

## Evaluation of antioxidant and neuroprotective effect of date palm (*Phoenix dactylifera* L.) against bilateral common carotid artery occlusion in rats

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The cerebral ischemia in rats was induced by occluding bilateral common carotid arteries (BCCAO) for 30 min., followed by 45 min reperfusion. BCCAO caused significant depletion in superoxide dismutase, catalase, glutathione, glutathione peroxidase, glutathione-S-transferase, glutathione reductase and significant increase in lipid peroxidation along with severe neuronal damage in the brain. All the alterations except depletion in glutathione peroxidase and glutathione-S-transferase levels induced by cerebral ischemia were significantly attenuated by 15 days pretreatment with methanolic extract of *P. dactylifera* fruits (100, 300 mg/kg), whereas 30 mg/kg dose was insignificant in this regard. These results suggest the possible use *P. dactylifera* against bilateral common carotid artery occlusion induced oxidative stress and neuronal damage.

**Keywords:** Bilateral common cerebral artery occlusion, Flavonoids, Oxidative stress, *Phoenix dactylifera*

Growing evidences support participation of oxidative stress in brain injury mediated by cerebral ischemia and stroke<sup>1,2</sup>. Tissue reoxygenation following ischemia provides oxygen for a variety of enzymatic oxidation in ischemic tissues and leads to overproduction of free radicals<sup>3-6</sup>. The apparent role of free radicals in the development of neuronal cell death has stimulated interest in the use of antioxidants and free radical scavengers as potential therapeutic agents for ischemia induced free radicals that may promote ischemic neuronal cell injury. Inhibition of free radicals, using scavengers or oxidative enzymes, results in significant neuroprotection in transient global ischemia<sup>7-9</sup>. In recent times, focus on plant research has increased globally and evidences show the immense potential of medicinal plants.

The polyphenolics including flavonoids, which are found in many herbal extracts, have been shown to be strong reactive oxygen species (ROS) scavengers, antioxidants and protectors of neurons from lethal damage *in vitro*<sup>10-12</sup>. In addition, the neuroprotective effects of flavonoids in herbal extracts and their physiologically relevant conjugates against ischemia/reperfusion (I/R)-induced oxidative damage have also been reported<sup>9</sup>. Phenolic antioxidants from medicinal plants have also been evaluated *in vivo* as neuroprotective agents in animal models of I/R induced oxidative stress<sup>13-14</sup>. *Phoenix dactylifera* L (date palm) belonging to family Arecaceae, called 'Nakhla' and the 'Tree of Life' by the Arabs, is considered as one of the oldest cultivated fruit trees. It is believed to be indigenous to the countries around the Arabian Gulf. Different parts of the plant are traditionally claimed to be used for the treatment of a broad spectrum of ailments including memory disturbances, fever, inflammation, paralysis, loss of consciousness and nervous disorders. Many Middle Easterners believe that consumption of date fruits, particularly in the morning on an empty stomach, can reverse the actions of any toxic material that the subject may have been exposed to. Earlier reports of phytochemical analysis of the whole plant have revealed the presence of carbohydrates, alkaloids, steroids, flavonoids, vitamins and tannins. The

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phenolic profile of date demonstrated the presence of mainly cinnamic acids (ferulic, sinapic and coumaric acids and their derivatives, such as 5-o-caffeoylshikimic acid also called as dactyliferic acid), flavonoid glycosides (luteolin, methyl luteolin, quercetin, and methyl quercetin) and flavanols (catechin, epicatechin). It is also scientifically proved to possess a variety of pharmacological activities which indicate its usefulness in various kinds of diseases and disorders. The aqueous extract of date is reported to possess antiulcer, hepatoprotective, antimutagenic and antidiarrhoeal effects. Whereas the methanolic and aqueous extracts of the date have exhibited antiinflammatory and antioxidant activity by significantly increasing the plasma levels of vitamin C, E and A,  $\beta$ -carotene and significantly decreasing the elevated malonaldehyde (MDA) levels due to the lipid peroxidation in adjuvant arthritis in rats. Various *in vitro* and *in vivo* antioxidant activities have been carried out on various extracts of different parts of *P. dactylifera*. The methanolic extract of the fruits demonstrated a linear relationship between antioxidant activity and the total phenolic content (TPC) of date fruit extract. Aqueous date extract inhibited significantly the lipid peroxidation and protein oxidation and also exhibited a potent superoxide and hydroxyl radical scavenging activity in a dose-dependent manner in an *in vitro* study<sup>15</sup>. However there is no report on the protective effect of the *Phoenix dactylifera* against brain injury induced by ischemia *in vivo*. Therefore, the present investigation has been carried out to evaluate the effect of *P. dactylifera* on cerebral ischemia induced by bilateral common cerebral artery occlusion (BCCAO) for 30 min followed by 45 min reperfusion.

