CHAPTER 1
INTRODUCTION

1.1. Cerebral Ischemia

Stroke is the second most common single cause of death in developed countries after ischemic heart disease and largest cause of disability worldwide (1, 2). Brain tissue is exquisitely sensitive to ischemia, such that even brief ischemic episodes to neurons can initiate a complex sequence of events that ultimately may culminate in cellular death. Different brain structures have varying thresholds for ischemic cell damage, with white matter being more resilient than gray matter (3). Brain exclusively dependents on the continuous delivery of oxygen and glucose through blood flow, and interruption of the cerebral blood supply exacerbates irretrievable damage (4). The major pathobiological mechanisms of ischemia/reperfusion (IR) injury include excitotoxicity, oxidative stress, inflammation and apoptosis (5-8).

Focal ischemia entails reduction in cerebral blood flow (CBF) to a specific vascular territory, usually encountered due to thrombotic/embolic or hemorrhagic blocks (9). The tissue in the center of ischemic area with severe CBF reduction is termed as ischemic core. Circumference of ischemic core where the blood flow is normal, the ischemic injury becomes progressively less severe. This peripheral region of the ischemic territory is called ischemic penumbra (Fig 1.1). In all cases, the stroke ultimately involves dysfunction or death of brain cells, giving rise to cerebral
infarction. Depending on the loci and size of the infarct, stroke may lead to neurological deficits or in severe cases, death.

World Health Organization (WHO) data shows 5.7 million deaths from cerebrovascular diseases out of 58 million global deaths in 2005. The number of people with transient ischemic attack (TIA) is estimated to be even greater (10). It is estimated that stroke is responsible for about 102000 deaths annually in India, which represent about 2% of the total deaths in the country (11).

Analysis of community surveys from different regions of India shows a crude stroke prevalence rate of about 203 per 100,000 populations above 20 years of age, amounting to a total of about 1 million cases with a male to female ratio estimated to be 7:1. Community survey in Kolkata, showed stroke prevalence rate of 545 per 100,000 populations is equal to or higher than that reported from developed countries (12). Though incidence of stroke and related mortality has declined by over 49% in developed countries, there exists growing concern over the emerging epidemic of stroke in low and middle income countries. Reports anticipates rise in life expectancy and in this aspect Indian economy will be burdened in socioeconomic prospect to meet the costs of managing stroke, hence current scenario demands the need for effective treatment regimens for the management of stroke.
Glutamate a major excitatory neurotransmitter in the brain plays important role in the pathogenesis of neuronal injury and death induced by cerebral ischemia (13). Stroke triggers a cascade of cellular and molecular events leading to delayed neuronal death. Release of glutamate and over-activation of glutamergic receptors are well-established mechanisms in the pathogenetic processes of neuronal death following ischemia. Sustained elevation in the intracellular calcium $[\text{Ca}^{2+}]_i$ levels is reported to trigger calcium dependent catabolic processes such as activation of phospholipases, proteases and endonucleases ultimately leading to cell death, otherwise known as “Excitotoxicity” (14). Further, elevated levels of $[\text{Ca}^{2+}]_i$ triggers substantial generation of reactive oxygen species (superoxide anion, hydroxyl radical and hydrogen peroxide) and reactive nitrogen species (NO, ONOO⁻) in ischemic – reperfusion (I/R) injury (15).
Brain is vulnerable to oxidative stress due to its high polyunsaturated fatty acids (PUFAs) content which are particularly susceptible to reactive oxygen species damage (16). Overproduction of reactive oxygen species results in oxidative damage, including lipid peroxidation, protein oxidation and DNA damage, which leads to cell death (17).

Reactive oxygen species (ROS) have been indicated as one of the earliest and most important components of tissue injury after reperfusion of ischemic brain and the extent of brain injury appears to depend on the experimental pattern of I/R. Free radical production is continuous during ischemia, while during reperfusion it is primarily confined to the early stage when fresh oxygen is supplied to the ischemic region (18). Intracellular Ca\(^{2+}\) elevation in the mitochondria further initiates downstream events, including activation of calpain, protease activity that effect structural integrity of both intra and extracellular structure of cellular membranes. Increased Ca\(^{2+}\) also induces nitric oxide synthase activity and expression, which favors formation of peroxynitrate. This eventually overwhelms the mitochondria, which leads to mitochondrial depolarization and swelling.

