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Original Article

Screening Indigenous Medicinal Plants of Northeast India for Their Anti-Alzheimer's Properties

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ABSTRACT

Introduction: Alzheimer's disease (AD) is a progressive neurologic disease of the brain that affects intellectual abilities, reasoning and memory. Acetylcholine (ACh) is involved in the maintenance of cognitive process. Pathologically, ACh production is compromised in the brains of AD affected people. Presence of acetylcholinesterase (AChE) in the synaptic cleft, which hydrolyzes ACh, further decreases the ACh-levels, and thereby, additionally compromises cognition. The tribal people of North East India have been using indigenous plants as traditional medicine for brain disorders. We assayed whether the plants used in the traditional tribal knowledge for the treatment of brain disorders might contain better AChE-inhibitors. **Methods:** We collected 10 traditional medicinal plants from Northeast India. A total of 39 plant extracts were prepared using three solvent systems. The Acetylcholinesterase (AChE) activity was measured with Ellman method. The experiment was done in triplicate for each level of inhibitor. The activity was measured at 412 nm wavelength using Plate Reader. The standard student t-test was used to show significant difference in IC50 values between extracts. **Results**: The result are reported based on Km, Vmax, IC_{50} ($\mu g/\mu l$), percentage inhibition and inhibition pattern. Two extracts had competitive inhibition, 11 extracts had mixed inhibition, 2 extracts had non-competitive inhibition, 11 extracts had uncompetitive inhibition and 4 extracts did not provide any proper pattern. The IC_{50} for these plant extracts were at the range of 0.51-12.4 $\mu g/\mu l$. Notably, Cinnamomum camphora (leaf: chloroform), Litsea glutinosa (stem; chloroform), and Litsea glutinosa (stem; methanol) showed IC_{50} values of 0.51, 0.53 & 0.81 $\mu g/\mu l$, respectively.

Key words: AChE-inhibition, Kinetics, Medicinal plants, Alzheimer's, Ellman assay.

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INTRODUCTION

Alzheimer's disease, the most common form of dementia, is a progressive neurologic disorder of the brain that leads to the irreversible loss of neurons. AD impairs cognitive and memory functions, communication, personality, behavior, and ability to function properly. The average duration of survival of AD patients after the onset of dementia is 5 to 9.3 years.[1,2] Because of the absence of a permanent cure, AD has become a major health problem, although there are some treatments that may slow down its advances.[3] It is estimated that there are 35.6 million people living with dementia worldwide and will increase to 65.7 million by 2030, whereby much of the increase will be in developing countries.[4]

Neurofibrillary tangles, Amyloid plaques and loss of cholinergic neurons are three pathological findings commonly observed in the cerebral cortex of Alzheimer's disease cases.[5-8] Based on the findings that there exist a correlation between cholinergic system abnormalities and intellectual impairment, "cholinergic hypothesis" has been put forward in the functioning of memory and accepted as the foremost hypothesis in the field of AD.[9-11] However, relationships between levels of acetylcholine and AD have been a challenge. It is now accepted that the acetylcholine dysfunction may not be the primary cause of AD but may be a consequence of the disease.[9]

The neurotransmitter acetylcholine is involved in active maintenance of novel information and enhancement of long-term potentiation, i. e. memory.[12] The action of acetylcholine is terminated in the synaptic cleft by AChE which hydrolyzes ACh into acetate and choline. Amyloid plaques formation in synapse prevents acetylcholine molecules to reach its cognate receptors on the post-synaptic neuronal membrane, thereby, leading to the gradual loss of communication between neurons.

Over the years several studies have focused on "cholinergic hypothesis" to ameliorate the acetylcholine deficiency in the brain of AD patients. These endeavors have led the scientists to discover various classes of molecules, for the treatment of AD, in the form of acetylcholine esterase inhibitors.[9] Drugs based on cholinergic hypothesis work in prolong-

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ing the duration of acetylcholine in the synaptic cleft by inhibiting AChE activity. Some of these drugs have been synthesized and/or derived from plants such as rivastigmine, huperzine and galanthamine etc.[13-15] However, these drugs only slow down the progression of the disease and have been reported for various side effects such as gastrointestinal disturbances and bioavailability problems.[16,17] These disadvantages make a room for finding newer drugs with better efficacy and lesser side effects.

In traditional practices of Ayurvedic medicine, numerous plants have been used and studied as treatment for cognitive disorders including neurodegenerative diseases such as AD, memory enhancement, anti-aging and preventing dementia.[18] For example, *Celastrus paniculatus* Wild. (CP) has been reported to be used in Ayurvedic medicine for stimulating intellect and sharpening the memory.[18] *Centella asiatica* (L.) Urb. (CA) is being used for anti-aging, prevention of dementia and mental exhaustion.[18,19] *Acorus calamus* L. (AC) extract is applied for the treatment of memory loss.[18,19] whiles the ripe fruit of *Terminalia chebula* Retz. (TC) is regarded to slow down the ageing process and to improve the cognition.[18,19] Recently *Mucuna pruriens* (L) DC has been shown to have potential for the treatment of Parkinson's disease[20] and *Withania somnifera* (L.) Dunal (WS) is routinely used for improvement of memory and cognition enhancement in Indian Ayurvedic medicine.[21] The indigenous tribal people of North East India have been using various plants as traditional medicine for treating brain & neurological disorders, over the millennium. Some of these plants have been shown to have AChE inhibition activity.[22] In addition, *Semecarpus anacardium* L. f. (SA) (from North-East India), has been shown to be neuroprotective especially to the hippocampal region in stress-induced neurodegeneration.[23]

