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Synergistic Effect of Selected Hydroxy Cinnamic Acid Derivative (HCA) With Rivastigmine in Dementia

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ABSTRACT

Background: *Alzheimer's disease (AD) is the most common form of dementia. AD is a progressive and irreversible neurodegenerative disease, which damages and kills brain cells.*

Expand design: *the study was designed to evaluate the anti-choline esterase inhibitory property of selected HCA derivative and observe its effects on AlCl3 induced dementia in rats. The four compounds Cinnamic acid, Protocatechuic acid, Sinapic Acid and Rosmarinic acid were tested for in vitro choline esterase activity and based on the results, and reported literature protocatechuic acid was selected to test in animals. Dementia was induced by aluminium chloride at a dose of 175 mg/kg, p.o. for a period of 25 days in rats and then divided into five different groups, i.e., rivastigmine, negative control and to see their additive effects two other groups were divided, which received rivastigmine (1.25 mg/kg. p.o+ 50 mg/kg PCA, p.o) and last group received PCA (50 mg/kg, p.o), where these groups treated and observed until the 35 day of experimental trial. The different behavioral and biochemical parameters were determined during or end of experiment.*

Results and Conclusion: *Aluminium chloride in a dose of 175 mg/kg, o.p. significantly induced the dementia and PCA acid, at a dose of 50 mg/kg, p.o., possessed potentially useful protective effect against aluminium chloride induced-dementia of AD type in rats and provide additive effects when given with rivastigmine*

Keywords: Dementia, Aluminium, Rivastigmine, Alzheimer's disease, Dichloromethane.

INTRODUCTION

Dementia is a group of brain disorders, which is chronic or progressive in nature. It's affecting cognitive functions and memory impairment, especially with cortical functions like judgement, judgment, decision-making, relationships with others, confusion, personality, emotional control, dysphasia, apraxia agnosia and behavioral changes. These symptoms interfere with the person's social and working life [1]. It is common in people over age of 65 [2]. There is no cure for dementia [3]. Cholinesterase inhibitors such as donepezil are often used and may be beneficial in mild to moderate disease [4]. Educating and providing emotional support to the caregiver is important. Exercise programs are beneficial with respect to activities of daily living and potentially improve outcomes [5].

The selective deficiency of acetylcholine in AD, has given rise to the "cholinergic hypothesis," which proposes that a deficiency of acetylcholine is critical in the genesis of symptoms of AD [3,6]. Therefore, a major approach for the treatment of AD has involved attempts to augment the cholinergic function within the brain. This involves the use of acetyl cholinesterase inhibitors such as rivastigmine, donepezil, tacrine and galantamine [7]. In addition to that more reactive oxygen species (ROS) will lead to imbalance between the formation of cellular oxidants and the anti-oxidative processes. This imbalance urges the use of compounds, which prevents the Oxidative metabolism of acetylcholine in treatment of AD pathogenesis [8,9].

MATERIALS AND METHODS

Drugs and chemicals

Rosmarinic acid, Protocatechuic acid, Cinnamic acid and Sinapic acid were purchased from Sigma Aldrich (S. & G. Lab Supplies). Rivastigmine was procured as a gift sample from Sun pharmaceutical Pvt. Ltd. India. Chemicals like aluminium chloride, DTNB, acetylthiocholine iodide, trichloroacetic acid, thiobarbituric acid, sodium carboxy methyl cellulose was procured from Merk and S.D. Fine Chemicals Ltd. (Mumbai). Solvents like methanol, chloroform, dichloromethane, tween 80, n-butanol and ethyl acetate were of analytical grade (AR) [10,11].

