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Anti-Acetylcholinesterase Activity of *Piper sarmentosum* by a Continuous Immobilized-enzyme Assay

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Abstract

Recent promising enzymatic methods are gaining attention for the screening of safe inhibitors. The physiologically significant enzymes can be obtained from natural resources used for clinical and pharmaceutical purposes. *Piper sarmentosum* is reported to have antioxidant activity. Its use in folk medicines suggests that it contains anti-acetylcholinesterase compounds, which have not been discovered yet. In this study we have used flow injection analysis (FIA) technique by immobilizing the enzyme in a column for AChE assay on the crude extract of plant for four different solvents. The results obtained in this research shows that ethanolic extract has strong inhibition (IC_{50}= 128ug/L), whereas methanol extract has medium inhibition (IC_{50}= 127 ug/L) while Ethyle acetate and water has weaker inhibitory activity (IC_{50}= 317 ug/L and IC_{50}= 348 ug/L). From the results, it is suggested that *piper sarmentosum* have the ability to be a potential inhibitor for the development of anti-Alzheimer disease treatment.

Keywords: Acetylcholinesterase inhibition, flow injection analysis (FIA) technique, Alzheimer's disease (AD)

1. Introduction

Alzheimer's disease (AD) is the most common cause of senile dementia in elderly population and is

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estimated to account for 50-60% of dementia cases in persons over 65 years of age [1]. An estimated 35.6 million people are living with dementia worldwide and the number is expected to increase up to 66 million by 2030. Nearly 66% cases live in low and middle income countries, with the sharpest increase in numbers compared to high income countries [2].The principal role of acetyl cholinesterase (AChE) is the termination of nerve impulse transmission at the cholinergic synapses by rapid hydrolysis of acetylcholine (ACh). Inhibition of AChE serves as a strategy for the treatment of AD, senile dementia, ataxia, myasthenia gravis and Parkinson's disease [3]. Current available drugs for the treatment of AD includ tacrine, donepezil and the natural product based rivastigmine for treatment of cognitive dysfunction and memory loss associated with AD [4]. These compounds have been reported to have adverse effects including gastrointestinal disturbances and problems associated with bioavailability which necessitates the interest in finding better AChE inhibitors from natural resources [5].

Nature is a rich source of biological and chemical diversity. The unique and complex structure of natural products cannot be obtained easily by chemical synthesis [6]. A number of plants are used as traditional medicine in Malaysia. For the study of drugs related to AD, we selected *Piper sarmentosum* which have been used by elderly people for improving memory. *Piper sarmentosum* was screened and studied for its AChE inhibitory activity using Ellman's method with new technique of Flow Injection Analysis (FIA).

2. Materials and Methods

2.1. Extraction

Plant leaves were collected from Pahang State in Malaysia and dried at room temperature in the shade. The dried material was ground to powder. Extraction was carried out using Soxhlet extraction method using the four solvents Ethanol, Hexane, Ethyl acetate and Water.

2.2. Chemicals

2.2.1 Buffers

Four buffers were used. Buffer A: 50mM Tris HCl, , pH 8; buffer B: 50mM Tris-HCl, pH 8, containing 0-1% bovine serium albumin (BSA) ; buffer C: 50mM tris -HCl, pH 8, containing 0.1M NaCl and 0.2 M MgCl2 .6H2O; and buffer D: 50mM NaH2PO4 - Na2HPO4, pH 7.6.

2.3. Enzyme

Acetylcholinesterase from electric eel (type VI-s, lyophilized powder) was purchased from sigma, USA.

2.4. Substrate

Acetylcholine iodide (ATCI) was purchased from sigma, USA. 1mM ATCI in buffer A was used for assay.

2.5. Ellman's reagent

5, 5'-Dinitrobis- (-2 nitro benzoic acid) (DTNB) was purchased from Sigma, USA. 1 mM solutionof DTNB in buffer A was used.

2.6. AChE reference inhibitor

Physostigmine (Eserine) was purchased from Sigma USA as a reference inhibitor for AChE.