Given that many current AD drugs are derived from plants and the North East India belongs to one of the major biodiversity hot-spots region in the world, it would be justifiable to explore whether the plants used in the traditional tribal knowledge for the treatment of various brain ailments might contain better AChE-inhibitors. To this end, we have collected several such plants and assayed their extracts for its capacity to inhibit AChE activity. The AChE-inhibition activities of these plant parts were compared with the standard AChE-inhibitor drugs and demonstrate that some of these plants contain a good amount of AChE inhibition activity that may be further investigated for isolation of active-inhibiting component in the AD drug discovery process.

MATERIAL AND METHOD

Plant material Collection & extraction

Under the aim of this study, different botanical books and journals were used to find the plants that are used either to treat any kind of brain disorder and/or improve the functions of brain by traditional healers in North-East India. A database of such plants was created that includes habitat, botanical and local name, purpose of use, picture of the plants and the journal or books where it was documented. Ten plants were collected from the gardens of Botanical Survey of India (BSI) which are located in the countryside of Shillong city in Meghalaya, India (with permission of Dr. B. K. Sinha, BSI Director on 03.10.2012). Proper identification and authentication of the collected plants were carried out with the help of specialist from BSI, and respective vouchers were provided. It is worth mentioning that although the plants which were collected from BSI and adjoining area of Shillong these plants may be found in other parts of India but we selected because these plants are being used by traditional healers of North East India for brain disorders.

The plants were first washed to remove dust and insects and then transferred to the clean room (out of sunlight) to dry gradually. When the plants were completely dried, different parts of the plants including, leaves, stem, root, bark, or the whole plant were pulverized and transferred to autoclavable bottle with some silica gel to protect them from moisture. The bottles were labeled by the name of the plants and its part.

The plant parts were extracted using three solvents including water, methanol and chloroform. The plant powder/solvent was used at the ratio of 1 g/20 ml. The plant powder was mixed with the solvent and the bottle was sealed completely. The mixture was kept in water bath overnight at the temperature of 318 K, 318 K and 338 K for methanol, chloroform and water extract, respectively. The mixture was filtered using Whatman filter 40 (110 mm) gently. The solvents were removed using the evaporator. In order to keep the same conditions and use the same amount of extracts in experiment, all extracts were lyophilized to remove moisture and get the extract completely dry. All extracts were sealed and kept at 4°C.

Buffer and reagents preparation

The phosphate buffer 0.1M was prepared at pH 7 and pH 8. Acetylcholine esterase enzyme and Acetylthiocholine (ATC) substrate were purchased from Sigma. The lyophilized AChE was dissolved in 1% gelatin to obtain 1000U stock enzyme. The final enzyme concentration in the assay buffer was 1.4 U. Eight different final concentrations of ATC were adjusted ranging between 35.476 µM and 354.770 µM. The stock was prepared in distilled water in buffer pH 8. The DTNB (5, 5'-dithiobis-(2-nitrobenzoic acid)) was obtained from SRL, India and used as a reporter reagent. The reaction between DTNB and free thiol group produces a mixed disulfide with thiols, liberating the chromospheres 2-nitro-5 thiobenzoate anion (TNB^{2-}) which gives an intense yellow color at 412 nm with a higher molar extinction coefficient. The DTNB was dissolved in 0.1M phosphate buffer pH 7 in which it is more stable and used at a final concentration of 0.33 mM.

The stock solution of each plant extract, 5 mg/ml, was prepared by dissolving the powder in the phosphate buffer pH 8. Extracts were dissolved by pipetting and vortexing gently, following a short spin to remove the particles. The supernatant was used as experimental solution. The extracts were used at final concentrations of 0.8298 µg/µl and 1.0373 µg/µl. However, we would like mention that, in most of the cases, extracts dissolved in the phosphate buffer to a limited extent ranging from 30 to 100%, and hence the final concentrations used (0.8298 µg/µl and 1.0373 µg/µl) might be much lower. Tacrine, a first generation FDA approved AD drug and is a known non-competitive AChE inhibitor[24,25] was used as a standard AChE-inhibitor control.

Experimental Procedure

The Acetylcholinesterase (AChE) activity was measured with the method developed by Ellman *et al.,* 1961 and modified by Laura *et al.*, 2010. The experiment was set up for the final volume of 120.5 µl using 96-well plate (Greiner bio-one). The activity was measured at the wavelength of 412 nm using Plate Reader (Synergy H1 422, BioTek). The raw data was analyzed using Origin-Pro8 and Microsoft Excel software.

