



A Kinetic Study of Acetylcholinesterase Inhibition by Fractions of *Oleo diox Roxb.leaf* and *Curculigo orchioides* Gaertn Rhizome for the Treatment of Alzheimer's Disease

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Authors' contributions

This work was carried out in collaboration between both authors. Author PGK has designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author MS conceived the study and were in charge of overall direction, planning and manuscript corrections. Both authors read and approved the final manuscript.

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ABSTRACT

Introduction and Aim: Acetylcholinesterase (AChE) has an important role in the biochemical studies indicating that AChE accelerates the formation of amyloid- β fibril and forms AChE-A β complexes in the brain. The medicinal plants of *Curculigoorchioides* Gaertn., and *Oleo diox Roxb.* collected from Western Ghat region of Kodagu, Karnataka, India have many medicinal values such as antioxidant, anti-cancer, anti-diabetic, neuroprotective activity and anti-inflammatory property. Aim of the study was to check whether *C. orchioides* Gaertn. and *Oleo diox Roxb.* fractions significantly inhibit acetylcholinesterase in different concentration-dependent manner (1-3 mg/mL). **Results:** Plant fraction concentrations resulted in >60%AChE inhibition as compared with the standard Galantamine. The IC₅₀ value was 0.15 mg/mL which was calculated from the equation of the percentage inhibition curve for the test or plant fraction. The Lineweaver-Burk plot indicated that

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the fraction followed competitive and non-competitive inhibition kinetics. Finally, we conclude that the type of plot exhibited by the *C. orchoides* Gaertn. and *Oleo diox Roxb. fractions* has a possible therapeutic application for improving memory and other cognitive functions. The medicinal plant-based drugs are known to have limitations due to their fewer side effects and problems associated with bioavailability, which necessitates the interest in finding better AChE inhibitors from plant sources.

Keywords: Acetylcholinesterase; amyloid- β fibril; *C. Orchoides Gaertn*; *Oleo diox Roxb fraction*; Lineweaver-Burk plot.

1. INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease distressing the brain. AD is the most frequent cause of dementia in an elderly population. Symptoms of AD are loss of memory, understanding, and speech, such as anxiety and dysphoria, unpleasant behavioral changes [1]. It has provided the intellectual structure for therapeutic intervention. It proposes that the development of deposition of the β -amyloid protein is the initial pathological event in AD, important to the creation of senile plaques and then to neurofibrillary tangles, neuronal cell death, and finally causes dementia. AD is globally recognized as the most common form of dementia. It will be increasing and by the year 2050, 115 million people will be affected throughout the world [2].

Acetylcholinesterase enzyme (AChE) is participating in cholinergic neurotransmission and stimulation of cholinergic receptors of acetylcholine released into the neuronal synaptic cleft by inhibiting acetylcholine hydrolysis by AChE through the use of acetylcholinesterase inhibitors [3]. Acetylcholinesterase enzyme is membrane-bound. The main role of acetylcholinesterase is the termination of nerve impulse transmission at the cholinergic synapses by fast hydrolysis of the neurotransmitter acetylcholine [4]. The AChEIs promote a boost in the concentration and duration of the act of synaptic acetylcholine [5].

Acetylcholinesterase has a significant role in the biochemical studies indicated that AChE accelerates the formation of amyloid- β fibril and form AChE-A β complexes in the brain. The neurotoxicity accelerates by AChE-A β complexes indicated that they prompt more neurodegeneration than those of the A β peptide [6].

Acetylcholinesterase activity is inhibited by many synthetic and natural compounds. There are two

main types of AChE inhibitors which can be distinguished from drugs and toxins [5]. The inhibitors are different compounds with different structural motives as they can bind to the etherification part of the active site by etherification of serine hydroxyl and the aromatic gorge and the peripheral anionic site [7].

Alzheimer's disease therapy is moderate and mainly based on acetylcholinesterase inhibitors (AChEIs) such as synthetic galantamine and donepezil. The plant-based drugs are known to have limitations due to their fewer side effects and problems associated with bioavailability, which necessitates the interest in finding better AChEIs from natural resources [8-14].

