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NEUROPROTECTIVE EFFECT OF PSIDIUM GUAJAVA (GUAVA) LEAF EXTRACTS ON CEREBRAL ISCHEMIC REPERFUSION INJURY INDUCED COGNITIVE IMPAIRMENT RATS

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Abstract:

Objective: *The present study was designed to investigate the neuroprotective effect of Psidium Guajava (PG) on bilateral common carotid artery occlusion (BCCAO) for 30 minutes, followed by 7 days reperfusion induced cognitive impairment in Wistar albino rats.*

Materials and methods: *Cognitive function was assessed by the Morris water maze, Rectangular maze test and locomotor activity test. To study the activity, rats weighing 250-300g were pretreated with successive extracts of n-hexane fraction (HF), ethyl acetate fraction (EAF), ethanol fraction (EF) and 50% hydro-ethanol fraction (HEF) of 400 mg/kg, 200 mg/kg, p.o of each for 10 days and the treatment was continued for another 7 days after cerebral ischemia. Biochemical parameters of oxidative stress were estimated in the brain after the treatment.*

Results: *There was significantly increased oxidative stress and cholinesterase activity with cognitive decline in the hippocampus in rats of BCCAO group as compared to normal group ($p<0.05$). The animals treated with Donepezil, HEF and EF of PG prevented the biochemical changes significantly ($p<0.001$) and there was significant improvement in cognitive parameters compared to BCCAO group. Whereas HF and EAF fractions of PG were shown poor significant improvement in cognitive and biochemical parameters.*

Conclusions: *BCCAO led to hippocampal oxidative stress with corresponding cognitive decline. Memory improving effect and antioxidant property of PG HEF and EF markedly improves in a dose dependent manner, which may be responsible for the prophylaxis and treatment of global cerebral ischemia.*

Keywords: *Antioxidant, bilateral common carotid artery occlusion, cognitive impairment, oxidative stress, Psidium guajava.*

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INTRODUCTION:

Worldwide, Ischemic stroke is a foremost cause of severe long-term disability and is characterized by a significant cognitive learning and memory deficit. Neuroprotective approaches are being investigated to reduce such deficits after an ischemic stroke. Significant depletion of oxygen supply in stroke may cause ischemia of the whole brain or of some cerebral regions depending on cerebral artery occlusion. There are rising evidences which suggest that involvement of oxidative stress in brain damage resulted in cerebral ischemia and stroke.[1] Cerebral ischemia followed by tissue reperfusion contributes oxygen for different types of enzymatic oxidative reactions in ischemic tissues and causes overproduction of free radicals. [2]

Cerebral ischemia/ Reperfusion injury (IRI), is characterized by inadequate oxygen availability and restoration of blood flow, involves complex mechanisms which can result in damage to the tissue. [3] It often contributes disturbances of blood flow which produces hypoxia and hypoglycemia, then stimulating neuronal cell death. Growing evidences to support that cerebral IRI cause neurological deficit with learning and memory impairment. [4] Hippocampal neurogenesis is observed in memory and learning. Pro-inflammatory cytokines delivered in the periphery and neuronal apoptosis resulted by cerebral ischemic reperfusion may cause possible injury to the hippocampal cells, resulting into learning and memory impairment. [5]

Not all the defence comes from antioxidants formed in the body; a considerable amount comes from nutritional components that also act as antioxidants. Hence antioxidants have been the center of studies for inventing neuroprotective agents to be used as remedy for cerebral injury by ischemic reperfusion, which is an acute and progressive neuro-degenerative disorder. In animal studies, flavonoids and Polyphenolic compounds have revealed to be neuroprotective in IRI induced brain damage. *Psidium guajava* leaves are rich in phenolic compounds and flavonoids with high antioxidant activity. [6]

The synthetic antioxidant agents are commercially accessible, but these produce adverse effects to human beings and animals. [7] There is a rising attention in herbal medicines because of their effectiveness and insignificant side effects. The herbal extracts have good antioxidant actions due to the existence of a variety of chemicals such as phenols, flavonoids, proanthrocyanidins and flavanols. *Psidium guajava (*PG*)* Linn is a medicative plant belongs to the family Myrtaceae. It is a popular conventional therapeutic plant used in various indigenous systems of medicine and widely distributed throughout India. Different parts of the *P. guajava* are evidenced to be used in general medicine. The main active substances in guava leaves are gallic acid, caffeic acid, guaijaverin, tannins, carotenoids, and triterpenoids. The leaves of PG are rich in flavonoids, especially, quercetin. Most of the PG*'s* curative activities are due to those flavonoids. [8] The various active substances of this plant leave have been extracted by using several solvents such as n-hexane, ethyl acetate, ethanol and (50 %) hydro ethanol (non polar to polar) in this study. The articles also suggesting the most efficient solvent for the antioxidant effectiveness of guava leaves. [9]