Initial Velocity (v_0)

The initial velocity (V_0) was calculated based on formation of the product at the particular time in the system. The rate or ($V_{0)}$ is: = $V0 = \Delta[P]/\Delta t$ where [P] is concentration of product at time t[27]. According to the Beer-Lambert Law $[P] = A/\varepsilon l$, where A is absorbance, ε is molar extinction coefficient and l is light path length. The original molar extinction coefficient of DTNB which was reported by Ellman is 13,600M^{-1} cm^{-1} at 412 nm. However, later studies have shown that the more accurate molar extinction coefficient value is 14,150 $M^{-1}cm^{-1}$ at 412 nm, which has been used in this study.[28,29] And l for 120.5 µl (Greiner bio-one plate) is 0.3374 cm. The initial velocity was calculated at the time of 5 min.

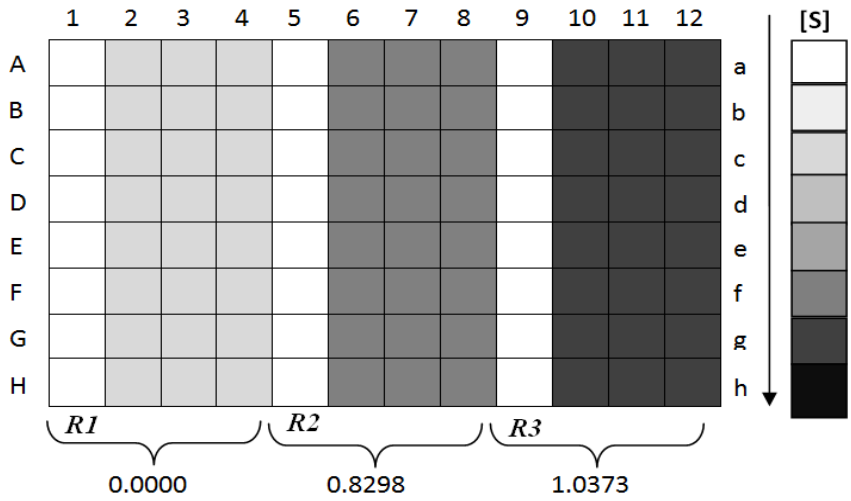

Figure 1: 96-well plate format for enzyme inhibition study. R1: Reference for A2-H4 (Zero inhibitor), R2: Reference for A6-H8 (0.8298 µg/µl of inhibitor) and R3: Reference for A10-H12 (1.0373 µg/µl of inhibitor).

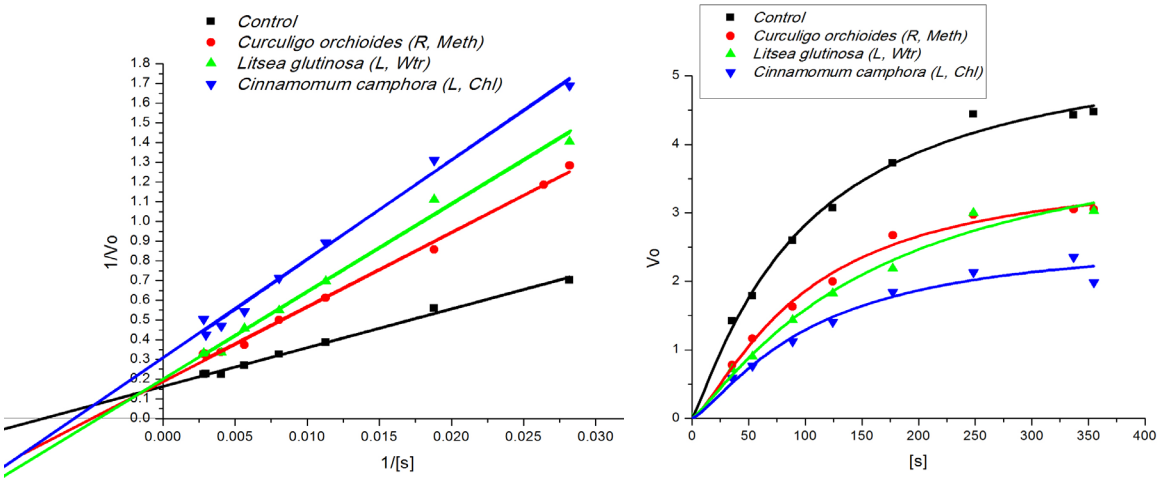

Figure 2: Lineweaver–Burk and Michaelis–Menten plot for five mixed inhibitors at the level of 1.0373 µg/µl. Control: (Zero inhibition), *Curculigo orchioides* (Root, Methanol), *Litsea glutinosa* (Leaf, Water), and *Cinnamomum camphora* (Leaf, Chloroform).

