# On-line Anti-Acetylcholine Esterase Activity of Extracts of *Oxystelma esculentum, Aerva javanica* and *Zanthoxylum armatum*

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Summary: Alzheimer's disease (AD), a disease of brain, resulting in memory impairment and the loss of thinking. The main reason of Alzheimer's disease is firmly associated with some impairment in cholinergic transmission. This impairment may be improved by diminishing the breakdown of acetylcholine at the synaptic site in the brain by inhibiting acetylcholinesterase (AChE). In this work, extracts of three different plants Oxystelma esculentum (OEM), Aerva javanica (AJM) and Zanthoxylum armatum (ZAA) have been screened for their anti-AchE activity. Results of the study demonstrate that of the studied extracts, ZAA [$IC_{50}$ 55.5 µg/ml] acquired stronger anti-AChE activity. While OEM with $IC_{50}$ 107.2 µg/ml showed moderate and ZAE and AJM showed weaker action [$IC_{50}$ 182.5 and 275.2 µg/ml].Galanthamine was used as a positive control [$IC_{50}$ 1.47 µg/ml].

Key Words: Acetylcholinesterase, Alzheimer's disease, Plant extracts, Flow Injection analysis.

## Introduction

Acetylcholine (ACh), a neurotransmitter, is hydrolyzed at a greater rate by the enzyme Acetylcholine esterase (AChE) than any other ester [1]. AChE hinders the passage of nerve impulse by the breakdown of acetylcholine (ACh) at the cholinergic synapse [2]. It results in the low concentration of ACh at these neurotransmitter junctions, which is the cause of Alzheimer's disease (AD). Another problem is ''myasthenia gravis'' in which muscle weakens due to low concentration of ACh [3]. A number of drugs like galantamine, nicotine, physiostigmine, morphine, neostigmine etc [4] are used for the inhibition of the enzyme but these may have side effects. Physiostigmine, a common memory improving drug, has many health hazardous effects like respiratory paralysis, cholinergic crisis, defecation. Similarly, Tacrine (tetrahydroaminoaccaridine) even more effective for the treatment of AD has similar problems [5, 6] and severe allergic reactions (rash; hives; itching; difficulty breathing; tightness in the chest). These drugs inhibit not only AChE but also butyrylcholinesterase (BuChE) which is useful in scavenging and detoxification of drugs like cocaine, amitriptyline, heroin [7]. Neostigmine, another drug for its treatment cannot cross the blood brain barrier [8]. Galantamine, more superior drug to all these, has high selectivity for AChE. However, its synthesis at commercial level is very difficult, costly and low-yielding. All these problems demand that there should be safer, easily available naturally occurring AChE inhibitors.

Medicinal plants, a significant natural resource for important drugs as drugs obtained from plants are relatively more safe, can play a vital role in pacifying human health. The potential side effects of allopathic medicine and their high cost encouraged people to use traditional medicines [9]. The increasing demand of plant extracts in food, cosmetics and pharmaceutical industries suggests that systematic studies of medicinal plants are very important in order to find active compounds and their use as a medicine for curing various diseases [10].

*Oxystelma esculentum* is used medicinally in Asia and Africa. Different parts of the plant have been claimed to be effective in a wide spectrum of diseases [11]. Its juice is used in gleets, gonorrhea, pain in the muscles, cough and given to children as an astringent. The milky sap forms a wash for ulcers. The herb also contains antiseptic properties and its decoctation is useful as a gargle in mouth and throat infection [12].

*Aerva javanica* (Amaranthaceae) is perennial hairy with broad leaves, distinguished by dense covering of star-like hairs on stems and on alternate to clustered leaves; flowers in terminal panicles of dense spikes to 10 cm long [13]. It is used to treat inflammation, kidney stone, antiplasmodial

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[14], antidiarrhoeal, antihyperglycaemic [15], diuretic, anticalculus, insecticidal [16]. It is widespread in northern Australian, tropical and warm areas of the Africa, Asia, India and Pakistan [17].

*Zanthoxylum armatum* (Winged Prickly Ash) commonly known as timur is a shrub or a small tree, up to 6m in height. Small shoots of timur are used as tooth brushes. They are very effective in toothache, can give flavor to food [18]. It is used in the treatment of various common diseases such as toothache, cold, cough, and fever. It gives warmth to the body. Its fruits and seeds are very effective in fever, dyspepsia and expelling roundworms. It has also stimulating effect upon the lymphatic system circulation and mucous membrane [19].

Flow Injection Analysis (FIA) is an automated, continuous flow approach to perform chemical analysis, based on injecting a small, well defined volume of sample into a continuously flowing carrier stream to which appropriate auxiliary reagent streams can be added whereby a concentration gradient of the sample is created. Incorporation of immobilized enzymes in this technique has made it even more attractive for a wide variety of analysis [20].