2.7. Enzyme Immobilization

Enzyme immobilization was carried out by the method described by Me-Leon Gonzales, 1990. Briefly, 0.2g of CPG-204 was boiled in 10mL of 5% nitric acid for 5 minutes. The CPG-240 was filtered on glass filter, washed with deionized water and dried in oven at 90^{O}C. The aqueous aminoalkylating agent was prepared by adding 1mL of 3-aminopropyletriethnosilane to 9mL of water and the pH was adjusted to 3.45 with 5M HCl. The dried glass was added in it and pH was adjusted to 3.45 and the mixture was kept at 75^{O}C on a water bath for 150 minutes; the mixture was stirred after every 15 minutes. The alkylaminated glass was filtered through a sintered glass filter (porosity G3), washed and dried as before. The Alkylamination process was repeated to ensure complete activation of glass. The dried alkylaminated glass was stored in an air-tight bottle and kept at room temperature till use.

The cross linking agent, glutaraldehyde (2.5% aqueous solution), was prepared by adding 2.5mL of 50% v/v glutaraldehyde solution (BDH) to phosphate buffer (0.1M, pH 7.0) and diluted to 50mL with the same buffer. Alkylamino glass (0.1g) was added to 1mL of the glutaraldehyde solution, in a well stoppard vessel through which nitrogen has been bubbled to remove oxygen. The reaction was allowed to proceed for 1 hour at room temperature with brief nitrogen deoxygenation every 10 minutes for first 30 minutes. The activated glass was washed well with distilled water. Acetylcholinesterase 4 mg , 765U)was dissolved in 2 mL of ice cold phosphate buffer (0.1M, pH 6.0) and added to the activated glass. Nitrogen was bubbled through the solution as before. The solution was kept at 4 OC for 2.5 hour. The immobilised enzyme derivative was washed first with cold phosphate buffer (pH 6.0) and then with cold water to ensure the removal of any unlinked enzyme. The resulting immobilised enzyme was packed into glass column.

2.8. Flow injection manifold and procedure

A simple flow injection procedure described by Ghous and Townshend, 1998 was adopted with slight modifications. The Ismatech-Reglo peristaltic pump was used for carrier stream propulsion while Rheodyne RH 5020 (Anachem) injection valve was used for injection of standard and sample. The manifold tubing was 0.5 mm i.e. PTFE. The volume of the sample and substrate mixture injection loop was 100 µl. Flow injection manifold used for the study is shown in Fig.1. Enzyme activity was measured by injecting standard substrate solutions into a carrier stream of the phosphate buffer (0.1M pH 8.5). The substrate was passed through the enzyme column. The reaction product was mixed with the chromogen (DTNB) in a second stream and absorbance (As) was measure at 405 nm in a flow cell (30 µl volume, 10 mm path length) using uv-visible spectrophotometer (Shimadzu 1700). For inhibition study, the sample (extract) was mixed with standard solution of substrate and the mixture (sample + standard) was injected into a stream of the phosphate buffer (0.1M, pH 8.5) passed through the immobilized enzyme column and decrease in absorbance (Ai) was recorded. The activity of immobilized enzyme was studied both in the presence and in the absence of the inhibitor. Percent inhibition by the extracts was calculated by the formula; % inhibition = (As – Ai)/As*100

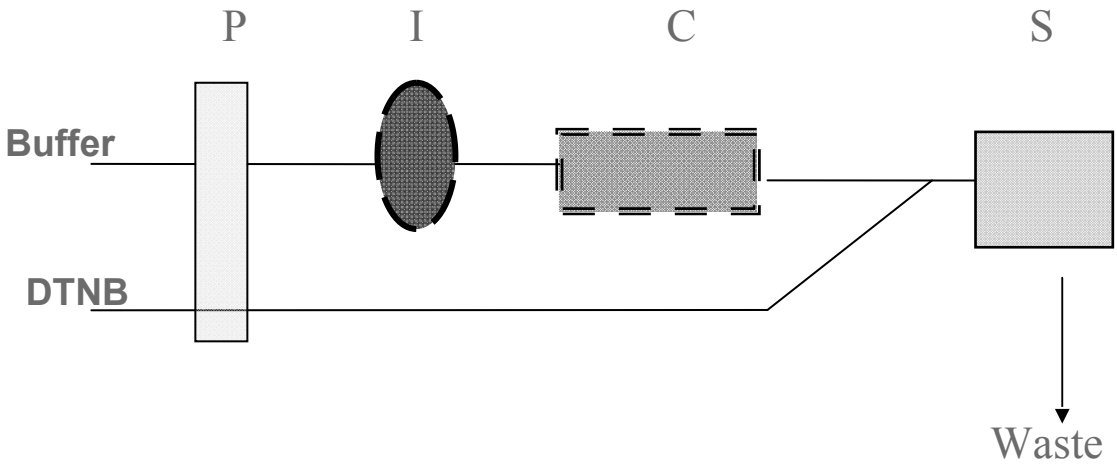

Fig. 1. Flow injection analysis (FIA) manifold used in study. P= peristaltic pump, I= Injection valve, C= Enzyme column, S= spectrophotometer.

