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Acetylcholinesterase inhibitory potential of *Bacopa monnieri* and acephate in heart of chick

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Abstract
The role of acetyl cholinesterase is the termination of nerve impulse transmission at the cholinergic synapses by rapid hydrolysis of acetylcholine. Hence inhibition of AChE serves as a reliable strategy for the treatment of Alzheimer's disease, in which acetylcholine is required to linger on in synapse.
Indian Medicine has a long history in treatment of disorders mostly by using medicinal plants. Since, acetylcholinesterase inhibitors (AChEIs) are the usual drugs for Alzheimer's disease; therefore, in order to find new AChEIs, the plants used in are good candidates. In the present investigation, AChEI activity [1] and AChE inhibitory kinetics of *Bacopa monnieri* and organophosphate pesticide (OP) Acephate in the heart of chick has been investigated**.** Chicks were dosed daily with ethanolic extract of *Bacopa monnieri* 100mg/kg b. w. for one week and acephate (34 mg/kg/b. w.) in ground nut oil for 4 days. Our results indicate significant AChE inhibition by acephate (55.8%) and *Bacopa monnieri (*31.6%). Our enzyme kinetic study showed that the acephate and *Bacopa monnieri* both yield competitive AChE inhibition. Acephate caused severe cardiotoxicity evidenced by damage of cardiomyocytes, necrosis and pyknosis in the heart muscles. However, *Bacopa monnieri* treated heart exhibited almost normal architecture, barring a little pyknosis, enlargement of nuclei and spaces among the cardiomyocytes fibers.

Keywords: Acetylcholinesterase Inhibition, Acephate, *Bacopa monnieri*, Chick, Heart, Kinetics.

1. Introduction
Cholinesterase inhibitors (ChE-I's) are commonly used as the therapy of AD (Alzheimer's disease) associated disorders and are the only class of permitted drugs by the Food and Drug Administration (FDA). In addition, acetylcholinesterase (AChE) is the target for many Alzheimer's dementia drugs which block the function of AChE but develop some side effects. Cholinesterase inhibitors are the only approved drugs for treating AD patients includes donepezil, galantamine and rivastigmine, which have limited effectiveness and some kind of side effect [2]. There has been worry among physicians regarding the potential for cardiac adverse effects related with acetylcholinesterase inhibitors in Alzheimer's disease. There is no agreement on how to manage this cardiovascular risk. Published information reveals that the incidence of cardiovascular side-effects is small and that serious adverse events are exceptional [3].
Aging is a major risk factor for dementia, which includes various types, such as vascular dementia, fronto temporal degenerative dementia, Lewy body dementia and Alzheimer disease. Dementia results secondarily from many neurodegenerative disorders appear in advancing aged people over 65 years [4-5]. In addition loss of memory and impairment of emotional functions are common symptoms of this disease [6]. The exact etiology of Alzheimer dementia is uncertain and controversial, but there is a general consensus about some of the factors that may be involved. It has been widely accepted that the occurrence of AD is based on loss of acetylcholine (ACh) synthesis in patients with AD [7]. Therefore, use of acetylcholinesterase inhibitors (AChEIs) to block the degradation of ACh is a rational approach that would lead to the accumulation of ACh in the synaptic cleft for transmission of nerve impulses [8]. Efforts are being made in search of new molecules with anti-AChE activity. The fact that naturally-occurring compounds from plants are considered to be a potential source of new inhibitors has led to the discovery of an important number of secondary metabolites and plant extracts with the ability of inhibiting the enzyme AChE [9-10]. A variety of plants have been reported to have significant AChE inhibitory activity [11]. Numerous phytoconstituents and promising plant species have been tested for their AChE inhibitory activity [12].
Indian herb Brahmi (*Bacopa monnieri*) is commonly used in Ayurvedic system of medicine for centuries. It is nervine tonic and memory enhancing herb.

The plant Bacopa is used as anxiolytic, relaxing, bronchodilatory, cognition enhancing antioxidant, anticancer, anti depressant, immune-modulating and anti inflammatory [13-14]. It contains different types of steroidal saponin, notably bacosides A and B, which were found to facilitate the capacity of mental retention [15].

However, Acephate is organophosphorus pesticide widely used in agriculture and veterinary practice. The effort has been made to establish the scientific validity to investigate and screening for acetyl cholinesterase inhibitory activity of ethanolic extract of *Bacopa monnieri* and compared to Acephate in the heart of chick.

