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Neuroprotective and antioxidant role of *Phoenix dactylifera* in permanent bilateral common carotid occlusion in rats

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ABSTRACT

Objective: To investigate neuroprotective and antioxidant effect of *Phoenix dactylifera* (*P. dactylifera*) (PD) fruits. **Methods:** Methanolic extract of *P. dactylifera* fruits (MEPD) at doses of 30, 100 and 300 mg/kg was studied against permanent BCCAO (long-term hypoperfusion) in rats. Chronic occlusion of bilateral common carotid arteries (BCCA) caused significant elevation in malondialdehyde levels due to increased lipid peroxidation as well as decrease in levels of other biochemical enzymes *i.e.* glutathione, glutathione peroxidase, glutathione reductase, glutathione-S-transferse, catalase and superoxide dismutase. **Results:** Post occlusion treatment for 15 d with 100 and 300 mg/kg doses of MEPD significantly reduced the enhanced malondialdehyde levels and reversed the alterations in the declined levels of antioxidant enzymes in brain homogenates of hypoperfused rats. Long-term cerebral hypoperfusion in rats caused a propensity towards anxiety and restlessness (open field paradigm) accompanied by deficits of spatial learning and memory (Morris water maze testing). Additionally, histopathological observations in hypoperfused brains revealed reactive changes like shrinkage and necrosis of neurons. 100 and 300 mg/kg doses of MEPD significantly alleviated these alterations. **Conclusions:** These results confirmed the protective role of *P. dactylifera* in ischemia hypoperfusion and thereby it's beneficial role in cerebrovascular insufficiency states and related complications.

1. Introduction

Chronic cerebral hypoperfusion, a moderate (20%-40%) but persistent reduction in cerebral blood flow (CBF), has been associated with many brain and vascular pathologies such as age-related disorders, cerebrovascular diseases, hypoxic-ischemic brain injury, carotid artery pathologies[1]. It has been suggested that chronic cerebral hypoperfusion plays a critical role in the pathological process of neurodegeneration and cognitive impairment, which negatively impact the quality of life in humans[2].

Permanent occlusion of bilateral common carotid arteries (Permanent BCCAO) in rats is a chronic cerebral hypoperfusion model that results in significant reduction of cerebral blood flow and can cause learning and memory impairment, neuronal damage as well as biochemical alterations due to oxidative stress resembling the effects observed in vascular dementia[3].

This model is useful to understand the pathophysiology of chronic cerebrovascular disorders and to screen drugs with potential therapeutic value in vascular dementia[4]. Long-term cerebral hypoperfusion induced by permanent BCCAO causes reduction of blood flow from about 30%-45% in the cortex and about 20% in the hippocampus with resultant decrease in glucose utilization by 20%-30% and 15%, respectively[5]. This chronic reduction in blood flow has been repeatedly shown to persist to a significant level for at least one month after permanent BCCAO and contributes to

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behavioral and cognitive deficits[6].

During the last two decades, pharmacotherapy with psychoactive drugs has been increasingly recognized as most effective in the management of anxiety, stress and psychosomatic and cerebral disorders. However, the prolonged use of synthetic drugs leads to a variety of autonomic, endocrine, allergic, hematopoietic and neurological side effects. Moreover, such agents primarily relieve the symptoms and offer a palliative relief of a temporary nature[7]. In recent years, there has been a phenomenal rise in the interest of scientific community to explore the pharmacological actions or to confirm the veracity of claims made about herbs in various official books of herbal medicine. In today's life of stress and strain, there is a need for agents having neuroprotective and neuropharmacological activity enhancing learning and memory function of the brain[8]. *Phoenix dactylifera* (*P. dactylifera*) (date palm) belonging to family Arecaecae is considered to be the most important fruit tree in most of the Arabian countries[9]. In local medicinal practices date fruits are considered as tonic and aphrodisiac, and in some communities they are thought to be useful against ulcer. In fact, muslims believe that "He who eats seven dates every morning will not be affected by poison or magic on the day he eats them"[10]. Different parts of this plant are traditionally claimed to be used for the treatment of a broad spectrum of ailments including memory disturbances, fever, loss of consciousness and nervous disorders[11,12]. Several studies indicate that consumption of fruits and vegetables is associated with reduced risk of several chronic diseases[13]. *P. dactylifera* (PD) fruits have been documented possess antioxidant activity due to the presence of water-soluble compounds with potent free radical-scavenging effects, such as phenolic compounds (mainly cinnamic acids) and flavonoids (flavones, flavonols and flavanones)[14]. PD fruits are proved to possess carotenoid content, consumption of which has been related to prevention of cancer, cardiovascular diseases and other degenerative processes involving oxidative stress. The plant is scientifically documented for its antiulcer, anticancer, anti-diarrhoeal, hepatoprotective, antimutagenic, antioxidant, aphrodiasiac, antiinflamatory, antimicrobial, antihyperlipidemic and nephroprotective properties but it has yet not been evaluated for its antioxidant and neuroprotective activity against permanent BCCAO[15]. Therefore the present investigation was carried out to evaluate the antioxidant and neuroprotective activity of Methanolic extract of *P. dactylifera* fruits MEPD against permanent BCCAO.

