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IN-VITRO ANTI-CHOLINESTERASE ACTIVITIES BY PIPERINE, AN ALKALOID FROM THE SPICE FAMILY PIPERACEAE

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Piperine, Acetylcholinesterase, Butyrylcholinesterase, Kinetics of Inhibition

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ABSTRACT: The alkaloid piperine from the spice family Piperaceae has been reported to possess poly-pharmacological activities including anti-depressant and cognitive enhancing effects. It has been suggested that its neurocognitive benefits may be via its activity on the cholinergic system, particularly on the enzyme acetylcholinesterase (AChE), a pharmacological target for neurodegenerative disease such as Alzheimer's disease (AD). The paucity of information on the potential mechanism of inhibition of acetylcholinesterase and butyrylcholinesterase (BuChE), also a primary target for drug development for the treatment of AD, prompted this *in vitro* investigation. Dose-dependent inhibition of AChE and BuChE by piperine was determined using a modified classic colorimetric method of Ellman. Kinetics of inhibition was determined by Lineweaver-Burk methods. Piperine inhibited both AChE and BuChE in a concentration dependent manner with IC_{50} values of 0.12 mM and 0.067mM, respectively. Piperine exhibited a higher selectivity towards BuChE with a BuChE/AChE ratio of 0.56mM. Kinetic values for AChE classify piperine as a competitive inhibitor whereas the values for BuChE classify it as a mixed inhibitor. Compared to galanthamine (a mixed competitive non-competitive AChEinhibitor, IC_{50} of 1.068 nmol/ml under similar assay conditions) we conclude that although the AChE inhibition by piperine is not as potent as that of galanthamine, in addition to its known antioxidant and anti-inflammatory activities, piperine could provide a novel poly-pharmacological lead of potential benefit for the symptomatic treatment of AD and therefore warrants further investigation.

INTRODUCTION: Alzheimer's disease (AD) is generally recognised as the most prevalent form of dementia. It is an irreversible and progressive disease which destroys memory and cognitive skills and eventually leads to death. [1] AD is a multi-aetiology disorder. Risk factors for AD include non-modifiable factors such as age and genetics, as well as modifiable factors such as dietary and lifestyle choices. [2] The current pharmacological options available for the treatment of AD include cholinesterase inhibition, glutamate receptor modulation, anti-oxidants and anti-inflammatory agents. [3]

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However, acetylcholinesterase inhibitors (AChEIs) have become the mainstay for the symptomatic treatment of AD. AChEI drugs approved in the US and UK are: donepezil (Aricept®), rivastigmine (Exelon®) and galantamine (Reminyl®). In Europe, availability differs from country to country. The general mechanism of AChEI is to increase the brain availability of the neurotransmitter acetylcholine (ACh) through the inhibition of the enzyme acetylcholinesterase (AChE) that plays a role in its hydrolysis. [3] Inhibiting AChE prolongs the time ACh molecules remain in the synaptic cleft, to combine with muscarinic receptors thus enhancing cholinergic neurotransmission.[3]

Two types of cholinesterases exist; AChE, which selectively hydrolyses ACh, and butyrylcholinesterase (BuChE), which hydrolyses other choline esters in addition to Ach. [3] BuChE is present in all human and mouse tissues, and is more

abundant than AChE in all body tissues except the brain. People who have no BChE activity due to a genetic variation are healthy, which has led to the hypothesis that BChE has no physiological function. [4] Over the course of AD however, AChE activity decreases while BuChE activity stabilizes and even increases. Xie and co-workers [5] have shown that cholinergic pathways lacking AChE are sensitive to the toxic effect of BuChE selective inhibitors. These data suggest that BuChE may act as a compensatory mechanism for Ach metabolism and support the potential of BuChE as a suitable target for the treatment of AD.

Black pepper (*Piper nigrum*) is the most widely used among spices and belongs to the family *Piperaceae*, cultivated for its fruit (berries) that are dried and used as a food seasoning. The spiciness of the pepper compound is due to the nitrogenous alkaloid substance piperine (1-piperoylpiperidine) (**Fig.1**), found both in the outer layer and seed inside the fruit berries. Piperine is present in black pepper (*P.nigrum*), long pepper (*P. longum*) and other piper spices (Family: *Piperaceae*). [6]

Piperine, as seen in the historic remedies, is the vital compound that exerts antipyretic and anti-inflammatory properties for medicinal uses. Other biological effects that piperine possess are; analgesic [7], anticonvulsant [8], anti-ulcer [9], antidepressant [10], cognitive enhancing [6, 11], cytoprotective and anti-oxidant. [12] The antioxidant properties in piperine have also been linked to improvements in cognitive function. In one study [11], piperine was administered to Wistar male rats, at doses ranging from 5, 10 and 20 mg/kg/day orally for 4 weeks and the neuropharmacological activity was determined after single, 1, 2, 3 and 4 weeks of treatment.