3. Result and Discussion

3.1. Determination of Anti-Acetylcholinesterase Activity

Nowadays natural product research is leading to obtain promising drugs in human ailments[7].So the researcher all over the world are actively engaged on screening natural products as AChE inhibitors. In this study four crude extracts from leaves were prepared after successive extraction. Solubility of all collected fractions was checked and then Ethonolic, Hexane, Ethyl Acetate and aqueous extracts were selected for anti-AChE activity study. The IC50 values of active fractions were calculated from straight line equations. Brief description of extracts, %inhibition and IC50 values are given in Table 1. Results of the study demonstrate that crud aqueous extracts (IC50, 348 µg/ml) have weaker action against AChE activity. While ethyl acetate extract (IC50, 317µg/ml) showed moderate action. However, hexane and ethanolic extracts with IC50 values of 127 and 128µg/ml respectively, showed very strong action against AChE activity.

Table 1. Anti-AChE activity of various extracts of leaves of *Piper sarmentosum*

Extract	Conc. tested µg/ml	% Inhibition	IC50ug/mL
Ethanol	38	12	
	96	32	128
	166	58	
Hexane	33	32	
	66	40	127
	166	70	
Ethyl Acetate	43.33	16.36	
	172	40	317
	354	57.57	
Water/ Aqueous	26.67	14	348
	266	38	
	366	52	

Vinuth et al., [8] have screened *Momordica charantia* (Bitter melon) using all the solvents that we used in our study. Results of the study show very less inhibition by aqueous extracts, while other extracts comply with that of our results. The difference might be because of the parts of plant they use. We used only leaves as it is the part of the *Piper sarmentosum* which is better source of the compounds to be found as compare to other parts. So we found that the alcohol is the best inhibitor for the alkaloids while other solvents like Ethyl acetate and water are not the efficient solvents if we do extraction for alkaloids, as shown in fig. 2.

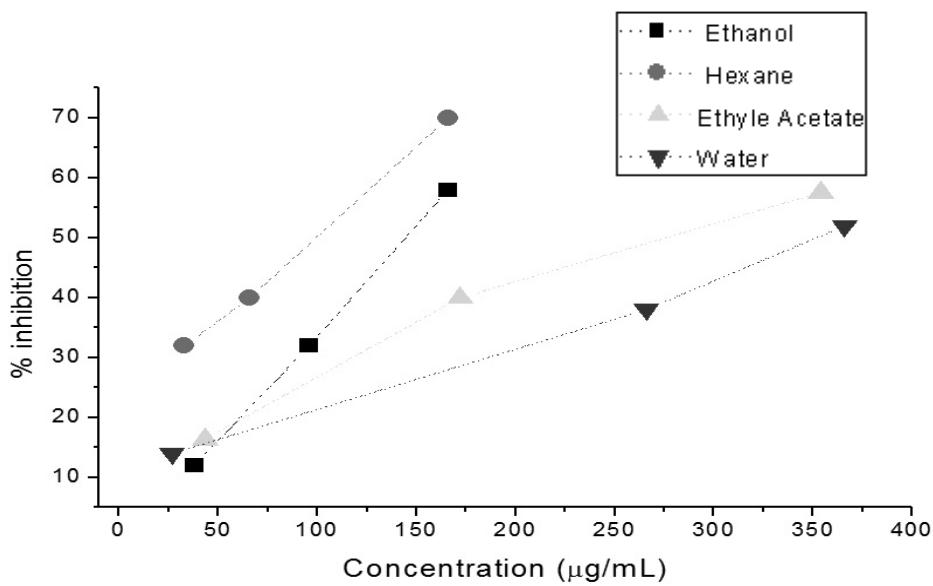

Fig. 2. Inhibition trends of different solvents with the change of concentration of plant sample. Hexanel and Ethanol are the best solvents for the extraction of alkaloids.

4. Conclusion

FIA method proves to be a very simple economical and rapid for the determination of Anti-cholinesterase activity. Bromocresol purple (BCP) has been found the most suitable indicator for routine Cholinesterase activity and inhibition studies. Our study proves that ethanol is the best solvent for the extraction of alkaloids from the plant material specifically the leaves.

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