2. Materials and methods

2.1. Plant material

Fresh fruits of *P. dactylifera* were collected from local market and authenticated by Botanical survey of India (Voucher specimen number: BSI/WC/Tech/2009/674). The methanolic extract of dried fruits was prepared in the approved laboratories of Green Chem, Bangalore, India using procedure mentioned below.

2.2. Preparation of extract

The *P. dactylifera* fruits were manually separated from the pits, crushed and cut into small pieces with a sharp knife and dry blended for 3 min using a stainless-steel blender. Then they were extracted with methanol-water (4:1, v/v), at room temperature (20 ℃ for 5 h using an orbital shaker). The extracts were then filtered and centrifuged at 4 000 *g*, for 10 min and the supernatant was concentrated under reduced pressure at 40 ℃ for 3 h using a rotary evaporator to obtain the methanolic extract. The crude extract was kept in dark glass bottles for three days inside the freezer until use.

2.3. Chemicals and drugs

Thiobarbituric acid and trichloroacetic acid were purchased from SD fine chemicals, Mumbai, India. Nicotinamide adenine dineuleotide phosphate (NADPH), oxidized glutathione and reduced glutathione were purchased from Vijay chemicals, Pune, India. Bovine serum albumin was purchased from Spacelab, Nashik India. Phenazine methosulphate, nitroblue tetrazolium, 1-chloro 2,4 dinitrobenzoic acid were purchased from Anand agencies, Pune, India. NADH, Folin Ciocalteau reagents were purchased from Bansal sales, Pune, India. Other chemicals and solvents used were of analytical grade purchased from commercial suppliers.

2.4. Animals

Wistar rats weighing 200-250 g were used. They were caged in a room under standard laboratory conditions (temperature (23±1) ℃, relative humidity (55±5)% and lighting 08:00-20:00 h). The rats were fed on a pelleted diet (Amrut feed, Pune, India) and water ad libitum. The rats were transferred to the laboratory at least 1h before the start of the experiment. The experiments were performed during day (08:00-16:00 h).

2.5. Ethical clearence

All the studies were carried out in accordance with the guidelines given by the Indian Council for Medical Research and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi (India) and the Institutional Animal Ethical Committee approved the study (Approval No.: CPCSEA/IAEC/PC-04/07-2K8).

2.6. 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[16].

2.7. Preliminary acute toxicity test

Healthy adult male albino mice (18-22 g) were subjected to acute toxicity studies as per guidelines (AOT 425) suggested by the organization for economic co-operation and development (OECD-2000). The mice 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[17].

2.8. 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 permanent BCCAO control group. Third, fourth and fifth groups served as test groups i.e. drug treated permanent BCCAO groups and received MEPD at doses of 30, 100 and 300 mg/kg respectively. All rats except sham operated were subjected to permanent BCCAO for 15 d. Treatment with either vehicle or MEPD (30, 100, 300 mg/kg) was started at 60 min after the surgery and continued for 15 d. On 15th day all rats were subjected to the behavioural studies i.e. open field test and morris water maze test. After behavioural study all the animals were sacrificed and their brains were isolated and subjected to biochemical analysis and histopathological evaluation[18].

2.9. Surgical procedure for permanent BCCAO

Surgical procedures were performed between 8:00 a.m. and 1:00 p.m. All the surgical equipments 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 anaesthetised by an intraperitoneal injection of 100 mg/kg ketamine 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 permanent BCCAO was performed by permanently occluding bilateral common carotid arteries. The carotid arteries were doubly ligated with 3-0 silk sutures and cut in between. The skin was then sutured with 3-0 silk suture. Sham control animals received the same surgical procedures, except bilateral common carotid arteries were not occluded. Core temperature (rectal) was maintained at (37.0±0.5) ℃ throughout the surgical process using a heating lamp. 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[18].

2.10. Behavioral testing

2.10.1. Open field test

Locomotor activity was evaluated in an open field paradigm[19]. The open field was made of plywood and consisted of a floor (96 cm×96 cm) with high walls (61 cm × 61cm). Entire apparatus was painted white except for 6 mm thick blacklines that divided the floor into 16 squares. During the experimentation the field was illuminated with two 40 W light bulbs. Each animal was placed at one corner of the apparatus. After 1 min adaptation, the behavior of each animal was recorded for 5 min by two observers 1 m away from the open-field area. During the trial intervals, the animals were returned to their home cages in the same room and the open field was wiped clean with a slightly damp cloth. The behavioral parameters were analysed as the following:

- Ambulations: the number of grids crossed in the arena during the observation period.
- Rearings: the number of times the mouse stands on its hind legs.
- Groomings: the number of times the mouse washes itself by licking, wiping, combing or scratching of any part of the

body.

