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Neuroprotective effect of *Acorus calamus* against middle cerebral artery occlusion-induced ischaemia in rat

Pradeep K Shukla[1], Vinay K Khanna[1]*, M Mohd Ali[1], Rakesh Maurya[2], MY Khan[3] and Rikhab C Srimal[1]

[1]*Industrial Toxicology Research Centre, PO Box 80, M.G. Marg, Lucknow 226001, India;*
[2]*Central Drug Research Institute M.G. Marg, Lucknow 226001, India;*
[3]*Dr. R.M.L. Avadh University, Faizabad 224001, India*

The neuroprotective potential of ethanol:water (1:1) extract of rhizomes of *Acorus calamus* (AC-002) has been investigated in middle cerebral artery occlusion (MCAO)-induced ischaemia in rats. A significant behavioural impairment in Rota-Rod performance and grid walking was observed in rats, 72 hours after MCAO as compared to sham-operated animals. These rats also exhibited an increase in lipid peroxidation (cortex – 157%, corpus striatum – 58%) and a decrease in glutathione levels (cortex – 59%, corpus striatum – 34%) and superoxide dismutase (SOD) activity (cortex – 64%, corpus striatum – 32%) as compared to sham-operated animals. Ischaemic rats treated with AC-002 (25 mg/kg, p.o.) exhibited a significant improvement in neurobehavioural performance viz. Rota-Rod performance and grid walking as compared to the MCAO group. Interestingly, treatment with AC-002 in MCAO rats significantly decreased malonaldialdehyde levels in cortex as compared to ischaemic rats. A significant increase in reduced glutathione levels and SOD activity was also observed both in cortex and corpus striatum in MCAO rats treated with AC-002 in comparison to MCAO rats. Treatment with AC-002 in MCAO rats also reduced the contralateral cortical infarct area (19%) as compared to MCAO rats (33%). Neurological function score was improved in the AC-002-treated rats as compared to the MCAO group. The results of the present study indicate the neuroprotective efficacy of *A. calamus* in the rat model of ischaemia. *Human & Experimental Toxicology* (2006) **25,** *187–194*

Key words: Acorus calamus; middle cerebral artery occlusion; neuroprotection; oxidative stress

Introduction

Ischaemic stroke is a leading cause of death throughout the world.[1] Brain is a soft target of stroke and depending upon the site of ischaemia the affected person shows neurological signs including loss of consciousness and memory, impaired muscle coordination and paralysis. Also, higher cortical functions including amnesia, delirium, language and speech may be impaired in stroke survivors.[2] Multiple mechanisms including excitotoxicity, calcium overload and enhanced oxidative stress have been suggested in the aetiology of ischaemic stroke.[3–8] Recently, increased intercellular adhesion molecule (ICAM-1) protein has been suggested to be involved in the aetiology of focal ischaemia, as antisense inhibition of ICAM-1 protein expression could decrease ischaemic brain damage.[9] It is largely accepted that ischaemia itself causes injury to the brain while reperfusion enhances the risk of injury significantly, possibly by increased formation of free radical species.[10,11]

Studies on experimental models of stroke have been carried out to investigate the neuroprotective efficacy of certain agents and drugs alone or in combination.[8,12–15] Also, various herbal drugs and plant extracts have been examined for their prophylactic efficacy in rat stroke model.[16,17] These studies have aroused significant interest among scientists to screen prophylactic efficacy of synthetic and herbal/plant preparations that could eventually be utilized for effective clinical use.

Acorus calamus Linn. (Araceae) rhizomes have been used for the treatment of insomnia, melancholia, neurosis, epilepsy, hysteria and loss of memory either as a single drug or as a component of certain compound drug preparations in the Indian Ayurvedic system of medicine.[18,19] Studies carried out by

*Correspondence: Vinay K Khanna, Industrial Toxicology Research Centre, Post Box 80, MG Marg, Lucknow 226 001, India
E-mail: vkkhanna1@gmail.com

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us earlier showed that treatment with *A. calamus* prevented damage from acrylamide neurotoxicity in rats.[20] Recently, Hazra and Guha reported that pretreatment with *A. calamus* extract in rats prevented the development of ferric chloride-induced epileptogenesis.[21] The present study was undertaken to investigate the protective/prophylactic effect of ethanolic extract of *A. calamus* in the middle cerebral artery occlusion (MCAO) in rat model.

