PRELIMINARY STUDY ON THE ACETYLCHOLINESTERASE INHIBITORY ACTIVITY OF *IPOMOEA MURICATA* (LINNAEUS) JACQUIN

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**ABSTRACT:** Acetylcholinesterase (AChE), a member of α/β hydrolase protein super family, is the key enzyme in the breakdown of acetylcholine (ACh) - an important neurotransmitter. Inhibition of this enzyme has been considered an auspicious strategy for the treatment of various neurological disorders such as Alzheimer’s disease (AD). In this study, crude methanolic *Ipomoea muricata* seed extract was subjected for analysis of its potential AChE inhibitory activity using a microplate assay based on Ellman’s method. *I. muricata*’s inhibitory activity was compared using Donepezil, a prescription drug for AD treatment. Two time intervals were used: 40 minutes and 24 hours. Based on the findings, Donepezil exhibited greater potency (IC50 > 0.005 μg/mL) at both time intervals in contrast to *I. muricata* (IC50 = 39.67 μg/mL) at 40 minutes and (IC50 > 57.44 μg/mL) at 24 hours. It is important to note that the crude methanolic seed extract of *I. muricata* has been shown to cause moderate but persistent inhibition of AChE in comparison to Donepezil which has shown a strong but short-lived action. With this concludes that in the near future followed by further studies, *I. muricata* can be a potential herbal alternative in the symptomatic treatment of AD.

**INTRODUCTION:** Alzheimer's disease (AD) is the major form of senile dementia, characterized by neuronal loss, extracellular deposits, and neurofibrillary tangles. It is the most common form of dementia which contributes to 60-70% of the cases with an estimated number of 35.6 million affected individuals worldwide according to the WHO 2012 report “Dementia: a public health priority”. These numbers are expected to double by 2030 and triple by 2050 with the greatest surges attributable to low and middle income countries. The disease is accompanied by a profound loss of cholinergic tone, and acetylcholine (ACh) levels in the brain, which were hypothesized to be responsible for the cognitive decline observed in AD. Also, acetylcholinesterase (AChE) activity is found to be enhanced around amyloid deposits (which are toxic to the brain) in the very early stages of amyloid deposition.

These events are then followed by the hydrolysis of the remaining ACh molecules in the synapse by AChE thereby impairing cholinergic neurotransmission. Since cholinergic function is required for short term memory, any cholinergic deficit in AD patients is believed to be responsible for much loss of intellectual abilities. Hence, blocking ACh degradation through the use of AChE may potentially reduce the severity of...
cognitive loss and slow down the progression of the disease.\(^3,4,5,6,7\)

Cholinesterase inhibitors are one of the classes of compounds to date that have consistently proven to be efficacious in managing the cognitive and functional symptoms of AD. Their administration often leads to the increased availability of ACh in the synaptic cleft leading to improved communication between nerve cells that utilize them as chemical messengers. This in turn improves cognitive performance and temporarily eases cognitive symptoms commonly associated with AD. Currently, three of these medications have been approved for the symptomatic treatment of AD namely Donepezil, Rivastigmine, and Galantamine.\(^8,9\)

Donepezil (Aricept) is an oral, highly selective, reversible acetylcholinesterase inhibitor (AChEI) that inaugurates a new class of AChEI’s with longer and more selective action. Administration results to increased ACh levels in the synapse leading to an improved cholinergic tone which in turn controls the symptoms associated with AD. However, as with any other AChEI that increase the levels of ACh in the brain, they can also increase ACh in the periphery causing potential side effects. Adverse events reported include nausea, vomiting, and diarrhea.\(^10\)

