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NEAR EAST UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

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Umar Ghali MUHAMMAD

MEDICAL BIOCHEMISTRY PROGRAM

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SUPERVISOR

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This study has been accepted by the thesis committee in Medical Biochemistry program as a Master of Science Thesis.

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DECLARATION

I hereby declare that this thesis work entitled: “INHIBITORY ACTIONS OF COMMON ORGANIC SOLVENTS ON HORSE SERUM BUTYRYLCHOLINESTERASE ACTIVITY” is the product of my own research work undertaken under the supervision of Prof. Nazmi ÖZER /Assoc. Prof. Kerem Teralı. No part of this thesis was previously presented for another degree or diploma in any University elsewhere, and all information in this document has been obtained and presented in accordance with academic ethical conduct and rules. All materials and results that are not original to this work have been duly referenced.

Name, Last Name:

Signature:

Date:

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ABSTRACT

Inhibitory Actions of Common Organic Solvents on Horse Serum Butyrylcholinesterase Activity. Near East University, Graduate School of Health Sciences, MSc Thesis in Medical Biochemistry Program, Nicosia, 2020.

Cholinesterases are a family of enzymes that regulate the undesired transmission of the neurotransmitter acetylcholine. Acetylcholinesterase and butyrylcholinesterase are the main classes of this family. The levels of these enzymes are altered in neurodegenerative conditions such as Alzheimer's disease (AD) and Parkinson's disease. In AD, the level of AChE decreases while the level of BChE was found to increase or remain normal. Several approved drugs have been in existence to curb the detrimental effects of these conditions on health. Synthetic drugs show some unwanted side effects. Alternative search for AD inhibitors that target BChE has also been on the rise. Some of the screened molecules are not soluble in water and therefore need to be solubilized in organic solvents. But these organic solvents may have either a positive or a negative influence on the enzymes.

In this study, the inhibitory effects of commonly used organic solvents on horse serum butyrylcholinesterase were investigated. The IC_{50} values for the solvents tested were determined in the presence of the substrate analog, BTC. Kinetic studies regarding the inhibitory actions of these solvents were also performed. Acetone shows the highest inhibitory potency on BChE assay and inhibited BChE competitively with an IC_{50} value of 707 mM and K_i and V_m values of 185.606 ± 14.879 mM and 134.027 U/mg protein, respectively.

Keywords: Alzheimer's disease, butyrylcholinesterase, organic solvents, acetone, competitive inhibition

ÖZET

Yaygın Organik Çözücülerin At Serum Bütirilkolinesteraz Aktivitesi Üzerindeki İnhibitör Etkileri. Yakın Doğu Üniversitesi, Sağlık Bilimleri Enstitüsü, Tıbbi Biyokimya Programı Yüksek Lisans Tezi, Lefkoşa, 2020.

Kolinesterazlar, bir nörotransmitter olan asetilkolinin istenmeyen salgısını düzenleyen bir enzim ailesidir. Bu ailenin iki esas sınıfını asetilkolinesteraz ve bütirilkolinesteraz oluşturur. Bu enzimlerin düzeyleri Alzheimer hastalığı ve Parkinson hastalığı gibi nörodejeneratif bozukluklarda değişmektedir. Alzheimer hastalığında AChE düzeyleri azalırken, BChE düzeylerinin arttığı ya da aynı kaldığı bulunmuştur. Bu hastalıkların sağlık üzerindeki zararlı etkilerini bertaraf etmek için birden fazla ilaca onay verilmiş olmasına rağmen bu sentetik ilaçların bazen istenmeyen yan etkileri olabilmektedir. BChE'yi hedef alan alternatif Alzheimer karşıtı ilaçlarla ilgili arayışlar günümüzde yükselişe geçmiştir. Taranan moleküllerden bir kısmı suda çözünmediğinden bunların organik çözücülerde çözünmeleri gerekmektedir. Buna karşın bu organik çözücülerin kolinesterazlar üzerinde olumlu veya olumsuz etkileri olabilmektedir.

Bu çalışma ile yaygın şekilde kullanılan organik çözücülerin at serum bütirilkolinesterazı üzerindeki olası inhibitör etkileri incelenmiştir. Test edilen çözücülere ait IC_{50} değerleri, substrat analoğu BTC'nin varlığında belirlendi. Ayrıca bu çözücülerin inhibitör etkilerini ölçen kinetik çalışmalar gerçekleştirildi. Bu deneyler ışığında asetonun BChE aktivitesi üzerindeki en yüsek inhibitör potensisine sahip olduğu ve IC_{50} = 707 mM, K_i = 185.606 ± 14.879 mM ve V_m = 134.027 U/mg protein değerleri ile enzimi yarışmalı şekilde inhibe ettiği bulundu.

Anahtar kelimeler: Alzheimer hastalığı, bütirilkolinesteraz, organik çözücüler, aseton, yarışmalı inhibisyon

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ABBREVIATIONS

Aβ: Amyloid Beta

ACh: Acetylcholine

AChE: Acetylcholinesterase

AD: Alzheimer's disease

BChE: Butyrylcholinesterase

BTC: Butyrylthiocholine

ChE: Cholinesterase

ChEI: Cholinesterase inhibitor

CNS: Central nervous system

dH_2O: Distilled water

DTNB: *5,5'*dithiobis (2-nitrobenzoic acid)

EqBChE: Equine butyrylcholinesterase

HuAChE: Human acetylcholinesterase

IC_{50}: Half maximal inhibitory concentration

PAS: Peripheral anionic site

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1.0 INTRODUCTION

Neurodegenerative diseases are genetical disorders associated with distortion in the structure of a neuron, its function, and finally, its loss (Roy 2018). Alzheimer's disease (AD) is a type of neurodegenerative disorder named after a German psychiatrist Alois Alzheimer. AD and other neurodegenerative diseases pose public health concerns as a result of their consistent rise (Holtzman *et al.*, 2011) due to the lack of standard treatment with no side effects. As of 2015, the prevalence of AD was estimated to be 44 million people globally, and that this value is predictably expected to double or triple by the year 2050 (Islam and Pillay, 2019; Van Cauwenberghe *et al.*, 2016). The onset of this disease is of two stages, termed early onset and late onset. Pathology of both early and late-onset of this disease is categorized as multifactorial. Most of the approved drugs for the treatment of AD by the Food and Drug Administration (FDA) such as rivastigmine, galantamine and donepezil are mainly acetylcholinesterase inhibitors (AChEIs). But recently, there is a rise in search for butyrylcholinesterase inhibitors (BChEIs) as well.

Butyrylcholinesterase (BChE) helps in regulating cognition, behavior, awareness and motor control in the cholinergic system by terminating the action of the neurotransmitter acetylcholine (Reid *et al.*, 2013). This is clear as it plays a role in nervous system development. It also functions in detoxifying toxins (both natural and synthetic) and hydrolyzes many drugs such as aspirin and bambuterol (Lockridge, 2015). The level of this enzyme was observed to rise in many studies of AD, and it has been used as a diagnostic biomarker of AD.

In search of BChEIs, potential molecules are dissolved in one of the many used solvents in a biochemical assay. The selection of solvent is paramount to the target of the experiment and has an effect on the final findings of an assay. Water is used mostly to dissolve many compounds. Its dipolar nature gives it the ability to dissolve ionic compounds; as a result, it was once regarded as 'universal solvent'. But many molecules have low solubility in water; therefore, alternative solvents such as organic solvents are used to dissolve compounds in experimental processes.

Organic solvents are a group of chemical compounds that contain at least a carbon atom linked to an atom of hydrogen, nitrogen, or oxygen, and has the ability to dissolve non-water-soluble materials (Sainio, 2015). There are two types of organic solvents: water-miscible and water-immiscible solvents. Organic solvents that is water-miscible plays a vital role in dissolving molecules that are suspected of having therapeutic potentials in the management of diseases. Most of these organic solvents are used as drug vehicle to deliver active pharmaceutical ingredient (API) to target sites so as to initiate their therapeutic action (Kelava *et al.*, 2011).

But there is a lack of insight into the effect of commonly used organic solvents on the activity of BChE. Therefore, this study investigates the inhibitory effect of acetonitrile, acetone, DMSO, ethanol, and methanol on the activity of BChE in an *in vitro* setting; so as to recommend the most suitable and safest solvent to use during screening of potential AD inhibitors. The five tested solvents were screened individually at set conditions.

1.1 Aims and Objectives

The aims and objectives of this study are to:

(a) estimate the IC_{50} values of each of the solvents;

(b) investigate the inhibitory effect of the common solvents on the activity of BChE;

(c) check the type of inhibition of the solvents against the enzyme;

(d) rank the order of their inhibition based on the obtained IC_{50} values.

2.0 GENERAL INFORMATION

Cholinesterases are a family of enzymes that regulate the undesired transmission of the neurotransmitter acetylcholine. The main classes are the acetylcholinesterase (AChE) and butyrylcholinesterase (BChE; also called pseudocholinesterase). AChE mainly functions to catalyze the hydrolysis of acetylcholine (ACh) which is an important process in the refurbishment of the cholinergic neuron (Pohanka, 2011). Cholinergic neurons regulate learning, cortex, blood flow, sleep-wake cycle, and cognition (Schliebs and Arendt, 2006).

Alzheimer's disease is a neurodegenerative disorder that is characterized by cognitive decline and affects most elderly. Hallmarks of AD progression lies in the deposition of neurofibrillary tangles (NFTs) and β-amyloid plaques at intracellular and extracellular neurons respectively; that leads to loss of synaptic function and neurotoxicity (Gupta and Mohan, 2014). At the synapse, the three FDA approved drugs (rivastigmine, donepezil, and galantamine) that are mostly used in the treatment of AD delay the hydrolysis of the neurotransmitter ACh which is found to be distributed in the central nervous system and the peripheral nervous system (Taslimi *et al.,* 2018). These drugs are known to be acetylcholinesterase inhibitors (AChEIs), but there also exist a non-cholinesterase inhibitor such as memantine that is approved by the FDA. Recent research also shifts to find butyrylcholinesterase inhibitors by screening for small molecules. These molecules are expected to have no/less side effect as the ones observed in AChEIs.

Butyrylcholinesterase (3.1.1.7) is a detoxifying enzyme found on mammalian tissues. As a tetrameric glycoprotein, it has a molecular weight of 85 kDa with 574 amino acids sequence on each of the four subunits (Lockridge *et al.,* 1987) and its concentration in the human

serum is about 5 mg/L and has a half-life of 12 days (Østergaard *et al.,* 1988). It functions as a proxy to acetylcholinesterase when AChE is depleted and neutralizes neurotoxins (Nordberg *et al.,* 2013). It has a slow rate of hydrolyzing choline when compared to AChE. HuBChE possessed properties such as immunogenicity to humans, the half-life of two weeks in circulation, and the long stability of years in solution. These properties make it a gold standard in studies that target nerve agents' toxicities (Dafferner *et al*., 2017; Nachon *et al*., 2013). There is a high identity between horse serum BChE and human serum BChE (Fig 4.23).

2.1 Pathogenesis of Neurodegenerative Diseases

Recently, there is a remarkable insight into the pathogenesis of AD with the cholinergic hypothesis as the most significant hypothesis to elucidate its mechanism (Rohit *et al.,* 2016). Perturbation in Ca^{++} is counted among major mechanisms in the pathology of AD. Aβ causes a distortion in Ca^{++} homeostasis, but this effect is ameliorated by medicinal plants such as the Gingko biloba extract by inhibiting oligomerization of Aβ 1-42 (Shi *et al.,* 2010) but long-term use of this extract does not depress cognitive decline (Vellas *et al.,* 2012).

2.2 Structure of Cholinesterases (AChE and BChE)

Cholinesterase active site gorge interact with ligand by two sites known as A-site (acylation) and P-site (peripheral) (Ahmed *et al.,* 2007). AChE catalyzes the hydrolysis of ACh at the neuromuscular junction to acetate and choline so as to regulate unwanted transmission.

Catalytic triad residues of BChE are Ser198, Glu325 and His438 (Fig 2.3) which gives the enzyme its esteratic function (Suarez *et al.,* 2006). This site is responsible for the catalytic activity.

Acyl binding pocket of AChE (Fig 2.2) and BChE differ by replacing F288 and F290 (of AChE) with L286 and Val288 (of BChE) respectively (Giacobini, 2004).

Each of the 85 kDa of BChE contains 574 amino acids. AChE and BChE are encoded by a gene on chromosome 7 (position 7q22) and chromosome 3 (position 3q26.1-q26.2) respectively. BChE active site is larger than AChE due to different amino acids that line the gorge.

BChE consists of four (tetrameric) subunits that are held by a polyproline rich peptides at the core of the enzyme (Fig 2.1). These polyproline rich peptides help to associate the individual monomers of BChE and are released through denaturation by heat or addition of the solvent acetonitrile (Larson *et al.,* 2014). Hydrogen bond holds the polyproline rich peptide at the middle core of BChE to several tryptophan residues and hydrophobic moieties.

BChE contains three major parts namely: peripheral active site (PAS), catalytic triad and the anionic site. Peripheral site of the gorge consists of D70, Y332, F329, and E325 which has an effect on the catalytic activity (Darvesh *et al.,* 2010; Darvesh *et al.,* 2008). The D70 residue direct substrate to catalytic serine moiety by stabilizing the cationic choline through pi interaction found on the tryptophan residue (W82) (Darvesh, 2016). The C-terminally truncated hBChE dimer is less stable than hAChE, and this is as a result of the four-helix bundle that helps in the dimerization of the ChEs (Novichkova et al., 2019).

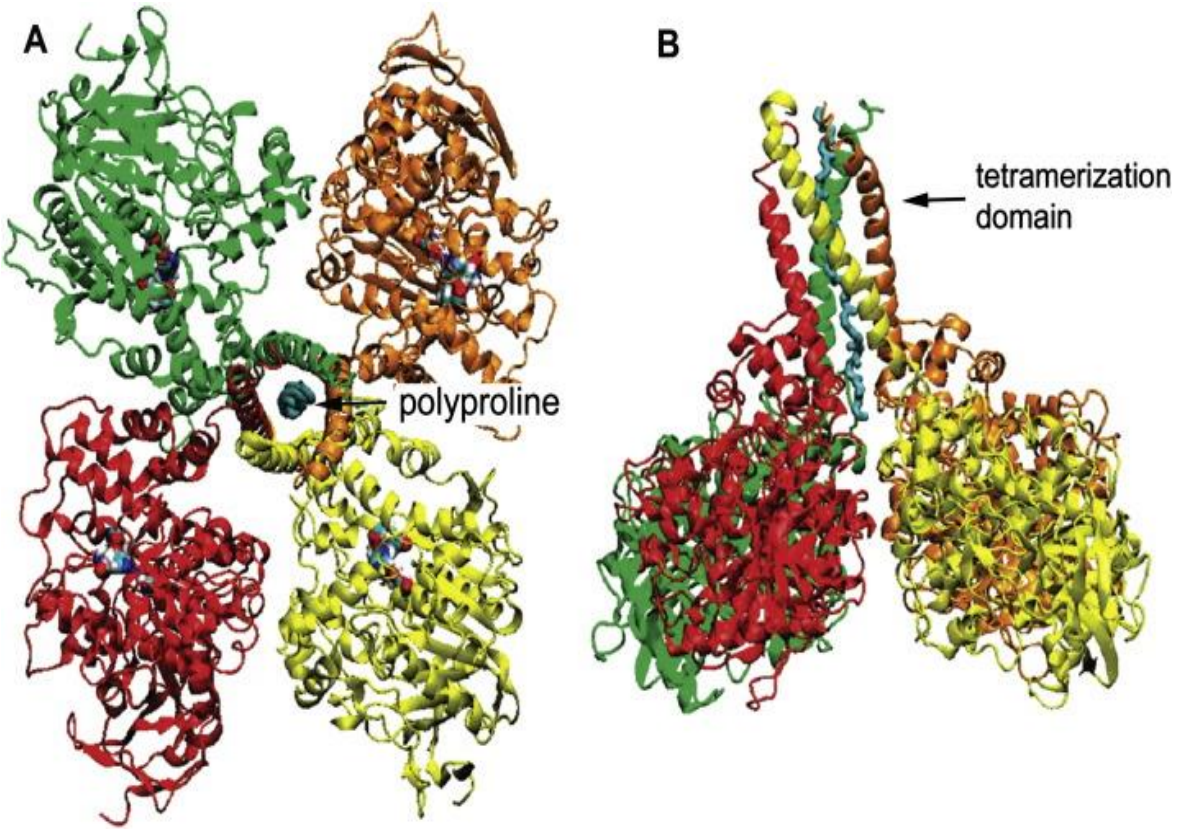

Figure 2.1: Structure of human butyrylcholinesterase with different ribbon colors to depict individual monomer (a) Top view of huBChE with polyproline peptide at the center (b) ribbon side view of human butyrylcholinesterase indicating the tetramerization domain (Lockridge, 2015)

