STUDIES ON POTENTIAL NEUROPROTECTIVE ACTIVITY OF SELECTED MEDICINAL HERBS

THESIS

SUBMITTED FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

APPLIED ANIMAL SCIENCE

BY

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DECLARATION

I hereby declare that the thesis entitled **"Studies on potential neuroprotective activity of selected medicinal herbs"** submitted by me under the guidance and joint-supervision of Dr.Venkatesh Kumar R. and Dr. Ràm Raghubir for partial fulfillment of the degree of PhD in Applied Animal Science in the Department of Applied Animal Sciences, School for Bioscience and Biotechnology, Babasaheb Bhimrao Ambedkar University (A Central University), Lucknow. It is outcome of my scientific efforts and an original piece of scientific work.

Date: 21/12/17

Sheeba Saji Samuel

Place: Lucknow

CERTIFICATE

This is to certify that the thesis titled "**Studies on potential neuroprotective activity of selected medicinal herbs**" submitted by **Mrs. Sheeba Saji Samuel**, is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

The thesis submitted to Babasaheb Bhimrao Ambedkar University, Lucknow satisfies all the requirements as stipulated in the Doctor of Philosophy (Ph.D.) regulations as amended in 2008/2010/2013 and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

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“God,

Thank You for Being My Peace

In World Full of Storms”

Dedicated to my dearly loved ones

****

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Sheeba Saji Samuel

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ABBREVIATIONS

ACA	anterior cerebral artery
ATP	adenosine triphosphate
BBB	blood-brain barrier
CBF	cerebral blood flow
CCA	common carotid artery
CLE	*Curcuma long* extract
CLEs	*Curcuma long* extracts
CNS	central nervous system
CV	cresyl violet
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
DNA	deoxyribo nucleic acid
DTNB	5,5'-dithio-bis(2-nitrobenzoic acid)
ECA	external carotid artery
FITC	fluorescein isothiocyanate
GABA	gamma amino butyric acid
GSH	glutathione
HE	hematoxylin and eosin
hr	hour
I/R	ischemia/reperfusion
ICA	internal carotid artery
kg	kilogram

MCA	middle cerebral artery
MCAO	middle cerebral artery occlusion
MDA	malonldialdehyde
mg	milligram
min	minute
MLE	mulberry leaf extract
MLEs	mulberry leaf extracts
mm	millimeter
nm	nanometer
nmol	nanomole
PBS	phosphate buffered saline
PCA	posterior cerebral artery
PCD	programmed cell death
PLA	poly-l-lysine
p.o.	per oral/oral administration
RNA	ribo nucleic acid
ROS	reactive oxygen species
SOD	superoxide dismutase
TIA	transient ischemic attack
TTC	2,3,5-triphenyltetrazolium chloride
TUNEL	terminaldeoxynucleotidyltransferase-dUTP nick end labeling
Veh	vehicle
µl	microliter
µ	micron

CHAPTER I

GENERAL INTRODUCTION

1. Introduction

1.1 Stroke: Incidence and Risk Factors

The cerebral stroke is also called brain attack, because it occurs rapidly and requires immediate treatment. Stroke is a major cerebro vascular disorder caused by impaired cerebral blood flow due to blockade or rupture of a brain blood vessel. It is a leading cause of adult disability in the United States and Europe and ranks third after cancer and heart diseases in causing death worldwide. It is a medical emergency, which can cause permanent neurobehavioral deficits owing to sudden loss of blood supply in neuronal tissue, which initiates a series of pathophysiological changes leading to the degeneration of neurons in affected brain regions. To meet the high oxygen and glucose demand, brain depends on continuous blood supply and slightest variation in the blood flow makes it all the more sensitive. These factors along with the fact that the brain has a high metabolic rate makes the brain so susceptible for stroke. Brain injury due to stroke is commonly associated with impaired sensory motor ability leading to coma, hemiplegia, monoplegia, multiple paralysis, speech disturbances and sensory impairment etc. According to the 'Internet Stroke Center', the most common sign of stroke is sudden weakness of the face, arm or leg, most often on one side of the body. The signs of stroke depend on the side of the brain that is affected, the part of the brain, and the severity of the injury. Therefore, stroke warning symptoms show up differently in every other individual. Stroke often occurs in patients with associated headache, or sometimes it is absolutely painless. Some functions may improve later, while others do not. Usually, loss of brain function is dehumanizing, and often makes individuals dependent on others for ordinary daily activities

Stroke occurrence worldwide is annually over 15 million people. Of these, 5 million die and another 5 million are left permanently disabled, placing a burden on family and community. Any other chronic disease does not have a greater disability impact on an individual as compared to stroke (Adamson *et al.*, 2004). Every five seconds, stroke takes a life worldwide and approximately 1 out of 8 (11.9%) deaths worldwide are caused by stroke (WHO 2014).

Though the risk of having a stroke doubles every decade after the age of 55 (Wolf *et al*., 1992), it can affect children too. Though men are more affected by stroke than women, mortality rate is higher in women. Studies have proven that by the age of 75, 1 out 5 women and 1out of 6 men would be a stroke sufferer (Seshadri *et al.*, 2006). The chances of stroke increase in case of family history of stroke. The chance of the occurrence of stroke also increases for a person who has had stroke, TIA or cardiac attack. There are many factors which put the people at high risk for stroke, such as hypertension, heart disease, sickle cell disease, transient ischemic attack, diabetes, alcohol/drug abuse, obesity, sedentary lifestyle and several other disorders. Many of the risk factors independently contribute to the precipitation of stroke. However, simultaneous occurrence of two or more risk factors may significantly increase the incidence of stroke.

Eastern Europe, northern Asia, central Africa are some of the regions which holds the highest mortality rates due to stroke. India compared to other developed countries has comparatively a less prevalence of stroke but with the increase in life expectancy this prevalence is expected to increase proportionally (Johnston *et al.*, 2009). In comparison to developed countries, the percentage of youngsters suffering from stroke is significantly more in India and stroke in India has already attained epidemic proportion; 145 per 100000 persons per year during 2003-05 and 2005-06 (Mishra and Khadikar, 2010).

1.2 Types of Stroke

Stroke can be either ischemic or hemorrhagic. As per American Heart Association report, the prevalence of ischemic and hemorrhagic stroke is 88% and 12%, respectively (Thom *et al*., 2006).

Ischemic strokes are caused by obstruction to blood flow, which mainly results from thrombus formation in a blood vessel supplying the brain (thrombotic stroke) or due to lodging of an embolus (embolic stroke). Thrombotic stroke can be either large vessel thrombotic stroke, which includes the carotid artery system, or small vessel thrombotic stroke (lacunar stroke) that comprises of the intra cerebral arteries, including the branches of the Circle of Willis and the posterior circulation. Large arteries are most often the occurrence site of thrombotic stroke, so the most common and best understood type of thrombotic stroke is the large vessel thrombosis. Cerebral artery bifurcating points especially in the internal carotid artery are the most common sites of thrombotic occlusion. Stenosis of the artery can cause unbalanced blood flow, because of which an increase in platelet adherence, thrombus formation or even atherosclerosis can happen. All these risk factors give way to formation of blood clots which can either lead to embolus formation or artery occlusion.

Intra or extra cranial arteries in the aortic arch or the heart can aid in emboli occurrence. The sources of cardiogenic emboli can be valvular thrombi, mural thrombi or atrial myxoma. Post myocardial infarction, in 85% cases, during the first month embolus occurs as myocardial infarction has a close association with embolic stroke (Witt *et al.,* 2006). Hence, the most alarming type of ischemic stroke is due to the formation of emboli (Luengo *et al.,* 2013).

When a superficial blood vessel or the vessel within the brain bursts, haemorrhagic strokes are caused. The damage caused by stroke due to a clot is far less than due to

haemorrhagic ones as the brain tissue experiences a high pressure blood leakage. Hemorrhagic strokes include subarachnoid hemorrhage, intra cerebral hemorrhage, and subdural hematomas. These types of hemorrhages are often associated with uncontrolled hypertension and sometimes to anti thrombotic or thrombolytic therapy. Most often, when a weakened small artery bursts due to chronic high blood pressure leading to bleeding in the brain parenchyma, an intra cerebral hemorrhage occurs. Whereas, subarachnoid haemorrhage is the result of bleeding on the surface of the brain, rather than inside it. As compared to ischemic strokes, haemorrhagic strokes are usually more severe and are linked to higher risk of mortality (Royal College of Physicians, 2014).

The ischemic stroke is of two types i.e. focal and global. Focal ischemia is caused when a very specific brain region, experiences a reduction in blood flow. Embolic middle cerebral artery occlusion is one such example. Whereas global ischemia occurs when most or all parts of the brain is affected due to reduced cerebral blood flow (CBF) example being cardiac arrest (Traystman, 2003; Smith, 2004). Since focal ischemia affects usually cortical and sub-cortical brain areas particularly striatum and global ischemia particularly affects neurons in the hippocampus, the mode of cell death varies significantly between the two subtypes of stroke. It was found in a study in rodents and humans that delayed neuronal death took place during short periods of global cerebral ischemia (Bonnekoh *et al*., 1990; Horn and Schlote, 1992). Whereas, in case of focal cerebral ischemia, necrosis is a common feature in nearly all of the cells in the ischemic core, and is exemplified by the abrupt decrease in cellular energy and inflammation and eventually the rupture of subcellular organelles.

To study focal cerebral ischemia, the most commonly used model is MCAO. Here, transiently or permanently the middle cerebral artery is occluded. Either an intra luminal suture (nylon filament) or a vascular clip can be used to induce MCAO. The cortex and the striatum are

primarily the worst affected regions in brain during focal cerebral ischemia. Due to the relevance to human embolic stroke, the MCAO model has been used extensively. A number of species including rabbit, baboon, rat, cat and dog has been used for the focal cerebral ischemia model, but the pathophysiological changes has been extensively investigated in rat and mice studies (Tamura *et al*., 1981; Bederson *et al*., 1986; Longa *et al*., 1989).

1.3 Pathophysiology of cerebral ischemic injury

When blood capillaries carrying oxygen and other nutrients to brain are blocked, a brain stroke occurs. There are two major zones of injury within the ischemic cerebrovascular bed, the ischemic core region and the ischemic penumbra (cerebral tissue which is ischemic but still viable). The cerebral blood flow in the area of severe ischemia ie. the core region, is 10% to 20% due to this there is inadequate supply of oxygen and glucose resulting in rapid depletion of energy stores. Within the severely ischemic area, necrosis of neurons and glial cells occurs.

