Original Article

In vitro Acetylcholinesterase Inhibitory Activity of Different Capsicum Varieties by using Chicken Brain Extract Prepared by Employing a Home Mixer-jar as an Alternative to Expensive Tissue Homogenizer

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ABSTRACT

Objectives: The present study aimed to investigate acetylcholinesterase (AchE) inhibitory activity of methanolic extract of different capsicum varieties using isolated chicken's brain homogenate as an alternative for rodent's brain homogenate by Ellman's *in vitro* assay. In this study, we used a home mixer-jar as a substitute for the expensive tissue homogenizer to mince the chicken brain along with conventional hand motor-pestle homogenization technique. **Materials and Methods:** This *in vitro* AchE inhibition assay was performed as described by Ellman *et al*. (1961) with minor modifications. **Results:** The IC_{50} values in ng/ml of the reference drug, neostigmine were found to be 149.92 ± 37.72 and 347.22 ± 81.50 using mortar and pestle (MP), and mixer-jar (MJ) method of tissue homogenization techniques, respectively. The IC_{50} values in μg/ml of the methanolic extract of different varieties of capsicum were found to be: red chilli by MP method 1786.1 ± 118.52; by MJ method 1858.5 ± 136.79, green chilli by MP method 1418.8 ± 94.16; by MJ method 1786.1 ± 118.52and green capsicum (bell pepper) by MP method 2022.8 ± 244.5; by MJ method1813.3 ± 45.63, respectively. Furthermore, it was noted that the yield of AchE in brain extract prepared using a mixer jar was high when compared with the brain homogenate prepared using the mortar and pestle. **Conclusion:** The use of chicken brain and employing mixer-jar for brain homogenization are evolved as good alternatives for unnecessary killing of laboratory animals and for the brain tissue homogenization. All capsicum varieties (green chilli, red chilli, and green capsicum) showed AchE inhibitory effect in *in vitro* Ellman's assay.

Keywords: Chicken brain, Mortar and pestle, Mixer jar, Neostigmine, Capsicum extracts, Acetylcholinesterase, Ellman's assay.

INTRODUCTION

Memory is one of the vital functions of the brain and involves multiple neuronal pathways and neuronal systems. Age, stress and emotions are the reasons that may lead to memory loss and often causing the threats like Alzheimer's disease, schizophrenia and dementia etc. Acetylcholinesterase (AchE) is a biologically important enzyme that hydrolyses acetylcholine thereby terminating the cholinergic neurotransmission.[1] The cholinergic neurotransmission in brain plays a vital role in the process of cognitive function. One of the important approaches to overcome the memory decline due to Alzheimer's disease involves the enhancement of acetylcholine level in the brain using AchE inhibitors.[1] Nature provides wonderful remedies to treat many disease conditions including memory decline or amnesia. Traditionally, a number of herbs have been employed in Ayurveda system of medicine to treat cognitive dysfunction that have yielded the positive and promising results. Certain reports have claimed that capsicum extracts enhanced learning and memory function by AchE inhibition.[2-3]

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Development of alternative model to effectively replace the use of laboratory animals in preclinical research is long standing demand of animal welfare communities. Scientists across globe are consistently working to develop an alternative to animal testing. *In vitro* method of animal testing is an excellent education and research tool that can extensively reduce number of laboratory animals. However, killing of laboratory animals just for collecting a piece of tissue for *in vitro* or *ex vivo* studies are not acceptable. Worldwide, nearly 50-100 million animals were used for the experimental purposes.[4] Rats, mice, guinea pigs, rabbits and hamsters are widely used laboratory animals in preclinical research and they are killed to extract various tissues to conduct *in vitro* or *ex vivo* assays as there are no judicial restrictions for the use of these animals, thereby, our ecosystem has been affected to a greater extent. The committee for the purpose of control and supervision of experimentation on animals (CPCSEA) of India set proper guidelines for the maintenance of laboratory animal facility along with humane care of laboratory animals used in research. The CPCSEA also recommends alternatives for use of laboratory animals in preclinical research.