2. Materials and Methods

2.1. Animals: 2 weeks old healthy chicks weighing 100-150 g were obtained from local poultry farm. 5 chicks were housed separately in polypropylene cages maintained under standardized condition (12 hours, light/dark cycles at 24 ˚C and 65% humidity) and provided free access to commercial food and clean drinking water ad libitum. Ethical clearance for the use of animals was obtained from the Institutional Animal Ethics Committee (IAEC).

2.2. Chemicals: Acetylthiocholine iodide (ATCI) (Himedia), 5, 5 Dithio-bis-2-nitrobenzoic acid (DTNB) (Sysco research laboratories Pvt. Ltd.), and other chemicals used were of analytical grade and Acephate (O-S Dimethyl acetylphosphor-dithiote 75% purity) was of technical grad.

2.3. Preparation of Stock Solution of Acephate: Fresh stock solution of acephate (10 mg/ml) was prepared in ground nut oil. This was orally administered to the experimental animals by canula.

2.4. Plant material and preparation of extract: Fresh Bacopa *monnieri* was procured from Jawaharlal Nehru Agricultural University, Jabalpur (M.P.), India. Fresh leaves and twigs were collected, authenticated and thoroughly washed in water. Shed dried leaves and twigs were grounded to powder. The powder obtained was extracted with ethanol (90%; 70-80˚C) in a Soxhlet apparatus. The residue obtained after removing the solvent was dried to semisolid mass and stored for further use.

2.5. Experimental design: Chicks were divided into 4 groups of 5 animals each. Group-I animals served as control and received 1ml/kg b.w. ground nut oil orally daily for 4 days. Group-II animals constituted intoxicated chicks and received 34 mg/kg b. w. oral dose of acephate (1/25thdose of LD_{50} of Acephate) daily for 4 days in ground nut oil through canula. The feeding was stopped before 12 hours of intoxication. Group-III animals were the herb-treated animals and received oral administration of 100 mg/kg b. w. ethanolic extract of *Bacopa monnieri* in double distilled water daily for 7 days. Group-IV animals were pretreated with oral dose of 100 mg/1kg b.w. ethanolic extract of *Bacopa monnieri* in double distilled water for 7 days followed by oral intubation of 34 mg/kg b. w. acephate daily for 4 days.

Animals were kept starved overnight on the 4th day. On the next day they were euthanized with Di-ethyl ether and the abdominal cavity was surgically opened, Hearts were removed, washed in ice cold 0.9% saline and blotted dry and 10% (w /v) homogenates were prepared in chilled 0.1 M phosphate buffer (pH 7.4) using Potter – Elvehjen type homogenizer followed by centrifugation at 5000 rpm at 4˚C for 10 minutes. Homogenates were kept in deep freeze for analysis of AChE activity, AChE inhibition, enzyme kinetic assays and Histology.

2.6. AChE Enzyme Assay: AChE activity was assayed spectrophotometrically in the heart homogenates using acetylthiocholin iodide (ATChI) as the substrate and 5'5'-dithiobis nitrobenzoic acid (DTNB) as the chromogen [1]. Homogenates were diluted with 2.6 ml 0.1M sodium phosphate buffer (pH 7.4) to which 100 mM DTNB and 75 mM ATChI was added. The rate of colour production was measured at 412 nm in SL 164 UV-VIS spectrophotometer. All measurements were done in duplicate. The AChE specific activity was expressed in µmoles of ATChI hydrolyzed/min./mg protein. AChE Percentage inhibition was calculated using the equation:

$$\text{Percentage inhibition} = \frac{(\text{Control} - \text{Extract})}{\text{Control}} \times 100$$

2.7. Enzyme kinetics: Lineweaver-Burk plots were drawn from assays using acephate (37.8 mg/kg) and *Bacopa monnieri extract* (100 mg/kg) at various substrate concentrations (0.66 mM, 0.44 mM, 0.33 mM, 0.26 mM ATChI). From these plots kinetic parameters (Km &Vmax) were determined for each assay by plotting the reciprocals of velocity and substrate concentrations.

2.8. Determination of Protein: Protein was determined according to Lowry *et al.* [16] using Bovine serum albumin as the standard. The tubes containing homogenates were kept at room temperature for 20 min. then 0.5 ml of Folin-phenol reagent was added and the colour after 30 min. read at 620 nm against the reagent blank. Final concentration of total proteins was expressed as milligrams per gram of wet weight of tissue.

2.9. Histopathological studies: Heart tissues from each group of chicks were fixed in aqueous Bouin's fluid, dehydrated and embedded in paraffin wax. Serial sections were cut at 5µ and stained with Haematoxylin and Eosin. The sections were micro photographed at 100X with computer-aided Leica microscope.