Therefore, this study was planned to explore the neuroprotective effect of n-hexane, ethyl acetate, ethanol and (50 %) hydro ethanol successive extracts of PG on cerebral ischemic reperfusion injury. However, no work has ever performed to assess the neuroprotective effect of these successive solvent extracts of PG on cerebral IRI. Thus, it was considered valuable to investigate the effect of *P. guajava* on BCCAO induced cognitive impairment and oxidative stress in rats.

MATERIALS AND METHODS:

Chemicals and drugs:

Donapezil, Thiopentone sodium, Hydrogen peroxide, povidone-iodine powder, 5% w/w (Sri medical & surgicals) ethanol, ethyl acetate, n-hexane (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).

Plant material:

The fresh leaves of *P. guajava* were collected from local market of Warangal district, Telangana state, India. The collected samples were authenticated morphologically (Voucher specimen no. ENM/SU 0019024) by Dr. E. Narasimha Murthy, Department of botany, Shatavahana University, Karimnagar-505002, Telangana state, India.

Plant material extraction and fractionation:

Shade dried leaves of *P. guajava* were powdered (500g). This fine powder was extracted in soxhlet apparatus with ethanol for about 36 hours. The ethanolic extract was cooled, filtered. The filtrate was concentrated by using a rotary evaporator under reduced pressure till the concentrated mass was obtained.

The concentrated ethanol extracts were further subjected to partial fractionation with solvents of increasing polarity viz. Hexane: Ethyl acetate: ethanol: water. Ethanol extracts were dispersed in the non-polar solvent, Hexane. Hexane dissolved part, which is called hexane fraction (HF), it was filtered and concentrated by evaporating the solvent under reduced pressure. The remainder thus obtained was fractionated subsequently with medium polar solvents such as ethyl acetate (EAF) and finally with polar solvent, 50% hydro ethanol (HEF). The extracts were analyzed by means of thin layer chromatography.

Experimental Animals:
Male Wister albino rats weighing 250-300 g were used in this study, which were procured from mahaveera enterprises, medchal district-98. They had free access to food, water and were maintained under standard laboratory conditions with alternating light and dark cycles of 12 h each. They were acclimatized to the laboratory environment for 2 days before experimental studies. All the readings were taken during the similar time of the day, which is between 10 am and 2 pm. The animal experiments were designed as per CPCSEA guideline 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, dated: 21/10/2017).

Acute toxicity studies:
The acute toxicity of HF, EAF, EF and HEF extracts of PG leaves were determined as per the OECD guideline no, 423 (acute toxic class method). It was observed that these leaf extracts were not fatal to the rats even at the 2000 mg/kg doses. Hence, $1/5^{th}$ (400mg/kg) and $1/10^{th}$ (200mg/kg) of these doses were selected for further studies. [10]

Groups and treatment:
The Wistar Albino rats (66) of 250-300 gm weight were randomly divided into 11 groups of 6 rats each. Vehicle and test substances of PG leaves of HF, EAF, EF and HEF successive extracts (400, 200 mg/kg of each) were prepared freshly and given (p.o.) once daily for 10 consecutive days prior to the cerebral ischemia. On day 11, 60 minutes after last dose, all the groups received BCCAO for 30 minutes followed by reperfusion for 7 days. From the second (13) day after induction the animals continued with the test substances for another week days, then the animals were assessed for behavioral parameters. After all behavioral assessments, the brains of different groups were removed and assessed for biochemical parameters and histopathological evaluation. [13] The detailed treatment schedule is as in table 1.

Drug administration:
Normal saline, Donepezil, PG leaves of HF, EAF, EF and HEF successive extracts were dissolved in 0.1% CMC solution. All drugs were prepared freshly every day. Doses were given according to the respective rat weights. The detailed treatment schedule is as in table 1.