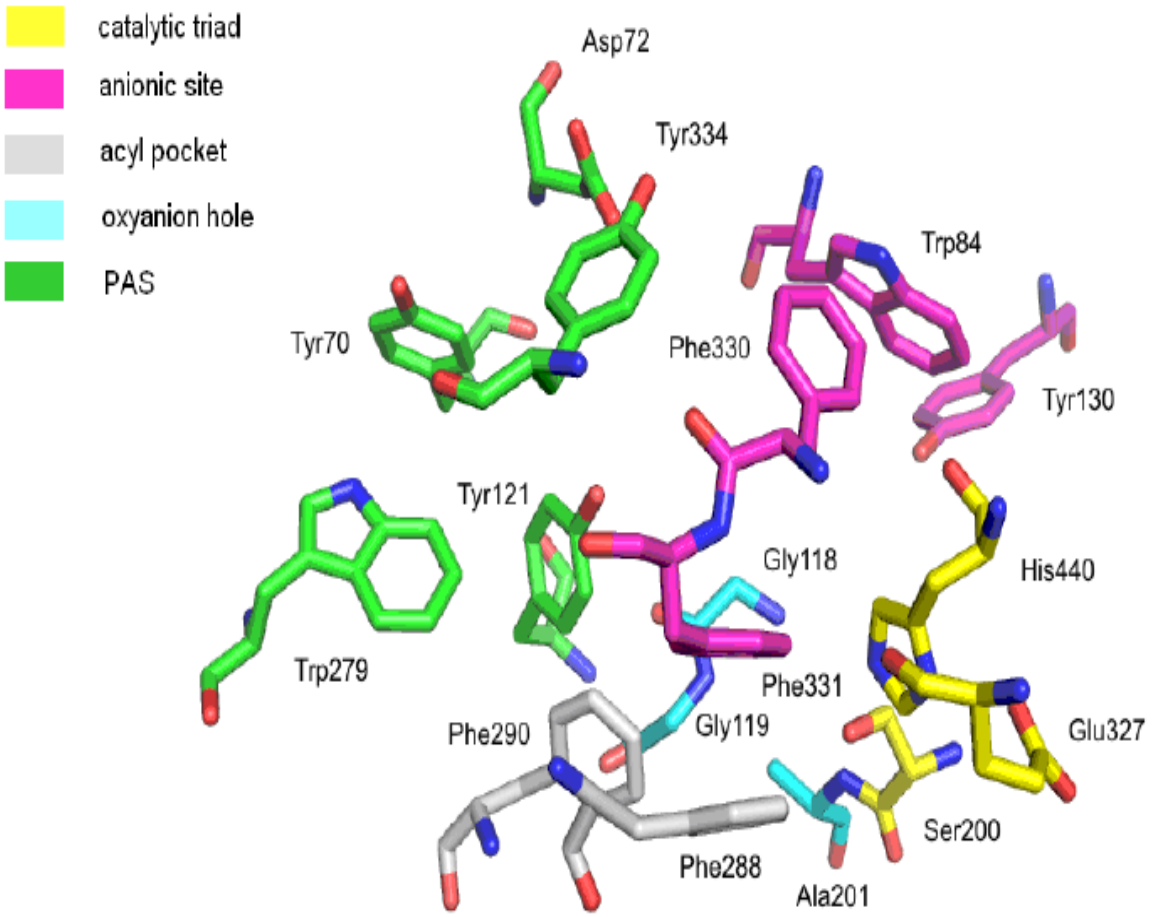

Figure 2.2: Active site of *Torpedo californica* acetylcholinesterase (TcAChE; PDB: 1EVE)

Source: Bajda et al. (2013)

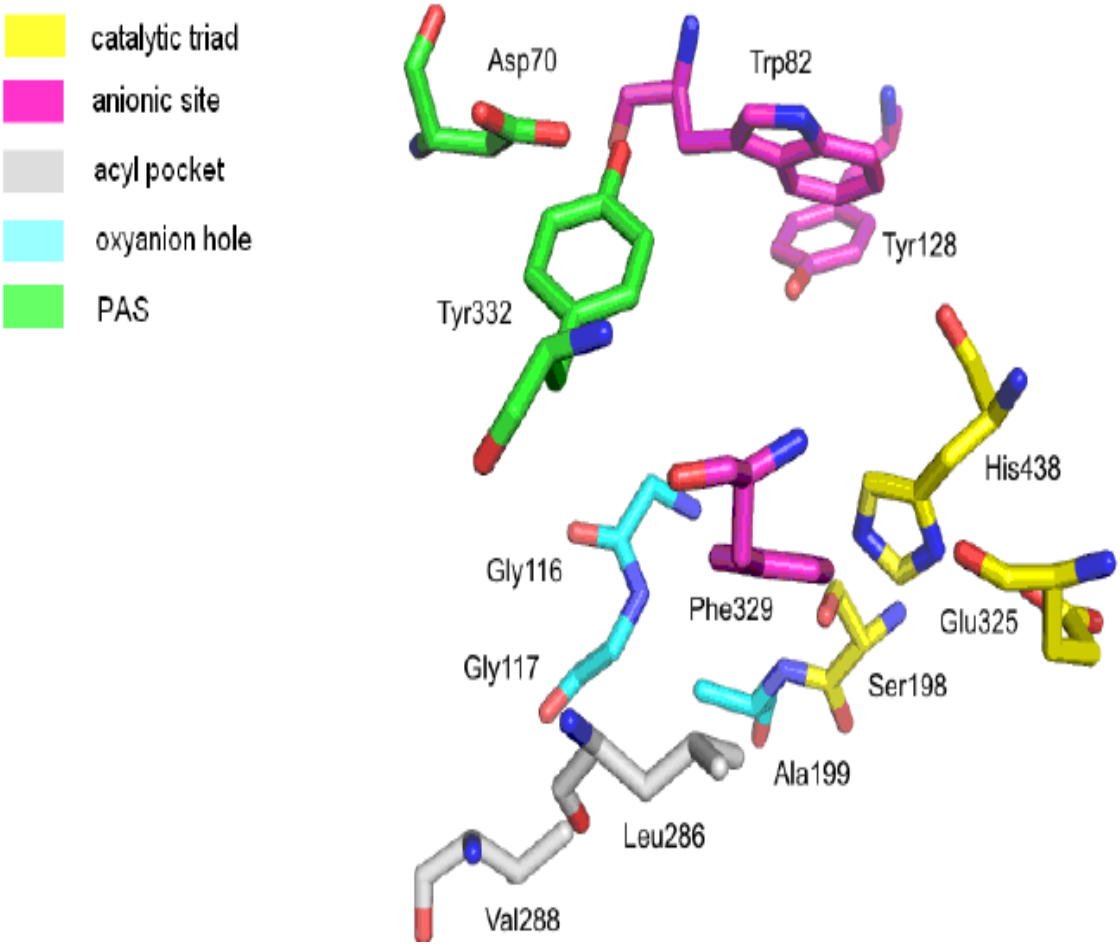

Figure 2.3: Active site of human butyrylcholinesterase (huBChE PDB: 1P0I) (Bajda et al., 2013).

2.3 Metabolic Functions of Butyrylcholinesterase

Butyrylcholinesterase has several metabolic functions and plays a role in many diseases. Aside hydrolyzing ACh, BChE hydrolyzes other compounds such as cocaine and heroin (Masson *et al.*, 1997).

2.3.1 BChE and fat metabolism

BChE activity has been associated with obesity and other metabolic syndromes (Randell *et al.*, 2005). BChE *gene* transfer help to restore resistance to high fat as it decreases the energy balance in obese mice (Chen *et al.*, 2016). This research further explains the function of BChE in regulating the hunger hormone (ghrelin) as its absence in mice increases food intake (Chen *et al.*, 2017). BChE regulate this hormone (ghrelin) via the peptide cleavage.

2.3.2 BChE as biomarker of disease

Enzymes have been used to detect diseases during diagnoses or monitored during therapy. BChE has shown the potential to be a good biomarker for several diseases. BChE was reported to be a good biomarker of liver disease that distinguishes liver-related diseases to non-liver related diseases (Ogunkeye and Roluga, 2006). Its level was found to be decreased in Parkinson's disease patients when compared to normal patients (Dong et al., 2017). Greenwood *et al.* (2015) have reported the role of BChE as a biomarker of neuroblastoma cells. Its activity was found to be decreased in the blood of neuroblastoma patients (Coulter *et al.*, 2017). In AD, there is a clear correlation between Alpha-Beta plaques and BChE. Previously, the level of BChE was weakly found to be correlated to pesticide-induced depression among workers (Strelitz *et al.*, 2014). Its level was monitored in AD using BChE knockout mice model in which BChE was found to be absent. BChE distinguishes AD pathology from other known dementia (Macdonald *et al.*, 2017).

2.4 Treatment

AD, as a multifactorial disease requires multiple therapeutic approaches that will have a profound effect than a single therapy approach (Bolognesi *et al*., 2009 N63). Inhibition of cholinesterase is one of the strategies taken in the management of AD. Among the approved cholinesterase inhibitors, tacrine is less used due to its hepatotoxicity effect. Its potency on BChE is higher than AChE but its homodimer (bis-7 tacrine) shows no toxic effect on subjects (Summers et al., 1986, Gupta and Mohan 2014). Memantine happens to be effective for the late stage of AD, while rivastigmine, donepezil, and galantamine are more effective at an early stage of treatment (Kumar *et al*., 2015).

Various proteins are targeted in the management of AD with tau protein among the most targeted protein. In this regard, tau protein is modified in the management of AD via different mechanisms such as its degradation, dephosphorylation deoligomerization, and immune system enhancement. Other therapeutic targets are amyloid transport, microtubule stabilization, secretase enzyme, amyloid aggregation, intracellular signaling cascades, etc. (Kumar *et al*., 2015).

Glycogen synthase kinase 3 (GSK3) is one of the enzymes that phosphorylate tau proteins, and it is targeted in the treatment of AD by compounds such as valproate and lithium (Engel *et al*., 2006). Inhibition of the secretase enzymes (α, β, γ) is also one of the approaches in therapeutic targets of AD (Strooper et al., 2010); but inhibition of γ-secretase is associated to toxicity risk.

2.5 Solvents

Organic solvents are lipophilic in nature, i.e they have the ability to pass through blood-brain barrier. They have a high affinity for the nervous system and accumulate in regions such as the brain, thereby exerting the neurotoxic effect (Sainio, 2015). Solvation properties of solvents differ significantly from one solvent to another. Based on solvophobic interactions of solvents, Ray (1971) classify solvents into three classes namely: class i (e.g. H_2O, ethylene glycol, glycerol), class ii (dimethylformamide and methyl formamide), and class iii (Ethanol, Methanol, Toluene). These solvents usually disposed of the water molecule from the hydration shell or bind to the active site of enzymes and caused the specific function of the enzyme (Szabó *et al.,* 2009).

Enantiomeric forms of enzymes may show different biological effect due to their specificity and interaction. Study on the effects of solvents on the enzyme lipase reveals a high correlation between the solvents properties and the enzyme enantioselectivity (Ueji *et al.,* 2001). Water helps in maintaining the structure and function of a protein, as it regulates the noncovalent interactions. Serdakowski *et al.* (2006) reported that the addition of small water molecule could help to activate enzyme activity as it alters the enzyme's environment, thereby affecting the transition state.

Properties such as appropriate melting and boiling point, low level of toxicity, purity, and good chemical stability make the organic solvents to be used as good solvents (Kenndler, 2009). The low solubility of compounds may be due to solvation energy or crystal disruption or a combination of both factors (Lipinski, 2004).

Organic solvents may have a positive or negative effect on proteins. Some organic solvents denature proteins (enzymes) at high concentrations while others increase the activity or stability of the protein. These conformational changes are as a result of distortion in the balance between the microenvironment and other forces/interactions that stabilize the protein. Charged molecules released by proteins and other factors help in stabilizing those proteins in an organic solvent. This occurs by changes that occur at the active site of the enzyme, thereby altering the microenvironment (Kajiwara *et al.,* 2019). Polar organic solvents penetrate the enzyme lipase and strip of critical water molecules from the enzyme active site and cause changes in certain amino acid residues to alpha-helix which is suspected to facilitate the activation of the enzyme in DMSO and methanol (Cao *et al.,* 2016). DMSO has also been reported to possesses dual mechanism in modulating avidin and $CYP_{142}A_1$ by reducing the charge distribution and simultaneously shows protective role in maintaining the heme group towards unfolding (Chan *et al.,* 2017). The different effect of DMSO on proteins differ based on the concentration used. Lower concentrations tend to strip off water molecule on a protein while higher concentrations bind to the protein.

Acetonitrile, just like DMSO, shows different effects on protein structure. Acetonitrile destabilizes the formation of alpha-helix (Shanmugam *et al.,* 2012) while it does the opposite in some proteins by promoting the formation of the helices (Naqvi *et al.,* 2013).

Organic solvents for example, methanol, ethanol, and DMSO help to increase the mobility of organic dye in non-aqueous capillary electrophoresis (Gu *et al.,* 2018). Recently, there is a great concern on the effect of solvents on enzymes during the biochemical assay. Polar solvents were reported to penetrate and perturb the 3D structure of proteins due to their high

polarity, unlike nonpolar solvents (Serdakowski and Dordick, 2008). At the same time, they enhance the emission of the sensitivity of DNA luminescence better than in water (Xu *et al.,* 2017).

Individuals/workers are exposed to organic solvents daily which are found in use in pharmaceutical products, agriculture and in textiles. These solvents are taken up via skin absorption or by inhalation, present mostly in paint spray and affect the elderly easily than the young age (Keski-Säntti *et al*., 2010).

Sometimes a ratio of water with a miscible organic solvent such as 1:1 are used to increase the solubility of a compound. Interaction of protein with any ligand is important, but there is a need for energy to drive the binding of substrate to an enzyme so as to initiate the catalytic potential of that enzyme. Alteration in this driving energy causes a decrease in enzymatic reaction (Doukyu and Ogino, 2010).

2.5.1 Stability of enzymes in organic solvents

Generally, proteins are denatured/inactivated in organic solvents at certain concentrations. But there are enzymes that are more stable in organic solvents and are popularly known as organic solvent-tolerant enzymes. Some of these enzymes are modified to be able to adapt to the organic solvent medium. The naturally solvent-tolerant enzymes are mostly extracellular and are secreted by organic solvent-tolerant microorganisms (Doukyu and Ogino, 2010).

2.5.2 Acetone

Acetone is a polar aprotic solvent with a molecular weight of 58.08 g/mol (Fig 2.4). It is a member of the ketone body family along with acetoacetate and 3-β-hydroxybutyric acid. Acetone and the other two ketone bodies can be produced during low glucose in the liver in the tricarboxylic acid cycle (TCA). This is as a result of enhancement of acetyl-CoA production from fatty acids that lead to its accumulation for the production of ketone bodies rather than moving into the TCA cycle (Fig 2.5). Its elimination in urine and breath has been used as a biomarker in diabetic patients, mostly type 1 diabetes. Some studies reported its elevation in type 2 diabetes while others report no difference; therefore, its association with diabetes still poses controversy (Storer *et al.*, 2011; Ueta *et al.*, 2009). Detection of acetone continues getting attention every day as several methods for its detection are proposed. A portable biosensor for the detection of acetone in the biomedical field was recently developed by Rauf *et al.* (2018). This method involves the immediate change of a dark yellow filter paper to slightly yellow upon addition of acetone.

Acetone is degraded anaerobically by carboxylation reaction, converting acetone to methane, carbon dioxide, and acetate that moves freely (Heider *et al.*, 2016). This process is usually carried out by carboxylase enzymes. It has also been confirmed by MALDI-ToF analysis to selectively modify proteins during its use in protein precipitation and concentration (Robert and Beynon, 2010). This process is a well-known approach to protein precipitation and concentration. Recently, Kostag *et al.* (2014) reported that a mixture of acetone with salt lowers cellulose viscosity and suggest that acetone can be used to dissolve and precipitate cellulose.

2.5.3 Acetonitrile

Acetonitrile is a colorless solvent with the formula C_2H_3N (Fig 2.6) that is also called methyl cyanide. It can affect cells as it produces hydrogen cyanide, but its biotransformation via two enzymatic pathways (nitrile hydratase and amidase) produces a less toxic compound acetate through its intermediate acetamide (Chuchat *et al.,* 2016). Naqvi *et al.* (2013) reported the potential of acetonitrile in promoting the formation of alpha-helices in certain proteins and shows selectivity to amino acid sequences.

