CHAPTER 2

THEORETICAL BACKGROUND

Highlights of the Chapter

- *Cerebral ischemia, pathophysiology and treatment*
- *Rodent model of cerebral ischemia*
- *Role of molecular docking for screening of inhibitors*
- *Chlorogenic acid*
- *Impedance spectroscopy for differentiation of normal and ischemic condition*

2.1 Cerebral ischemia, pathophysiology and treatment

Stroke or Cerebrovascular accident (CVA) is the second leading cause of death worldwide [49]. A cerebral stroke (brain attack) happens when blood circulation to the brain fails [5]. The CVA is of two types i.e. ischemic and hemorrhagic. Ischemic stroke (Cerebral ischemia or cerebrovascular ischemia) occurred when a blood vessel that supply blood to the brain become blocked or narrowed, causes severe reduction in oxygen and glucose to the nerve cells [2]. It is most frequent and is liable for about 85% of the strokes [50]. The blockages in blood vessels can be categories in thrombosis (when a clot is formed within an artery of the brain or neck), embolism (when a clot is formed in another part of the body and translocated to the vessel of the brain or neck) and stenosis (severe narrowing of an artery of the brain) [51]. Hemorrhagic stroke caused when a blood vessel bursts within or the spaces surrounding the brain [52]. In ischemic stroke, a sudden loss of blood flow triggers a complex molecular cascade of detrimental physiological and biochemical events (Fig. 2.1) including overactivation or abnormal activity of NMDARs, Rip1K, nNOS, Calpain, MMP2/9, TNFR-1 and PARP-1 resulting in neuronal cell death [53].