Materials and methods

Animals
Male Wistar albino rats weighing 275–300 g from the Industrial Toxicology Research Centre animal breeding colony were used in the experiments. The animals were housed in polypropylene cages in standard animal house conditions and fed pellet diet (Hindustan Lever Ltd. Ghazibad India) and water *ad libitum*. The body weight of each animal was recorded before and after the treatment. The animals were divided into three groups of 30 each as given below:

1. sham-operated (normal group);
2. MCAO (control group);
3. AC-002 treated with MCAO.

Experimental model
Rats were subjected to 2 hours of MCAO. Focal cerebral ischaemia was induced following the standard procedure of Longa *et al.*[22] Briefly, the right common carotid artery of a pentobarbitone anaesthetized (50 mg/kg, ip) rat was exposed through a midline incision in the neck under the operating microscope. A 4-0 nylon suture, the tip of which was rounded by heating over the flame, was introduced into the right external carotid artery and advanced into the internal carotid artery for a length of 16 mm from the bifurcation. The tip of the suture was placed at the origin of the anterior cerebral artery, thereby occluding the middle cerebral artery. The suture was left like this for 2 hours. The animal was allowed to recover from the anaesthesia after closure of the operation site and the suture was gently removed 2 hours after MCAO. The weight of each animal was recorded before MCAO and 72 hours after the ischaemic insult.

Neurological score Neurological evaluation of rats was carried out after 30 min to verify successful MCAO and immediately before they were sacrificed after 72 hours. An eight-point behavioural rating scale, modified from the scale as described by Rogers *et al.*,[2] was used to score the neurological deficits.

0, no neurological deficit;
1, failure to extend right forepaw fully;
2, decreased grip of the right forelimb while tail gently pulled;
3, spontaneous movement in all directions, contralateral circling only if pulled by the tail;
4, circling or walking to the right;
5, walks only when stimulated;
6, unresponsive to stimulation with a depressed level of consciousness;
7, dead.

Preparation of aqueous ethanolic extract of Acorus calamus and treatment
The freshly collected *A. calamus* was dried in the shade, powdered (200 g), placed in a glass percolator and then submerged in ethanol:water (1:1). After standing for 16 hours (overnight) at room temperature, the aqueous ethanolic extract was drained off. This process of extraction at ambient temperature was repeated four times. The combined extract was filtered through filter paper and evaporated to dryness under reduced pressure in rotavapour at 45°C and finally it was dried in high vacuum, which furnished a viscous extract (47.5 g), named AC-002.

Rats were pretreated with AC-002 (25 mg/kg/day, po) for 5 days before MCAO. The treatment with AC-002 was continued for another 3 days after MCAO, following which behavioural, biochemical and histological studies were carried out. The dose of the extract was based on published reports.

Behavioural studies

Rota-Rod performance Rats were conditioned to the accelerating Rota-Rod before MCAO. Each animal received training on the Rota-Rod (rotating at a constant speed of 8 rpm). They were trained until they achieved a criterion of staying on the rod for 60 s. The rats then received single baseline training on the Rota-Rod in which the speed was increased from 4 to 40 rpm over a period of 5 min. At 24 hours postocclusion, each rat received another trial on the Rota-Rod and scoring was carried out by a person blind to the condition.[2]

Grid walking Rats were acclimatized for 1 min to an elevated level of stainless steel grid with mesh size of 30 mm before MCAO. At 24 hours postocclusion, they were placed on the grid again for 1 min and the total number of paired steps (placement of both fore limbs) were counted. The scoring was

carried out by a person blind to the condition. Foot-fault error (in which the animal made a mistake in placing the forelimbs or fell from the grid) was monitored and the total number of errors in placing the forelimbs was recorded.[2]

Biochemical estimations
Rats in all groups were sacrificed 72 hours after MCAO by cervical decapitation and the brains were immediately removed. The brain regions, corpus striatum and cerebral cortex, were dissected following the method of Glowinski and Iverson,[23] and processed for biochemical assays the same day.