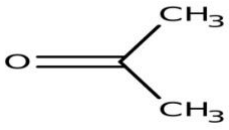

Figure 2.4: Structure of acetone

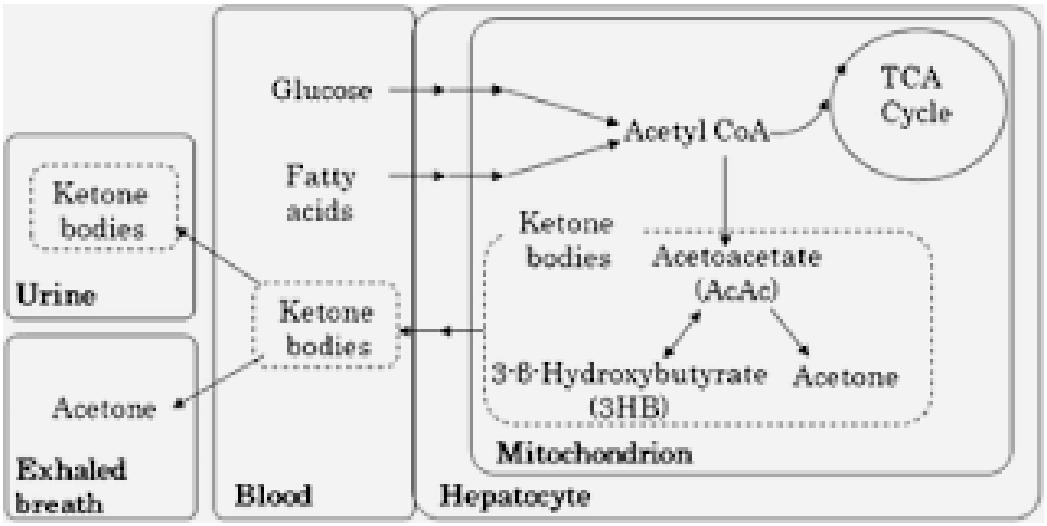

Figure 2.5: Ketone bodies production from TCA cycle intermediate in exhale breath and urine (Yamada *et al.*, 2016)

Figure 2.6: Structure of acetonitrile

2.5.4 DMSO

Dimethyl sulfoxide (DMSO) is a polar aprotic solvent that is used in dissolving many water-insoluble compounds in biochemical assays. It has a small molecular weight of 78.13 g/mol and contains sulphur and methyl group in its structure (Fig 2.7). The sulphur-oxygen bond accepts hydrogen from the donor molecule while the methyl group participates in hydrophobic interaction. DMSO was reported to lower the aggregation of lipase enzyme under high temperature (Vaezzadeh *et al.*, 2017); this indicates the role of DMSO in decreasing the rate of lipase denaturation at high temperature. Previously, Ghosh *et al.* (2014) also reported the potential of DMSO in unfolding lysozyme at a different site of the protein with alteration of pH of the protein from 7 to a basic pH (pH 8). In a simulation study, DMSO decreases the water of hydration in an increasing concentration with little uniformity when compared to acetonitrile (Yadav and Choudhury, 2019).

It affects the nervous system and polarizes the blood-brain barrier, but also affect nuclear factor-kappa B in a positive manner (Bini *et al.*, 2008). Respiratory toxicity of DMSO was reported by Takeda *et al.* (2015) as it has been shown to inhibit lung ventilation in a dose-dependent manner. During exocytosis, DMSO helps to stabilize pore by destabilizing actin and regulate dynamin function; which help in closing and opening of pore respectively. This stabilization enhances the release of catecholamines and increases the length of the pores (Majdi *et al.,* 2017). Other positive effects of DMSO include its role in ameliorating the toxicity effect of acetaminophen when applied in low doses (Kelava *et al.,* 2010).

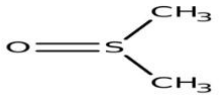

Figure 2.7: Structure of DMSO

2.5.5 Methanol

Methanol (Fig 2.8) is used frequently in the industrial process as an additive to gasoline and in the production of raw materials (Sibirnyj *et al.,* 2015). Methanol in a dose-dependent manner, penetrate the active site of an organic solvent-stable enzyme (protease) and result in the destruction of the secondary structure by loosening the hydrophobic interaction, and therefore lowers the stability of the enzyme (Gu *et al.*, 2019). NiÓgain *et al.* (2012) reported that methanol/n-butyl acetate mixture does not inactivate the enzyme trypsin, in a spray-drying of the enzyme. Also, a previous study reports the stability and enhancement of protease activity in methanol compare to control (Hemke and Dhundal, 2017). This ambiguity may be due to the reaction medium used for the analysis or the source of the enzyme.

Figure 2.8: Structure of methanol

2.5.6 Ethanol

Ethanol (Fig 2.9) is among the most frequently used solvent in various processes and also in the household. Ethanol serves as a depressant in the CNS as it enhances the inhibitory effect of GABA, and also shows other neuroprotective effects (Qi *et al.,* 2009). But this is at a low dosage as the high dose may be detrimental to macromolecules. Ethanol shows different effects on the same enzyme isolated from a different source. Anesthetic role of ethanol still remains controversial as some studies reported its positive role while others reported no significant effect (Fathi, 2014).

Figure 2.9: Structure of ethanol

Properties of the 5 commonly used organic solvents are shown in Table 2.1 below.

Table 2.1: Properties of the organic solvents used

Solvent	**MW (g/mol)**	**VISCOSITY (Pa.s)**	**DENSITY (g/mL)**	**Polarity**
Acetonitrile	41.05	0.369	0.786	Polar aprotic
Acetone	58.08	0.306	0.791	Polar aprotic
DMSO	78.13	1.987	1.100	Polar aprotic
Ethanol	46.07	0.983	0.789	Polar protic
Methanol	32.04	0.544	0.791	Polar protic

2.6 Effect of Organic Solvents on Vision

Degeneration or inflammation of the eye in a phenomenon known as chemical eye burns, result in the accumulation of reactive oxygen species (ROS) (Altan and Oğurtan; 2017). An organic solvent such as DMSO has been known to possess anti-inflammatory and analgesic effect that could ameliorate the degenerative effect of chemicals on eyes. It has been previously reported to treat inflammation of the cornea (keratitis) at 50% concentration in dogs (Balicki, 2012).

2.7 Lipinski's Rule of 5

Lipinski rule help to determine a drug-like molecule from a large library of molecules.

1. Water/octanol partition coefficient (log P) ≤ 5
2. Hydrogen bond donors ≤ 5
3. Hydrogen bond acceptors ≤ 10
4. Molecular weight ≤ 500

When one or more of the Lipinski's rule is violated, a compound is not regarded as drug-like due to its lack of bioavailability (Gupta and Mohan 2014). However, some compounds violate one of the Lipinski's rules of five but still show a drug-like nature. For example, abamectin with large molecular weight (873 Da) was recently reported to inhibit the active-site gorge of equine BChE in a reversibly competitive manner (Terali *et al*., 2018), which may be due to its macrocyclic nature that has multiple complexity that shows several functionalities (Driggers *et al*., 2008). All the solvent used in the experiment do not violate Lipinski's rule of 5.

Effects of some commonly used organic solvents were recently investigated on AChE by Kumar and Darreh-Shori (2017); since both AChE and BChE are related and have been targeted in a search for AD inhibitors, our study aimed at investigating the inhibitory effects of these commonly used organic solvents on BChE activity.

3.0 MATERIALS AND METHODS

3.1 Materials

Butyrylcholinesterase from equine serum (eqBChE; Catalog no.: C1057), substrate analog *S*-butyrylthiocholine iodide (BTCh; Catalog no.: B3253), chromogenic reagent 5,5′-dithio-2-bis-nitrobenzoate (DTNB; Catalog no.: D8130), 3-(*N*-morpholino)propane sulfonic acid (MOPS; Catalog no.: M1254), NaOH (Catalog no.: S8045) BSA (Catalog no.: A3294), Methanol (Catalog no.: 34885), DMSO (Catalog no.: D2438), Acetone (Catalog no.: 3294), Acetonitrile (Catalog no.: 271004), and Ethanol (Catalog no.: 240697) were all purchased from Sigma-Aldrich, Inc., St. Louis, MO, USA.

3.2 Methods

3.2.1 Preparation of 200 mM MOPS/NaOH buffer pH 7.5 in 250 mL:

10.46 g MOPS was weighed on an electronic balance and dissolved in 200 mL distilled water. With constant shaking, a clear solution was obtained.

3.2.2 10 M NaOH

16 g NaOH was weighed and dissolved in 40 mL distilled water and stirred constantly to obtain a solution. A warm solution was obtained and allowed to cool at room temperature. 10 M NaOH was added dropwise to adjust the pH of the 200 mM MOPs to 7.5. This gives a concentration of 200 mM MOPS/NaOH buffer pH 7.5, and then distilled water was added to obtain a final volume of 250 mL.

3.2.3 20 mM MOPS/NaOH buffer 7.5:

200 mM MOPS/NaOH buffer 7.5 was diluted 10 times using distilled water, i.e. 25 mL of 200 mM MOPS/NaOH buffer pH 7.5 was picked and diluted with 225 mL of distilled water.

3.2.4 Chromogen (DTNB) 2.5 mM:

14.86 mg DTNB was weighed and dissolved in 20 mM MOPS/NaOH buffer pH 7.5 and make to a final volume of 15 mL. Thus, making a final concentration of 0.25 mM DTNB from which 100 µl was picked and added to each reaction mixture.

3.2.5 Substrate 50 mM:

1. 95.16 mg butyrylthiocholine iodide was weighed and dissolved in 6 mL of distilled water. This gives a concentration of **50 mM** BTCh.
2. **3 mL** of **50 mM** was picked, and **3 mL** of dH_2O was added and made a final volume of **6 mL**. This makes the concentration **25 mM**
3. **3 mL** of **25 mM** was picked, and **3 mL** of dH_2O was added to make a final volume of **6 mL**. This makes the concentration **12.5 mM**
4. **3 mL** of **12.5 mM** was picked, and **3 mL** of dH_2O was added to make a final volume of **6 mL**. This makes the concentration **6.25 mM**
5. **3 mL** of **6.25** mM was picked, and **3 mL** of dH_2O was added to make a final volume of **6 mL**. This makes the concentration **3.12 mM**

3.3 Enzyme:

1mg of lyophilized equine BChE was weighed and dissolved in 1 mL of 20 MOPS/NaOH buffer pH 7.5 + 0.5 mg/mL BSA. This was aliquoted and stored at -20 ^{o}C.

3.3.1 Bovine serum albumin

0.25 mg/ml of BSA was weighed and added to 20 mM MOPS/NaOH buffer pH 7.5.

20 µL of the enzyme was picked and added to 4980 µL of 20 mM MOPS/NaOH + 0.25 mg/mL BSA.

3.3.2 Enzyme activity determination

BChE activity was assayed in a 1-cm pathlength quartz cuvette darkened on both sides on a PerkinElmer Lambda 25 UV/VIS spectrophotometer according to the colorimetric method of Ellman (Ellman *et* al., 1961) with slide modifications. The final volume of the reaction mixture in a cuvette is 1000 µL and consists of components as shown in the table above. The blank tube contains all components and 50 µl of 20 mM MOPS/NaOH buffer pH 7.5 was added instead of the enzyme. Increased in absorbance at 412 nm as a result of color development was monitored for 20 seconds at 37 ^{o}C and data obtained were in triplicates. Specific activity was calculated from the averaged of the triplicates.

Specific activity (U/mg protein) = $\frac{\Delta Abs412 \text{ X } Vt}{13.6 \text{ X } Vs \text{ X } [protein]}$

ΔAbs_{412}/min: change in absorbance at 412 nm per minute

Vt: total volume of the reaction mixture (1000 µL)

13.6: Extinction coefficient of 5-thio-2-nitrobenzoate (mM^{-1} cm^{-1})

Vs: sample volume (50 µL)

[protein]: protein concentration.

3.4 IC_{50} Determination

Effect of solvents at various concentrations was determined. The reaction mixture contains 500 µL of 200 mM MOPS/NaOH buffer pH 7.5, 100 µL of DTNB, 20 µL of BTCh. and 50 µL of BChE added last to initiate the reaction with subsequent rapid mixing. The volume of distilled water along with the solvent was varied to make the final reaction volume 1000 µL. The final concentration of BTC and DTNB are fixed at 1mM and 0.25 mM respectively.

3.5 Inhibition Kinetics

Inhibitory kinetic experiments were performed in the presence of different concentrations of solvents (0.25% v/v, 0.5% v/v, 1% v/v, 2% v/v, and 4% v/v) so as to determine the kinetic parameters (K_m, V_m, and k_{cat}), inhibition constant (K_i) and mode of inhibition. The different concentrations of solvents [I] were tested individually against five different BTCh concentrations **0.0625 mM, 0.125 mM, 0.25 mM, 0.5 mM, 1 mM**, the initial stock concentrations correspond to **3.125 mM, 6.25 mM, 12.5 mM, 25 mM, 50 mM** respectively. The volume of distilled water along with the solvent was varied so as to make the final reaction volume 1000 µL. A blank tube contains all components and 50 µL 20 mM MOPS/NaOH buffer pH 7.5 was added instead of BChE. Increased in absorbance at 412 nm as a result of color development was monitored for 20 seconds at 37 ^{o}C and data obtained were in triplicates. Specific activity was calculated from the average of the triplicates to plot related graphs.

4.0 RESULTS

4.1 IC_{50} Determination

IC_{50} of all solvents were determine at increasing each solvent concentration (0.25% v/v, 0.5% v/v, 1% v/v, 2% v/v, and 4% v/v) in an *in vitro* setting as described by the colorimetric method of Ellman *et al*. (1961). Substrate analog (BTC) for the enzyme was kept at a constant concentration (1 mM) throughout the IC_{50} determination experiment. BChE activity in the absence of the solvent is considered to be 100%. All experiments were performed at 37 ^{o}C and at 412 nm wavelength. Each data point was recorded as results of triplicate readings. Graph of % remaining activity was plotted against each of the inhibitor (solvent) concentration (Fig 4.1). The individual IC_{50} value of each solvent is presented in Table 4.1.

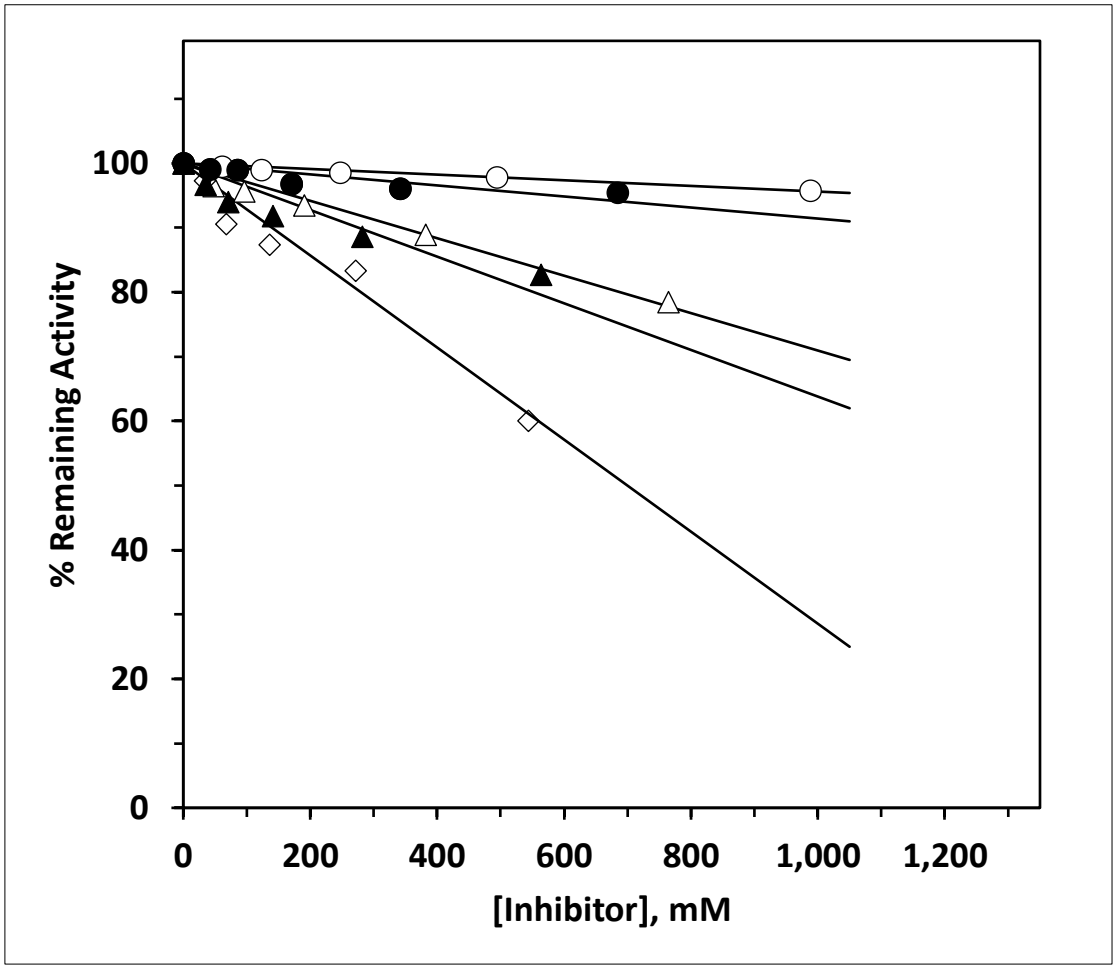

Figure 4.1: Estimated IC_{50} values of the commonly used organic solvents on BChE activity at 1 mM substrate concentration and different solvent concentrations (0.25% v/v, 0.5% v/v, 1% v/v, 2% v/v, and 4% v/v).

(○: methanol, ●: ethanol, △: Acetonitrile □: DMSO, ◇: Acetone)

4.2 Inhibitory Kinetics

Inhibitory kinetics of the solvents on butyrylcholinesterase activity were determine in the presence of different substrate concentrations (0.0625 mM, 0.125 mM, 0.25 mM, 0.5 mM, and 1 mM) and constant chromogen concentration (0.25 mM). Each solvent concentration was set at 0.25% v/v, 0.5% v/v, 1% v/v, 2% v/v, and 4% v/v, respectively, to test its inhibitory effect on the enzyme. Triplicate measurements were carried out at 412 nm.

Inhibitory activity of acetonitrile against BChE was determined using five different acetonitrile concentrations. A hyperbola curve was obtained that obeys Michaelis-Menten (MM) kinetics graph (Fig 4.2). The MM plot was transformed into Lineweaver-Burk (reciprocal) plot to obtain a straight-line graph on which the maximum velocity (V_m) was obtained (Fig 4.3). The lines passing through the ordinate and intersecting at the second quadrant showed an increase in the K_m value and decrease V_m. This indicates a mixed-type (partially competitive and pure noncompetitive) inhibition. from the slope (1122mM) and αK_i from intercept (4076 mM) was graphically estimated (Fig 4.4). The Dixon plot was produced to determine the specific type of inhibition (Fig 4.5), and the K_i was estimated from the graph using the same acetonitrile concentrations. Secondary replot of Dixon (Fig 4.6) indicates that the type of inhibition is competitive as line nearly passes through the centre but deviate a little due to experimental error.