The results showed that across the entire dosage range piperine possessed anti-depression-like activity and a cognitive enhancing effect during the entire treatment duration. Although the precise mechanism is unknown, it was suggested that it might be through the inhibition of lipid peroxidation and the acetylcholinesterase enzyme and that further studies were essential to understand the precise mechanism of piperine's activity [6], hence the objective of this *in vitro* study.

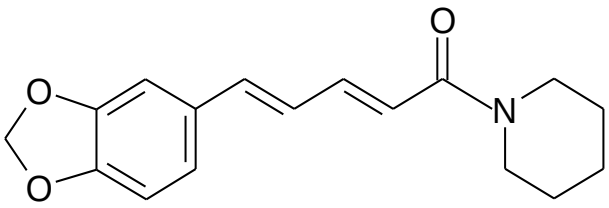

FIG.1: CHEMICAL STRUCTURE OF PIPERINE

MATERIALS AND METHODS:

Chemicals/reagents:

Acetylcholinesterase (EC 3.1.1.7) from *Electrophorus electricus* – homologous to human, acetylthiocholine iodide (ATChI), butyrylcholinesterase (EC 3.1.1.8) from equine serum, butyrylthiocholine (BTCh I), 5:5-dithiobis-2-nitrobenzoic acid (DTNB) sodium bicarbonate and Piperine (97% purity) (P49007) were purchased from Sigma Chemical Co., UK.

Acetylcholinesterase activity:

Assessment of AChE inhibition was carried out using a calorimetric method of Ellman, *et al*.[13] as modified by Okello *et al*.[14] A typical run consisted of 5 µL of AChE solution, at a final assay concentration of 0.03 U/ml; 20 µL of 0.1M phosphate buffer pH 8; 5 µl of DTNB, at a final concentration of 0.3 mM prepared in 0.1 M phosphate buffer pH 7 with 0.12 M of sodium bicarbonate; and 5 µl of a piperine extract solution in 95% ethanol (EtOH). The reactants were mixed in a 96-well, flat bottom polystyrene microtitre plate. The mixture was shaken and pre-incubated at 30^{o}C for 10 minutes. The reaction was initiated by adding 5µl of ATChI at a final concentration of 0.5 mM. Each sample was assayed in triplicate. The wells were again shaken before incubation at 30^{o}C for 10minutes. As a control 5 µl of the extract solution was replaced with 5 µl of 95% ethanol.

The control was also assayed in a triplicate. To monitor the non-enzymatic hydrolysis in the reaction mixture, a blank consisting of 5 µl buffer pH 8 replaced the AChE enzyme and 5 µlbuffer, pH 8 replaced the piperine solution. A kinetic run absorbance at 405nm was measured on a Thermo Labsystems Multiskan (Ascent software version 2.6) 96-well plate reader for a period of 16 minutes at 30^{o}C. The first 6 minutes were maintenance time for problems reported by [15, 16, 17] that EtOH prevented inhibition of *E. electricus* AChE. Using a low concentration of 1.2% EtOH in the assay and

maintaining the mixed reagents in the plate for an extra 6 minutes prevented this inhibition.

Butyrylcholinesterase activity:
Assessment of BuChE inhibition was performed as described above, with the exception of 5µlBuChE replacing AChE and 5µlBTChI replacing ATChI.

Determination of dose response curve and kinetic parameters:
The concentration of piperine that inhibited the hydrolysis of substrate by 50% (IC_{50}) was determined by monitoring the effect of various concentrations (0.03125 to 0.5mM). The per cent inhibition of AChE and BuChE was calculated using the following formula:

((Control (abs/min) – Piperine concentration (abs/min))/ control (abs/min)) * 100 = % inhibition.

Each concentration was run equivalent to *n*=6. Dose-response curves were fitted to the data points using Microsoft Excel software and Sigma Plot 11. The IC_{50} value was calculated from the standard curve equations using Sigma Plot 11. For inhibition kinetic studies, the serial dilutions of the piperine were pre-incubated with different substrate concentrations ranging from (0.0625 mM to 0.5 mM). Piperine concentration of 0.0625 mM and 0.125 mM were chosen for the kinetics studies as they were concentrations similar to the specific BuChE and AChEIC_{50} values respectively. The data for substrate kinetics were analysed using the Lineweaver-Burk models for the determination of K_m and V_{max} constants.

