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In vitro Acetylcholinesterase Inhibitory Activity of Selected Sri Lankan Medicinal Plants

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Abstract

Acetylcholinesterase (AChE) inhibition is a well-accepted therapeutic strategy for Alzheimer's disease and many categories of dementia. Medicinal plants are promising sources of useful AChE inhibitors and have been used to treat Alzheimer's disease by people around the world. This investigation was carried out to assess the AChE inhibitory activities of the crude organic extracts of nine Sri Lankan medicinal plants. Air dried, powdered samples of different plant parts were sequentially extracted with 3 organic solvents to yield a total extract of the individual plant part. These extracts were tested for AChE inhibitory activity using Ellman's assay in 96-well microplates. Galantamine (IC_{50} 1.57 ±0.01 µg/ml) was used as the standard acetylcholinesterase inhibitor and all the tests were done in triplicates. Potent AChE inhibitory activities were shown by the leaf extracts of *Wrightia antidysenterica* and *Flueggea leucopyrus* with IC_{50} values of 64±0.5 µg/ml and 107±0.1 µg/ml, respectively. Furthermore, *Zingiber cylindricum* rhizome extract and *Areca concinna* seed extract also exhibited considerable AChE inhibitory activities with IC_{50} values of 189±1.4 µg/ml and 217±1.2 µg/ml, respectively. Hence, it can be concluded that *W. antidysenterica* and *F. leucopyrus* possess potent anti-cholinesterase activity and can be used to isolate drug leads with anti-acetylcholinesterase activity.

Keywords: Acetylcholinesterase; *Wrightia antidysenterica*; *Flueggea leucopyrus*; Ellman's assay

Introduction

Alzheimer's disease is the most prevailing neurodegenerative disease and is becoming one of the major human mental health concerns today. Neuronal death and great synaptic loss in the brain regions accountable for cognitive functions, especially the hippocampus, the entorhinal cortex, cerebral cortex and the ventral striatum are the main characteristics of Alzheimer's disease (1). Previous research works have revealed that the malfunction of the cholinergic system can cause memory deficiency (2). Brains of the affected patients with Alzheimer's disease had shown deterioration of acetyl-cholinergic neurons. Enzyme acetylcholinesterase (AChE) cause activities of cholinergic neurons to be lessened in cerebral cortex and modification of acetylcholinesterase activity in the parietal and frontal cortex has a relationship with dementia (3). Pharmacological treatment for this disease has not yet at a satisfactory level for controlling neurodegeneration or any other alternative.

Medicinal plants have been used to treat neurodegenerative diseases including Alzheimer's disease and memory related disorders for many years in different parts of the world (4). Converse to synthetic drugs, it

is reported that drugs of plant origin are not coupled with many side effects and have a vast therapeutic potential to cure various diseases (5). Several scientific investigations have revealed the importance of medicinal plants in the enhancement of nervous system function (6). Therefore, numerous compounds having potential anticholinesterase activity have been isolated from plant families such as Arecaceae, Apocynaceae, Zingiberaceae and Lycopodiaceae (6,7).

Huperzine A, a natural compound isolated from the medicinal plant *Huperzia serrata*, is a selective inhibitor of AChE. Subsequently, Huperzine A was subjected to extensive studies as a lead compound for the expansion of novel effective anti-acetylcholinesterase medications for the treatment of Alzheimer's disease comparative to those approved synthetic drugs by the Food and Drug Association in USA, such as galanthamine, rivastigmine and donepezil (7).

In Sri Lanka traditional physicians prepare various medications utilizing endemic and native medicinal plants to treat several neurological diseases (8,9). However, only a few research studies had been carried out in Sri Lanka on identification of potential drugs for Alzheimer's disease and no reported compounds have been isolated as potential drug leads for Alzheimer's disease from plants in Sri Lanka (10,11). In the current study, Sri Lankan medicinal plants from seven different families, Balsaminaceae, Arecaceae, Apocynaceae, Zingiberaceae, Dilleniaceae, Phyllanthaceae, and Rubiaceae were investigated for their AChE inhibitory activity.