Freshly dissected wet tissues were homogenized in chilled 0.1 M phosphate buffer saline (10% w/v) using a glass homogenizer with a Teflon pestle under cold conditions for assay of reduced glutathione and lipid peroxidation. Activity of superoxide dismutase (SOD) was measured in mitochondrial fractions for which the homogenate was centrifuged at 14 000 × g (10 min, 4°C). The pellet obtained was suspended and used for the assay of SOD activity.

Assay of lipid peroxidation As a measure of lipid peroxidation, malonaldialdehyde (MDA) levels were estimated by measuring thiobarbituric acid reactive substances (TBARS) following the standard protocol of Bohme *et al.*[24] Equal volumes (120 μL) of EDTA (10 mM), ascorbate (10 mM), and a mixture of EDTA (16.7 mM) and $FeSO_4$ (16.7 mM) were mixed and to this a homogenate of different brain regions (0.6 mL) was added. The reaction mixture was incubated at 37°C for 60 min. The reaction was stopped by adding 1 mL of ice cold 10% trichloroacetic acid (TCA). The deproteinized homogenate was centrifuged at 2000 × g for 10 min and supernatant was aspirated out. The supernatant was mixed with an equal amount of 0.67% TBA and kept in a boiling water bath for 15–20 min. The intensity of pink colour developed was read at 532 nm on a spectrophotometer.

Assay of reduced glutathione levels The levels of reduced glutathione (GSH) in corpus striatum and cortex were measured spectrophotometrically using 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) as the colour reagent following the method of Hasan and Haider.[25] Briefly, brain homogenate (1 mL) was deproteinized with an equal volume of 10% TCA and allowed to stand at 4°C for 2 hours. This mixture was centrifuged at 2000 × g for 15 min and an aliquot of the supernatant (1 mL) was added to 2 mL of Tris–HCl buffer (0.4 M, pH 8.9) containing EDTA (0.02 M). Finally 0.1 mL of DTNB (0.01 M) was added. The mixture was diluted with 0.5 mL of distilled water, stirred and the absorbance was recorded at 412 nm. A range of glutathione (2–10 μg) as standard was also run in parallel to plot the standard curve.

Assay of superoxide dismutase activity The activity of SOD was assayed following the method of Kakkar *et al.*,[26] using NADH as a substrate. The assay mixture in a final volume of 3 mL contained sodium pyriophosphate buffer (0.082 M, pH 8.3), phenazine methosulphate (186 μM), nitro blue tetrazolium (300 μM), NADH (780 μM), enzyme preparation and distilled water. The reaction was initiated by addition of NADH followed by incubation at 37°C for 90 s. The reaction was stopped by 1 mL of glacial acetic acid and the reaction mixture was vigorously shaken with 4 mL of n-butanol. The mixture was allowed to stand for 10 min, centrifuged, and the butanol layer was separated. The colour intensity of the chromogen in butanol was measured at 560 nm against butanol using a spectrophotometer. A mixture without enzyme preparation was run in parallel to serve as control. The SOD activity was expressed in units per milligram of protein. One unit of the enzyme was the amount required to inhibit the rate of chromogen formation by 50%.

Protein estimation Protein content was measured following the method of Lowry *et al.*,[27] using bovine serum albumin as a reference standard.