Brain tissue lying between the normally perfused area and the area in which infarction is evolving, forms the mild to moderately ischemic penumbra region. The brain cells within this region remain viable for several hours due to collateral blood supply (Fisher *et al*., 1998; Fisher *et al*., 2006). To maintain the demand for oxygen and glucose in the neurons for long, the collateral circulation is inadequate so eventually if during the early hours, reperfusion is not established the cells in this region will die (Smith *et al*., 2004).

1.4 Cascade of ischemic events

If a stroke patient is left untreated, every minute around 1.9 million neurons in the brain are lost (Saver, 2005). A complex sequence of pathophysiological changes or the ischemic cascade demonstrating overlapping features occurs within minutes of vascular occlusion (Fig. 1).

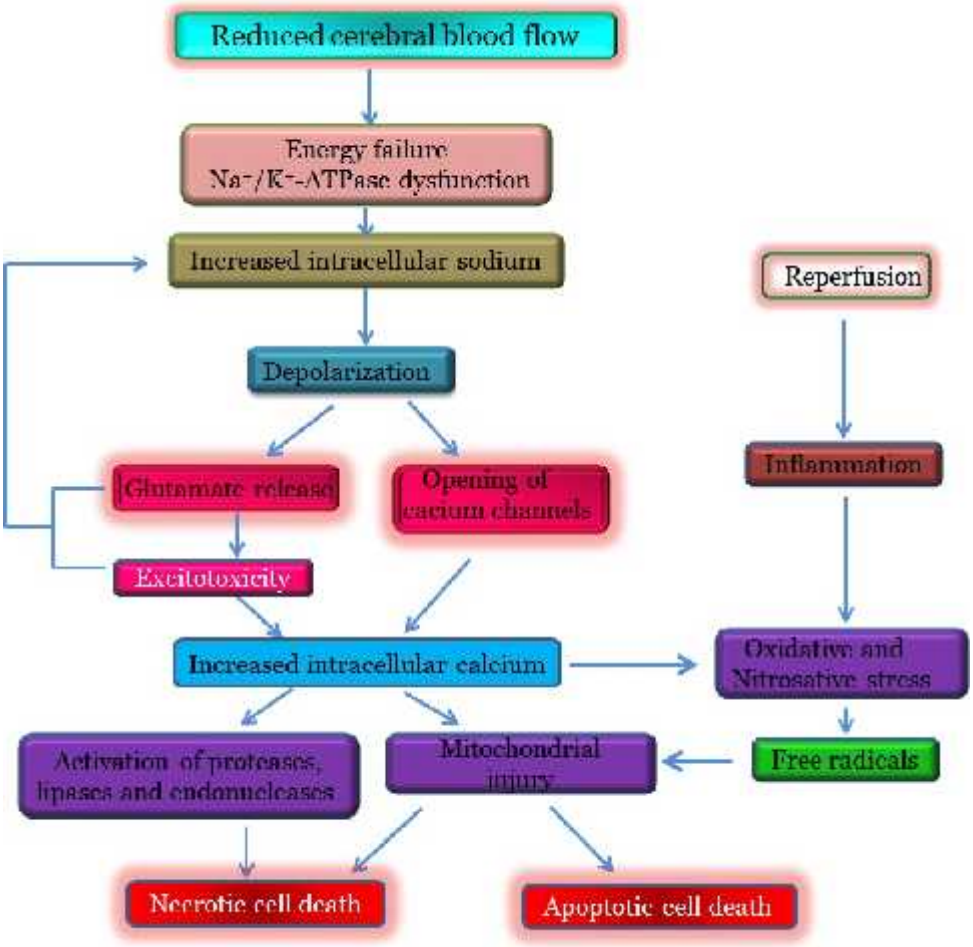


Fig. 1 Schematic representation showing the ischemic cascade of events during the Ischemic/Reperfusion injury.

It is a time-dependent cascade, which may last hours or even days. Major molecular events includes the rapid depletion of intracellular energy stores, anaerobic glycolysis, lactic acidosis, membrane depolarization, glutamate excitotoxicity, intracellular calcium overload, activation of calcium-stimulated enzymes (phospholipases, proteases, protein kinases, nitric oxide synthase or NOS, endonucleases), mitochonral dysfunction, free radical production, activation of the immune system (neutrophils, monocytes/macrophages), over expression of genes and neuronal death.

1.4.1 Glutamate and Calcium Overload

Brain has no stores of energy as it is exclusively dependent on the continuous supply of blood providing glucose and oxygen to undergo oxidative phosphorylation for energy production. Energy deprivation occurs within minutes and the first consequence of CBF reduction is the depletion of substrates, particularly oxygen and glucose that causes accumulation of lactate via anaerobic glycolysis. Unavailability of ATP leads to failure of Na+/K+ ATPase activity. Therefore, the membrane potential is lost since sodium and potassium ion gradients at the plasma membrane are no longer maintained and consequently neurons and glia depolarize (Dirnagl et *al*., 1999). The ensuing waves of depolarization in the very early phase of ischemia, result in release of especially glutamate, the excitotoxic amino acids, into the extracellular compartment from presynaptic neurons in large amounts. Extracellular glutamate accumulation is further aggravated by failure of glutamate transporters to recycle glutamate back into the cells. Accumulation of glutamate gives way to overstimulation and activation of the post synaptic receptors which in turn aid in entry of calcium into neuronal and some glial cells. Besides direct neurotoxicity of glutamate on neurons, increase in the intracellular Ca^{2+}, Na^{+}, and Cl^{-} levels due to the activation of glutamate receptors (NMDA, AMPA, and metabotropic glutamate receptors) occurs and this in turn due to excess intracellular Ca^{2+} production increases the edema and toxicity.

Generally the fine balance of free intracellular calcium Ca^{2+} is maintained via calcium influx, efflux, buffering and cellular distribution maintained by multiple channels. However, during cerebral ischemia decrease in ATP levels impairs the calcium exchangers and pumps, thereby disturb the intraneuronal Ca^{2+} homeostasis (Pringle, 2004). Thus, the high cytosolic Ca^{2+} leads to activation of a variety of calcium stimulated enzymes including proteases, lipases,

nucleases, nitric oxide synthases, protein kinases. Moreover, Ca^{2+} also regulates gene transcription and phophorylation and dephosphorylation of various proteins in nucleus and cytoplasm thereby modulating various signaling such as apoptosis. Both mitochondrial and ER calcium overload also leads to the activation of certain caspases. In addition to this, calcium dependent neuronal nitric oxide synthase (nNOS) activation through NMDA receptor linkage and other signaling pathways however also contribute to more extensive cell death (Aarts *et al*., 2002).

1.4.2 Oxidative stress

Oxidative stress is associated with excessive production of reactive oxygen species (ROS). The brain is a major target for oxidative stress induced damage because it contains high concentrations of peroxidisable lipids, low levels of protective antioxidants, high oxygen consumption, and high levels of iron that act as pro-oxidants under pathological conditions. It is a condition caused by an imbalance between the production of ROS and endogenous antioxidant system, which readily detoxifies the reactive intermediates or easily repairs the resulting damage. A constant input of metabolic energy preserves the reducing environment within the cells of all forms of life by certain cellular enzymes aiding in maintenance of this reduced state. Significant cellular effects like lipid peroxidation, protein denaturation, inactivation of enzymes, nucleic acid and DNA damage, release of $Ca^{2+}$from intracellular stores, damage to the cytoskeletal structure and function are caused when there is a disturbance in this normal redox state , resulting in tissue damage and cell death (Fig. 2).