In-vitro ache assay

The acetyl-cholinesterase inhibition assay was deter-mined by Ellman colorimetric method [12] as modified by Albano et al. [13]. In a total volume of 1 ml, 415 µl of Tris–HCl buffer 0.1 M (pH 8), 10 µl of solution of Test compounds in methanol with different concentrations and 25 µl of enzyme (acetyl-cholinesterase, Sigma–Aldrich, St. Louis, USA) solution containing 0.5 U/ml was incubated for 15 min at room temperature. 75 µl of a solution of AChI (acetyl-thiocholine) (Sigma–Aldrich, Steinheim, Germany) 1.83 mm and 475 µl of DTNB (5,5-dithiobis-2-nitrobenzoicacid), 3 mm (Sigma–Aldrich, Steinheim, Germany) was added and the final mixture incubated for 30 min, at room temperature. Absorbance of the mixture was measured at 412 nm in an X-Rite 640B spectrophotometer. Rivastigmine was used as positive control. The percentage inhibition of enzyme activity was calculated by comparison with the negative control:

$$\text{Inhibition } \% = A_0 - A_1/A_0 \times 100$$

Where A_0 was the absorbance of the negative control (enzyme + methanol), and A_1 was the absorbance of the sample (enzyme + solution of Test). Tests were carried out in triplicate, and data were analyzed using descriptive statistics.

Estimation of IC_{50} values

The IC_{50}values (concentration of test compounds that inhibits the hydrolysis of substrates by 50%) were deter-mined by spectrophotometry measurement of the effect of increasing concentrations of test compounds (test and positive controls) on AChE activity. Determinations were carried out in triplicates. To calculate the IC_{50} values, each sample was assayed at 5 concentrations (30, 20, 10, 5 and 2.5 mg/ml). IC_{50}values were obtained from dose effect curves by linear regression.

Inhibition factor

This variable is defined as the inhibitory strength to a test relative to the reference inhibitor. The values give the number of times the test compound is more potent or less potent than the reference inhibitor.

It is calculated as follows:

IC_x of reference inhibitor/IC_x of test compound

Where, IC_x is the concentration of test substance that inhibited x% of AChE activity.

Animals

Age matched young wistar albino rats of either sex, weighing 120-150 g were selected as the study. The animals were kept in the paddy husk as bedding material. Husk changed every day. The animals were housed in a group of 6 (both sex) per polypropylene cages kept under controlled room temperature (25 ± 1°C) in 12 hours light – dark cycle. The rats were allowed free access to food (Standard pallet) and water. The experiment was conducted in a noise-free environment between 9:00 AM to 2:00 PM. All procedures were approved and carried out as per the official guidelines of experimentation on animals.

Acute toxicity study (LD50)

LD_{50} was determined according to the guidelines of organization for economic cooperation & development (OECD) following the up and down method (OECD guideline no. 423) and fixed dose method (OECD guideline no. 420). Based on this guideline a limit test was to categorize the toxicity class (LD_{50}) of the compound. The limit test was performed at 2000 mg/kg, p.o. A dose range of 50 mg/kg, 100 mg/kg was selected for the pharmacological activity. For all the studies overnight fasted animals were used. Further the dose of PCA (50 mg/kg) was selected as the basis of previous reported activities related its potential Neuroprotective effects in brain cells.

Experimental design

Aluminium chloride-induced Dementia: On prove day (day 5-consider as day 1), Randomized animals were divided randomly into experimental groups (n=6) (overall protocol- (5+36) =42 days). Control group received a normal saline (5 ml/kg, p.o). $AlCl_3$, Rivastigmine and Protocatechuic Acid suspensions were made freshly at a time of dosing. Morris water mazes (MWM), test was performed on 5, 16, 26 and 36 day to access learning, memory and ambulatory movements. Later, the animals were sacrificed for

biochemical studies. Extent of oxidative stress was measured by estimating the levels of Glutathione (GSH), Superoxide dismutase (SOD), Nitrite, Catalase and, Brain acetylcholine esterase (Ache) activity [3] (Figures 1-5 and Table 1).