Histological studies
Five coronal sections (2 mm thick) of the whole brain were taken from the region beginning 1 mm from the frontal pole and ending just rostral to the corticocerebellar junction and stained with 2,3,5-triphenyl tetrazolium chloride (TTC, 2%) following the procedure of Tortella *et al.*[5] The damage following MCAO and protection by AC-002 treatment was quantitated.

Statistical analysis
Data were analysed by comparing the mean (±SE) of each group and subjected to Student's *t*-test and $P < 0.05$ was considered significant.

Results

Body weight
A significant decrease in body weight was observed in ischaemic rats 72 hours after MCAO. However, a marginal change in body weight was observed in the sham group (Table 1). Treatment with AC-002 in

Table 1 Effect of AC-002 on body weight and neurological score 72 hours after ischaemic reperfusion injury

Group	*Initial weight (g)*	*Post-treatment weight (g)*	*Neurological score*
Sham ($n=10$)	295 ± 6.4	298 ± 2.3	0
MCAO ($n=6$)	311 ± 5.1	272 ± 4.3*	4.96 ± 0.8*
MCAO + AC-002 ($n=8$)	310 ± 3.8	298 ± 4.0	2.10 ± 0.6**

*Significantly differs from control ($P < 0.05$).
**Statistically significant from ischaemic group ($P < 0.05$).
n represents the number of animals in each group.

MCAO rats did not show significant change in body weight.

Neurological score
Rats exhibited focal neurological deficits following MCAO with failure to fully extend the forepaw (range 0–7). Forty per cent of animals died within 24 hours due to severe brain infarction/haemorrhage. No neurological deficit was observed in sham-operated animals. Treatment with AC-002 showed a significant decrease in neurological score (range 0–7) (Table 1).

Behavioural changes

Effect on Rota-Rod performance The time of fall from the rotating rod was significantly decreased in rats 72 hours after MCAO as compared to sham-operated rats, suggesting an impairment in the Rota-Rod performance. Treatment with AC-002 in MCAO rats gave significant protection, as evident by an increase in the time of fall in comparison to ischaemic rats (Figure 1).

Effect on grid walking A significant impairment in grid walking was observed following MCAO. These rats exhibited a decrease in total steps covered per minute and also showed error in placement of fore limbs during the grid-walking test as compared to the sham group. Treatment with AC-002 in ischaemic rats caused a significant protection both in the total number of steps covered per minute and errors in placement of fore limbs (Figure 2).

Biochemical changes

Effect on lipid peroxidation A significant increase in MDA levels was observed in corpus striatum (58%) and cortex (157%) 72 hours after MCAO. Interestingly, treatment with AC-002 significantly decreased the MDA levels in cortex as compared to the ischaemic group. No significant change was, however, observed in MDA levels in corpus striatum in the MCAO group treated with AC-002 extract as compared to the ischaemic group (Figure 3).

Effect on reduced glutathione levels Reduced glutathione levels were significantly decreased in ischaemic rats both in corpus striatum (34%) and cortex (59%) as compared to the sham group. Treatment with AC-002 in the MCAO group increased the reduced glutathione levels significantly in both regions as compared to ischaemic rats (Figure 4). Levels of reduced glutathione in cortex

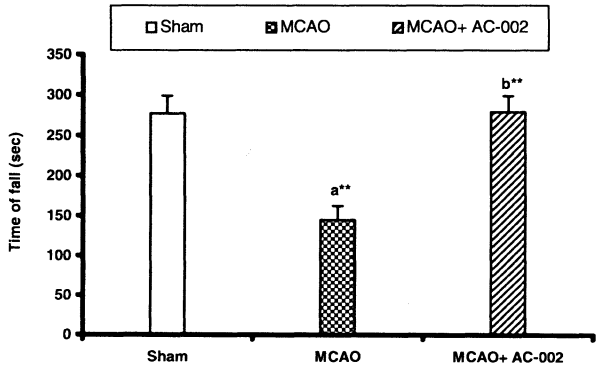

Figure 1 Protective effect of AC-002 on Rota-Rod performance. Values are mean ± SE of six animals in each group. a, Significantly differs from sham. b, Significantly differs from ischaemic group. **$P < 0.01$.