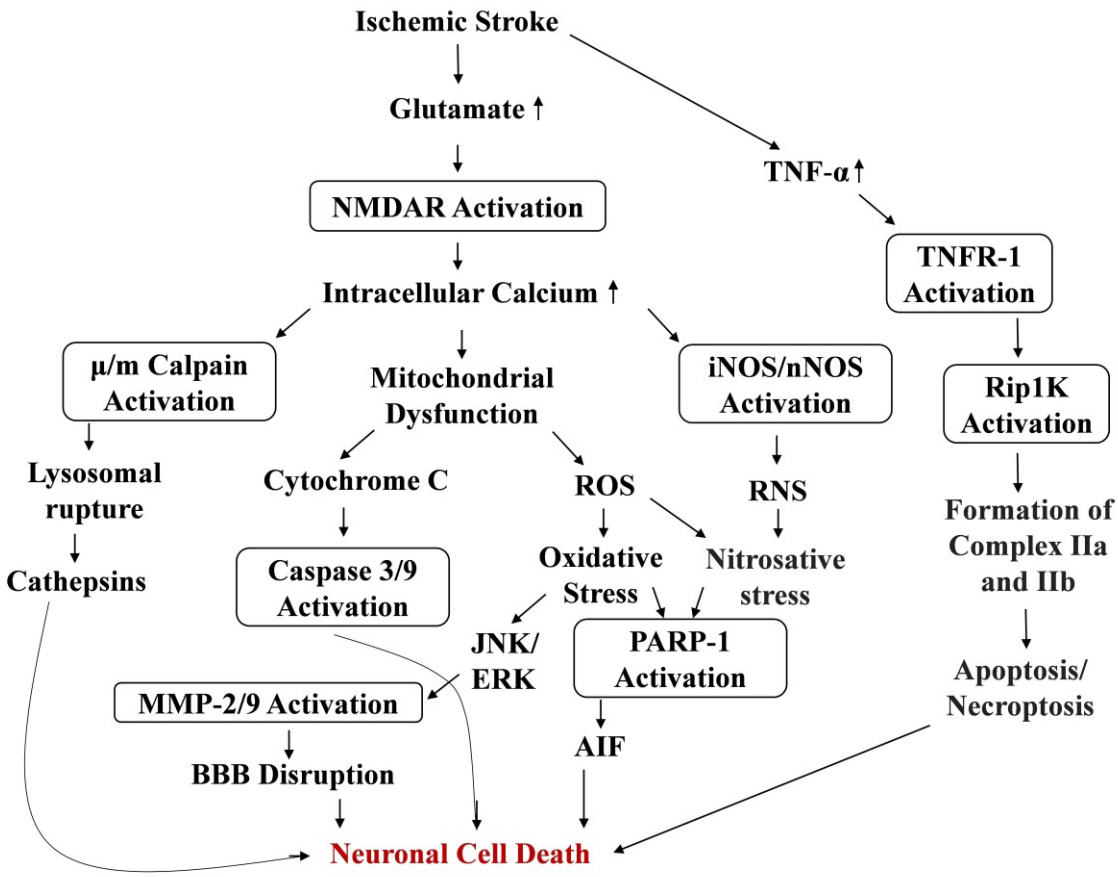

Fig. 2.1: Pathophysiology of ischemic stroke and mechanism of neuronal cell death.

The various mechanisms involved in neuronal cell death are release of excitatory amino acids especially glutamate into the synaptic space [54]. An uncontrolled release of glutamate in extracellular space activates N-methyl-d-aspartate (NMDA), kainite receptors, and α-amino-3-hydroxy5-methyl-4-propionate (AMPA) that triggers the sodium and calcium influx [5]. Accumulation of intracellular calcium ions initiate a series of cytoplasmic and nuclear events, including the triggering of the intrinsic apoptotic pathway [55]. Activation of GluN2B containing NMDARs leading to intracellular calcium overloads resulting activation of calpain, neuronal nitric oxide synthase (nNOS) and mitochondrial oxidative stress [56]. Calpain activation engaging both cathepsins and calpain-dependent cell death [57]. Activation of nNOS leading to peroxynitrite ($ONOO^-$) production that causes lipid peroxidation and mitochondrial

dysfunction [58]. Leakage of cytochrome-c, an inner mitochondrial membrane protein into the cytosol activates caspase-3 resulting neuronal cell death. Formation of reactive oxygen species (ROS) causes oxidative stress leading to production of apoptosis inducing factors (AIF) which promote apoptotic cell death [59]. Following an ischemic event, those neuronal and supporting glial cells received lowest blood supply (10–12 ml/100 g/min or less), immediately dies due to necrosis and formed inner core of infarction. While cells surrounding inner core received relatively high blood supply (less than 18–20 ml/100 g/min) by collaterals appeared as penumbra and can be retrieved after re-perfusion (Fig. 2.2).

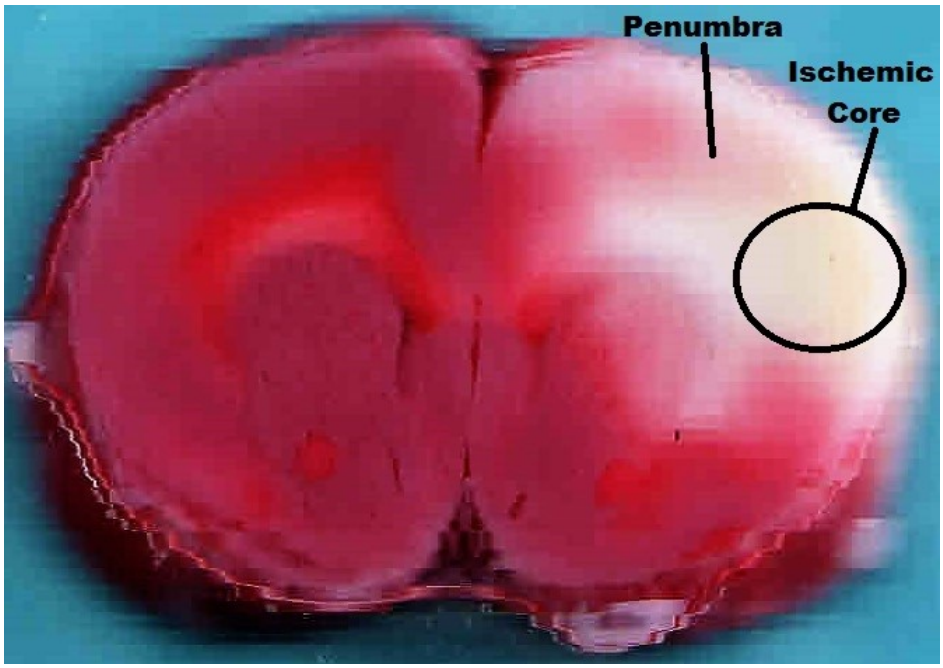

Fig. 2.2: Ischemic core and penumbra in a TTC stained coronal brain section of rat.