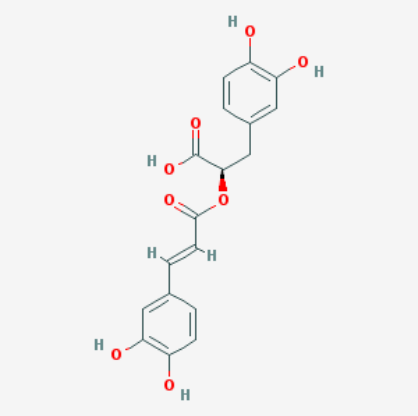

Figure 1: Structure of rosmarinic acid.

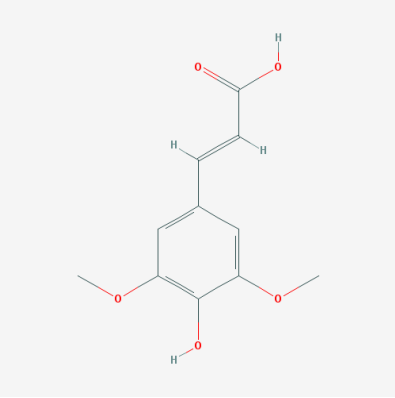

Figure 2: Structure of cinnamic acid.

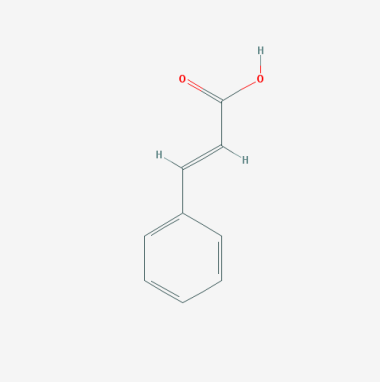

Figure 3: Structure of sinapic acid.

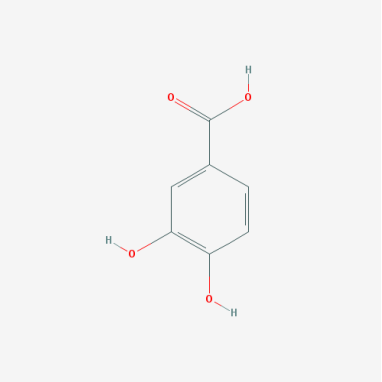

Figure 4: Structure of PCA.

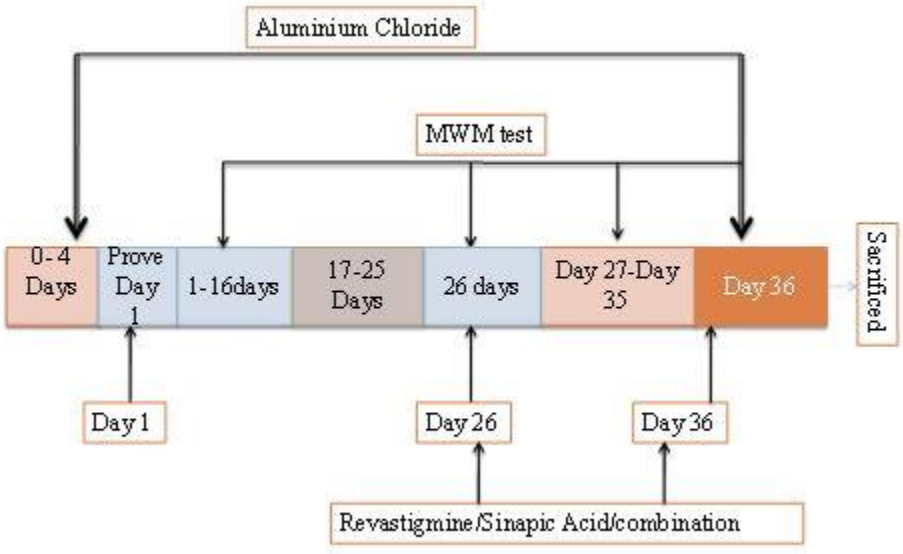

Figure 5: Experimental protocol for aluminum chloride induced dementia.

Table 1: Summary of *invitro* AchEI by HCE derivatives.

