioxidant activity with an IC_{50} value of 47.39 ± 0.43, 401.45 ± 18.52, 80.23 ± 0.70, and 316.47 ± 3.56 μg/ml for DPPH, hydroxyl, nitric oxide radical, and hydrogen peroxide, respectively. Total polyphenolic and flavonoid contents in methanolic extract were observed to be 598.13 ± 1.85 μg of gallic acid equivalent and 48.85 ± 0.70 μg of rutin equivalent/mg of extract. Compound characterization illustrated (+)-catechin as the bioactive compound responsible for cholinesterase inhibitory and antioxidant activities.
Conclusion: The presence of rich source of flavonoids, in particular catechin, might be responsible for its cholinesterase inhibitory and antioxidant activities.

Keywords

Alzheimer's disease, butyryl cholinesterase, catechin, enzyme kinetics, total polyphenolic conent

History

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Introduction

Alzheimer's disease (AD) is the most common type of dementia in aging adults, and a substantial burden to patients, caregivers, and the healthcare system (Marchesi, 2012). Multiple etiological factors such as amyloid-β (Aβ) peptide and/or Tau protein aggregation, excessive metal ions (Cu^{2+}, Fe^{2+}, and Zn^{2+}), oxidative stress, and reduced acetylcholine (ACh) level play a major role in the pathogenesis of AD (Povova et al., 2012). Inhibition of acetylcholinesterase (AChE), the prime enzyme in the degradation of acetylcholine (ACh), is considered as one of the approaches for the treatment of mild to moderate AD. As approved by Food and Drug Administration tacrine, donepezil, rivastigmine, and galanthamine were used for the treatment of mild to moderate AD (Mehta et al., 2012). A great deal of research has implicated that oxidative stress is an important contributor to Aβ accumulation and Tau protein hyperphosphorylation, suggesting that it plays an essential role in the pathogenesis of AD (Madeo & Elsayad, 2013; Zhao & Zhao, 2013). Involvement of reactive oxygen species in the pathogenesis of AD raises the possibility of the therapeutic use of free radical scavengers and antioxidants (Chauhan & Chauhan, 2006). Diverse pathogenic factors and safety limitation of the currently used drugs necessitates the exploration of multi-potent drug from natural source to hit more than one target implicated in AD. The tropical plant *Rhizophora mucronata* Lam. (Rhizophoraceae) widely distributed along the coastal region of Indo-Pacific region, South, and East Africa has been selected based on its use in traditional medicine and nutritional value (Bandaranayake, 2002). Traditionally, bark and leaf extract of *R. mucronata* have been used as an astringent, antiseptic, and hemostatic with antibacterial, anti-ulcerogenic, and anti-inflammatory activities (Duke & Wain, 1981; Kokpol et al., 1990b). Furthermore, this Asiatic mangrove has been used in the treatment of elephantiasis, hematuria, and diarrhea (Bandaranayake, 1998). Stem extracts of *R. mucronata* have been used to treat constipation and to cure fertility related and menstruation disorders

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(Liebezeit & Rau, 2006). Leaves of *R. mucronata* have been used traditionally as an alternate source of tea and as animal feed (Kathiresan, 1995). Pharmacological studies showed that leaves exhibited antiviral, anti-inflammatory, and anti-diabetic activities (Gaffar et al., 2011; Premanathan et al., 1999). Preliminary screening for cholinesterase and antioxidant activity of mangroves has shown that *R. mucronata* leaf extract exhibited excellent antioxidant (Suganthy et al., 2009a) and anticholinesterase activity (Suganthy et al., 2009b). Since the preliminary work was promising, further study was carried out to evaluate the therapeutic role of *R. mucronata* against AD and to identify the bioactive compound responsible for the neuroprotective effect.

Materials and methods

Preparation of plant extract

Rhizophora mucronata (voucher number AU1723) used for the present study was collected during spring season (May 2011) from Pichavaram, Thondi, Tamil Nadu, India. The mangroves were identified by Prof. K. Kathiresan, Centre for Advanced Studies in Marine Biology, Annamalai University, India. Fresh leaves were washed with water, air dried, and ground to fine powder using a kitchen blender. The powdered sample (100 g) was successively extracted with solvents (250 ml) of different polarities, such as petroleum ether (PE), hexane (HEX), benzene (BEN), dichloromethane (DCM), chloroform (CHL), ethyl acetate (EA), acetone (ACET), methanol (MET), and water (H_2O) in Soxhlet apparatus for 12 h. The extracts were concentrated to dryness in rotary vaccum evaporator (Martin Christ, Osterode am Harz, Germany) at 40 °C and the dried extracts were stored in air tight container at −20 °C for further studies. The percentage of yield was calculated using the formula given below and the results are tabulated in Table 1.

$$\text{Yield of the extract} = \frac{\left\{\begin{array}{l}\text{Wt. of the beaker with extract}\\ -\text{Wt. of the empty beaker}\end{array}\right\}}{\text{Wt. of the sample in grams}} \times 100$$

Determination of AChE and BuChE inhibitory activities

AChE and BuChE inhibitory activities were measured by slightly modifying the spectrophotometric method of Ingkaninan et al. (2000). Donepezil, the standard anti-cholinesterase drug, was used as a reference. AChE/BuChE (10 U/ml) solution (10 µl) was incubated with various concentrations of test solution (100–500 µg/ml) in 0.05 M Tris-HCl buffer (pH 8.0/7.4) for 45 min at RT. Briefly, 125 µl of 3 mM DTNB was added and the total volume was made up to 300 µl with Tris-HCl buffer (pH 8.0). Enzyme activity was initiated with the addition of 15 mM ATCI/BTCI. The hydrolysis of ATCI and BTCI was monitored by the formation of the yellow colored 5-thio-2-nitrobenzoate anion at 405 nm in Micro Elisa plate reader (Biorad Model no. 680). Percentage inhibition of AChE/BuChE was determined by comparing the rate of reaction of test samples relative to blank (Tris-HCl buffer) using the formula $(E - S)/E \times 100$, where E is the activity of enzyme without test sample and S is the activity of enzyme with test sample.

In vitro antioxidant assay

Total anti-oxidative power

Total antioxidative power of *R. mucronata* was assessed by FRAP (ferric reducing antioxidant power) assay according to the method of Benzie and Strain (1996). FRAP assay depends on the ability of sample to reduce Ferric tripyridyltriazine (Fe (III)-TPTZ) complex at low pH to intensive blue colored Ferrous tripyridyltriazine (Fe (II)-TPTZ), which is read at 593 nm. Different concentrations of various solvent fraction of *R. mucronata* leaf (100–500 µg/ml) in distilled water were treated with 1.5 ml of FRAP reagent (containing 10 mM/L TPTZ in 40 mM/L of HCl, 20 mM/L of ferric chloride in 300 mM acetate buffer pH 3.6). The absorbance was monitored for 4 min (every 10 s) at 593 nm. Aqueous solution of known $FeSO_4 \times 7H_2O$ (100–1000 µg/ml) was used as a standard for the calibration. The relative activity of the sample was compared with standard ascorbic acid (100–500 µg/ml).

Table 1. Acetylcholinesterase inhibitory activities of different solvent extracts of *R. mucronata*.