2.2 Ischemic stroke treatment strategies

The treatment strategies for ischemic stroke in the acute phase involves use of thrombolysis, antiplatelet, anticoagulants and neuroprotecting agents [60].

- **Thrombolysis**

The goal of thrombolytic therapy is to restore blood flow using either pharmacological agent or mechanical procedure or both. The administration of recombinant tissue plasminogen activator (rtPa) intravenously within 4.5h of symptom onset lyse the thrombus by converting the serine protease zymogen plasminogen into plasmin- a major enzyme responsible for clot breakdown and improves the patient outcome at 3–6 months [61]. Till date, rtPA is single FDA approved drug available for treatment of acute ischemic stroke [60]. Administration of rtPA outside of the recommended window (4.5 h) upturns the risk of intracerebral hemorrhages and makes worse the injury [8]. Therefore, this narrow therapeutic window of 4.5 hours limits its use to a major population of patients worldwide. Recent studies suggest that mechanical thrombectomy is superior to intravenous thrombolysis alone [62]. In this method, a blood clot of large anterior circulation is mechanically pulled out [63]. This procedure also has a therapeutic windows of up to six hours after onset of stroke [64].

- **Antiplatelet**

Antiplatelets are drugs that reduces the platelet aggregation and decrease the formation of thrombus and blood clot. This strategy is effective to prevent the ischemic stroke. Although a number of antiplatelets agents are in advanced stage of clinical trials but aspirin is widely used and accepted molecule for the treatment of acute ischemia in early stage [65]. When a patient becomes intolerant for the aspirin then another antiplatelet (clopidogrel or ticlopidine) might be used for the treatment but the efficacy of latter drugs has not proven in ischemic stroke subjects till date. Also they have severe side effects compared to aspirin [66].

- **Anticoagulants**

Anticoagulants is also used for the treatment of cerebral ischemia. These drugs block the production of vitamin K-dependent clotting factors which is required for blood clotting hence prevent the clot formation. It includes heparinoids, heparins, and thrombin inhibitors [67]. Several clinical studies suggest that administration of anticoagulants increases the risk of bleeding and intracranial hemorrhages hence increases the rate of mortality of stroke patients [68] [69].

- **Neuroprotective agents**

Neuroprotective agents are compounds that can diminish neuronal injury caused due to ischemia and reperfusion through varied targets. It can shield the brain from venerable damage and can preserve the neuronal functions by suppressing the neural apoptosis, decreasing the local cellular metabolism, and reducing the production of neurotoxin in the penumbra. It may also combined with existing thrombolytic rtPA treatment to enhance the therapeutic window. The preclinical studies have demonstrated that overexpression or abnormal function of GluN2b containing NMDARs [70], Calpain [71], Matrix metalloproteinase (MMP)-2/9 [72], [73], nNOS, iNOS [74][75][76], poly ADP ribose polymerase-1 (PARP-1) [77], Receptor-interacting serine/threonine-protein kinase 1 (RIPK1) [78], IkB kinase beta (IKK-β/IKK-2) [79] and tumor necrosis factor-α (TNF-α) converting enzyme (TACE) [80] play detrimental role in neurological disorders; and inhibition of these molecular mediators might be a useful therapeutic strategy for neuroprotection. Although several findings have shown great promise in animal model of ischemic stroke [81], but most of them failed during translating for human application in clinical trials [82]. However, many clinical trials of neuroprotective drugs fails

in the past [83], the inhibition of molecular mediators of neurological dysfunction still hold promise in the treatment of ischemic stroke.