### Materials and Methods

**Plant material**—Fresh fruits of *Phoenix dactylifera* L. were collected from local market and authenticated by Botanical Survey of India (Voucher specimen number: BSI/WC/Tech/2009/674).

**Preparation of extract**—The fruits were manually separated from the pits and dried at room temperature and ground into powder using a stainless-steel blender. The powder was then extracted with methanol-water (4:1, v/v), at room temperature (20°C for 5 h using an orbital shaker). The extracts were then filtered and centrifuged at 4000 g, for 10 min and the supernatant was concentrated under reduced pressure at 40°C for 3 h using a rotary evaporator to obtain the methanolic extract of the date.

**Chemicals and drugs**—Thiobarbituric acid and Trichloroacetic acid were purchased from SD Fine Chemicals, Mumbai, India. Nicotinamide adenine dinucleotide phosphate (NADPH), oxidised 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 and folin ciocalteau reagent were obtained from Bansal Sales, Pune, India. The other chemicals and solvents used were of analytical grade purchased from commercial suppliers.

**Animals**—Male swiss albino mice weighing 25-30 g were used for acute toxicity test. Male wistar albino rats weighing 200-250 g were used for cerebral ischemia induction. These animals were obtained from Yash Farms, Aundh, (Pune district) India. They were caged in the animal house under standard laboratory conditions (23 $\pm$ 1°C, 55 $\pm$ 5% RH and 08:00-20:00 hrs lighting) and fed on a pelleted diet (Amrut Feed, Pune, India) and water *ad libitum*. They were transferred to the laboratory at least 1h before the start of the experiment. The experiments were performed during day (08:00- 16:00 hrs).

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 experimental design (Approval No.: CPCSEA/IAEC/PC-04/07-2K8). The extract as well as distilled water was administered by oral route to the rats as well as mice.

**Preliminary acute toxicity test**—Healthy adult male albino mice (25-30 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 behavioural and autonomic profiles and for any sign of toxicity or mortality for seven days<sup>16</sup>.

**Experimental protocols**—Wistar albino rats were divided into 5 groups of 6 each for histopathological as well as biochemical estimations respectively. 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 control group. Third, fourth and fifth groups served as

test groups i.e. drug treated BCCAO groups and received methanolic extract of *P. dactylifera* at 30, 100 and 300 mg/kg doses respectively. Rats were treated for 15 days. On last day 60 min after last dose, all rats except sham operated were subjected to 30 min of BCCAO followed by 45 min reperfusion. Thereafter, all rats were sacrificed and their brain removed and subjected to biochemical analysis and histopathological evaluation.

**Surgical procedure for bilateral common carotid artery occlusion**—Surgical procedure was performed according to the method of Iwasaki *et al.*<sup>32</sup> between 8:00-13:00 hrs. All the surgical equipments and surgical pad were disinfected with 70% ethanol before the surgery to avoid any kind of infection and sepsis. Before the experiment, food to the rats was withheld overnight but water was freely available. They were anaesthetised by an ip 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 ischemia was performed by occluding bilateral common carotid arteries with microaneurysmal clips (Bulldog Clamp, 25 mm straight) for 30 min, followed by 45 min reperfusion and the skin was closed with stitches using 3-0 silk suture. Core temperature (rectal) was maintained at  $37^{\circ}\pm 0.5^{\circ}\text{C}$  throughout the surgical process using a heating lamp. Sham control rats received the same surgical procedures, except the bilateral common carotid arteries were not occluded. After the completion of reperfusion period the rats were assessed for neurological outcome and then sacrificed<sup>17</sup>.

**Biochemical analysis**—The rats were sacrificed by decapitation under deep anaesthesia after the completion of reperfusion. The brain was immediately removed, weighed and homogenised 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 2000 rpm at 4°C for 20 min and the supernatant was used for measurement of total protein<sup>18</sup>, lipid peroxidation (LPO)<sup>19</sup>, superoxide dismutase (SOD)<sup>20</sup>, catalase (CAT)<sup>21</sup>, reduced

glutathione (GSH)<sup>22</sup>, glutathione peroxidase (GPx)<sup>23</sup>, glutathione-S-transferase (GST)<sup>24</sup>, glutathione reductase (GR)<sup>25</sup>.