		% of inhibition ± S.D.[a]					
Solvent extract	% of Yield	100 µg/ml	200 µg/ml	300 µg/ml	400 µg/ml	500 µg/ml	IC_{50} (µg/ml)
Donepezil	1.2	82.91 ± 1.8	83.86 ± 1.8	88.6 ± 1.89	88.60 ± 5.02	94.30 ± 0.00001	5.75 ± 0.04
Petroleum ether	0.19	NI[b]	NI	NI	NI	NI	Nil
Hexane	0.28	NI	NI	NI	NI	NI	Nil
Benzene	1.41	NI	NI	NI	NI	NI	Nil
Dichloromethane	0.16	NI	NI	NI	NI	NI	Nil
Chloroform	0.06	39.39 ± 0.13	98.78 ± 0.13	99.09 ± 0.033	99.8 ± 0.000019	99.88 ± 0.000019*	117.86 ± 2.61
Ethyl acetate	0.99	72.23 ± 0.95	78.08 ± 0.47	78.48 ± 2.44	79.98 ± 3.74	81.12 ± 2.67*	69.21 ± 1.98
Acetone	4.77	34.97 ± 3.4	47.53 ± 4.52	56.43 ± 5.42	62.25 ± 1.76	62.32 ± 1.71*	259.56 ± 5.69
Methanol	4	84.30 ± 1.22*	84.55 ± 0.34	87.43 ± 1.14	88.49 ± 0.302	92.73 ± 0.54*	59.31 ± 0.35
Water	1.2	NI	NI	NI	NI	NI	Nil

[a]Results were expressed as mean ± SEM ($n = 3$).
[b]NI, no inhibition.
*$p < 0.05$.

Free radical scavenging assay

The free radical scavenging activity of *R. mucronata* was assessed by the DPPH· method according to Shimada et al. (1992). Different concentrations of *R. mucronata* leaf extracts (100–500 μg/ml) in water were incubated with 0.1 mM DPPH and the mixture was shaken vigorously, allowed to stand at room temperature for 30 min and the absorbance was read at 517 nm in UV-visible spectrophotometer (UV 2450, Shimadzu, Kanagawa, Japan). Lower the absorbance of the reaction mixture higher the free radical scavenging activity. BHT (100–500 μg/ml) was used as a positive control. The percent DPPH· scavenging effect was calculated from the following equation:

$$\text{DPPH·\ Scavenging effect}\,(\%) = \frac{A_{\text{cont}} - A_{\text{test}}}{A_{\text{cont}}} \times 100$$

where A_{cont} and A_{test} are the absorbance of the control reaction and test samples.

Hydroxyl radical scavenging activity

The ability of mangrove plants to scavenge OH· was assessed using the classic deoxyribose degradation assay as described by Halliwell et al. (1987). Different concentrations of various solvent fractions of *R. mucronata* leaf (100–500 μg/ml) in distilled water was treated with 1 ml of reaction buffer (containing 1 mM EDTA, 10 mM $FeCl_3$, 10 mM deoxyribose, 10 mM H_2O_2, 1 mM ascorbic acid, and 50 mM phosphate buffer pH 7.4). The mixture was incubated at 37°C for 1 h. The incubated mixture (1.0 ml) was mixed with 1 ml of 10% TCA and 1 ml of 0.4% TBA (in glacial acetic acid pH 3.5) to develop the pink chromagen which is measured at 532 nm. BHT (100–500 μg/ml) was used as a positive control. The hydroxyl radical scavenging activity of the extract is reported as % inhibition of deoxyribose degradation and was calculated as above.

Hydrogen peroxide scavenging activity

The ability of the *R. mucronata* to scavenge hydrogen peroxide (H_2O_2) was determined according to the method of Gülçin et al. (2003). Different doses of *R. mucronata* leaf extract (100–500 μg/ml) in distilled water were mixed with 40 mM H_2O_2 in phosphate buffer (pH 7.4). Ascorbic acid (100–500 μg/ml) was used as a positive control. The absorbance of H_2O_2 at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without H_2O_2. The percentage scavenging of H_2O_2 of samples and standard were calculated as above.

Nitric oxide radical ($NO^{\bullet}$) scavenging activity

Nitric oxide (NO) generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which was measured by the Griess reaction (Green et al., 1982). The reaction mixture (1 ml) containing sodium nitroprusside (10 mM), phosphate buffered saline (0.25 ml), and various concentrations of *R. mucronata* leaf extracts (100–500 μg/ml) was incubated at 25°C for 150 min. After incubation, 0.25 ml of the reaction mixture was mixed with 0.5 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization. Then, 0.5 ml of naphthyl ethylene diamine dihydrochloride (0.1%) was added, mixed, and allowed to stand for 30 min at 25°C. A pink colored chromophore is formed in diffused light. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions. BHT (100–500 μg/ml) was used as a positive control. The NO· scavenging activity of the mangrove extract is reported as % inhibition and was calculated as above.

Total reduction power

Total reducing capacity of mangrove plants was determined according to the method of Oyaizu (1982). Different doses of *R. mucronata* leaf extracts (100–500 μg/ml) in 0.25 ml of distilled water were mixed with phosphate buffer (0.5 ml, 0.2 M pH 6.6) and potassium ferricyanide (0.5 ml, 1%) and the mixture was incubated at 50 °C for 20 min. 0.5 ml of 10% TCA was added to the reaction mixture and centrifuged at 1000 *g* for 10 min. The upper layer of solution (0.5 ml) was mixed with distilled water (0.5 ml) and $FeCl_3$ (0.1 ml, 0.1%) and the absorbance was measured at 700 nm. Ascorbic acid (100–500 μg/ml) was used as a positive control. Higher the absorbance of the reaction mixture, greater the reducing power.

DNA nicking assay

DNA nicking assay was performed using super-coiled pUC 18 plasmid DNA according to the Lee et al. (2002) method. Plasmid DNA (0.5 μg) was added to Fenton's reagents (30 mM H_2O_2, 50 μM ascorbic acid, 80 μM $FeCl_3$) containing 10 μl (1 mg/ml) of *R. mucronata* leaf extracted with different solvents and the final volume of the mixture was brought up to 20 μl. The mixture was then incubated for 30 min at 37 °C and the DNA was analyzed on a 1% agarose gel electrophoresis followed by ethidium bromide staining.

Metal chelating activity

The metal chelating ability was determined according to the method of Haro-Vicente et al. (2006). Briefly, different concentrations (100–500 μg/ml) of various solvent extract of *R. mucronata* were added to 0.15 mM ferrous sulfate solution and the reaction was initiated by the addition of ferrozine (0.5 mM). The mixture was shaken vigorously and incubated for 20 min at room temperature; the absorbance was measured at 562 nm. EDTA (20–100 μg/ml) was used as a positive control.