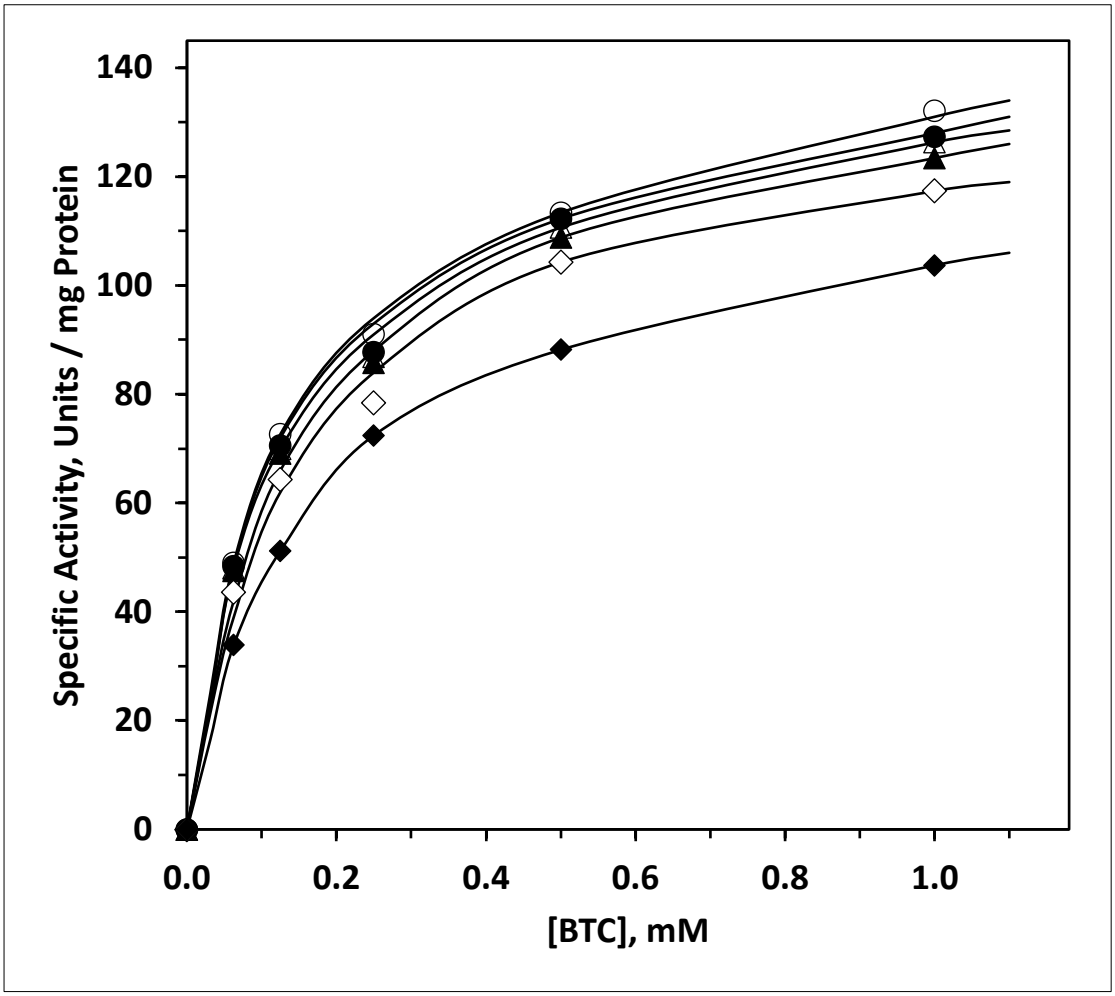

Figure 4.2: Michaelis-Menten plot of the inhibitory effect of acetonitrile on BChE activity in the presence of five different substrate concentrations (0.0625 -1 mM). Each data point is an average of triplicate readings.

Acetonitrile (○: Control, ● :0.25% (v/v), △: 0.5% (v/v), □: 1% (v/v), ◇: 2% (v/v), ♦: 4 % (v/v)).

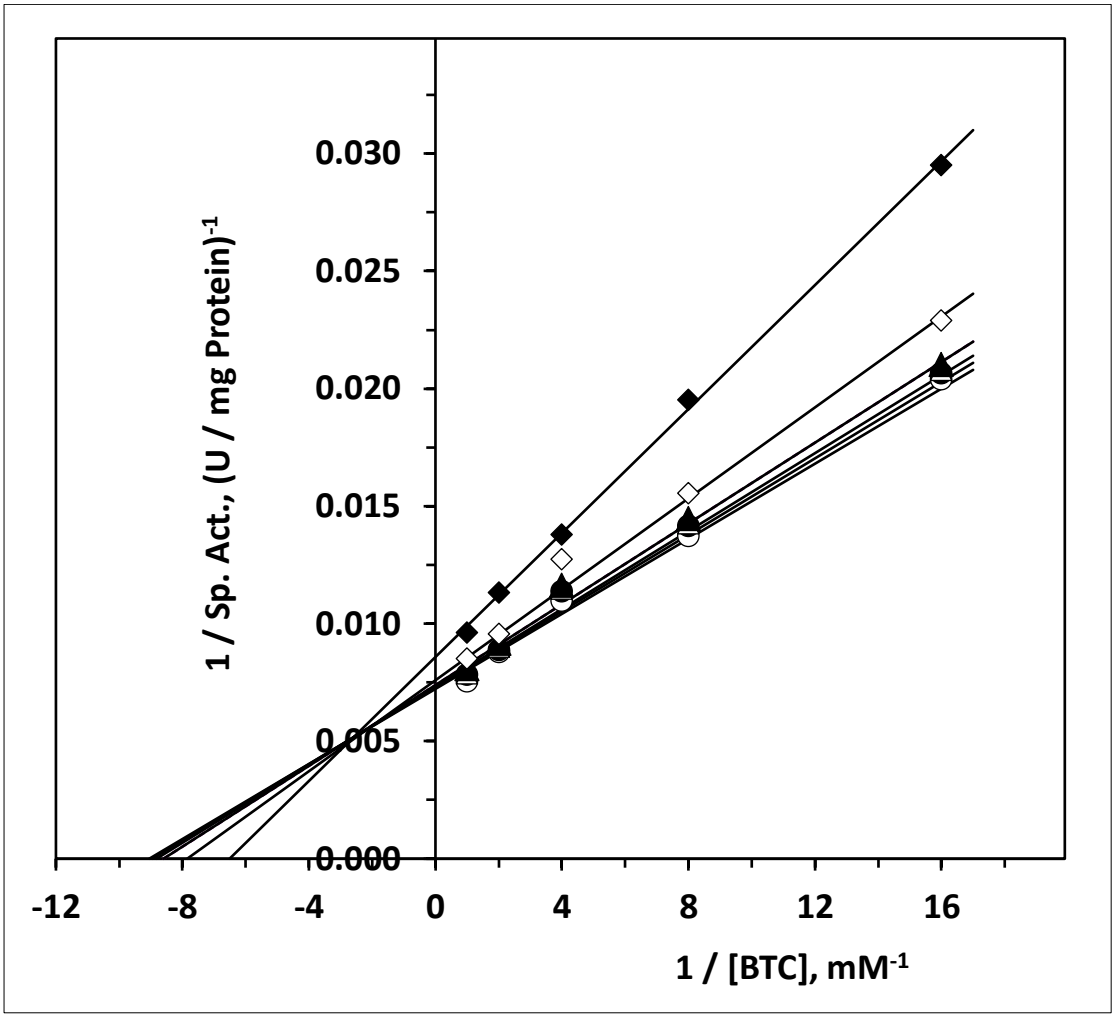

Figure 4.3: Lineweaver-Burk plot of the inhibitory effect of acetonitrile on BChE activity. Acetonitrile concentrations (○: control, ●: 0.25% (v/v), △: 0.5% (v/v), □: 1% (v/v), ◇: 2% (v/v), ■: 4% (v/v))

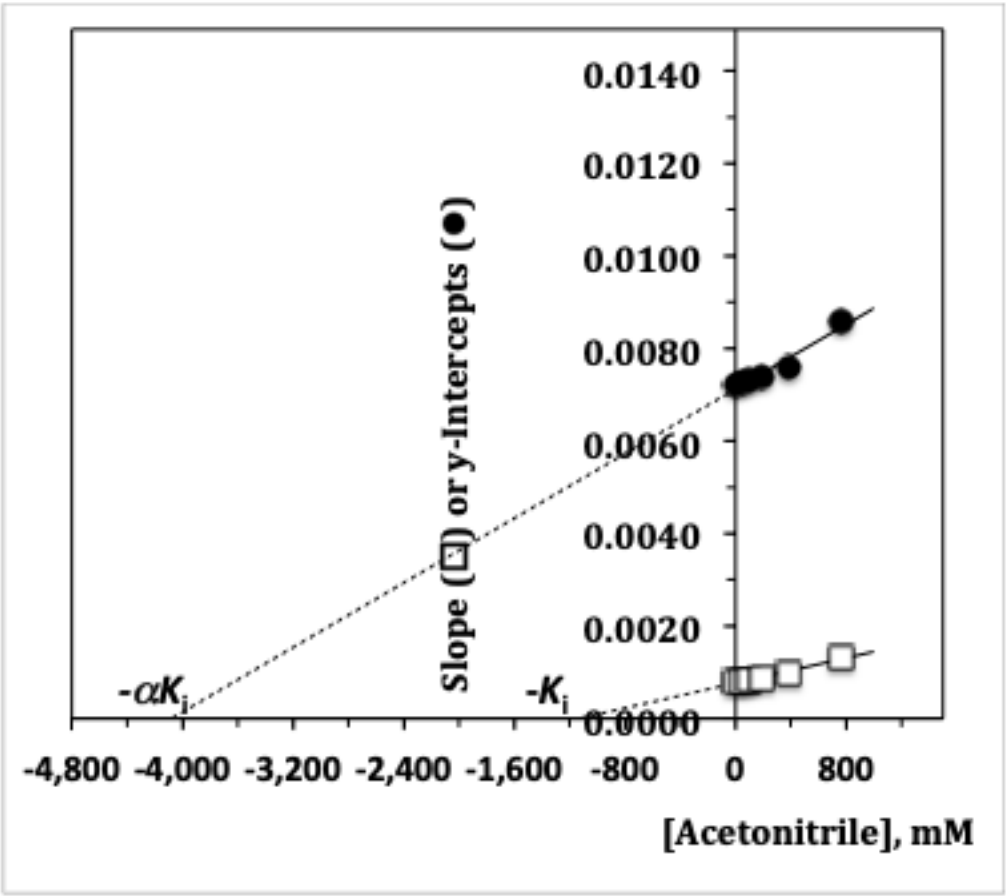

Figure 4.4: Slope vs [acetonitrile] or Intercept vs [acetonitrile] from Lineweaver-Burk plot. Data points represent average of triplicate measurements.

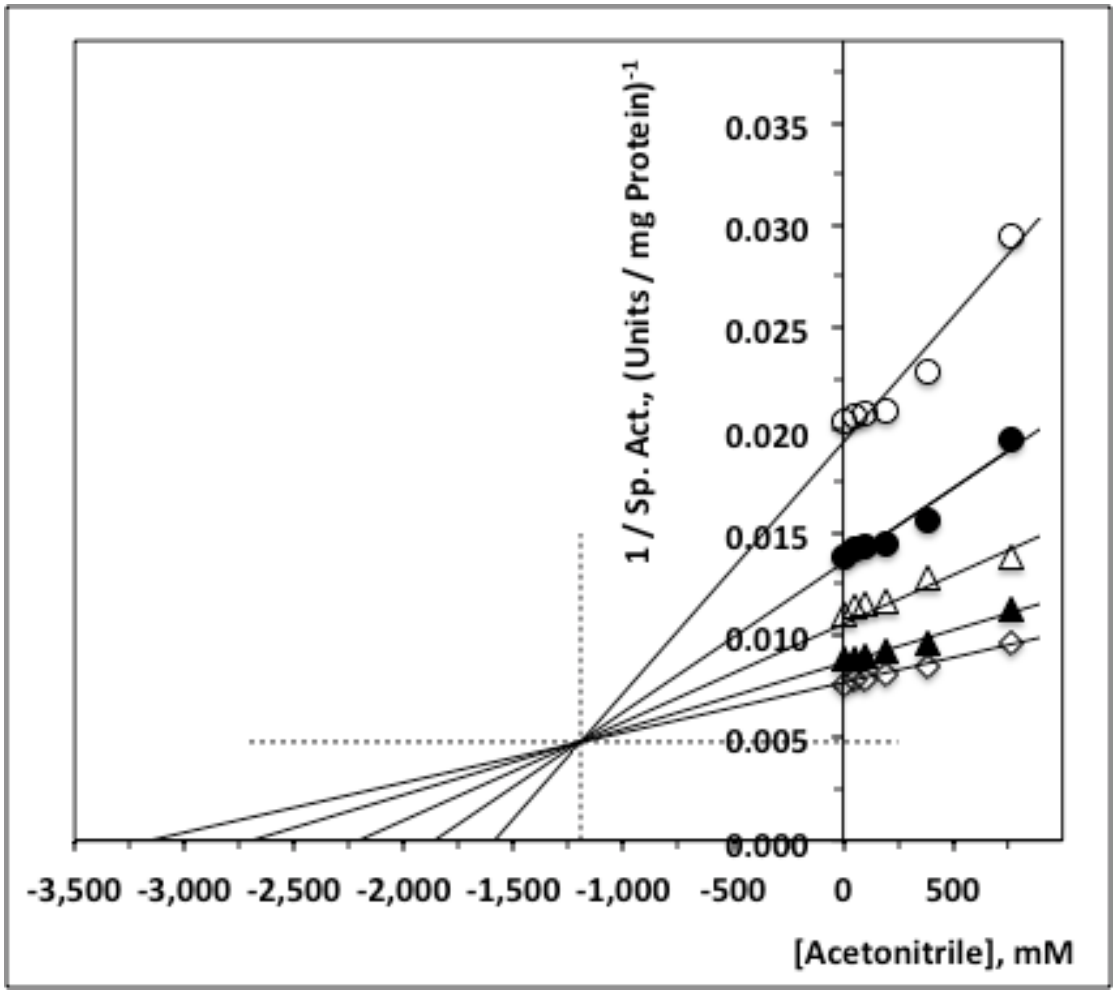

Figure 4.5: Dixon plot of BChE activity in the presence of acetonitrile concentrations. Line intersecting at the negative axis indicate mixed-type inhibition.

Acetonitrile concentrations (○:0.25% (v/v), ●: 0.5% (v/v), △: 1% (v/v), □: 2% (v/v), ◇: 4% (v/v)).

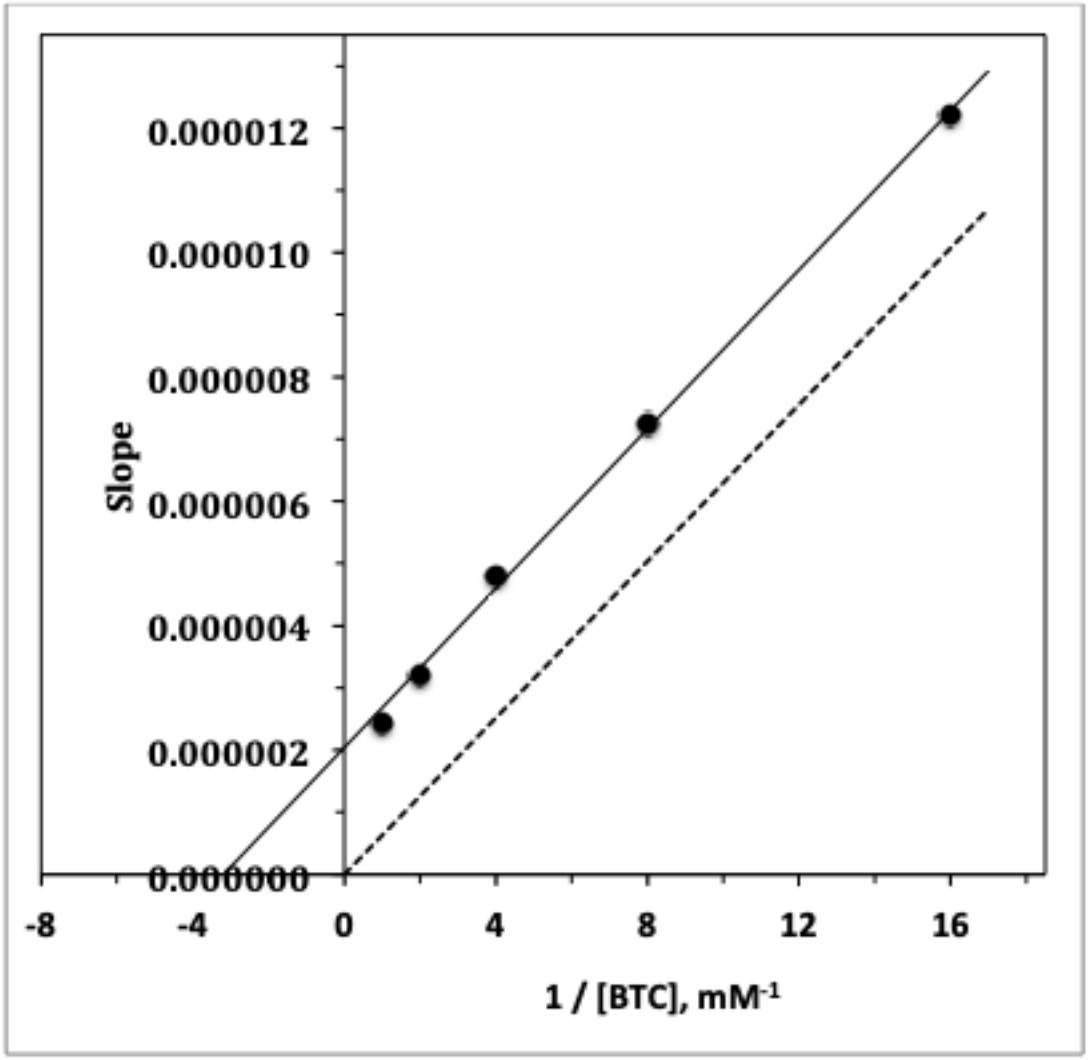

Figure 4.6: Secondary replot of Dixon for acetonitrile obtained by plotting slope against 1/[BTC]. Data point represent average of three measurements.