Materials and Methods

Sample collection

Fresh plant parts of *Wrightia antidysenterica, Impatiens repens, Zingiber cylindricum, Dillenia retusa, Curcuma albiflora, Phoenix pusilla, Areca concinna, Flueggea leucopyrus* and *Knoxia zeylanica* (leaves, stem bark, roots and fruit) were collected from different locations of Sri Lanka. All the plants were authenticated by the Bandaranaike Memorial Research Institute Herbarium, Nawinna, Sri Lanka. The collected plant samples were washed under running tap water, chopped and air dried in shade for about 72 hours and then ground into fine powders using a grinder. These powders were stored in sealed bottles at 4 ºC until used.

Preparation of plant extracts

All the solvents used for extraction purposes were analytical grade solvents (Sigma Aldrich, Germany). Powdered plant parts were extracted first with 250 ml of methanol and the extraction was repeated with the same volume of methanol. These extracts were filtered with Whatman No1 filter paper. Then the residual powder was extracted using 250 ml of Methanol:CH_2Cl_2 1:1 mixture and the extraction was repeated with 250 ml of the same. Next, the residual powder was extracted twice with 250 ml of dichloromethane. Finally, the filtrates were combined to obtain the total extract of each plant part. The organic solvent was evaporated to dryness under reduced pressure at room temperature using a rotary evaporator (BUCHI-R-200). The obtained crude extracts were further dried using N_2 gas. All the samples were stored in sealed bottles at 4 ºC until further use.

Determining percentage yield

The masses of the powdered plant specimen and the resulting dry crude extract were used to calculate the percentage yield, using the following equation (12),

$$\text{Percentage Yield}(\%) = \frac{W_2 - W_1 \times 100}{W_0}$$

Where W_2 is the mass of the dry plant extract and the glass bottle, W_1 is the mass of the glass bottle and W_0 is the mass of the dried powder of the plant specimen.

Reagents for ellman's assay

5,5′-Dithio-bis(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide (ATCI), acetylcholinesterase type VI-S, Galantamine were purchased from Sigma-Aldrich, Germany. All the solvents and reagents used were analytical grade. For the screening of acetylcholinesterase inhibitory activity of plant parts, plant extracts were dissolved in methanol, to be used in modified Ellman's assay (13, 14).

Ellman's assay

Acetylcholinesterase was dissolved in 0.1 M phosphate buffer (pH 8.0) to prepare an 18 U/mL stock solution and it was stored at 4 °C. The stock acetylcholinesterase was diluted to 0.3 U/mL before use. Acetylthiocholine iodide of 6.2 mM and 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) of 7.6 mM solutions were prepared. Freshly prepared reagents were used for each acetylcholinesterase inhibition assay. Ellman's assay was adjusted to final volume of 240 µl in 96 well microplates. 0.3 U/mL acetylcholinesterase (50 µl) (0.015 AChE Units), 0.1 M pH 8.0 potassium phosphate buffer (125 µl), 7.6 mM DTNB (25 µl) and crude extract/ sample dissolved in methanol (10 µl) were used in the modified Ellman reaction mixture. Reaction mixture was incubated for 30 min at 30 °C, and the enzymatic reaction was initiated by adding 30 µl of 6.2 mM acetylthiocholine iodide (ATCI). Absorbance was measured at 412 nm in every 11 seconds for a duration of 220 seconds (SPECTROstar Nano Plate Reader). Enzyme inhibition was calculated as a percentage compared to an assay with 10 µl of methanol instead of the sample extract (SB). All the experiments were done in triplicate. pH 8.0 potassium phosphate buffer was used as the blank instead of acetylcholinesterase enzyme. Galantamine was used as the positive control.

Percentage AChE inhibition was calculated by using equation -1 (13, 14),

Percentage AChE inhibition = [(SB - S) / SB] × 100 ---------- 1

SB = variation in the absorption of the blank sample (0 s – 220 s)

S = variation in the absorption of the test sample extract (0 s – 220 s)

Statistical analysis

Data were analyzed using GraphPad Prism 9 software. All the assays were carried out in triplicates. Values were expressed as means ± standard deviations. IC_{50} (Concentration providing 50% inhibition) values were estimated from the plot of AChE inhibition percentage against extract concentration.

Results and Discussion

It is well-documented that the deterioration in mental and cognitive functions associated with Alzheimer's disease is due to the depletion of cortical acetyl-cholinergic neurotransmission. Acetylcholinesterase inhibitory compounds inhibit or stop AChE enzymes from cleaving acetylcholine.