Taking into account these side effects, many research groups have averted their gaze into developing new, more effective, targeted therapeutics for the treatment of AD that have fewer adverse reactions. For this reason, the gain in popularity of harnessing naturally-occurring compounds from plants became undeniable in the recent years and not without a reason that there is a possibility to slow down the brain’s degeneration caused by AD.\(^5\) Several reviews regarding newly discovered AChEI’s found in plants, fungus, and marine organisms have been published throughout the years. Majority of these AChEIs belong to the alkaloidal group, including indole, isoquinoline, quinolizidine, piperidine, physostigmine and its analogs, xanthostigmine and its analogs, lycorine, carbazole, and steroidal alkaloids. On the other hand, potent non-alkaloidal AChEI’s have also been found from natural sources such as terpenoids, flavonoids, and other phenolic compounds.\(^11,12,13,14,15\)

Ipomoea muricata (Linn.) Jacquin of the family Convolvulaceae, locally known as “tonkin/tunkin”\(^16\), has various therapeutic applications such as being used as a purgative, febrifuge, spasmyloytic, hypotensive, antibacterial and antifungal agent.\(^17,18\) In addition, the Dominican parochial community has been known to exploit its seeds, stems, and leaves for the treatment of skin ailments for instance chronic and gangrenous wounds, cuts, and blisters due to burns.\(^19\) It is found effective in the treatment of pain, insect and snake bites, ulcer, tumor, and cancer.\(^20\)

I. muricata contains an array of biologically active compounds in which alkaloids comprise of (0.49%). For example, Lysergol, which constitutes ~53% of these alkaloids, has been demonstrated to have hypotensive, psychotropic, and uterus and intestine-stimulating properties.

Also, Chanoclavine, which comprises ~37% of these alkaloids, may have AChE inhibitory capability according to a literature but its mechanism still remains unclear.\(^18\) The seeds were reported to contain ethyl caffeate and muricatin that have antibacterial activity.\(^20\) The presence of ipomine, ipalbidine, ipalbine, and ipalbinum was also confirmed in which ipalbidine displayed analgesic activity.\(^22,23\) Lastly, caffeic acid which can be found in the seeds of I. muricata has been known to be hepatoprotective\(^24\) and is effective in scavenging 2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ABTS\(^{++}\), 1,1-diphenyl-2-picryl hydrazyl radical (DPPH\(^{+}\)), and superoxide anion radical. Alongside, I. muricata has high total reducing power and metal chelating activity on ferrous ions.\(^25\) Following this, a recent study showed that crude I. muricata extract bears strong antioxidant activity against DPPH\(^{+}\), nitric oxide, and lipid peroxides.\(^26\) The study aimed to investigate the probable AChE inhibition of the crude methanol seed extract of I. muricata Linn. Jacquin.

MATERIALS AND METHODS:
Plant Material: Seeds were given by the director of the Research Center for the Natural and Applied
Sciences (RCNAS), Prof. Mafel C. Ysrael Ph.D. for study. Seeds were collected from Bocaue, Bulacan, Philippines and were identified by botanist Ophelia S. Laurente M.Sc. of University of Santo Tomas, Research Center for the Natural and Applied Sciences, Manila, Philippines. A voucher specimen (USTH-012720) was deposited at the University of Santo Tomas Herbarium.

Chemicals and Reagents:
Analytical grade solvents were used in all of the experiments and were purchased from RCl Labscan Limited, Bangkok, Thailand. All standards and reagents used in the study were purchased from Sigma-Aldrich (Singapore) and Merck (Germany).

Extraction:
Two hundred grams of I. muricata seeds were made into a coarse powder and were later subjected to solvent extraction by adding 500mL methanol. It was then placed on an orbital shaker (J.P. Selecta Rotabit) at 135 opm for approximately 6-8 hours and was left standing at room temperature for a day. The extract was filtered using Whatman Filter Paper Grade no. 1 and concentrated by using a rotary evaporator (EYELA). The extraction was repeated thrice. The accumulated extract was further concentrated by placing it under a fume hood for a week. Extraction yield was 17.15%. The concentrate was then transferred in an amber bottle and stored at -20°C until use.