Chicken, sheep and fish are commonly killed species for the procurement of meat worldwide. The freshly collected tissues from these species when they are killed as per the government norms are better option for conducting *in vitro* and *ex vivo* assays. In view of this option, we used chicken's brain which is a waste product easily available from slaughter houses instead of rodent's brain to study AchE inhibitory activity of various capsicum varieties in which neostigmine was used as a reference drug.

Generally, the tissue homogenizer is used to mince the tissue which is not affordable to many researchers from middle income countries. In this study, we introduced a new technique of brain tissue homogenization for the first time i.e., affordable tissue homogenizer (home mixer-jar). The present *in vitro* investigation aims to evaluate and compare the AchE inhibitory activity among different varieties of capsicum (*Capsicum annum*) by using isolated chicken brain. In addition, the different tissue homogenization techniques such as manual homogenization (by motor and pestle) and electrical homogenization (by home mixer-jar) were compared to propose home mixer-jars an alternative to expensive homogenizers for the tissue homogenization.

MATERIALS AND METHODS

Drugs and Chemicals

DTNB (Dithiobisnitrobenzoicacid) and acetylthiocholine iodide were obtained from Sigma-Aldrich, India. Neostigmine methyl sulphate injection (Myostigmin®, NEON Lab Ltd., Palghar, India), potassium dihydrogen phosphate, and sodium hydroxide were purchased from local suppliers and all the other chemicals used were of analytical grade.

Plant Material

Three different varieties of capsicum (red chilli, green chilli and capsicum) were collected from a local market and were authenticated by a botanist Dr. Suguna Kumari, Department of Botany, BHHS Girl's Junior College, Guntur and a voucher specimen was deposited in Chalapathi Institute of Pharmaceutical Sciences, Guntur for future reference. The collected capsicum samples were washed in running water and later cut into small pieces and kept for shade drying at room temperature of 27-35°C for 2 weeks.

Preparation of Capsicum extracts

The different capsicum varieties (red chilli, green chilli and capsicum) were size reduced and grinded into coarse particles using mechanical grinder. The grinded coarse material of different varieties of capsicum was subjected for further methanolic extraction as (a). Red chilli methanolic extract: 46g of red chilli powder was taken and macerated in 450 ml of methanol for 72 hr with occasional stirring, (b). Green chilli methanolic extract: 87g of green chilli powder was weighed and macerated in 960ml of methanol for 72 hr with occasional stirring and (c) Capsicum (green) methanolic extract: 38g of capsicum dried powder was weighed and macerated in 450 ml of methanol for 72 hr with occasional stirring. After maceration, the resultant solution was filtered using muslin cloth and the filtrate was kept for evaporation under Rotary-evaporator at 100rpm for 30 min. Later, the concentrated product obtained from Rotary-evaporator was kept in fume-hood at room temperature for further evaporation. The total weight of the concentrated product was noted and the percentage yield of the methanolic extracts of three different varieties of capsicum were calculated as: the percentage yield of red chilli extract-17.8% w/w; the percentage yield of green chilli extract- 8.9% w/w; and the percentage yield of capsicum (green)- 10% w/w.

Phytochemical screening

The methanolic extracts of 3 varieties of capsicum were screened for different secondary metabolites like carbohydrates, proteins, glycosides, tannins, flavonoids, steroids and alkaloids,[5] and found that all the three variants of capsicum were tested positive for carbohydrates (Molisch's test, Benedict's test, Fehling's

test, and Barfoed's test), alkaloids (Mayer's test, and Wagner's test) and tannins (Bromine water test).

Animals and tissues

Chickens (*Gallus domesticus*) head samples were freshly obtained from a slaughter house. The collected head samples of chickens were immediately wrapped in an aluminium foil and transferred to the pharmacology research laboratory in a chiller box by maintaining the ice- cold condition. Then the whole brains were immediately dissected out from the chicken heads under ice cold condition.