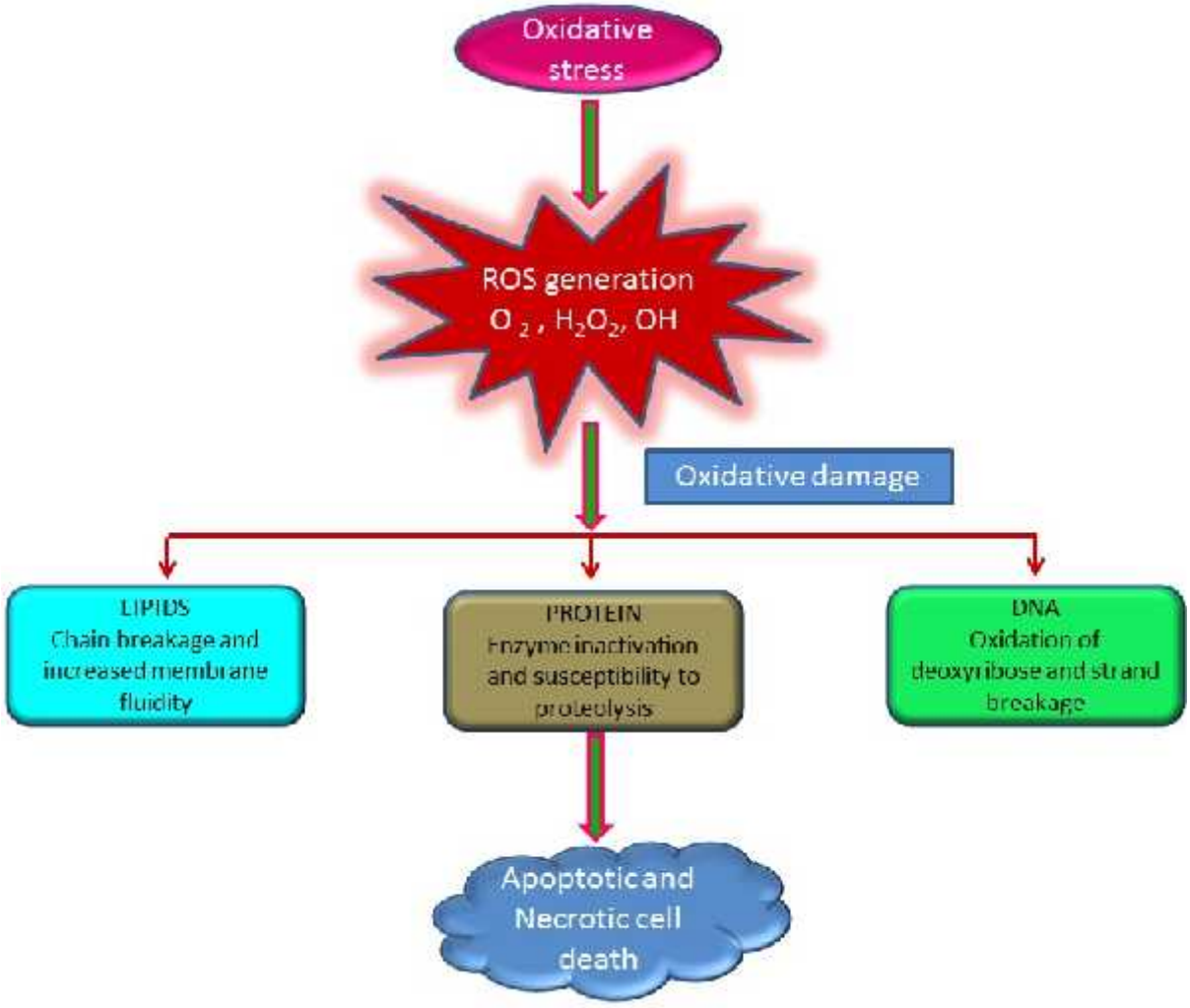


Fig. 2 Oxidative stress and the cellular damage in cerebral stroke

The reduction of molecular oxygen to H_2O leads to ROS generation. The reduction of the dioxygen molecule generates the superoxide anion radical (O_2^-) and hydrogen peroxide. In the presence of transition metals such as Fe^{2+}, H_2O_2 can undergo the Fenton reaction, which leads to increased levels of the hydroxyl radical. The highly reactive radical which is to be blamed most for oxidative damage is the hydroxyl radical. A number of antioxidant enzymes, including glutathione peroxidase (Gpx), catalase (CAT), and superoxide dismutase (SOD) comes into action for providing defense against the destructive free radicals. SOD converts O_2^- to H_2O_2,

whereas Gpx and CAT convert H_2O_2 to H_2O. Levels of ROS are found to be reduced by the rescue action of enzymes along with other antioxidants, such as ascorbic acid and α-tocopherol and GSH. Oxidative stress occurs when a shift in balance occurs, either by the increase of free radical formation or by decrease of antioxidant defense mechanism, leading to lipid, protein, and DNA damage and cell death eventually leads to apoptotic or the necrotic pathway.

Since 1970s, ROS have been implicated in cerebral ischemic damage and it contributes significantly to ischemic injury in the brain but there is still debate about its source, mechanism and time course of ROS generation during ischemia/reperfusion. Potential sources of ROS includes mitochondria, NADPH oxidases, uncoupled nitric oxide (NO) synthases, xanthine oxidase, cytochrome P450- based enzymes, and infiltrating inflammatory cells. Abramov *et al*. (2007) examined the kinetics of ROS production during hypoxia and re-oxygenation in primary cultures of neurons from rat cortex and hippocampus. Their findings suggest that three distinct phases of ROS generation were identified, during early hours of ischemia the major source is mitochondria and production of ROS that eventually diminishes with membrane depolarization, a delayed production of ROS through activation of XO, as determined by inhibition with oxypurinol ensues. The penultimate phase of ROS generation occurs with re-oxygenation and this was blocked by NADPH oxidase inhibitors and was Ca^{2+}dependent. More recently, it has been shown that in endothelin-1-induced stroke in rat, the important mediator of oxidative stress is NADPH (McCann *et al*., 2008). It was significantly elevated in the ipsilateral cortex and the striatum from 6 hr and up to 7 days post-stroke (Singh, 2010). The oxidative stress induced by NADPH oxidase following cerebral ischemia/reperfusion injury is a major source and is mainly responsible for the brain damage. Therefore, it requires most attention to counteract oxidative stress to avoid brain damage as a result of stroke.

1.4.3 Inflammation

Inflammation is the endogenous host defense response of the body to tissue damage like ischemia and autoimmune disease response or infectious agents. Although acute inflammatory response is designed to limit the tissue damage and protect against infection, in many cases, this response can be detrimental, especially if it is uncontrolled and prolonged. In stroke, inflammation may be caused by endogenous cells (brain tissue) or exogenous cells (blood cells that migrate across the blood brain barrier). Following stroke, inflammatory reactions at the blood–endothelium interface within the arterial wall play a major role in progression of disease (Elkind *et al*., 2002; Chamorro and Planas, 2004).

Immediately after the onset of ischemia, irreversible damage in the form of inflammatory injury are caused by the production of cytokines (IL-1, IL-6, TNF-α, and TGF-β), adhesion molecules (selectins, integrins, and immunoglobulins), eicasanoids and iNOS. These molecules are produced by endothelial cells, astrocytes, microglial cells and leukocytes (granulocytes, monocyte/macrophages, lymphocytes).

Cellular mediators of subsequent micro vessel obstruction, edema formation, cellular necrosis, and tissue infarction attributes to the infiltration of leukocytes, a major requirement of inflammatory reaction when tissue injury begins after the interruption of CBF (Huang *et al*., 2006). Within the parenchyma, inflammatory cascades are triggered and amplified tissue damage in ischemic stroke related brain injury (delZoppo *et al*., 2000). Inflammatory mediators are generated by the reactive microglia, macrophages and leukocytes which are recruited into ischemic brain and also by the neurons and astrocytes at ischemic area.

Cell to cell interaction for leukocyte migration is mediated by the immunoglobulin gene family's cell adhesion molecules (selectins, integrins and proteins). It is reported that upregulation of selectins (P-, E-, and L-) in leukocytes and endothelial cells after focal cerebral

ischemia and reperfusion mediates the accumulation of neutrophils and subsequent brain injury (Okada *et al*., 1994; Huang *et al*., 2000). Similarly, in cerebral ischemia, inflammatory reactions can also be mediated by the cytokines such as IL-1, IL-6 and TNFα (Becker, 2001; delZoppo *et al*., 2000).

Post transient or permanent cerebral ischemia, in astrocytes, microglia, and neurons, expression of IL-1 increased (Liu *et al*., 1993; Buttini *et al*., 1994; Zhang *et al*., 1998). An important role in inflammation has also found to be played by iNOS and COX-2 (Iadecola G, 1997; Khan *et al*., 2004). The function of the brain has been found to be influenced by COX2 along with iNOS (Iadecola *et al*., 2001; Sugimoto and Iadecola, 2003). A huge number of phagocytically active inflammatory molecules are produced by the microglial cells. Immuno modulatory molecules are generated when proliferation, chemotaxis, morphological alterations of activated microglial cells takes place.

Once ischemia is mediated, detrimental and potentially beneficial pathways get stimulated by the inflammatory cascades via these cytokines. The stage of tissue injury or the prevalence of signalling cascade forms the basis of the net effect of these mediators among multiple divergent pathways. In general, to precisely understand the complex role of inflammation in cerebral ischemia, further study needs to be undertaken and also to foster protective molecules and specifically block the damaging proteins during the inflammation process in cerebral ischemia.

1.4.4 Cell death

Subsequent necrotic cell death after getting fatally injured occurs in the part of brain tissue which gets the most reduced blood flow within minutes of a focal ischemic stroke. And adjacent to this necrotic core is surrounded by metabolically active yet functionally silent region of brain tissue which due to reduced blood flow is far less severely affected (Ginsberg *et al*.,1997).

Recent research has revealed that many neurons are potentially recoverable for some time after the onset of stroke in the ischemic penumbra region but after several hours or days it may undergo apoptosis. To dispose off surplus cells, the energy-dependent programmed cell death, apoptosis appears to be a comparatively systematic process in which cells undergoing apoptosis are dismantled from within with minimal damage and disruption to neighboring cells in contrast to necrosis, (Taylor *et al*., 2008). Even though independent multiple pathways are involved in apoptosis, there can be the occurrence of crosstalk between these pathways (Liou *et al*., 2003). The two pathways, one involving the activation of caspases and the other involving the caspase-independent release of apoptotic factors from mitochondria can be segregated in the neuronal apoptosis (Zhang *et al*., 2002).

1.5 Strategy for treatment of stroke

Thus it is logical to suggest that if one or more of the processes involved in the propagation of these cascades are timely interrupted, some of the affected brain tissue may be rescued. Neuroprotective therapies designed to intervene the multiple cascades at various points of the cell death signaling mechanism may provide protection in reperfusion injury. Successful treatment with thrombolysis or spontaneous recanalization of the occluded vessel leads to re-oxygenation of the ischemic brain tissue but in majority of the cases it leads to further injury of the brain cells therefore it is essential to administer the thrombolytic drug as soon as possible so that the brain cells do not enter the phase of no return as after that it is impossible to save the tissue from dying. Despite the enormity of the problem, there are few effective therapeutic treatments for stroke. At present, the only US Food and Drug Administration approved treatment is to provide rtPA (recombinant Tissue Plasminogen Activator) to restore blood flow in occluded

blood vessels. However, due to a narrow therapeutic time window (3 hours), only a very small number of patients gets the opportunity to get this treatment.

In view of the lack of effective drugs with large therapeutic window, traditional and herbal medicines, which are believed to have fewer side effects and multiple modes of action, have gained lot of interest in recent years as potential candidates for the prophylactic treatment of stroke. It is reported that these, or their products, may improve microcirculation in the brain (Gong and Sucher, 2002; Wang *et al.,* 2005), provide protection against ischemic reperfusion injury (Lee *et al*., 2005), and posses neuroprotective properties (Kim, 2005) and inhibit apoptosis (Bei *et al*., 2004) as well, thus justifying detailed investigations for their use in prevention of ischemic stroke. The effect and potential health benefits of traditional herbal medicine in treatment of stroke has been studied extensively and published in recent years, (Shiflett, 1999; Zeng, 2005).

Substantial awareness in investigating the ability of naturally occurring antioxidants (phytochemicals) has been generated in recognition of the participation of oxidative stress (produced by the imbalance between the free radical production and the antioxidative defense system) involved in neuronal damage associated with stroke (Fig. 3). The oxidation of membrane lipids, DNA, protein, carbohydrate, and other biological molecules and increased inflammation and apoptosis by free radicals (Chan, 2001) generated during I/R injury due to mitochondrial dysfunction and activation of the enzymes xanthine oxidase and NADPH oxidase may cause DNA mutation and cellular damage; this often results in cell death. The causal relationship between oxidative stress and the pathology of cerebral ischemia has generated considerable interest in developing antioxidant therapies like uric acid and NXY-059 to combat ischemia induced damage.

