5.1 Introduction

Alzheimer’s disease (AD) is a progressive and irreversible neurodegenerative disease and the most common cause of dementia in the elderly population (Ahmad et al., 2016). AD is considered as a complex syndrome where different factors are responsible for its etiology such as β-amyloid aggregation, tau protein aggregation and low levels of acetylcholine (Forst and Kurz, 1999; Melnikova, 2007). AD is characterized by a loss of basal forebrain neurons and reduced cortical and hippocampal levels of acetylcholine (ACh). The relation between the observed cholinergic dysfunction and AD severity provides a rationale for the therapeutic use of acetylcholinesterase (AChE) inhibitors (Hitzeman, 2006). The association between cholinergic neurotransmission deficiency and AD provides a base for the development of acetylcholinesterase (AChE) inhibitors as a therapeutic agent (Inestrosa et al., 1996). The main function of AChE is to breakdown acetylcholine and terminates the impulse transmission at cholinergic synapses, moreover, AChE can enhance the deposition of senile b-amyloid plaques in brain (Citron, 2010). It has been observed that use of AChE inhibitors can relieve some behavioral and cognitive symptoms of AD (Sabbagh, 2009; Adewusi et al., 2011). Cholinesterase inhibitors are not normally utilized as a part of allopathy and current medications do not lead adequate creation of acetylcholine to help in the management of AD. The research in the field of phytochemicals has formed into examination of characteristic compounds responsible for antioxidative and antiaging properties that can be valuable for neurodegenerative disorders (Fusco et al., 2007).

Vincamine, a peripheral vasodilator, is useful in accelerating the rate of blood flow towards the brain. Vincamine is a monoterpenoid indole alkaloid found in the leaves of Vinca minor (Fandy et al., 2016). Vincamine is an alkaloid which is better-known for the neuroprotective
qualities. In this work, in vitro enzyme inhibition and enzyme kinetic studies have been undertaken as an attempt to explore the ability of vincamine to act as potent inhibitor of AChE and to elucidate the possible mechanism of action. Since the inhibitory mechanisms against AChE are still unclear, therefore, the binding mechanism of vincamine inhibitor is studied by multiple approaches consisting of enzyme inhibition analysis and molecular docking studies in chapter 4. We strongly believe that this study would be useful for scientists involved in drug designing in their ongoing search for potent and versatile AChE inhibitors. In addition, the implications of possible inhibition by a compound may help in the development of new drugs that exhibit the anti Alzheimer’s activity.

5.2 Materials and methods

5.2.1 Chemical used for enzyme assay

Dimethyl sulfoxide (DMSO), (5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), Tris Buffer, Acetylcholine iodide (AChI) was obtained from Sigma–Aldrich Co., Acetylcholinesterase enzyme (AChE) was obtained from Sigma–Aldrich Co., Magnesium Chloride (MgCl₂), Sodium Chloride (NaCl), Vincamine.

5.2.2 Determination of AChE activity

The principle involves the measurement of the rate of production of thiocholine as acetylthiocholine is hydrolysed by AChE. Hydrolysis is accompanied by a continuous reaction between thiocholine and DTNB which produces the yellow anion of 5-thio-2-nitrobenzoic acid. The rate of anion production is measured by absorbance at 405 nm.

5.2.3 In vitro inhibition studies on AChE

The enzymatic activity was measured using an adaptation of the method described by Ingkaninan method (Ingkaninan et al., 2003). 1000 µl of DTNB (3 mM), 200 µl of AChI (15
mM), 700µl of Tris–HCl buffer (50 mM/pH 8), 50 µl AChE (0.25 Uml⁻¹) were added to a 3 ml cuvette. This cuvette was used as blank. In the reaction cuvette, the different concentrations of vincamine were added in above mentioned cuvette. The reaction was monitored spectrometrically for 10 min at 405 nm. The percentage inhibition of acetylcholinesterase activity was determined with the help of following formula:

\[
\text{Percentage inhibition} = \frac{(\Delta \text{OD of Control} - \Delta \text{OD of Drug})}{\Delta \text{OD of Control}} \times 100
\]

5.2.4 Kinetic Assay

The enzyme kinetic studies of vincamine against AChE were performed by using five different concentrations (5, 5.5, 7.5, 8.5 and 10 mM) of acetylcholine iodide (AChI) in the presence or absence of three different concentrations (150, 200 and 250 µM) of vincamine in three different reactions. The progress of the reaction was monitored on spectrophotometer for 10 minutes. After each minute OD was taken and used for kinetic analysis. Then the data were plotted on a Lineweaver–Burk diagram to reveal the mechanism of inhibition.

5.3 Results and Discussion

The inhibitor kinetics studies were attempted to explain how an inhibitor acts on enzyme and predict its efficacy. The kinetic constants Km and Ki are critical to understand enzymatic action on controlling metabolism of an organism. We studied enzyme inhibition kinetics of Vincamine on human AChE.

Selected inhibitor displayed a significant concentration dependent inhibition of AChE using AChI as a substrate. The Michaelis-Menten constant Km was determined by Lineweaver-Burk plot (Lineweaver and Burk, 1934) in which the reciprocals of substrate hydrolysis (1/V)
for inhibitor concentration were plotted against the reciprocals of the substrate concentrations by fitting the resulting data in ORIGIN 6.1 (Fig 5.1). Lineweaver and Burk equation (Equ 5.2) can be obtained by inverting the Michaelis-Menten equation (Equ 5.1) as following:

\[ V = \frac{v_{\text{max}} [S]}{K_m + [S]} \]  
\[ \text{...... Equ5.1} \]

\[ \frac{1}{V} = \frac{K_m}{v_{\text{max}}} \frac{1}{[S]} + \frac{1}{v_{\text{max}}} \]  
\[ \text{...... Equ.5.2} \]

The competitive property of selected inhibitor can be checked by using following equation (Equ 5.3).

\[ K_m' = \frac{K_m}{K_i} [I] + K_m \]  
\[ \text{...... Eq} \text{.5.3} \]

**Figure 5.1** Lineweaver–Burk plot of acetyl cholinesterase inhibition by different concentration of vincamine inhibitor.