2. MATERIALS AND METHODS

2.1 Chemicals

Acetylthiocholine iodide (ATCI), Acetylcholinesterase (AChE) from electric eel, 5,5'-dithiobis [2-nitrobenzoic acid] (DTNB), Galantamine, purchased from Sigma. Ethanol and all other organic solvents (analytical grade) were purchased from Merck.

2.2 Collection of Sample

Rhizomes of *C. orchoides* Gaertn., and leaves of *Oleo diox Roxb.* were collected from the Western Ghat region of Kodagu, Karnataka, India.

2.3 Sample Preparation

Freshly collected rhizomes and leaves of the plants were washed 2-3 times with tap water and then with distilled water, followed by ethanol and then allowed to dry at room temperature for 23 days and finally mechanically powdered. 50 g of powder was Soxhlet extracted with ethanol and methanol, dried at room temperature and collected the sample in brown bottles until further analysis. Extracts of both plants were

reconstituted in ethanol and methanol and then subjected to column chromatography. Active fraction (*C. orchoides* Gaertn. F3 Fraction and *oleo diox Roxb.*, F2 fraction) have exhibited positive results.

2.4 Estimation of the IC₅₀ Value

The different concentrations of the fraction inhibited 50% of AChE activity (IC₅₀). The estimation of IC₅₀ was carried out by the method of Kamal et al. [14]. It was performed by plotting % activity and % inhibition of acetylcholinesterase by the plant fraction (inhibitor) concentrations on the graph. The concentrations at the cross-section of these two curves was the IC₅₀ value. The assay mixture contained briefly 0.2 mL of 0.1 M Tris-HCl buffer (pH 8.0), 20 µL of fraction at different concentrations (1.2, and 3 mg/mL) and 20 µL AChE (3 U/mL) solutions were mixed and incubated for 15 min at room temperature and added 50 µL of 3 mM DTNB to this mixture. The reaction was then initiated by the addition of 50 µL of 15 mM AChI (acetylthiocholine iodide) substrate at different concentrations (0.5-4 mM). At this stage, yellow colour was formed. Galantamine was used as a positive control (1.5 mg/mL). The absorbance of the mixture was measured at 412 nm in a UV-Visible spectrophotometer (Beckman Coulter, USA). The final volume was adjusted to 2 mL with the 50 mM sodium phosphate buffer pH 8 [11]. IC₅₀ values were obtained from (%) activity of the compound concentration graph by measuring the enzyme activity at different inhibition concentrations. In the mixture with

inhibitor, the enzyme activity was measured at different concentrations of the substrate [4].

2.5 AChE Enzyme Kinetic Studies

2.5.1 Effects of various concentrations of the enzyme on the rate of reaction

The activity of acetylcholinesterase was assessed for the enzyme at different concentrations [12], ranging from 25-150 µL of AChE (3 U/mL) and 50 µL substrates were taken in all the test tubes. The assays were performed in triplicate.

2.5.2 Effects of varying concentrations of ATCI (acetylthiocholine iodide) substrate on the rate of reaction

The activity of acetylcholinesterase was assessed for acetylthiocholine iodide substrates at different concentrations [13], which ranged from 25-150 µL (ATCI, 15 mM) and uniform (AChE 25 µL, 3 U/mL) remains the same for all the test tubes. The assays were performed in triplicate.

2.5.3 Effects of various concentrations of the substrate on the rate of reaction

The activity of acetylcholinesterase was assessed for substrates at different concentrations. The substrate concentration ranged ATCI from 0.5-4 mM and the AChE enzyme concentration (3 U/mL) remains the same for all the tests [12]. The assays were performed in triplicate.

