Screening of siddha medicinal plants for its *in-vitro* acetylcholinesterase and butyrylcholinesterase inhibitory activity

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ABSTRACT

Background: The plants selected for the study were traditionally used in siddha system of medicine in neurological disorders. **Aim:** The aim of the following study isto screen the plant species for both acetylcholinesterase (AchE) and butyrylcholinesterase (BuchE) inhibition by in-vitro Ellman's method and a thin layer chromatography bioautographic assay for newer drug candidates for the treatment of Alzheimer's disease. **Materials and Methods:** Ellman's colorimetric method was performed in a 96 well micro plate for cholinesterases inhibition using galantamine as standard drug. **Results:** Present studies confirmed that out of all the tested extracts Hemidesmus indicus R.Br (HI) showed considerable IC_{50} values for AchE (28.40 ± 0.92 µg/mL) and BuchE (43.47 ± 0.64 µg/mL) inhibition which indicates that HI extract has considerable specificity toward AchE and BuchE compared with all the tested extracts and the activity was followed by Vernonia anthelmintica (VA) Willd and Saussurea lappa Clarke (SL). The bioautograms also confirmed the activity potent extracts. **Conclusion:** Besides various bioactivities HI, VA and SL exhibited considerable cholinesterases inhibition making it to consider these species for further investigation of new compounds.

Key words: Acetyl cholinesterase, Alzheimer's disease, butyrylcholinesterase, *Hemidesmus indicus* R.Br, siddha system of medicine

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INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, which results in impaired memory and behavior manifestations and most prevalent in the aged population above 65.[1] Currently, cholinesterase inhibitors were accepted as effective drugs for treating mild to moderate AD.[2] Cholinergic theory for treating AD came into light from the post-mortem studies conducted on brain.[3] In normal healthy brain acetylcholinesterase (AchE) plays a major role when compared with that of butyrylcholinesterase (BuchE), but whereas in AD brain BuchE activity outweighs AchE by alteration in the ratio from 6 (normal brain) to about 11 (diseased brain) in the cortex regions where generally the impact of the disease is high.[4] Drugs in the current market are either directly or indirectly derived from herbal sources were recognized as an outstanding source for cholinesterase inhibitors such as huperzine, galantamine (GA), tacrine, rivastigmine, donepezil etc., However these drugs exhibit low bioavailability, shorter half-life, pharmacokinetic parameters, permeability through BBB and hepatotoxicity.[2] Due to these limitations search for new drug candidates for AD from herbal sources was still in demand because they were not only effective for treating AD, but also effective in the treatment of other forms of dementia such as vascular dementia, lewy bodies dementia and Down's syndrome etc.[5]

The present study was aimed to screen the selected herbs used by traditional healers of siddha system of medicine (SSM) for neurological disorders. The screening of these plants will provide a platform for drug discovery process. As there were no reports on the selected plants from SSM, the purpose of this study was to determine the *in-vitro* activity of selected species for AchE and BuchE inhibition.

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MATERIALS AND METHODS

Selection of plant species for study

The plants selected for the study rely upon the work of Indian ethnobotanist.[6] This reference work gives

the details of usage of these plants for various medical conditions from the past decade and the plant species selected were based on their usage for neurological conditions [Table 1].

Plant materials

The plant species were collected [Table 1] from in and around The Nilgiris hills and Himalayan region of India and authenticated by Dr. S. Rajan, field botanist, Survey of Medicinal Plants and Collection Unit, Central Council for Research in Homeopathy, Department of AYUSH, Emerald, The Nilgiris, Tamilnadu, India. The voucher specimens for each of the herbal materials were deposited at Department of Pharmacognosy and Phytopharmacy, JSS College of Pharmacy, Udhagamandalam 643001, India.

Chemicals and standards

Acetylcholinesterase enzyme (AchE) from *Electrophorus electricus*, butyrylcholinesterase enzyme (BuchE) from equine serum, acetylthiocholine iodide (ATCI), butyryl thiocholine iodide (BTCI), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and GA (purity ≥ 94%) were obtained from Sigma (Poole, UK). Methanol, tris-hydrochloride (HCl) buffer and potassium dihydrogen orthophosphate ($KH_2PO_4.2H_2O$) of analytical grade were purchased from SD Fine Chem Ltd. (Mumbai, India).