Determination of total phenolics, flavonoids, and flavonol contents

Total soluble phenolic compounds in different solvent extracts of leaves of *R. mucronata* were determined with the Folin–Ciocalteu reagent according to the method of Singleton and Rossi (1965) using gallic acid as a standard. The sample (100 μl) (1 g of dry sample in 10 ml of acetone) in duplicates was incubated with 1 ml of diluted Folin's–Ciocalteu's reagent (1:2 with water) at RT for 5 min. 7% Na_2CO_3 (1 ml) was added to the reaction mixture and incubated at RT for

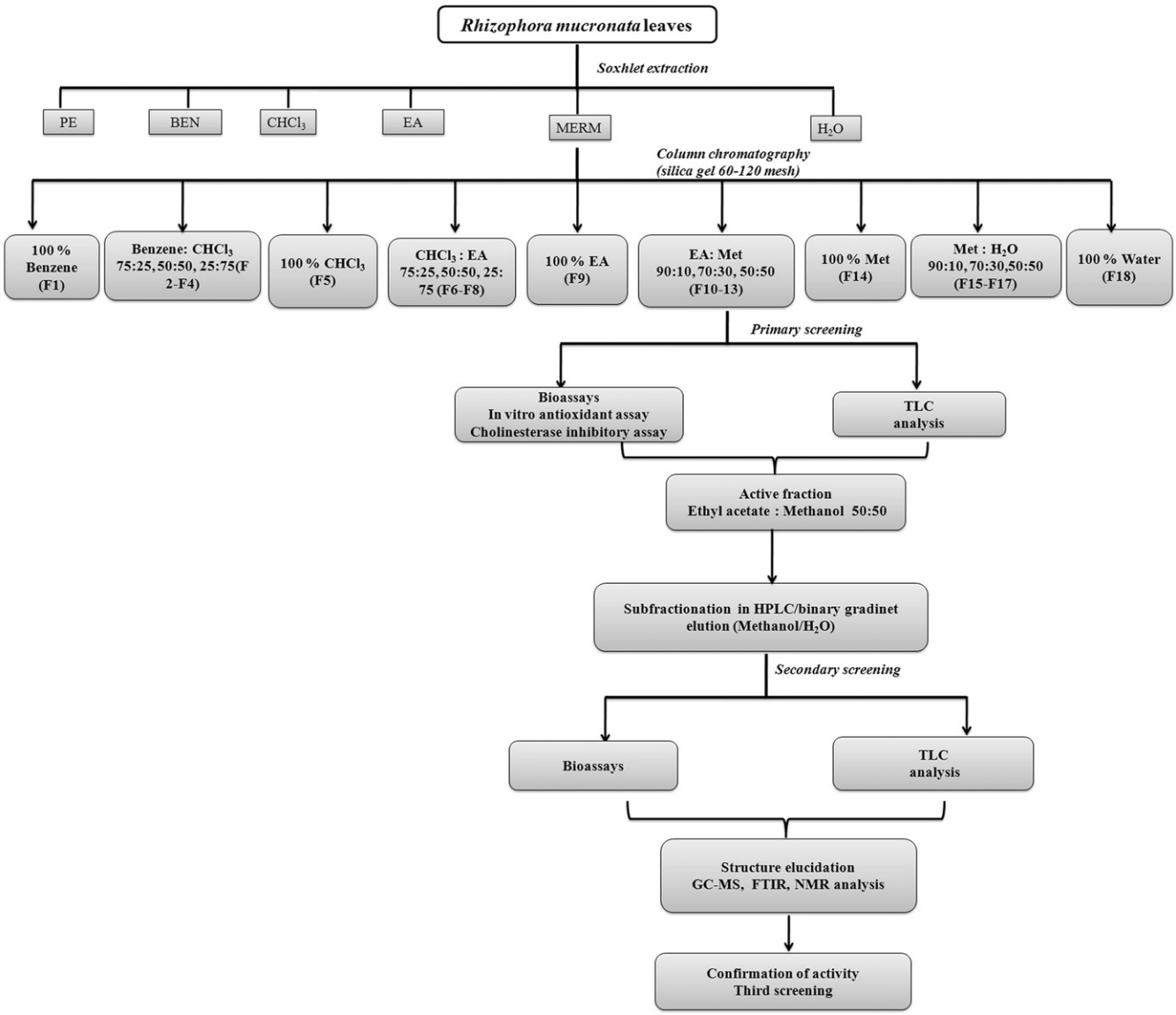

Figure 1. Schematic representation of bioactive guided fractionation of methanolic extract of *Rhizophora mucronata*.

90 min and the absorbance was read at 750 nm. The total phenolic content was expressed as gallic acid equivalent (GAE) in milligram per gram of dry sample. Total flavonoids were estimated using the method of Ordonez et al. (2006). To 0.5 ml of methanolic extract, 0.5 ml of 2% $AlCl_3$ ethanol solution was added and incubated for 1 h at RT. The absorbance was measured at 420 nm. A standard calibration curve was prepared using rutin and the flavonoids were expressed in mg rutin equivalents (RE) per gram DW.

LC-MS analysis

Mass spectrum of the methanolic extract of *R. mucronata* was recorded in LC-MS (LCMS-2010 A, Shimadzu, Kanagawa, Japan). Electron spray ionization with full scan including both positive and negative modes of ionization was used for analyzing the compound. The identity of the compound and its molecular mass was confirmed by comparing their *m/z* ratio with those on the stored library (Metwin version 2.0, MetLife India Insurance Company, Chennai, India).

Purification and characterization of compound

Fractionation of methanolic extract by column chromatography

Freeze dried bioactive methanolic extract (35 g) was subjected to silica gel column ($100 \times 35\,mm^2$) and eluted with linear gradient of solvent (benzene, chloroform, ethyl acetate, methanol, and water) with increasing polarity (Figure 1). Fractions were collected and freeze dried, and the bioactivity was screened based on antioxidant and cholinesterase inhibitory activities.

Sub-fractionation of active fraction using HPLC

Active fraction with similar R_f value was pooled and subjected to further purification using UFLC (Shimadzu, Kanagawa, Japan) with Luna reverse phase preparative C18 column ($250 \times 10\,mm^2$ dimension), PDI detector, and LC6AD pumping system. Samples were prepared in HPLC grade methanol and filtered using 0.2 µm filter. The injection

volume was set to 1 ml. Elution was carried out by the binary gradient method using methanol:water as the mobile phase with a flow rate of 1 ml/min. The active fractions were subjected to TLC to analyze the number of phyotconstituents.

Analysis of active subfraction by TLC

TLC was performed on the $20 \times 20\,cm^2$ plates precoated with silica (Silica gel GF 254, Merck, Darmstadt, Germany). One-dimensional TLC was performed with butanol:acetic acid: water – 14:1:3.5 as the mobile phase. The developed plates were sprayed with 1% vanillin sulfuric acid and air dried. The plates were then heated at 120 °C for 10 min and visualized in day light. For flavonoid detection, the plates were sprayed with 20% aluminum chloride and visualized at 366 nm. Anisaldehyde sulfuric acid was used for the detection of terpenoids. For glycoside detection, the chromatogram was sprayed with 25% antimony trichloride in chloroform and heated at 105 °C for 5 min and viewed under UV at 366 nm.