2.3 Rodent model of cerebral ischemia

The experiment al models for cerebral ischemia can be categorized as focal, global and multifocal ischemia (Fig 2.3) [84]. Global ischemia mimics the neuronal injury caused due to cardiac arrest and occurs when cerebral blood flow (CBF) of whole or most of the brain is reduced [85]. The focal cerebral ischemia is closely mimics the condition of human ischemic stroke that represents by occlusion in middle cerebral artery (MCA) resulting reduced blood flow to very specific region [86]. The multifocal model of cerebral ischemia occurs due to reduction of CBF in different location of the brain [87].

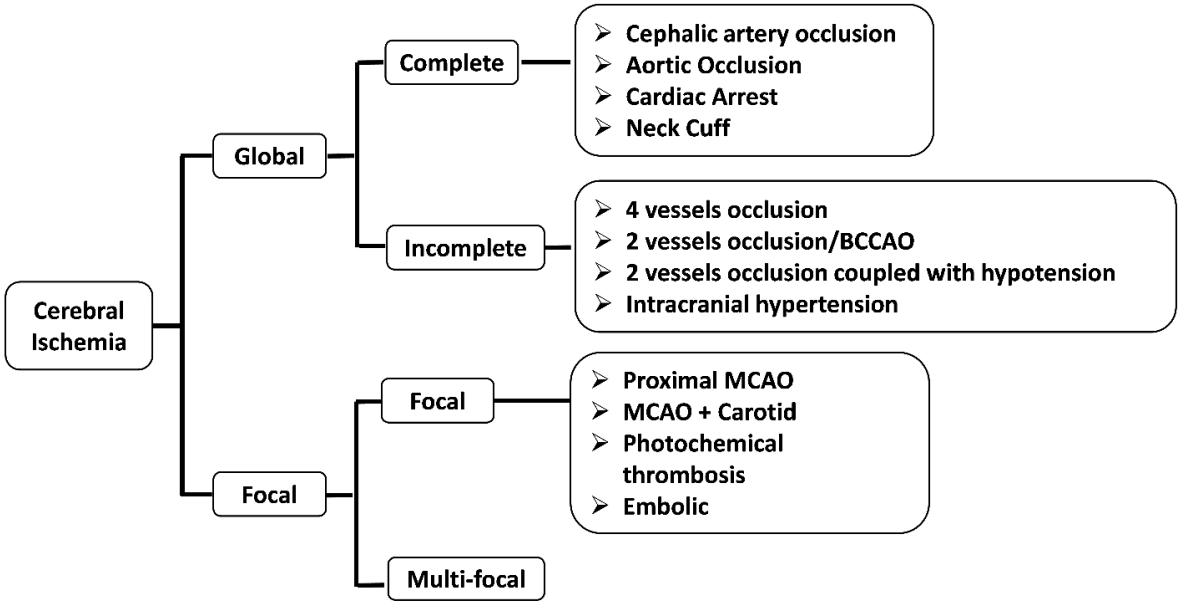

Fig 2.3: Types of cerebral ischemia. BCCAO: Bilateral Common Carotid Artery Occlusion; MCAO: Middle Cerebral Artery Occlusion. (Adopted with permission to [84])

Though clinically significant, focal brain ischemia is reported as not so appropriate method for studying brain damage due to cardiac arrest/resuscitation [88][89]. The modeling of this clinical phenomenon requires ischemia induction in the whole brain followed by resumption of cerebral blood flow, which is often mimicked using cardiac arrest induction closely followed by Cardiopulmonary resuscitation (CPR) and defibrillation [90]. Also, this model requires a large number of animals for studying a hypothesis as it is responsible for high mortality, thus necessitating a more dependable, consistent, reproducible model with better survival chances for examining the outcomes of global cerebral ischemia. Induction of global cerebral ischemia and consequent preservation of systemic blood flow reduces mortality and allows better possibilities of exploring the changes occurring due to brain damage. Interruption of blood flow via vascular structure Circle of Willis, composed of internal carotid arteries and vertebral arteries, is necessary for the production of global cerebral ischemia (Fig. 2.4) [91]. These four vessels are responsible for cerebral blood supply and the anastomotic loop formed by these arteries helps to maintain the perfusion in case of proximal vascular occlusion. So, interruption of blood flow via all contributory vessels is required to induce global ischemia in the brain [39]. Carotid arteries can be occluded using aneurism clips after exposing them by a minimal invasive cut in the ventral neck region, but due to encasement of vertebral arteries inside transverse foramina of the vertebral column, the obstruction of blood flow via these vessels are difficult. A 24-48 h prior electrocauterizing of the vertebral arteries to common carotid arteries occlusion in 4VO model can overcome this problem for the induction of cerebral ischemia. But due to surgical complexity, more invasive nature of the model resulting in high mortality, 2VO is generally preferred for the studying cerebral ischemia in laboratory condition (Fig. 2.5).

