

EVALUATION OF SAFETY AND NEUROPROTECTIVE EFFECT OF POLYHERBAL EXTRACT AGAINST COGNITIVE IMPAIRMENT

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Abstract

The present study was aimed to investigate the neuroprotective effect of polyherbal extract (PHE) of three (*Citrus lanatus* seeds, *Cucumis sativus* peel and *Psidium guajava* leaves) plant parts on cholinergic dysfunction and oxidative stress. The objective of the study is to carry out in vivo tests to evaluate the cognitive enhancing effect of PHE against bilateral common carotid artery occlusion (BCCAO) induced cognitive impairment in rats. Cognitive impairment was induced by BCCAO for 30 min, followed by 7 days reperfusion in male wistar rats. The rats weighing 200-250gms were pretreated with 200mg/kg and 100mg/kg of PHE for 10 days and the treatment was continued for another 7 days after cerebral ischemia, following which the Morris water maze and elevated plus maze were performed to assess learning and memory. Then the animals were sacrificed for biochemical tests such as acetylcholinesterase (AChE) activity, antioxidant enzymes and histopathological analysis. Results were expressed in mean $\pm$ SD which were analyzed by one-way ANOVA followed by Dunnett's test. The study demonstrates that BCCAO resulted in learning and memory deficits which were significantly ($p < 0.001$) ameliorated by the PHE. The PHE counteracted BCCAO induced increase in AChE activity and decreases in activities of the antioxidant enzymes. From the above results, it could be postulated that PHE exerts a neuroprotective effect against oxidative damage induced by BCCAO which might be associated with its antioxidant and anti-cholinesterase activities. These results suggest that PHE might offer a useful therapeutic choice in either the prevention or the treatment of Alzheimer's disease.

Key words: Antioxidant, bilateral common carotid artery occlusion, cognitive impairment, oxidative stress.

1. Introduction

Alzheimer's disease (AD) is a progressive form of dementia that characterized by impairment of memory and loss of intellectual ability which results in irreparable dysfunction and loss of neurons in distinct brain areas. AD has been estimated to account for 50%–60% of dementia cases in people over the age of 65 years [1]. Aging is a phenomenon leading to the dysfunction of normal cellular regulation including cognitive

function. As the age advances, the cognitive capability is declined. Since the cognitive decline is the costliest, in terms of the financial, personal, and societal, it is regarded as a major health and social issue burden. Therefore, age-related cognitive memory impairment is one of the important health problems that should be concerned. Characteristic pathological features of the central nervous system in AD include the accumulation of plaques and neurofibrillary tangles, aberrant oxidative and inflammatory processes, and neurotransmitter disturbances [2]. The etiology of this disease is very complex and is characterized by symptoms that include memory and language impairment, cognitive dysfunction, and behavioral disturbances (i.e., depression, agitation and psychosis) [3]. In the later stages these symptoms become progressively more severe, resulting in incontinence, gait and motor disturbances that eventually lead to loss of speech and locomotion. Due to its debilitating nature, an enormous social and economic burden is placed on family members and society. India had an estimated 1.8 million patients suffering from dementia in the year 2000 and by 2040, the number of cases in North America alone is predicted to rise to 9.2 million [4].

Severity of AD and associated memory loss has been correlated with neuropathological occurrences due to cholinergic deficits [5]. Since the present strategy has not yet been met by effective symptomatic treatments or preventative strategies. Thus, a rational approach for the symptomatic treatment of AD is to restore cholinergic functions by prolonging the availability of acetylcholine in the neuronal synapse. This has been achieved by inhibitors of the enzyme acetylcholinesterase (AChE) which plays a role in the degradation of this neurotransmitter. Donepezil, Rivastigmine, and Galantamine are AChE inhibitors (Ache I) used for the treatment of AD. The glutamate receptor antagonist Memantine that can also be used alone or in combination with Ache I [6]. These therapies however only address symptoms but not disease progression. Therefore, treatment options that can disrupt the progression of AD are urgently needed, however, the exceptionally high failure rate of AD candidates during clinical trials highlights the complexity of this disease and the need for innovative approaches to effectively treat this condition [7].