Spectroscopic analysis

Freeze dried bioactive fraction (100 mg) was subjected to Fourier infra red spectroscopy (FTIR) analysis (JASCO 1400, JASCO, Tokyo, Japan). For FTIR analysis, the samples were prepared in potassium bromide discs and scanned within 500–4000 cm^{-1} range. Samples were dissolved in deuterated methanol and subjected to ^1H and ^{13}C NMR (Ultrashield-400 mHz, Bruker, Fallanden, Switzerland). Sample in methanol was subjected to UV shift analysis in UV–visible spectroscopy (U-2450, Shimadzu, Kanagawa, Japan). Melting point of the active compound was assessed using melting point apparatus (Tempo equipment Pvt Ltd., Mumbai, India).

Compound (1)

Buff-colored needles, molecular formula $C_{15}H_{14}O_6$; MS: negative ES-MS, *m/z*: 290; melting point 176°; $[\alpha]_D^{20} = +16$ (CH_3OH); UV $\lambda_{max}^{CH_3OH}$ nm: 210, 272, 311 (Figure S1). IR ν_{max}^{KBR} cm^{-1} 3344 (hydroxyl gp), 2922, 2851, 1629, 1521, 1469, 1285, 1146, 1078, 1031, 815, 765, 721, 704 (aromatic gp) (Figure S2). ^1H NMR (δ, CH_3OH, 400 MHz) 5.86 (1H, d, $J = 2.0$ Hz, H-6), 5.93 (1H, d, $J = 2.0$ Hz, H-8), 4.56 (1H, d, $J = 7.6$ Hz, H-2), 3.98 (1H, m, H-3), 2.50 (1H, dd, $J = 16.0$ and 8.0 Hz, H-4 ax), 2.84 (1H, dd, $J = 16.0$ and 5.2 Hz, H-4 eq), 6.84 (1H, d, $J = 2.0$ Hz, H-2′), 6.72 (1H, dd, $J = 8.0$, H2, H-5′) (Figure S3). ^{13}C NMR (δ, CH_3OH, 100 MHz); 82.84 (C-2), 68.82 (C-3), 28.51(C-4), 157.58 (C-5), 96.35 (C-6), 157.80 (C-7), 95.57 (C-8), 156.92 (C-9), 100.89 (C-10), 132.22 (C-1′), 115.29 (C-2′).

Statistical analysis

All determinations were done in triplicate, and the results are reported as mean ± S.D. Significant differences between means were determined by one-way ANOVA followed by Duncan's multiple range tests and p value <0.05 were regarded as significant. IC_{50} value was calculated by the Probit analysis method.

Result and discussion

Alzheimer's disease (AD) and Parkinson's disease (PD) are the most prevalent neurodegenerative disorders of the twenty-first century affecting mainly the elderly population (Vangilder et al., 2011). AD is mainly characterized by cognitive deficits due to the decrease in the level of acetylcholine followed by degeneration of cholinergic neurons in the basal forebrain (Giacobini, 2003). Accumulating evidence suggests that oxidative stress is involved in the mechanism of Aβ-induced neurotoxicity. Increase in calcium influx followed by Aβ-induced oxidative stress increases the activity of AChE, which promotes the assembly of Aβ peptide into fibrils thereby inducing cytotoxicity of cholinergic neurons (Devore et al., 2010; Feng & Wang, 2012; Melo et al., 2003). Currently, AChE inhibitors (AChEI) have proven to be the most viable therapeutic target for symptomatic improvement of AD, as AChEIs enhance neuronal transmission (Loizzo et al., 2008). In addition, antioxidant therapy has also been proven to be successful in improving cognitive function and behavioral deficits in AD animal models (Danta & Piplani, 2014). Based on these observation, it has been hypothesized that natural antioxidants with potent

Table 2. Butyrylcholinesterase inhibitory activities of different solvent extracts of *R. mucronata*.

	% of inhibition ± S.D.[a]					
Solvent extract	100 µg/ml	200 µg/ml	300 µg/ml	400 µg/ml	500 µg/ml	IC_{50} (µg/ml)
Donepezil	90.35 ± 0.53	95.24 ± 0.306	97.52 ± 0.505	98.05 ± 0.11	98.92 ± 0.23	5.53 ± 0.06
Petroleum ether	80.99 ± 2.74	82.59 ± 2.09	90.41 ± 2.82	90.64 ± 0.34	94.54 ± 0.72*	60.21 ± 0.23
Hexane	NI[b]	NI	NI	NI	NI	Nil
Benzene	74.45 ± 0.07	86.10 ± 8.9	86.31 ± 6.68	88.13 ± 3.17	90.38 ± 1.12*	67.154 ± 2.9
Dichloromethane	88.07 ± 2.88	87.13 ± 2.0	91.14 ± 0.35	95.16 ± 1.13	95.75 ± 0.70*	57.06 ± 1.20
Chloroform	95.98 ± 0.54	96.93 ± 1.59	97.04 ± 0.20	97.16 ± 0	97.75 ± 0.20*	52.09 ± 0.29
Ethyl acetate	93.50 ± 0.20	93.50 ± 0.20	94.80 ± 0.89	96.45 ± 1.97	97.52 ± 1.84*	53.48 ± 0.40
Acetone	97.27 ± 0.30	97.88 ± 0	98.18 ± 0.30	98.02 ± 0.46	98.48 ± 0.000013	51.64 ± 0.24
Methanol	96.66 ± 1.09	98.07 ± 0.17	98.15 ± 0.52	98.88 ± 0.76	98.98 ± 0.17*	51.72 ± 0.33
Water	77.49 ± 0.76	77.90 ± 8.47	79.40 ± 3.02	79.71 ± 3.78	84.66 ± 1.55*	64.58 ± 1.7

[a]Results were expressed as mean ± SEM ($n = 3$).
[b]NI, no inhibition.
*$p < 0.05$.