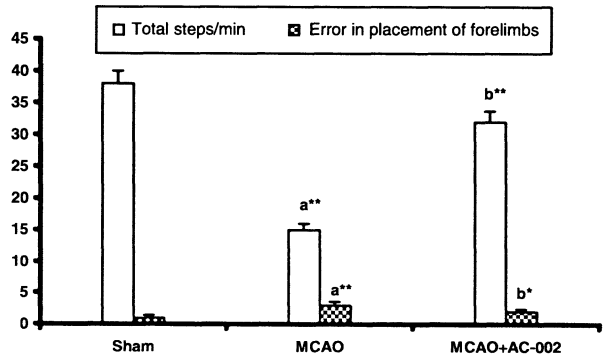

Figure 2 Protective effect of AC-002 on grid-walking performance. Values are mean ± SE of six animals in each group. a, Significantly differs from sham. b, Significantly differs from ischaemic group. *$P < 0.05$, **$P < 0.01$.

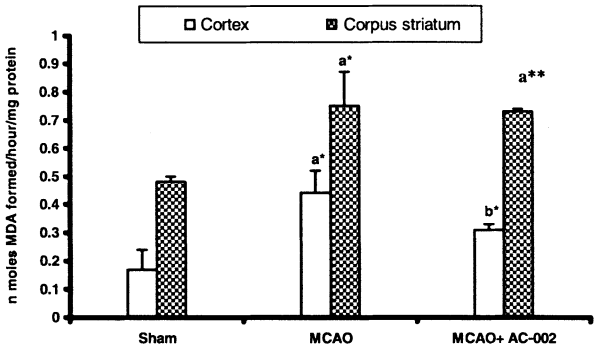

Figure 3 Protective effect of AC-002 on lipid peroxidation in ischaemic rats. Values are mean ± SE of six animals in each group. a, Significantly differs from sham. b, Significantly differs from ischaemic group. $^{*}P < 0.05$.

were also found to be increased in MCAO rats treated with AC-002 in comparison to the sham group.

Effect on superoxide dismutase activity A significant decrease both in the corpus striatum (32%) and cortex (64%) was observed in SOD activity in ischaemic rats as compared to the sham group. The activity of enzyme was significantly increased following treatment with AC-002 in both the brain regions as compared to ischaemic rats (Figure 5).

Histological studies
Contralateral hemispheric infarction was well demarcated (33%) in rats 72 hours after MCAO as revealed by histological analysis using TTC staining of coronal sections of the brain. Treatment with AC-002 in MCAO rats resulted in only 19% contralateral hemispheric infarction. No ischaemic damage was observed in brain sections of the sham-operated rats (Figure 6).

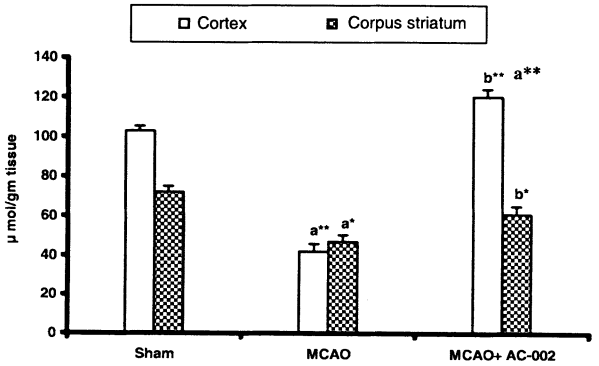

Figure 4 effect of AC-002 on reduced glutathione levels in ischaemic rats. Values are mean ± SE of six animals in each group. a, Significantly differs from sham. b, Significantly differs from ischaemic group. $^{*}P < 0.05$, $^{**}P < 0.01$.