AD is associated with several pathologies, including oxidative stress, inflammation, and loss of cholinergic neurons; a multi-targeted approach may therefore be essential to effectively treat this condition. The herbal medicine can be used either by single herb or by polyherbal formulation. In the traditional system of Indian medicine, plant formulations and combined extracts of plants are chosen rather than individual ones. It is known that Ayurvedic herbals are prepared in a number of dosage forms, in which mostly all of them are PHE [8,9]. Even though the active phytochemical constituents of individual plants have been well established, they usually present in minute amount and always, they are insufficient to achieve the desirable therapeutic effects. For this, scientific studies have revealed that these plants of varying potency when combined may theoretically produce a greater result, as compared to individual use of the plant and also the sum of their individual effect. This phenomenon of positive herb-herb interaction is known as synergism. Certain pharmacological actions of active constituents of herbals are significant only when potentiated by that of other plants, but not evident when used alone. Due to synergism, polyherbalism confers some benefits not available in single herbal formulation. It is evident that better therapeutic effect can be reached with a single multi-constituent formulation. For this, a lower dose of the herbal preparation would be needed to achieve desirable pharmacological action, thus reducing the risk of deleterious side-effects. Besides, polyherbal formulations (PHF) bring to improved convenience for patients by eliminating the need of taking more than one different single herbal formulation at a time, which indirectly leads to better compliance and therapeutic effect. All these benefits have resulted in the popularity of PHF in the market when compared to single herbal formulation [10].

We recently reported on the in vivo positive effects of single plant parts (*Citrulus lanatus* seeds, *Cucumis sativus* peel and *Psidium guajava* leaves) on cognitive deficit rats. These single plant part extracts also demonstrated antioxidant and neuroprotective properties as determined by measurements of relevant biochemical and behavioral estimations [11-13]. Based on these investigations and evidences, the present study was aimed to investigate the neuroprotective effect of polyherbal extract (PHE) of those three plants

on cholinergic dysfunction and oxidative stress in bilateral common carotid artery occlusion (BCCAO) induced cognitive impairment in rats.

2. Materials and methods

2.1. Chemicals and drugs

Donapezil, Thiopentone sodium, Hydrogen peroxide, povidone-iodine powder, 5% w/w (Sri medical and surgical) Ethanol (Venkateshwara agencies) Acetylthiocholine iodide (Sigma Aldrich), Perchloric acid Formalin 10% (Finar Chemicals), DTNB (5,5- dithiobis (2-nitrobenzoic acid) reagent, DPPH (1,1-diphenyl-2- picrylhydrazyl) radical reagent (Sigma Aldrich).

2.2. Plant material

The fresh three plant materials were collected from the local market of Warangal district, Telangana state, India. The individual plant parts were shade dried. The collected samples were authenticated morphologically (Voucher specimen nos. ENM/SU 0019022, ENM/SU 0019023, ENM/SU 0019024 respectively) by Dr. E. Narasimha Murthy, Department of botany, Satavahana University, Karimnagar-505002, Telangana state, India.

2.3. Extraction and preparation of polyherbal drug formulation

Each one gram of a PHE contains equal amount of *Citrus lanatus* (seeds), *Cucumis sativus* (peel) and *Psidium guajava* (leaves). 100g of the dried powder of each plant was taken and the extraction of these plant materials were carried out using soxhlet assembly with ethanol for about 36 h. The ethanolic extract were cooled, filtered. The filtrate was concentrated by using a rotary evaporator under reduced pressure till the concentrated mass was obtained. The yield of crude extract is called as polyherbal extract (PHE) and were stored in an air-tight desiccator's and used for further analysis [14].

2.4. Experimental animals

Male albino rats of Wistar strain weighing about 150- 200 g obtained from the animal breeding station, mahaveera enterprises, medchal district-98, India were used for the study. The animals were maintained under standard conditions of humidity, temperature ($25 \pm 20^{\circ}\text{C}$) and light (12 hours light/dark). They were acclimatized to animal house conditions and were fed on a commercial pellet rat chow and water ad libitum. Experimental animals were handled according to the Institutional Legislation, regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and protocol of the experiments after the authorization of the Institutional Animal Ethical Committee (IAEC), Vaagdevi College of Pharmacy, Warangal (A. P) and India (1047/PO/Re/S/07/CPCSEA).

2.5. Preliminary phytochemical analysis

The extract was subjected to preliminary screening, for various active phytochemical constituents such as alkaloids, carbohydrates, steroids, protein, tannins, phenols, flavonoids, gum and mucilage, glycosides, saponins and terpenes by standard procedures [15].

2.6. Preliminary acute toxicity test

Healthy male albino rats of Wistar strain weighing about 150- 200 g were subjected to acute toxicity studies as per guideline no, 423 (acute toxic class method) suggested by the organization for economic co-operation and development. The rats were observed continuously for 2 h for behavioral and autonomic profiles and for any sign of toxicity or mortality up to a period of seven days [16]. It was observed that these seed extracts were not fatal to the rats even at the 2000 mg/kg doses. Hence, 1/10th (200 mg/kg) and 1/20th (100 mg/kg) of these doses were selected for further studies.