Standard Curve Preparation:
Analytical grade L-cysteine hydrochloride monohydrate was used as a standard. The reaction of 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB) with the liberated thiocholine from the hydrolysis of acetylthiocholine may be estimated in a sample by comparison to a standard curve comprising known concentrations of sulfhydryl-containing compounds such as cysteine. Stock standard solution were initially made by mixing 250μL of a standard concentration, 50μL of 3.96mg/mL DTNB, and 2.5mL of 0.1M sodium phosphate buffer pH=8.0 with 0.1mM ethylenediaminetetraacetic acid (EDTA) then was incubated at room temperature for 15 minutes. The hydrolysis process was confirmed by the formation of TNB from the interaction of DTNB with thiocholine, producing a yellow coloration. The rate of hydrolysis was monitored by Corona Electric SH1000 microplate reader at 412nm for 40 minutes with 2 minute intervals and at the 24th hour of the assay.

The experiment was done in triplicates to ensure the repeatability of the results. The median inhibitory concentration (IC₅₀) was determined by linear regression analysis.

Statistical Analysis:
One-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test was used to determine if there are significant differences between the concentrations of the crude methanolic seed extract of I. muricata in inhibiting AChE with p<0.05 being considered as significant. Graph Pad Prism software was used.

RESULTS AND DISCUSSION: AD is a neurological disorder characterized by the death of brain cells that causes memory loss and cognitive decline in affected individuals. Although the exact mechanism remains unknown, a complex interplay of genetics, environment and aging are likely the culprits in the course of disease progression. Given the enormity of this field, this research paper
mainly focuses on AChE and its inhibition and a slight deviation to oxidative stress, both shown involved in areas of study.

The crude methanolic extract from the seeds of *I. muricata* were analyzed together with 0.005μg/mL Donepezil, a potent reversible AChE inhibitor, as positive control in order to assess its stance in contrast to AD prescription drugs. The results were expressed in rate of TNB formation (mmol/min), percent inhibition (%) and as median inhibitory concentration (IC₅₀) values.

**Fig.1** shows the buildup in the production of TNB over time. A slower buildup of TNB signifies a slower hydrolysis of the substrate acetylthiocholine. The hydrolysis process generates thiocholine which reacts with DTNB to produce TNB, a colored product which can be detected at 412nm. The negative control which contains methanol as sample has shown the greatest surge in the rate of formation of TNB (0.0508mmol/min) representing uninhibited AChE activity. On the other hand, 0.005 μg/mL of Donepezil showed the least increase in TNB formation (0.0049mmol/min) demonstrating the greatest inhibition of AChE which was followed by 100μg/mL of the crude methanolic seed extract of *I. muricata* (0.0174mmol/min) with a concentration dependent inhibition.

In relation to the development of yellow color as a result of TNB creation as shown in Figure 1, a lighter colored well implies less TNB formed as a result of the slower hydrolysis of acetylthiocholine indicating AChE inhibition. As shown in **Fig.2**, Donepezil achieved close to colorless wells followed by the light tinges of yellow of the various concentrations of crude *I. muricata* extract.
This has shown that Donepezil prevented the formation of thiocholine (a product in the hydrolysis of acetylthiocholine) that reacts with DTNB to form TNB that produces the yellow coloration to a greater extent in contrast to the crude extract. On the other hand, the color of Donepezil wells became more intense in comparison to 100μg/mL of the extract that maintained its color at the 24th hour mark indicating the extract’s stability and AChE inhibitory persistence.

Fig. 3 shows the highest inhibitory activity recorded was that of Donepezil (positive control) having 90.36% inhibition at 40 minutes. It was subsequently followed by I. muricata at 100µg/mL having 65.62% inhibition with decreasing inhibitory activity as the concentration decreases having a concentration dependent behavior. The extract’s level of inhibition is considered mild in comparison to pure isolated AChE inhibitors. However, it is important to note that 100µg/mL of the extract maintained its inhibitory activity (64.94%) in a span of 24 hours compared to Donepezil which declined (58.17%) over the course of the assay. At the same time, concentrations ≤ 100μg/mL had decreased inhibitory activity presenting the same behavior as the positive control.