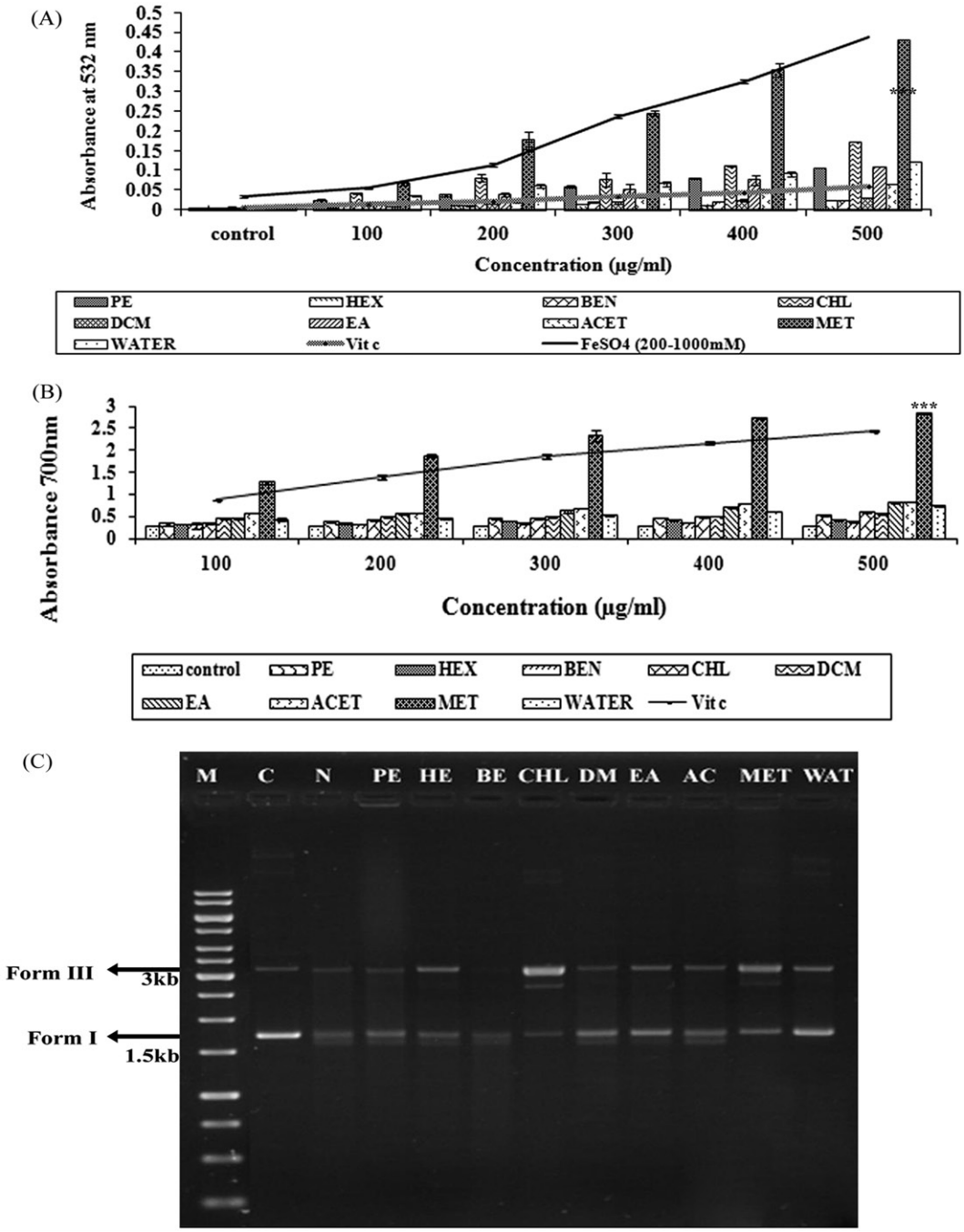

Figure 2. (A) Ferric reducing antioxidative power of different solvent extracts of *R. mucronata* (100–500 µg/ml) in comparison with L-ascorbic acid (100–500 µg/ml). (B) Reducing power of different solvent extracts of *R. mucronata* (100–500 µg/ml) in comparison with standard L-ascorbic acid, (C) inhibitory effects of *R. mucronata* extract on DNA nicking caused by hydroxyl radicals, 0.5 µg of pUC18 plasmid DNA to Fenton's reaction solution in the absence (lane 3) or presence of different solvent fractions of *R. mucronata* (1 mg/ml) for 30 min at 37 °C [lane 4 (PE), lane 5 (HE), lane 6 (BE), lane 7 (CHL), lane 8 (DM), Lane 9 (EA), lane 10 (Ac), lane 11 (Me), lane 12 (Water). Lanes 1 and 2 show the DNA molecular marker and native plasmid DNA, respectively].

cholinesterase inhibitory activity can act as better drug for the treatment of AD and other age-related neurodegenerative disorders (Zhao & Zhao, 2012). Till date studies on terrestrial plants involving its cholinesterase inhibition and antioxidant activities have been reported enormously, but reports on marine resources especially mangroves are still at its infancy, which prompted us to study the antioxidant and cholinesterase inhibitory activities of *R. mucronata*. In the present study, we investigated the antioxidant and cholinesterase inhibitory activities of different solvent extracts of leaf components of *R. mucronata* in cell-free *in vitro* assays. The percentage yield of *R. mucronata* leaf extracted with different solvents is shown in Table 1.

Acetylcholinesterase and butyrylcholinesterase inhibitory activity

Experimental evidences illustrate that AChE accelerates aggregation of Aβ peptide and formation of Aβ–AChE complex at the synaptic region of hippocampus leading to

Table 3. Antioxidative properties of *R. mucronata* leaves extracted with different solvents.

Sample	Total phenolic content/mg of extract (μg gallic acid equivalent/mg)[a]	Total flavonoid (μg of rutin equivalent/mg of extract)[a]	% of inhibition				
			DPPH	$OH^•$ scavenging assay	$NO^•$ scavenging assay	H_2O_2 scavenging assay	Metal chelating activity
Std BHT	–	–	96.6 ± 0.07	95.99 ± 0.2	87.77 ± 0.01	98.8 ± 0.06	85.08 ± 0.20
PE	50.65 ± 0.5	–	20.21 ± 0.05	90.65 ± 0.07	54.21 ± 0.02	96.47 ± 0.04	100.0 ± 0.02
HEX	12.36 ± 0.31	–	87 ± 0.02	86.31 ± 0.08	55.84 ± 0.01	26.6 ± 0.02	36.04 ± 0.04
BEN	30.81 ± 0.27	–	67.2 ± 0.05	84.9 ± 0.03	65.48 ± 0.07	76.99 ± 0.02	43.39 ± 0.05
DCM	79.25 ± 0.35	–	89.54 ± 0.0	60.97 ± 0.03	50.63 ± 0.01	90.84 ± 0.09	34.03 ± 0.05
CHL	115.2 ± 0.62	20.9 ± 0.04	74.79 ± 0.01	84.76 ± 0.1	83.69 ± 0.04	41.99 ± 0.08	24.25 ± 0.07
EA	596.75 ± 0.35	42 ± 0.05	60.75 ± 0.07	83.41 ± 0.02	94.86 ± 0.01	95.13 ± 0.04	9.94 ± 0.03
AC	65.68 ± 0.59	42.50 ± 0.03	48.34 ± 0.06	85.54 ± 0.04	84.58 ± 0.19	83.46 ± 0.06	59.41 ± 0.01
MET	598.13 ± 1.85*	48.85 ± 0.70*	91.05 ± 0.07*	95.16 ± 0.02*	89.13 ± 0.06*	96.4 ± 0.04*	90.21 ± 0.02*
H_2O	64.99 ± 0.06	41.50 ± 0.04	39.64 ± 0.05	60.45 ± 0.09	57.95 ± 0.01	45.30 ± 0.04	98.02 ± 0.02

[a]Each experiment was performed in triplicate and the results are mean ± SD. Values in the table are significantly different (*$p < 0.05$).

neuronal degeneration (Dinamarca et al., 2010; Reyes et al., 2004). Symptomatic treatment for AD involves potentiation of cholinergic activity through the inhibition of AChE (Rahman & Choudhary, 2001). Therefore, the current study was carried out to assess the AChE inhibitory activity of different solvent extracts of *R. mucronata*. Results showed that among the different solvent extracts, chloroform and methanolic extracts (500 μg/ml) showed significant ($p < 0.05$) AChE inhibitory activity with IC_{50} values of 59.31 ± 0.35 and 67.86 ± 2.61 μg/ml, respectively, when compared with positive control (IC_{50} value of 5.75 ± 0.04 μg/ml) (Table 1).