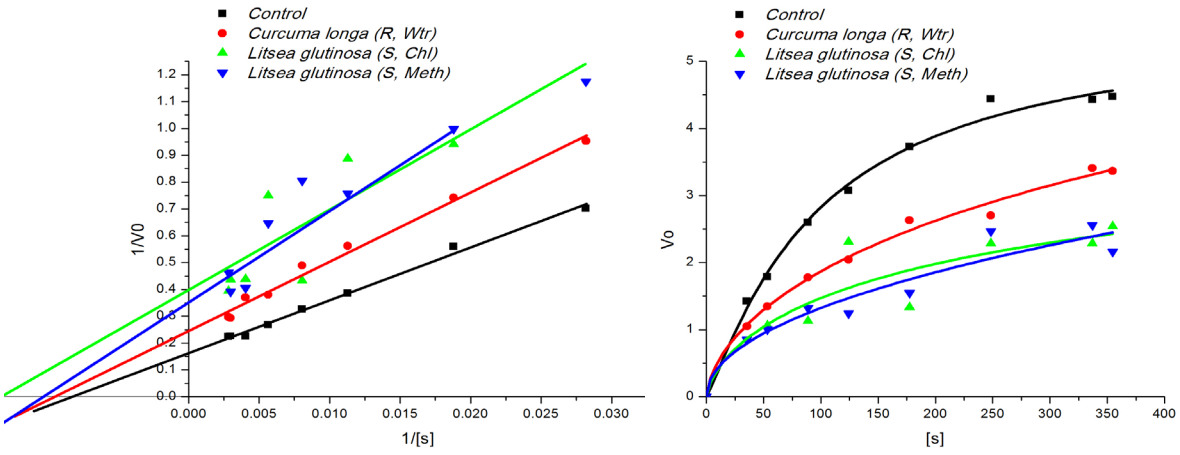

Figure 3: Lineweaver–Burk and Michaelis–Menten plot for Uncompetitive inhibitors at the level of 1.0373 µg/µl. Control: (Zero Inhibition), *Curcuma longa* (Root, Water), *Litsea glutinosa* (Stem, Chloroform) and *Litsea glutinosa* (Stem, Methanol).

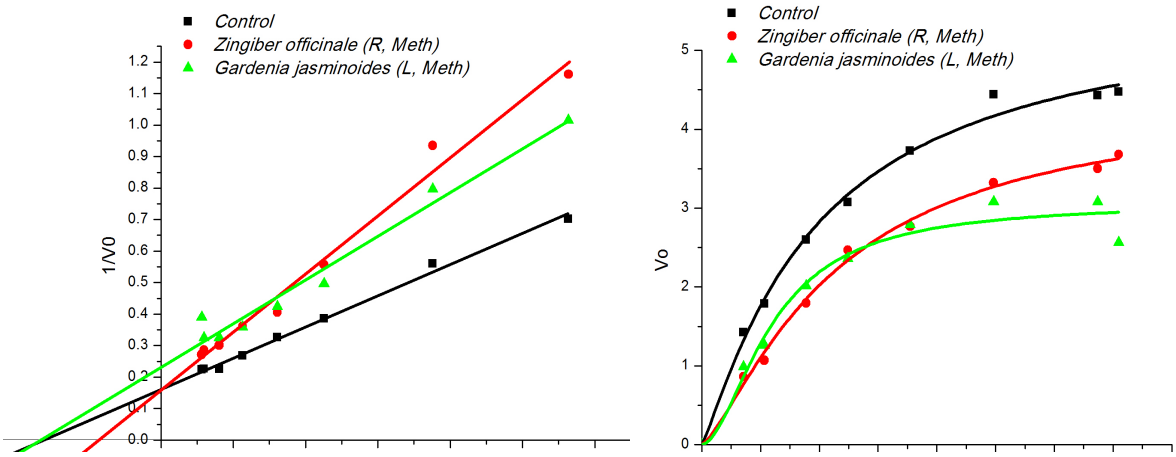

Figure 4: Lineweaver–Burk and Michaelis–Menten plot for competitive and non- competitive inhibitors at the level of 1.0373 µg/µl. Control: (Zero Inhibition), *Zingiber officinale* (Root, Methanol competitive inhibition) and *Gardenia jasminoides* (Leaf, Methanol, non-competitive inhibition).

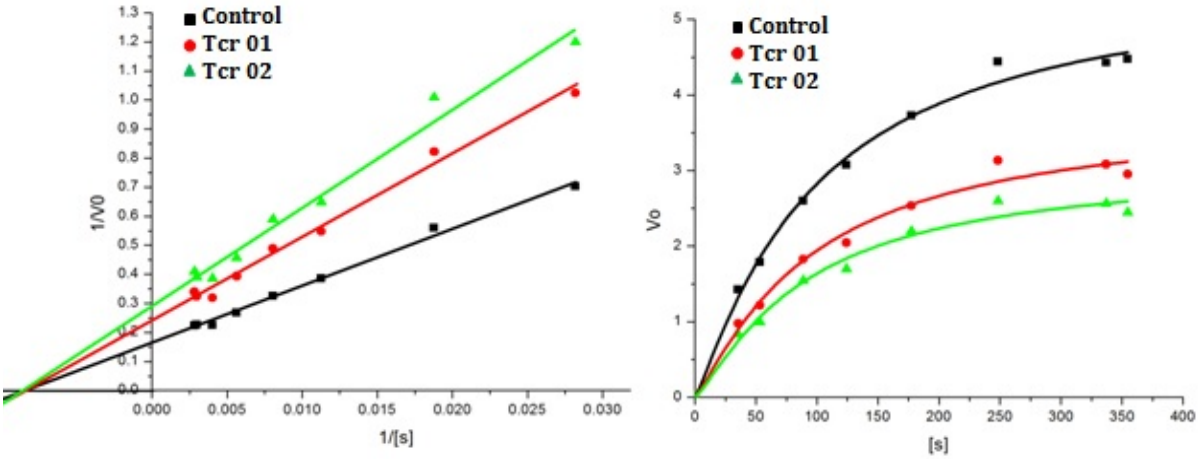

Figure 5: Lineweaver–Burk plot and Michaelis–Menten plot for Tacrine. Non-Competitive inhibition at two levels. Tacrine was used as a known AChE inhibitor that shows non-competitive inhibition to prove that the assay has been set up correctly. Control (Zero Inhibition), Tcr 01: 16.59 nM and Tcr 02: 33.19 nM.