Further to mitochondrial dysfunction the penumbral neurons undergo apoptotic damage rather than necrotic cell death which depends on the magnitude of initial injury. Overwhelming evidences suggest that besides necrosis, apoptosis do contributes significantly to the cell death subsequent to I/R injury (19). Cerebral ischemia triggers two general
pathways of apoptosis: the intrinsic pathway, originating from mitochondrial release of cytochrome c and associated stimulation of caspase-3; and the extrinsic pathway, originating from the activation of cell surface death receptors, resulting in the stimulation of caspase-8 (20).

Neuron – astrocyte interactions plays major role in the metabolism of excessive glutamate. Astrocytes regulate synaptic levels of glutamate via the excitatory amino acid transporters present in the astrocyte preventing accumulation of glutamate in the synaptic cleft and thus protect neurons from excessive activation of glutamate receptors and excitotoxic injury (21). During cerebral ischemia, impairment of astrocytic Na⁺K⁺ATPase leads to accumulation of extracellular K⁺, leading to astrocytic swelling. The swollen astrocytes may release glutamate in to extracellular space contributing to excitotoxic neuronal death in surrounding tissue (22). Astrocyte is involved in glutamate, cysteine and glycine cycle where it synthesis glutathione (23). Excess of glutamate inhibits the transport of cysteine thereby blocking the glutathione formation. On the other hand, oxidative stress collapses the glutamate-cystine antiporter which leads to depletion of glutathione level (24).

Experimental models of cerebral ischemia have been developed to understand the deleterious mechanisms involved in brain ischemic damage and to the development of therapeutic strategies. One of the most clinically relevant models of focal ischemia is the development of middle cerebral artery occlusion/reperfusion using intraluminal nylon
monofilament in rats (25). Transient MCA occlusion (MCAO) mimics the problem of both ischemia and reperfusion, whereas permanent MCAO models the problem of long term vessel blockade (26). Transient focal ischemia produces varying degrees of ischemic damage depending on the duration of ischemia (27). Importantly, after transient ischemia, brain damage results from both the ischemia and the effects of reperfusion (reperfusion injury). Compared to permanent occlusion, which mimic only a minority of human strokes where there is no recanalization, transient models better correlate with conditions such as therapy-induced thrombolysis, spontaneous thrombolysis, and transient ischemic attack (28).

Ischemia is associated with a high incidence of deficits in sensorimotor function and cognitive ability (29). Thus, studies of functional outcome after experimental stroke have focused primarily on sensory, motor, or cognitive deficits. The sensorimotor tasks are most commonly used in MCAO studies assess postural abnormalities, coordinated movements, balance, forelimb strength, locomotor activity or sensory capabilities. In addition stroke is associated with an increased incidence of anxiety although the etiology remains unknown (30). Damage to the cortex and caudate putamen is not typically associated with increase in anxiety, however, it is necessary to rule out the possibility that experimental stroke causes increased anxiety in rodents which could then affect performance in additional testing. Anxiety is commonly assessed using an open field test or the elevated plus maze. Further, earlier studies have reported that
Neuroprotective role of total oligomeric flavonoids of Cyperus rotundus in rat model of transient focal ischemia

Rats subjected to permanent occlusion of bilateral common carotid artery (BCCA) show impaired spatial learning/memory capabilities and/or structural alterations (31).

Several in vitro experimental models have been used to mimic brain ischemic conditions and to elucidate the protective activities of test agents against ischemic damage. Rat pheochromocytoma (PC12), a cell line derived from a pheochromocytoma of the rat adrenal medulla has been used as an in vitro model as it possesses much of the biochemical machinery associated with synaptic neurons. PC12 cells can differentiate into neuronal-type cells following treatment with nerve growth factor (NGF) (32). These cells have been used as a model system for studying the regulation of neuronal differentiation (33), peripheral neuropathies (34) and neurosecretion (35) as well as evaluating neurodegenerative disorders such Parkinson’s disease or Alzheimer’s (36). Nerve growth factor, a prototypical neurotrophic factor and a member of the neurotrophin family, promotes a wide range of responses in its target cells. These ranges from neuronal differentiation, maintenance of survival, and regulation of metabolic activities (37). Several marker proteins for PC12’s differentiate into sympathetic neuron-like cells and studies of synthesis of various mRNAs in PC12 cells treated with NGF seem imminent. PC12 cells differentiate into neuronal phenotype and attain morphology by formation of neurofilaments, the major structural components of neurons, both at the level of mRNA and protein expression. The three mammalian neurofilament subunits, NF-L (68 kDa), NF-M (145 kDa) and NF-H...
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(≈200 kDa), are believed to form heterodimers consisting of NF-L in combination with either NF-M or NF-H (38).