Therefore, AChE inhibitors can suppress the degradation of acetylcholine and improve concentrations of acetylcholine, which leads to improved communication between nerve cells. Consequently, this stabilises or reduces the symptoms of Alzheimer's disease (15). AChE inhibitor drugs, including physostigmine and donepezil show some increase in the cognitive functions of Alzheimer's patients. However, due to the less selectivity of acetylcholinesterase inhibitory drugs in the market, patients experience numerous side effects (16,17). Of the 18 plant extracts investigated the highest percentage yield (16.07 %) was obtained for the organic extract of fruit of *D. retusa* and the lowest percentage yield (5.91 %) was given by *W. antidysenterica bark* extract (Table 1). These changes in the percentage yield can be due to the differences in chemical composition, the solubility of the solvent, and the bioactive compound density.

Table 1. Percentage yields of plant extracts

Plant species	Family	Plant part	Sample Code	Percentage Yield (w/w %)
I. repens	*Balsaminaceae*	Whole plant	IR-P	9.44
P. pusilla	*Arecaceae*	Leaves	PZ-L	10.31
		Bark	PZ-B	7.21
W. antidysenterica	*Apocynaceae*	Leaves	WA-L	11.45
		Bark	WA-B	5.91
		Seeds	WA-S	9.39
Z. cylindricum	*Zingiberaceae*	Leaves	ZC-L	6.15
		Rhizome	ZC-R	7.45
C. albifora	*Zingiberaceae*	Rhizome	CA-R	8.99
		Leaves	CA-L	8.36
A. concinna	*Arecaceae*	Seeds	AC-S	14.22
D. retusa	*Dilleniaceae*	Leaves	DR-L	11.28
		Bark	DR-B	8.33
		Fruit	DR-F	16.07
F. leucopyrus	*Phyllanthaceae*	Leaves	FL-L	11.38
		Bark	FL-B	6.94
K. zeylanica	*Rubiaceae*	Roots and stem	KZ-RS	7.52
		Leaves	KZ-L	11.23

The standard AChE inhibitor Galantamine displayed notable activity even at 1 µg/ml concentration (41.1% ±0.1 µg/mL). IC_{50} value of Galantamine was 1.57 ±0.01 µg/mL and achieved maximum 68% AChE inhibition at 8 µg/ml concentration (Table 2).

Table 2. AChE inhibition of Galantamine

Galantamine (µg/ml)	AChE inhibition (%)	Galantamine IC_{50} (µg/ml)
0.1	8.0±0.0	1.57 ±0.01
0.2	13.2±0.2	
0.4	22.3±0.3	
1	41.1±0.1	
2	54.1±0.2	
4	60±0.0	
8	68.2±0.1	

Data are given as the mean of at least three independent experiments ± S.D.

The analyzed 8 plant extracts displayed considerable anti-AChE activity and achieved 50% AChE inhibition before reaching the highest concentration (400 µg/ml) used in the study (Table 3). Only *W. antidysenterica* (Leaf), *D. retusa* (Fruit and leaf), *A. concinna* (Seeds) and *F. leucopyrus* (Leaf) extracts were active at 40 µg/ml concentration. The highest percentage inhibition was achieved by the *W. antidysenterica* with a value of 85±0.6% at 400 µg/mL concentration. *Phoenix pusilla* and *D. retusa* bark extracts did not show any inhibition