Brain AchE Enzyme Preparation

The dissected brains were weighed and suspended in ice cold 0.1M phosphate buffer (pH 7.2) at g/10ml and homogenized using two different techniques (a) Mortar and pestle and (b) Mixer-jar, respectively. The brain tissue homogenates were then centrifuged (5000g/30min/5°C) by using a cooling centrifuge (Model No.: C 24 B; REMI, India) and the resulting supernatant solution was collected and stored at-20°C till use.

In vitro AchE enzyme inhibitory activity using chicken brain

AchE inhibitory activity of methanolic extract of different capsicum varieties (red chilli, green chilli and green capsicum) was measured by following the method proposed by Ellman *et al.* (1961),[6] with slight modifications. Acetylthiocholine iodide was used as a substrate and DTNB was used as a chromogenic agent for the measurement of acetylcholinesterase activity and the chicken brain homogenate was used as the source of AchE enzyme.

Assay procedure

Various concentrations of the reference drug, neostigmine (0.0005, 0.005, 0.05, 0.5 and 5µg/ml) was prepared in distilled water. A stock solution of different varieties of capsicum extracts was freshly prepared in methanol at a concentration of 10 mg/ml. From the stock solutions of different capsicum extracts, various concentrations (1, 10, 100, 1000 and 2000µg/ml) of working solutions using distilled water by serial dilution were prepared. In a UV cuvette, 50µL of the chicken brain homogenate was taken and 100µL of distilled water/ neostigmine or capsicum extract was added to which 10µL of acetylthiocholine iodide and 100µL of DTNB reagent were added. Finally, 3 ml of phosphate buffer (pH 7.2) was added to the cuvette and incubated at 28°C for 5min. For blank, the cuvette was added with substrate, DTNB reagent, phosphate buffer and water. The UV absorbance values were noted at 412 nm by using UV/VIS-Spectrophotometer (Model No. UV 3092, LABINDIA, India).

The percentage inhibition of AchE was calculated using the formula:

$$\% \text{ Inhibition} = \left[\frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}} \right] \times 100$$

Data analysis

All tests were carried out in triplicate and the data are expressed as mean ± SEM. The inhibitory concentration by 50% (IC_{50}) was graphically determined by using graphPad Prism (ver.5.0) software.

RESULTS

In vitro AchE enzyme inhibition assay

In vitro AchE inhibition assay was performed by following Ellman's method,[6] using chicken brain extracts prepared by different homogenization techniques using mortar and pestle (MP) and mixer-jar (MJ), respectively. The control UV absorbance of different homogenization techniques using MP and MJ revealed a significant (p <0.001) higher UV absorbance for MJ when compared with MP which implies the presence of high amount of AchE in MJ method (Figure 1).

The methanolic extracts of different capsicum varieties (red chilli, green chilli and green capsicum) dose-dependently inhibited AchE enzyme activity using chicken brain homogenates (MP and MJ) by Ellman's assay which was similar to the AchE inhibition by the reference drug, neostigmine as plotted in Figure 2(a-d). The IC_{50} values of capsicum methanolic extracts

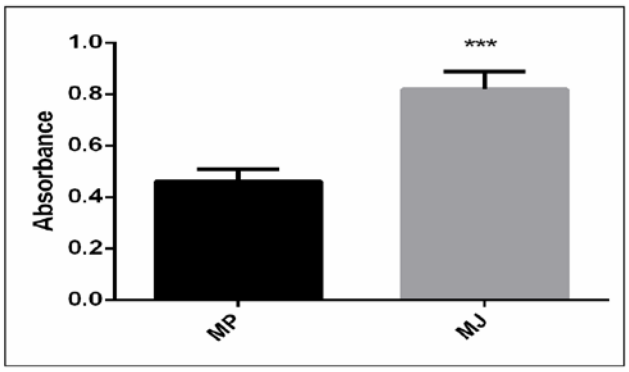

Figure 1: Comparison of UV absorbance at 412nm of chicken brain AchE enzyme preparations obtained by mortar and pestle (MP) and mixer-jar (MJ) homogenization techniques using Ellman's assay.