A lower IC$_{50}$ value means greater potency and better inhibition of the enzyme. Based on interpolated values: IC$_{50}$=39.67μg/mL (40 minutes) and IC$_{50}$ = 57.44μg/mL (24th hour) in comparison to Donepezil IC$_{50}$ < 0.005μg/mL at both time intervals, it has been shown unequivocally that Donepezil has higher potency and better AChEI activity. After all, the methanolic sample used is of crude nature and purification may most likely increase its effectiveness and strength.

One of the characteristic neuropathological alterations that occur in AD is the decrease of AChE activity in both cholinergic and noncholinergic neurons in the brain. Despite this loss of AChE activity in the brain, AChE activity is found to be enhanced around amyloid plaques, promoting the assembly of amyloid beta-peptides into fibrils and intensifying the cytotoxicity of these peptides. Accompanied by the decrease of ACh synthesis, cognitive impairment is further exacerbated by the hydrolysis of the remaining ACh in the synapse by the enzyme AChE. Hence, restoration of cholinergic function by inhibiting AChE to increase ACh levels in the synapse may potentially reduce the severity of cognitive loss and slow down the progression of the disease.

Current approved AChEIs for AD treatment increase ACh levels in the synapse which results to the temporary improvement of cognitive function while at the same time increase ACh levels in the periphery causing unwanted side effects that may develop to clinically significant problems in the long run. Consequences of AChEI administration has resulted to the need for the development of targeted effective therapeutics with fewer adverse reactions. Herbal medication has gained the attention of the scientific community as medicinal herbs contain various phytochemicals which may hold the answer for this overwhelming disease. In addition, these herbs are inexpensive and can easily be obtained which gives us the possibility to reduce the costs attributed to medications used for AD treatment.

Based on the findings, the crude methanolic seed extract of I. muricata has been shown to cause moderate but persistent inhibition of AChE in comparison to Donepezil which has shown strong but short-lived action. Chanoclavine which has been reported to be present in the seeds of I. muricata is suspected to be responsible for
inhibiting AChE. Several species in the *Ipomoea* family have been discovered to exhibit potent anticholinesterase activity *in vitro* such as the leaves of *Ipomoea asarifolia* and *Ipomoea aquatica* Forsk. In addition, isoquinoline alkaloids isolated from *Hippeastrum* species demonstrated significant AChE inhibitory activity which also opens the possibility of the active component in *I. muricata* being an alkaloid.

Admonishing the fact that the medium used in this study are seeds rather than using leaves makes *I. muricata* unique among its family. This may also explain why *I. muricata* presents this kind of inhibitory activity and may contain several similar to identical biochemicals responsible for these effects as the other species of *Ipomoea*. Despite all of this, the underlying mechanism on how the plant executes its AChE inhibitory activity *in vivo* as well as the responsible biological components remains unclear and need further studies. Overall, the crude methanolic seed extract of *I. muricata* should be considered for its application in the prevention and management of neurodegenerative disorders such as AD.

**CONCLUSION:** *I. muricata*’s moderate but persistent natural inhibitory activity implies that it can be a possible natural alternative source for an AChE inhibitor even though it is less potent compared to Donepezil that has intermittent action. Then again, it is imperative to consider that the methanolic extract used is crude which when purified, is expected to display higher inhibitory activity and better potency. Further studies regarding the responsible active component that inhibits AChE, the type of interaction that exists between the inhibitor and the enzyme, the use of brain tissue homogenates and whether it can pass through the blood brain barrier are recommended. With this concludes that in the near future *I. muricata* can be used as a potential herbal alternative in the symptomatic treatment of AD.

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