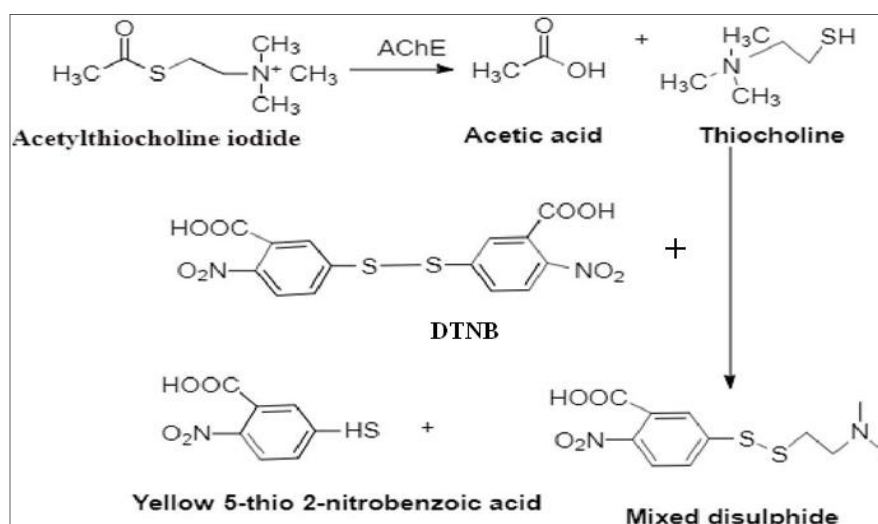


Fig. 1. Ellman's reaction

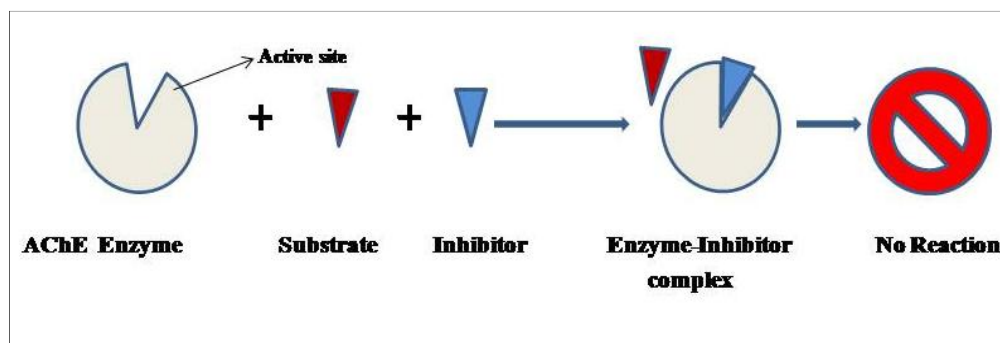


Fig. 2. Inhibitor binds to the active site and directly blocks the active site

2.5.4 AChE enzyme saturation level

The saturation curves were constructed for the calculated acetylcholinesterase enzyme performance at various time(S) intervals [14,15]. Saturation curves were determined at room temperature. The 1mL of reaction mixture contains 50 μ l of AChE enzyme (3 U/mL +500 μ l DTNB (3 mM) +100 μ l of substrate (15 mM, ATCI) 200 μ l phosphate buffer (pH 8.0). All assays were performed three times. The absorbance at 412 nm was determined by UV-spectro- photometer. The absorbance was recorded for every 30 seconds up to 4min. The assays were performed in triplicate.

2.6 Lineweaver-Burk (LB) plot and Michaelis-Menten Equation (K_m and V_{max})

The kinetic inhibition of ethanolic and methanolic extracts of plant fraction was analyzed [16]. Ellman reaction mixture consisted 0.2 mL of 0.1 M Tris-HCl buffer (pH 8.0), 20 μ l of fraction at different concentration (1.2, and 3 mg/mL) and 20 μ l AChE (3 U/mL) solution which were mixed and incubated for 15 min at room temperature and added 50 μ l of 3 mM DTNB. The reaction was then initiated by the addition of 50 μ l of 15 mM AChI(acetylthiocholine iodide) substrate at different concentrations (0.5-4 mM) when yellow colour was formed. Galantamine was used as a positive control (1.5 mg/mL). The absorbance of the mixture was measured at 412 nm in a UV-Visible spectrophotometer (Beckman Coulter, USA). Each assay was repeated three times. The final volume was adjusted to 2 mL with the 50mM sodium phosphate buffer with pH 8. The kinetic parameters K_m and V_{max} were determined from Lineweaver and Burkmethod [17]. Acetylcholinesterase enzyme concentration was kept constant with different concentrations of