•Immobility: Total time for which the animal freezes without making any movement.

•Number of feacal pellets.

2.10.2. Morris water maze

Cognitive function was tested in the Morris water maze[20]. The maze consisted of a black circular pool (1.2 m diameter, 40 cm height) filled to a depth of 20 cm with water (22± 1) ℃. At the beginning of each day, the water was made opaque by adding milk to prevent the animals from seeing the submerged platform. On 14th day after surgery the rats received habituation (exposure in water maze for 1 min) in which there was no platform present. Then, on day 15th, a circular platform (14 cm diameter) was submerged 2 cm below the surface of the water in the center of one of the quadrants and hidden from the rat's view. Four points, equally spaced along the circumference of the pool, were arbitrarily assigned as North, South, East, and West. The pool, therefore, was divided into four quadrants (Northeast, Southeast, Southwest, and Northwest). These points served as the starting positions for the rat being gently lowered into the water, with its head facing the wall of the water maze. The platform remained in the same position during training days (reference memory procedure). All animals followed this sequence for that session. Each rat was placed in the water facing the wall at the start location and was allowed 90 s to find the hidden platform. The animal was allowed a 20 s rest on the platform. The latency to reach the platform was recorded. If the rat was unable to locate the hidden platform, it was lifted out and placed on the platform for 20 s. The procedure was repeated for all the four start locations. Two sessions of four trials each were conducted on the first day of testing separated by 4 h and one session of four trials was conducted on the next day. After that, the platform was removed and a probe trial (without platform) was conducted 4 h later. Each rat was placed in the pool at the same randomly selected starting pole and swimming path was observed and time spent in the quadrant of pool which initially contained platform was measured. On completion of the probe trial, a black platform that extended 1 cm above the surface of water was placed in a quadrant other than that chosen for the submerged platform. Each rat was then given four trials of 90 s to locate it. The latency to reach the platform was recorded (working memory procedure).

2.11. Biochemical analysis

2.11.1. Preparation of brain homogenate

The rats were sacrificed by decapitation under deep anaesthesia on 15th day after behavioural testing. The brains were immediately removed, weighed and homogenized with 10 times (w/v) ice cold phosphate buffer saline (50 mM pH 7.0) in a teflon glass homogeniser. The homogenate was centrifuged at 2 000 rpm at 4 ℃ for 20 min and the supernatant was used for measurement of various biochemical parameters[21].

2.11.2. Estimation of total protein in the brain

The total protein content in tissue homogenates was measured following the method of Lowry et al. using bovine serum albumin as a standard[22].

2.11.3. Measurement of lipid peroxidation (LPO)

As a measure of lipid peroxidation, malonaldialdehyde (MDA) levels were estimated spectrophotometrically by measuring thiobarbituric acid reactive substances (TBARS) at 532 nm[23].

2.11.4. Estimation of superoxide dismutase (SOD)

Superoxide dismutase activity was determined spectrophotometrically by measuring the inhibition of reduction of NBT to blue-colored chromogen in the presence of PMS and NADH by SOD at 560 nm[24].

2.11.5. Estimation of catalase (CAT)

Catalase measurement was carried out spectrophotometrically by measuring the ability of CAT to oxidize hydrogen peroxide (H_2O_2) at 240 nm[25].

2.11.6. Estimation of reduced glutathione (GSH)

Glutathione was measured spectrophotometrically according to the method of Ellman (1959) at 412 nm[26].

2.11.7. Estimation of glutathione peroxidase (GPx)

Selenium-dependent glutathione peroxidase activity was measured spectrophotometrically by monitoring the continuous decrease in NADPH concentration using H_2O_2 as a substrate at 340 nm[27].

2.11.8. Estimation of glutathione-S-transferase (GST)

Glutathione-S-transferase activity was determined by monitoring the formation of the thioether product from the reaction between GSH and CDNB (1-chloro, 2,4-dinitrobenzene) at 340 nm[28].

2.11.9. Estimation of glutathione reductase (GR)

Glutathione reductase activity was assayed by measuring the disappearance of NADPH at 340 nm the method of Carlberg and Mannervik (1975) at 340 nm[29].

2.12. Histopathological examination

The rats were sacrificed by decapitation under deep anaesthesia on 15th day after behavioural testing by decapitation under deep anesthesia and the brains were removed. The intact whole brains were fixed in formalin (10%, v/v). The tissue was cut into 3 mm thickness and its blocks were embedded in paraffin. The forebrain sections (5-10 m) thickness were prepared using a rotary microtome and stained with hematoxylin and eosin[21].

2.13. Statistical analysis

Results were expressed as mean±S.E.M. Statistical analysis was performed using oneway analysis of variance (ANOVA). If the overall P-value was found statistically significant ($P<0.05$), further comparisons among groups were made according to post hoc Tukey's test. The diagrammatic representation of the data was performed by using instat software (Graph Pad Instat).

3. Results

3.1. Preliminary phytochemical analysis

Preliminary phytochemical analysis of MEPD showed the presence of steroids, alkaloids, glycosides, flavonoids, proteins and carbohydrates.