BuChE was found mainly in the gilal cells and subcortical neurons also co-regulate cholinergic neurotransmission through hydrolysis of ACh. Reports have shown that as AD progresses, the level of BuChE increases, which promotes the transformation of ''benign'' plaques to ''malignant'' plaques ultimately leading to neuronal degeneration (Darvesh et al., 2012; Guillozet et al., 1997). Inhibition of BuChE not only increases the level of ACh in the brain but also impedes the formation of beta amyloid plaques (Greig et al., 2005). Hence, in the present study, the BuChE inhibitory activity of various concentrations of solvent extracts of *R. mucronata* was evaluated. Results showed that all the extracts except hexane showed significant ($p < 0.05$) inhibitory activity when compared with control (Table 2). Comparing the inhibitory activities of the extracts for BuChE, methanolic and acetone extracts showed significant ($p < 0.05$) inhibition with the IC_{50} value of 51.64 ± 0.24 and 51.72 ± 0.33 μg/ml, respectively. Hodges (2006) and Zimmermann et al. (2004) reported that inhibition of AChE and BuchE not only plays a key role in enhancing the cholinergic neurotransmission but also in reducing the aggregation of Aβ peptide, the key pathogenic step in AD. From the results, it is clear that the methanolic extract of *R. mucronata* can act as a potent neuroprotectant by effectively inhibiting both AChE and BuChE, thereby restoring the level of ACh in synaptic junction.

In vitro antioxidant assays

Multiple evidence has shown strong implications that oxidative stress-mediated damages play an essential role in the pathogenesis of several neurodegenerative diseases such as AD. Therefore, development of approaches to prevent or reduce oxidative damages may provide therapeutic efficacy, and antioxidants have been identified as part of these therapeutic strategies for AD (Devore et al., 2010; Feng & Wang, 2012). In the present study, the antioxidative property of different solvent extracts of *R. mucronata* was assessed using a battery of *in vitro* antioxidant assays (DPPH, hydroxyl radical, nitric oxide, H_2O_2 scavenging activity, metal-chelating, reducing power, and FRAP assays) and the results are shown in Figure 2 and Table 3. Results of total antioxidative power and reducing capacity showed that among the extracts, methanolic extract (500 μg/ml) showed significantly ($p < 0.05$) highest ferric reducing capacity and reducing power when compared with positive control ascorbic acid (Figure 2A and B). Highest $DPPH^•$, H_2O_2, $NO^•$ scavenging activity, and metal chelating activity were observed in methanolic extract followed by ethyl acetate extract (Table 3). DNA nicking assay illustrated that methanolic leaf extract exhibits excellent $OH^•$ scavenging activity when compared with other extracts (Figure 2C). Total polyphenolic and flavonoid contents of *R. mucronata* extract are shown in Table 3. Total polyphenolic and flavonoid contents in methanolic extract were observed to be 598.13 ± 1.85 μg of gallic acid equivalent and 48.85 ± 0.70 μg of rutin equivalent/mg of extract, respectively. High polyphenolic and flavonoid contents in methanolic extract might be responsible for the potent antioxidative and anticholinesterase activities as reported by Rice-Evans et al. (1996). Classical TLC analysis showed spots with chromatographic behavior identical to terpenoids, flavonoids, and tannins (Figure 3A(i–iii)).

LC-MS analysis of methanolic extract

LC-MS analysis showed the presence of compounds belonging to flavonoids, terpenoids, and sugar (Table 4 and Figure S5). Flavonoids (catechin, 3′,4′,5,7-tetrahydroxy-3,6,8-trimethoxy flavone), phenolic acids (syringic acid, catechol, coumaric acid, and methyl ellagic acid), lignans (coumaryl alcohol, caffeoyl alcohol, scopoletin, magnolol, and xanthotoxin), alklaoids (hygrine and synephrine), and

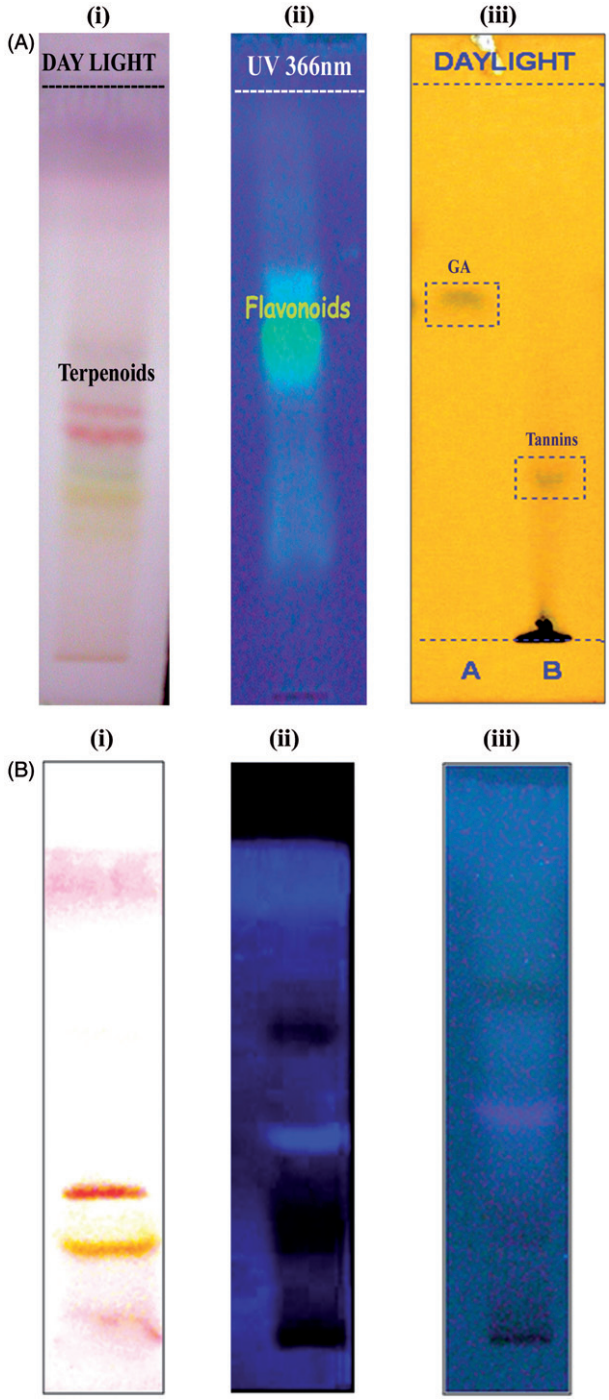

Figure 3. (A) HPTLC chromatogram of methanolic fraction of leaves of *R. mucronata* (i) *p*-anisaldehyde sulfuric acid; (ii) plates sprayed with 20% aluminum chloride; (iii) plates sprayed with 10% ferric chloride. (B) HPTLC chromatogram of column fraction F13. Plates sprayed with (a) *p*-anisaldehyde sulfuric acid; (b) 20% aluminium chloride; (c) 25% antimony trichloride in chloroform.

terpenoids (β-amyrin, myrcene, and lupeol) were the main constituents present in methanolic leaf extract of *R. mucronata*.