2.10. Statistical analysis: For the data of statistical comparison between different treatments and control groups, data were analyzed by Student's t-test to determine the effect of the treatment. The level for the accepted statistical significance was $P>0.05$.

3. Results

3.1. AChE Inhibition in the Heart of Chick: In group I (control) the AChE activity in chick heart was observed 18.9 µM. However, in group II animals' 34 mg/kg b. w. of acephate elicited 55.8% AChE inhibition. While, in group III animals, treatment of 100 mg/kg b. w. of ethanolic extract of *Bacopa monnieri* for 7 days resulted into 31.6% AChE inhibition. We find notable increase in heart AChE inhibition in the group IV of chicks those were pretreated with 100 mg/kg b.w. ethanolic extract of *Bacopa monnieri* for 7 days followed by intubation of 34 mg/kg b. w. acephate for 4 days. There was 91.38% inhibition observed in AChE activity in heart of animals of this group (Table -1).

3.2. AChE Inhibitory kinetics: AChE inhibition kinetics in heart of chicks due to acephat, ethanol extract *of Bacopa monnieri* and combined treatment were determined by plotting Lineweaver-Burk plots (Fig. 1). Km of heart AChE of group I (control) was 0.83 X 10^{-3}M, which was increased to 1.53 X 10^{-3}M in group II (acephate treated). Group III (Bacopa extract treated) the Km rise to 1.08 X 10^{-3}M from 0.83 X 10^{-3}M (group I control). Group IV (pretreatment of Bacopa extract followed by acephate) also showed elevation in Km to 1.29 X 10^{-3}M against control Km value. Vmax values of all groups did not change from the Vmax of control and remained same i.e. 0.2 A /mg protein/ min. (table-1 and figure 1). The *K*m and Vmax values were derived from the trend line equation of the graphs. The acephate and *Bacopa monnieri* both showed competitive AChE inhibition. This is evident from the series of lines crossing the y (1/v) axis at the same point - i.e. *V*max is unchanged, but with a decreasing value of 1/*K*m (and hence a higher *K*m) in the presence of the inhibitor. The result as shown in Table -1 revealed that ethanol extract of *Bacopa monnieri* followed by oral dose of acephate to chicks caused remarkable increase in *K*m in chick heart (Fig.1).

3.3. Histopathological observation: Histopathological study of heart from group I animals showed a normal cardiac architecture (Fig. 2-A). In acephate treated group II severe cardiotoxicity was evidenced by damage of cardiomyocytes, necrosis and pyknosis in the heart muscles. Small spaces were also observed in the chick heart of this group (Fig.2-B). In group III animals treated with ethanolic extract of *Bacopa monnieri*, the heart exhibited almost normal architecture, barring a little pyknosis, slight enlargement of nuclei and spaces among the cardiomyocytes fibers (Fig.2-C). In group IV animals several changes were observed, which include little damage in cardiomyocytes fibers, fiber necrosis and the enlargement in few nuclei in the heart of chick (Fig. 2-D).

4. Discussion

Medicinal plants or their derived compounds such as *Gingko biloba* and Huperzine A are presently undergoing clinical trials for the symptomatic treatment of dementia and Alzheimer's disease [17]. The present investigation was undertaken to examine the effect of oral administration of an organophosphorus pesticide acephate, ethanolic extract of *Bacopa monnieri* and their combined effect on AChE activity, AChE kinetics and histology of the heart of chick. We have observed significant 55% and 31% acetylcholinesterase inhibition in the heart of chick due to acephate and ethanolic extract of *Bacopa monnieri* respectively. Our findings evidenced neurotoxic potential of acephate and *Bacopa monnieri* by inhibition of AChE activity in the heart of chick. It was interesting to note that the pretreatment of *Bacopa monnieri* followed by acephate dose to chick intensified the AChE inhibition. Ethanolic extract of *Bacopa monnieri* contained some active compounds that exhibited AChE inhibition [18]. However, this result might be due to synergistic effect of many compound present in this compound. Our results are in conformity with the result of Duysen *et al*. [19]. In spite of great potential of *Bacopa monnieri* in the treatment of neurological disease, effect of the plant on AChE has not been investigated widely except few investigators screened 27 crude methanolic extracts belonging to the Asteraceae, Euphorbiaceae, Melastomataceae, Rubiaceae, and Solanaceae families for their AChE inhibitory activities [10, 20].