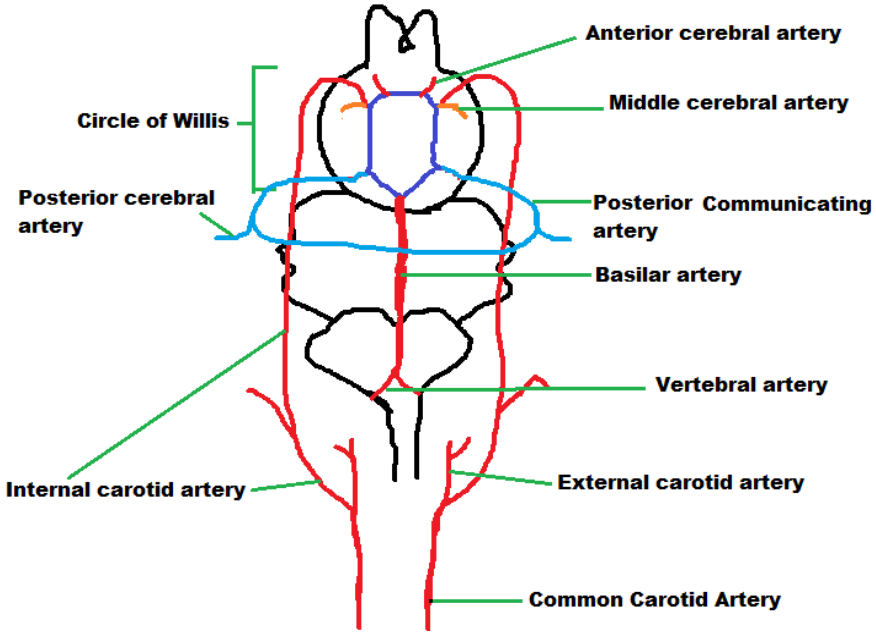

Fig. 2.4: Arterial system of rat brain

The 2VO model involves occlusion of bilateral common carotid arteries to induce global cerebral ischemia, but the method has remained controversial. A relatively high reproducible model of induction of brain tissue damage with the variation of severity was demonstrated by Iwasaki et al. by occluding the bilateral carotid artery [92]. A two-fold increase in activity of superoxide dismutase and lipid peroxidation in rat brain due to transient bilateral common carotid artery occlusion (tBCCAO) of 30mins followed by 45 min reperfusion has been reported [93]. BCCAO also decreases the length of dendrites, dendritic spine density, and branching in the CA1region of the hippocampus [94]. BCCAO also reportedly caused impairment of long-term potentiation (LTP) and spatial memory [95]. A recent report by Khoshnam et al. suggests a significant increase in cell death in the hippocampal CA1 region by 30 minutes of tBCCAO and a subsequent 72 hours of reperfusion [96]. In contrast to the studies mentioned above, it was recently suggested that acute BCCAO alone might not be a suitable strategy to induce ischemic condition in rats [38]. Also, according to a study by Faezi