Acetone inhibit BChE at different concentrations (0.25% v/v, 0.5% v/v, 1% v/v, 2% v/v, and 4% v/v). Substrate were varied at 0.0625 mM, 0.125 mM, 0.25 mM, 0.5 mM, and 1 mM, respectively. Chromogen was kept constant at a final concentration of 0.25 mM. Michaelis-Menten curve was generated (Fig 4.7) showing the decrease in enzyme activity at increasing solvent concentrations. Lineweaver-Burk plot was produced to obtain a straight line (Fig 4.8). V_m and K_m were estimated from the graph. V_m value was unaltered while K_m increases; this indicates a competitive type of inhibition. Secondary plot of the Lineweaver-Burk plot was produced using slope vs [acetone] and $K_{m(app)}$ vs [acetone] plots. Line merging at a point on the x-axis indicate a competitive inhibition (Fig 4.9).

Dixon plot (Fig 4.10) was generated, and the K_i value was estimated (Table 4.1). Slope vs 1/[S] was plotted and a line passing through the origin of the graph indicates a competitive inhibition (fig 4.11).

Michaelis-Menten curve of DMSO (Fig 4.12) was produced by using different DMSO concentrations at different substrate concentrations. Lineweaver-Burk plot (Fig 4.13) was generated using the reciprocal of the MM graph. Competitive type of inhibition was noted as V_m value was not altered, and the corresponding K_m value increases. Secondary replots of Lineweaver-Burk; $K_{m(app)}$ vs [DMSO] and Slope vs [DMSO] was generated (Fig 4.14) which indicate a competitive inhibition. Dixon plot was generated to further support this result, and K_i value was estimated graphically (Fig 4.15). K_i and V_m values were further estimated statistically (Table 4.1). Slope versus 1/[BTC] plot indicate a pure competitive inhibition as line passes through the origin (Fig 4.16).

Inhibitory kinetics of ethanol and methanol were investigated, and Michaelis-Menten graphs (Fig 4.17 and Fig 4.20 respectively) were generated. The double reciprocal plot of 1/specific activity against 1/[S] was plotted for ethanol (Fig 4.18). V_m value 110.98 ± 0.96 U/mg protein was obtained (Table 4.1).

Plots of slope vs [ethanol] and $K_{m(app)}$ vs [ethanol] were generated and K_i **(**3280.5 ± 28.5 mM) value was statistically estimated and are shown graphically (Fig 4.19).

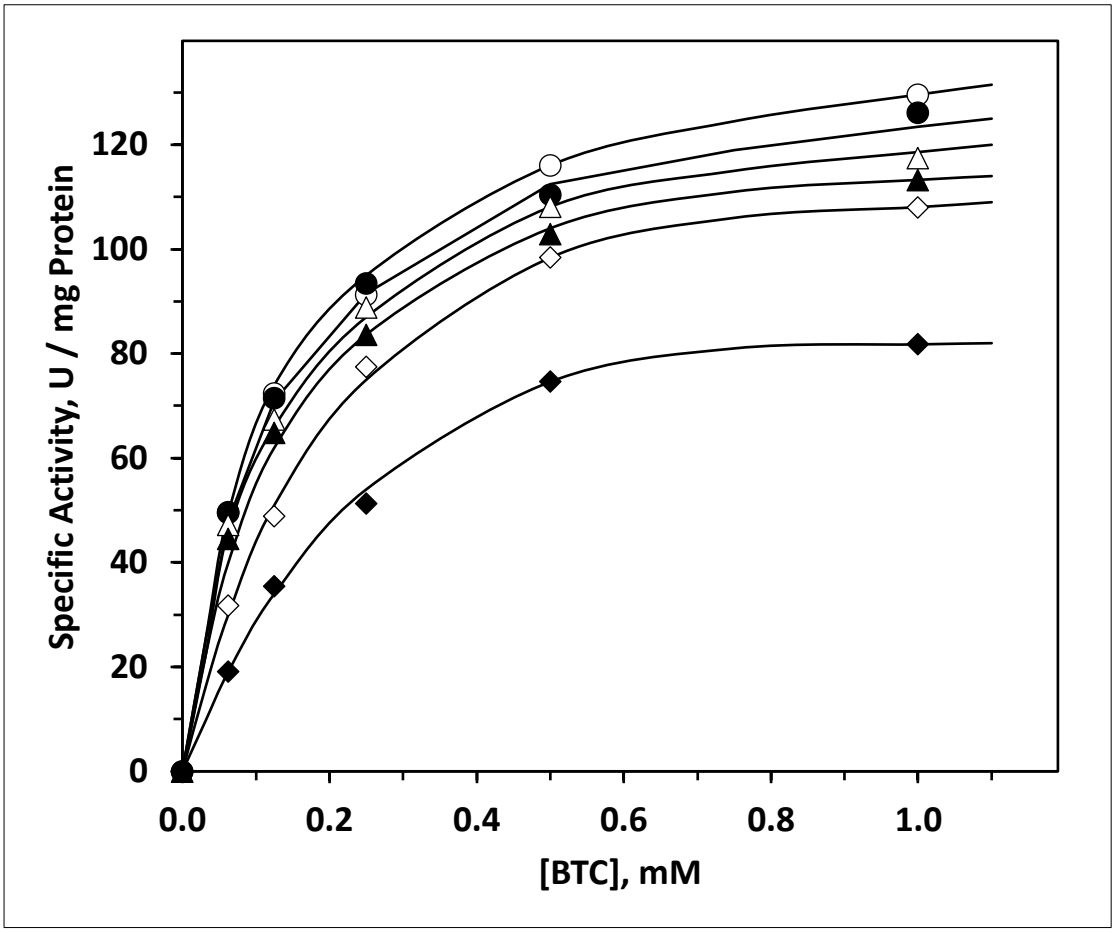

Figure 4.7: Michaelis-Menten graph of acetone inhibitory effect on BChE activity. Data points represent the average of three individual readings.

(○: control, ●: 0.25% v/v, △: 0.5% v/v, □: 1% v/v, ◇: 2% v/v, ■: 4% v/v)

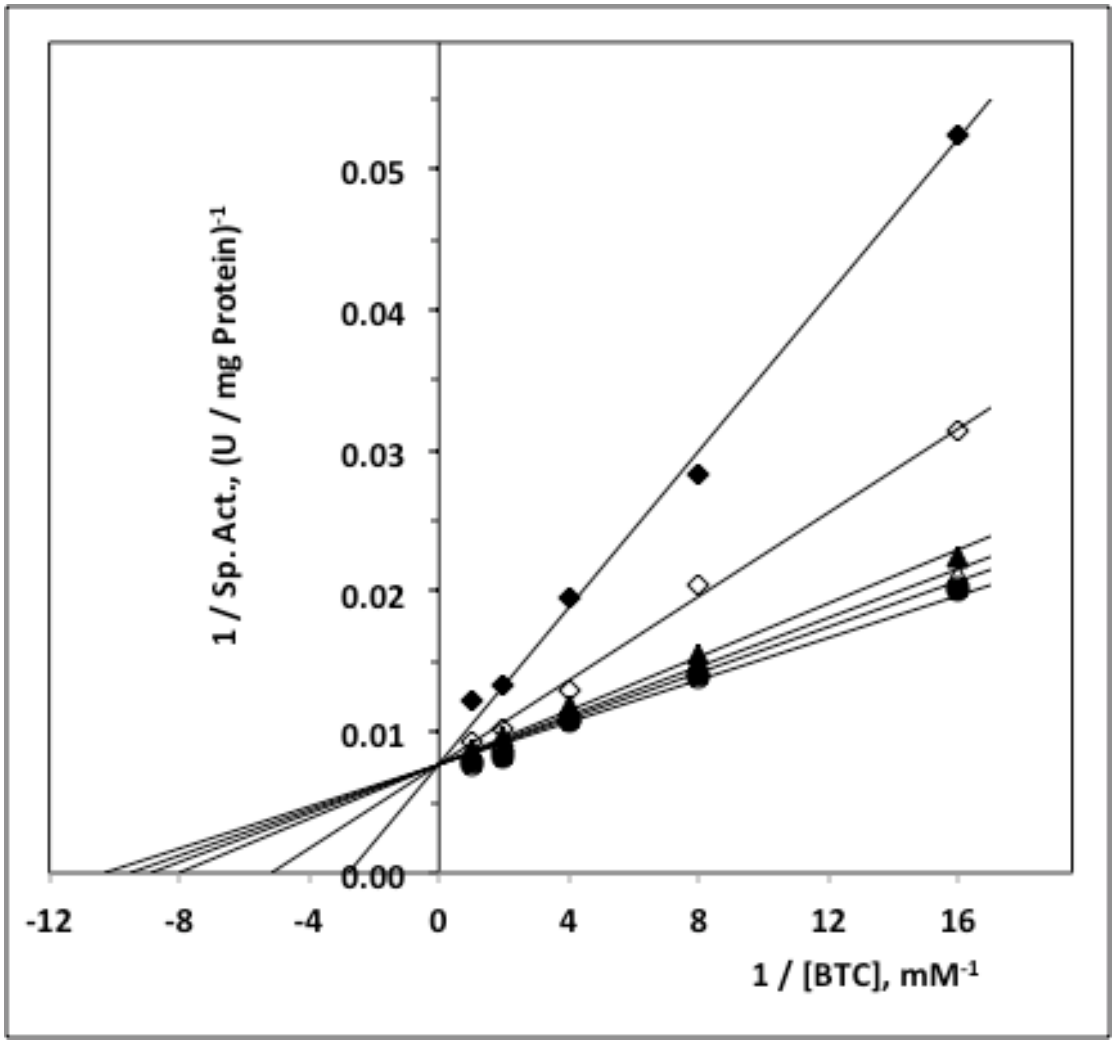

Figure 4.8: Lineweaver-Burk plot of the inhibitory effect of acetone on BChE activity. Acetone concentrations (●: control, ○: 0.25% (v/v), □: 0.5% (v/v), △: 1% (v/v), ◇: 2% (v/v), ■: 4% (v/v)).

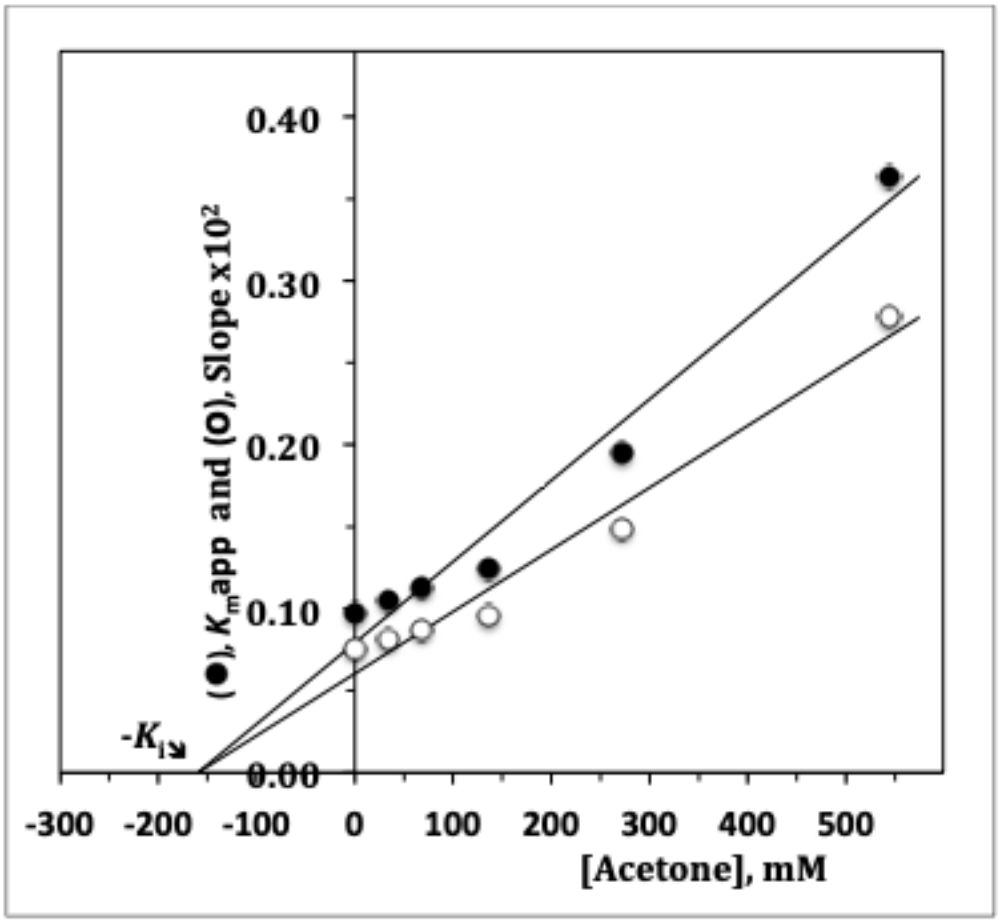

Fig 4.9: Secondary plot obtained from Lineweaver-Burk. Slope vs [acetone] and $K_{m(app)}$ vs [acetone] plots

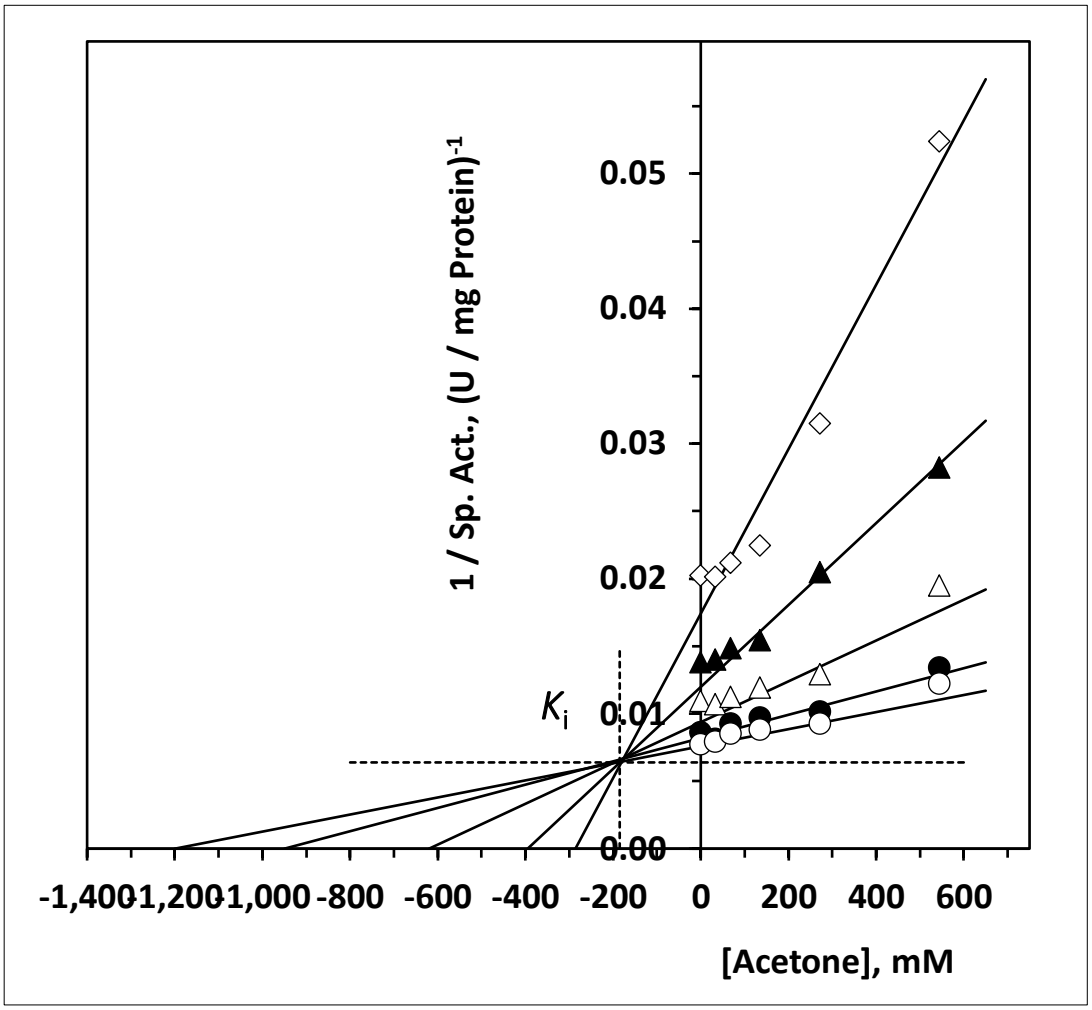

Figure 4.10: Dixon plot of BChE inhibition by acetone; 1/specific activity against [acetone].