1.2. Mechanism of ischemic brain injury

Ischemic stroke is a complex phenomenon which involves numerous processes, including energy failure, loss of ion homeostasis, acidosis, increased intracellular calcium levels, excitotoxicity, free radical-mediated toxicity, generation of arachidonic acid products, cytokine mediated cytotoxicity, complement activation, disruption of the blood-brain barrier (BBB), activation of glial cells, and infiltration of leukocytes (39). Within few minutes of cerebral ischemia, the core of brain tissue exposed to the most dramatic blood flow reduction and subsequently undergoes necrotic cell death (20). Each of the above pathophysiological processes has a distinct time frame, some occurring over minutes, others over hours and days, causing injury to neurons, glia and endothelial cells (Fig 1.2).

Focal impairment of cerebral blood flow restricts the delivery of metabolic substrates, particularly oxygen and glucose, leading to depletion of energy stores required to maintain ionic gradients (40). Na⁺K⁺ATPase found on the plasma membrane of neurons, consumes 70% of the energy supplied to the brain (41). After ischemia, reduction in mitochondrial ATP synthesis leads to neuronal plasma membrane depolarization, release of potassium into the extracellular space and entry of sodium into cells (42). Loss of membrane potential and subsequent depolarization of neurons and glia triggers excitotoxic amino acid efflux from presynaptic neurons in
large amounts, which is very early event in ischemia. Concomitantly, an energy-dependent process such as presynaptic reuptake of excitatory amino acids is impeded, which increases the accumulation of glutamate in the extracellular space. This direct neurotoxicity of glutamate on neurons, activates glutamate receptors N-methyl-D-aspartate (NMDA) and α-amin-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors leading to further increase in intracellular Ca$^{2+}$,Na$^+$ and Cl$^-$ levels exacerbating the amount of edema and toxicity via intracellular Ca$^{2+}$ overload (43). Increased intracellular calcium overload in mitochondria and uncontrolled degradation of cellular structures activates many, if not all calcium dependent proteases, lipases and DNases (41).

As a consequence to the calcium overload in mitochondria during focal ischemia and particularly when reperfusion occurs, oxygen radicals are produced during enzymatic conversions, such as cyclooxygenase-dependent conversion of arachidonic acid to prostanoids and degradation of hypoxanthine (44) leading to a burst of free oxygen radicals and release of proapoptotic proteins (45). Free radicals cause lipid peroxidation, membrane damage and dysregulation of cellular processes. These events lead to necrosis or programmed cell death depending on the intensity of insult and the metabolic state of the neurons.
1.3. Treatment strategies in stroke

Catastrophic temporal and anatomical nature of stroke conspires with realities to produce consequences that are difficult to treat with medicines, and thus far, this has been a challenge beyond the capacities of modern medicine. Neuroprotection for stroke refers to strategies, applied singly or in combination, that antagonise the alterations in biochemical and molecular events (46). There are two major classes of therapeutic approaches to acute stroke: neuroprotectants that target biochemical pathways controlling cellular fate to preserve brain function and/or enhance neuronal repair and recovery and thrombolytics that...
restore cerebral blood flow (47). A wide variety of neuroprotective agents have been studied which interfere at various points in ischemic cascade; the most extensively studied agents are antagonists of voltage gated calcium channels, sodium channel blockers, excitatory amino acid receptor antagonists and free radical scavengers (48).

Excitatory amino acid receptor antagonists mediate the action through glutamate receptor subtypes, including NMDA and AMPA receptors. Experimentally, the prototypic agents like NMDA antagonists have shown significant neuroprotective effect but unfortunately, they did not show protective effect in experimental focal ischemia (49). During reperfusion, or late phase of permanent focal ischemia, AMPA antagonists have been demonstrated to protect CA1 region (hippocampal zone) and to reduce infarct volume (50). Systematic review of trials tested on excitatory amino acid antagonists showed no improvement in rates of either death or favorable outcomes in stroke (51). Although the prospects for 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 improving the overall outcome (52). This necessitates the exploration of novel therapeutic regimens, including herbal based drugs for the treatment of stroke.