A very important aspect of immobilized enzyme is that enzyme is reactivated after inhibition by the sample, using high concentrated injection of substrate "acetylcholine" [21]. In this way same enzyme is used throughout the process.

In this study we have elaborated this technique for Anti-AChE activity study by organic extracts of some selected medicinal plants.

**Results and Discussion**:

We used four extracts ZAE (*Zanthoxylum Armatum* in ethanol), AJM (*Aerva javanica* in methanol), OEM (*Oxystelma esculentum* in methanol), ZAA (*Zanthoxylum Armatum* in acetone) for the inhibition of AChE using flow Injection technique. Under prescribed conditions calibration curve for each extract was constructed Fig.1 and $IC_{50}$ values for the extracts were evaluated from calibration curves. Results of the study demonstrate that of the studied extracts, ZAA [$IC_{50}$ 55.5 µg/ml] acquired stronger anti-AChE activity. While OEM with $IC_{50}$ 107.2 µg/ml showed moderate and ZAE and AJM showed weaker action [$IC_{50}$ 182.5 and 275.2 µg/ml]. Results are depicted in Fig.2. Galanthamine was used as a positive control with $IC_{50}$ value of 1.47 µg/ml.

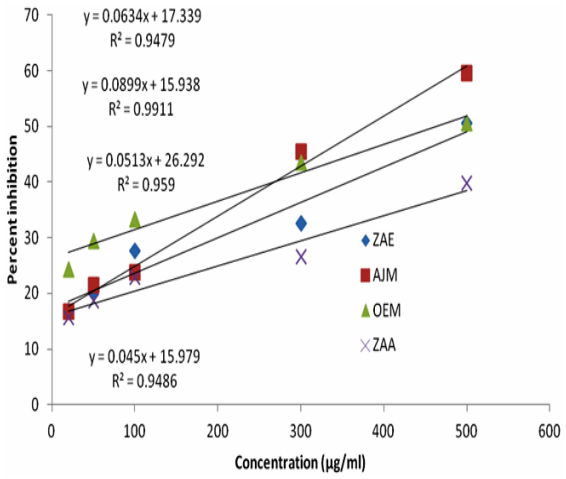


Fig. 1: Calibration Curves for the studied extracts. Concentration range studied 20-500 µg/ml.

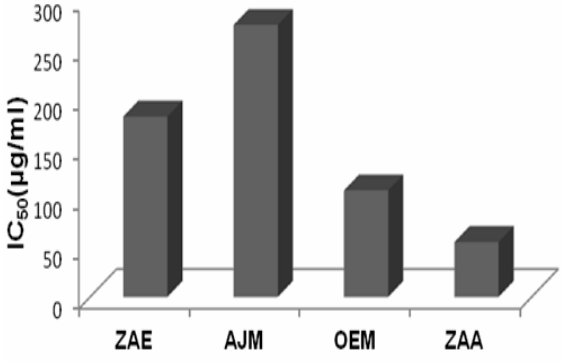


Fig. 2: $IC_{50}$ (µg/ml) values of studied extracts.

**Experimental**

*Reagents and Materials*

Acetylcholinesterase (E.C. 1. 1. 3. 7) from electric eel, controlled porosity glass (CPG-24, 80-120 mesh, mean pore diameter 22.6 nm) were purchased from Sigma-Aldrich.Sodium dihydrogenphos-phate,13.7$gL^{-1}$, was used to prepare 0.1M buffer, pH was adjusted with 0.1M NaOH solution, A stock solution of acetylthiocholine iodide (Sigma- Aldrich) ($2x10^{-3}$M) was prepared by dissolving 0.057g in 100 mL of water. Further dilutions were made in the pH 8.5 buffer. A l $x10^{-3}$M solution of 2,2 dithiobis (2-nitrobenzoic acid (DTNB) (Sigma-Aldrich) was prepared by dissolving 39mg in 100 mL of the buffer. 3-aminopropylethoxysilane and glutaraldehyde were also purchased from Sigma-Aldrich. All reagents used were of analytical-reagent grade. Distilled and deionised water was used throughout the experiment.

*Enzyme Immobilisation*

Enzyme immobilization was carried out by the method described by Me-Leon Gonzales [22] and Ghous [23, 24]. Briefly, a 0.2 g amount of CPG-240 was boiled in 10 mL of 50% nitric acid for 30 min. The CPG-240 was boiled in water bath, washed with deionized water and dried in an oven at 70 °C for 2 h. The aqueous aminoalkylating agent was prepared by adding 1 mL of 3-aminopropylethoxysilane (Sigma-Aldrich) to 9mL of water and the pH was adjusted to 3.45 with 5 M HCl. The dried glass was added, the pH was readjusted to 3.45 with 5M HCl and the mixture was kept at 75 °C on a water bath for 150 min; the mixture was stirred every 15 min, 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 the glass. The dried alkylaminated glass was stored in an air-tight bottle and kept at room temperature until needed.