Compound	**IC10 (mg/l)**	**IC50 (mg/l)**	**IC90 (mg/l)**	**Inhibition factor (IF) (IC50)**	**Inhibition factor (IF) (IC90)**
Rivastigmine	ND	9.52	12.51	1	1
Cinnamic Acid	ND	9.41	13.01	1.01	0.99
Sinapic Acid	1.52	8.61	11.98	1.10	1.07
Rosmarinic Acid	ND	10.51	14.52	0.90.	0.89
Protocatechuic Acid	1.92	6.52	11.45	1.46	1.12

1. **Group I**
    - **Control group**: (Normal saline (0.9% NaCl) - 5 ml/kg, p.o., from 6^{th} to 36^{th} day):
2. **Group II**
    - **Negative control group:** Rats were administered with $AlCl_3$ suspension (175 mg/kg) orally from day 0 (i.e., 24 h after the completion of retention trial on day 5) to 36 days.
3. **Group III**
    - **Rivastigmine-treated group:** After 25 days, rats were administered standard drug suspensions (Rivastigmine -2.5 mg/kg), (1% aqueous solution of Tween 80) orally from day 26 to 36 day.
4. **Group IV**
    - **Protocatechuic acid -treated group- affected rats:** After 25 days, rats were administered Protocatechuic Acid suspension (50 mg/kg) (1% aqueous solution of Tween 80) orally from day 26 to 36 days.
5. **Group V**
    - **Rivastigmine + PCA-treated group:** After 25 days, rats were administered Standard drug suspensions (Rivastigmine -1.25 mg/kg), (1% aqueous solution of Tween 80) along with PCA (50 mg/kg) orally from day 26 to 36 day.

Memory assessment (Water Maze Test)

The water maze consisted of a circular tank (150 cm diameter and 40 cm height) [14]. Water pool was divided into four equally spaced quadrants [North-East (NE), south-east (SE), South-West (SW) and North-West (NW)] along the circumference of the pool. An escape platform (10 cm diameter) submerged 2cm below the water surface and was placed in NW quadrant. Rats were trained to find the hidden platform at a fixed location in NW quadrant. All rats were subjected to one session of four trials per day for five consecutive days (0-5^{th} day). During each trial, the animal was placed in each quadrant to eliminate quadrant effects. All rats were left in the platform for 30 s and then removed, and towel dried. Rats failing to find the platform within 60 s were guided to the platform (Figures 4 and 5). In day 5 (Probe day/ Zero day), 24 h after previous training, escape platform was removed and probe trial was conducted. The cut-off time for animal to swim was set to 60 s before the end to the session. Similarly, the retention trials were conducted on day 5, 16, 26 and day 36 on different groups to evaluate memory. Time elapsed in escaping to the NW quadrant, i.e., escape latency time (ELT) and total time (TT) time spent in NW quadrant, was measured during the retention trials [3,14] (Figure 5).

EVALUATION

Brain Tissue sampling and preparation

After 24 h of the experimental period (after 35 days), the animals were sacrificed and their brains were removed and weighed. The whole brain of was washed thoroughly with ice-cold isotonic saline. A 10% issue homogenate was prepared in 0.1 M phosphate buffer (pH 8, stored 2-8°C) for various neurochemical estimations and other anti-oxidative parameters.

Biochemical assessments

Acetyl choline esterase (AchE) activity

This activity was measured by Ellman method [12].

Estimation of glutathione (GSH) activity

GSH level was measured by the method described by Butler [15,16] with slight modifications. The absorbance was measured spectrophotometrically at 412 nm (X-Rite 640B spectrophotometer). Different concentration of GSH standard was also processed similarly to prepare a standard curve (1–50 µg) simultaneously. Results were expressed as nmol of GSH/mg of protein.

Estimation of catalase

This activity was determined by Luck method [17].