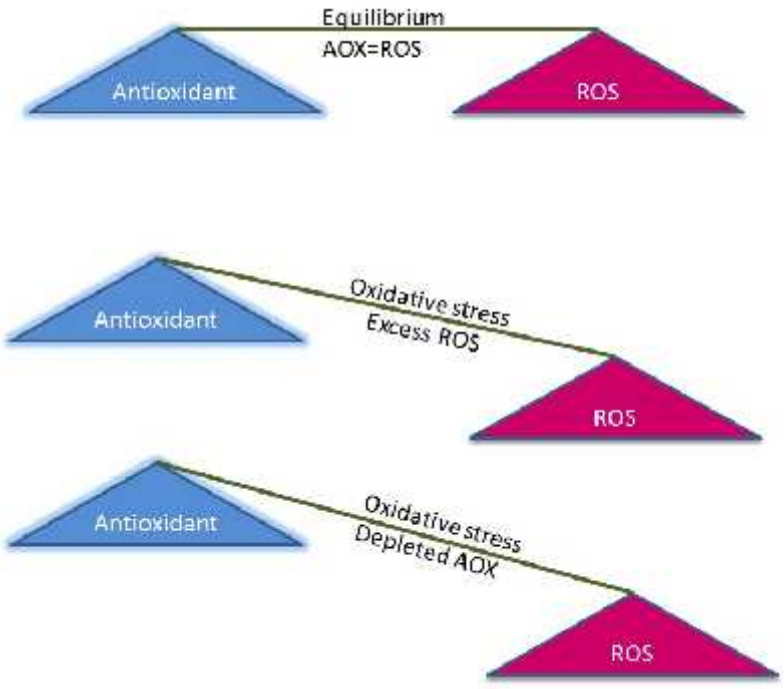


Fig. 4 Shows the state of equilibrium and imbalance between antioxidants and ROS to induce oxidative stress.

1.6 Anti oxidant strategy in stroke

An endogenous antioxidant defence mechanism balances the continuously produced ROS during physiological processes in normal brain tissue. However, post cerebral ischemic injury, the natural endogenous antioxidant system gets disequilibrium in redox because of increase in free radical production and oxidants are overproduced and detoxification mechanisms are inactivated. Post cerebral ischemia, subsequent neuronal injury and increased oxidative stress results due to an increase in ROS levels (Facchinetti, 1998). Hence much research has been focused on assessing the therapeutic effects of antioxidants with free radicals as a potent therapeutic target. There are five main strategies by which antioxidants work; (i) inhibition, (ii) scavenging of free radical production; (iii) increasing free radical degradation (Margaill, 2005); iv) targeting the mitochondrial antioxidants; or v) upregulating of endogenous antioxidants

1.6.1 Free radical inhibitors

Specific inhibitors of ROS generating enzymes are targeted in this approach. The main sources of ROS production in cerebral ischemia/reperfusion injury are the NADPH oxidases (NOXs) and Xanthine Oxidases (XO). Inhibition of NADPH oxidase complexes with the pharmacological agent apocynin (Tang *et al*., 2007; Singh, 2010; Genovese *et al.*, 2011) and Xanthine Oxidases with allopurinol (Khan *et al.*, 2008), has highlighted the contributory role of NOX and XO in brain injury as demonstrated by the cerebral ischemic parameters getting attenuated in rat models of experimental stroke.

1.6.2 Free radical scavengers

Here, the free radicals produced during oxidative stress are being scavenged. Though for treatment of cerebral ischemic stroke some compounds capable of this have been developed but not much success has been attained. Tirilazadmesylate, an inhibitor of lipid peroxidation (Sena *et al*., 2007), NXY-059 (Kuroda *et al*., 1999; Zhao *et al.*, 2011; Sydserff *et al*., 2002), Edaravone (Ikeda *et al*., 2013) are the free radical scavenger that has been found to terminate radical chain reactions.

1.6.3 Free radical degradation

Increasing antioxidant SOD levels in experimental models of stroke can be one of the strategies aimed at reducing oxidative stress as integral role of ROS has been demonstrated in lesion progression (Sheng *et al*., 1999). Pre treatment with ebselen, in pre-clinical studies on focal cerebral ischemia in rodent models (Imai *et al.*, 2001), Normobaric oxygen (Esposito *et al.*, 2013), Lubeluzole (Aronowski *et al*., 1996) in occlusion models showed improvement in ischemic damage and neurological deficit, respectively.

1.6.4 Mitochondrial targeted antioxidants

Oxidative damage is potentially ameliorated by targeting specifically the interior of the mitochondria by certain antioxidants. Such mitochondrial targeted antioxidants on the other hand inhibits mitochondrial complex I thereby inhibiting both ischemic and reperfusion-mediated oxidative damage protecting the mouse brain from hypoxic/ischemic (HI) injury (Niatsetskaya *et al*., 2012). Moreover, mitochondrial targeted antioxidants like vitamin E (Dhanasekaran *et al.*, 2004), Mitoquinone (Murphy and Smith, 2007) signifies in the treatment of stroke as an imperative emerging therapeutic strategy.

1.6.5 Upregulation of endogenous antioxidants

Natural defence mechanism showcased by our body against oxidative stress are through the antioxidant vitamins like Vitamin C and E which are the most studied natural antioxidants. Dietary Vitamin C has an important biological role as a hydrogen donor to reverse the oxidation process, so it should be made available to the body by the consumption of good amount of vegetables and citrus fruits (Yokoyama *et al.*, 2000).

1.7 Naturally occurring antioxidants to combat stroke

There are countless herbs grown all across the globe, delivering a host of health benefits. Some of the herbs like sage, basil rosemary, thyme, garlic, ginger, onion, hibiscus, cinnamon, mulberry, turmeric, fenugreek, ginseng etc. hold a plethora of antioxidants, phytosterols, vitamins, essential oils and other nutrient substances that prepare our body to combat deadliest of the chronic conditions. When our body processes oxygen, free radicals are produced with potential dangerous effects resulting in cellular damage. To counter this effect, naturally occurring endogenous antioxidants plays an important role. Antioxidants are therefore, a potential treatment for many diseases especially cerebral stroke because they either inhibit the

free radical production, or act as free radical scavengers, or account for free radical degradation, or even upregulate the endogenous antioxidant system thereby reducing the cellular damage.

There is a plethora of plants that are important resource of antioxidants which can scavenge free radicals (per oxide, hydro peroxide or lipid peroxyl, super oxide anion radicals, hydroxyl radicals, hydrogen peroxide and the singlet oxygen) produced during normal and pathological cell metabolism and thus increase the antioxidant capacity and thereby reducing the occurrence of certain life threatening diseases such as cardio vascular diseases, cancer, and stroke (Mathew *et al*., 2006). These naturally occurring antioxidants in these plants may act, as as a reducing agent, free radical scavenger, as complexes of pro-oxidant metals, or as a quencher of singlet oxygen thereby preventing or attenuating the damages caused by free radicals. The presence of polyphenolic compounds and secondary metabolites in various herbals attributes to the pharmacological properties especially as antioxidant potential (Elfalleh *et al*., 2011; Kaisoon *et al*., 2011).

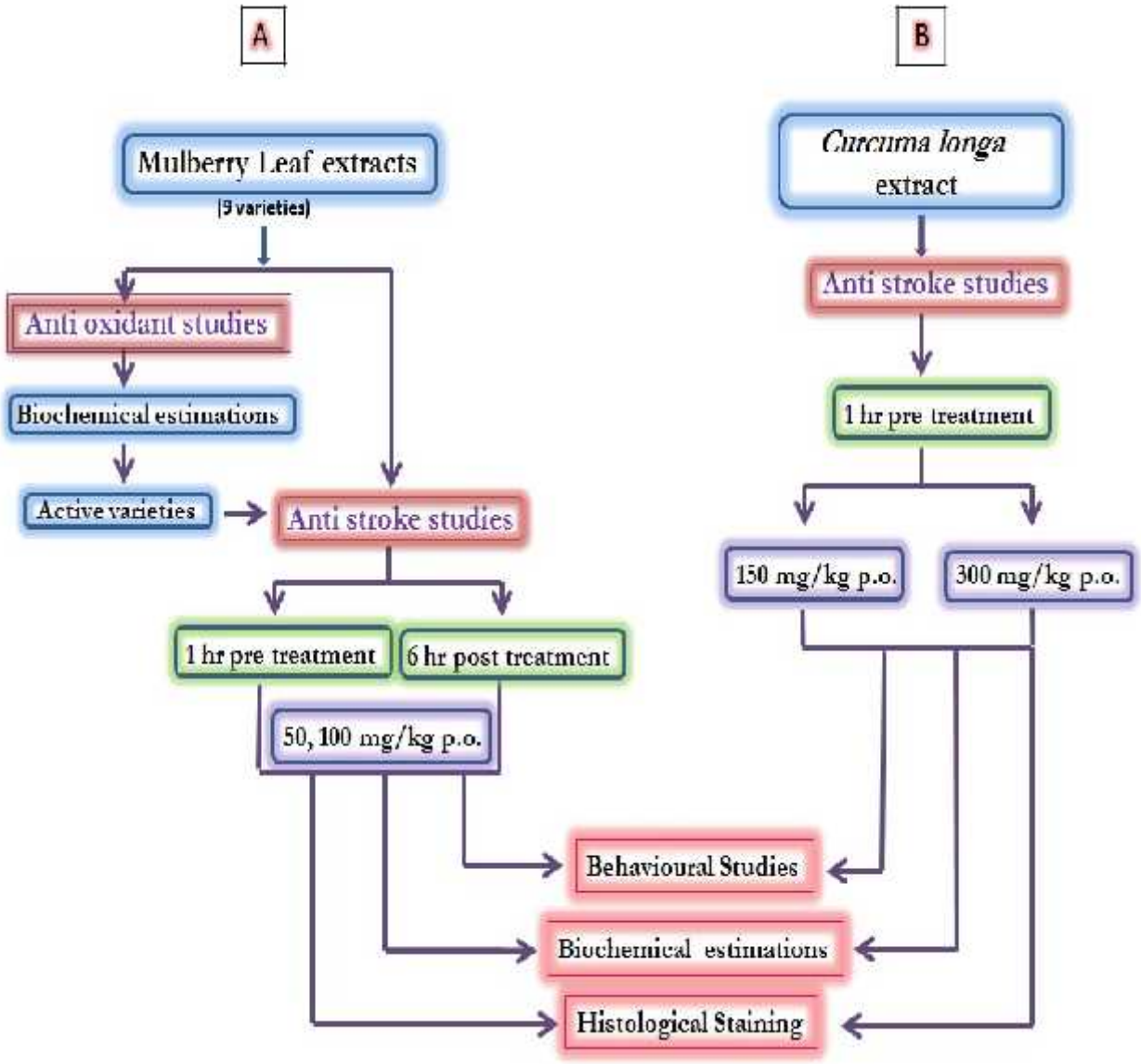
Oxidative stress and inflammation are central to ischemic pathology and also since mulberry and curcuma extracts having shown anti-inflammatory and anti oxidative properties due to the presence of abundant phytophenols in different models under varied conditions. This study has been undertaken to investigate the potential of the extracts of mulberry for offering neuroprotection against cerebral stroke in focal cerebral ischemia model in rats. This study will also focus on establishing the neuroprotective properties of curcuma extract against cerebral stroke and also evaluating the possible mechanism of neuroprotection and/or attempt to establish a possible co-relation in its effects on the functional deficits induced by middle cerebral artery (MCA) occlusion with the alterations in tissue damage.