substrate (ATCI) ranging from 0.5-4 mM in the presence of AChE (3 U/mL) activity at the different concentration of inhibitor and measured the rate of reaction (1/V). At lower concentrations of substrate, the plot suggested less competitive reaction and at higher concentrations of substrate, I showed the competitive mode of production. The K_m and V_{max} were determined from Lineweaver and Burk double reciprocal plot analyzed over a different range of AChI concentrations (0.5 to 4 mM). AChE enzyme velocity is the dependent variable and AChI substrate is the independent variable. This experiment generates the K_m and V_{max} (Michaelis-Menten) plot.

3. RESULTS

3.1 Estimation of IC_{50} Value

Acetylcholinesterase is inhibited in a concentration-dependent manner. The IC_{50} values of rhizome of *C. orchoides* Gaertn., and *Oleo diox Roxb fraction* 1-3 mg/mL respectively. Galantamine was used to constant the standard curve. By increasing the concentration of plant fractions of *C. orchoides* Gaertn., and *Oleo diox Roxb.*, the acetylcholinesterase inhibition rate was increased by >50% for 5 min incubation whereas that of rhizome of *C. orchoides* Gaertn., from >60% for 5 min incubation. The potency of *C. orchoides* Gaertn., and *Oleo diox Roxb* was compared to that of standard galantamine, the drug which is being used for the treatment of Alzheimer disease. Galantamine was found to have an IC_{50} value of 1.5 mg/mL *O. diox Roxb.*, is at least 30 times more potent than galantamine, and *C. orchoides* Gaertn., is 50 times more potent than *O.diox Roxb.*, and Galantamine. The AChE enzyme inhibition kinetics of *C. orchoides* Gaertn., and *Oleo diox Roxb.*, fraction were determined from LB plots,

and both showed non-competitive and competitive inhibitions.

3.2 Effects of Various Concentrations of the Acetylcholinesterase Enzyme (AChE) and Acetylthiocholine Iodide (ATCI) Substrate on the Rate of Reaction

The varying AChE enzyme concentration with constant substrate concentration was taken. The effects of varying AChE enzyme concentrations on the rate of reaction, substrate concentrations remaining the same were being determined [Graph1]. The graph indicates that with an increase in AChE concentration using constant ATCI substrate concentration, the rate of reaction increased the formation of thiocholine and acetic acid. The AChE can take up to numerous catalytic reactions per second. To determine the maximum speed of an enzymatic reaction, the substrate concentration is the same until the increasing rate of reaction and product formation. This is the V_{max} of the AChE enzyme, active sites of which are saturated with ATCI, Michaelis and Mentent [18-21].

3.3 Effects of Varying Concentrations of ATCI (Acetylthiocholine iodide) Substrate on the Rate of Reaction

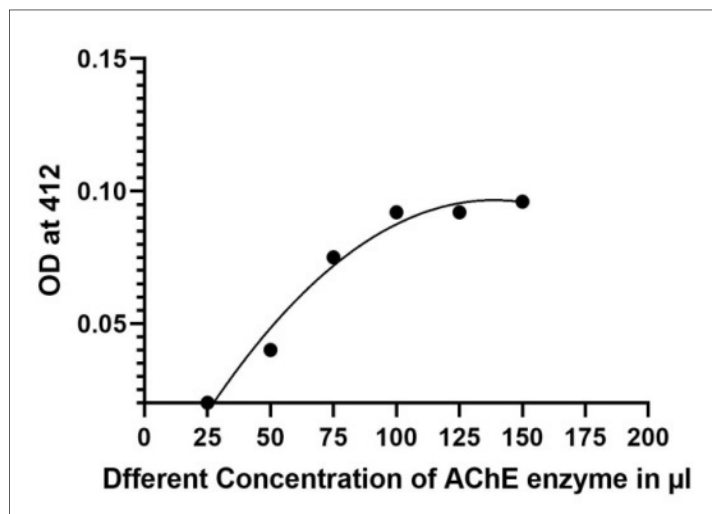
The varying ATCI substrate concentrations and constant AChE concentration were taken and the effects were determined [Graph2]. The graph indicates that with an increase in substrate concentration, using AChE concentrations remaining the same, the rate of reaction increases with the formation of thiocholine and acetic acid. The substrate attentiveness is increased until a stable rate of product formation [18-21]. This is the V_{max} of the enzyme. In this state, all AChE sites are active and saturated with the substrate (acetylthiocholine iodide). Since the substrate concentration at V_{max} value exactly cannot be measured, AChE enzymes are categorized by the substrate concentration at the rate of reaction is half its maximum.