Table 1: The relationship between [I] / IC_{50} and %I for enzyme inhibition [27]

[I]/IC_{50}	% Inhibition
0.25	20
0.33	25
0.43	30
0.50	33
0.67	40
1.00	50
1.50	60
2.33	70
3.00	75
4.00	80
5.00	83
6.00	86
9.00	90
19.00	95

Table 2: The inhibition pattern and values of different parameters (%I, IC_{50}, [I]/IC_{50}, V_{max} and K_m) for each extract

No.	Name of Plant	Part	Solvent	% I	IC50 µg/µl	[I]/ IC50	Vmax	Km	Inhibition Pattern	Sample No
	Cinnamomum camphora	Leaf	Chloroform	53.9	0.51	1.17	3.3	168	Mixed	06_c
	Litsea glutinosa	Stem	Chloroform	47.6	0.53	0.91	*2.8	*97	Uncompetitive	27_c
	Litsea glutinosa	Stem	*Methanol*	48.7	0.81	0.95	2.5	74	Uncompetitive	26_m
	Litsea glutinosa	Leaf	*Water*	41.5	1.31	0.71	5.0	226	Mixed	28_w
	Cassia occidentalis	Leaf	Chloroform	28	1.71	0.39	4.0	73	Uncompetitive	03_c
	Curculigo orchioides	Root	Methanol	34.5	1.76	0.52	5.1	194	Mixed	11_m
	Ficus religiosa	Leaf	Chloroform	20.1	2.09	0.25	5.2	142	Mixed	21_c
	Cinnamomum camphora	Leaf	Methanol	32.8	2.11	0.49	4.8	172	Mixed	05_m
	Curcuma longa	Root	Water	29	2.14	0.41	4.0	104	Uncompetitive	13_w
	Gardenia jasminoides	Leaf	Chloroform	13.8	2.19	0.14	5.2	117	N-Competitive	24_c
	Ficus religiosa	Leaf	Methanol	30.7	2.34	0.44	4.6	144	Mixed	20_m
	Sapindus mukorossi	Leaf	Chloroform	21.3	2.46	0.27	4.3	96	Uncompetitive	36_c
	Gardenia jasminoides	Leaf	Methanol	29.4	2.48	0.41	4.2	119	N-Competitive	23_m
	Litsea glutinosa	Stem	Water	28.7	2.57	0.4	4.7	153	Mixed	25_w
	Curcuma longa	Root	Methanol	21.9	2.59	0.28	4.4	106	Uncompetitive	14_m
	Zingiber officinale	Root	Methanol	23.8	2.97	0.31	7.5	286	Competitive	38_m
	Zingiber officinale	Root	Chloroform	25.8	3.12	0.23	4.0	107	Uncompetitive	39_c
	Curculigo orchioides	Root	Chloroform	12.7	3.71	0.11	*6.0	*154	Mixed	12_c
	Cassia occidentalis	Leaf	Water	18.5	4.4	0.23	4.3	89	Uncompetitive	01_w
	Cassia occidentalis	Leaf	Methanol	15	5.25	0.19	*5.9	*164	Mixed	02_m
	Gardenia jasminoides	Leaf	Water	13.2	6.79	0.15	4.5	84	Uncompetitive	22_w
	Litsea glutinosa	Leaf	Methanol	13	6.92	0.14	4.7	95	Uncompetitive	29_m
	Sapindus mukorossi	Leaf	Water	12.2	7.44	0.13	5.6	132	Mixed	34_w
	Sapindus mukorossi	Leaf	Methanol	11.6	7.9	0.13	5.6	132	Mixed	35_m
	Cinnamomum camphora	Leaf	Water	10.2	9.03	0.11	5.2	108	Uncompetitive	04_w
	Litsea glutinosa	Leaf	Chloroform	7.71	12.4	0.08	*6.2	*151	Competitive	30_c
	Curculigo orchioides	Root	Water	NA	NA	NA	NA	NA	NA	10_w
	Curcuma longa	Root	Chloroform	NA	NA	NA	NA	NA	NA	15_c
	Ficus religiosa	Leaf	Water	NA	NA	NA	NA	NA	NA	19_w
	Zingiber officinale	Root	Water	NA	NA	NA	NA	NA	NA	37_w
	Tacrine					0.74	3.45	117	N-Competitive	

V_{max} and K_m of control assay were 6.1 and 120 respectively (8 substrate concentrations). V_{max} and K_m of control assay were 6.5 and 139 for those which have been marked with star (*) (7 substrate concentrations). K_i has not been mentioned here as it was not useful to compare the inhibition activity between two different extracts. Tacrine has been reported based on nanomolar. Water: ($_w$), Methanol: ($_m$) and Chloroform: ($_c$).