In another recent study conducted in animal model, Bacopa has been shown to decrease whole brain AChE activity which reflects that Bacopa might prove to be a useful memory restorative agent in the treatment of Alzheimer's and dementia [21]. Strongest AChE inhibitory activities has been exhibited by the methanolic extract of *Solanum leucocarpum, Dunal*, *Sinforosi, Solanum* species and Witheringia species. Limpeanchob *et al*. [22] found *Bacopa monnieri* to protect against β-amyloid-induced (but not glutamate-induced) neurotoxicity in rat cortical neurons. The mechanism was attributed to greater anti-oxidant activity and AChE inhibition in the BM group (0–500 μg extract verified to contain 5.045%±0.400 bacoside A_3, bacopaside II, bacop asaponin C isomer, and bacopa saponin C). The inhibitory AChE activities could be attributed to their high alkaloid contents [23]. We have also determined toxicity of acephate and *Bacopa monnieri* on AChE kinetics of heart of chick. Our results indicate a significant increase in Km values of Heart due to exposure of acephate and *Bacopa monnieri*. We have calculated Km by applying data to Lineweaver-Burk plots. The increase in the Km in presence of pesticide indicated inhibition in enzyme activity [24-25]. We have observed different range in Km value with treatment of acephate to chick. We found increase in the Km from the control chick Heart (0.83 X 10^{-3}M). Our findings revealed that acephate treatment did not change maximum velocity (Vmax) and remained unchanged from the control animals. However, there were no inhibition kinetic data available in the literature using similar tissue preparation. The Km of AChE of rat, pigeon and fish was increased in presence of increasing monocrotophos (MCP) concentration, relatively [26].

Our results are also consistent with the finding of other co-worker who have reported that some of the organophosphate pesticide comply with the substrate of AChE to be similar in structure. It is clearly indicated that acephate and *Bacopa monnieri* are reversible inhibitor of AChE of heart of chick. Although the kinetic study of enzymes have been considered as one of the important tools for diagnosing the effect of various compound on rate of reactions. Now-a-days various herbal extracts are being investigated to asses for their pharmacological potencies [27-28].

In this study we have shown for the first time that ethanolic extract of *Bacopa monnieri* exert its inhibitory effect on AChE kinetics in a competitive manner therefore, it is suggested that it may be used in pharmacology for the purpose of synthesis of drug for Alzheimer disease. The results of the present investigation also revealed changes in the histological structure of the heart due to acephate treatment to chick. This was represented by the damage in cardiomyocyte, necrosis in the heart muscles, pyknosis and edema. These results were in agreement with the findings of [29]. Our results clearly showed that *Bacopa monnieri* exert slight changes in the heart of chicks, like pyknosis enlargement of nuclei and spaces among the cardiomyocytes fibers. Dapar *et al*. [27] observed histopathological effect of *Securidaca longepedunculata* on the heart of rats. They reported mild wavy contraction bands unremarkable of any significant changes in the histology of myocardium.

5. Conclusion

Bacopa monnieri and acephate were screened and compared for inhibitory activity on AChE in the heart of chick. The results show that extracts from the aerial parts of *Bacopa monnieri* could inhibit the activity of AChE. Our study suggests that *Bacopa monnieri* may be considered as an important target for isolation and characterization of phyto

compounds responsible for its anticholinesterase biological activity. The results show that these plants could be very interesting for further isolation of acetylcholinesterase inhibitors, which are widely used in the treatment of Alzheimer's disease. Therefore, further work is required to confirm that this medicinal plant has a high potential for the discovery of new and valuable compound to their pharmaceutical application.

Table 1: Acetylcholinesterase activity (Activity/mg protein/min.), inhibition, Km x 10^{-3} M and Vmax of AChE of Heart of Chick treated with sub lethal dose of Acephate (34 mg/kg b. w.), *Bacopa monnieri* (100 mg/kg b. w.) and pretreatment of *Bacopa monnieri* (100 mg/kg b. w.) followed by Acephate (34 mg/kg b. w.). The AChE specific activity is expressed in µ moles of ATCI hydrolysed / mg protein /min.

Parameters	Control	Acephate (34 mg/kg b.w.)	*Bacopa monnieri* (100 mg/kg b.w.)	*Bacopa monnieri* (100 mg/kg body wt.) and Acephate (34 mg/kg b.w.)
AChE specific activity	18.92 ± 5.48	1.35 ± 5.10***	12.94 ± 6.47**	1.63 ± 0.44*
AChE inhibition %	-	-55.85%	-31.6%	-91.38%
Km x 10^{-3} M	0.83 ± 0.45	1.53 ± 0.58* +84.3%	1.08 ± 0.47* +30.1%	1.29 ± 0.50* +55.4%
Vmax (A / min/mg protein)	0.2	0.2	0.2	0.2

Values are expressed as mean ± S.D. of 5 individual animals in each group, analyzed by student's t-test
P<0.01*; *P*<0.02**; *P*<0.05***, compared with control vs. treated.