The AChE activity was measured at different concentrations of substrate AChI (5, 5.5, 7.5, 8.5 and 10 mM). Line Weaver–Burk reciprocal plot showed that inhibitor was a
competitive inhibitor of AChE in which the Km value increases without affecting the Vmax. The Km value for the AChE with AChI was found to be 0.598mM and same was further confirmed by secondary graph (Fig5.5b), Eadie- Hofstee (Eadie, 1942) and Hanes plots (Hanes, 1932). Eadie-Hofstee plot is a graphical representation of enzyme kinetics which is plotted in between V against V/[S] for the Km value as negative slope which was 0.598mM (Fig 5.2) using Equ 5.4.

\[
\frac{V}{[S]} = \frac{V_{\text{max}} - V}{K_m} \quad \ldots \text{Equ 5.4}
\]

**Figure 5.2** Eadie- Hofstee plot used for the determination of $K_m$. Hanes Plot is the ratio of the initial substrate concentration $[S]$ to the reaction velocity $V$ is plotted against $[S]$ for the determination of Km value (fig 5.3).
Figure 5.3 Hanes plots used for the determination of $K_m$.

The value of $K_i$ was determined by Dixon (Dixon, 1953) method in which the reciprocals of substrate hydrolysis $(1/v)$ were plotted against the inhibitor concentration and fitting the data in software (Fig 5.4) using Equ5.5.

$$\frac{[S]}{v} = \frac{[S]}{V_{max}} + \frac{K_m}{V_{max}}$$

.....Equ 5.5

Figure 5.4 Dixon's Plot: inhibition of AChE by Vincamine.
The obtained value of Ki from Dixon plot was found to be 239µM and it was also confirmed by secondary plots (Fig 5.5.a,b).

**Figure 5.5** Secondary plot (a) used to calculate the inhibition constant Ki and (b) used to calculate the inhibition constant Ki & Km.

The AChE activity was measured at different concentrations of Vincamine (150, 200 and 250 µM). The inhibitor was found to inhibit AChE with an IC\(_{50}\) value (50% inhibitory concentration) of 239µM by fitting the data using ORIGIN 6.1 in fig5.6.
Figure 5.6 AChE inhibition curves for Vincamine.

Similarly, one of the alkaloid hamayne isolated from Nigerian Crinum species showed IC50 value of 250 µM (Houghton et al., 2004). In a recent study, tetrahydroquinolines derivative also showed an IC50 value of 215 µM (Gutie´rrez et al., 2015) and glimepiride showed dose dependent inhibitory activity against AChE enzyme with IC50 value of 235 µM (Rizvi et al., 2016). Curcumin inhibited AChE, with an IC50 value of 67.69 µM (Ahmed & Gilani, 2009). All the predicted values from different plots were summarized in table 5.1.
**Table 5.1** The obtained value of $K_m$, $K_i$ and $IC_{50}$ from different plots using the substrate AChI.

<table>
<thead>
<tr>
<th>Plots Name</th>
<th>Plots in between</th>
<th>$K_m$ Value</th>
<th>$K_i$ Value</th>
<th>$IC_{50}$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lineweaver-Burk Plot</td>
<td>$1/V$ Vs $1/[S]$</td>
<td>0.598 mM</td>
<td>____</td>
<td>____</td>
</tr>
<tr>
<td>Dixon Plot</td>
<td>$1/V$ Vs $[I]$</td>
<td>____</td>
<td>239 µM</td>
<td>____</td>
</tr>
<tr>
<td>Eadie- Hofstee Plot</td>
<td>$V$ Vs $V/[S]$</td>
<td>0.598 mM</td>
<td>____</td>
<td>____</td>
</tr>
<tr>
<td>Hanes Plot</td>
<td>$[S]/V$ Vs $[S]$</td>
<td>0.598 mM</td>
<td>____</td>
<td>____</td>
</tr>
<tr>
<td>secondary plot</td>
<td>$K'_m$ Vs $[I]$</td>
<td>0.598 mM</td>
<td>239 µM</td>
<td>____</td>
</tr>
<tr>
<td>secondary plot</td>
<td>Slope of primary graph Vs $[I]$</td>
<td>____</td>
<td>239 µM</td>
<td>____</td>
</tr>
<tr>
<td>Inhibition assay</td>
<td>% inhibition Vs $[I]$</td>
<td>____</td>
<td>____</td>
<td>239 µM</td>
</tr>
</tbody>
</table>

**5.4 Conclusion**

In this study, we have selected a bioactive compound that possesses inhibitory activity toward human AChE enzyme. We investigated the kinetic mechanism of this enzyme and the interaction mechanisms of vincamine with AChE. Results showed that vincamine significantly inhibited AChE activity with an $IC_{50}$ of 239 µM, and its inhibition was a competitive type inhibition with a $K_i$ of 239µM. The Michaelis-Menten constant $K_m$ was preliminary determined by Lineweaver-Burk plot which was found to be 0.598mM and same was further confirmed by Eadie- Hofstee and Hanes plots. Lineweaver- Burk reciprocal plot showed that inhibitor was a competitive inhibitor of AChE where the value of $K_m$ increases with the increase in the concentration of inhibitor without affecting the $V_{max}$. These findings partly explain the beneficial effects of vincamine against AD and provide a basis for the design and development of AChE inhibitors of anti Alzheimer’s disease.