**Histopathological examination**—At the end of reperfusion the rats were sacrificed by decapitation under deep anaesthesia and the brains were removed. The intact whole brains were fixed in formalin (10%, v/v) for 24 h and then embedded in paraffin. The forebrain sections (5–10 nm) thickness were prepared using a rotary microtome and stained with hematoxylin and eosin and observed under 40X magnification.

**Statistical analysis**—Results were expressed as mean $\pm$ SE. Statistical analysis was performed using one-way 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.

## Results

**Acute toxicity test**—The mice treated with methanolic extracts of date fruits were found to be free of any toxicity upto the 2000 mg/kg dose and exhibited normal behaviour. 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.

**Biochemical analysis**—Results of biochemical analysis are present in (Fig. 1).

**Histopathological examination**—From the histopathological study it was observed that 30 min of BCCAO followed by 45 min of reperfusion produced shrinkage, atrophy and necrosis of neurons along with the vacuolisation and inflammatory infiltration in the forebrains of BCCAO control group rats compared to sham operated rats. The reactive changes were significantly attenuated in methanolic extract of *P. dactylifera* (100 and 300 mg/kg) pretreated rats as compared to BCCAO control group rats (Fig. 2).

## Discussion

The present investigation showed the neuroprotective potential of methanolic extract of date fruit against ischemia/reperfusion (I/R) induced oxidative stress as well as histopathological alterations. The activities of extract appeared to work by restoring the altered antioxidants enzymes as well as decreasing the lipid peroxidation in various brain

regions induced by BCCAO. There is considerable evidence which supports the role of ROS in the pathogenesis of I/R induced oxidative stress in brain<sup>26,27</sup>.

Brain reperfusion after ischemia frequently results in neuronal death, which occurs preferentially in some brain regions. This neuronal degeneration has been associated with ROS, which react with cellular

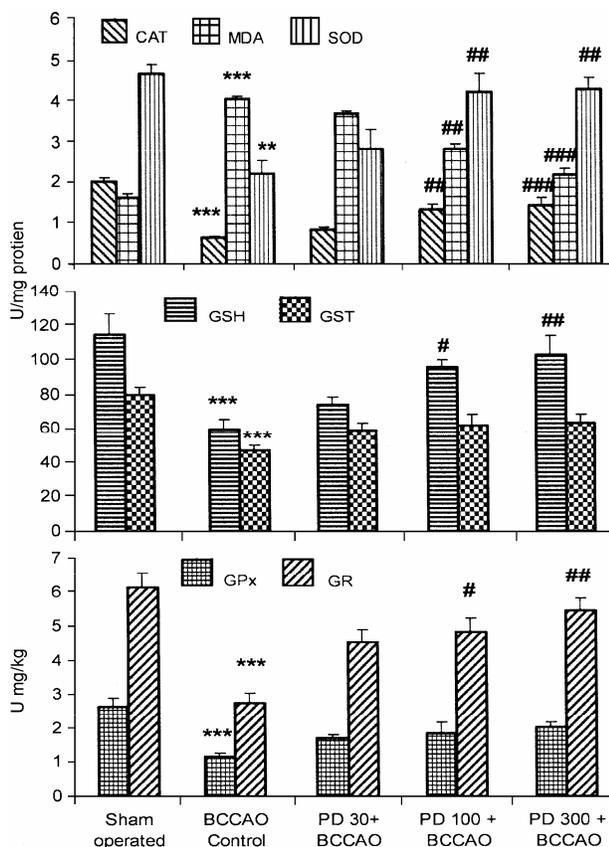


Fig. 1—Effect of methanolic extract of *P. dactylifera* on CAT, MDA, SOD, GSH, GST, GPx and GR levels in the brains of rats subjected to bilateral common carotid artery occlusion (BCCAO) [Values are mean±SE from 6 observations each. Data were analysed by ANOVA followed by Tukey's test. *P* values: \*,#<0.05; \*\*,##<0.01, \*\*\*,###<0.001. \*: BCCAO control group rats compared against sham operated rats; #: PD extract pretreated BCCAO group rats compared against BCCAO control group rats. CAT=catalase (U/mg of protein, where 1 U=  $\mu$ M of CAT formed/min); MDA=malonaldehyde (U/mg of protein, where 1 U= nM of MDA formed/h); SOD=superoxide dismutase (U/mg of protein, where 1 U= mU of activity of SOD/min); GSH=glutathione (U/mg of protein, where 1 U= nM of GSH formed); GST=glutathione-S-transferase (U/mg of protein, where 1 U= amount of enzyme that synthesizes 1  $\square$ mol of product i.e. thioether/min); GPx=glutathione peroxidase (U/mg of protein, where 1 U= amount of enzyme oxidizing 1  $\square$ M of NADPH/min); GR= glutathione reductase (U/mg of protein, where 1 U= nM NADPH oxidized/min); PD= methanolic extract of *P. dactylifera*]