Table 1- Experimental Design

Groups		Treatment
Group-I	Normal control	No ischemia; 0.1% of CMC in normal saline (10ml/kg)
Group-II	BCCAO treated	BCCAO; 0.1% of CMC in normal saline (10ml/kg)
Group-III	Standard	BCCAO + received Donepezil (5mg/kg) oral
Group-IV	Hexane -I	BCCAO + HF (400mg/kg) orally
Group-V	Hexane –II	BCCAO + HF (200mg/kg) orally
Group-VI	Ethyl acetate-I	BCCAO + EAF (400mg/kg) orally
Group-VII	Ethyl acetate-II	BCCAO + EAF (200mg/kg) orally
Group-VIII	Ethanol-I	BCCAO + EF (400mg/kg) orally
Group-IX	Ethanol-II	BCCAO + EF (200mg/kg) orally
Group-X	Hydro-ethanol (50%)-I	BCCAO + HEF (400mg/kg) orally
Group-XI	Hydro-ethanol (50%)-II	BCCAO + HEF (200mg/kg) orally

Surgical procedure for bilateral common carotid artery occlusion:
All surgical apparatus and surgical pad were sanitized with 70% ethanol before the surgery to keep away from any kind of infection and sepsis. Before the experimentation, food to the rats was withheld during the night, but the water was liberally available. Thiopentone sodium was used to anaesthetize rats at a dose of 50mg/kg, (i.p). A midline opening was made in the area of the ventral part of the neckline and subcutaneous adipose tissue was dissected avoiding the thyroid. The omohyoid muscle was incised from

side to side a median incision and a dissection was made among the sternocleidomastoid and the sternohyoid muscles parallel to the trachea. Adventitial sheath and vagus nerve were cautiously separated from both the left and right common carotid arteries. Ischemia was induced by clamping both the arteries with the help of microaneurysmal clips (bulldog clamps). After 30 min of ischemia, the clamps were detached from both the arteries to permit the reflow of the blood through carotid arteries, which was verified visually. The neck cut area was then sutured and antiseptic powder (povidone-iodine powder, 5% w/w) was applied. The body temperature of the animals was maintained at about 37°C until recovery to prevent post-ischemic hypothermia. Without occlusion of the common carotid arteries normal control rats were received the same surgical procedure. [11,12]

Behavioral assessments:
All animals were trained for 10 days prior to the BCCAO and drug administration.

Morris water maze test:
The standard Morris water maze test was used to measure the capability of hippocampal-dependent spatial navigation learning and memory in rats.[14]The water maze was a 120-cm circular pool, filled 45 cm deep with 28±2˚C opaque water. Four equally spaced points around the border of the pool were designed as Q_1, Q_2, Q_3 and Q_4 (Quadrants). A movable circular platform (9 cm diameter) was located 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. The animals were trained to locate the platform. 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. A trial began by releasing the animal into the maze facing towards the wall of the pool. 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 rat to reach the platform was taken as the initial acquisition latency (IAL). Following 24 hours (day 13) and 8 days (day 21) after IAL, the rat was released randomly from one of the edges facing the wall of the pool. The time taken to find the hidden platform was recorded and termed as first retention latency (1^{st} RL) and second retention latency (2^{nd} RL) on day 13 and day 21after cerebral ischemia, respectively.[15]

Rectangular maze test:
The rectangular maze 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. All animals were acclimatized with a rectangular maze for a period of 10 minutes for 2 hours prior to the experiment. The rats received 2 consecutive trials of training per day in the maze for about four days. 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). 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 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. [16,17]

Locomotor activity:
The locomotor activity (horizontal activity) can be an evidence for the alertness of cerebral activity. The actophotometer was used to evaluate this activity, it consists of photoelectric cells and they are connected with a counter in the circuit. An actophotometer consists of a square arena in which the animal placed, the beam of light falling on the photocell gives cut off by animal, that count was recorded. [18] Each rat of all groups was observed on days 1, 7 before induction and 13, 21 after induction with respective treatment was placed individually in the actophotometer for 5 minutes, activity score was noted.

Homogenization, biochemical and histopathological examination:
On the day 21, after all behavioral assessments, the rats of different group were sacrificed by decapitation under deep anesthesia and brains were removed, weighed and homogenized with 10 times ice cold 0.1M phosphate buffer (pH 7.4). Then it was centrifuged at 3000 rpm at 4°C for 15 minutes and the supernatant was used for biochemical estimations

Measurement of Acetyl Cholinesterase (ACHE):

Acetyl cholinesterase (AChE) is a marker of extensive loss of cholinergic neurons in the forebrain. The AChE activity was assessed by Ellman method. The change in absorbance was measured for 2 minutes at 30-second interval at 412nm using spectrophotometer. Results were expressed as micromoles of acetylthiocholine iodide hydrolyzed per minute per mg protein. [19,20]