2.7. Experimental protocols

The rats were divided into five groups of 06 each. First group served as sham-operated control group and received vehicle i.e. distilled water. Second group also received distilled water and served as BCCAO treated control group. Third, fourth and fifth groups served as test groups i.e. drug treated BCCAO groups and received donepezil and PHE at doses of 200 and 100 mg/kg respectively. The treatment with vehicle, donepezil and PHE (200, 100 mg/kg) was started once daily for 10 consecutive days prior to the cerebral ischemia. On day 11, 60 min after the last dose, all the groups received BCCAO for 30 min followed by reperfusion for 7 d. From the second (13) day after induction, the animals continued with the test substances for other weekdays, then the animals were assessed for behavioral parameters i.e Morris water maze, rectangular maze and elevated plus maze. After behavioural study all the animals were sacrificed and their brains were isolated and subjected to biochemical analysis and histopathological evaluation [17].

2.8. Drug administration

Donepezil (5 mg/kg), the PHE (dissolved in 0.1% CMC) were administered at a dose of 200 and 100 mg/kg oral. Rats in the sham group and BCCAO group administered solely with an equal amount of normal saline. All drugs were prepared freshly every day. Doses were given according to the respective rat weights.

2.9. Induction of cerebral ischemia

Surgical procedures were performed between 8:00 a.m. and 1:00 p.m. All the surgical equipment and surgical pad were disinfected with 70% ethanol before the surgery to avoid any kind of infection and sepsis. Rats were fasted overnight and at the time of experiment, they were anaesthetized with thiopentone sodium at a dose of 50 mg/kg, (i. p.) and supplemented as needed. A median incision was performed in the skin of the ventral part of the neck and the subcutaneous adipose tissue was dissected avoiding the thyroid. The omohyoid muscle was cut through a median incision and a dissection was made between the sternocleidomastoid and the sternohyoid muscles parallel to the trachea. Each carotid artery was freed from its adventitial sheath and vagus nerve, which was carefully separated and maintained. The induction of BCCAO was performed by clamping both of the arteries with two microaneurysmal clips (bulldog clamps). The reperfusion of blood through the carotid arteries allowed after 30 min of cerebral ischemia by removing both the clips. Sham control animals received the same surgical procedures, except bilateral common carotid arteries were not occluded. During the surgery, the body temperature was monitored with a temperature probe and maintained at 37.0–37.5 °C using a heating pad [18, 19]. The skin was then sutured with 3-0 silk suture. After the survival surgery the antiseptic povidone iodine was applied to the neck incision area of all the animals to avoid infection and they were returned to their home cages with free access to feed and water.

2.10. In-vivo Pharmacological behavioral studies

2.10.1. Morris water maze: Morris water-maze test was employed to assess learning and memory of the animals [20, 21]. It consists of large circular pool (120 cm in diameter, 60 cm in height, filled to a depth of 40 cm with water maintained at 28±2°C). The water was made opaque with white colored non-toxic dye. A submerged platform (9 cm diameter), was placed inside the pool 2 cm above the water level during the

acquisition phase. Similarly, for the maze retention phase, the platform was placed in the pool 2 cm below the water level. Four equally spaced locations around the edge of the pool (Q1, Q2, Q3, and Q4) were used as starting points and this divided the pool into four equal quadrants. Each animal was subjected to four consecutive training trials on each day with inter-trial gap of 5 min. From the second day after induction, the animals received a training session consisting of 4 trials on day 12. In all 4 trials, the starting position was different. The latency to find the escape platform was recorded to a maximum of 90 seconds. If the rat did not escape onto the platform within this time, it was guided to the platform and was allowed to remain there for 20 seconds. The time taken by the rat to reach the platform was taken as the initial acquisition latency (IAL). Following 24 h (day 13) and 8 d (day 21) after IAL, the rat was released randomly from one of the edges facing the wall of the pool. The time is taken to find the hidden platform was recorded and termed as first retention latency (1st RL) and second retention latency (2nd RL) on day 13 and day 21 after cerebral ischemia, respectively.

2.10.2. Rectangular maze test: This test was used to evaluate the learning ability. The maze consists of a fully enclosed rectangular box with an entry and a reward chamber added at opposite ends. The box is partitioned like twisting corridor leading into blind passages from the entry to the reward chamber with wooden slats. From the second day after induction the animals received a training session consisting of 4 trials on day 12. In each trial the rat was placed in the entry chamber and the timer was activated as soon as the rat leaves the chamber. The time taken by the animal to reach the reward chamber from the entry chamber was recorded as the initial transfer latency (ITL). The animal was allowed to explore the maze for 20 seconds after recording the ITL and then returned to the home cage. If the animal did not enter the reward chamber within 90 seconds, it was guided on the back to reach the reward chamber and the ITL was given as 90 seconds. Retention of memory was assessed by placing the rat in an entry chamber and the retention latency was noted on day 13 and day 21 of ITL and was termed as the first retention transfer latency (1st RTL) and second retention transfer latency (2nd RTL) respectively. Lower scores of the assessment indicate efficient learning while higher scores indicate poor learning in animals [22, 23].