RESULTS:

Effect of piperine on AChE and BuChE activity:
Analysis of data showed that piperine inhibited AChE activity in a concentration dependent manner (**Fig.2**). The maximum inhibition (100%) was observed at the final assay concentration of 0.5 mM. The IC_{50} value calculated from the equation obtained from the log inhibition curve was 0.12mM. This contrast with an IC_{50} value of 1.068 nmol/ml for galanthamine, as previously determined under identical assay conditions. [18] Piperine also exhibited a concentration dependent inhibition of BuChE activity (**Fig. 3),** maximum inhibition (67%) was observed at the final assay concentration of 0.5 mM. The IC_{50} value from the equation obtained from the log inhibition curve was 0.067mM. There was a difference in efficiency between the two enzymes with piperine inhibiting BuChE 47% more effectively than AChE at the same concentration; despite AChE reaching 100% maximum inhibition and BuChE reaching 67% maximum inhibition. The ratio between the IC_{50} values is 0.56mM, of which a higher ratio would have supported a higher selectivity for the AChE enzyme.

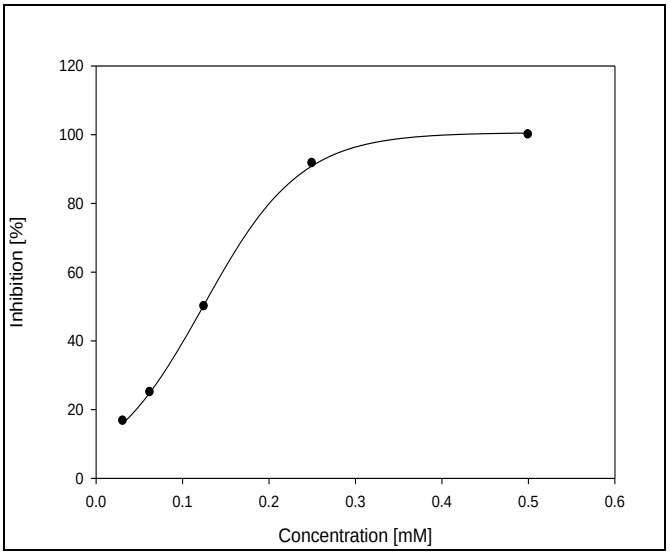

FIG.2: DOSE-DEPENDENT INHIBITION OF AChE BY PIPERINE

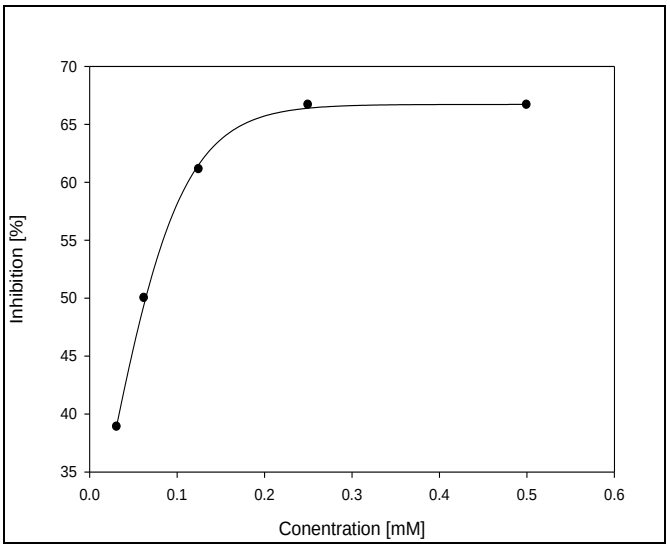

FIG.3: DOSE-DEPENDENT INHIBITION OF BuChE BY PIPERINE

Determination of the kinetic profile of AChE and BuChE against piperine:
The mechanism by which piperine inhibited the enzymes AChE and BuChE was investigated using the Lineweaver-Burk plots (Figures 4 & 5) and

Lineweaver-Burk constants (**Table 1a** and **1b**). The control (no piperine), 0.0625mM and 0.125 mM concentrations were used. The kinetic values for AChE classified piperine as a competitive inhibitor; where the V_{max} values remained more or less constant while the K_m values increased. However, the kinetic values for BuChE classified piperine's as a mixed-inhibitor; where the V_{max} values are variable whereas the K_m values increase. Compared to galanthamine (a mixed competitive non-competitive inhibitor, piperine is not as potent as this positive reference (IC_{50} of 1.068 nM/ml) as previously tested under the same assay conditions. [18]