The medicinal benefits of both the plants have been extensively studied, and their remarkable health promoting pharmacological activities can be attributed due to the presence of

phyto-nutrient compounds like antioxidants, minerals and vitamins. Poised to be the newest sensation in health-conscious crowds, both the mulberry and turmeric tackle an impressive range of illness and diseases as reviewed in the following section.

Therefore, at the outset prior to assessing the precise neuroprotective activity of mulberry in middle cerebral artery occlusion model of focal cerebral ischemia, an attempt was made first to find out the anti oxidant potential of the various mulberry leaf extracts in rotenone induced oxidative stress model. Further this study will also highlight the neuroprotective effect of curcuma extract and provide information about neuronal survival in various brain regions following I/R stress. The understanding of anti oxidant activity of the extracts during energy deficiency in case of cerebral ischemia may generate knowledge about the extent of ischemic brain damage as a consequence of oxidative stress. The study may also be helpful in developing neuroprotective strategies for clinical exploitation in cases of cerebral stroke in humans.

1.8 Plan of work



1.9 Objectives of the study

The experimental study was designed to investigate the neuroprotective effect of selected herbal plants *viz.* mulberry and curcuma extracts in MCAO model of focal cerebral ischemia. The presence of phytophenols in the herbal plants plays a prominent role in defining its pharmacological effects. Hence, the antioxidant activity due to the presence of various antioxidants was also elucidated in the rotenone induced oxidative stress model of rats.

The specific aims of this study are as follows:

1. To prepare extracts from Mulberry and Curcuma herbs.

2. To standardize the middle cerebral artery occlusion model of transient focal cerebral ischemia using male Sprague-Dawley rats.

3. To compare the possible neuroprotection offered by the mulberry extracts in the MCAO model.

4. To establish neuroprotective properties of curcuma extract, also to highlight its neuroprotective mechanism and/or to co-relate its effect on behavioral deficits with the alteration in tissue damage in MCAO model.

CHAPTER II

STUDIES ON NEUROPROTECTIVE EFFECT OF MULBERRY LEAF EXTRACTS

2 Introduction

Mulberry is a deep-rooted perennial plant with highly branching root and shoot systems. Mulberry belongs to the family moraceae. It is a moderately sized (three to six metres high) deciduous tree (Fig. 5). It is a native of India, China and Japan. It can grow both in the tropics and in the temperate regions and can be cultivated in different soil types with either rain-fed or irrigated conditions. Environmental fluctuations, pests and diseases relatively do no harm to the plant. It has been enumerated that there are about 24 species of mulberry and its known cultivars accounting to more than 100 (Koidzumi, 1917).

The eastern, central, and southern Asia has grown this plant for sericulture since ages. And till date, the most important commercial use of this plant is for Sericulture. Silkworm larvae feed on the tender, fully developed leaves. The availability of soil water in a mulberry plantation accounts for the quality and quantity of leaves harvested for silkworm feeding. The nutritive value of leaf varies from variety to variety as it depends on the photosynthetic and respiratory activities of the leaf.

Mulberry trees can be dioecious or monoecious, and but sometimes will change from one sex to another. They are wind pollinated and some cultivars do not require the process of pollination. Even on the same plant, one can find thin, glossy, light green leaves which are variously lobed with some being unlobed while others having the shape of a clover. The leaves are alternatively placed and are broad oval shaped and 2 to 4 inches long with toothed margins. The upper surface of the leaf has a smooth smooth and shiny texture while the lower leaf surface is pale green, generally smooth, with hairs only along the main veins.

Fig. 5 An aerial view of mulberry plantation

The mulberry fruits are juicy, sharp-tasting and sweet and are indeed rich source of phenolic flavonoid phytochemicals called anthocyanins. Around the central axis, small fruits arrange concentrically in an aggregated manner to form the complete mulberry fruit. The berries contain the polyphenolic flavonoid antioxidant, resveratrol in addition to vitamin A, C and E. Iron which is rarely found in other berries is abundantly present in the mulberry. They are also good source of niacin, folic acid, magnesium, vitamin B6, manganese, potassium and riboflavin. Juice, stews, wine and tarts can be made out of the fruits apart from having it fresh. Ripe fruit juice is useful in treating heart diseases, bleeding disorder, burning sensation, weakness and anti aging. Traditionally, the mulberry fruit has been used as a medicinal agent to purify the blood, to treat kidney disorders and weakness, combat fatigue, anemia, urinary incontinence, tinnitus, dizziness, premature graying of hair and constipation in the elderly patient (Nadkarni, A.K. 1976).

2.1 Background of the study

Since time immemorial, almost all parts of the mulberry plant have been extensively used in Chinese medicine (Bown, 1995) to reduce blood pressure, treat fever, protect the liver, improve eyesight, strengthen joints, facilitate urine discharge (Zhishen *et al*., 1999). As the root of mulberry exhibits antibacterial activity, in Asian countries, it has been used as an herbal medicine against food poisoning micro organisms. In a study, on the methanolic extract of mulberry roots, it was found to possess adaptogenic activity, indicative of its effectiveness as an anti stress agent (Nade, 2009).

Many studies done in various parts of the world by top medical institutions prove mulberry leaf as one of the most powerful natural herbal supplements having potent solution to many health issues. Study conducted by Srivastava *et al*. (2006) analysed the basic composition of mulberry leaves, and found that the leaves had 15-30% of protein, 2-7.9% fat, 13-17% ash and carbohydrate content and fiber was present between 9.9-13.8%. White mulberry leaves are also rich in minerals like iron, zinc, calcium, phosphorus and magnesium. Among the vitamins included in the white mulberry were labeled ascorbic acid and β carotene. The presence of essential unsaturated fatty acids (palmitic acid, oleic acid, linoleic acid, eicosanoids) was also identified in the mulberry leaf (Memon et al., 2010). In terms of the medicinal value of white mulberry, the most important role is played by polyphenols. Some researchers identified the phenolic compounds (Apigenin, quercetin, morin, luteolin, gallic acid, caffeic acid, chlorogenic acid, syringic acid, coumaric acid, ferulic acid, rutin, umbelliferone, astragalin, magniferin, sanggenon J and K, morusin, cyclomorusin, atalantoflavone, kaempferol) present in the leaf (Chu *et al*, 2006; Zou *et al.*, 2012, Dat *et al*., 2010). Apart from these the mulberry leaf also posses

enzyme inhibitors (Moranoline, 1-deoxynojirinmycin) and lectins (hemagglutinin, phytoagglutinin, phytohemagglutinin and phytosins) (Ratanapo *et al*., 1998). Mulberry leaves also contain GABA that strengthens the blood vessel membranes and thereby lowers the blood pressure and increases circulation of blood. In chronic diabetic patients, vascular dysfunction is induced by free radicals leading to its pathogenesis. And when diabetic rats were administered with Mulberry leaf extract, it was found that the extract helped in restoring the vascular reactivity of diabetic rats (Naowaboot *et al.*, 2009). As the leaves of mulberry contain 1-deoxynojirimycin, a potent α-glycosidase inhibitor, they are given to patients with diabetes mellitus as anti hyperglycemic nutraceutical foods in Korea and Japan (Kim *et al*., 2003). Streptozotocin-induced diabetic mice when administered with mulberry leaf extract restored impaired glucose metabolism and also attenuated the hyperglycemic conditions (Nojima *et al*., 1998; Kimura *et al*., 1995). In a study conducted by Jamshid and Prakash, the effect of *Morus alba* leaf extract on the pancreas of diabetic rats were studied. And it was found that the extract of this plant was able to regenerate β cells in pancreas thereby reducing the blood glucose levels (Jamshid and Prakash, 2012).

The antihyperglycemic, antioxidant and antiglycation effects of ethanolic extract of mulberry leaf has already been proven in some of the studies conducted in chronic diabetic rats, so it can be used as a food supplement for diabetics (Naowaboot, 2009). In addition, to aid blood sugar control, the polyphenols, isoquercitrin and astragalin present in the leaf extract can be a powerful natural supplement. Mulberry leaf contains moranoline (1-Deoxynojirimycin / 1-DNJ) which inhibits the enzyme, α-glycosidase present in the intestinal tract involved in the digestion of carbohydrates. The complex carbohydrates, maltose, sucrose starches are generally broken down into glucose. But Moranoline or 1-DNJ aids in inhibiting this process (Kimura *et al*., 2007)

which actually prevents sugar from entering into the blood stream. It should also be noted that apart from inhibition of alpha glycosidase, extracts of mulberry stimulate insulin secretion (fagomyns) and lower cholesterol (phytosterols). Extracts of white mulberry also reduce the number of adverse side effects resulting from the development of diabetes, such as cataracts and abnormalities in the functioning of neurotransmitters (El-Sayyad *et al.*, 2011).