2.10.3. Elevated plus maze test: The elevated plus maze is designed to study behavioral patterns (such as sensitivity to external stimuli, anxiety, exploration, and learning and memory) of experimental animals and was performed as described in previous studies. The maze consisted of four arms (two open and two closed), each 49 cm × 10 cm. The closed arms consisted of 40 cm high walls with an open roof. The whole structure was elevated 50 cm above the ground. From the second day after induction, the animals received a training session consisting of 4 trials, on the 12th day, 60 min after treatment, each rat was placed at the end of an open arm, facing away from the central platform. The time taken by the rat to enter any of the closed arms was recorded and considered as the transfer latency, and served as the initial acquisition latency (IAL). If the rat did not enter into any one of the closed arms within 180 s, it was gently pushed into one of the two closed arms and the transfer latency was assigned as 180 s. For the next 15 s the rat was allowed to explore the maze before returning it to its home cage. On the next day (day 13) and 8 d (day 21) after IAL, the transfer latency was recorded again and served as first retention latency (1st RL) and second retention latency (2nd RL) on day 13 and day 21 after cerebral ischemia, respectively. Between each session, the maze was carefully cleaned with 30% ethanol to remove any olfactory cues [24, 25].

2.11. Biochemical analysis

After the behavioral testing, on the day 21 following the start of the treatment, animals were sacrificed by decapitation and the brain tissues were quickly removed, forebrain was dissected out, and cerebellum was discarded. Hippocampus dissected out from the brain and it was put on ice and rinsed with ice-cold isotonic saline. A (10% w/v) homogenate was prepared with 10 times ice cold 0.1M phosphate buffer (pH 7.4). Now the obtained homogenate was centrifuged at 3000rpm for 15 min and aliquots of supernatant were separated

and used for biochemical estimation. AChE activity was estimated in the hippocampus according to the method described by Ellman [26]. Briefly, tissue homogenate was incubated along with the Ellman reagent [5,5'-Dithiobis (2-nitrobenzoic acid)] and phosphate buffer, freshly prepared acetylthiocholine iodide (pH 8.0) was then added following which the absorbance was measured at 412 nm. The extent of lipid peroxidation in the brain was determined as described by Wills [27]. The amount of malondialdehyde (MDA) was measured by reaction with thiobarbituric acid at 532 nm using a spectrophotometer. Catalase activity was assessed by the method of Luck [28], wherein the breakdown of hydrogen peroxide is measured. Briefly, the assay mixture consisted of 3 ml of H₂O₂ phosphate buffer and 0.05 ml of the supernatant of the tissue homogenate. The change in absorbance was recorded for 2 min at the 30-second interval at 240 nm using a spectrophotometer. The results were expressed as micromoles of H₂O₂ decomposed per minute per mg protein. The free radical scavenging activity of the test drug was measured *in vitro* by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay [29]. In this, measurement is made from the bleaching of a purple coloured methanol solution of DPPH. To the 1000 L of diverse concentration of the homogenate, 4 ml of 0.004% methanolic solution of DPPH was added. After 30 min incubation in dark, absorbance was read at 517 nm. Inhibition of free radical by DPPH in percentage was calculated.

2.12. Statistical analysis

Results were expressed in mean $\pm$ SD. The significance of the difference in means between disease control and test drug-treated animals for different parameters was evaluated by using One-way Analysis of Variance (ANOVA) followed by multiple comparisons Dunnett's test. Data were measured statistically, significant at $p < 0.05$ and highly significant at $p < 0.01$. Statistical analysis was executed using GraphPad Prism 7 statistical software.

3. Results

3.1. Effect of PHE on the Morris water maze

During the acquisition sessions day12, BCCAO treatment showed significantly longer escape latencies in the mean IAL when compared to the sham control indicating BCCAO induced impaired acquisition of water maze task; this effect was mitigated by co-administration of the PHE (Fig. 1). Following training on days 13–21 after induction, the mean retention latencies (1st and 2nd RL) to escape onto the hidden platform were significantly ($P < 0.001$) decreased in sham control, donapezil (5 mg/kg, PO) and PHE (200 and 100 mg/kg, PO) group rats, as compared to IAL, but not in the BCCAO treated rats, instead this rats showed the significant increase in mean retention latencies compared to IAL on day 12. The results suggest that BCCAO caused significant cognitive impairment, which indicates improved the retention performance of the water maze task in both groups in a concentration-dependent manner as compared to BCCAO treated rats.