Table 3: Student t-test to compare IC_{50} values for those which have less than 3 µg/µl

	05_m	06_c	11_m	13_w	14_m	20_m	21_c	23_m	24_c	25_w	26_m	27_c	28_w	36_c	38_m
03_c	0	1^*	0	0	0	0	0	0	0	0	0	1^*	0	0	1^*
05_m		1^{**}	0	0	0	0	0	0	0	0	1^{**}	1^{**}	0	0	0
06_c			1^{**}	1^{**}	1^{**}	1^{**}	1^{**}	1^{**}	1^{**}	1^{**}	0	0	0	1^{**}	1^{**}
11_m				0	0	0	0	0	0	0	1^*	1^{**}	0	0	1^*
13_w					0	0	0	0	0	0	1^{**}	1^{**}	0	0	0
14_m						0	0	0	0	0	1^{**}	1^{**}	1^*	0	0
20_m							0	0	0	0	1^{**}	1^{**}	1^*	0	0
21_c								0	0	0	1^{**}	1^{**}	0	0	0
23_m									0	0	1^{**}	1^{**}	1^*	0	0
24_c										0	1^{**}	1^{**}	0	0	0
25_w											1^{**}	1^{**}	1^*	0	0
26_m												0	0	1^{**}	1^{**}
27_c													0	1^{**}	1^{**}
28_w														1^*	1^{**}
36_c															0

Not significant: (0), significant for α = 0.05: (1^*) and for α = 0.01: (1^{**}). The table shows the difference not only between two different plants but also between two different solvent for same plant.

Plots

The Michaelis-Menten plot was graphed using V_0 versus [S]. The Line weaver-Burk plot was used to calculate V_{max} (maximum velocity) and K_m (Michaelis constant). The initial velocity of AChE was calculated for each substrate concentration individually. V_{max} (No inhibitor), V_{max}^{app} (with Inhibitor), K_m (No inhibitor) and K_m^{app} (with Inhibitor) were calculated by plotting the graph of $1/V_0$ versus $1/[s]$. The proper inhibition pattern for each level of inhibitor was plotted. K_i (Inhibition constant), %I (%Inhibition) and IC_{50} was calculated accordingly.

To calculate the %I the following equation was used:

$$\% \text{ inhibition} = 100 * [1 - (V_i / V_0)] \quad \text{(E1)}$$

Where (V_i / V_0) is the fractional activity therefore $[1 - (V_i / V_0)]$ is the fraction of enzyme occupied by inhibitor. V_i and V_0 are reaction velocity at the presence and absence of inhibitor.[27]

The following equations were used to calculate IC_{50} and relationship between $[I]/ IC_{50}$ and %I of enzyme activity:[27]

$$V_i / V_0 = 1 / 1+([I] / IC_{50})^h \quad \text{(E2)}$$

$$\%I = 100 / 1+(IC_{50} / [I])^h \quad \text{(E3)}$$

Where [I] is the concentration of inhibitor (extract) and *h* is Hill coefficient which is related to number of active site of the enzyme and interaction between inhibitor and enzyme.

After rearranging the equation E2 the following equation can be used to calculate IC_{50}:

$$[I] = IC_{50} [(V_0 / V_i) - 1]^{1/h} \quad \text{(E4)}$$

Where, (V_0 / V_i) is reciprocal of fraction activity.

Looking at the equation E4 we see that at the point of 50% inhibition, the fractional activity will be 0.50 and its reciprocal will be equal to 2.00. It means when 50% inhibition is achieved then $[I] / IC_{50}$ is equal to 1.00. And if the $[I] / IC_{50}$ is calculated for different %I (i.e. 25%, 30%, 50%, 75% and etc.), given h is equal to 1, the standard values as mentioned in Table 1 can be obtained.[27]

By rearrangement of the standard Langmuir isotherm equation the relationship between $[I] / IC_{50}$ and %I for enzyme inhibition was calculated:[27]

$$[I] / IC_{50} = \%I / (100 - \%I) \quad \text{(E5)}$$

The standard values mentioned in Table 1 were used to evaluate whether our calculation is correct[27] (Table 1).

In order to show that how large a difference in ***IC*$_{50}$** between two extracts can be considered significant, the standard student *t*-test was used:[27,30]

$$\text{t-value} = |IC_{50}{}^a - IC_{50}{}^b| / \sqrt{S^2_a/n_a + S^2_b/n_b} \quad \text{(E6)}$$

Where, a and b identify two inhibitors (extracts), and S_a and S_b are standard errors of each IC_{50} value, n_a and n_b are number of data points for two inhibitors (extracts) respectively.

As our experiment was set up in triplicate, therefore, the degrees of freedom for testing the significance of *t*-value was calculated using following equation:[27,30]

$$df = \{[(n_a*3) - 1] + [(n_b*3) - 1]\} \quad \text{(E7)}$$

Where n_a and n_b are number of data points for two inhibitors (extracts) respectively.

The experiment was set up for three levels of inhibitors (extract) 0.0000, 0.8298 and 1.0373 µg/µl. The activity without any inhibitor was negative control and Tacrine as known inhibitor was used as positive control. The experiment was done in triplicate for each level of inhibitor plus one column (eight wells) as reference (***R***) without enzyme. Because of non-enzymatic reaction between DTNB and substrate or inhibitors some absorbance will be detected as background which must be subtracted from the activity columns. Therefore, the reference column must include all reagents present in activity columns except enzyme (Figure 1).

