CHAPTER 1

INTRODUCTION

Highlights of the Chapter

- *Identification of the problem*
- *Objectives and contribution of the thesis*
- *Outline the thesis organization*

1.1 Identification of the problem

Ischemic stroke is a serious neurological disease and a major cause of mortality and morbidity worldwide [1]. It occurs due to occlusion in blood vessels that supply oxygen and glucose to the nerve cells [2]. In ischemic stroke, acute loss of brain cells (astroglia, oligodendroglia and neurons), and synaptic architecture disruption occurs due to occlusion in the cerebral artery [3]. After reperfusion of the ischemic tissue (metabolically compromised), the oxygenated blood rapidly increases cerebral pO_2 causes the production of reactive oxygen species that leads to oxidative damage [4]. An interruption in cerebral blood flow (CBF) triggers a complex cascade of detrimental physiological and biochemical events including ATP production failure, disruption in ion homeostasis and electrical activity resulting in neuronal cell death via necrosis, apoptosis, necroptosis, and autophagy [5]. Treatment strategies for acute ischemic stroke encompasses two approaches i.e. clot-busting (thrombolysis) using streptokinase, tissue plasminogen activator (tPA: a single FDA approved drug) and mechanical clot retrieval (thrombectomy) [6]. Nevertheless, therapy with tPA has significant limitations, notably the narrow therapeutic window of 4.5 hours [7], which limits its use to a small population (2% to 4%) of patients. Administering the tPA outside of the recommended window upturns the risk of intracerebral hemorrhages makes worse the injury [8]. If tPA is not received by the patient

in time, there is no treatment reported till date that provide neuroprotection and stimulates the tissue regeneration after it is lost from the stroke [9]. Research from last few years has been carried out for the development of novel treatments to protect the brain from the damage following the ischemic insult [10][11][12]. These studies have shown great promise in animal model of ischemic stroke but unfortunately, translation failed so far. Recent studies provide strong evidence that inhibition of molecular targets involved in neurodegeneration is an effective therapeutic approach. The preclinical studies have demonstrated that overexpression or abnormal function of N-methyl-D-aspartate receptor (NMDAR), Matrix metalloproteinase (MMP)-2/9 and inducible nitric oxide synthase (iNOS)/neuronal nitric oxide synthase (nNOS) play detrimental role in neurological disorders. Thus, inhibition of these molecular mediators might be a useful therapeutic strategy for neuroprotection, and a compound that possesses inhibition potential for molecular mediators of neurodegeneration may be developed as a neuroprotective drug [13].

Natural sources are attracting significant attention towards neuroprotection strategies. Natural products possess unique chemical diversity, and some of them have polypharmacology property, i.e., they can modulate several molecular targets simultaneously [14]. They have abundant active constituents and act synergistically with neurotrophic factors to promote the reformation of neuronal networks in the diseased brain or may act as potent inhibitors for mediators of neurodegeneration [15]. Therefore, more importance has been placed on the use of phytochemicals for neuroprotection and neuroregeneration strategies that provides new understandings for the drug development for the treatment of the neurological disorders [14]. Ayurvedic medicinal herbs viz., *Withania somnifera* (ashwagandha), *Bacopa monnieri* (Brahmi), *Mucuna pruriens* (velvet bean), and *Centella asiatica* (Gotu kola) have been in use