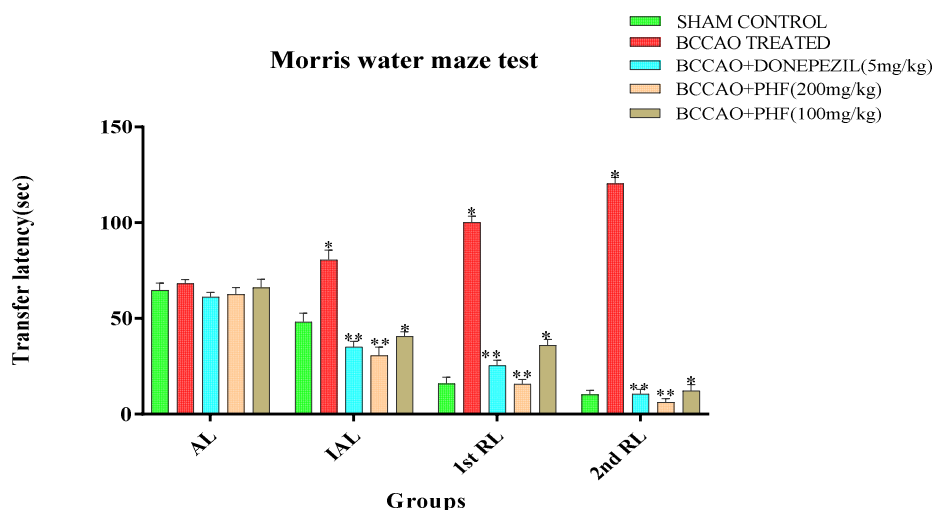


Figure 1. Effect of Donepezil, PHE on memory performance in water maze compared to the BCCAO group.

Values are expressed as Mean $\pm$ SD (n = 6); * p<0.01, ** p<0.001 as compared with corresponding values of BCCAO treated. (One-way ANOVA followed by dunnett's test)

3.2. Effect of PHE on the rectangular maze

In the present experiment, mean ITL on day 12 for each rat was relatively stable and showed no significant variation among different groups. All the rats entered the reward chamber within 90 seconds. Following training, Sham control, donepezil (5 mg/kg, PO) and PHE (200 and 100 mg/kg, PO) rats entered the reward chamber quickly as compared to BCCAO treated rats. Mean retention transfer latencies (1st RTL and 2nd RTL) to enter the reward chamber on days 13 and 21 were shorter as compared to ITL on day 12 of each group, respectively. In contrast, BCCAO treated rats performed poorly throughout the experiment and did not show any change in the mean retention transfer latencies on days 13 and 21 as compared to pretraining latency on day 12, demonstrating that BCCAO induced marked memory impairment. Chronic administration of PHE (200 and 100 mg/kg, PO) beginning prior to cerebral ischemia significantly decreased the mean retention latencies on days 13 and 21 after induction (Fig. 2).

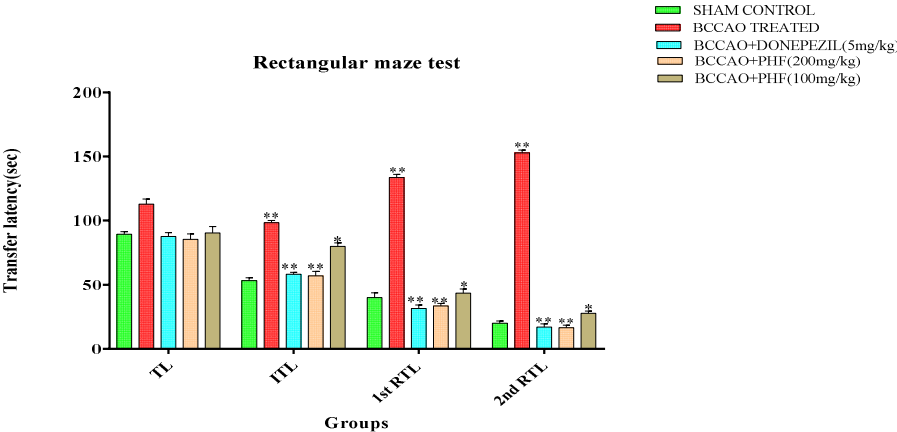


Figure 2. Effect of Donepezil and PHE on memory performance in rectangular maze compared to the BCCAO group