et al., reduction in the neurological deficit scores and infarct volume in rats was observed as a result of preconditioning with prolonged BCCAO and subsequent intermittent BCCAO [97]. The conditions of cerebral ischemia have been observed in an animal model in which hypotension has been induced artificially. Smith et al. demonstrated that a systemic lowering of mean arterial blood pressure (MAP) to 40 mmHg causes reduction of blood flow via the vertebral arteries leading to highly lowered or no blood flow. The condition mentioned above when coupled with occlusion of carotid arteries produced forebrain ischemia [98]. The resulting brain damage due to this process exhibits a pattern similar to patients surviving cardiac arrest [39,98]. But, the model remains complex due to the requirement of blood withdrawal from either a femoral artery or jugular vein. Cerebral ischemia has a very complex cellular cascades that are responsible for neuronal injury. Till date none of the rodent models closely mimic the human cerebral ischemia pathophysiology. The fact that human stroke patients also suffer from other pathologies like hypertension, arrhythmia and diabetes that affect the pathophysiology of stroke in its own way.

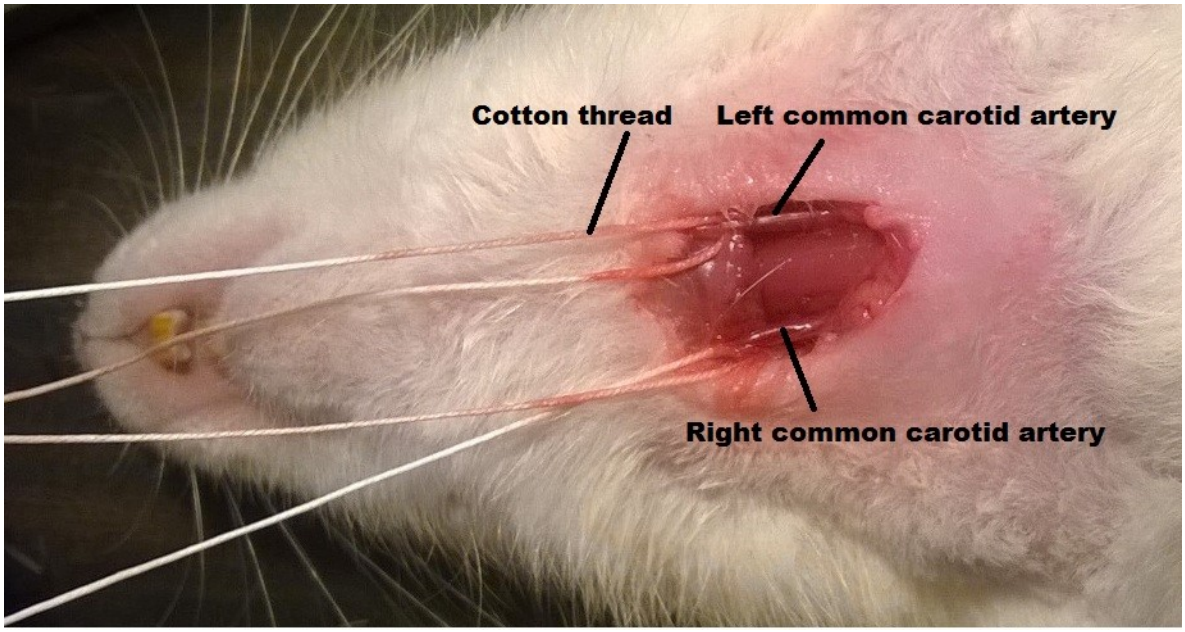

Fig. 2.5: Bilateral common carotid artery occlusion

2.4 Role of molecular docking for screening of inhibitors

Structure-based drug designing and virtual screening have now emerged as valuable tools for the identification of potent inhibitors against receptors, enzyme, and proteins [99]. Protein data bank comprises crystal/solution structures solved by X-ray crystallography and NMR that are co-crystallized with various ligands (substrate, inhibitor) which provides significant understanding for their catalytic/allosteric site and mode of binding [100]. These data provide information about the ligands-residues interactions near the active site and can be used for designing of potent and selective drug-like inhibitors. The molecular docking plays a significant role to model the interaction between a lead compound and a protein at the molecular level. It allows researchers to explain the basic biochemical processes as well as characterize the activity of lead molecules in the active/catalytic site of target proteins [101]. The docking simulation consists prediction of the ligand binding conformation, position as well as orientation within binding sites and computation of the binding affinity in terms of formation of hydrogen bonds and hydrophobic interaction with residues of the target. Molecular docking has two main steps ligand conformation sampling into the docking site and ranking of the sampled conformations through a scoring function [102]. Some sampling algorithms are Monte Carlo, Genetic algorithms, Molecular dynamics, Multiple Copy Simultaneous Search and Incremental construction which is widely used in molecular docking software [103]. The scoring function involves estimation of the binding affinity in between ligand and protein using force-field or empirical or knowledge based function and adopting a kind of simplification and assumptions. In the present work, we used AutoDock 4 that adopts a semi-empirical free energy force field for the evaluation of ligand conformations during docking simulations [104]. It allows users to use three sampling algorithms viz. Lamarckian