3.2. Acute toxicity test

The mice treated with MEPD were found to be free of any toxicity upto the dose of 2 000 mg/kg and exhibited normal behavior. Mice were alert with normal grooming, touch response, pain response and there was no sign of passivity, stereotypy and vocalization. There was no abnormal change in motor activity, secretory signs as well as their body weight and water intake.

3.3. Behavioural testing

3.3.1. Open field test

In open-field paradigm, rats of permanent BCCAO control gorup showed marked alterations in exploratory behavior as compared to those of sham operated group. The number of ambulations were significantly ($P<0.001$) decreased in permanent BCCAO control group rats as compared to sham operated group rats. This decrease was significantly ($P<0.01$, $P<0.001$) ameliorated by treatment with MEPD 100 and 300 mg/kg. Permanent BCCAO for 15 days produced a significant ($P<0.01$) reduction in number of rearings in permanent BCCAO control group rats as compared to sham operated group rats. Treatment with MEPD 100 and 300 mg/kg significantly ($P<0.05$) attenuated this decrease. A significant ($P<0.01$) reduction in number of groomings was also observed in permanent BCCAO group rats in comparison with sham operated group rats. This reduction was significanlty ($P<0.05$) overcomed by treatment with MEPD 300 mg/kg. Accompanying this alteration in exploratory activities, the period of immobility was significantly ($P<0.001$) increased in permanent BCCAO control group rats and they exhibited more freezing behavior as compared to sham operated group rats. Treatment with MEPD 100 and 300 mg/kg significantly ($P<0.05$, $P<0.01$) attenuated increase in duration of immobility. However, no difference between the groups was observed for number of fecal pellets. The dose 30 mg/kg was nonsignificant in reversing the behavioural alterations (Table 1).

3.3.2. Morris water maze test

In the first session of escape latency trial, wherein the latency to locate the submerged platform was assessed, no significant difference was observed between the groups. The escape latencies of sham operated group rats, permanent BCCAO control group rats, MEPD 30, 100 and 300 mg/kg treated permanent BCCAO group rats in the first session of morris water maze test were (63.00±10.78), (75.90±6.67), (68.63±7.35), (66.38±9.57) and (66.77±7.68) respectively. In the

Table 1
Effect of MEPD on the ambulations, rearings, groomings and time of immobility in open field test of rats subjected to permanent BCCAO.

Group	Ambulations	Rearings	Groomings	Duration of immobility	Fecal pellets
Sham operated	74.33±5.76	30.16±4.30	8.66±1.05	25.16±5.10	2.00±0.57
Permanent BCCAO control	24.83±2.86***	13.16±2.38***	19.00±1.26***	102.33±9.02***	2.83±0.60
PD 30 + Permanent BCCAO	30.83±3.53	27.50±4.85	13.16±2.05	74.00±11.81	2.50±0.42
PD 100 + Permanent BCCAO	48.16±2.62##	29.50±3.33#	12.33±2.26#	66.50±6.26#	2.00±0.57
PD 300 + Permanent BCCAO	56.66±4.37###	36.67±3.30#	13.00±2.28##	58.33±8.42##	2.50±0.42

Results are expressed as mean + SEM. Comparison between the groups was made by one way analysis of variance (ANOVA) followed by Tukey's test *,#-$P<0.05$, **,##-$P<0.01$, ***,###-$P<0.001$; *- Permanent BCCAO control group against sham operated; #- MEPD treated ischemia group against permanent BCCAO control group.

second session, the latency to reach the submerged platform *i.e.* escape latency in permanent BCCAO control group rats was significantly (P<0.01) higher as compared to sham operated group rats. The escape latencies of sham operated group rats, permanent BCCAO control group rats, MEPD 30, 100 and 300 mg/kg treated permanent BCCAO group rats in the second session of morris water maze test were (29.47 ±3.99), (71.931±5.67), (63.64±9.67), (40.17±8.92) and (33.51 ±6.99) respectively. Whereas, MEPD 100 and 300 mg/kg treated permanent BCCAO group rats required significantly (P<0.05, P<0.01) less time to locate the submerged platform when compared with permanent BCCAO control group rats. Similarly, in third session also permanent BCCAO control group rats took longer time to find the submerged platform as compared to sham operated group rats, and this increase in escape latency was attenuated significantly (P<0.01) by the treatment with MEPD 100 and 300 mg/kg. The escape latencies of sham operated group rats, permanent BCCAO control group rats, MEPD 30, 100 and 300 mg/kg treated permanent BCCAO group rats in the third session of morris water maze test were (27.51±4.52), (69.31±4.80), (46.93±9.05), (33.21±6.55) and (32.92±6.53) respectively (Figure 1).