RESULTS

A total of 30 extracts of 9 plants were screened for AChE inhibition activity. The inhibition pattern was found for each extract by plotting $1/V_0$ versus 1/[S] (Lineweaver–Burk plot). The following were the distribution of inhibition patterns: Two extracts had competitive inhibition, 11 extracts had mixed inhibition, 2 extracts had non-competitive inhibition,

11 extracts had uncompetitive inhibition and 4 extracts did not provide any proper pattern (Table 2). AChE has Michaelis constant of 90 μM.[31] In this study, the maximum velocity and Michaelis constant for control assay (zero-inhibitor) were obtained as 6.1 and 120 μM, respectively, using eight different substrate concentrations. However, only for four assays we used seven different substrate concentrations and their V_{max} and K_m were of 6.5 and 139 μM. Based on obtained initial velocities and substrate concentrations, six different parameters including %I, IC_{50}, $[I]/IC_{50}$, Ki, V_{max} and K_m were calculated for each extract. V_{max} and K_m were used to prove that the inhibition pattern has obtained correctly. Three parameters including %I, IC_{50} and $[I]/IC_{50}$ were used to compare the inhibition activity of extracts.

We calculated a very important parameter ($[I]/IC_{50}$) and its relationship with %I. We compared our results for ($[I]/IC_{50}$) and %I with the standard values mentioned in Table 2. We found that relationship values between ($[I]/IC_{50}$) and %I of our data was in good agreement with the standard values. This indicates that our calculation for %I and IC_{50} are correct. In order to show that how much difference between two values of IC_{50} is considered significant, the Student t-test was done for those which have shown more than 20% inhibition. The t values were calculated at $\alpha = 0.05$ and $\alpha = 0.01$ (2 tail) (Table 3).[27, 30]

The Lineweaver–Burk and Michaelis-Menten plots have been shown in Figures 2, 3 and 4 for mixed inhibition, uncompetitive inhibition, competitive inhibition and non-competitive inhibition. The plots for Tacrine, as a known AChE inhibitor, have been shown in Figure 5 to demonstrate that the enzyme assay was working correctly.

DISCUSSION

Pathologically, the formation of Amyloid plaques in synapse impedes the access of acetylcholine molecules to reach their cognate receptors on the post-synaptic membrane to deliver their message. In fact, gradual build up of Amyloid plaques in the synapse slows down the action of acetylcholine and leading to the loss of communication between neurons. Inhibiting AChE would mean that more acetylcholine molecules would be available in the synaptic cleft to deliver the message before they are being catalyzed by AChE.

Therefore, to increase the acetylcholine levels in the brains of AD patients, search for AChE-inhibitors has led to the discovery of plant derived drugs such as Rivastigmine, huperzine and Galanthamine. Rivastigmine and Galanthamine have been isolated from two different plants, Calabar bean (*Physostigma venenosum*) and bulbs of snowdrop (*Galanthus woronowii* Los.), respectively, while huperzine A (HupA) has been isolated from moss (*Huperzia serrata* (Thunb. Ex Murray) Trev.). HupA is selectively potent and reversible inhibitor with a better therapeutic index than physostigmine and tacrine.[32] In fact, HupA is clinically prescribed in China for symptomatic treatment of AD. In spite of their excellent AChE-inhibition capacity, the above drugs have serious side effects such as Nausea, vomiting, diarrhea, weight loss, loss of appetite and muscle weakness.[3,18] Many other plant compounds have shown remarkable AChE inhibitory capacity, for example, withanolides from *Withania somnifera* Dunal. (ashwaganda or Indian ginseng), curcuminoids from *Curcuma longa* L., tanshinones from *Salvia miltiorrhiza* Bunge and quercetin from *Quercus* sp. (oak) and their full potentials has yet to be investigated.[33-37] Therefore, it seems that plants have a vast kingdom of natural sources of several compounds for combating neurodegenerative diseases.

In this study, 30 plant extracts were assayed at two levels of plant extract concentrations, 0.829 μg/μl and 1.037 μg/μl. Two extracts have shown competitive inhibition in which inhibitor only binds to free enzyme and V_{max} remains same but K_m increases. Eleven extracts have shown mixed inhibition in which inhibitor can bind to either free enzyme or enzyme-substrate both and herein V_{max} decreases while K_m increases. Two extracts have shown non-competitive inhibition. Non-competitive inhibitors bind to enzyme and enzyme-substrate both with equal affinity and K_m remains same but V_{max} decreases. Eleven extracts have shown uncompetitive inhibition in which inhibitor only binds to enzyme-substrate complex. In uncompetitive pattern V_{max} and K_m both decrease. And finally, 4 extracts have not given any proper pattern (Table 2).

Working with natural plant inhibitors is very cumbersome as different molecules are present in plant extract. Hence, using different concentration of the same crude extract might show variant inhibition patterns. Therefore, it is often difficult to find an inhibition pattern that may be appropriate for a certain plant extract and unlike pure compounds it is impossible to use K_i to compare the inhibition activity of the plant extracts. In fact, even in pure compounds if they do not have similar inhibition pattern, K_i cannot be a proper parameter to compare. Based on the type of reaction between inhibitor and enzyme, there are different types of inhibition patterns and different types of calculations for K_i. Having said that, it seems it won't be correct if we use K_i as a comparison parameter to select the inhibitor. That is why we have not mentioned K_i values in the Table 2, although the values have been calculated for all extracts.