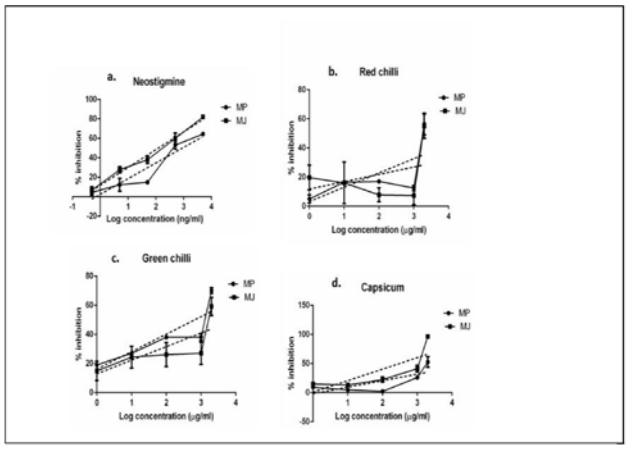

Figure 2: AchE enzyme inhibitory activity of (a) neostigmine (b) red chilli (c) green chilli and (d) green capsicum using chicken brain homogenates obtained by mortar and pestle (MP) and mixer-jar (MJ) in Ellman's assay.

Table 1: The IC_{50} values of the different capsicum methanolic extracts and neostigmine against AchE using chicken brain homogenates prepared by mortar and pestle (MP) and mixer-jar (MJ) in Ellman's assay.

Treatment	IC_{50} (values in Mean ± SEM of triplicates)	
	MP brain homogenization	MJ brain homogenization
Neostigmine (ng/ml)	149.92±37.72	347.22±81.50
Red chilli (µg/ml)	1786.14±118.52***	1858.47±136.79****
Green chilli (µg/ml)	1418.78±94.15***	1786.14±118.52****
Green capsicum (µg/ml)	2022.80±244.5****	1813.28±45.63****

One-way ANOVA followed by *post hoc* Tukey's multiple comparison test revealed a significant difference was observed at ***p<0.001 and ****p<0.0001 when compared with neostigmine. However, IC_{50} values of different capsicum extracts were not significantly altered.

(red chilli, green chilli and green capsicum) along with neostigmine against AchE by Ellman's assay using chicken brain homogenates (MP and MJ) were determined as shown in Table 1.

DISCUSSION

AChE is a specific metabolic enzyme, serine hydrolase which rapidly hydrolyses ACh to choline and acetate at a rate of approximately 25000 ACh molecules/second.[7] ACh is not only a neurotransmitter in autonomic nervous system but also in central nervous system (CNS) as present in short interneuron and long cholinergic innervation from the basal forebrain to the neocortex and related limbic structures.[7] ACh plays a vital role in the processing of various cognitive functions such as attention, working memory, spatial memory, and episodic memory encoding.[8] Loss of cholinergic neurons in the brain thereby decrease in ACh can lead to cognitive dysfunction as seen with Alzheimer's disease (AD). Reversible AchE inhibitors such as donepezil, rivastigmine and galantamine (FDA approved) are known to improve cognitive function without halting AD progression. Researchers are continuously working to find novel AchE inhibitors with halting AD progression. *In vitro* Ellman's assay,[6] was extensively used for decades to screen AChE inhibitory activity of new chemical entities (NCEs) using rodent's brain. Many rodents were killed just for collecting the brain tissue to perform this *in vitro* assay. In the present study, we made an attempt to use the chicken brain obtained from slaughter house instead of rodent brain thereby adhering to one of the 3Rs principles of humane use of animals in scientific research (i.e. replacing the use of animals with alternatives). In present study, AchE inhibitory effect (IC_{50} value) of neostigmine methyl sulphate was found to be 150-350 ng/ml. In earlier study,[9] using electric eel, AchE inhibitory effect of neostigmine bromide (IC_{50} value) was found to be 11.3 nM (equivalent to 3.43 ng/ml). This study also tried with a new method of tissue homogenization (i.e. using mixer-jar) as an alternative to the expensive tissue homogenizers and found that AchE enzymatic activity was higher in mixer-jar method when compared with conventional mortar-pestle method of brain tissue homogenization based on higher UV absorbance value (Figure 1) at 412 nm in *in vitro* Ellman's assay using chicken brain.