For the treatment of rheumatism, arthritis and gout, the branch of *Morus alba* is used in several traditional Chinese preparations (Shia *et al.*, 2012). Various constituents of the drug were found to have significant anti hyper-uricemic potential, including mulberroside A, a stilbene glycoside (Wang *et al.*, 2011) and a number of flavonoids, primarily morin (Yu *et al*., 2006, Yu *et al*., 2007).

Mulberry leaf extracts has also been known to possess antibacterial activity. Kuwanon G obtained from the methanolic leaf extract inhibits the activity of periodontitis causing oral pathogen *Streptococcus mutans* and cariogenic bacteria *Streptococcus sobrinus, Streptococcus sanguis and Porpyromonas gingivalis* at a concentration of 8 mg/ml (Park *et al.*, 2003). In yet another study, it was found that kuwanon G completely inactivated S. mutans at a concentration of 20 mcg/ml. Antibacterial activity of isolated leaf compounds against S. mutans was elucidated in some similar studies (Islam *et al.*, 2008). Ayoola *et al*. (2011) studied the antibacterial activity of ethanol extracts of M. alba from leaves. They showed that the ethanolic extract inhibits the growth of Gram-negative bacteria *Escherichia coli, Pseudomonas aeruginosa, Neisseria gonorrheae* and gram-positive bacteria *Proteus vulgaris, Staphylococcus aureus* and *Streptococcus faecium*, and fungi *Aspergillus tamari, Aspergillus niger, Fusarium oxysporum* and *Penicillium oxalicum.* Due to the presence of broad spectrum antibiotic compounds in the leaf extract, antibacterial and antifungal activity was exhibited in one of the study (Srinivasan *et*

al., 2001). And these activities along with antioxidant activity and wound healing activity could be attributed due to the presence of flavonoids, triterpenoids, alkaloids, steroids, phenolic compounds and tannins.

Kim *et al*. (2010) showed that extracts from mulberry inhibit the action of lipases, while increasing the lipolysis in adipocytes, leading to a reduction in the level of intracellular triglycerides. A supply of powdered mulberry leaves together with pioglitazone to mice inhibited weight gain, reduced lipid peroxidation and decreased triglyceride levels. Hence an anti-obesity effect of 1-deoxynojirimycin from mulberry was determined (Tsuduki *et al*., 2013).

Traditional medicine recommends the use of mulberry water infusions to treat high blood pressure, reduce cholesterol and protect against atherosclerosis (Priya, 2012). Japanese scientists have isolated certain biologically active compounds which are effective in suppressing the progression of atherosclerosis, inhibiting the oxidation of LDL-cholesterol and reducing the build-up of cholesterol-rich plaque in the arteries (Doi *et al*., 2000).

In a study, the gene expression in vascular endothelial cells of pro inflammatory stimuli was suppressed when treated with white mulberry leaf extract (Shibata *et al.*, 2007). Rat aorta experienced a dual vaso active effect in a study when leaf ethyl acetate extract was administered (Xia *et al.*,2008). In the pathophysiology of atherosclerosis, the leaf extracts play an important role as it inhibits the activity of matrix metalloproteinases, protein expression and phosphorylation, and signaling pathways in the cells present in the rat thoracic aorta smooth muscle (Chan *et al*., 2009). As white mulberry leaf contains quercetin, its dietary consumption proved to reduce the development of atherosclerotic lesion by increasing the LDL resistance to oxidative modification in LDL receptor deficient mice (Enkhmaa *et al*., 2005). Administration

either with white mulberry leave constituent, DNJ or DNJ enriched leaf extract proved to reduce the accumulation of lipid in rat liver (Tsuduki *et al.*, 2009).

In traditional medicine, mulberry was used to restore normal liver function after ingestion of toxic substances. Hogade *et al*. (2010) demonstrated that administration of the ether (petroleum ether), chloroform, alcoholic and aqueous mulberry extract to rats with carbon tetrachloride-induced tetrachloride (CCl4) hepatotoxicity has a protective effect on the liver.

Antihelmintic activity of the leaf extract was found when alcoholic, petroleum ether and aqueous extracts of leaves at varying concentration was able to immobilize the worms in rat intestine. The optimum concentration being 50 mg/ml (Maheshwar *et al*., 2010). The methanolic leaf extract can find itself in potential clinical application by helping in managing the psychiatric disorders. The evaluation was based on the catalepsy, aggression, stereotyped behavior study and sleeping behavior in mice (Adhikrao and Vandana, 2008). Hole-board test; elevated plus-maze paradigm and open field test with light/dark paradigm was used as parameters to study the anxiolytic effect of a methanolic extract of mulberry leaves in mice (Yadav *et al*., 2008).

Many laboratory studies demonstrated the protective role of leaf extracts of mulberry against many neurological disorders. This may be attributed due to the presence of oxyresveratrol, a natural hydroxystilbene and an inhibitor of tyrosinase. Oxyresveratrol has a neuroprotective effect against Alzheimer's disease, vascular dementia and stroke. Strong antioxidant activity was exhibited by oxyresveratrol in some of the studies (Zhang and Shi, 2012). In yet another study, its antioxidant activity was expressed when it was found effective in scavenging free radicals such as 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) and 2,2-diphenyl-1-pieryl-hydrazyl (DPPH) (Zhao *et al*., 2008). In a study done by Horn *et al* , Oxyresveratrol was found to be a potent neuroprotectant and they even suggested it as a

potential drug for the treatment of acute ischemic stroke (Horn *et al*., 2003). It was able to reduce the neurological deficits, the infarct volume and apoptotic DNA fragmentation in MCAO induced rats administered with mulberry leaf extract (Andrabi *et al*., 2004). Likewise, Breuer *et al.* (2006) demonstrated that oxyresveratrol was able to cross the blood-brain barrier exerting direct protective effects on the brain and hence can be used as a complementary drug in the treatment of neurodegenerative diseases like stroke involving oxidative/nitrosative stress.

Many laboratory studies similarly demonstrated the protective role of mulberry extracts in Alzheimer's disease owing to the presence of oxyresveratrol. Oxyresveratrol from mulberry extract demonstrated neuroprotective activity in cortical neuronal cells (culture cortical neurons), in SHSY5Y cells as well as in models of *in vitro* and *in vivo* (Chang *et al*., 2010). It has showed that the methanol extract of mulberry leaves exerts anti-dopaminergic effect by blocking D2 receptors. In rats treated with the ethanol extract of mulberry, resulted in an improvement in memory test performance in the water maze and decreased activity of acetyl-cholinosterase and an increase in the density of neurons in the hippocampus was also observed (Chen *et al*., 2006; Kaewkaen *et al.*, 2012). The plant leaf extract has also been used in various studies to prove its neuroprotective effect in stroke as well. Kang *et al*., (2006) pointed out the possibility of the brain protection against adverse changes by introducing a source of GABA to your diet. The authors showed that the administration of an extract containing GABA is neuroprotective on PC12 cells, protecting from hydroxyl radicals, and delays the ischemic stroke.

The accumulation of GABA in mulberry leaf as a result of the anaerobic treatments enhances the neuroprotective effect against cerebral ischemia (Tong *et al*., 2006). In a study, it was demonstrated that the antioxidative flavanoid, quercetin-3-O-β-D-glucopyranoside significantly decreased the stroke parameters like hemoglobin, strokin, cortexin, frontalin,

temporalin, parietalin, occipitalin, brain venticulin, hemorraghic clot in rabbit thereby providing various oppurtunities for new vegetable drug development concerning with stroke parameters (Vahdettin, 2009). The above results clearly showed neuroprotective activity of extracts of mulberry. Therefore, a search for new natural and non toxic compounds with the neuroprotective effects is of particular interest.

2.2 Materials used

Chemicals, Biochemicals, Instruments used

Table 1 The list of chemicals and biochemical used in the experiments.

Sl. No.	Chemical/Biochemical	Manufacturer
1.	2,3,5-Triphenyl tetrazolium chloride (TTC)	Sigma
2.	2-Thiobarbituric acid (TBA)	Sigma
3.	5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB)	Sigma
4.	5-Sulphosalicylic acid	Qualigens
5.	Absolute alcohol	Qualigens
6.	Acetone	Qualigens
7.	Anaesthetic ether	TKM Pharma
8.	Benzene	Merck
9.	Calcium chloride	Sigma
10.	Chloral hydrate	Sigma
11.	Cresyl violet	Sigma
12.	Disodium hydrogen phosphate (Na_2HPO_4)	Sigma
13.	DPX mountant	Fluka Biochemika
14.	Eosin	HiMedia
15.	Ethylene diamine tetra acetic acid	Sigma

	(EDTA)	
16.	Fenton Reagent	San Jose Scientific
17.	Formaldehyde	Merck
18.	Glutathione	SRL
19.	Gum accacia	HL Pharma
20.	Hematoxylin	HiMedia
21.	Hydrochloric acid	Merck
22.	Liquid paraffin	Merck
23.	Magnesium chloride ($MgCl_2$)	USB TM Chemicals
24.	Magnesium sulphate ($MgSO_4$)	Qualigens
25.	OCT embedding matrix	Leica
26.	Paraffin wax	SD fine chemicals
27.	Paraformaldehyde	Merck
28.	Poly-L-lysine	Sigma
29.	Potassium chloride	Qualigens
30.	Potassium dihydrogen phosphate(KH2PO4)	Sigma
31.	Resveratrol	Sigma
32.	Sodium carbonate (Na_2CO_3)	Merck
33.	Sodium chloride (NaCl)	Merck
34.	Sodium citrate	SRL
35.	Sodium hydrogen carbonate (NaHCO3)	SD fine chemicals
36.	Sodium phosphate	Sigma
37.	Sucrose	Sigma
38.	Tetra methoxy propane (TMP)	Sigma
39.	Trichloro acetic acid (TCA)	Sigma
40.	Xylene	Rankem

Table 2 The list of instruments used in the experiments.