genetic algorithm (LGA), traditional genetic algorithm, and simulated annealing. Among these three algorithm, LGA is most efficient that provides reproducible results [105].

2.5 Chlorogenic acid

Medicinal plants provide is a tremendous source of novel compounds that has therapeutic potential and low side effects compared to the synthetic drugs. The phytochemicals presents in these plants possess polypharmacology property and has unique chemical diversity. Plant extracts and parts of some plants viz. *Withania somnifera, Bacopa monnieri, Convolvulus pluricaulis, Coffea Arabica, Mucuna pruriens, Centella asiatica, Nardostachys jatamansi and Tinospora cordifolia* has been used as Nootropics and neuroprotective agents as well as regarded as brain tonic [106–113].

Using molecular docking as a tool, about hundred phytochemicals present in the different plants were docked into the active/catalytic sites of NMDAR, nNOS, iNOS, MMP-2 and MMP-9 to identify a potent photochemical that has good binding affinity for two or more targets. After screening, chlorogenic acid appeared as multi-target inhibitor by showing higher affinity toward above-mentioned targets in terms of hydrogen binding and hydrophobic interaction as well as binding energy. This is reason behind selection of chlorogenic acid for the present work (*extensively discussed in the next chapter*).

Chlorogenic acids are broad group of phytochemicals which are synthesized after esterification of hydroxycinnamic acid with 1L-(−)-quinic acid [21]. The major hydroxycinnamic acid are caffeic, sinapic, p-coumaric and ferulic acids. The term ‘chlorogen acid’ coined by Payen who isolate a crystalline potassium caffeine chlorogenate from *Coffea arabica* (green coffee) beans after observing its conversion into a green pigment on alkaline oxidation [21]. Till date about 400 types of CGAs have been reported. Among them 5-Ocaffeoylquinic acid (5-CQA) is the

most abundant and possibly only one commercially available CGA (Fig 2.6) [114]. The biosynthesis of 5-CQA involves two enzymes cytochrome P450 oxidase p-coumaroyl-3′-hydroxylase (C3H) and hydroxycinnamoyl CoA:quinate hydroxycinnamoyl transferase (HQT) that convert p-coumaroyl-CoA into 5-CQA [21]. 5-CQA is a major polyphenolic component of *Coffea Arabica, Coffea canephora and Ilex paraguariensis A. St-Hil.* The green coffee beans contain about 5-12% of CGA by weight while only 1% of caffeine [115]. It has been estimated that per 200 ml cup dietary intake of Arabica and Robusta coffee contain 70–200 mg and 70–300 mg CGA respectively as well as per 200 ml cup of green maté provides 107–133 mg CGA [116]. The roasting of coffee bean decreased the CGA content at about 90%. It is also present to some extent in other plants including fruits, tea, vegetables [21], *Withania somnifera* (ashwagandha*)* [22].