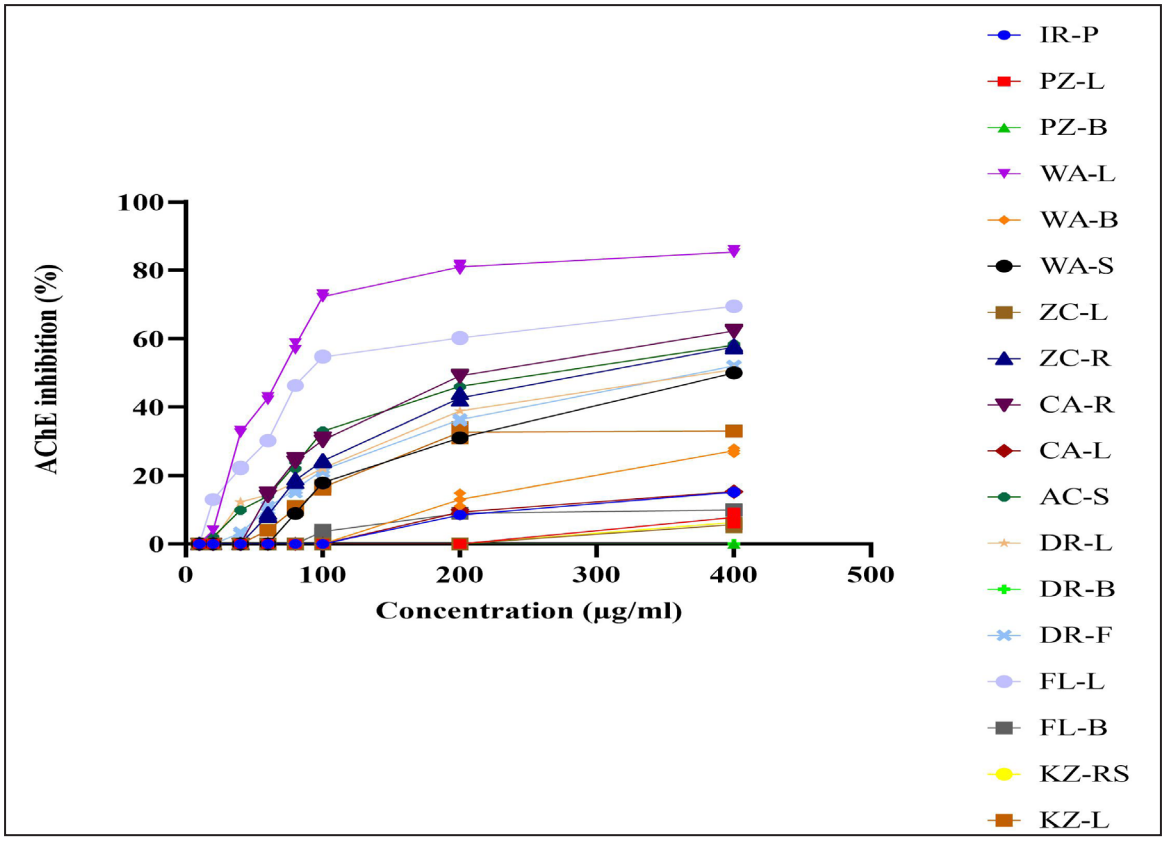
of AChE enzyme even at 400 µg/mL. Crude organic extracts obtained from barks of the tested plants did not show 50% inhibition. In the Zingiberaceae family, both *Z. cylindricum* and *C. albiflora* showed moderate activity toward AChE (below 25%) at 80 µg/ml concentration. However, both rhizome samples of *Z. cylindricum* and *C. albiflora* achieved 50% AChE inhibition below 250 µg/ml concentration. Out of these two plants in the Zingiberaceae family, *C. albiflora* rhizome was more active having an IC_{50} value of 189±1.4 µg/ml. *Phoenix pusilla* plant parts did not show any AChE inhibition even at 200 µg/ml. However, *P. pusilla* leaves extract showed low inhibition of the AChE enzyme (7.7±1.6%.) at 400 µg/ml.

Table 3. Anti-acetylcholinesterase activities of crude extracts

Plant species	Family	Plant part	Sample Code	AChE inhibition (%)				
				40 (µg/ml)	80 (µg/ml)	100 (µg/ml)	200 (µg/ml)	400 (µg/ml)
I. repens	*Balsaminaceae*	Whole plant	IR-P	-	-	-	8.4±0.1	15.1±0.2
P. pusilla	*Arecaceae*	Leaves	PZ-L	-	-	-	-	7.7±1.6
		Bark	PZ-B	-	-	-	-	-
W. antidysenterica	*Apocynaceae*	Leaves	WA-L	32.6±0.5	58±1.1	72±0.6	81±0.9	85±0.6
		Bark	WA-B	-	-	-	13±1.9	27.26±0.7
		Seeds	WA-S	-	8.9±0.1	18±0.1	31±0.1	50±0.0
Z. cylindricum	*Zingiberaceae*	Leaves	ZC-L	-	-	-	-	5.7±0.6
		Rhizome	ZC-R	-	18.7±0.6	24.3±0.3	42.7±1.2	57.6±0.4
C. albifora	*Zingiberaceae*	Rhizome	CA-R	-	24.3±0.6	30.4±0.6	49.1±0.2	62.3±0.2
		Leaves	CA-L	-	-	-	9.3±0.6	15.3±0.1
A. concinna	*Arecaceae*	Seeds	AC-S	10±0.1	23.3±1.1	33.0±0.1	46.1±0.1	58.1±0.2
D. retusa	*Dilleniaceae*	Leaves	DR-L	12.3±0.1	18.0±0.0	22.2±0.2	38.9±0.1	51.0±0.1
		Bark	DR-B	-	-	-	-	-
		Fruit	DR-F	3.2±0.3	16.0±1.0	21.7±0.6	36.3±0.3	52.1±0.1
F. leucopyrus	*Phyllanthaceae*	Leaves	FL-L	22.2±0.3	46.4±0.1	54.7±0.3	60.2±0.2	69.5±0.1
		Bark	FL-B		-	3.7±0.6	9.0±0.0	10.0±0.1
K. zeylanica	*Rubiaceae*	Roots and stem	KZ-RS	-	-	-	-	6.3±0.6
		Leaves	KZ-L	-	10.5±0.5	16.6±0.5	32.7±1.5	33.0±0.0