Therefore, molecules that provoke multiple protective and regenerative mechanisms in combating pathological events like oxidative stress and apoptosis gain much importance. Oxidative stress leading to ischemic cell death involves the formation of reactive oxygen species.
through multiple injury mechanisms, such as mitochondrial inhibition, intracellular ca\(^{2+}\) overload and inflammation (53). Reactive oxygen species produced during dysregulated antioxidant homeostasis not only injure other cellular macromolecules, but also activate molecular signaling cascades leading to recruitment of inflammatory cells to the site of injury thereby enhancing neuronal death (6, 54). In this context, free radicals can be regarded as an important therapeutic target for improving the outcome of an ischemic stroke. Several compounds with significant antioxidant properties including ebselen (55) and resveratrol (56), a natural phytoalexin found in dietary sources such as grapes and red wine have been demonstrated to reduce brain damage in animal models of stroke.

In recent times, more attention in the field of drug discovery has been focused on the neuroprotection of natural compounds from traditional medicinal herbs. Studies have focused on the possible capacity of natural compounds extracted from fruits, vegetables and beverages to prevent neurological disorders especially polyphenols such as quercetin, catechin and resveratrol (57). Indian medicinal plants have been extensively studied against various neurological disorders like stress, learning and memory diseases, depression and anxiety (58). Many herbal drugs and prescriptions including *Ginkgo biloba* extract (GBE) (59), gypenosides, green tea extract (60), *puerariae* (61) and garlic extracts (62) have been used clinically for the treatment of various neurological disorders. Exploration of health benefits of flavonoids is tremendously increasing due to their broad spectrum of biological activities with evidences emphasizing remarkable neuroprotective effects of dietary flavonoids. Flavonoids are ubiquitous plant polyphenols increasingly treasured as chemopreventive
agents against pathophysiological conditions, such as cancer, cardiovascular disease and neurodegenerative diseases (63, 64). The primary activity of plant flavonoids is believed to reside in their free radical scavenging capacity (65). Large body of evidences shows that dietary flavonoids have remarkable neuroprotective effect. They were shown to improve learning and memory processes and also have protective effect against cerebral ischemia induced damages via the modulation of critical neuronal signaling pathways (66).

*Cyperus rotundus* (CR) (Family: Cyperaceae), a traditional Indian medicinal plant is used as a nerve tonic, nootropic and sedative in Ayurvedic system of medicine (67). Phytochemical reports revealed the presence of β-sitosterol, cyperene, cyperol, flavonoids, sesquiterpenoids, ascorbic acid and polyphenols in the roots of *C. rotundus* (68). Methanolic extract of rhizome of CR showed inhibition of nitric oxide (NO) production in RAW 264.7 cell lines and demonstrated for its anti-inflammatory potential (69). CR is shown to contain high flavonoid content (70) and its total oligomers flavonoids (TOFs) were demonstrated for various biological activity (71). Cyperi Rhizoma, the rhizome of *Cyperus rotundus* were reported to play a major role in protection of neurodegenerative disorders, such as Parkinson’s disease (PD) where neuroprotective effect of *Cyperi Rhizoma* against 6-OHDA-induced toxicity through antioxidant and anti-apoptotic activities in an *in vitro* PC12 PD model was investigated (72).

However the role of CR and TOFs in the ischemic reperfusion have not been studied yet and hence the present study was under taken to evaluate the neuroprotective effect of TOFs *in vitro* PC12 cell line model and *in vivo* rat model of Transient focal ischemia.
1.4. **Aim of the present study**

To investigate the neuroprotective role of total oligomeric flavonoids (TOFs) of *Cyperus rotundus* (CR) using *in vitro* and *in vivo* studies.

1.5. **Objectives of the present study**

- To determine *in vitro* free radical scavenging activity and *in vitro* cytotoxic activity of CR extract and TOFs.
- To unravel the role of TOFs against N-methyl-D-Aspartate (NMDA) induced neuronal damage in PC 12 cells lines.
- To ascertain the role of TOFs on ROS production and apoptotic signaling in PC12 cells.
- To evaluate the role of TOFs on neurochemical and biochemical alterations following MCAO/R in rats.
- To determine the significance of TOFs on neurological and behavioural alterations in MCAO/R in rats.
- To ascertain the extent of neuroprotective effect of TOFs through histopathological examinations.