3.4 Pre-steady-state Kinetics

The AChE is mixed with the substrate, at zero seconds. There is no product formed and there are no intermediates exist. The next few seconds of the reaction mixture is called pre-steady-state

Table 1. IC_{50} of fraction of *C. orchoides* Gaertn and *Oleo diox Roxb*

Inhibition $IC_{50} \pm SD$ (mg/mL)			
Galantamine	1.5 mg/mL 1.23±0.53		
Name of the plant	1 mg/mL	2 mg/mL	3 mg/mL
<i>C. orchoides</i> Gaertn	2.29±0.73	3.59±0.80	5.36±1.11
<i>Oleo diox Roxb</i>	2.21±0.43	3.25±1.08	2±0.81



Graph 1. Different concentrations of Enzyme Vs Substrate

kinetics, which is shown that the formation and consumption of AChE and ATCI substrate intermediates pending their steady-state kinetics are reached. In case of AChE, an intermediate is formed by an attack on the active site of the substrate and the formation of the product [21,22]. In the first few seconds, the AChE enzyme forms the product. The final when steady state is reached the amount of product released is slowdown which indicates the saturation curves of AChE [Graph-3]. The amount of product formed is shown in the Y-axis and x-axis shows the steady-state time or saturation level time.

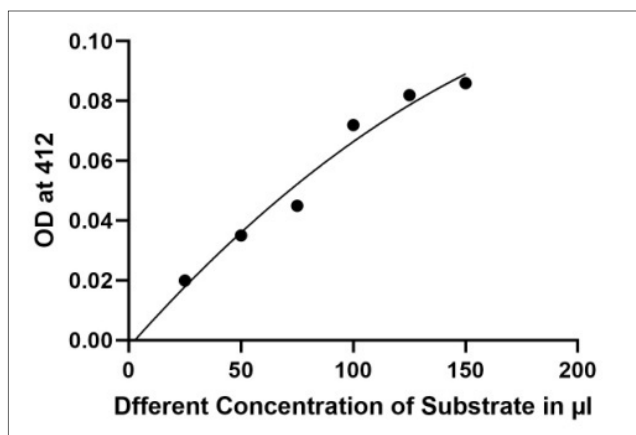
3.5 Effects of Varying Concentrations of ATCI Substrate in mM on the Rate of Reaction

The rate of a reaction concerning AChE also increases as the substrate concentration increases. The limited number of active sites are

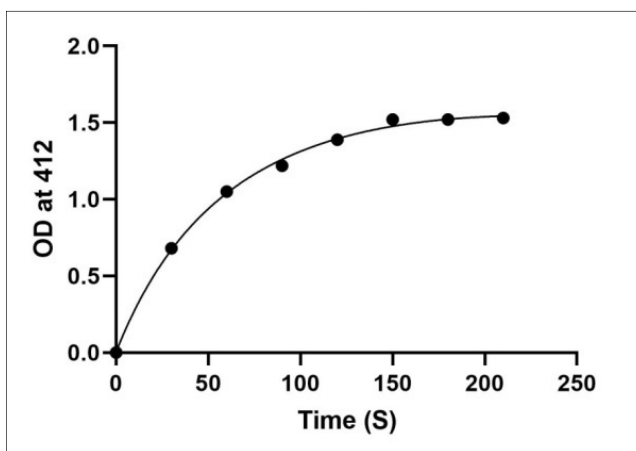
available in the enzyme. The available enzyme active sites are occupied with substrates. Consequently, increasing the substrate concentration it is observed that it will not alter the rate of diffusion [23]. Some maximum rate of reaction (V_{max}) when all the enzyme active sites are occupied by the substrate. The rate of reaction increases with increasing substrate concentration, but must asymptotically approach the saturation rate or level [Graph4]. V_{max} is always directly proportional to the total enzyme concentration, and the catalytic constant of the enzyme [24].