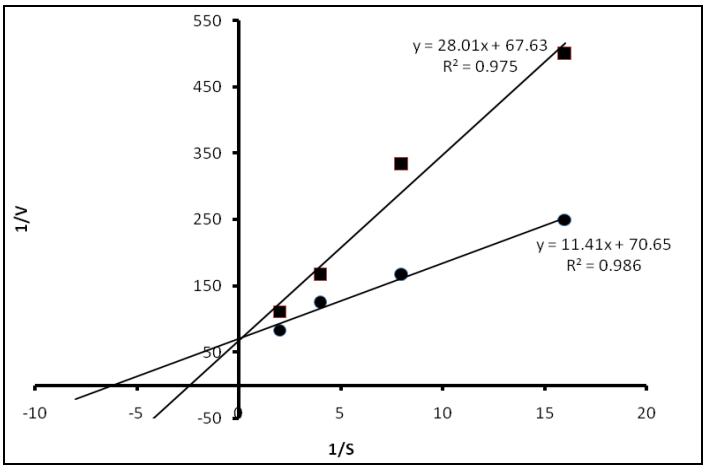

FIG.4: LINEWEAVER-BURK PLOT FOR AChE inhibition(● No piperine, ■Piperine 0.0625 mM)

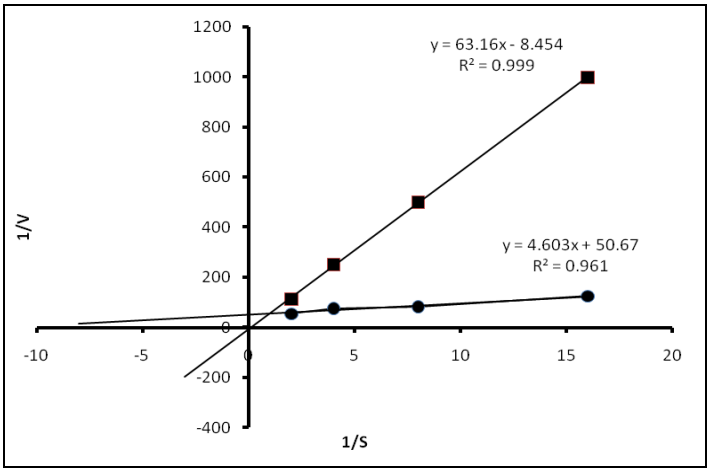

FIG. 5: LINEWEAVER-BURK PLOT FOR BuChE inhibition (● No piperine, ■Piperine 0.125mM)

TABLE 1a: KINETIC CONSTANTS FOR AChE

	Kinetic Constants			
	$^{1}/V_{max}$[mM/min]	V_{max}[mM/min]	$^{-1}/K_m$ [mM]	K_m [mM]
No piperine	70.652	0.014	6.19	0.16
Piperine 0.0625 mM	67.633	0.015	2.41	0.41

TABLE 1 b: KINETIC CONSTANTS FOR BuChE

	Kinetic Constants			
	$^{1}/V_{max}$[mM/min]	V_{max}[mM/min]	$^{-1}/K_m$ [mM]	K_m [mM]
No piperine	50.678	0.020	11.01	0.09
Piperine 0.125 mM	8.45	0.118	0.13	7.47

DISCUSSION: Previous studies on piperine have used animal models to assess the protective [6] and cognitive enhancing effects [11] against neurodegeneration and cognitive deficit. However further research was essential in order to understand the precise inhibitory mechanism piperine exhibits against AChE and BuChE. This study therefore focussed on identifying the

cholinesterase inhibitory property of piperine via kinetic study. Our study demonstrated that piperine exhibited anti-cholinesterase activity in a dose dependent manner. Furthermore, analyses of the Lineweaver-Burk kinetics showed that piperine followed a competitive inhibition pattern for AChE and mixed-inhibition for BuChE. There was a higher affinity for BuChE with the IC_{50} value over 50 fold that for AChE inhibition. This is in contrast to galanthamine which has a 50 fold more selective inhibition for AChE over BuChE. [19]