Lipid Peroxidation (MDA):

The extent of lipid peroxidation in the brain was determined as described by Wills. The amount of malondialdehyde (MDA) was measured by reaction with thiobarbituric acid at 532nm using spectrophotometer. The values were calculated using the molar extinction coefficient of chromophore (1.56 × 105 (mol/L) $^{-1}$cm^{-1}). [21,22]

Catalase (CAT):

Catalase activity was assessed by the method of Luck [21], wherein the breakdown of hydrogen peroxide is measured. Briefly, the assay mixture consisted of 3mL of H_2O_2 phosphate buffer and 0.05mL of the supernatant of the tissue homogenate. The change in absorbance was recorded for 2 minutes at 30-second interval at 240nm using spectrophotometer. The results were expressed as micromoles of H_2O_2 decomposed per minute per mg protein. [23]

Free Radical Scavenging Activity (DPPH):

The free radical scavenging activity of the test drug was measured *in vitro* by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay [24]. In this, measurement is made from the bleaching of purple coloured methanol solution of DPPH. To the 1000 L of diverse concentration of the homogenate, 4mL of 0.004% methanolic solution of DPPH was added. After
30 min incubation in dark, absorbance was read at 517nm. Inhibition of free radical by DPPH in percentage was calculated in the following way:

$\% = (A_{blank} - A_{sample} / A_{blank}) \times 100$

A_{blank}: absorbance of control reaction. A_{sample}: absorbance of test sample. Values of inhibition were calculated

Histopathological examination:

The hippocampal region of the brain separated and was fixed in (10% v/v) formalin for 24 hours and were embedded in paraffin and stained with Heamtoxylin-Eosin. They were assessed microscopically at 40x magnification.

Statistical analysis:

Results were expressed in mean ± 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 $p < 0.05$. Statistical analysis was executed using Graph pad Prism 7 statistical software.

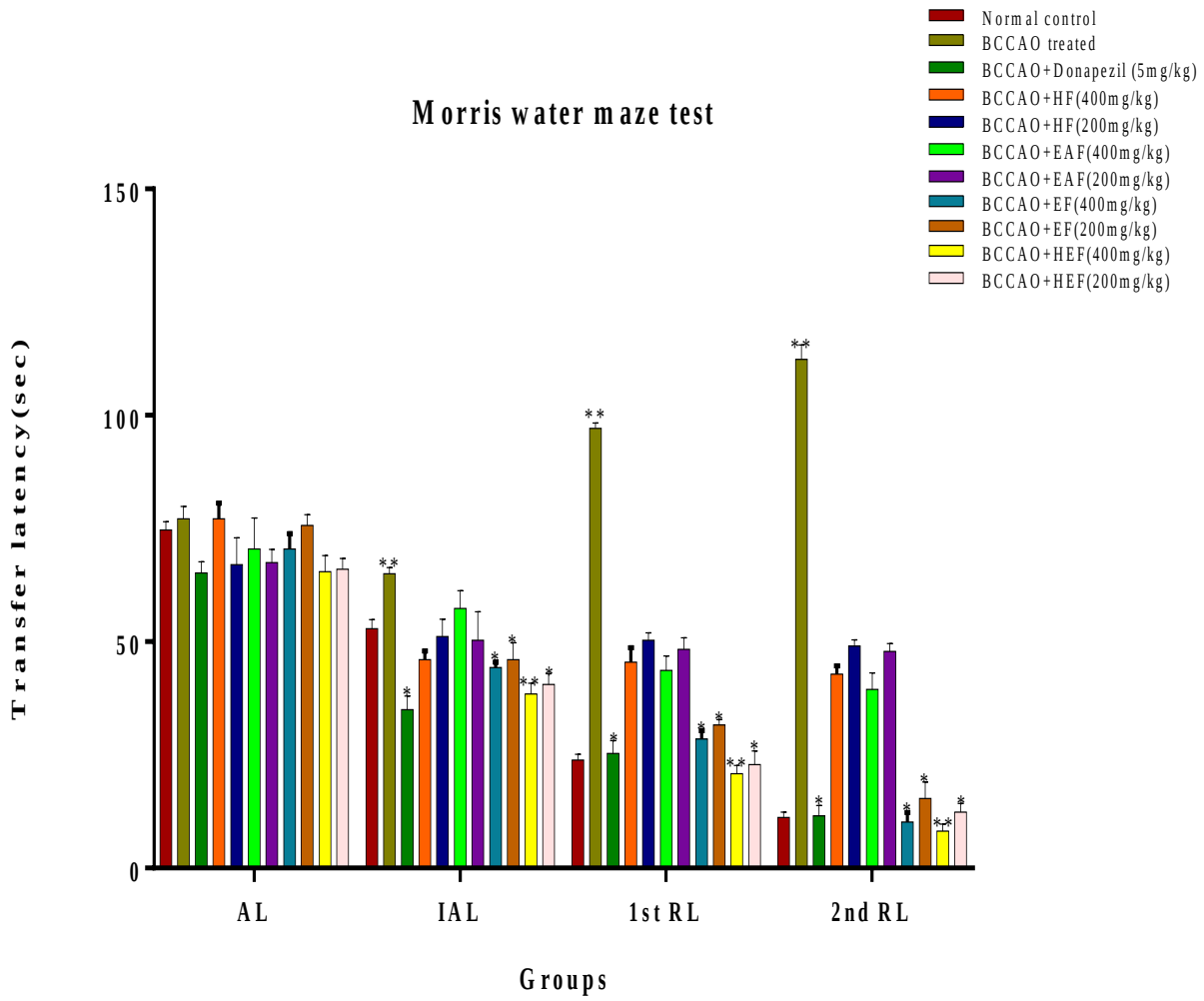
RESULTS:

Behavioral tests:

Sham-operated, donapezil (5mg/kg, PO) and PG various extracts (HF, EAF, EF and HEF of 400 and 200 mg/kg, PO) group of animals quickly learned to swim directly to the platform and passage from entry to reward chamber in the Morris water maze and rectangular maze respectively on day 12. From figure 1, 2 & 3 there was a significant difference in the mean IAL and ITL of BCCAO treated group compared to normal control group on day 12 indicating BCCAO induced impaired acquisition of spatial navigation task ($P < 0.05$). In contrast, PG various extracts (HF, EAF, EF and HEF of 400 and 200 mg/kg, PO) treatment after cerebral ischemia significantly decreased the IAL and ITL to reach the platform and reward chamber respectively in the pretrained rats as compared to BCCAO treated rats on day 12. Following training, the mean retention latencies (1st and 2nd RL) were significantly decreased in sham-operated, donapezil (5mg/kg, PO) and PG various extracts (HF, EAF, EF and HEF of 400 and 200 mg/kg, PO) group rats on days 13 and 21, as compared to IAL on day 13 after induction. On the contrary, the performance in the BCCAO treated rats was changed after initial training in the water and rectangular maze on days 13 and 21, with significant increase in mean retention latencies compared to IAL and ITL respectively on day 12. The results suggest that BCCAO caused significant cognitive impairment. The mean scores of locomotor activity for each rat were showed significant variation among different groups. The mean scores in sham-operated, donapezil (5mg/kg, PO) and PG various extracts (HF, EAF, EF and HEF of 400 and 200 mg/kg, PO) rats cause significant increase in locomotor activity compared to BCCAO treated rats on days 13 and 21 as compared to pretraining locomotor activity on day 1 and 7. Especially, the donapezil, HEF and EF treated rats exhibited significant ($p<0.001$) increase in locomotor activity and decrease in latency times compared with the BCCAO treated group. But there were no significant differences were observed in HF, EAF treated animals of behavioral parameters as compared with BCCAO treated group. This result representing that HEF and EF significantly repaired