3.6 Lineweaver-Burk (LB) plot and Michaelis- Menten equation (K_m and V_{max})

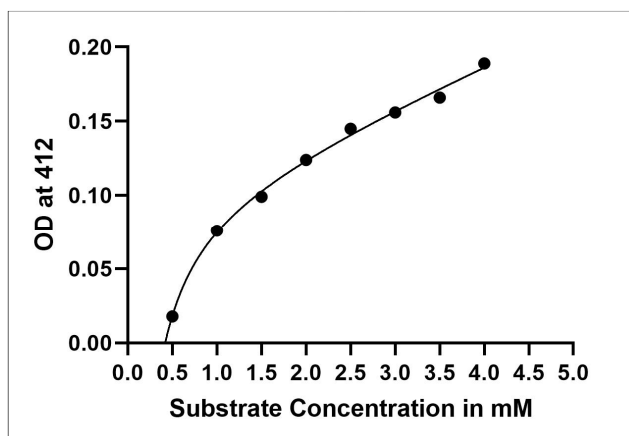
The substrate specificity of acetylcholinesterase (AChE) was evaluated by testing AChI as a substrate varying from 0.5 to 4 mM. AChE hydrolyzes acetylthiocholine. Substrate kinetic



Graph 2. Different concentration of Substrate Vs Enzyme



Graph 3. The saturation curve for acetylcholinesterase



Graph 4. Varying concentrations of substrate in mM

parameters for AChI were calculated by K_m and V_{max} values by Lineweaver-Burk plot [5A & 6A] values [25,26]. The Lineweaver-Burk plot was used to calculate V_{max} and K_m (Michaelis constant). The initial velocity of AChE was calculated for each substrate concentration individually. This K_m value is also called Michaelis-Menten constant. The enzyme-substrate complex is converted to a product. How quickly enzyme active sites become saturated can be described by the variable K_m , [Graphs 5B & 6B] the substrate concentration at which the reaction rate is V_{max} .

4. DISCUSSION

The medicinal plant extracts of *O. diox Roxb.* (F2 fraction) and *Curculigo orchoides Gaertn.* Rhizome (F3 fraction) caused significant inhibition of AChE activities *in vitro* studies. The highest inhibition in acetylcholinesterase activity was noticed in the *Curculigo orchoides Gaertn.* rhizome *in vitro* methods. Acetylcholinesterase is found in the cell membrane it degrades which the neurotransmitter acetylcholine into choline and acetate, is the important reaction for the regulation of synaptic activity in the nervous system [27].

The rate of product formation increases with increasing substrate concentration [Graphs 1,2&3]. However, in the presence of enzymes, the enzyme reaction has higher values and a much steeper slope. This implies enzymes greatly increase the rate of reaction. However, as the enzymes become saturated, the reaction rate levels remain constant [Graph3] [28]. The LB plot was used to study enzyme inhibition kinetics.