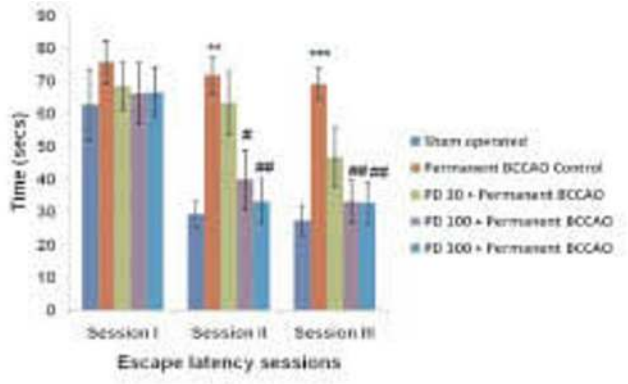

Figure 1. Effect of MEPD on the escape latency in morris water maze test of rats subjected to permanent BCCAO.

Results are expressed as mean + SEM. Comparison between the groups was made by one way analysis of variance (ANOVA) followed by Tukey's test *,#–P<0.05, **,##–P<0.01, ***,###– P<0.001; *–Cerebral Ischemia control group against sham operated; #– MEPD treated ischemia group against Cerebral Ischemia control group.

In the probe trial, a significant difference in the spatial bias of rats towards the quadrant of pool that contained the submerged platform during the escape latency sessions was observed. The time spent by sham operated group rats, permanent BCCAO control group rats, MEPD 30, 100 and 300 mg/kg treated permanent BCCAO group rats in the quadrant of the initial platform position were (53.60±5.99), (20.24±3.34), (27.23±4.07), (51.31±11.06) and (52.61±9.55) respectively. The permanent BCCAO control group rats spent a significantly (P<0.05) less time in the quadrant of the initial platform position than sham operated group rats. Treatment with MEPD 300 mg/kg significantly increased the time spent in the quadrant of the initial platform position in comparison with permanent BCCAO control group rats (Figure 2).

The new platform trial demonstrated that the permanent BCCAO control group rats took significantly (P<0.01) longer time in reaching the submerged platform as compared to sham operated group rats. Whereas, the latency to reach submerged platform was significantly lessened in the MEPD 100 and 300 mg/kg treated rats. The escape latencies of sham operated group rats, permanent BCCAO control group rats, MEPD 30, 100 and 300 mg/kg treated permanent BCCAO group rats in the new platform trial of morris water maze test were (27.38±5.78), (69.73±4.12), (51.35±10.61), (34.26±9.93) and (25.21±6.36) respectively (Figure 2).

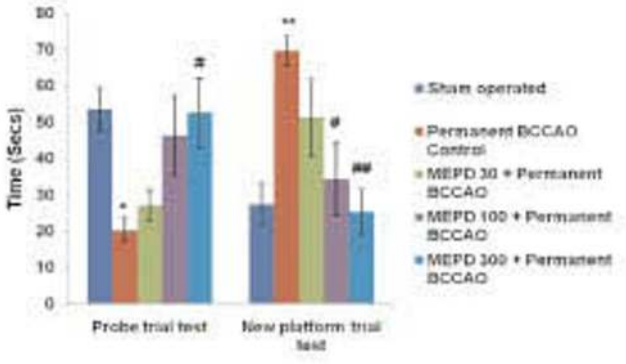

Figure 2. Effect of MEPD on the escape latency in probe trial and new platform trial tests in morris water maze test of rats subjected to permanent BCCAO.

Table 2

Effect of MEPD on MDA, CAT and SOD levels in the brains of the rats subjected to permanent BCCAO.

Groups	MDA (U/mg protien)	CAT (U/mg protien)	SOD (U/mg protien)
Sham operated	1.56±0.43	1.81±0.05	4.68±0.28
Permanent BCCAO control	3.84±0.52***	0.73±0.04***	1.82±0.11***
MEPD 30 + Permanent BCCAO	4.13±0.71	0.95±0.04	3.64±0.62#
MEPD 100 + Permanent BCCAO	2.15±0.82#	1.37±0.11###	3.63±0.37#
MEPD 300 + Permanent BCCAO	1.74±0.85##	1.70±0.16###	4.18±0.35##

Results are expressed as mean ± SEM. (n = 6). Data was analysed by one way analysis of variance (ANOVA) followed by Tukey's test. *,#P<0.05, **,##P<0.01, ***,###P<0.001. Permanent BCCAO control group rats compared against sham operated rats; #–MEPD treated permanent BCCAO group rats compared permanent BCCAO control group rats.

Results are expressed as mean + SEM. Comparison between the groups was made by one way analysis of variance (ANOVA) followed by Tukey's test *,#-$P<0.05$, **,##-$P<0.01$, ***,###-$P<0.001$; *-Permanent BCCAO control group against sham operated; #- MEPD treated ischemia group against permanent BCCAO control group.

3.4. Biochemical analysis

3.4.1. Measurement of lipid peroxidation (LPO)

The permanent BCCAO for 15 days produced a significant ($P<0.001$) rise in the malonaldialdehyde (MDA) levels of permanent BCCAO control group as compared to sham operated group, MEPD (100 and 300 mg/kg) treated permanent BCCAO group showed significantly ($P<0.05$, $P<0.01$) lowered MDA levels in comparison with permanent BCCAO control group rats. The dose 30 mg/kg was found to be ineffective in this regard (Table 2).