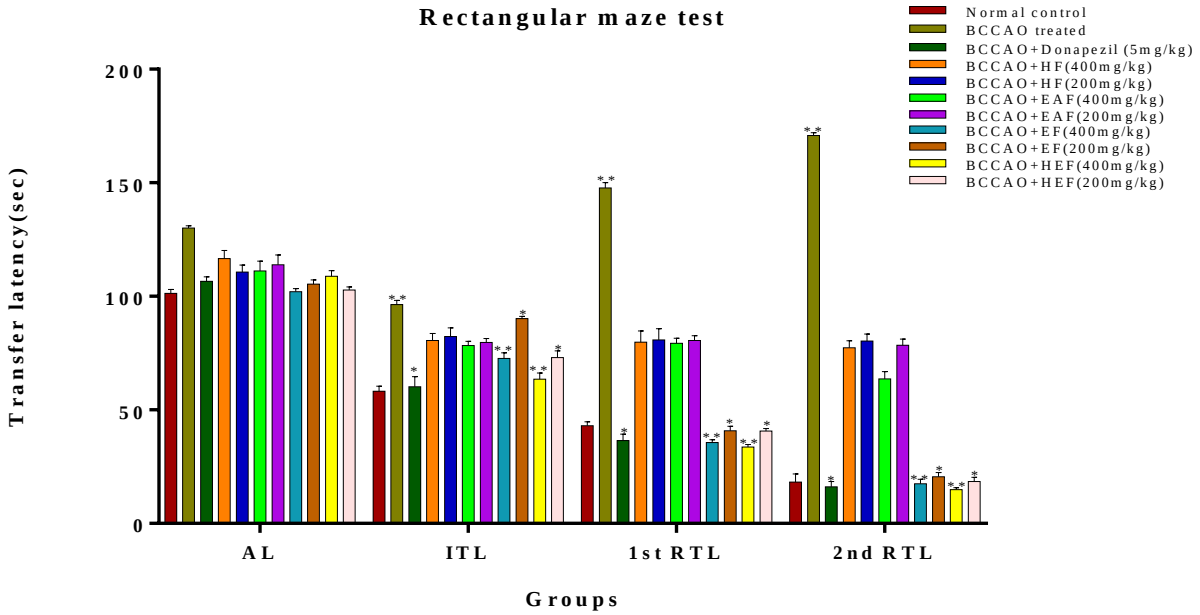
the spatial cognitive and memory deficits induced by ischemia.

Fig 1- Effect of Donepezil, PG leaves of HF, EAF, EF and HEF successive extracts on escape latency time compared to the BCCAO treated group.



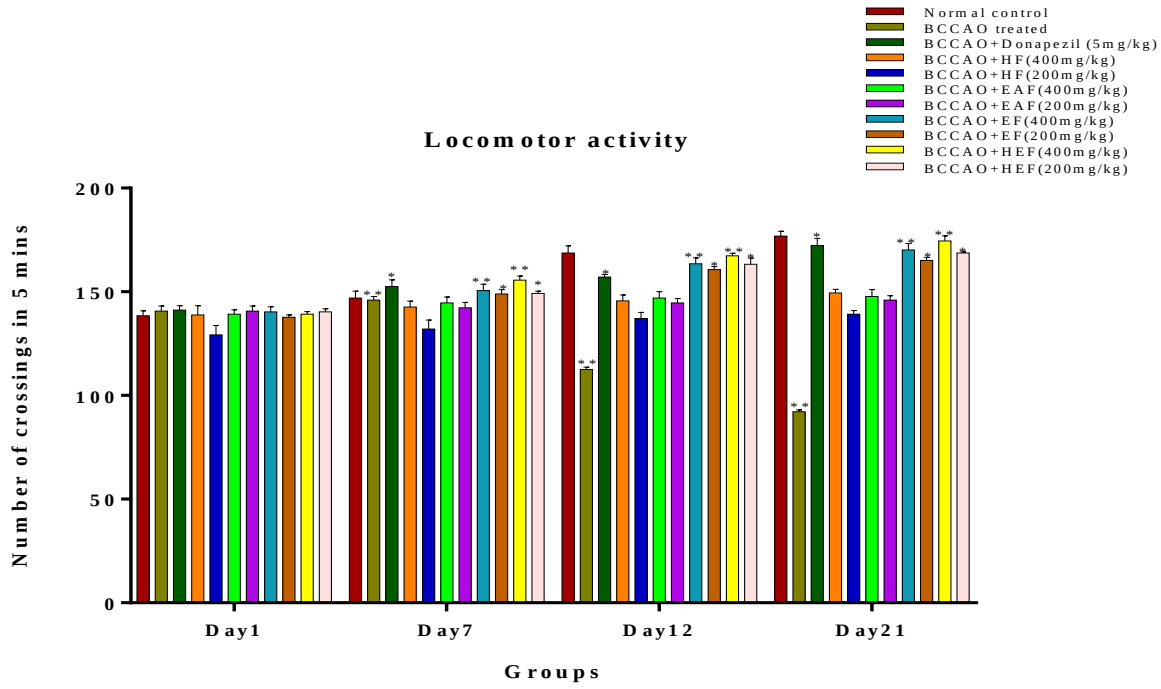
Values are expressed as Mean ± SD (n = 6) of latency time in seconds. * p<0.05, ** p<0.01 as compared with corresponding values of disease control group. (One-way ANOVA followed by dunnett's test)

Fig 2- Effect of Donepezil, PG of HF, EAF, EF and HEF successive extracts on transfer latency time compared to the disease BCCAO treated group.