for treating neurological disorders since ages in Indian subcontinent [16][17][18][19]. Chlorogenic acid (CGA, 5-O-caffeoylquinic acid) is a major polyphenolic component of *Coffea canephora*, *Coffea arabica* L., and Mate (*Ilex paraguariensis* A. St-Hil.) [20]. It is also present to some extent in other plants including fruits, tea, vegetables [21], *Withania somnifera* (ashwagandha*)* [22]. The green coffee beans contain about 5-12% of CGA by weight [23]. The clinical studies revealed that consumption of green coffee decreased the mental fatigue, headaches and positively modulates the mood-related processes [24], as well as proven beneficial for the brain aging [25]. Evidence suggests that CGA has neuroprotective [26–28], neurotrophic [29], anti-oxidative [30] and anti-inflammatory [31] activities. Previously, *in-vivo* studies using CGA effectively confirmed that it increased the survival of dopaminergic neurons [32], reduced anxiety, improved motor function [33], and improved spatial learning and memory [34]. It also reduced the oxidative stress and neuroinflammation in MPTP-intoxicated mice [23]. Regardless of various studies demonstrating the neuroprotective ability of CGA, the exact mechanisms of the neuroprotective potential of CGA in ischemic insult are yet to be explored. Earlier, finding using *in vitro* blood brain barrier (BBB) model suggest that CGA exhibits a very low rate of BBB permeation and cannot considered for a direct effect on the central nervous system (CNS) [35]. No known study has been carried out to investigate the brain distribution and plasma pharmacokinetics profiles of chlorogenic acid after intranasal administration. Therefore, an in vivo study is also required to analyze the brain penetration potential of chlorogenic acid.

Study of global cerebral ischemia demands simple and reproducible rodent models with fewer chances of mortality. 2-vessel and 4-vessel occlusion models are commonly used for induction of global cerebral ischemia [36][37], but the surgical complexity and high mortality rate

associated with the 4-vessel occlusion model makes it an unpopular model of choice for laboratory experiments. The 2-vessel occlusion (2VO) or bilateral common carotid artery occlusion (BCCAO) model is relatively simple and reproducible to induce global cerebral ischemia, but it has spurred controversies [38]. The 2VO with hypotension is a widely accepted model that produces marked forebrain lesions and used for the evaluation of neuroprotective agents [39]. This model requires lowering of blood pressure by withdrawing blood from a femoral artery or jugular vein which makes it complicated [36]. No known study has been reported for the analysis of cerebral infarction and blood-brain barrier (BBB) disruption in rats after inducing hypotension (using vasodilator drug) coupled with bilateral common carotid artery occlusion.

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 demonstrated that the 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.

Under the ischemic condition, NO is produced in high concentration by the activation of neuronal NOS (nNOS) and inducible NOS (iNOS) that lead to neuronal cell death [45]. Since NO has a very short half-life (estimated to be a few seconds), its direct measurement is quite a challenge [46]. It is a highly reactive molecule and in the presence of oxygen, rapidly metabolized into nitrate (NO^{3-}) and nitrite (NO^{2-}). Nitrate is a stable, long-lasting end product of NO is used as a reliable biomarker of NO production [47]. Whereas, nitrite is usually a

short-lived ion with a half-life of 110 seconds in whole blood [48]. Estimation of NO^{2-} and NO^{3-} is a reliable strategy for indirect measurement of total NO production. Although, till date, no known simple method has been reported to determine the nitrate and nitrite level in the various brain samples and CSF of rats with bilateral common carotid artery occlusion using only HPLC UV.

The present thesis consists of the identification of chlorogenic acid as a potent dual-inhibitor using in silico and in vivo studies; evaluation of its pharmacokinetics, brain penetration and neuroprotective potential when administered via intranasal route; development of a rapid, simple and cost-effective method for the determination of nitrate and nitrite level in the various brain regions and CSF of ischemic rat model using HPLC coupled with UV detector; exploring the effect of administration of amlodipine and metoprolol (vasodilator drugs) followed by tBCAAO in the induction of cerebral ischemia in rat brain; as well as investigating the changes in impedance during ischemia with electrolytes concentration of rat brain.

1.2 Objectives of the Thesis

The ultimate objective of the thesis is to understand the pathophysiology of global cerebral ischemia and evaluation of neuroprotective potential of chlorogenic acid. In this view, the thesis has following objectives:

1. Identification of chlorogenic acid as a multi-target inhibitor by virtual screening of various phytochemicals presents in medicinal plants using molecular docking
2. Evaluation of pharmacokinetics and brain penetration efficiency of chlorogenic acid after intranasal and intravenous administration.
3. Exploring the neuroprotective potential of chlorogenic acid in bilateral common carotid artery occlusion-reperfusion induced cerebral ischemia in rats

4. Development of a rapid, simple and cost-effective method to determine the nitrate and nitrite level in the various brain regions and CSF of albino rats with bilateral common carotid artery occlusion using HPLC coupled with UV detector
5. Investigating the possibilities related to changes in impedance during ischemia-reperfusion condition is dependent on changes in concentrations of various electrolytes present in rat brain.
6. Development of a simple and reproducible ischemic rat model for studying both acute cerebral ischemia and the associated pathophysiological changes due to brain damage.

