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Research report

Total oligomeric flavonoids of *Cyperus rotundus* ameliorates neurological deficits, excitotoxicity and behavioral alterations induced by cerebral ischemic–reperfusion injury in rats

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ABSTRACT

Interactions between neurons and astrocytes play a critical role in the central nervous system homeostasis. *Cyperus rotundus* (family: Cyperaceae), a traditional Indian medicinal herb, used as nervine tonic and nootropic in the Ayurvedic system of medicine. The present study was undertaken to investigate the neuroprotective effect of total oligomeric flavonoids (TOFs), prepared from *C. rotundus*, in rat model of cerebral ischemia and reperfusion. Male Sprague Dawley rats (290–340 g) were subjected to middle cerebral artery occlusion (MCAO) for 2 h and reperfusion for 70 h. Experimental animals were divided into four groups: Group I – sham operated ($n = 7$); Group II – vehicle treated ischemic–reperfusion (IR) ($n = 9$), and Group III and IV – TOFs treated (100 and 200 mg/kg body weight, p.o., respectively; $n = 7$ in each group). Vehicle or TOFs were pretreated for four days before the induction of ischemia and continued for next three days after the ischemia i.e. treatment was scheduled totally for a period of 7 days. MCAO surgery was performed on day 4, 1 h after TOFs administration. Neuroprotective effect of TOFs was substantiated in terms of neurological deficits, excitotoxicity (glutamate, glutamine synthetase and Na^+K^+ATPase levels), oxidative stress (malondialdehyde, super oxide dismutase, and glutathione) and neurobehavioral functions in the experimental animals. TOFs decreased glutamate, glutamine synthetase (GS) and increased Na^+K^+ATPase activity in a dose dependent manner when compared to the IR rats. Treatment with TOFs significantly reduced the neurological deficits and reversed the anxiogenic behavior in rats. Further, it also significantly decreased MDA and increased superoxide dismutase (SOD) and glutathione content in brains of experimental rats. Histopathological examination using cresyl violet staining revealed the attenuation of neuronal loss by TOFs in stroke rats. The present study demonstrates the unswerving involvement of TOFs on ischemia–reperfusion triggered biochemical alterations in MCAO/R rats. Hence, TOFs might be an attractive candidate for further studies in the development of new drugs for cerebral stroke treatment.

Abbreviations: AMPA, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate; ANSA, amino napthol sulphonic acid; ATP, adenosine triphosphates; BHT, butylated hydroxyl toluene; CA, cornu ammonis; CR, *Cyperus rotundus*; DNPH, dinitrophenyl hydrazine; DTNB, 2,4-dithionitrobenzene; EAAT, excitatory amino acid transporters; GluR, glutamate receptor; GS, glutamine synthetase; GSH, reduced glutathione; HPTLC, high performance thin layer chromatography; IAEC, Institutional Animal Ethical Committee; IR, ischemic–reperfusion; MCAO, middle cerebral artery occlusion; MCAO/R, middle cerebral artery occlusion and reperfusion; MDA, malondialdehyde; mGLUR, metabotropic glutamate receptor; Na.EDTA, ethylene diamine tetra acetic acid.sodium salt; NAD, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide reduced; NBT, nitro blue tetrazolium chloride; NMDAR, N-methyl-D-aspartate receptor; NO, nitric oxide; $O_2^{\bullet}$, superoxide; $ONOO^-$, peroxy nitrite; PMS, phenazonium methosulphate; SO, sham-operated; SOD, superoxide dismutase; SSA, sulphosalicylic acid; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reacting substance; TCA, trichloro acetic acid; TLC, thin layer chromatography; TOFs, total oligomeric flavonoids.