Bioactivity guided fractionation and structural elucidation

Bioactive methanolic extract was subjected to column fractionation and the results illustrated that among the 18 fractions eluted, F13 (E.A.:MET–1:1) showed significantly ($p < 0.05$) highest antioxidative capacity and reducing power, but similar when compared with the same concentration of standard ascorbic acid (Table 5). Highest DPPH radical scavenging activity was observed in F13 ($94.42 \pm 0.02\%$) when compared with standard BHT ($88.52 \pm 0.02\%$). Cholinesterase inhibitory activity showed that among the 18 fractions eluted, F13 (50–100 µg/ml) showed significantly ($p < 0.05$) highest inhibitory activity against both AChE and BuChE (97.83 ± 0.0003 and $98.58 \pm 0.003\%$, respectively). Cholinesterase inhibitory activity of F13 was similar to the inhibitory activity of standard reference donepezil (Table 5). TLC chromatogram of F13 revealed the presence of terpenoids (pink bands, an R_f value of 0.51), flavonoids (yellow fluorescent bands with an R_f value of 0.81), and glycosides with an R_f value of 0.88 (Figure 3B(i–iii)).

Table 4. Compounds identified by LC-MS analysis in methanolic extract of *R. mucronata*.

S. no.	Compound name	Molecular mass
1.	Syringic acid	198.18
2.	Catechol	290.28
3.	Beta-amyrin	426.73
4.	Coumaric acid	116.08
5.	Octanal	128.22
6.	Oxal acetic acid	132.08
7.	Myrecene	136.24
8.	Hygrine	141.22
9.	Coumarin	146.15
10.	Coumaryl alcohol	150.18
11.	Caffeoyl alcohol	166.18
12.	Synephrine	167.21
13.	Scopoletin	192.17
14.	Glucuronic acid	194.15
15.	Magnolol	266.34
16.	Linolenic acid	278.44
17.	Rutinose	342.31
18.	Methyl ellagic acid	344.28
19.	Lupeol	426.73
20.	Cysteine sulfoxide	177.22
21.	Xanthotoxin	216.20
22.	Catechin	290.27
23.	3′,4′,5,7-Tetrahydroxy-3,6,8-trimethoxyflavone	376.32

Sub-fractionation of F13 in reverse phase UPLC showed the presence of eight peaks indicating the presence of eight different phytoconstituents (Figure 4). Among the sub-fractions, F3 (retention time (RT) 6.2 min), F7 (RT 32.37 min), and F8 (RT 32.74 min) showed potent dual cholinergic activity and antioxidant activity (Table 5). TLC analysis of F3 showed single yellow fluorescence band in UV at 366 nm indicating the presence of flavonoids (Figure 5A). The structural determination of compound in F3 was established using spectral methods and their spectroscopic data were in full agreement with those of standard samples.

Characterization of compound 1

Compound **1** (Figure 5B), buff-colored needle (melting point: 176 °C), showed positive reaction with alcoholic ferric chloride (blue color) and shinoda test. On the basis of ^{1}H and ^{13}C NMR data, the molecular formula for compound **1** was identified as $C_{15}H_{14}O_6$ (M^+, *m/z*: 290.27). The UV

Table 5. Antioxidant and cholinesterase inhibitory activity of fractions and (+)-catechin.

Sample	Antioxidative property			% of inhibition	
	DPPH (% inhibition)	FRAP assay (absorbance 532 nm)	Reducing power (absorbance 700 nm)	Acetyl cholinesterase	Butyryl cholinesterase
Fraction 13[a] (ethyl acetate:methanol 1:1)	94.42 ± 0.005	0.092 ± 0.012	1.753 ± 0.014	97.83 ± 0.0003	98.58 ± 0.003
Fraction 3 (catechin)[b]	89.89 ± 0.005	0.314 ± 0.002	2.01 ± 0.013	93.20 ± 0.0012	92.50 ± 0.0043
Fraction 7[b]	80.41 ± 0.001	0.168 ± 0.0004	1.61 ± 0.008	64.78 ± 0.006	88.60 ± 0.001
Fraction 8[b]	85.49 ± 0.006	0.131 ± 0.0002	1.11 ± 0.013	75.39 ± 0.001	89.26 ± 0.001
Standard donepezil	–	–	–	99.25 ± 0.00014	94.03 ± 0.00012
Standard BHT/ascorbic acid	84.98 ± 0.005	0.386 ± 0.0015	2.02 ± 0.070	–	–

[a]Fraction eluted using column chromatography.
[b]Fraction eluted using RPHPLC.