* Galantamine was used as positive control (IC_{50} 1.57 ±0.01 µg/mL). Data are given as the mean of at least three independent experiments ± S.D.

It is notable that leaf extracts of *W. antidysenterica* and *F. leucopyrus* showed potent acetylcholinesterase inhibitory activity at lower concentrations. These two extracts showed AChE inhibition even at 20 µg/ml concentration with a percentage inhibition value of 4±0.0% and 13±0.0%, respectively (Figure 1). Both achieved over 50% AChE inhibition when the concentrations were increased to 100 µg/ml. At this concentration, *W. antidysenterica* displayed potent inhibition with a percentage inhibition value of 72±0.6%.



Further purification of *W. antidysenterica* and F. *leucopyrus* organic plant extracts would be useful in isolating compounds with higher AChE inhibitory activities due to their potent activities at low concentrations. From the tested organic extracts of plant parts, the highest AChE inhibitory activity with an IC_{50} value of 64 ±0.5 µg/mL was reported from the leaf extract of *W. antidysenterica* (Table 4).

Table 4. The IC_{50} value of percentage AChE inhibition of plant extracts

Plant species	Family	Plant part	Sample code	IC_{50}/ (µg/ml)
W. antidysenterica	*Apocynaceae*	Leaves	WA-L	64±0.5
		Seeds	WA-S	400±0.2
Z. cylindricum Family	*Zingiberaceae*	Rhizome	ZC-R	249±1.3
C. albiflora	*Zingiberaceae*	Rhizome	CA-R	189±1.4
A. concinna	*Arecaceae*	Seeds	AC-S	217±1.2
D. retusa	*Dilleniaceae*	Leaves	DR-L	373±0.6
		Fruit	DR-F	359±1.1
F. leucopyrus	*Phyllanthaceae*	Leaves	FL-L	107±0.1

Data are given as the mean of at least three independent experiments ± S.D.

Furthermore, leaf extract of *Flueggea leucopyrus* showed good AChE inhibitory activity with an IC_{50} value of 107 ±0. µg/mL. *Curcuma albifora* rhizome extract and *Areca concinna* seed extract exhibited AChE inhibiting activities with IC_{50} values of 189±1.4 µg/mL and 217±1.2 µg/mL, respectively. The results of this study suggest that these Sri Lankan plants may have potential as therapeutic agents for the treatment of Alzheimer's disease. The next step will be to isolate the active drug leads of the crude organic extracts. Subsequently, IC_{50} values can then be compared with known AChE inhibitors. This can lead to an evaluation of whether these medicinal plants could be a novel source of potential drugs for Alzheimer's disease.

Conclusion

Of the nine plants investigated *W. antidysenterica, Z. cylindricum, D. retusa, C. albiflora, A. concinna* and *F. leucopyrus* showed promising anti-cholinesterase activity and achieved 50% AChE inhibition at 400 µg/ml. The crude extract of *W. antidysenterica* leaf exhibited the most potent AChE inhibition activity with an IC_{50} value of 64 ±0.5 µg/ml. Leaf extracts of *F. leucopyrus* and *W. antidysenterica* exhibited more than 20% AChE inhibition even at 40 µg/ml concentration. Thus, this initial screening provides valuable information for further studies to isolate anti-cholinesterase active compounds from Sri Lankan medicinal plants.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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