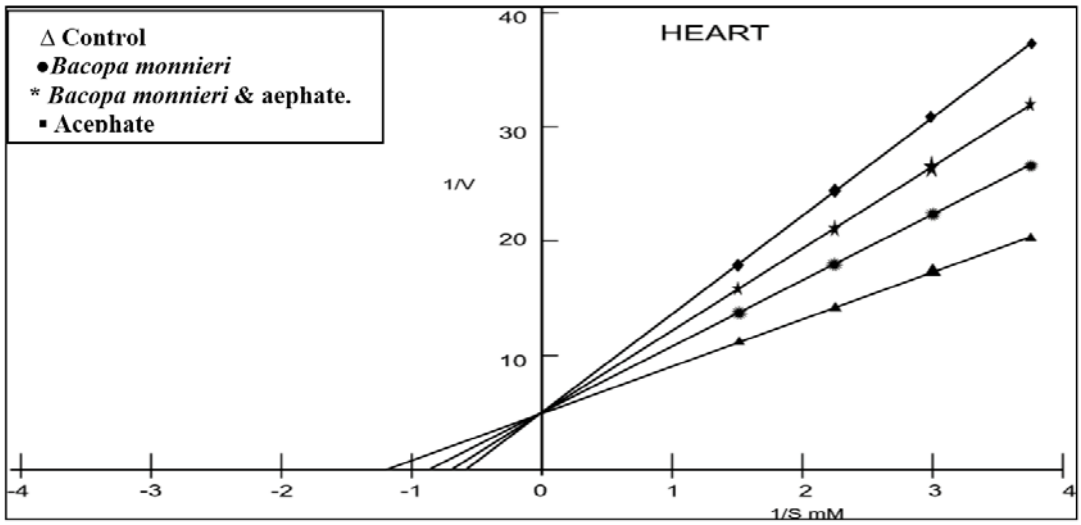

Fig 1: Lineweaver-Burk plot of in vivo inhibition of acetylcholinesterase in the Heart of Chick exposed to Acephate, *Bacopa monnieri* and pretreatment of *Bacopa monnieri* followed by treatment of Acephate. Each point represents the mean of five assays.

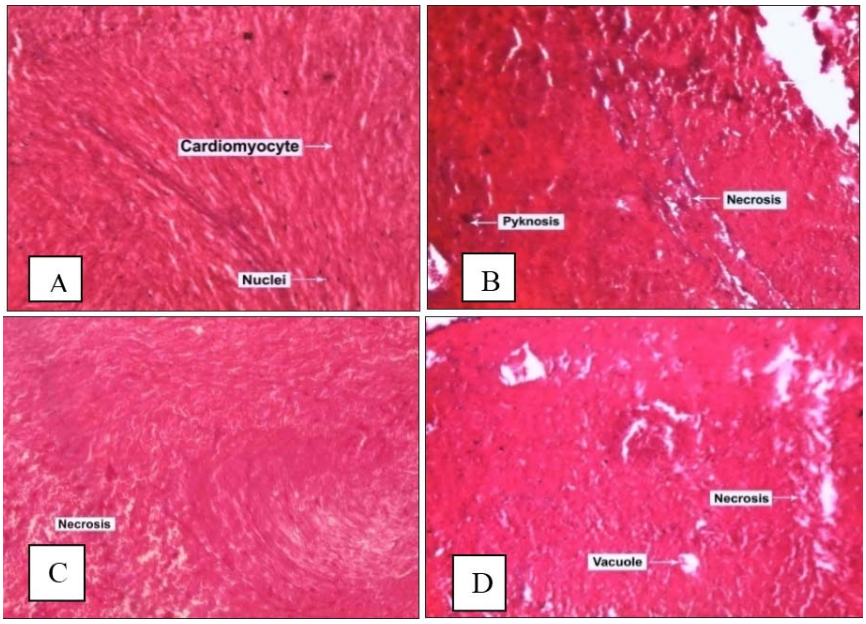

Fig 2: Photomicrograph of T. S. of Heart of Chick. H. E. X 100. : A – group I(Control) showing the normal structure. B – group II (Exposed to acephate 34 mg/kg b.w.) showing damage of cardiomyocyte. C-group III(Exposed to *Bacopa monnieri* 100 mg/kg b.w.) showing enlargement of nuclei and necrosis. D – group IV (Exposed to pretreatment of *Bacopa monnieri* 100 mg/kg b.w. followed by acephate 34 mg/kg b.w.) showing damage in cardiomyocyte fibers and vacuoles.

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