Therefore, it is necessary to have a particular comparison parameter which should have two important aspects. First, it should be independent parameter that is it should not be influenced by the type of reaction between inhibitor and enzyme. Secondly, it should be applicable for both pure and crude samples. Practically in inhibition kinetics, it seems percentage inhibition (%I) and IC_{50}/EC_{50} are the best parameters which can be used to select inhibitors. The %I and IC_{50}/EC_{50} are using velocities directly (as first step values) and they are independent from the type of reaction between inhibitor and enzyme while K_m and V_{max} (as second step values) are necessary to calculate K_i and they are not independent.

When we work with crude samples, we have to consider the following things. Firstly, we assayed the crude plant extract so we do not have any idea of what would be the real percentage of the active compound (that inhibits AChE) in the crude extract. Generally, percentage of any active compound in plant extract is much less than 1%. Secondly, because the crude contains several other compounds, these compounds will effectively inhibit and/or dampen the access of the active compound for AChE site and thus may display lesser inhibition capacity. Third, the active compound might be in complex with other compounds which will prevent the reaction between active compound and AChE and reduce the inhibition activity. Fourth, being in complex condition might change the solubility of active compound leading to reduction of active compound concentration in the stock solution. Fifth, as we are using crude plant extracts there might be some compounds which can increase the AChE activity, thereby reducing inhibitory potential of the active-inhibiting compound. Therefore, keeping all these in mind, it is not fair to compare the inhibition-activity of a pure compound that with crude extract.

In this study the best IC_{50} with 0.51 μg/μl belongs to *Cinnamomum camphora* (leaf: chloroform) following by *Litsea glutinosa* 0.53 μg/μl (stem; chloroform), *Litsea glutinosa* 0.81 μg/μl (stem; methanol) and *Litsea glutinosa* 1.31 μg/μl (leaf; Water) (Table 2). We found 20 plant extracts having more than 20% inhibition and specifically the above mentioned plants displayed % inhibition range varying from 40-54%. Other studies on different plants have reported more than 70% inhibition using plant extract concentration of 1 mg/ml.[16,38] But first, none of them clarified for how they have done the analysis and calculations for %I and IC_{50} using the velocities and inhibitor concentrations and what was the relationship between all these parameters. Secondly, %I is not a good parameter to compare two different plant extracts because it is almost impossible to use the same amount of different plant extracts due to

their solubility in phosphate buffer. That is why we should use %I only to calculate the IC_{50} for each extract independently and then use IC_{50} for comparison. As we have found during our analysis, although we use the same data to calculate all these different parameters (%I, IC_{50}, V_{max}, K_m and K_i), if we do not care about the relationship between parameters the calculation will be wrong and there is a high chance of getting a higher percentage of inhibition. We have searched through literatures but did not find such a study which had examined the plants we studied and given all the kinetic parameters that we calculated. Hence, we could not compare our results with others.

We have compared the IC_{50} values for those which have less than 3 µg/µl of IC_{50} by Student t-test in Table 3 in order to see how much difference between two IC_{50} is considered significant.[27,30] For each IC_{50} there is a particular range to be significant from others, up and down. Calculation of the t value and knowing the difference between two extracts can be very useful if we use the extracts for further separation and purification and/or experiments using cell line or mouse model. It can be helpful when the toxicity and availability of the plant extract is considered.

In nutshell, our results demonstrate that selected plants from North East India collected and assayed under the current study contain appreciable amount of AChE-inhibitors. It is therefore imperative that more such plants of North East India are screened for potential AChE-inhibitors, which could pave the way for the development of new classes of chemical compounds. Currently our work is under way for further separation and assaying of the extract fractions which might provide much better AChE-inhibition profile. The comparative study of the isolated fraction against known AChE-inhibiting drugs could provide valuable insight for development of newer lead compounds.

CONCLUSION

Plants used by the traditional Tribal healers of Northeast India for the treatment of brain disorders contain appreciable amount of AChE-inhibitors. These inhibitors might have better safety profile since they have been used by the tribes for millennium. Importantly, these inhibitors may belong to new classes of compound paving the way to develop lead compound for better AChE-inhibitors. Further screening of other plants used in tribal medical interventions in the Northeast India may provide better AChE-inhibitors.

ABBREVIATION

AD: Alzheimer's Disease; ACh: Acetylcholine; AChE: K_m^{app} V_{max}^{app} Acetylcholinesterase; : Apparent Michaelis Constant; : Apparent maximum velocity; IC_{50}: inhibitory concentration; BSI: botanical survey of India; ATC: Acetylthiocholine; DTNB: 5, 5'-dithiobis-(2-nitrobenzoic acid; TNB^{2-}: 2-nitro-5 thiobenzoate anion; V_0: initial velocity; [P]: Product concentration; [S]: Substrate concentration; Ki: Inhibitory constant; %I: percentage of inhibition; [I]: Inhibitor concentration; R: Reference (Blank); HupA: Huperzine A.

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