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1. Introduction

Cerebral stroke is the second most common cause of death in the developed countries after ischemic heart disease and the largest cause of disability in patients [7,41]. Stroke registries have shown that 87% of strokes are due to cerebral ischemia [32]. Stroke triggers a cascade of cellular and molecular events leading to delayed neuronal death. The massive release of glutamate and over-activation of glutamergic receptors are the well established mechanisms in the pathogenetic processes of neuronal death following ischemia. Sustained elevation in the intracellular calcium $[Ca^{2+}]_i$ levels reported to trigger calcium dependent catabolic processes such as activation of phospholipases, proteases and endonucleases or free radicals generation ultimately leading to cell death, otherwise known as "Excitotoxicity" [21]. Further, elevated levels of $[Ca^{2+}]_i$ triggers substantial generation of reactive oxygen species (superoxide anion, hydroxyl radical and hydrogen peroxide) and reactive nitrogen species (NO, $ONOO^-$) in ischemic–reperfusion injury [26]. Alarmingly, the post-ischemic status have significant prospects of problems that can lead to impaired cognition and memory, progressing to serious neurological disorders such as sensorimotor deficits and behavioral alterations [20,28,42]. Neuron–astrocyte interactions plays major role on the metabolism of excessive glutamate following ischemia. Na^+K^+ATPase and glutamine synthetase play major role in the transport and breakdown of glutamate in brain [5]. Thus Na^+K^+ATPase and glutamine synthetase can serve as better research markers in ischemic–reperfusion injury and also in the screening of newer therapeutic agents for neuroprotective property.

Although the prospects for the development of neuroprotective agents for treatment of stroke are promissory at preclinical level, the translational studies ranging from basic research to clinical applications shows no convincing evidence either in reducing the infarction size or in improving the overall outcome [12]. This necessitates the exploration of novel therapeutic regimens, including herbal based drugs for the treatment of stroke. Exploration of health benefits of flavonoids is tremendously increasing due to their broad spectrum of biological activities. Large body of evidences shows that dietary flavonoids have remarkable neuroprotective effect. Keli et al. demonstrated that dietary flavonoids improve learning and memory processes and also have protective effect against cerebral ischemia induced damages via the modulation of critical neuronal signaling pathways [16].

In the Ayurvedic system of medicine, *Cyperus rotundus* L (family: Cyperaceae) is used as nervine tonic, nootropic and sedative [37]. Root extracts of *C. rotundus* (CR) showed the presence of β-sitosterol, cyperene, cyperol, flavonoids, sesquiterpenoids, ascorbic acid and polyphenols [39]. Methanolic extract of CR rhizome showed to inhibit nitric oxide (NO) production in RAW 264.7 cells and also potential anti-inflammatory effect [36]. The high flavonoids content [44], especially total oligomeric flavonoids (TOFs) of CR was correlated to various biological activities [17,18]. Recently, Lee et al., demonstrated the neuroprotective role of CR in an in vitro model of Parkinsonism [19]. The present study demonstrates the neuroprotective potential of TOFs in the middle cerebral artery occluded and reperfused (MCAO/R) rats. The neuroprotective effect of TOFs was evaluated in terms of neurological deficits, anti-excitotoxicity (glutamate, glutamine synthetase, Na^+K^+ATPase – the key players in excitotoxicity) and neurobehavioral functions in MCARO/R rats. Efficacy of TOFs was also substantiated by the histopathological examination using cresyl violet stain.

2. Materials and methods

2.1. Plant material and chemicals

Rhizomes of *C. rotundus* was a kind gift of M/s. SKM Siddha and Ayurvedic Medicines India Private Limited, Tamil Nadu, India and authenticated by Dr. P. Jayaraman, Botanist, Plant Anatomy Research Center, Chennai, India. A Voucher specimen of the herb is deposited in phytochemistry division of CEFT, SRU for future reference. L-Glutamic acid and 1,1,3,3-tetra ethoxypropane were purchased from Sigma, USA. 4-0 nylon monofilament Ethicon® was procured locally in Chennai, India. Precoated HPTLC plates, amino napthol sulphonic acid (ANSA), sodium pyrophosphate, and sulphosalicylic acid (SSA) were procured from M/s. Merck, Mumbai, India. Ninhydrin, guanidine HCl, nitro blue tetrazolium chloride (NBT), phenazonium methosulphate (PMS), nicotinamide adenine dinucleotide reduced (NADH), 2,4-dithionitrobenzene (DTNB), butylated hydroxyl toluene (BHT), naphthyl ethyldiamine, adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NAD) were supplied by M/s. SISCO Research Laboratories, Mumbai, India. Thiobarbituric acid (TBA) and sulphanilamide were procured from M/s. Himedia laboratories, Mumbai, India. Dinitrophenyl hydrazine (DNPH) was procured from M/s. Loba Chemie, Mumbai, India. All other chemicals, reagents and solvents were of analytical grade unless mentioned.