Preparation of plant extracts

The plant materials freshly collected were shade dried and ground to a coarse powder. About 1000 g of powder was extracted with methanol and water (1:1) by cold-maceration technique for 72 h; the hydro-alcoholic extract was drained and the procedure was repeated for 5 times and the pooled extract was filtered and concentrated in rotavapor at 40°C. Thus the obtained extracts were freeze-dried and stored at − 20°C until they were used for the further studies and the percentage yields were calculated [Table 1].

Phytochemical screening

The phytochemical screening of hydro-alcoholic extracts was tested for all the major groups by standard methods.[7]

Cholinesterases inhibition assays

The cholinesterases (AchE and BuchE) inhibition was performed by Ellman's assay and a thin layer chromatography (TLC) bio autographic assay using GA as a positive control.

Ellman's assay for AchE and BuchE activity

The principle underlying the assay was that the enzyme AchE hydrolyses the substrate ATCI resulting in the product thiocholine. This thiocholine in turn reacts with DTNB (Ellman's reagent) and produces 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitrobenzoate a yellow colored substance detected at 405 nm. The principle for BuchE is also same except that the enzyme hydrolyses BTCI substrate.[8] The assay was performed by the addition of 3 mM of 125 µL DTNB, 15 mM of 25 µL of ATCI/BTCI, buffer (50 µL) and sample in increasing order of concentrations (10, 20, 40, 80, 160 and 320 µg/mL) were dissolved in phosphate buffer (pH 8; 50 mM) were added to wells of 96 well plate. Absorbance was noted down at 405 nm for 65 s at every 13 s time interval. The reaction was started by the addition of 0.22 U/mL of 25 µL AchE/BuchE to every well and again absorbance was measured at 405 nm for 104 s for every 13 s time interval. Any increase in absorbance due to sudden hydrolysis of substrate was nullified by subtracting the absorbance from before and after addition of enzyme. The percentage inhibition was calculated by comparing rates of samples to that of control (50% Hydroalcohol in phosphate buffer [pH 8]). GA (positive control) was used as standard drug and the samples were analyzed in triplicate to calculate IC_{50} values. The percentage inhibition for AchE and BuchE were calculated using the formula:

%Inhibition of AchE and BuchE =
(A [Control] – A [Sample]/A [Control]) *100.

Where A (Control) is Absorbance of the control reaction (except test extracts) and A (Sample) is the absorbance of sample reaction.

TLC bioautographic assay

The hydroalcoholic extracts were dissolved in methanol and water (1:1 v/v) to obtain a solution of 10 µg/mL. Drug loaded

Table 1: AchE and BuchE inhibition of the tested extracts

Plant	Family	Part used	Common name	Voucher specimen no.	Extract yield (%)	AchE inhibition IC_{50} values (µg/mL)	BuchE inhibition IC_{50} values (µg/mL)
Calotropis gigantea Linn	Asclepiadaceae	Aerial parts	Crown flower	JSSU-379	6.45	111.80±1.3	148.74±0.16
Caesalpinia bonduc (L) Roxb	Caesalpiniaceae	Seeds	Fever nut	JSSU-352	5.47	98.58±0.98	137.33±0.91
Hemidesmus indicus R.Br	Asclepiadaceae	Roots	Indian Sarsaparilla	JSSU-348	4.82	28.40±0.92	43.47±0.64
Saussurea lappa Clarke	Astraceae	Roots	Costus	JSSU-359	7.59	58.68±0.86	94.46±0.5
Vernonia anthelmintica Willd	Astraceae	Seeds	Iron weed	JSSU-391	5.21	36.44±0.89	74.62±0.84
Galantamine	Positive control					0.0036±0.79	0.0098±0.45

The IC_{50} values of AchE and BuchE inhibition were expressed as triplicate of mean±SEM (n=3). AchE: Acetylcholinesterase; BuchE: Butyrylcholinesterase; SEM: Standard error mean

TLC plate was developed in a mobile phase of chloroform, methanol and water (60:35:5 v/v) and anisaldehyde sulfuric acid as spray reagent. The plate was completely dried and saturated by spraying with 5 mm ATCI, DTNB (dissolved in 50 mM of TrisHcl buffer, pH 8 and 37°C) and dried. Then 3 U/mL of AchE (50 mM of TrisHcl buffer, pH 8 and 37°C) was sprayed on to the plate which produced yellow colored background with white spots which are AchE inhibiting extracts observed after 5 min. The false positive reactions were identified.[9] Another plate was developed and sprayed with 5 mm of DTNB (50 mM of TrisHcl buffer, pH 8, 37°C) and after drying it was sprayed with 5 mm of ATCI and 3 U/mL of AchE dissolved in 50 mM of TrisHcl buffer, pH 8, the appearance of white spots on a yellow background resembles false-positive results. In the test for BuchE inhibition, AchE was replaced by BuchE and ATCI by BTCI respectively.