Lipid peroxidation assay (TBARS)

Thiobarbituric acid reactive substances (TBARS) measurement is an index of lipid peroxidation in the brain. For the estimation of TBARS, ten percent (w/v) tissue homogenate were mixed with sodium dodecyl sulfate, acetate buffer (pH 3.5), and aqueous solution of thiobarbituric acid. After heating at 95°C for 60 min, the red pigment produced was extracted with *n*-butanolpyridine mixture and estimated by the absorbance at 532 nm. As an external standard, tetramethoxypropane was used, and lipid peroxide level was expressed in terms of nmol malondialdehyde [18].

Estimation of Superoxide dismutase (SOD)

The SOD activity was determined using the RANDOX Ransod enzyme kit. This method employs xanthine and xanthine oxidase (XOD) generated superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitropheno)-5- phenyltetrazolium-chloride to form the red formazon dye. The SOD activity was measured by the degree of inhibition of this reaction [19].

Estimation of nitrite

It was measured by Najmun method [20].

Statistics and data analysis

Dose relationship plots and dose response equation were generated using the percentage inhibition of each compound at each given concentration of each compound. The 10% (IC_{10}), 50% (IC_{50}) and 90% (IC_{90}) inhibitory concentration values were calculated from the dose response equation. All dose response plots and calculations were achieved using M.S. Excel software. The experiment data was expressed as Mean ± SEM. In all the tests, the criterion for the statistical significance was set at $p<0.05$. The data for all studies was analyzed using one-way ANOVA followed by Tukey-Kramer Multiple Comparisons test.

RESULTS

Effects of HCA derivatives on in vitro AchEI Capacity

The summary of AchE inhibition by compounds used for this study is given in Table 1. The AchE inhibition of each compound was expressed by its IC_{50} value and complimented by the IC_{10} and IC_{90} as a lower and upper limit to their inhibition capacity. From the observed IC_{50} values, cinnamic acid showed an AchEI capacity which was matched by the reference compound

rivastigmine. PCA showed a potent AchEI Capacity at lower concentration with less IC_{50} value. Other compounds also showed the AchEI capacity but not found as potent as PCA. So based on these results PCA has been selected for further study to test in animals.

Effect of PCA on Aluminium chloride induced behavioral parameters

Effect of PCA acid on Aluminium chloride induced dementia of Alzheimer's type in rat using the Morris Water Maze:

The rat spends significantly more time in the target quadrant (NW) in search of the missing platform as compared with the time spent in other quadrants (SE, SW and NE) during the retention trial conducted on the 5th day (probe day). Control group rats (Group I) showed the normal retrieval of memory on 36th day ($p<0.001$). The rats administered with $Alcl_3$ showed significant memory deficit accessed by raised ELT and reduced time spent in the target quadrant (NW) i.e. TT markedly in search of the missing platform during the retention trial on 16th, 26th and 36th day [ELT ($p<0.001$) and TT ($p<0.001$)] as compared to control group rats (Group, I). The animals treated with rivastigmine significantly ($p<0.01$) reversed the effect on ELT and TT as compared group II. The animals showed reversal of ELT and TT, when treated with PCA acid at a dose 50 mg/kg, p.o. and this effect were found more significant ($P<0.01$)when treated in combination with rivastigmine (Figures 6 and 7).