3.4.2. Estimation of superoxide dismutase (SOD)

SOD levels in the brains of permanent BCCAO control group rats were significantly lowered as compared to sham operated rats. 15 days post occlusion treatment with MEPD (30, 100 and 300 mg/kg) significantly ($P<0.05$, $P<0.05$, $P<0.01$) reversed this decrease in comparison with permanent BCCAO control group rats (Table 2).

3.4.3. Estimation of catalase (CAT)

There was a significant ($P<0.001$) reduction in the CAT levels in the brains of permanent BCCAO control group rats as compared to sham operated rats. MEPD (100 and 300 mg/kg) significantly ($P<0.001$) attenuated this reduction in comparison with permanent BCCAO control group rats. The dose 30 mg/kg was found to be ineffective in this regard (Table 2).

3.4.4. Estimation of reduced glutathione (GSH)

Reduced glutathione (GSH) levels in brains of sham operated rats, permanent BCCAO control group rats. Permanent BCCAO for 15 d produced a significant ($P<0.001$) decrease in GSH levels in the brains of permanent BCCAO control group rats as compared to sham operated rats. 15 days post occlusion treatment with MEPD (100 and 300 mg/kg) significantly ($P<0.05$, $P<0.05$) attenuated this decrease in comparison with permanent BCCAO control group rats (Table 3).

3.4.5. Estimation of glutathione peroxidase (GPx)

A significant decrease was found in GPx levels in the brains of permanent BCCAO control group rats as compared to sham operated rats but none of the doses of MEPD showed the ability to increase the decreased levels of GPx significantly (Table 3).

3.4.6. Estimation of glutathione-S-transferase (GST)

Permanent BCCAO for 15 d produced a significant ($P<0.001$) decrease in GST level in the brains of permanent BCCAO control group rats as compared to sham operated rats but none of the doses of MEPD were significant ($P<0.001$) in reversing the decreased levels of GST (Table 3).

3.4.7. Estimation of glutathione reductase (GR)

There was a significant reduction in the GR concentration in the brains of permanent BCCAO control group rats as compared to sham operated rats. MEPD (100 and 300 mg/kg) significantly ($P<0.05$, $P<0.01$) reversed this decrease in comparison with permanent BCCAO control group rats (Table 3).

3.5. Histopathological examination

From the histopathological study it was observed that 15 d of permanent BCCAO and the effect of MEPD treatment on the changes induced. 15 d of permanent BCCAO produced severe neuronal damage by atrophy and necrosis of the neurons along with vacuole formation in the forebrain regions of permanent BCCAO control group rats when compared with the sham operated group rats. The treatment

Table 3
Effect of MEPD on GSH, GST, GPX and GR levels in the brains of the rats subjected to permanent BCCAO.

Groups	GSH (U/mg protien)	GST(U/mg protien)	GPX(U/mg protien)	GR(U/mg protien)
Sham operated	119.18±6.94	67.41±5.18	3.44±0.19	6.02±0.20
Permanent BCCAO control	80.59±6.12***	36.60±3.06***	1.03±0.03***	2.34±0.29***
PD 30 + Permanent BCCAO	86.90±5.41	49.72±6.25	1.80±0.39	2.81±0.24
PD 100 + Permanent BCCAO	106.39±5.01#	54.75±5.41	1.85±0.15	3.91±0.45#
PD 300 + Permanent BCCAO	107.26±4.15#	54.07±4.49	1.91±0.22	4.39±0.34##

Results are expressed as mean ± SEM. (n=6). Data was analysed by one way analysis of variance (ANOVA) followed by Tukey's test. *,#$P<0.05$, **,##$P<0.01$, ***,###$P<0.001$. Permanent BCCAO control group rats compared against sham operated rats; #-MEPD treated permanent BCCAO group rats compared permanent BCCAO control group rats.

with MEPD 100 and 300 mg/kg attenuated these changes as compared to the permanent BCCAO control group. MEPD 30 mg/kg was found inefficient in reversing these changes (Figure 3).