Values are expressed as Mean ± SD (n = 6); * p<0.01, ** p<0.001 as compared with corresponding values of BCCAO treated. (One-way ANOVA followed by dunnett’s test)

3.3.Effect of PHE on the elevated plus maze

Significantly higher transfer latencies were observed in the BCCAO treated rats compared to the sham controls during both the acquisition (day 12) and retention (day 13 & 21) sessions (Fig. 3). Additionally, unlike the controls, no significant differences between the transfer latencies measured in the acquisition and retention sessions were observed in the BCCAO treated rats. Administration of the PHE in BCCAO treated rats significantly (P < 0.001) lowered the transfer latencies with training from days 13-21 after induction in the retention session when compared to the acquisition session.

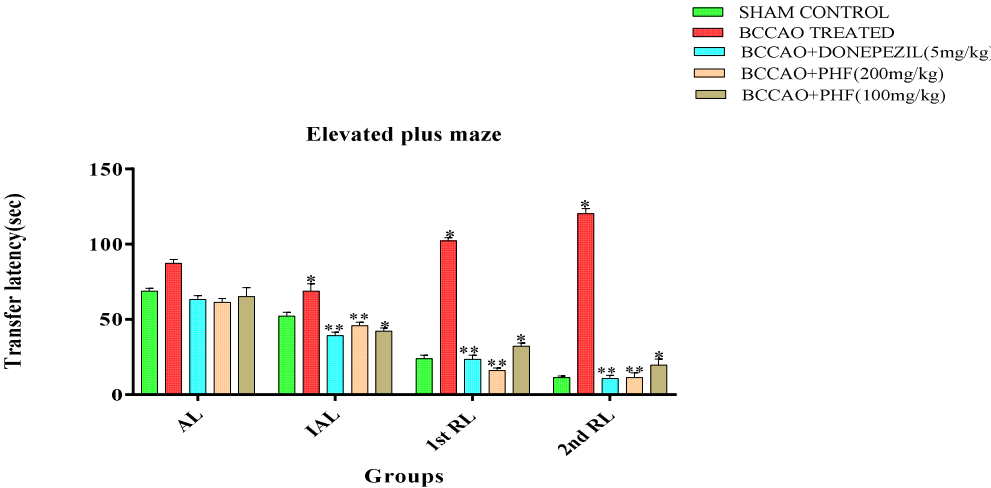


Figure 3. Effect of Donepezil, PHE on memory performance in elevated plus maze compared to the BCCAO group.

Values are expressed as Mean ± SD (n = 6); * p<0.01, ** p<0.001 as compared with corresponding values of BCCAO treated. (One-way ANOVA followed by dunnett’s test)

3.4. Effects of the PHE on anti-oxidant enzymes and AChE activity

Cerebral ischemia followed by reperfusion caused a significant rise in brain MDA levels and depletion of CAT, DPPH levels in BCCAO treated animals as compared to sham control animals. However, chronic PHE (200 and 100 mg/kg, PO) treatment significantly ($P<0.0001$) attenuated the increase in MDA levels and also caused a significant ($P<0.0001$) increase in CAT and DPPH levels when compared to the BCCAO treated rats.

In order to test whether the positive effects of the PHE on learning and memory in BCCAO treated rats were mediated by the cholinergic system, AChE activity were measured in the hippocampus of the experimental animals. BCCAO treatment caused a significant increase in AChE activity when compared to sham control. PHE (200 and 100 mg/kg, PO) administration in BCCAO treated rats caused a significant decrease in AChE activity in the hippocampus. However, chronic PHE (200 and 100 mg/kg, PO) treatment significantly ($P<0.0001$) attenuated enhanced AchE activity compared to BCCAO treated rats (Fig. 4).