1.3 Contribution of the Thesis

This thesis contributes to the area of global cerebral ischemia and its neuroprotection by chlorogenic acid. Specifically:

1. In the field of structure-based drug designing using molecular docking simulation study. By using this method, I have screened several phytochemicals against the five protein targets and identified "chlorogenic acid" as a dual-inhibitor of NMDAR and nNOS.
2. Here, for the first time, we report the brain penetration ability of chlorogenic acid when administered via the intranasal route and intravenous route.
3. Here, for the first time, we report the brain impedance behavior during ischemia has a relation with brain electrolytes concentration.
4. Here, for the first time, we report a simple, rapid, and reproducible method for the determination of nitrate and nitrite in the ischemic brain.
5. We reported for the first time that the administration of amlodipine and metoprolol, followed by tBCAAO, could induce a massive infarction in rat brain.

1.4 Organization of Thesis

The remainder of this thesis describes in more detail what has been anticipated in this introduction. The structure of the thesis is given as follows:

Chapter 2 provides the background and literature review. This chapter discusses about the cerebral ischemia, its pathophysiology, treatment strategies and use of animal models. The chapter presents a brief overview of chlorogenic acid, intranasal drug administration and molecular docking. This chapter also discusses about use of impedance spectroscopy for the differentiation of normal and ischemic rat brain. Further, importance of nitrate determination and hypotension coupled with 2 vessels occlusion rat model has been discussed.

Chapter 3 reports the identification of chlorogenic acid as a multi-target inhibitor by virtual screening of various phytochemicals presents in medicinal plants using molecular docking. A set of phytochemicals was docked with catalytic/active site of GluN2b containing NMDA receptor, MMP-2/9, nNOS and iNOS and its binding energy and interactions was compared with their inhibitors.

Chapter 4 demonstrates the pharmacokinetics and brain penetration study of chlorogenic acid after intranasal and intravenous administration. Till date this work reports for the first time that chlorogenic acid can reach to the brain after intranasal administration.

Chapter 5 describes the development of a rapid, simple and cost-effective method for the determination of the nitrate and nitrite level in the various brain regions and CSF of albino rats with bilateral common carotid artery occlusion using HPLC coupled with UV detector.

Chapter 6 investigates the relation of changes in brain impedance during ischemia-reperfusion condition with changes in concentrations of various electrolytes present in rat brain. In this study, the changes in the bio-impedance spectroscopy using two electrodes at different

frequencies 100, 500, 1K, 5K, and 10K Hz has been assessed in a model of global cerebral ischemia in the anesthetized rat in normal, occlusion and reperfusion conditions.

Chapter 7 reports a simple and reproducible hypotensive ischemic rat model for studying both acute cerebral ischemia and the associated pathophysiological changes due to brain damage. This study designed to explore the effect of administration of amlodipine and metoprolol, followed by 2 vessels occlusion in the induction of cerebral infarction in rat brain.

Chapter 8 explores the possible mechanism of neuroprotection by chlorogenic acid in bilateral common carotid artery occlusion-reperfusion induced cerebral ischemia in rats. In this study dual-inhibition potential of chlorogenic acid is investigated by comparing the level of calcium ions with ifenprodil (a potent NMDA receptor inhibitor) and the level of nitrate is compared with 7-nitroindazole (a selective nNOS inhibitor). This chapter also includes dose optimization, histological, biochemical and immunohistochemistry studies using chlorogenic acid.

Chapter 9 summarizes the overall contribution and point out the main achievement of the thesis along with its future directions which might be the interest in further research.