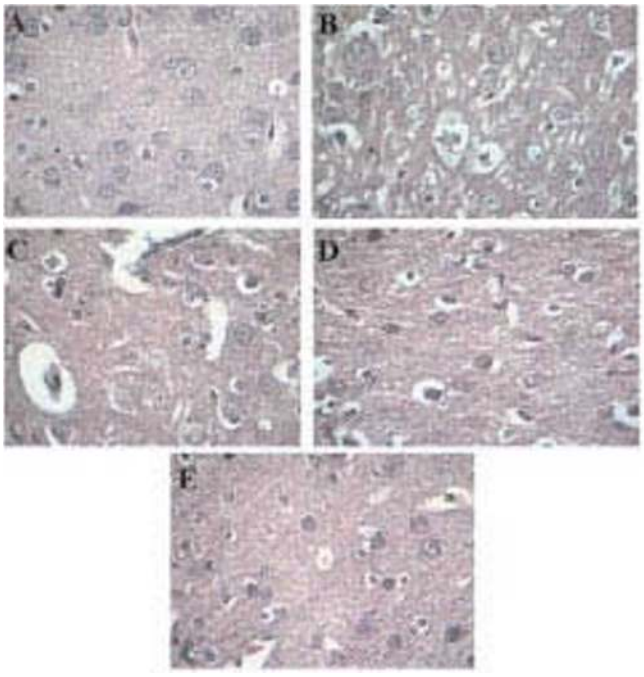

Figure 3. Representative photomicrographs (H & E stain) of forebrain sections of sham operated rats (A), permanent BCCAO control group rats (B), MEPD 30, 100 and 300 mg/kg treated permanent BCCAO group rats (C, D, E). Rats subjected to permanent BCCAO showed morphological alterations suggestive of shrinkage and necrosis of neurons and vacuolisation which was attenuated by treatment with MEPD 100 and 300 mg/kg.

4. Discussion

Chronic cerebral hypoperfusion is considered to be a factor contributing to the memory dysfunction in neurological diseases such as cognitive decline in aging, Alzheimer's disease and vascular dementia. A sudden disruption of the blood supply to distinct brain regions leads to stroke, while a moderate but persistent reduction in regional cerebral blood flow (CBF) compromises memory processes and contributes to the development and progression of dementia. The association of decreased CBF, particularly in the temporal and parietal cortices, with Alzheimer's disease (AD) has been firmly established[3,30,31]. Additionally, the degree or pattern of cerebral hypoperfusion in mild cognitive impairment has been suggested as a predictive marker for the progression to AD. Chronic BCCAO has also been used in behavioral studies as an abstracted model of reduced cerebral perfusion (chronic hypoperfusion) in aging. Furthermore, cerebral hypoperfusion can occur in patients who have suffered a cardiac arrest or those who undergo complex cardiac surgery, and this condition can lead to a poor neurologic outcome[32]. For an understanding of the role of the cerebrovascular pathology in the development of a cognitive dysfunction and dementia, it is important to explore the cerebral hypoperfusion-related metabolic changes, the distinct neurodegenerative and cognitive correlates of hypoperfusion and the causal relationships between these factors. Furthermore, recognition of particular mechanisms in the chain of events from chronic cerebral hypoperfusion to a cognitive decline may identify potential targets for effective therapies. The reconstruction of a pathological condition in animal models is a suitable approach to the unraveling of causal relationships. For this reason, permanent BCCAO in rats has been established as a procedure to investigate the effects of chronic cerebral hypoperfusion on cognitive dysfunction and neurodegenerative processes. Over the years, this model has generated a large amount of data, revealing the permanent BCCAO related pattern of cerebral hypoperfusion and metabolic changes, cerebrovascular insufficiency states, white matter lesions, neurodegenerative conditions and learning and memory disturbances, failure of neuronal signaling and the neuropathological changes in the hippocampus and thus it is considered to be suitable for the development of potentially neuroprotective strategies in neurodegenerative diseases[32-34].

The present study was aimed at investigating the potential beneficial effects of *P. dactylifera* on ischemia-induced oxidative stress as well as functional and anatomical integrity following long-term cerebral hypoperfusion in rats. It is very well documented that long-term cerebral hypoperfusion produces abnormal levels of reactive oxygen species which may initiate and sustain the cascade of neuropathological events underlying vascular dementia. Clinical research findings also indicate reduced antioxidant defense involved in the pathophysiology of vascular dementia[35]. Therefore we examined the antioxidant enzyme levels like MDA, SOD, CAT, GSH, GPx, GST and GR which served as oxidative indices in various brain regions of the permanent BCCA occluded rats. Excessive generation of reactive oxygen species (ROS) results in the lipid peroxidation of the cell membrane and subsequent damage is reflected by accumulation of MDA, a neurotoxic by-product of lipid peroxidation[36]. Neural tissue concentrations of MDA were assayed as an index of membrane oxidative damage. A marked increase in lipid peroxidation indicated by increased MDA levels in brain tissues, resulting from free-radical generation induced by chronic cerebral hypoperfusion, was observed in the present study. These increased levels were significantly reversed by chronic postischemic treatment with MEPD (100, 300 mg/kg) for 15 d

which indicates the protective role of MEPD against lipid peroxidation. In the present study, decrease in the levels of SOD and CAT were noted in brains of the ischemic rats that indicated the participation of superoxide radical which is known to produce highly toxic hydroxyl radical through its reaction with H_2O_2 (Haber-Weiss reaction) in turn leading to decreased SOD through a modification in histidine residue located in the active site of the enzyme. On the other hand this over production of H_2O_2 can be scavenged further by tissue catalase which leads to reduction in the tissue catalase levels[35-37]. MEPD (100 and 300 mg/kg) was found to elevate the activity of two major oxygen radical species metabolizing enzymes *i.e.* SOD and CAT in brains of the ischemic rats which shows that MEPD possess antioxidant activity against hypoperfusion induced oxidative stress. Sulfahydryl groups represent an important endogenous antioxidant mechanism. They have role in maintenance of cellular proteins and lipids in their functional states. When these are consumed, the toxic effects of oxidative insult are exacerbated resulting in increased membrane and cell damage. Glutathione and other protein/nonprotein sulphahydryls are scavengers of ROS and help to maintain macromolecular components of the cell in their functional status.