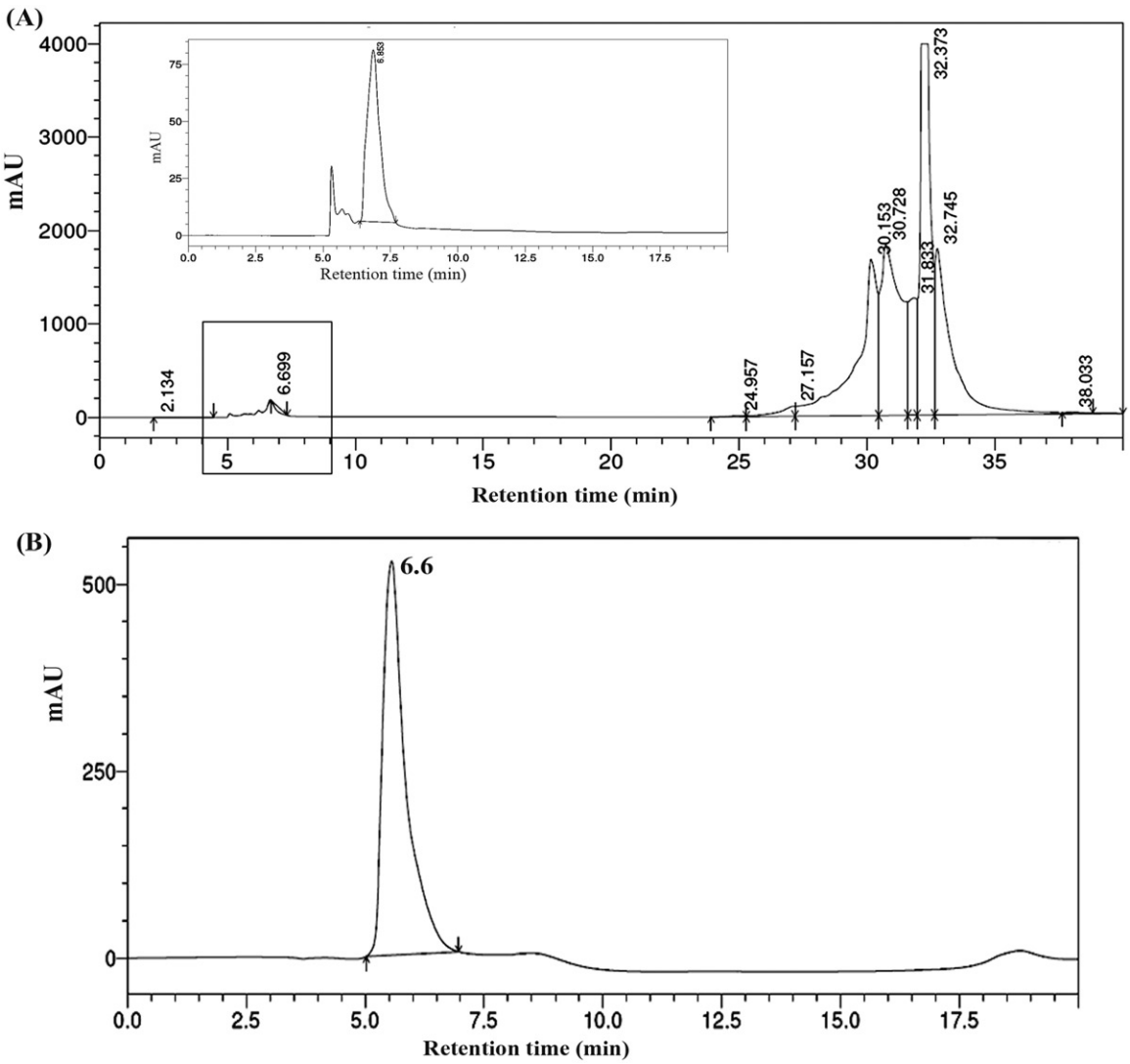

Figure 4. HPLC chromatogram of (A) fraction 13 eluted by column chromatography and (B) purified active fraction 3.

spectrum of the compound **1** in methanol exhibited three peaks 210, 270, and 310 nm with a maximum peak at 270 nm suggesting the presence of flavan skeleton. Specific rotation of the compound **1** was observed to be +16. The IR spectrum showed the presence of the hydroxyl group (3344 cm^{-1}) and aromatic system (1629, 1521, 1469, 815, 765, 721, 704 cm^{-1}). No carbonyl peak was detected showing the absence of flavan carbonyl moiety. The identity of the compound (+)-catechin (**1**) was confirmed by the comparison of the physical and spectroscopic data (UV, IR, ^{1}H and ^{13}C NMR, ESI-MS) with those reported in the literature (Hye et al., 2009; Sarg et al., 2011).

Enzyme kinetic studies

Pharmacological screening of the purified compound (+)-catechin (**1**) revealed potent antioxidant, AChE, and BuChE inhibitory activities with IC_{50} values of 25.25 ± 0.02, 36.13 ± 0.01, and 20.02 ± 0.02 µg/ml, respectively. Enzyme kinetic studies of methanolic extract of *R. mucronata* and catechin (**1**) against AChE and BuChE were assessed using the Lineweaver burk plot and the results are tabulated in Table 6. In AChE inhibitory studies, (+)-catechin (**1**) and methanolic extract showed significant decrease in the K_m value with no change in the V_{max} value, which indicates the type of inhibition as competitive. Donepezil and galanthamine the long-acting AChE inhibitor used for the treatment of AD showed a similar type of competitive type inhibition toward AChE (Khan et al., 2009; Sugimoto et al., 2000). In the case of BuChE, catechin (**1**) showed significant changes in both K_m and V_{max} value, which indicates it as mixed-type inhibition. A similar type of mixed inhibition was observed in cardiovascular drugs and benzodiazepines against BuChE (Chiou et al., 2005). Several reports have shown that green tea catechins and its derivatives exhibited potent neuroprotective effect in AD by attenuating oxidative stress and inflammatory mediated damage (Stevens et al., 2002; Sutherland et al., 2006). Recent studies have shown that green tea catechins enhance cholinergic function by inhibiting AChE activity and prevent neuronal damage by effectively blocking the aggregation of Aβ peptide (Okello et al., 2012; Wang et al., 2012). Moreover, Wu et al. (2012) reported the blood–brain permeability of catechins, which illustrates that catechins can act as drug for the treatment of AD.

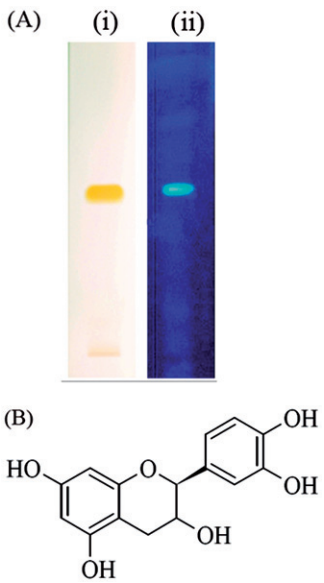

Figure 5. (A) HPTLC chromatogram of purified compound **1** – (i) ammonia vapors viewed under visible light and (ii) alcoholic aluminum chloride viewed under UV 366 nm. (B) The structure of purified compound **1** – (+)-catechin.

Scientific evidences have illustrated AChE inhibitor as most viable therapeutic target for symptomatic improvement in AD because AChE not only enhances the cholinergic neurotransmission in the brain but also reduce the aggregation of β-amyloid, the key factor in AD (Dinamarca et al., 2010). Recent reports have showed that in addition to cholinergic deficit, oxidative stress also plays a pivotal role in the cognitive impairment of AD. Hence, intake of antioxidants may reduces the risk of AD and minimize neuronal degeneration (Feng & Wang, 2012). Drugs that combine inhibition of AChE and BuChE with the ability to reduce oxidative and nitrative stress may be more efficacious than those that only inhibit ChE in preventing and/or treating AD (Yanovsky et al., 2012). In the present study, it has been observed that (+)-catechin (**1**) isolated from methanolic extract of *R. mucronata* leaves showed dual cholinergic activity and antioxidant activities dose dependently, which according to the previous report (Feng & Wang, 2012) can act as potent neuroprotectant.

Conclusion

Overall the results conclude that (+)-catechin, isolated from *R. mucronata* leaves can act as multipotent drug for the treatment of AD through its cholinesterase inhibitory activity, antioxidant, and metal-chelating activities. Blood–brain permeability of (+)-catechin makes it a suitable candidate for the development of neuroprotective agents. Further work is underway to assess the neuroprotective effect of (+)-catechin against beta amyloid induced toxicity under *in vitro* and *in vivo* conditions.

Declaration of interest

The authors have declared that there is no conflict of interest. K. P. D. wishes to thanks DST, India, and N. S. wishes to thanks CSIR, India, for the financial assistance. The authors gratefully acknowledge the computational and bioinformatics facility provided by the Alagappa University Bioinformatics Infrastructure Facility (funded by Department of Biotechnology, Government of India; Grant no. BT/BI/25/015/2012).

Table 6. Enzyme kinetic study of MERM and bioactive compound.

	AChE			BuChE		
Kinetic parameters	Control	MERM	Purified compound **1**	Control	MERM	Purified compound **1**
V_{max} (mM/min/mg of protein)	0.05 ± 0.002	0.043 ± 0.001	0.04 ± 0.0001	0.002 ± 0.0001	0.005 ± 0.0008	0.004 ± 0.0003
K_m (mM)	21.49 ± 0.10	9.75 ± 3.30	11.71 ± 6.54	0.97 ± 0.18	10.59 ± 2.01	8.53 ± 0.49
K_i (mM)		0.36 ± 0.001	49.58 ± 0.04	–	127.11 ± 1.07	24.11 ± 2.56
Type of inhibition	–	Competitive	Competitive	–	Mixed type inhibition	Mixed type inhibition

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Supplementary material available online
Supplementary Figures S1, S2, S3 and S5.