In present study, AchE inhibitory activity of different capsicum extracts (methanolic extract of red chilli, green chilli and green capsicum) was assessed and found that AchE inhibition was observed with all capsicum varieties equally in both tissue homogenization techniques (MP and MJ) using chicken brain by Ellman's method as depicted in Table 1. In earlier study by Sugunama *et al.* (1999) reported that the senescence-accelerated mouse (SAM) fed with dietary red bell pepper (*Capsicum annuum* L.) exhibited an improved learning performance in passive avoidance tasks.[10] This earlier study also highlighted the enhancement of choline acetyl transferase (ChAT) activity in the parietal cortex that led to pronounced cholinergic activity in red bell pepper-dieted SAM, thereby, it improved the cognitive function.[10] Therefore, it is postulated that capsicum extracts exhibited learning and memory enhancing activity by inhibition of AchE and enhancement of ChAT activity. Further studies are required to identify the bioactive principles of capsicum that could be responsible for its memory enhancing activity.

CONCLUSION

Based on present findings, it is suggested that the chicken brain which is available as a waste product in slaughter houses as a good alternative for conducting *in vitro* Ellman's assay instead of killing the laboratory rodents like mice, rats and Guinea pigs. This study also highlighted the use of home mixer-jar for brain tissue homogenization as an alternative for expensive tissue homogenizer which is not affordable to all. Finally, the different varieties of capsicum (red chilli, green chilli and green capsicum) showed AchE inhibitory effect in *in vitro* Ellman's assay using chicken brain and thereby it suggests that different capsicum varieties could be used as promising memory boosters from the nature.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

Ach: Acetylcholine; **AchE:** Acetylcholinesterase; **AD:** Alzheimer's disease; **ANOVA:** Analysis of variance; **ChAT:** Choline acetyl transferase; **CNS:** Central nervous system; **CPCSEA:** The committee for the purpose of control and supervision of experimentation on animals; **DTNB:** Dithiobisnitrobenzoicacid; **FDA:** The Food and Drug Administration; **IC_{50}:** The half maximal inhibitory concentration; **MJ:** Mixer-jar; **MP:** Mortar and pestle; **NCEs:** New chemical entities; **SAM:** The senescence-accelerated mouse; **UV:** Ultraviolet.

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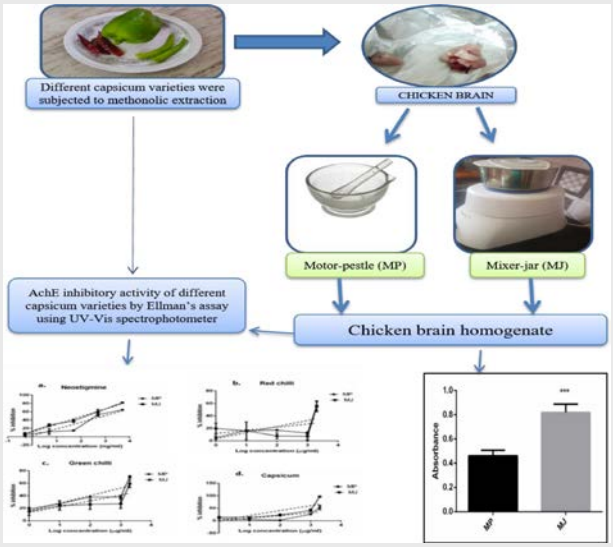
PICTORIAL ABSTRACT



SUMMARY

- In this study, the methanolic extract of different capsicum varieties (red chilli, green chilli and capsicum) were evaluated for acetylcholinesterase (AchE) inhibitory activity using isolated chicken's brain homogenate as an alternative for rodent's brain homogenate.
- A home mixer-jar was used to mince the chicken brain as a substitute for the expensive tissue homogenizer along with conventional hand motor-pestle homogenization.
- *In vitro* Ellman's assay revealed all capsicum varieties showed AchE inhibitory effect.
- The use of chicken brain and employing mixer-jar for brain homogenization are good alternatives for performing conventional *in vitro* Ellman's assay.

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