Acetone concentrations (◇: 0.25% (v/v), □: 0.5% (v/v), △: 1% (v/v), ●: 2% (v/v), ○: 4% (v/v)).

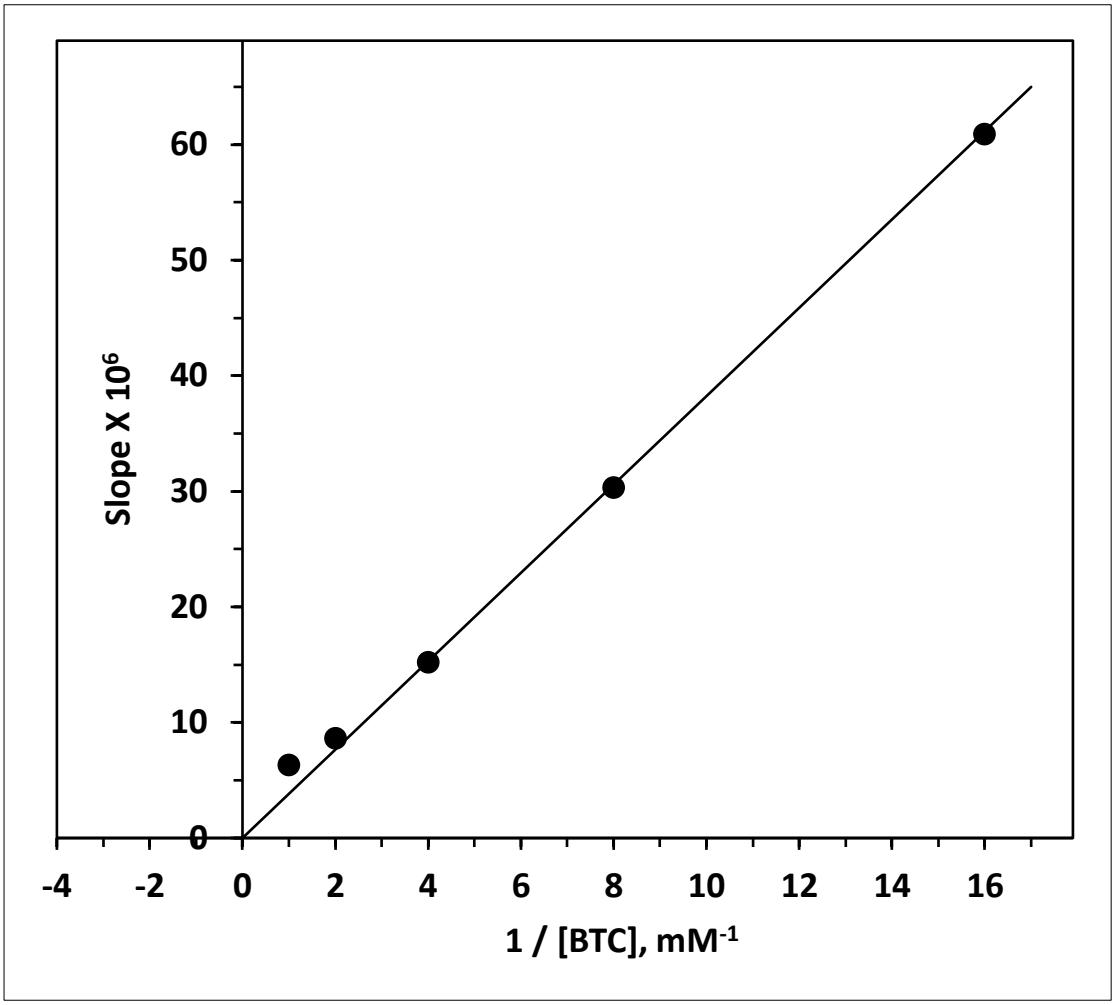

Fig 4.11: Slope vs 1 / [Substrate] from Dixon plot of acetone. Line passing through the origin indicate pure competitive inhibition.

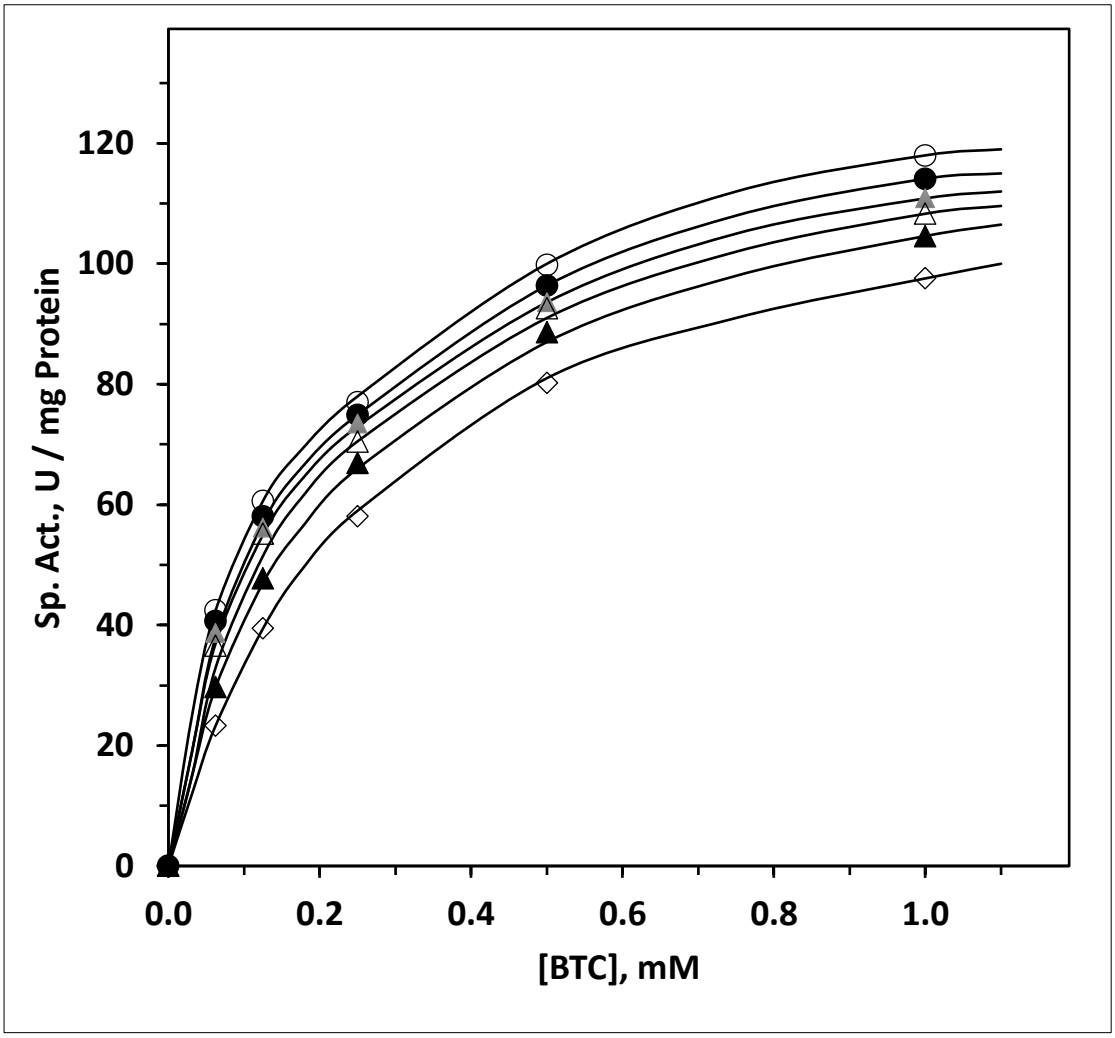

Fig 4.12: Michaelis-Menten plot of DMSO inhibitory action on BChE activity.

DMSO concentrations (○: control, ●: 0.25% (v/v) △: 0.5% (v/v), ▲: 1% (v/v), □: 2% (v/v), ◇: 4% (v/v)).

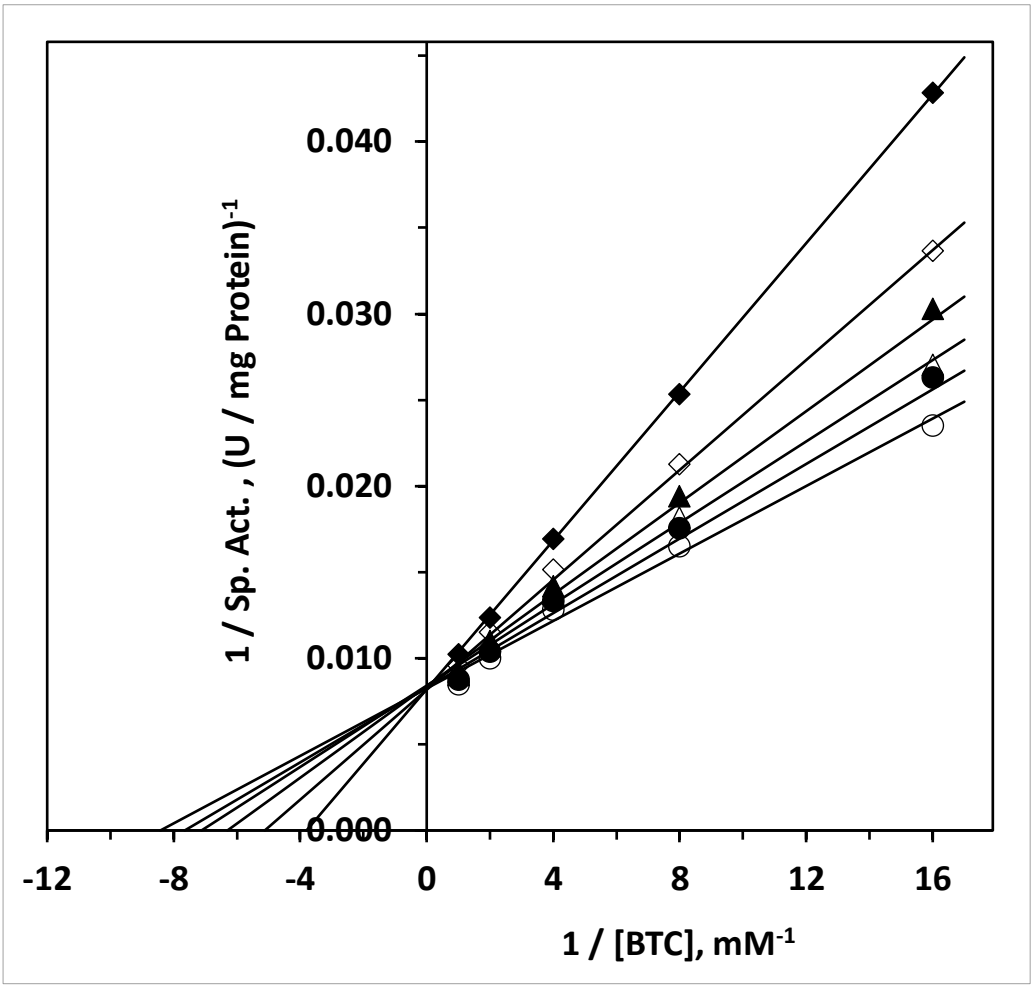

Figure 4.13: Lineweaver-Burk plot of DMSO inhibitory effect at increasing concentration on BChE activity. DMSO concentrations (○: control, ●: 0.25% (v/v), △: 0.5% (v/v), □: 1% (v/v), ◇: 2% (v/v), ■: 4% (v/v)).

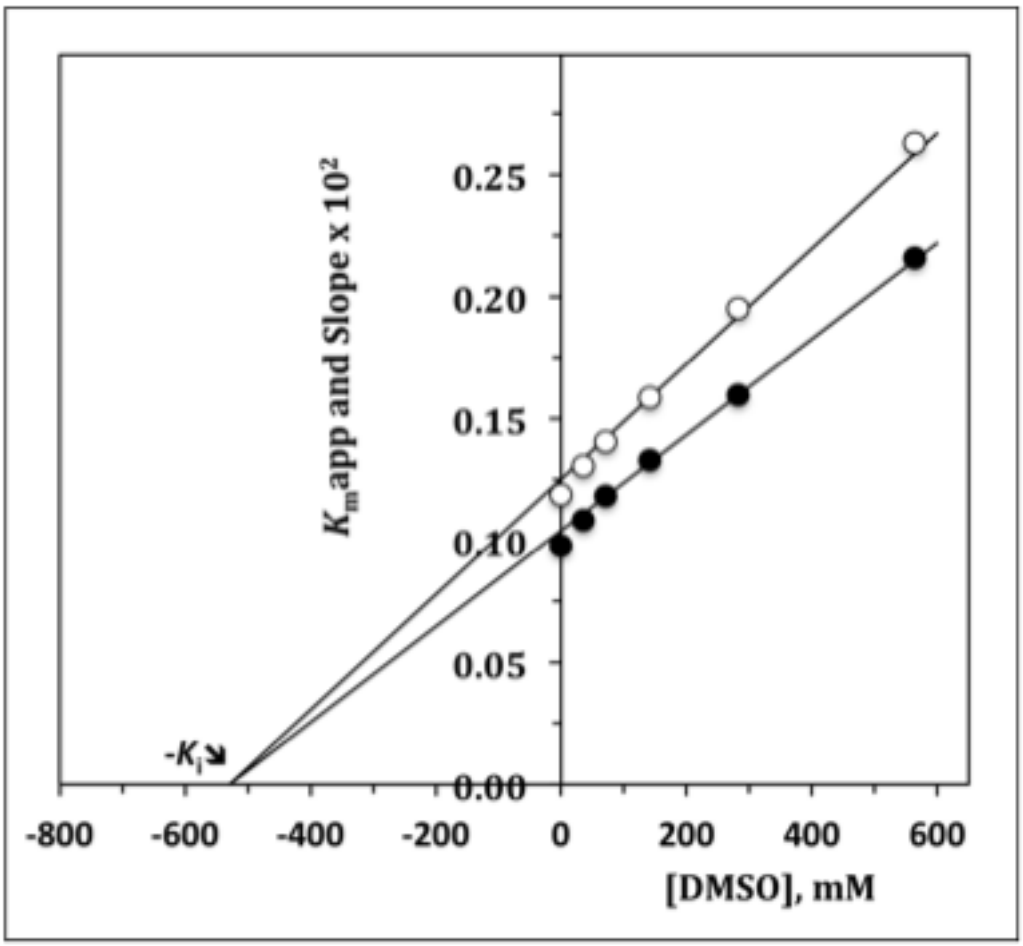

Figure 4.14: $K_{m(app)}$ vs [DMSO] and Slope vs [DMSO]; ○: K_{mapp} graph ●: Slope vs [DMSO]

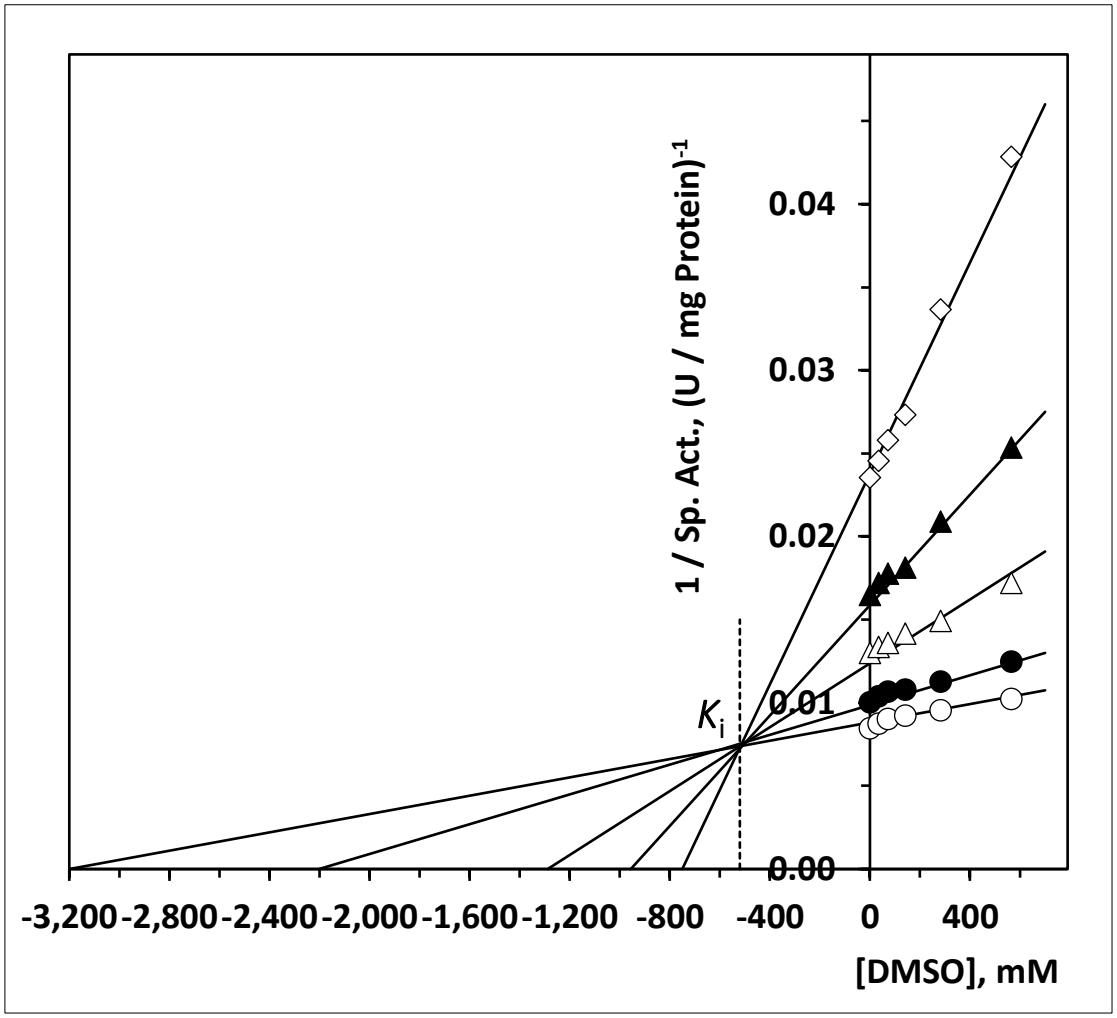

Figure 4.15: Dixon plot of DMSO replotted from Lineweaver-Burk plot data. DMSO concentrations (◇: 0.25% (v/v), □: 0.5% (v/), △: 1% (v/v), ●:2% (v/v), ○: 4% (v/v)).