Values are expressed as Mean ± SD (n = 6) of latency time in seconds. * p<0.05, ** p<0.01 as compared with corresponding values of disease control group. (One-way ANOVA followed by dunnett's test)

Fig 3: Effect of Donepezil, PG leaves of HF, EAF, EF and HEF successive extracts on locomotor activity compared to the BCCAO treated group.



Values are expressed as Mean ± SD (n = 6) of latency time in seconds. * p<0.05, ** p<0.01 as compared with corresponding values of disease control group. (One-way ANOVA followed by dunnett's test)

Biochemical assays:

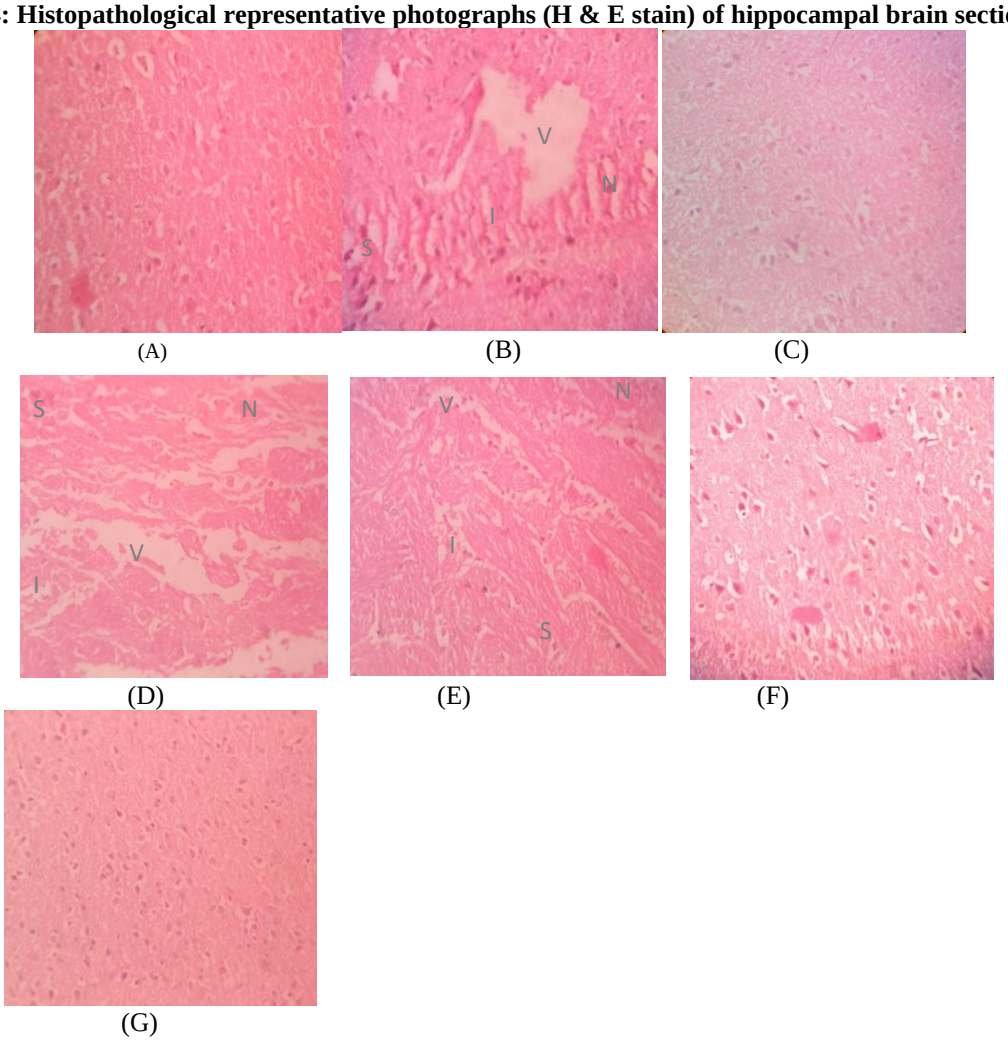
After cerebral ischemia followed by reperfusion, there was a significant increase in oxidative stress in BCCAO treated group, which is indicated by increase in LPO, AchE and decreased in CAT, DPPH assay in the brain as compared to a normal control group. The animals of HEF, EF treated had showed significant ($p< 0.01$) decrease in MDA, AchE levels and increase in CAT, DPPH activities when compared with animals. However, there were no significant differences were observed in HF, EAF treated animals of oxidative stress parameters like LPO, AchE, CAT and DPPH activities as compared with disease control group, which have given in table 2.