2.2. Preparation of TOFs

TOFs was prepared as described by Kilani et al. [17]. The rhizome was shade dried and made into coarse powder. It was then macerated in 1:2 (v/v) ratio of water/acetone and incubated in dark for 6 h with intermittent stirring. Acetone was evaporated under low pressure and the extract was filtered. Filtrate was precipitated with excess of NaCl for 24 h at 5 °C to separate tannins. The supernatant was collected and extracted with ethyl acetate, concentrated and precipitated with excess of chloroform. Precipitate was separated and the TOFs fraction was dissolved in water for further investigation.

2.3. Animals

Male Sprague Dawley rats of 290–340 g body weight range were used in the study. Animals were housed individually in polypropylene cages in a room (with 15 air cycles per minute in the ratio of 70:30 air exchanges) under an ambient temperature of 25 ± 2 °C and 40–65% relative humidity, with a 12-h light/12-h dark cycle. Animals were provided with standard rodent pellet diet (Nutrilab Rodent, Tetragon Chemie, India) and purified water ad libitum (RIOS, USA). Animals were acclimatized for 7 days to the laboratory conditions prior to initiation of experiment. Guidelines of "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, National Academic Press 1996; NIH publication number #85-23, revised 1996) were strictly followed throughout the study. Study protocol was approved by Institutional Animal Ethical Committee (IAEC), Sri Ramachandra University, Chennai, India (EC-XI/SRMC&RI/62/2006).

2.4. Experimental design and drug treatment

Schematic overview of the experimental schedule is shown in Fig. 1. The experimental animals were divided into four groups consisting of 7–9 animals in each group and treatment was carried out as mentioned below. Vehicle or TOFs was administered once a day for a period of 7 days (i.e. 4 days prior to induction of ischemia and continued for next 3 days following the induction of ischemia). The experimental animals were sacrificed following the behavioral tests.

Group 1: Sham Operated (SO) rats; received double distilled water as vehicle (5 ml/kg, p.o., $n = 7$).
Group 2: IR (IR) rats; received double distilled water (5 ml/kg, p.o., $n = 9$).
Group 3: TOFs low dose rats; received TOFs (100 mg/kg, p.o., $n = 7$).
Group 4: TOFs high dose rats; received TOFs (200 mg/kg, p.o., $n = 7$).

2.5. Surgical procedure

Focal cerebral ischemia was induced by middle cerebral artery occlusion as described by Longa et al. [24] with minor modifications. Rats were anesthetized with chloral hydrate (350 mg/kg, i.p.) and right common carotid artery was exposed at the level of external and internal carotid artery bifurcation. The tip of 4-0 nylon monofilament was made round headed by flaming and then coated with 0.01% poly-L-lysine and inserted into the external carotid artery and advanced to the internal carotid artery for a length of about 20–21 mm until a slight resistance was felt. After occlusion, the filament was held in place with a ligature and skin was temporarily sutured. After 2 h of ischemia the rats were anesthetized, suture was released, the filament was withdrawn to establish reperfusion that was visually ensured. Throughout the surgical procedure, body temperature was monitored by inserting rectal thermometric probe and maintained at 37 ± 0.5 °C by a thermostatically controlled heating blanket. Animals were then housed in a cage with heating lamp to maintain the temperature at 29 ± 1 °C for another 1 h to counteract any possible hypothermic effect. In sham-operated group, external carotid artery was surgically prepared for insertion of filament, but filament was not inserted. Since the study was focused mainly on the biochemical events and neurobehavioral alteration following MCAO/R, regional blood flow monitoring was not kept as one of the objectives. Excluding the primary author who performed the surgery, all others involved in the study are blinded to the study protocol.