macromolecules such as lipids, proteins and nucleic acids leading to oxidative damage of the neurons<sup>28,29</sup>. Thus the endogenous antioxidant enzyme activity of the brain impaired by I/R is particularly important and measurement of those antioxidant enzymes after reperfusion can assess the vulnerability of the particular areas of the brain<sup>30</sup>. Novel therapeutic neuroprotective strategies support the applications of ROS scavengers and induction of endogenous antioxidants drugs, such as natural antioxidants, e.g. plant derived polyphenolic compounds, in monotherapy, or as part of an antioxidant cocktail formulation, for the treatment of neurodegenerative diseases. Therefore, the potent radical scavenging properties or LPO inhibiting ability of polyphenolic natural compounds protecting the neurons from oxidative stress may provide useful therapeutic agent for the treatment of neurodegenerative diseases such as I/R induced oxidative stress<sup>31-35</sup>.

In the present study pretreatment with date extract (100 and 300 mg/kg) markedly reduced the MDA level and inhibited the neuronal injuries from propagating chain reaction of LPO. During cerebral ischemia, a number of events predispose the brain to the formation of ROS such as rapid decrease in adenosine triphosphate levels, calcium release from intracellular stores, loss of  $Ca^{2+}$  homeostasis, excitotoxicity, arachidonic acid release and metabolism, mitochondrial dysfunction, acidosis and edema<sup>36-38</sup>. This spectrum can modulate the antioxidants enzymes and its gene expression in I/R induced oxidative stress<sup>30</sup>.

Therefore the antioxidants like CAT, GSH, GR, GST, GPx and SOD, which served as oxidative indices in brains of the BCCA occluded rats were examined. Decrease in the levels of CAT, GSH, GR, GST, GPx and SOD were noted in brains of the ischemic rats that indicates participation of superoxide radical which is known to produce highly toxic hydroxyl radical through its reaction with  $H_2O_2$  (Haber-Weiss reaction)<sup>27</sup>. These in turn decrease the SOD through a modification in histidine residue located in the active site of the enzyme<sup>39</sup>. On the other hand this overproduction of  $H_2O_2$  can be inactivated by catalase enzyme and thereby reduction in CAT<sup>40</sup>. The extract (100 and 300 mg/kg) was found to elevate the activity of two major oxygen radical species metabolizing enzymes in various regions of ischemic brain. Such peroxidation processes and overproduction of free radicals may lead to