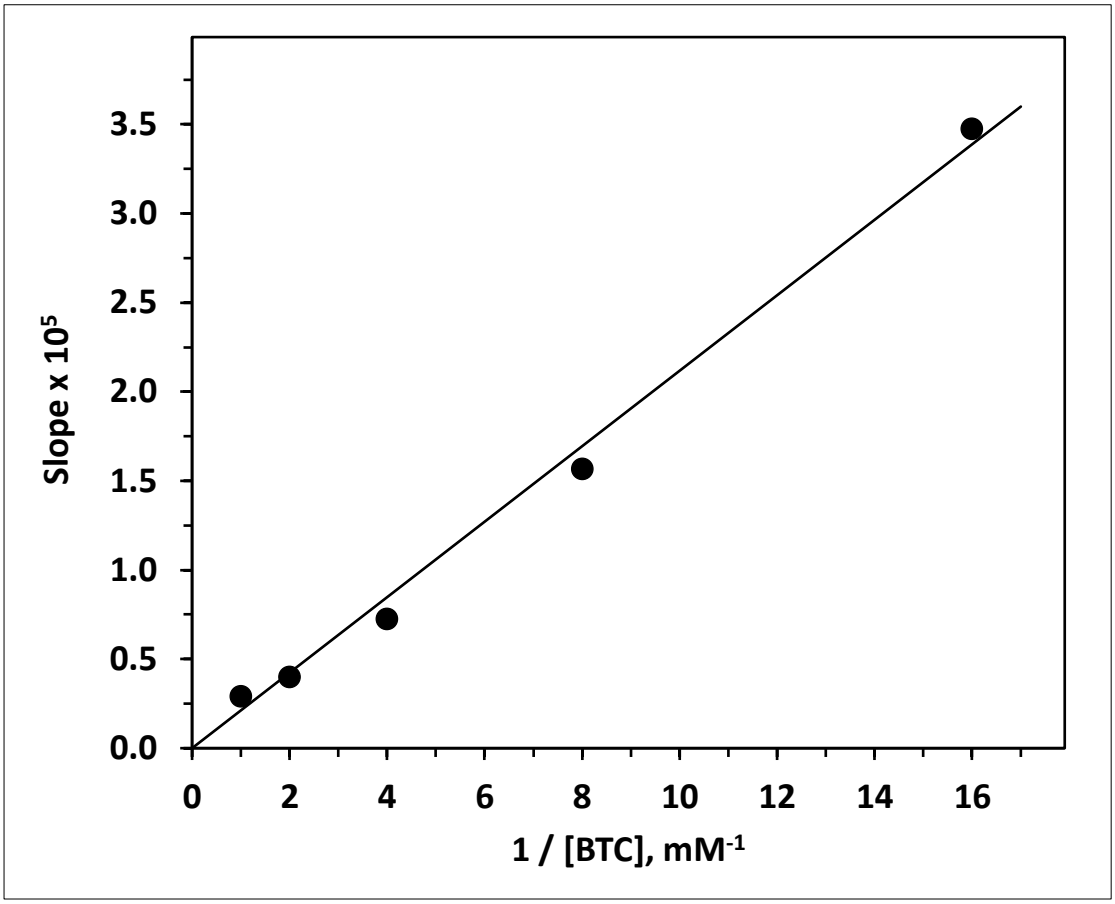

Figure 4.16: Slope vs 1 / Substrate graph for the effect of DMSO on BChE activity. Line passing the origin indicate a pure competitive inhibition.

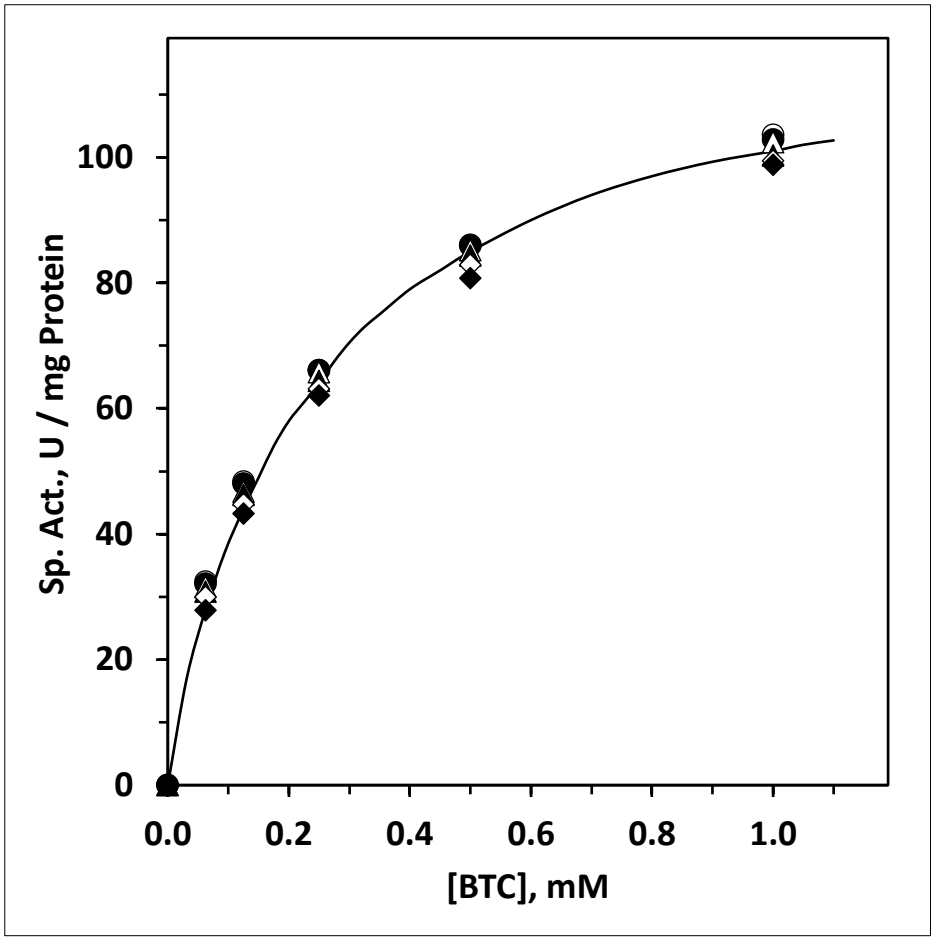

Fig 4.17: Michaelis-Menten graph of the inhibitory action of ethanol on BChE activity at five different concentrations of ethanol.

BTC concentrations (0.0625 mM, 0.125 mM, 0.25 mM, 0.5 mM, and 1 mM)

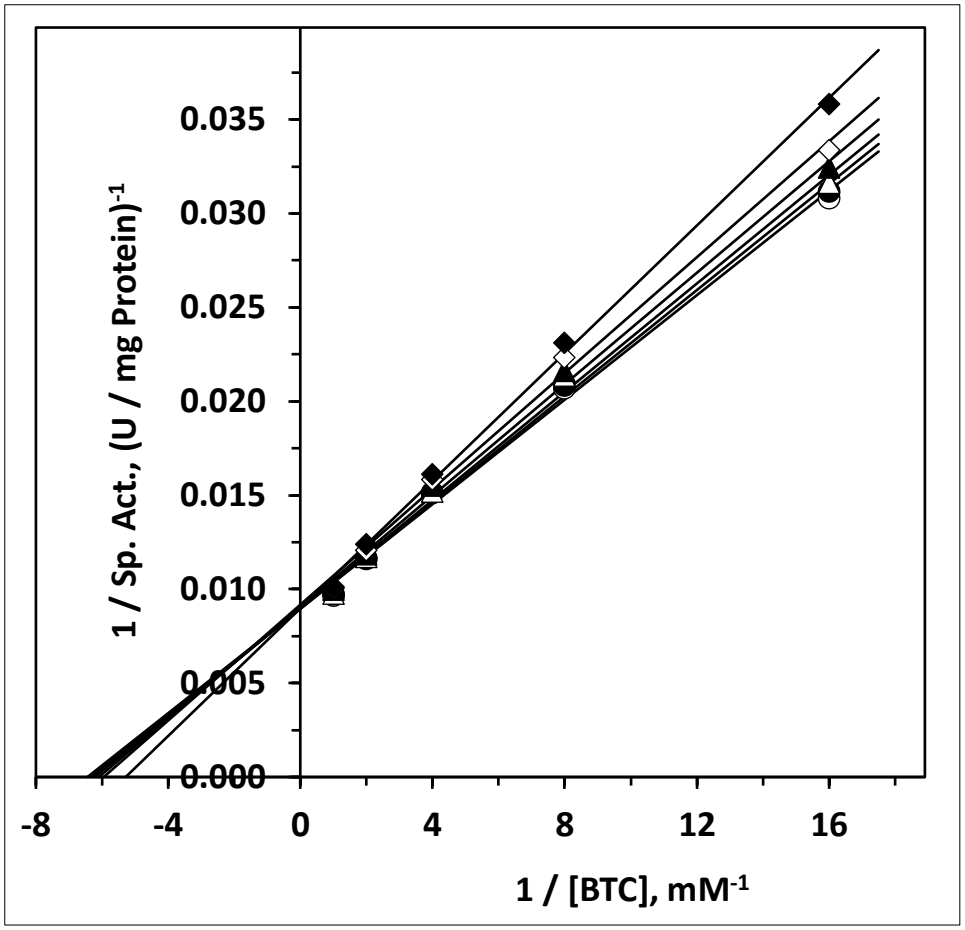

Figure 4.18: Lineweaver-Burk plot of butyrylcholinesterase in the presence of ethanol at different BTC concentrations (0.0625 mM, 0.125 mM, 0.25 mM, 0.5 mM, and 1 mM).

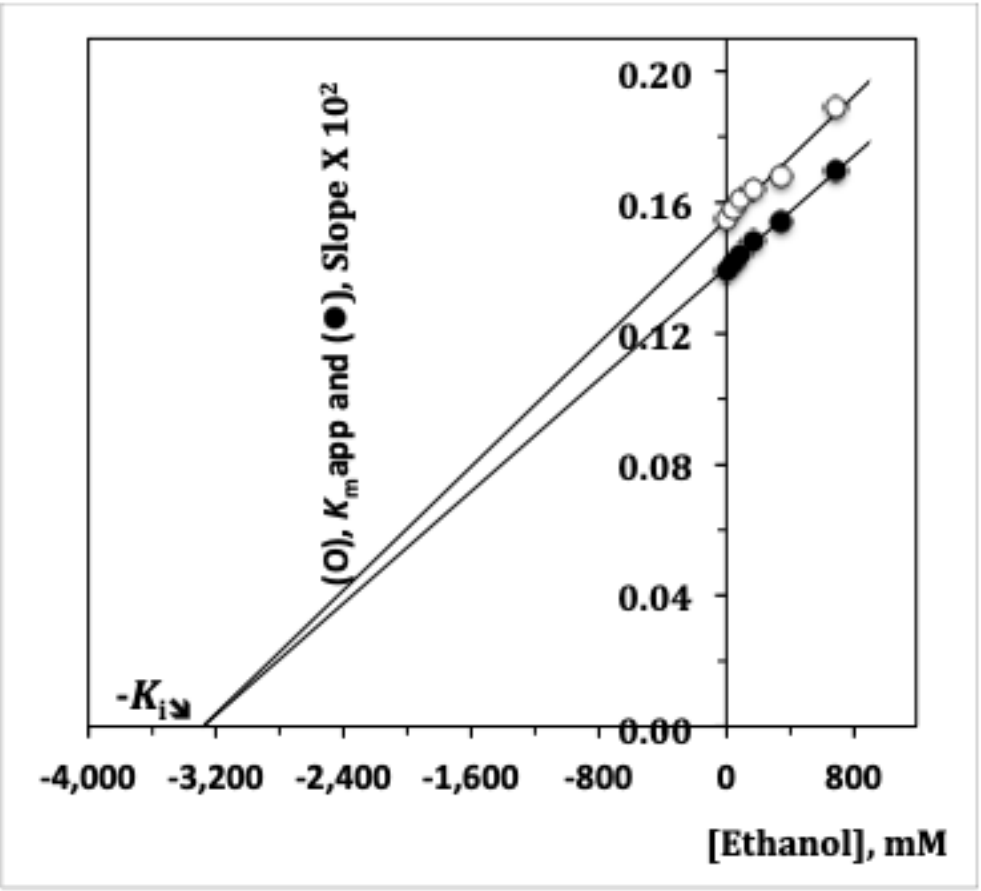

Figure 4.19: K_{mapp} vs [DMSO] and Slope vs [DMSO]; ○: K_{mapp} graph ●: Slope vs [DMSO]

The last solvent tested was methanol. Effect different methanol concentrations (0.25% v/v, 0.5% v/v, 1% v/v, 2% v/v, and 4% v/v) were tested on BChE at different substrate concentrations. A hyperbola curve that obeys Michaelis-Menten kinetics was obtained (Fig 4.20). Lineweaver-Burk plot (1/specific activity vs 1/[BTC]) was replotted to obtain a straight-line graph (Fig 4.21) that gives the V_m. Line merges at the negative part of the graph indicate a noncompetitive type of inhibition. K_m is unaltered while V_m decreases. Slope vs [methanol] plot was generated, giving a K_i of 22.9 M (fig 4.22).

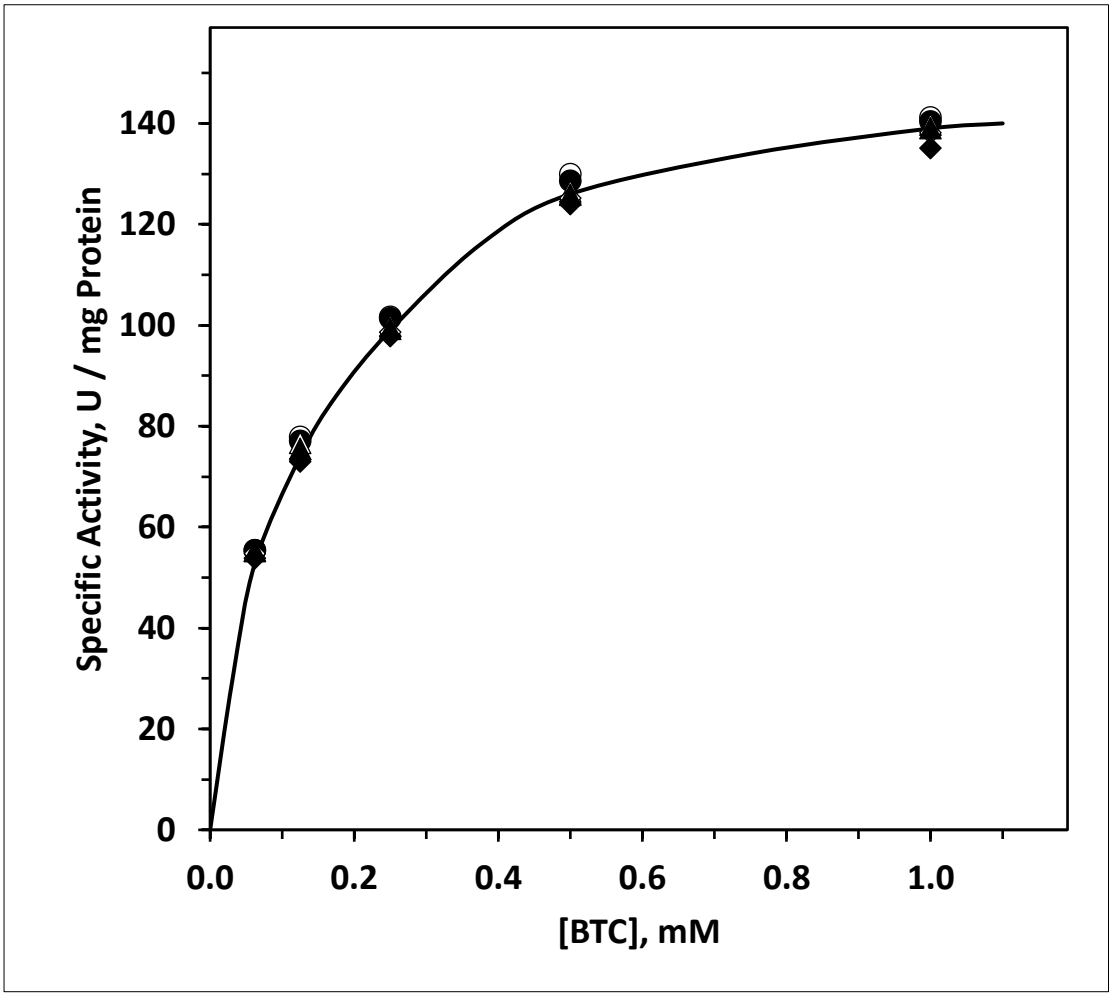

Fig 4.20: Michaelis-Menten plot of inhibitory action of methanol on the activity of BChE. BTC concentrations (0.0625 mM, 0.125 mM, 0.25 mM, 0.5 mM, and 1 mM).