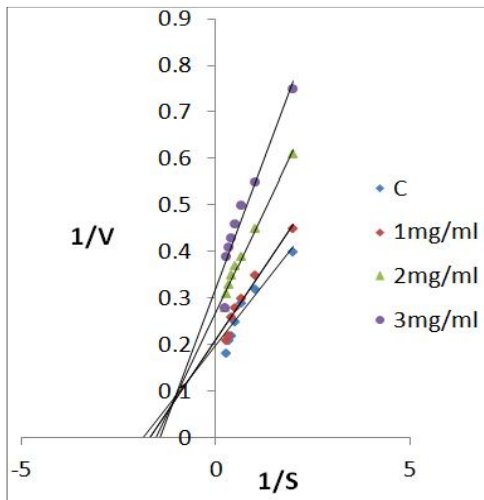
A total of 2 semi-purified plant fractions of 2 different plants were screened for AChE inhibition activity. The inhibition sample was initiated for each semi-purified fraction by plotting $1/[V]$ versus $1/[S]$ (LB plot). The following were the distribution of inhibition patterns: Two fractions at different concentrations had competitive inhibition, mixed inhibition, and non-competitive inhibition. Hence Lineweaver-Burk and Michaelis-Menten plots have been shown in Graphs 5A, 5B and 6A, 6B, showed mixed inhibition, uncompetitive inhibition, and competitive inhibition. The plots for galantamine, as a known AChE inhibitor, have been shown in graphs 5A, 5B and 6A,6B.

In this study, 2 plant fractions were assayed at one is *O. diox Roxb.* levels another one is *Curculigo orchoides Gaertn.* Rhizome extract at different concentrations (1-3 mg/mL), fraction have shown competitive inhibition in which inhibitor only binds to free enzyme and V_{max} and K_m increase. F2 fraction 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. F2 and F3 Fraction have shown uncompetitive inhibition in which inhibitor only binds to enzyme-substrate complex. In uncompetitive pattern V_{max} and K_m both decrease [Table 2].

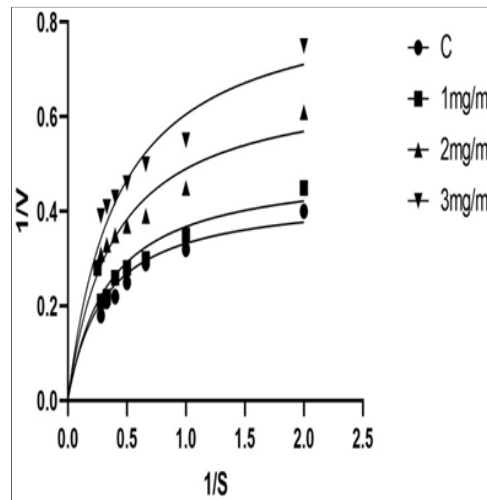
In this study, the best IC_{50} with 2mg/mL belongs to *Curculigo orchoides Gaertn.* (Rhizome: ethnoal) following by *O. diox Roxb* [Leaves; Methanol; Table 2].

Table 2. The plant name, part used, the solvent used, Km and Vmax values from Linweaver- Burk plot, and inhibition pattern CI-competitive inhibition; MI-Mixed inhibition; NCI-Non-Competitive inhibition

Sl. no	Plant name	Part used	Solvent used for extraction	V_{max}			K_{max}			Inhibition pattern		
				1.5 mg/mL	2 mg/mL	3 mg/mL	1 mg/mL	2 mg/mL	3 mg/mL	1 mg/mL	2 mg/mL	3 mg/mL
1	Galantamine	-	-	5.291			0.645			CI		
2	<i>Oleo diox Roxb.</i>	Leaf	Methanol	4.424	4.310	4.081	0.778	0.896	0.971	NCI	CI	MI
3	<i>Curculigo orchioides</i> Gaertn.	Rhizome	Ethanol	4.784	3.745	3.134	0.593	0.655	0.699	NCI	CI	MI