The proportion of the two enzymes, AChE and BuChE, present in the human brain are strongly altered during the course of Alzheimer's disease. In the cortex AChE activity decreases progressively to 10-15% of normal values, while BuChE activity is unchanged or even increased by 20%. This may be the consequence of a combination of an accumulation of BuChE in neuritic plaques and of reactive gliosis.[20] Given the close spatial relationship between glial cell protoplasm and synaptic gap, it is likely that extracellularly-diffusing ACh may come in contact with glial BuChE and be effectively hydrolysed as a result.[21]An important feature distinguishing BuChE from AChE is the enzymes kinetics toward concentrations of ACh. An excess of this substrate (µM) will inhibit only AChE but not BuChE. Consequently, because of the difference in K_m of the two enzymes, glial BuChE is less efficient in hydrolysing ACh at low substrate concentrations (sub-µM) than neuronal AChE.[22] The mechanism of inhibition caused by the excess of substrate has been clarified.[23]

Comparing the synaptic function between the two enzymes has shown variations between studies. An extensive investigation found that AChE inhibition was more efficient than BuChE in elevating cortical ACh using a non-selective inhibitor compound eptastigmine. [24] Interestingly when comparing these results against a BuChE specific inhibitor MF 8622, a high elevation of ACh similar to that of eptastigmine, without the typical cholinergic side effects was demonstrated.[25] The results of these experiments support the concept of two pools of functional cholinesterases in the brain, one neuronal and AChE-dependent acting mainly within physiological conditions and one mainly glial and BuChE-dependent acting within conditions of decreased AChE activity such as in the Alzheimer's disease brain. Two pools show different kinetic properties with regard to regulation of brain ACh and can be separated by selective inhibitors.[24] In the absence of AChE, BuChE can hydrolyse ACh and potentially substitute for AChE without producing peripheral or central cholinergic side effects. Considering the drastic decrease in AChE activity taking place in the brain and the large pool of BuChE still available both in glia and neurons, it may not institute an advantage for a cholinesterase inhibitor to be selective for AChE. In contrast, a good balance between AChE and BuChE inhibition should result in higher therapeutic efficacy.[22]

Compounds with preferential cholinergic activity could potentially be used therapeutically according to the severity of Alzheimer's disease. Dual-cholinergic inhibitors are appropriate for a mild stage of the disease where the K_m of ACh is low enough to keep BuChE inactivated.[26]

The present knowledge of the molecular configuration of the two enzymes would allow researchers to design compounds possessing well-balanced AChE-BuChE specificity, high central nerve system (CNS) penetration and low peripheral and central cholinergic toxicity. Some of the second generation cholinesterase inhibitors have demonstrated some of the advantages of these characteristics.[22] Comparing this study's findings with the current literature on approved cholinesterase inhibitors; piperine evidently inhibits both cholinergic enzymes and has an added affinity for BuChE, which is seemingly a pertinent characteristic to have for higher therapeutic efficiency.

Research findings have revealed piperine to exhibit a range of pharmacological effects, particularly with neurodegenerative diseases like Alzheimer's. A study by Chonpathompikunlert *et al.*[6] infused piperine at doses 5, 10, 20 mg/kg/body weight for 3 weeks bilaterally via intracerebro ventricular route as a bypassing strategy into the CNS. The findings were significantly improved spatial memory and neurodegeneration, however there was a lack of dose response relationship of piperine and AChE,

indicating the dose range was too high, therefore a saturation effect was observed. A more recent investigation into the antidepressant and cognitive effects of piperine-encapsulated liposomes (PL) employed a randomly assigned oral dose of 5 mg/kg/body weight or an intranasal administration of 48 ng/kg/body weight in male Wister rats for 14 days. [27]

The results showed a 60% efficiency of PL when administered intra-nasally, delivering piperine to the hippocampus at a faster rate and higher extent than oral does. Intranasal delivery of piperine from the liposomes could therefore reduce the dose of piperine intake while giving similar cognitive and antidepressant effects as the oral dosage.[27] The various routes of administration shown by these studies, has provided substantial research and findings toward the development and implementation of piperine as a drug.

CONCLUSION: Findings of this research have provided a preliminary insight into the mechanism of AChE and BuChE inhibition by piperine, concluding that it inhibits both cholinesterase enzymes and exhibits more selectivity toward BuChE. The study also demonstrates competitive inhibition for AChE and mixed-inhibition for BuChE. Compared to galanthamine (a mixed competitive non-competitive inhibitor), we conclude that although the AChE inhibition by piperine is not as potent as that of galanthamine, in addition to its known antioxidant and anti-inflammatory activities, piperine could provide a novel poly-pharmacological lead of potential benefit to the treatment of AD and therefore warrants further investigation. Further *in silico* docking studies and *in vivo* enzyme evaluation are therefore warranted in order to confirm our mechanistic studies and to determine the safe dose of which limitations were found [6] and whether intranasal or oral administration are a practical approach for human trials.

CONFLICT OF INTEREST: None

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