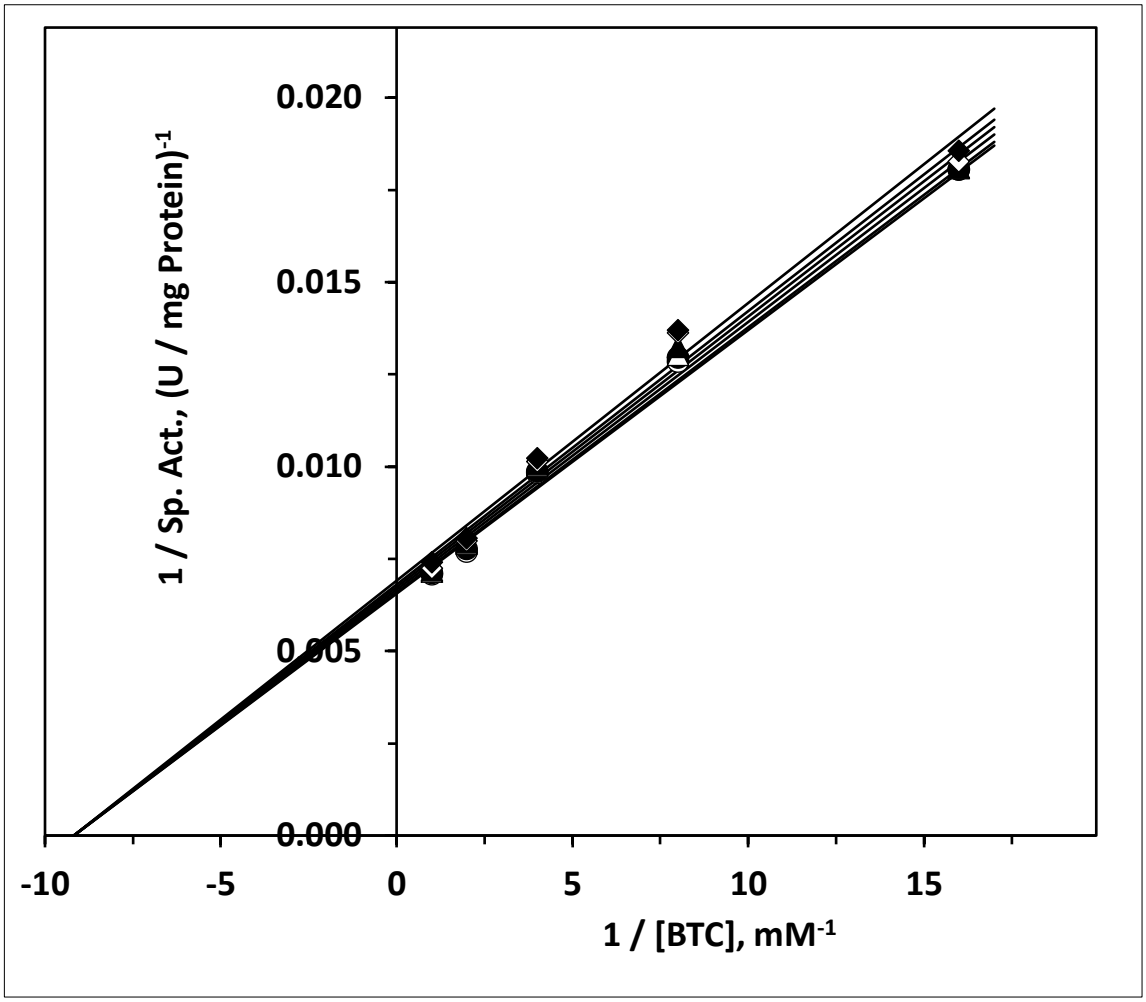

Figure 4.21: Lineweaver-Burk Plot of inhibitory action of methanol on BChE activity. Lines intersecting at the second quadrant indicate a noncompetitive inhibition**.** BTC concentrations (0.0625 mM, 0.125 mM, 0.25 mM, 0.5 mM, and 1 mM)

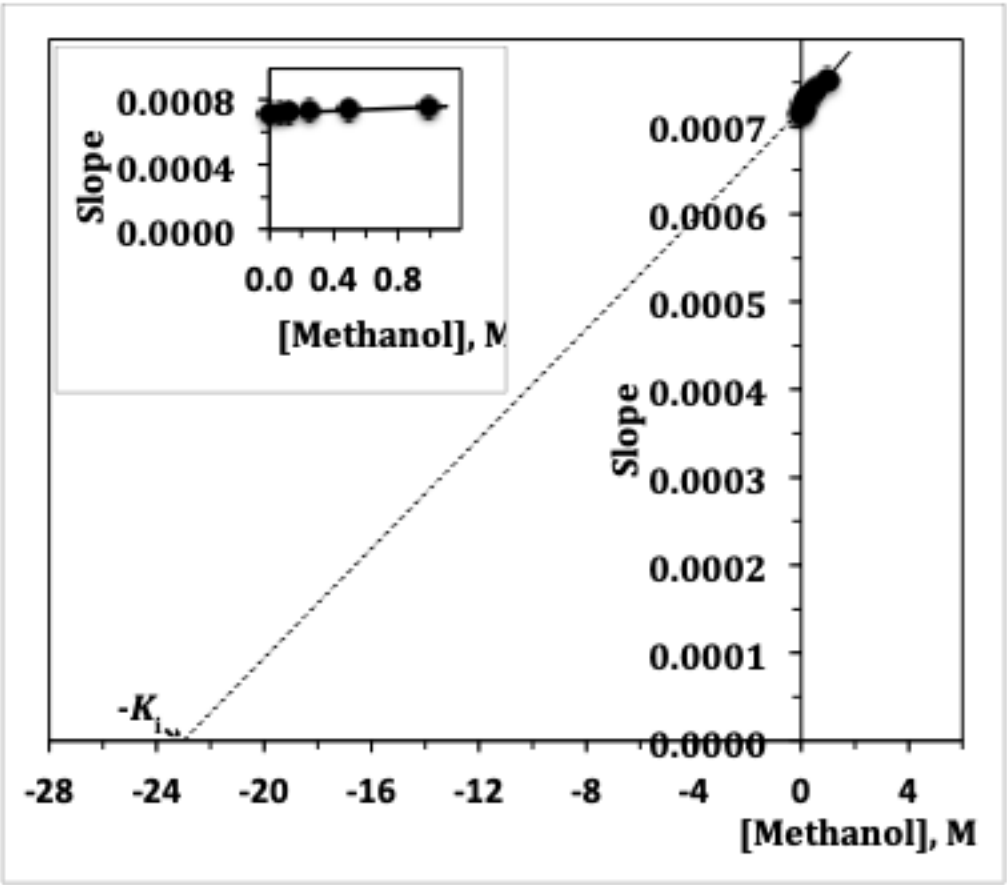

Figure 4.22: Slope vs [methanol]

4.1: Summary of kinetic parameters and inhibition type

Solvents →	Acetone	DMSO	Acetonitrile	Ethanol	Methanol
IC_{50}, mM	707	1719	1848	7741	12199
Inhibition type	Competitive	Competitive	Mixed-type, Competitive	Competitive	Noncompetitive
Vm, U/mg protein	134.03 ± 1.645	127.19 ± 1.045	137.719 ± 1.341	110.98 ± 0.96	158.66 ± 1.348
*K*i, mM	185.61±14.88	529.31 ± 35.41	724.96 ± 66.90	3280.5 ± 28.5	22990.64 ± 6534.98
K_m, *mM*	0.092 ± 0.005	0.140 ± 0.004	0.110 ± 0.004	0.155 ± 0.35	0.128 ± 0.003

4.3 Comparison of Human BChE and Horse BChE (Sequence alignment)

HuBChE and hBChE sequence alignment shows 90.22% identity with about 30 similar positions and 518 identical positions (Figure 4.5).

```
Human BChE      EDDIIIATKNGKVRGMNLTVFGGTVTAFLGIPYAQPPLGRLRFKKPQSLTKWSDIWNATK      60
Horse BChE      EEDIIITTKNGKVRGMNLPVLGGTVTAFLGIPYAQPPLGRLRFKKPQSLTKWSNIWNATK      60
                *:****:********** *:*********************************:******

Human BChE      YANSCCQNIDQSFPGFHGSEMWNPNTDLSEDCLYLNVWIPAPKPKNATVLIWIYGGGFQT      120
Horse BChE      YANSCYQNTDQSFPGFLGSEMWNPNTELSEDCLYLNVWIPAPKPKNATVMIWIYGGGFQT      120
                ***** ** ******* *********:**********************:**********

Human BChE      GTSSLHVYDGKFLARVERVIVVSMNYRVGALGFLALPGNPEAPGNMGLFDQQLALQWVQK      180
Horse BChE      GTSSLPVYDGKFLARVERVIVVSMNYRVGALGFLALSENPEAPGNMGLFDQQLALQWVQK      180
                ***** ****************************  **********************

Human BChE      NIAAFGGNPKSVTLFGESAGAASVSLHLLSPGSHSLFTRAILQSGSFNAPWAVTSLYEAR      240
Horse BChE      NIAAFGGNPRSVTLFGESAGAASVSLHLLSPRSQPLFTRAILQSGSSNAPWAVTSLYEAR      240
                *********:********************** *: ********** *************

Human BChE      NRTLNLAKLTGCSRENETEIIKCLRNKDPQEILLNEAFVVPYGTPLSVNFGPTVDGDFLT      300
Horse BChE      NRTLTLAKRMGCSRDNETEMIKCLRDKDPQEILLNEVFVVPYDTLLSVNFGPTVDGDFLT      300
                ****.***  ****:****:*****:**********.*****.* ***************

Human BChE      DMPDILLELGQFKKTQILVGVNKDEGTAFLVYGAPGFSKDNNSIITRKEFQEGLKIFFPG      360
Horse BChE      DMPDTLLQLGQFKRTQILVGVNKDEGTAFLVYGAPGFSKDNNSIITRKEFQEGLKIFFPR      360
                **** **:*****:*********************************************

Human BChE      VSEFGKESILFHYTDWVDDQRPENYREALGDVVGDYNFICPALEFTKKFSEWGNNAFFYY      420
Horse BChE      VSEFGRESILFHYMDWLDDQRAENYREALDDVVGDYNIICPALEFTRKFSELGNDAFFYY      420
                *****:******* **:**** *******.*******:********:**** **:*****

Human BChE      FEHRSSKLPWPEWMGVMHGYEIEFVFGLPLERRDNYTKAEEILSRSIVKRWANFAKYGNP      480
Horse BChE      FEHRSTKLPWPEWMGVMHGYEIEFVFGLPLERRVNYTRAEEILSRSIMKRWANFAKYGNP      480
                *****:*************************** ***:*********:*************

Human BChE      NETQNNSTSWPVFKSTEQKYLTLNTESTRIMTKLRAQQCRFWTSFFPKVLEMTGNIDEAE      540
Horse BChE      NGTQNNSTRWPVFKSTEQKYLTLNTESPKVYTKLRAQQCRFWTLFFPKVLELTGNIDEAE      540
                * ****** ****************** :: ************ *******:********

Human BChE      WEWKAGFHRWNNYMMDWKNQFNDYTSKKESCVGL                           574
Horse BChE      REWKAGFHRWNNYMMDWKNQFNDYTSKKESCSDF                           574
                 ****************************** .:
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Identical positions                              518
Identity                                         90.244%
Similar positions                                30


An asterisk (*) indicates positions which have a single, fully conserved residue.
A colon (:) indicates conservation between groups of strongly similar properties.
A period (.) indicates conservation between groups of weakly similar properties.
```

Figure 4.23: Sequence alignment of HuBChE and hBChE

5.0 DISCUSSION

A high rise in the incidence of Alzheimer's disease has created a global concern. In 2016, Alzheimer's disease and other related dementia estimated to hit 43.8 million globally, with the highest incidence in women (Nichols *et al.,* 2019). This value has been predicted in many studies to increase periodically. As a result, several calls for prevention or curative approach has been made. Compounds with suspected therapeutic potentials have been screened in organic solvents at different concentrations. The commonly used concentrations of organic solvents lie between 1-4%. But these solvents may alter the structure of enzymes either positively or negatively. Therefore, this study aimed at investigating the effect of commonly used organic solvents in the screening of potential drug targets.

The partial or low solubility of compound leads to a shift in the concentration curve giving a higher IC_{50} that connotes low inhibitory effect of the compound (Di and Kerns, 2006). Commonly used organic solvents in this study showed different IC_{50} values. The order of the potency of the solvents decreases as follows acetone (707 mM) > DMSO (1719 mM) > acetonitrile (1848 mM)> ethanol (7741 mM) > methanol (12199 mM). Inhibitory kinetics study of the individual solvents revealed different types of inhibition.

Acetone and ethanol were reported to enhance the activity of cellobiohydrolase I and endoglucanase II possibly by increasing the binding of the substrate to the enzyme active site (Nan *et al.,* 2019). But herein, acetone in the presence of different BTC concentrations (0.0625 mM, 0.125 mM, 0.25 mM, 0.5 mM, 1 mM) inhibit the activity of BChE in increasing concentration manner. Based on the obtained IC_{50} value (707 mM), acetone is the most potent inhibitor among the commonly used organic solvent in this study. It binds to BChE

competitively with an inhibition constant (K_i) of 185.606 ± 14.879 mM and V_m of 134.027 ± 1.645 U/mg. The mode of inhibition of acetone was revealed to be competitive.

Inhibitory action of acetonitrile against BChE was determined in a dose-dependent manner. The plotted graph shows an increase in K_m value and decreases V_m that indicate a mixed-type (partially competitive and pure noncompetitive) inhibition. This result was then further plotted and a clear noncompetitive inhibition was seen (fig 4.6) as the replots of slope vs [ethanol] and *K*mapp vs [ethanol] revealed straight lines that intersect at a single point that gives the K_i value.

DMSO decreases BChE activity in a dose-response manner as K_m increases and V_m value unaltered. The observed decrease in enzyme activity by DMSO in this study could be due to the interaction of the methyl group with hydrophobic residues. DMSO shows a mixed type competitive inhibition on AChE (Kumar and Darreh-Shori, 2017) and mixed-type noncompetitive inhibition on aldose reductase (Misuri *et al.*, 2017), but here it shows a pure competitive inhibition of BChE (Fig 4.16). It can be seen that DMSO behaves differently with enzymes as the results of Kumar and Darreh-Shori (2017) shows an IC_{50} for DMSO against human recombinant AChE (rAChE) to be ~258.4 mM, which is contrary to the result of this study that shows a triple of the value. This could be due to the differences in the active sites of the enzymes and also shows that DMSO is more potent to AChE than BChE.

Ethanol and methanol activate hydrogenase enzymes above the commonly used concentrations and shows a decrease at higher concentrations (>40%), with a similar effect for DMSO (Serebryakova *et al.,* 2009). An enzyme such as thermostable laccase was

reported to be more stable in 50% ethanol than in water, and this highlight its importance in industrial applications that involve the use of organic solvents (Maijala *et al.,* 2012). It has also been shown to enhance the inhibition of collagen-bound proteases by chlorhexidine than in water with no loss of mass by the enzyme (Ekambaram *et al.*, 2015). Methanol and ethanol as representatives of the alcohol family show minimal effect even at the highest concentration on butyrylcholinesterase in this study. Methanol has the highest K_i value of 22.99 M, indicating low binding potency to BChE.

CONCLUSION

Inhibitory effects of commonly used solvents were assayed on equine serum butyrylcholinesterase at different solvent concentrations. Each of the solvent IC_{50} was obtained graphically. From the result of this study, it can be deduced that there is no universality on the effect of solvents on the same class of enzymes as AChE (from different study; Kumar and Darreh-Shori, 2017) and BChE shows different behavior towards the solvents. Acetone shows the highest potency among the solvents with a half-maximal inhibitory concentration of approximately 5% (v/v) and binds the enzyme competitively in the presence of BTC. Other solvents such as acetonitrile and DMSO also inhibit BChE but with lower potency than acetone. Ethanol and methanol show minimal inhibition of BChE. Therefore, the use of these solvents (especially acetone) to screen for potential AD inhibitors should be given careful considerations on the concentrations to be used, as their presence may interfere with the final findings of the study.

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