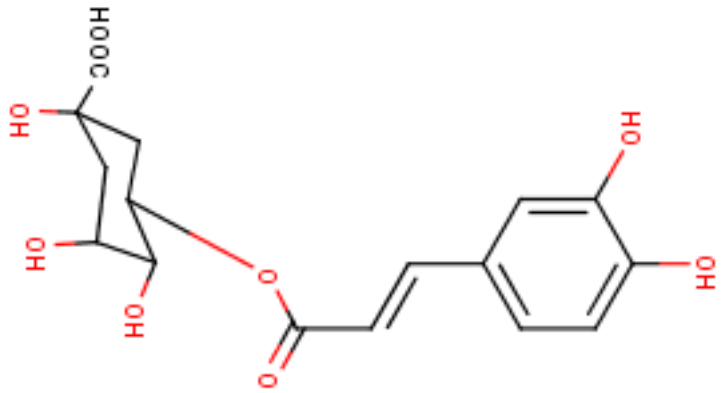

Fig. 2.6: Chemical structure of CGA (5-O-caffeoylquinic acid; 5-CQA)

Several studies have demonstrated that CGA has antioxidant [117], anti-inflammatory [31], hepato-protective [118], anti-obesity [119], Anti-diabetic [120], Antihypertensive [121], Anti-hepatitis [122], Antitumor [123] and neuroprotective [28] properties. The neuroprotective studies revealed that CGA have anti-amnesic activity by inhibition of malondialdehyde and acetylcholinesterase in the frontal cortex and hippocampus [124]. Evidences suggest that CGA

has neurotherapeutic effect for Parkinson's disease [125], dementia [126], Alzheimer's disease [127], Mental Diseases [27] and cerebral ischemia [128]. Regardless of various studies demonstrating the neuroprotective ability of CGA, the exact mechanisms of neuroprotective potential of CGA in ischemic insult are yet to be explored.

2.6 Impedance spectroscopy for differentiation of normal and ischemic condition

The bio-impedance analysis is a non-invasive method, which is used to measure the linearly varying electrical response across a specific dielectric medium (such as the cell, tissue, etc.), to yield useful information of that particular medium [129]. In bioengineering, based on the source of production, bio-impedance can be classified into Active and Passive type. (i) If the biological medium itself acts as a source of signal generation in determining its bioelectrical properties, then this type of response is termed as "Active type bio-impedance." Example, Biological signal (EMG, EOG, ECG, EEG, etc.) and source of signal generation is "*ionic activities of the cells*" [130]. (ii) If an external voltage controlled current source (VCCS) is used to excite and determine the bioelectrical properties such as electrical resistivity, conductivity, and permittivity of any biological cells/tissues, then this type of response is termed as "Passive type bio-impedance" [131]. Electrical Impedance Spectroscopy is a well-known technique used to determine the physiological condition of any biological medium by measuring the electrochemical interactions between intracellular and extracellular cell-membrane of the system [132]. The electrical impedance of any two terminal electrode system is the measure of resistance offered to resist the flow of an alternating current through that medium. Quantitatively, electrical Impedance (Z) can be represented by a resistance R and its reactance X_c with an imaginary term j in a Cartesian domain (equation 2.1), whereas, its magnitude (equation 2.2) and phase angle (equation 2.3) is in a polar domain.

$$Z = R + jX_C \quad (2.1)$$

$$|Z| = \sqrt{R^2 + X_C^2} \quad (2.2)$$

$$\phi = \tan^{-1}\left[\frac{X_C}{R}\right] \quad (2.3)$$

Generally, at the lower frequency range (10Hz to a few kHz), the maximum amount of current will flow in the extracellular region, and this can be termed as α-dispersion. At mid-frequency range (>1 kHz to few MHz), the maximum amount of current will flow through the extracellular region while some portion of current will enter into the cell membrane and intracellular region, and this can be termed as β-dispersion. At higher frequency range (>10 MHz), all the current enters into the intracellular region and lesser amount of current will pass through the extracellular region, this can be termed as γ-dispersion [133]. The α- and β-dispersion have been widely used as the region of interest to study the parametric changes in the biological tissue/medium [134]. *In-vivo* bio-impedance measurement studies had been carried out to differentiate between normal and ischemic rat brain [40], ischemic and hemorrhagic rat brain [41], cerebral edema in piglets [42], as well as for the detection of ischemic condition in normoglycemic and hyperglycemic rats [43]. Previous studies have established that the electrical impedance of rat brain is increased during occlusion and reversed during reperfusion [41] [44] but no known study till date has demonstrated and compared the changes in bio-impedance with change in electrolyte concentration of rat brain.