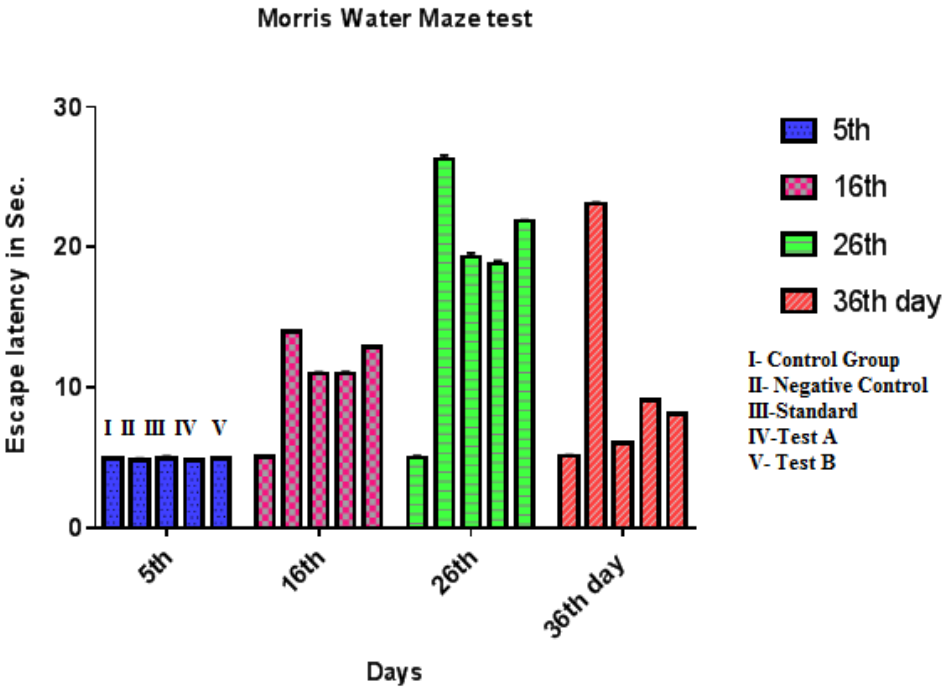

Figure 6: Effect of PCA acid on aluminium chloride induced dementia of Alzheimer's type in rats.

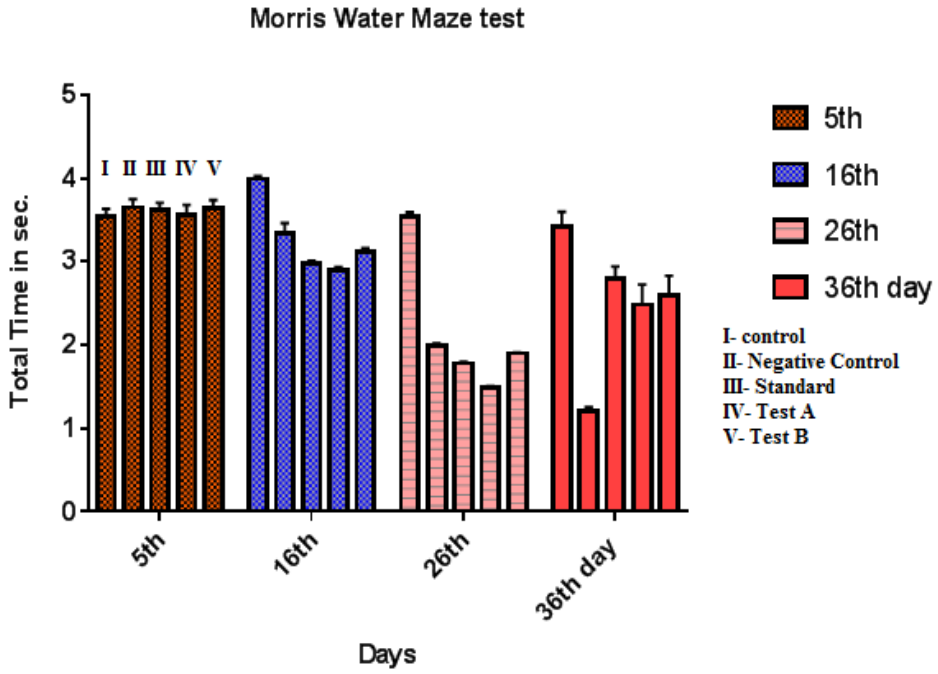

Figure 7: Effect of PCA acid on aluminium chloride induced dementia of Alzheimer's type in rats.

Effect of PCA on AchE

The AchE level was significantly increased in negative control rats as compared to control group. The elevated level was decreased with the standard drug treatment (Rivastigmine at 3.25 mg/kg). The response observed with a sub maximal dose of rivastigmine along with PCA (50 mg/kg) was almost twice of the response observed by giving PCA alone. The response observed with PCA treated group (AchE level) found significant when compared to negative control, and this will indicates the interaction of PCA with rivastigmine to synergize the anti-choline esterase effect.