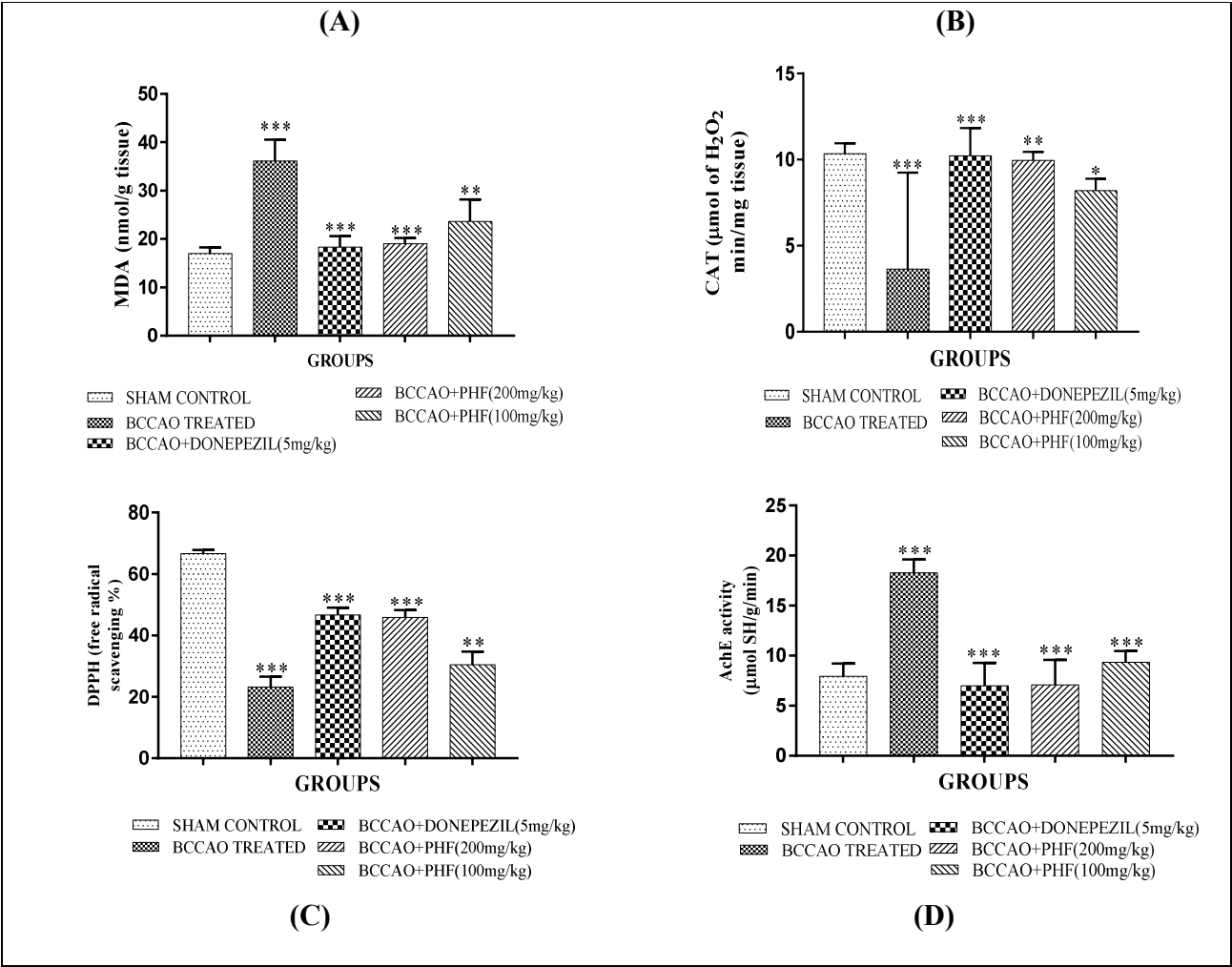


Figure 4. Effect of Donepezil, PHE on anti-oxidant enzymes and AChE activity compared to the BCCAO group.

- A. Graph represents malonaldehyde assay B. Graph represents Catalase activity
C. Graph represents DPPH assay D. Graph represents AchE activity

Values are expressed as Mean $\pm$ SD (n = 6); * p<0.01, ** p<0.001, *** p<0.0001 as compared with corresponding values of BCCAO treated. (One-way ANOVA followed by dunnett's test)

3.5. Histopathological results

From the figure 5 histopathological study, it was observed that 30 min of BCCAO followed by 7 days reperfusion in BCCAO group produced marked congestion of blood vessels and neutrophil infiltration and increased intracellular spaces. These effects were further augmented by reperfusion i.e. lymphocytic proliferation and neuronal necrosis. There is significant reversal of damage observed in PHE (200 & 100mg/kg) treated groups and also in donepezil treated group. The sham control group maintained the normal architecture.