Sl. No.	Instruments	Manufacturer
1.	Cryostat	Microm GmbH, Germany
2.	Histocentre 2 workstation	Shandon
3.	Image Acquisition System (Biovis Image Plus)	Expert Vision Labs Pvt Ltd, India
4.	Incubator (Fine PCR Combi SV12)	Fine PCR, Korea
5.	Lambda 35 UV/VIS spectrophotometer	Perkin Elmer, Germany
6.	Light/Fluorescent Microscope (Leica DM5000B)	Leica Microsystem, Germany
7.	Magnetic stirrer	Genie, Bangalore
8.	Microtome (Leica RM2255)	Leica Microsystem, Germany
9.	Incubator (Fine PCR Combi SV12)	Fine PCR, Korea
10.	pH meter	Systronics, India
11.	Polytron Homogenizer (PT 3100)	Kinematica AG, Switzerland
12.	Table top Centrifuge (Sigma 3K30)	Sigma, Germany
13.	Ultracentrifuge	Beckmann, USA
14.	Vortex Mixer	Spinix, Tarsons, India
15.	Water Bath (Julabo SW22)	Julabo, Germany
16.	Weighing Balances (Precisa)	Toledo, Germany

Fig. 6 Nine varieties of mulberry plant used in the study.

2.3 Preparation of aqueous mulberry leaf extracts

The mulberry leaf extracts were prepared using the protocol as follows:

Fresh mulberry leaves of both (*Morus alba* and *Morus indica*) nine varieties designated as- V-1, S-1, S-13, S-146, AR-12, AR-14, S-1635, BR-2 and TR-10 (Fig. 6) were obtained from mulberry garden of Babasaheb Bhimrao Ambedkar University, Lucknow. The leaves were cleaned, dried and grinded into a fine powder. The freshly prepared powder was then passed through an 80-mesh sieve and the filtered powder thus obtained was kept in a sealed aluminum foil in the refrigerator, till further use.

The aqueous extract of powder was prepared following the method of Katsube *et al*. (2006). Briefly, 2 g of mulberry leaves powder was soaked in 200 ml of boiling water for 20 minutes. At room temperature the mixture was cooled and Whatman No.1 filter paper was used for filtering and the residue obtained was then lyophilized. The freeze-dried solid extract was transferred into plastic tubes and stored at −20 °C to protect from light. The extract was re-dissolved in double distilled water in desired concentration prior to use in all the experiments. The final dissolved extract is denominated as mulberry leaf extract (MLE) in the text.

2.4 Experimental protocol

Adult male Sprague-Dawley (SD) rats were procured from the National Laboratory Animal Centre of CSIR-Central Drug Research Institute (CDRI), Lucknow, India and used throughout the study. After necessary approval of Institutional Animal Ethical Committee (IAEC Approval no. **86/08/Pharmacology/IAEC/Renew 03(70/09)**), all experimental procedures and laboratory animal handling were carried out carefully in strict accordance. Throughout the study rats were allowed food and water *ad libitum*.

The animal groupings and the number of rats in each group were according to the type of experiment for each single dose of extract used in varying studies.

2.4.1 Study of anti oxidant activity of MLE

Adult male Sprague Dawley rats, 200±10g, were used in the experiments. Eleven experimental groups comprising of 8-10 male rats were used in the each study. Group I was taken as sham. Animals of Group II were subjected to oxidative stress by rotenone treatment alone. DMSO was used to dissolve rotenone was dissolved in and administered orally at a dose of 75 mg/kg to induce state of oxidative stress in rats. The group III to XI animals received pretreatment with nine varieties of mulberry leaf extracts (100mg/kg, p.o.) each one hour prior to challenge with same dose of rotenone. Animals were sacrificed after one hour post rotenone challenge and brain tissue was gently removed in ice-cold chilled conditions for further studies.

2.4.2 Study of neuroprotective activity of MLE

Adult male Sprague Dawley rats, weighing 260±20g, were used in the experiments. Two different sets of eight experimental groups were made consisting of 8-10 rats in each group. Resveratrol,a polyphenolic compound found in certain plants and in red wine, which has antioxidant properties has been here used as a standard against MLE. It was administered at a dose of 50 mg/ kg p.o. and 100 mg/kg p.o.both pre and post ischemic insult. The control groups of ischemic rats were given 1% gum acacia suspension in equal volume.

First set had the groups which includes sham, MCAO placebo (gum accacia), test substance MLE-AR-14 (50 and 100 mg/kg p.o.) and resveratrol (50 and 100 mg/kg p.o.). Second set had the same groups but were administered with the extract, MLE-S-146 for assessing of neuroprotective activity. The test substances were administered pre and post I/R insult.

The focal cerebral ischemia/ reperfusion injury of 2/24 hrs was used throughout the study. The schedule of treatment for preventive effect includes treatment with test substance or standard compound 1 hour prior to ischemia and for curative effect, 6 hour post reperfusion injury.

2.5 Animal models used

Anti oxidant and neuroprotective activity of the extracts were assessed using the rotenone induced oxidative stress model and focal cerebral ischemia model respectively which are described as follows:

2.5.1 Rotenone induced oxidative stress model

Rotenone, a phyto-toxin obtained from roots of *Derris sp* belonging to *Leguminosae* family is a broad-spectrum pesticide and is generally used to induce oxidative stress. Rotenone interferes with the electron transport chain in mitochondria thus affecting the ATP synthesis. It causes neurotoxicity by inhibiting the oxidation of NADH to NAD, which blocks the oxidation of substrates such as glutamate, α-ketoglutarate and pyruvate, thereby generating ROS and other free radicals leading to various adverse effects in cellular physiology (Uversky, 2004; Swarnkar *et al*., 2009).

2.5.2 Focal cerebral ischemia model

Several animal models of cerebral ischemia have been developed to mimic cerebral ischemic pathology in humans. However, the MCAO in rat is the most preferable for inducing focal cerebral ischemia, because majority of human ischemic strokes results from occlusion of the middle cerebral artery, hence this model is the most acceptable animal model simulating to the clinical situation in humans.

The cerebral ischemia/ reperfusion in rats was induced as per the protocol given below:

- The rats were examined grossly for any ill health signs.
- Focal cerebral ischemia was induced by (MCAO) using intraluminal suture method with slight modifications (Longa *et al*., 1989, Raghubir *et al.*, 2011).
- Male Sprague Dawley rats weighing, 260±20g were anaesthetized with chloral hydrate (300mg/kg i.p.) and then the animal was placed in supine position over a preheated operation table and a thermostatic device was used to maintain the body temperature at about 37ºC±0.5ºC.
- In the neck region, through a midline incision, the left common carotid artery (CCA) was exposed. The external carotid artery (ECA) and internal carotid artery (ICA) was isolated after the neck muscles were gently separated.
- A curved micro vascular clip was placed across the CCA adjacent to the ECA origin and a knot with silk suture was tied loosely close to the bifurcation of ICA.
- Through a small nick in the ECA, a (3-0) nylon monofilament suture (Ethicon, Johnsons & Johnsons Ltd, Mumbai) was introduced into the ECA lumen and gently advanced 20-22 mm passing through the CCA bifurcation into the ICA till the resistance was felt; confirming that it has reached the proximal segment of the anterior cerebral artery (ACA) (Fig. 7)
- The silk suture around ECA stump was tightened together with nylon suture to prevent bleeding and then the micro vascular clip was removed. The intraluminal filament blocked the origin of the MCA thereby occluding all sources of the blood flow from the ICA, anterior cerebral artery (ACA) and posterior cerebral artery (PCA).
- The filament was pulled back after 2 hrs of ischemia to re-establish the cerebral blood flow

(CBF) and rats were allowed reperfusion for 24 hrs.

- In the group of sham operated rats all the surgical procedures were performed except the insertion of the nylon suture.
- As the animals recovered from anaesthesia, they were kept for regaining the righting reflex. Then they were transferred into cages and placed in an experimental animal room and the temperature was maintained at 25±0.1ºC. Animals were provided food and water *ad libitum* and were closely monitored for any gross behavioural disturbances.

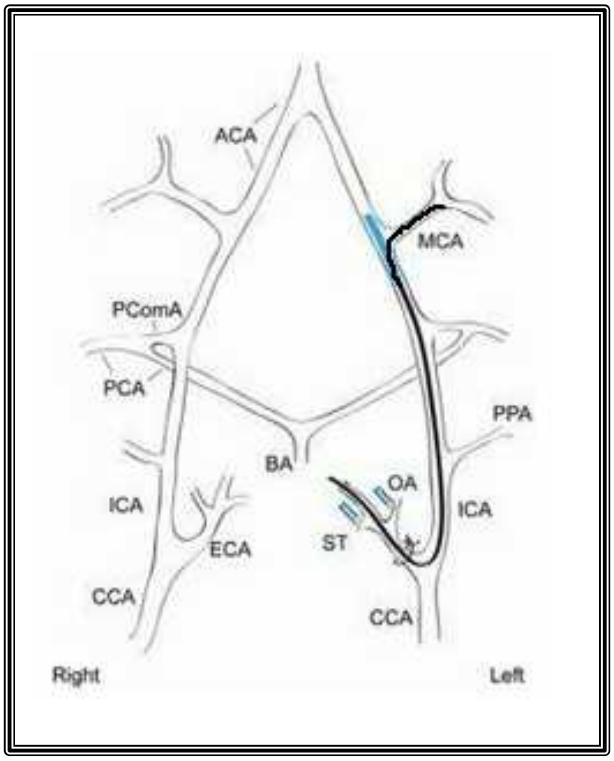


Fig. 7 The diagram showing the course of intraluminal filament to block the MCA origin.

- Rats showing tremor and seizures were not included in the studies.
- After the 2/24 hour of ischemia/reperfusion, rats were examined for neurologic deficit. Thereafter, re-anaesthesized with ether and about 3.5ml of blood was taken from retro

orbital plexus to determine the MDA and GSH levels in the blood. Finally the brains were dissected out and horizontal brain sections of 2 mm thickness were cut and stained with TTC to assess the extent of brain damage and cerebral infarct area.

2.6 Estimation of oxidative stress markers

Excessive production of free radicals is a consequence of oxidative stress that contributes to cellular damage. Free radicals directly oxidize various macromolecules as well as activate or directly participate in all the degenerative processes of the cell. The markers of oxidative stress, MDA and SOD were biochemically estimated.

After one hour of rotenone challenge, rats were transcardially perfused with normal saline under deep anesthesia and the brains were carefully isolated in chilled condition. The brain tissue was then homogenized in 5 volumes of 0.1M phosphate buffer, pH 7.0. The tissue homogenate was spun at 1500g for 15 min at 4^{o}C and supernatant was used to analyze MDA by method of Colado *et al*. (1997), and SOD by the method described by Misra and Fridovich (1971).