Values are expressed as Mean ± SD (n=6) * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns- non significant as compared with corresponding values of disease control group (one-way ANOVA followed by dunnett's test).

Histopathological studies:

From the figure 4 histopathological study, it was observed that 30 minutes of BCCAO followed by 7 days reperfusion produced shrinkage, atrophy and necrosis of neurons along with the vacuolization and inflammatory infiltration in the hippocampal regions of BCCAO treated animals when compared with normal control animals. The reactive changes were significantly attenuated in the HEF, EF and donapezil pretreated animals as compared to BCCAO treated animals. However, no significant changes were observed in the HF and EAF treated animals when compared to BCCAO treated animals.

Fig 4: Histopathological representative photographs (H & E stain) of hippocampal brain sections.



(A) (B) (C)

(D) (E) (F)

(G)

(A) normal control (B) disease control (C) Donapezil (D, E, F, G) *P. guajava* extracts of HE, EAF, EF and HEF 400mg/kg treated ischemic groups respectively, which were observed under 40X magnification.[inflammatory infiltration (I), Necrosis (N), shrinkage (S) vacuolization (V)

DISCUSSION:

The successive extracts of PG at 2000mg/kg b. wt. po dose during acute toxicity study were found devoid of mortality of any animals, which revealed the safety of the HF, EAF, EF and HEF in doses up to 2000 mg/kg b. wt. po. Hence, an optimal dose of 400 and 200mg/kg b. wt. po of successive extracts of PG was selected here for the experimental study.

In the present investigation showed the neuroprotective potential of the HEF and EF of PG extracts against BCCAO for 30 minutes, followed by reperfusion for 7 days induced oxidative stress as well as histopathological alterations. The activities of fractional extracts appeared to work by restoring the altered antioxidant enzymes. There is extensive evidence which supports the responsibility of ROS in the pathogenesis of BCCAO induced oxidative stress in brain,[25]

IRI produces marked and most likely, everlasting decrease of cortical and cerebral blood flow in various areas of the brain, specifically which damages hippocampal region and is associated with behavior alterations that are similar to the clinical symptoms observed in stroke patients. [26] Therefore, the model of BCCAO and reperfusion was used in this research work to induce global cerebral ischemia.

Dementia is frequently observed after stroke. The 4-year follow up evaluation revealed that, about 1/3 rd of stroke survivors predictable during hospitalization was observed to meet the criteria for dementia. [28] Cerebral IRI impairs memory function because of its influence on hippocampal neurons. [28] The major finding of the present study is that the BCCAO followed by reperfusion injury was related with oxidative stress, as indicated by the increase in brain MDA, AchE levels and depletion of cerebral endogenous antioxidant status (CAT, DPPH) in the BCCAO treated group compared to normal control PG extracts of the HEF and EF treated groups were showed significant decrease in brain MDA, AchE levels and increase in antioxidant enzyme activity. This supports the antioxidant actions of test drugs.

Donepezil is one of the drugs belongs to reversible AchE inhibitor that increases the concentration of acetylcholine especially in the surviving neurons of cerebral cortex and hippocampal regions of brain by blocking the enzyme AchE which destroys acetylcholine. [29] This increase is believed to be responsible for the improvement of memory. Thus, 5mg/kg b. wt., p.o donepezil was used for the improvement in memory. It is clearly evident from the present study that the latency time of the Morris water maze, rectangular maze were significantly ($p< 0.05$) decreased while locomotor activity was significantly ($p<0.05$) increased in donepezil, HEF and EF treated animals as compared with BCCAO animals indicating the stimulatory action of these drugs on the cholinergic system. Hence, the memory enhancing effect PG extracts of HEF and EF can be attributed to its anti-AchE activity. Recently, natural antioxidants playing a major role as a cancer chemo preventive agent, which are able to postpone or inhibit the oxidative damages produced by free radicals. [30]

CONCLUSION:

Thus, the present study suggests that chronic administration of PG extracts of HEF and EF reduced oxidative stress and improved cognition which may be responsible for PG mediated protection of hippocampal cholinergic neurons in BCCAO animals. However, further investigation is acceptable to explore the possible involvement of other neurotransmitters which may responsible for the memory improvement.

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Conflicts of interest:

The authors declare no conflicts of interest.

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