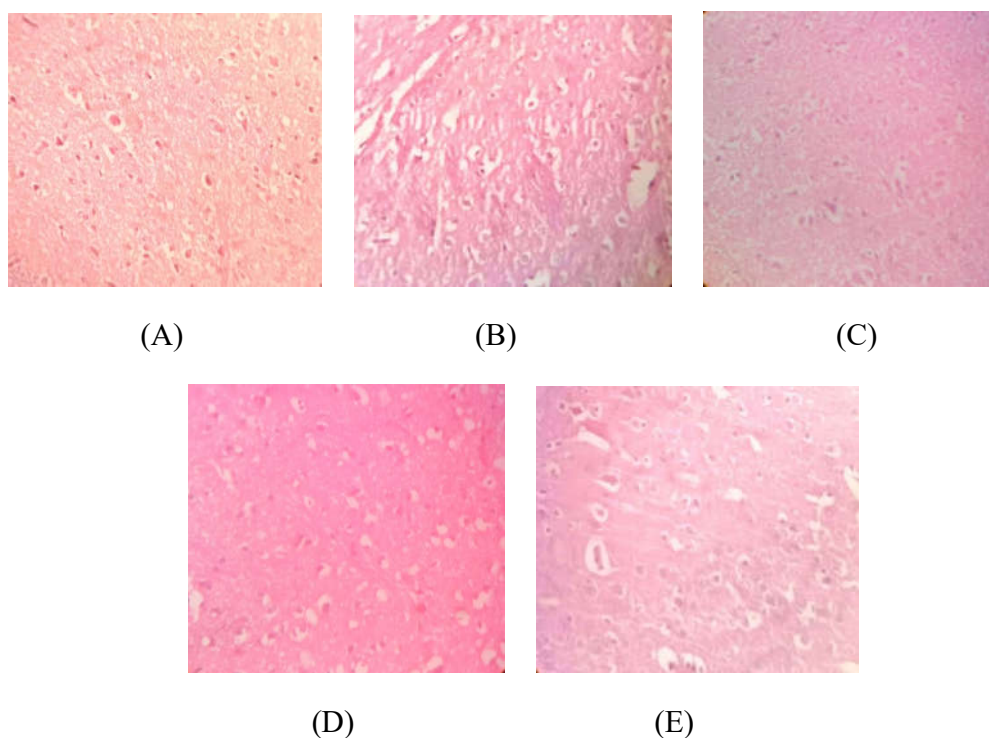


Figure 5. Histopathological representative photographs (H & E stain) of hippocampal brain sections.

(A) Normal control (B) BCCAO treated (C) Donapezil and (D&E) PHE 200mg/kg and 100mg/kg treated ischemic groups respectively, which were observed under 40X magnification.

4. Discussion

Medicinal plants have long been used in traditional mythology in various cultures throughout the world. Recently, a scientific interest for phytotherapy has increased in various aspects especially the researches targeting at justifying the reputations of medicinal plants in traditional customs and at their possible underlying mechanism.

PHE showed not only cognitive enhancing effect but also neuroprotective effect. This formulation could attenuate the neurodegeneration and could disturb the function of the affected areas. It has been reported that neurodegeneration occurs as the result of various factors including oxidative stress. Previous evidences demonstrated that oxidative stress is strongly scavenged by polyphenolic compounds including flavonoids which are found in herbal extracts [30]. Our data showed that PHE significantly enhanced the activities of CAT, DPPH and decreased in MDA, AchE levels in the hippocampus area. The decrease in MDA level reflected the decreased attack of oxidative stress at lipid component, the main component of membrane including neuronal membrane resulting in the increased survival of neuron and cholinergic neuron in the hippocampus.

Hippocampus is regarded as a brain region essential for intact cognitive abilities and appears to be particularly vulnerable to the oxidative stress during aging [31,32]. The neurodegeneration and the degeneration of cholinergic neuron in hippocampus contribute the important role on the spatial memory or hippocampal dependent memory [33-37]. therefore, the enhanced neuron density in hippocampus might also responsible in part for the cognitive enhancing effect of PHE.

Therefore, our results suggested that the cognitive enhancing effect of PHE might produce through mechanisms like, the suppression of AchE leading to the elevation of Ach which is a neurotransmitter playing an important role on learning and memory and the enhanced neuron density in hippocampus via decreased oxidative stress induced by the increased antioxidant enzyme activities as shown in figure 5. The possible active ingredients which contributes the role on the neuroprotective and the cognitive enhancing effect of PHE might be polyphenolic compounds especially quercetin where previous evidences showed both the cognitive enhancing and neuroprotective effect [38,39].

5. Conclusion

The results obtained from this study proves that PHE, a polyherbal ethanolic extracts of parts *Citrus lanatus* seeds, *Cucumis sativus* peel and *Psidium guajava* leaves which provides beneficial effect on the nervous system. It can enhance learning and memory in cognitive decline states. In addition, it also shows neuroprotective effect. The possible underlying mechanism occurs partly through the enhanced Ach and the decreased oxidative stress. However, this study is only preliminary study and further studies are necessary too fully elucidate the possible active ingredients, the detail mechanism of action of the PHE. Moreover, further development of the standardized product of PHE so that it can be used easily at home when required is still necessary.

6. Acknowledgment

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7. Conflicts of interests

The authors declare no conflicts of interest

8. References

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