Calculation of IC_{50} values

A graph was plotted for % inhibition against concentration of the samples for each extract separately and IC_{50} values for each test extract were calculated from the regression equation of the each tested extract individually for AchE and BuchE. The concentration at which the tested extracts inhibited 50% of either AchE or BuchE was determined as IC_{50} value and represented as mean ± standard error mean of three experiments performed individually in triplicate.

RESULTS

Phytochemical analysis

The phytochemical analysis of hydroalcoholic extracts revealed that all the tested extracts showed presence of glycosides, carbohydrates, phytosterols, flavonoids and tannins. Apart from the above HI showed presence of coumarins, saponins and fixed oils; VA showed coumarins and fixed oils; SL showed lignans; CB showed alkaloids, proteins and saponins; and CG was positive for alkaloids and coumarins.

Extractive values

The extractive values of all the extracts were calculated and given in Table 1.

Ellman's assay for AchE and BuchE activity

The tested extracts exhibited inhibition for AchE and BuchE in the order of HI > VA > SL > CB > CG which followed a dose dependent fashion for the hydroalcoholic extracts. The IC_{50} values of the extracts were given in Table 1.

TLC bioautographic assay

The plant extracts which are showing the most potent activity by enzyme assay were tested for the inhibitory activity of AchE and BuchE by TLC bioautographic assay and confirmed whether the reaction is due to inhibition by the enzymes or not. No false-positive reactions were observed instead white spots of inhibition were observed for the potent active extract [Figure 1].

DISCUSSION

Though the herbal medicines were used widely very sparse scientific studies were accomplished to ascertain the mechanistic ways and efficacy of traditional formulation, remedies.[10]

Hydroalcoholic extract of *Hemidesmus indicus* R.Br (Asclepiadaceae) roots showed better inhibition against cholinesterases with IC_{50} values of 28.40 ± 0.92 μg/mL and 43.47 ± 0.64 μg/mL compared with other tested extracts. Traditionally it is being used for the treatment of epileptic fits in children.[6] Methanolic roots extract exhibited significant ($P < 0.05$) nociception at a dose of 50 mg/kg in mice.[11] Even the methanolic root extract of *H. indicus* was also proven to be an effective neuro-protectant and reduce the cerebral infraction when tested in rats. At dose levels of 200 and 400 mg/kg significant ($P < 0.01$) improvement in functions of complex neuromuscular and vestibulomotor were observed and an increment in dopamine and serotonin levels was observed and decrease in mono amino oxidase-B, glutamate and acetyl cholinesterase was observed.[12] Hence, the neuro-protection of the extract may be co-related to the cholinesterases inhibition but still further studies are required to confirm the activity.

Vernonia anthelmintica (VA) Willd (Astraceae) is traditionally used for mental disorders.[13] The inhibition of the targeted cholinesterases, i.e. AchE and BuchE IC_{50} values were reported as 36.44 ± 0.89 μg/mL and 74.62 ± 0.84 μg/mL

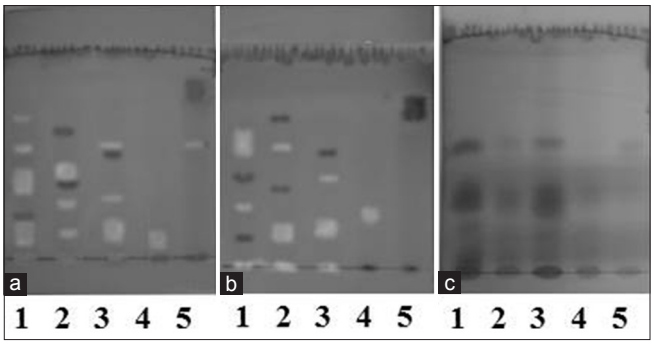

Figure 1: Bioautogram of acetylcholinesterase and butyrylcholinesterase inhibition of the tested extracts (a) acetylcholinesterase inhibition for the compounds (1) *Hemidesmus indicus* R.Br (2) *Vernonia anthelmintica* Willd (3) *Saussurea lappa* Clarke (4) *Caesalpinia bonduc* (L) Roxb. (5) Calotropis gigantea Linn. (b) Bioautogram representing the butyrylcholinesterase inhibition (same as mentioned in a). (c) Thin layer chromatography of hydroalcoholic extracts (same as mentioned in a) sprayed with anisaldehyde sulfuric acid spray reagent

respectively. Though drugs such as GA, physostigmine which were alkaloid in nature were potent cholinesterases inhibitors[2] apart from these even other chemical compounds such as glycosides, terpenoids, flavonoids[2,14] lignans[15] coumarins[16] and ursolic acid[17] also showed inhibition along with alkaloids. The phytochemical evaluation of the tested extracts revealed that the first three active extracts showed negative result for alkaloids but still the activity may be attributed due to presence of other constituents such as glycosides, phytosterols, flavonoids and tannins which are commonly present in all those extracts.