In the present investigation fall in the levels GSH, GPx, GR and GST was observed in the rats subjected to cerebral hypoperfusion. Decline in the levels of GSH can be attributed to several factors such as cleavage of GSH to cysteine, decrease in synthesis of GSH and the formation of mixed disulfides with GSSG, causing their cellular stores depleted. Glutathione peroxidase along with catalase removes hydrogen peroxide and limits the hydroxyl radical formation during chronic hypoperfusion whereas and glutathione-S-transferase and glutathione reductase removes toxic products of ROS damage leading to depletion in their levels[38]. Postischemic treatment with MEPD (100, 300 mg/kg) significantly increased the levels of GSH and GR and thus exhibited its ability to restore the altered endogenous antioxidant defense system. None of the doses of MEPD were found to elevate the decreased levels of GPx and GST.

In evaluations of the potential neuroprotective drug, the need for assessing the drug effects on the histological and/or functional measures of neuronal damage has always been emphasized[39,40]. Extensive investigations report that rats subjected to permanent occlusion of BCCA show impaired spatial learning/memory capabilities and/or structural alterations and neuronal damage accompanying compromised brain homeostasis resembling the effects observed in vascular dementia[41,42].

In the present study, hypoperfused animals, when tested in open-field paradigm, exhibited decreased ambulations, rearings and groomings with an increase in period of immobility. These findings, according to accepted tenets, indicate that the hypoperfused animals were more susceptible to develop anxiety when exposed to novel environment[19]. MEPD significantly attenuated these alterations in exploratory behavior, suggesting that MEPD protects against hypoperfusion-induced anxiety.

Learning was mainly explored by the Water Maze test that investigates the ability of a rat to learn a spatial route to find a platform hidden in a pool, after a training phase. In general, cognition includes at least three primary processes: acquisition, consolidation, and retention. The Morris water maze is a hippocampus-dependent memory task that has been used to assess cognitive deficits in the ischemic brain. In the present study, the hidden platform trial measured acquisition, the reversal trial measured relearning ability, and the probe trial measured retention[20,43,44]. The results exhibited that both learning capacity and memory were gradually impaired in the rats with chronic cerebral hypoperfusion. Long term hypoperfused animals consistently took longer time to find the submerged platform reflecting a defective learning process. This when combined with the results of probe trial and new platform trial reflected the disturbances in reference and working memory in long term hypoperfused rats. These observations were in accordance with earlier reports of hypoperfusion-induced spatial learning and memory deficits. It has been suggested earlier that such behavioral disturbances may be due to subcellular alterations, purely functional and/or transmission failure[45-47]. MEPD significantly attenuated these alterations suggesting its positive role in learning and memory in long term hypoperfused rats. Extensive investigations report that rats subjected to permanent BCCAO show structural alterations and neuronal damage in their brains[48-50].

Histopathological changes like shrinkage and necrosis of neurons, vacuolisation and inflammatory infiltration reflect hypoperfusion induced reactive changes in rat brain. These alterations were alleviated by postischemic administration of MEPD (100, 300 mg/kg). Attenuation of reactive morphological changes as well as behavioral deficits by MEPD indicates that it reduces neuronal insult in the settings of permanent BCCAO.

At dose of 30 mg/kg, MEPD was found to be ineffective in attenuating any of the biochemical, histopathological as well as behavioural changes. Previous reports of phytochemical investigations of *P. dactylifera* fruits have revealed the presence of flavonoids which was confirmed in the present study by the results of preliminary phytochemical analysis. These pharmacophores have been shown to possess potent

antioxidant and anti-inflammatory activity[51,52]. The present findings suggest that the palliative effect of MEPD on cognitive deficits and structural changes is associated with its antioxidant activity.

In conclusion, in the present study MEPD exhibited significant neuroprotection against oxidative stress, neuronal damage and spatial learning and memory impairment induced by permanent BCCAO and validated its claim as neuroprotective drug. The neuroprotective role can be attributed to the polyphenolic compounds such as flavonoids and plant sterols, ascorbic acid present in the plant. These findings suggest a promising role of *P. dactylifera* in stroke and vascular dementia in the Alzheimer's disease and age related memory disturbances. However, further studies are needed to elucidate the effects of MEPD on cerebral microcirculation in the ischemic brain.

Conflict of interest statement

We declare that we have no conflict of interest.

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