Effect of PCA acid on biochemical parameters

The table explicates the levels of TBARS, Nitrite, GSH and Catalase, and SOD level in brain homogenate of control and experimental groups of rats. The levels of TBARS, and Nitrite were significantly elevated in Untreated $AlCl_3$-affected rats as compared to a control group. The animals treated with rivastigmine showed a significant reduction in the levels of AchE, TBARS, and Nitrite on 36^{th} day of trial as compared with Untreated $AlCl_3$-affected group. The elevated levels of TBARS, and Nitrite were declined up on treatment with PCA during the experimental trial as compared with Untreated $AlCl_3$-affected group.

The levels GSH, Catalase and SOD were significantly decreased in Untreated $AlCl_3$-affected rats as compared to a control group. The animals treated with rivastigmine showed a significant increase in the levels of GSH, Catalase and SOD on 36^{th} day of trial as compared with Untreated $AlCl_3$-affected group. The reduced levels of GSH, Catalase and SOD were increased significantly with a dose of 40 mg/kg of PCA acid as compared with Untreated $AlCl_3$-affected group (Table 1) [21-35].

DISCUSSION

Acetyl cholinesterase inhibitors are the only agents approved by the Food and Drug Administration (FDA) for the treatment of AD. Rivastigmine was used as standardized drug as it is the only proven pharmacological therapy for the symptomatic treatment of AD [35]. Impaired cholinergic transmission is one of the complications seen in the etiopathogenesis of memory deficit in AD. The neurodegeneration in frontal cortex and hippocampus areas within the brain [36] resulting in impaired cholinergic transmission occurs by two ways. Firstly, in AD patients, it occurs either due to (I) decline in Ach release (ii) decreased choline acetyltransferase activity (ChAT), which results in the scarcity of Acetylcholine [36-38]. Secondly, elevated acetyl cholinesterase (AchE) enzyme further adds to scarcity of Ach at the synapse by degrading the available Ach. This degradation of Ach is abolished by rivastigmine (AchE inhibitor) so it's effective in AD through improvement in cholinergic transmission.

Polyphenols are the most voluminous antioxidants in human diets. These polyphenols are to be categorized in different classes as phenolic acids, flavonoids, lignins, and stilbene. Phenolic acids are naturally-occurring compounds found throughout the plant kingdom with unique structural similarities, presence of the carboxylic group as in caffeic acid, gallic acid, p-coumaric acid, vanillic acid, ferulic acid, and protocatechuic acid (PCA) [39-41]. PCA belongs to the class of phenolic acids with bioactive carboxylic acids; the class mainly includes caffeic acid, ferulic acid, and sinapic acid [37,38]. These are potent inhibitors of an enzyme AchE [42,43] and another possible mechanism of them by reducing cerebral hypoxia [44] and improved memory disturbance by activating cholinergic function [Acetylcholine (Ach) and choline acetyltransferase (ChAT)] [45].

In our study, aluminium treatment resulted in behavioral changes such as a spatial memory deficit, indicated by increased escape latency and decreased in time spent in target quadrant [NW]. Rivastigmine and PCA antagonized the spatial memory deficit caused by aluminium. This suggests the synergistic role of PCA in correcting cognitive dysfunction associated with aluminium exposure.

So, in the present study the synergistic effect of Protocatechuic acid with Rivastigmine may be due to its chelating effects of the enzyme which is supported by a study published by Erica stated the electrostatic interaction of PCA with AchE and confirms its AchEI property. Thus, Protocatechuic acid may be considered as a potential candidate in treatment of various memory disorders.

CONCLUSION

The treatment with Protocatechuic acid showed improvement in the memory impairment, motor activity as well as muscle strength in $AlCl_3$ induced model. This reversal of memory loss induced behavioral alteration by Protocatechuic acid may be attributed to its brain AchE activity. Further, molecular-level investigation is to be done using more biochemical parameters to assess the possible mode of action and active principle responsible for synergistic activity of Protocatechuic acid.

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