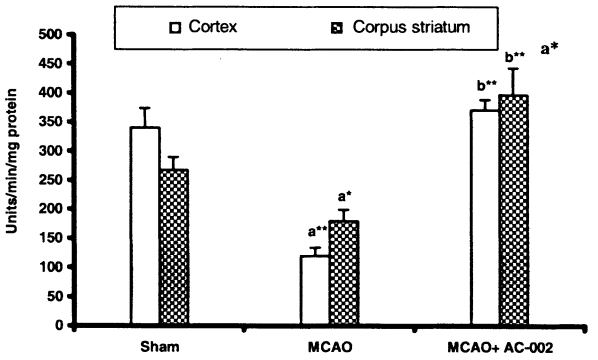

Figure 5 Protective effect of AC-002 on SOD activity in ischaemic rats. Values are mean ± SE of six animals in each group. a, Significantly differs from sham. b, Significantly differs from ischaemic group. $^{*}P < 0.05$, $^{**}P < 0.01$.

Discussion

Enhanced oxidative stress has been reported to modulate ischaemic reperfusion injury.[3,7] Reactive free oxygen species including superoxide radicals generated during and after ischaemia appear to play a crucial role in development of neuronal damage.[3,28,29] In the present study, an increase in lipid peroxidation and decrease in SOD activity and reduced glutathione levels both in cortex and corpus striatum is consistent with earlier reports and suggest an enhanced oxidative stress following ischaemia.

Plant and herbal extracts have been reported to inhibit free radical generation and lipid peroxidation. Some of these have been used in the management of ischaemia and found to attenuate reperfusion injury following cerebral ischaemia.[30–32] Recently, Wang *et al.* reported reduction in cerebral infarction by dietary supplementation with blueberries, spinach and spirulina.[33] Chaudhary *et al.* reported that pretreatment with *Withania somnifera* had significant neuroprotection in MCAO rats, which was attributed to anti-oxidant properties of the herb.[17] Plant extracts and formulations with anti-oxidant properties have also been found to be neuroprotective in ischaemic injury.[12–14] Moreover, agents with lipid peroxidation inhibitory or anti-oxidant properties have been found to be neuroprotective in ischaemia.[34,35]

Alcoholic and aqueous extracts of *A. calamus* root have been reported to be pharmacologically active. These extracts have been found to increase the latency of seizures and reduce mortality.[18] Recently, Hazra and Guha reported that pretreatment with *A. calamus* and diazepam prevented the development of ferric chloride-induced epileptogenesis in rats.[21] The present study shows that AC-002 inhibits lipid peroxidation in MCAO rats, which confers substan-