Saussurea lappa Clarke (Asteraceae) was used for brain tonic, headache, epilepsy and paralysis in traditional system of medicine and its root oil, root were used commonly in the formulations for these indications. The smoke of roots powder which is used as a substitute for opium when inhaled makes the patient to fall asleep fast.[6,18] In the present study, both cholinesterases inhibitory activity was reported with an IC_{50} value of 58.68 ± 0.86 μg/mL for AchE and 94.46 ± 0.5 μg/mL for BuchE inhibition. Petroleum ether roots extract of *S. lappa* at doses of 100, 300 mg/kg i.p increased the seizure threshold by means of GABAnergic mechanistic pathway.[19]

Extract from the seeds of *Caesalpinia bonduc* L. Roxb (Caesalpiniaceae) exhibited inhibitory activity with an IC_{50} values for both AchE (98.58 ± 0.98 μg/mL) and BuchE (137.33 ± 0.91 μg/mL). The plant is reported for its various potential usages in the traditional systems of medicine for various ailments, including potential for CNS related disorders such as paralysis, convulsions and similar nerve complaints.[20,21] *C. bonduc* seeds while elucidating various seed extracts (Petroleum ether, ethanol, methanol and water) for pentylenetetrazole (PTZ), strychnine and maximal electro shock (MES) induced convulsions. The petroleum ether extract at 600 and 800 mg/kg doses delayed the onset of tonic convulsions and showed 66.66% and 83.33% protection against convulsions.[6]

The IC_{50} values were reported as given in Table 1. *Calotropis gigantea* Linn (Asclepiadaceae) aerial parts extract exhibited inhibitory activity with IC_{50} of 111.80 ± 1.3 μg/mL and 148.74 ± 0.16 μg/mL for AchE and BuchE respectively. The plant was used traditionally by many ethnic groups for wide range of diseases, including CNS disorders in various ways such as the powdered dried leaves mixed with sweet oil and turmeric is applied on the paralyzed parts and the oil of boiled leaves is applied to the affected body parts will be useful for the treating paralysis.[6,13,22,23] In an article in 2011, Sureshbabu and Karki[24] had proven that the methanolic leaves extract of *C. gigantea* at a dose of 180 mg/kg showed 32% of mortality, 68% of protection and 45% of mortality, 55% of protection for MES and PTZ induced seizures. Hence, the methanolic leaves extract showed potent anticonvulsive activity though it was used for various CNS related indications.

The TLC bio autographic assay revealed that the active extracts did not show any false positive reactions for the active hydroalcoholic extracts (HI, VA and SL) for both AchE and BuchE inhibition which confirms that the reaction was due to the enzyme reaction and not due to the reaction between thiocholine and DTNB. Though marketed drugs such as GA, Physostigmine which are alkaloids were potent cholinesterases inhibitors, apart from these even other chemical compounds such as glycosides, terpenoids, flavonoids, lignans, coumarins and ursolic acid[2] also showed potent inhibition. The phytochemical evaluation of the tested extracts revealed that the first three active extracts showed negative result for alkaloids but still the activity may be attributed due to presence of other constituents such as glycosides, phytosterols, flavonoids, saponins and tannins which are commonly present in all the tested extracts. Hence, the active constituents may be responsible for the cholinesterases inhibition either individually or synergistically. The study results confirms that out of all the tested plant extracts HI, VA and SL exhibited the highest order of inhibition against AchE and BuchE enzymes. The other plant species which are not having potent activity may comprise of higher active inhibiting compounds. Hence the studies confirm the flora collected is having great potential for novel compounds with various grades of biological applications.

CONCLUSION

HI, VA and SL which exhibited the highest inhibitory activity against both the cholinesterases however, further investigation of these plants need to be embarked upon for potent active molecules and hence that they serve as starting compounds for synthesis. The plant extracts, which showed weaker results for cholinesterases inhibition was may be due to other course of action.

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