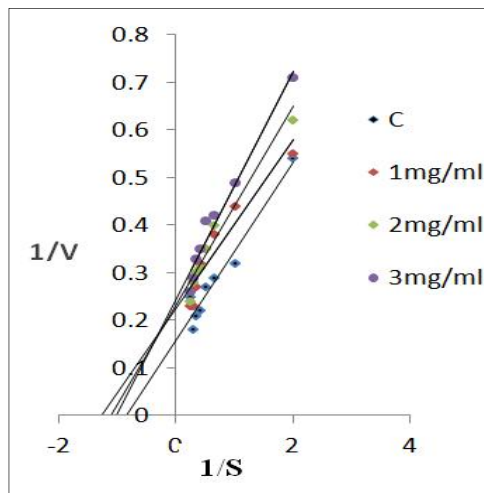


5A

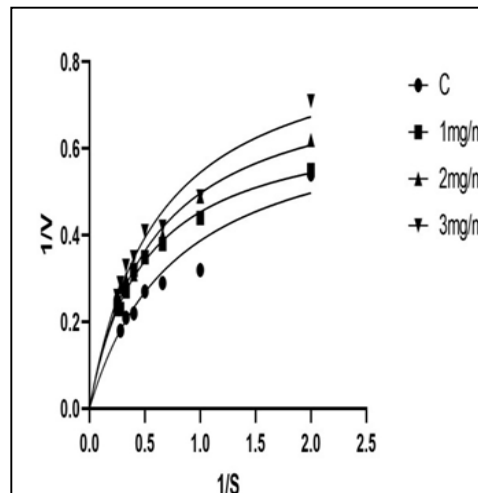


5B

Graph-5(A) The Lineweaver-Burk plot, representing the initial enzyme velocity vs AChI concentration for *Km* determination of *Curculigo* F3 fraction. Graph-(5-B) shows Michaelis-Menten constant



6A



6B

Graph 6A: The Lineweaver-Burk plot, representing the initial enzyme velocity vs AChI concentration for *Km* determination of *Oleo diox* Roxb., Graph 6B: shows Michaelis-Menten constant

At the lower concentrations of a plant fractions suggested uncompetitive inhibition and at higher concentrations (3 mg/mL) of fractions showed the non-competitive mode of inhibition [13].

Hence, overall from LB plot, it was a mixed type of inhibition, which is very common in medicinal plants due to the presence of a different type of

compounds present in the fractions [29-32]. In *Oleo diox Roxb.* F2 fraction and *Curculigo orchoides* Gaertn. Rhizome F3 fraction inhibited AChE in a concentration-dependent manner (1 to 3 mg/mL). The F3 fraction showed maximum inhibition of AChE enzyme at 3 mg/mL final concentration. The IC_{50} value obtained from the inhibition curve was 2 mg/mL and *Oleo diox*

Roxb. F2 fraction shows minimum inhibition compare to F3 fraction of *Curculigo orchioides* Gaertn. Rhizome.

5. CONCLUSION

Plant fractions (F2 and F3) contained certain bioactive compounds that exhibited the AChE inhibition. However, this might be due to the synergistic effect of many compounds in this plant fraction. Further purification and identification should be accomplished for understanding the exit mechanism of AChE inhibitory activity. The screening of AChE inhibition in *O. diox Roxb.*, and *Curculigo orchioides* Gaertn., fractions was carried out using semi-purified fraction which might contain bioactive compounds such as the flavonoids, Phenolics and tannins. The type of bioactive compounds and the percentage of compounds which inhibit AChE in the fraction are yet to be explored. Generally, active compound percentage of any plant fraction is <1% and moreover, several types of bioactive compounds would effectively inhibit the AChE site and thus may display inhibition capacity. It is likely that the bioactive compound complexing with some other compounds would stop the reaction between bioactive compounds and AChE and thus decrease the inhibition activity probably due to change in solubility of bioactive compound to reduce its concentration in the final stock solution. Because of this reason, it is not fair to compare the inhibition activity of a pure compound with that of a plant fraction.

In the present study, we report that *Oleo diox Roxb.*, F2 fraction and *Curculigo orchioides* Gaertn., rhizome F3 fraction showed significant AChE inhibitory activities which may contribute to the development of a more effective drug from these plants. The *Curculigo orchioides* Gaertn. rhizome fractions showed more specific and effective inhibitors against acetylcholinesterase enzyme which is useful in designing new drugs for the treatment of AD. Further examination of finding new bioactive compounds in *Curculigo orchioides* Gaertn., plant is going on.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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