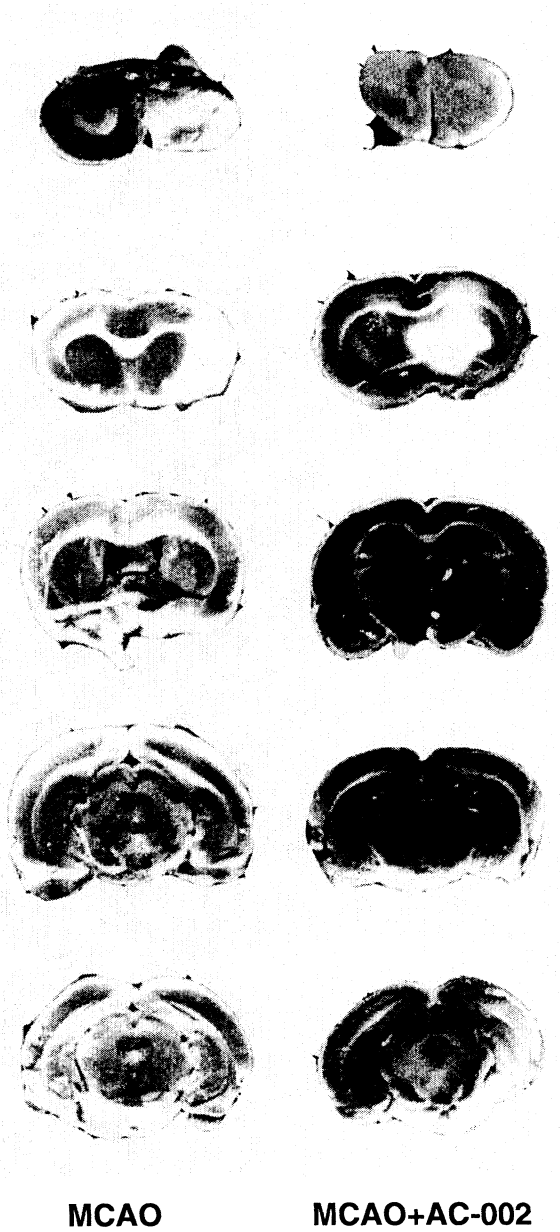

Figure 6 A representative photograph exhibiting histological infarction in rat brain coronal sections after staining with TTC in MCAO rats and those treated with AC-002.

tial neuroprotection, that is evident both on neurobehavioral examination and on quantitative histopathology. It is difficult to explain the selective changes at present, as the decrease in lipid peroxidation in cortex is remarkable, while no such change was observed in corpus striatum in MCAO rats treated with AC-002. The dose of AC-002 used in this study was chosen on the basis of our previous study with this extract.[20]

It has been largely accepted that both glutathione and SOD are significant modulators of oxidative stress. Treatment with AC-002 has been found to significantly enhance the reduced glutathione levels in rat brain and could prevent acrylamide-induced neurotoxicity.[20] A number of reports indicate that SOD, an enzymatic scavenger of superoxide radicals, effectively diminishes ischaemic neuronal damage in focal ischaemia animal model. A significant increase in reduced glutathione levels and SOD activity in MCAO rats treated with AC-002 in the present study exhibit the antioxidative and protective effect of the plant extract.

Observation of neurological deficits is important not only in clinical cases of stroke but also in experimental cerebral ischaemia models. Various neurological and behavioural parameters have been employed to evaluate functional impairment in rats after cerebral ischaemia. Grid walking is one of the sensitive tests used to monitor spontaneous locomotor activity and muscle co-ordination. Any deficit in placement of forelimbs and foot-fault error is easily detected through the grid-walking test. Rats with ischaemic reperfusion injury following transient and permanent occlusion of middle cerebral artery have been reported to have decreased grid walking and grip test results.[2,16] Errors in placement of foot and impaired co-ordination following MCAO for 2 hours were observed in the present study. Rota-Rod performance was affected following MCAO, which provides further evidence of impaired balance and muscle co-ordination due to ischaemia–reperfusion injury. It has been reported that Rota-Rod performance in rats is significantly affected following focal cerebral ischaemia.[36] A linear relationship between the duration of ischaemia and time during which rats stay on the accelerating rod has been reported. Further, treatment with AC-002 in the MCAO rats caused an improvement in grid walking and co-ordination, as evident by the score of foot-fault errors. The time of fall from the rotating rod was also significantly increased in these animals, suggesting that AC-002 treatment could significantly prevent impairment of motor functions, which has been observed in earlier work as well.[37–39] The behavioural tasks in this study were chosen to address specific functional deficits associated with the brain areas damaged by MCAO (ie, striatum and dorsolateral neocortex).[40,41] In the present study, treatment with AC-002 in MCAO rats significantly improved the neurological score compared to the sham group. Interestingly, treatment with AC-002 in MCAO rats reduced the contralateral hemispheric infarction to 19%, which was well demarcated (33%) in MCAO rats.

Results of the present study exhibit neuroprotective efficacy of *A. calamus* by modulating the

antioxidant capacity in rats with MCAO. Further, it would be interesting to study whether AC-002 can reverse the neurological damage, although it seems unlikely, as neurons, once dead, cannot be revived. Identification of the chemical moiety responsible for the beneficial effects of AC-002 requires further investigation.

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