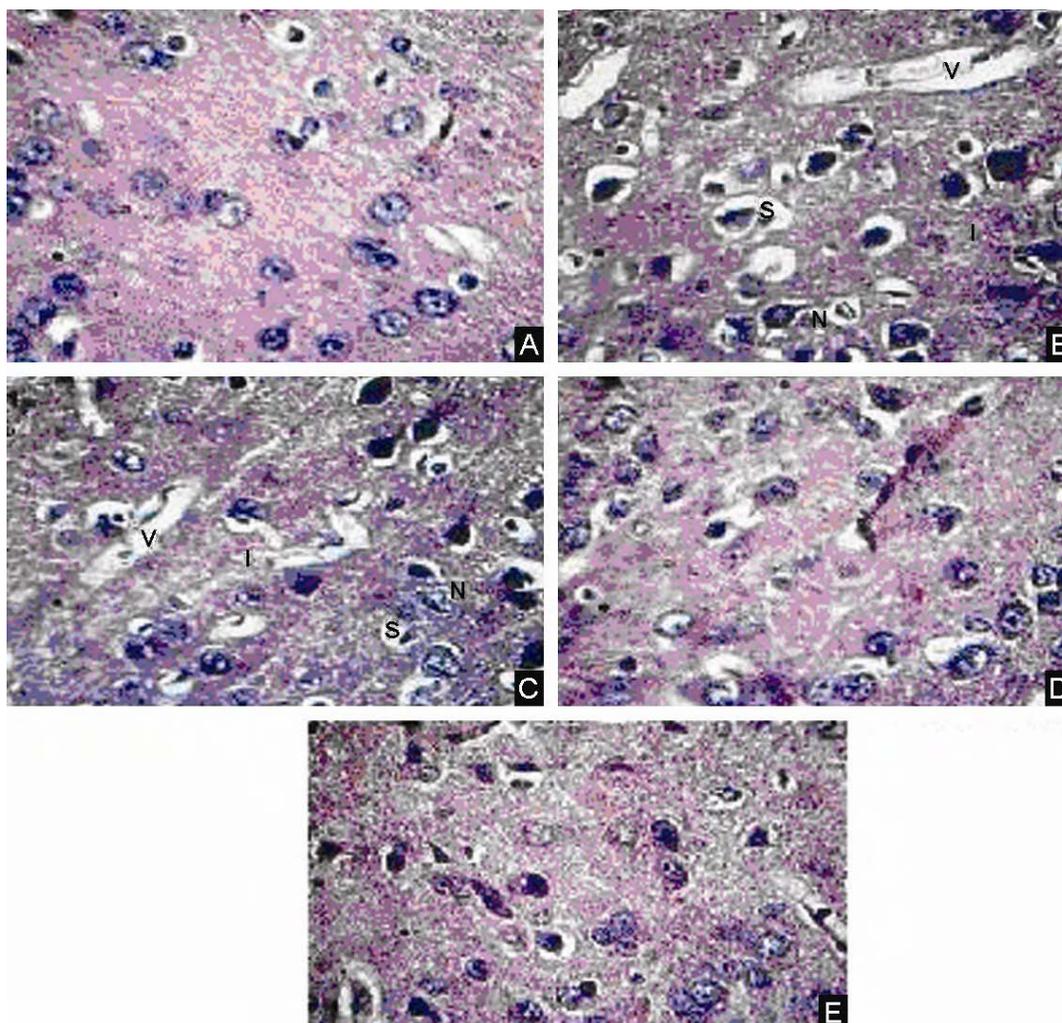


Fig. 2—Representative photomicrographs (H & E stain) of forebrain sections of (A) sham operated rats (B) BCCAO control group rats (C, D, E) MEPD 30, 100 and 300 mg/kg pretreated BCCAO group rats observed under 40X magnification [BCCAO control group showed morphological alterations suggestive of shrinkage (S) and necrosis of neurons (N), vacuolisation (V) and inflammatory infiltration (I) which were found to less in PD (100 and 300 mg/kg) treated groups]

consumption of detoxifying endogenous antioxidants such as GSH, GPx, GR, and GST causing their cellular stores to be depleted<sup>41</sup>. These are the primary endogenous antioxidant defense systems in the brain, which remove H<sub>2</sub>O<sub>2</sub> and lipid peroxides<sup>42</sup>. Decline in their levels could either increase or reflect oxidative status<sup>43</sup>. In the present study, depletion in these enzyme levels was observed in brains of ischemic rats. This could be explained by the consumption of these defensive enzymes due to scavenging of the rapidly generated hydrogen peroxide and lipid peroxides. Interestingly, those rats fed with date extract (100 and 300 mg/kg) showed increased the brain levels of GSH and GR. None of the doses of extract was able to reverse the decreased levels of GPx and GST.

The severe neuronal loss, observed as shrinkage of neurons and atrophy, was observed in the histopathological brain sections of the I/R group. Interestingly this effect was attenuated by the administration of date extract at 100 and 300 mg/kg doses.

At dose of 30 mg/kg, the extract was neither effective in reversing the altered enzyme levels nor in attenuating the histopathological changes in the brain regions of I/R. This may be explained by its insufficient concentration and antioxidative capacity to defend adequately against oxygen free radicals generated in the brain region during ischemia.

### Conclusion

The results demonstrate that methanolic extract of *P. dactylifera* (100 and 300 mg/kg) provides

significant neuroprotection against cerebral ischemia induced by bilateral common carotid artery occlusion and post ischemic reperfusion by reversing the changes in the biochemical parameters of the brain produced due to the oxidative stress and histopathological alterations associated with ischemia reperfusion and validated its claim as neuroprotective agent. The neuroprotective effect of the drug may also be contributed to the polyphenolic compounds such as flavonoids and plant sterols, ascorbic acid present in the drug. These findings suggest a potential role of *Phoenix dactylifera* may have the potential to be used as a protective agent against a variety of conditions where cellular damage is a consequence of oxidative stress.

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