The cross-linking agent, glutaraldehyde (2.5% aqueous solution), was prepared by adding 2.5 mL of 50% v/v glutaraldehyde solution (BDH) to phosphate buffer (0.1 M. pH 7.0) and diluted to 50ml with the same buffer. Alkylamino glass (0.1 g) was added to 1ml of the glutaraldehyde solution, in a well-stopper vessel. The reaction was allowed to proceed for 1 h at room temperature. The activated glass was washed well with distilled water. Brain extracts supernatant (2 mL) containing AChE was added in 2 mL of ice-cold phosphate buffer (0.1M, pH 6.0) and added to the activated glass. The solution was kept at 4 °C for 2.5 h. The immobilized enzyme was washed first with cold phosphate buffer (pH 6.0) and then with cold water to ensure the complete removal of any unlinked enzyme and rest of the contents. The resulting immobilized enzyme was packed into a glass column for use in the flow injection manifold.

*Preparation of Extract*

The aerial parts of *Aerva javanica, Oxystelma esculentum* and *Zanthoxylum Armatum* were dried under shade to constant weight. Then the dried material was ground mechanically. The powdered material (50 g) of each plant was macerated for 3 days with 500 ml of solvent at room temperature, by occasional shaking. The extract was dried under reduced pressure using a rotary evaporator. The dry extract was stored in glass voiles at room temperature for further use. Four different extracts were obtained from these plants using different solvents. ZAE (*Zanthoxylum Armatum* in ethanol), ZAA (*Zanthoxylum Armatum* in acetone), AJM (*Aerva javanica* in methanol), OEM (*Oxystelma esculentum* in methanol).10 mg of each extract was dissolved in 10ml of distilled water. It was centrifuged at 6000rpm for 1hour using a Hettich centrifuge machine. Supernatants were collected and labeled as above. Each extract was further filtered through ultrafilitration syringe filters (pore size 0.2µm) to remove any insoluble material. Further dilutions were made from the stocks as required, in 0.1 M sodium phosphate buffer (pH 8.5).

*Flow Injection Manifold and Procedure*

The flow injection manifold used for AChE inhibition study is shown in Fig. 3. Flow injection method described by Ghous and Townshend 1998 was adopted with slight modifications(13, 20). The peristaltic pump for carrier stream propulsion was an Ismatech-Reglo, and Rheodyne RH 5020 (Anachem) injection valve was used for injection of standard and sample. The manifold tubing was 0.5 mm i.d. PTFE. The volume of the sample and substrate mixture injection loop was 100 µl. Enzyme activity was measured by injecting standard substrate solutions into a carrier stream of the phosphate buffer (0.1 M 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 was measured at 405 nm in the flow cell (30 µL volume, 10 mm path length) using Shimadzu 1700 spectrophotometer. For inhibition study, sample (extract) was mixed with standard solutions of substrate and the mixture (sample+standard) was injected into a stream of the phosphate buffer (0.1 M, pH 8.5) passed through the immobilized enzyme column. Decrease in absorbance was recorded. The behavior of immobilized enzyme was studied both in the presence and in the absence of the inhibitor.

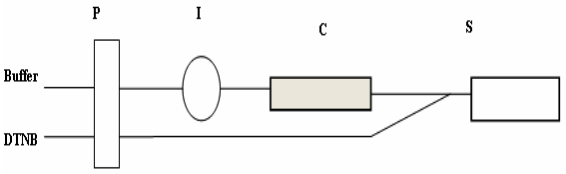


Fig. 3: Flow Injection manifold used in this study. P = peristaltic pump, I = injection valve, C = enzyme column, S = spectrophotometer

**Conclusion:**

Plants have been used for treating ailments and diseases since ages. Owing to their use as medicines many plants have been discovered having

antioxidants, anti-inflamatory, neuroprotective agents and enzymes inhibition acivity. However, the pharmacological properties of memory enhancing plants have not been extensively investigated in the perspective of recent models of Alzheimer's disease. The limited effectiveness of so-called rationally designed therapies for Alzheimer's disease, it is appropriate to re-explore historical archives for new directions in drug development. With current important progress in understanding the neurobiology of Alzheimer's disease, the use of complementary medicines such as plant extracts in the therapy should of great importance to be explored. The plants used in this work *Zanthoxylum Armatum* show very good activity while *Oxystelma esculentum* show moderate activity against acetylcholine esterase, which is major cause of the disease. The method used is simple and sensitive. It could open new dimensions to use natural products for the treatment of the disease.

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