2.6.1 Malondialdehyde estimation in brain

Cerebral ischemia is associated with cell membrane damage which occurs due to increased production of the superoxide anions which in turn leads to lipid peroxidation. In particular, lipid in brain tissue are susceptible to peroxidation as it has large amount of lipids. MDA is one of the byproduct of lipid peroxidation and may serve as important biomarker of membrane damage.

MDA is also called thiobarbituric acid reactive substances (TBARS). Thiobarbituric acid (TBA) undergoes reaction with MDA and it forms the basis of estimation. Upon reacting with TBA, MDA gives pink color, the intensity of which is read spectrophotometrically at 532 nm. To

the tissue homogenate (500 µl), 30% trichloroacetic acid (300 µl), 5 M HCl (150 µl) and 2% (w/v) thiobarbituric acid in 0.5 M NaOH (300 µl) was added with mixing after each addition. With triple distilled water, the final reaction volume of 3 ml was made. Vortexing of the reaction mixture was done and then for 15 mins it was incubated in a water bath at 90^{o}C and centrifuged at 13000g for 10 min. The pink color thus developed, and intensity of which was read at 532 nm by using spectrophotometer (Shimadzu, Japan). A standard plot using different concentrations of tetra methoxy propane (TMP) was used to determine the MDA levels in brain. In nmol/mg protein the values were expressed.

2.6.2 Superoxide estimation in brain

In *in vivo* condition, short-lived and highly ROS (·OH (hydroxyl radical), O_2^- (superoxide) and H_2O_2 (hydrogen peroxide)) are continuously generated. To prevent the disruption of normal physiological functions, the equilibrium between the oxidants and the antioxidants, is sufficient. On the other hand, if this balance is disrupted either by increase in oxidants or decrease in antioxidants, ROS level increases. The endogenous antioxidant enzymes keep a check on the cellular levels of ROS. Superoxide dismutase is one such antioxidant enzyme as it scavenges ROS. And when there is a decrease in SOD activity, there arises an elevated level of superoxide which in turn leads to NO reduction and increase in peroxynitrite levels.

To assay the SOD levels in brain of experimental animals, auto-oxidation of epinephrine was inhibited using an indirect method. On adding epinephrine to a 1.5 ml mixture of Fenton reagent, Na_2CO_3, EDTA, and 0.5 ml of supernatant (tissue homogenate) and deionized water, epinephrine's auto-oxidation process was initiated. The auto-oxidation was recorded every 30 sec for 5 min in a spectrophotometer at 480 nm. This was repeated for every sample obtained

from different group of animals. The initial rate of auto-oxidation was calculated after plotting a graph of absorbance against time. The concentration of the SOD enzyme (mg protein/ml) in the brain that causes decrease in the auto-oxidation of epinephrine by 50 % is equal to one unit of SOD activity (Jewett and Rockling, 1993). Each sample was tested for SOD activity and was calculated subsequently.

2.7 Assessment of cerebral ischemia/reperfusion induced injury

The brain damage following I/R was assessed using various parameters, which included gross behavioral, biochemical and histological studies.

2.7.1 Neurobehavioral studies

Brain undergoes cellular damage in cerebral ischemia leading to severe sensori-motor dysfunction. Assessment of neurological deficits was carried out 24 hr post I/R injury to assess influence of I/R injury on motor performance. A 10-point scale (Table 3) following the method of Longa *et al*., 1989 and Bederson *et al*. (1986) was used to score the neurological deficit.

Table 3 ND scoring on a 10 point scale

Sl. No.	Neurobehavioral Parameters	Scores
1	No deficit	0
2	Flexion	1
3	Circling	2
4	Hemi paresis	3
5	Non-spontaneous movement	4

1. No neurologic deficit:

The rats were held gently by their tail at one meter height from the ground. Those rats that extended both their forelimbs downwards indicating no neurological deficit were assigned a score of zero **(0)**.

2. Flexion:

Contra lateral to the affected hemisphere, rats consistently flexed the forelimb, when held in upright position by the tail. The neurologic score of one **(1)** indicated relative difference in the movement of forelimb on the contralateral side (Fig. 8).

Fig. 8 A typical flexion response in MCAO rat.

3. Circling:

The ischemic rat shows circling behavior usually consistently towards contralateral side wheras, the normal rat moves translationally on the wooden platform (Fig. 9).

Score **1** - if rat shows intermittent circling behaviour.

Score **2** - when rat shows consistent circling movement.

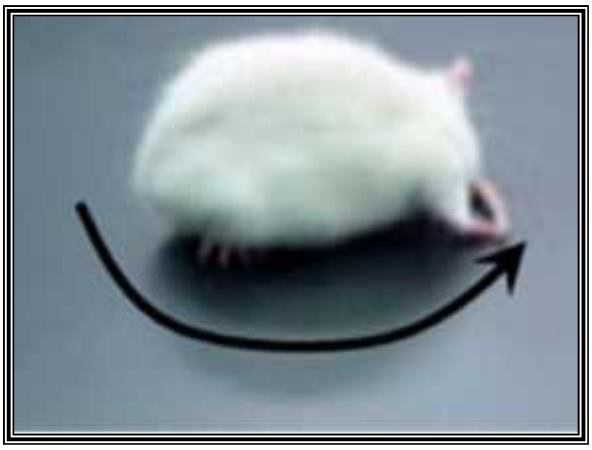


Fig. 9 Circling behaviour in MCAO rat.

4. Hemi-paresis:

The rats were placed on vertical steel wire grid and the gripping strength of the rats were noticed carefully. Normal rat uses all the limbs to climb up, whereas affected rats failed to do so and the score is given as follows:

Score **1**- rats rarely moved up and downwards and stayed with slightly impaired gripping strength.

Score **2**- gripping strength is moderately reduced and rats were able to stay on the grid for only some time and eventually fell down.

Score **3**- gripping strength is maximally affected and the rat falls on the ground in<5-10 seconds.

5. Spontaneous movements

Evaluation of spontaneous motor activity was done by observing the rats in the cage with normal environmental conditions for 5 minutes. The scores were given as follows:

Score **0**- the environment was explored by the free movement of the rats in the cage.

Score **1-** rats moved in the cage and approached at least three sides of the cage.

Score **2-** rats movement was drastically reduced and limited to only one side.

Score **3-** movement in the cage was meager and the rats showed postural abnormalities like tilt towards the contralateral side.

Score **4**- The rat remained in the same position as placed indicating complete motor deficit.

Thus, an impact of ischemic injury is indicated by enhancement in the behavioral deficit scores. The neurobehavioral scores obtained after careful assessment of each rat were averaged as neurological deficit scores for each experimental group. The neurological deficit score was also used as an inclusion/exclusion criterion and I/R rats showing no sign of neurological deficits were excluded from the study.

2.7.2 Biochemical estimations

Cerebral ischemia is associated with a decrease in the tissue energy potential and overproduction of free radicals causing lipid peroxidation of sub cellular membranes and accumulation of toxic products during the reperfusion period. MDA level was assessed to estimate the lipid peroxidation and to depict the endogenous antioxidant level GSH was estimated.

At the end of reperfusion, biochemical estimations were done in the blood as well as in the affected brain samples. Rats with ND $\geq$ 5 were anesthesized and by using heparin coated glass capillaries (Mucap, India), retro-orbital plexus was punctured to collect the blood GSH and MDA estimation.

2.7.2.1 Estimation of malondialdehyde

The malondialdehyde is one of the major by-product of lipid peroxidation and hence is an important biomarker of lipid peroxidation. It is based on its reaction with TBA giving rise to a pink pigment that has absorption maximum in acidic solution at 532 to 535 nm. Blood MDA content was determined by the method of Colado *et al.* (1997) to assess the level of lipid peroxidation owing to brain ischemic injury.

Rats were anaesthetized with ether and 3ml blood (1.5ml in each 2ml tube) was withdrawn from the retro-orbital plexus using the glass capillary. Samples were spun down at 13,000 rpm for 10 min at 25^0 C. Serum (500µl) was separated and used for MDA estimation. Trichloroacetic acid (30% w/v, 300µl) was added in the serum followed by addition of HCl (5N, 150µl), 2-thiobarbituric acid (2% w/v, 300µl) and triple distilled water (1750µl). Vigorous vortexing was done after every step. Samples were incubated at 90^0C for 15 min in the water-bath, and then spun down at 3000 rpm for 10 min to settle down the particulate matter. Absorbance was taken at 532nm and nmol/mg protein was the unit given to the the results.

2.7.2.2 Estimation of glutathione

Found in high concentration in brain, GSH is a major intracellular antioxidant and involved in protection against oxidative damage caused by ROS. GSH peroxidase catalyzes the GSH-dependent reduction of H_2O_2. The GSH was estimated based on the intensity of yellow color developed following its reaction with DTNB (5, 5'-dithio-*bis*(2--nitrobenzoic acid) as described earlier by Anderson *et al.* (1985).

Rats were deeply anaesthetized with ether and 500µl blood was withdrawn from the retro-orbital plexus using the glass capillary. Immediately an anticoagulant, sodium citrate (100µl) was added and mixed vigorously. 500µl blood was taken in another tube and 100µl 6%

acetic acid and 400µl 10% sulphosalysilic acid was added. Vigorous vortexing was done after every step. The sample was spun down at 13,000 rpm for 10 min at 25^0 C. Plasma (500µl) was taken out and was added into a mixture of 2ml phosphate buffer (pH 8.0), 480µl TDW and 20µl DTNB (5,5'-Dithiobis(2-nitro-benzoic acid) followed by vortexing. Samples were incubated at 37^0C for 10min leading to development of yellow chromogen. The absorbance was read at 412nm using spectrophotometer and the results were expressed as nmol/mg protein.

2.7.3 Histological studies

Transcardiac Perfusion of the rat- The skin on the ventral aspect of body was incised and the peritonium was carefully dissected out. The thoracic cage was exposed so that there is easy access to the heart. During perfusion, to ensure that the fluid spreads throughout the body blood vessels should be kept intact. The cardiac perfusion was done using chilled normal saline through the left ventricle's apex region. A small incision was made with a fine scissors in the left auricle, to serve as an outlet. For histological staining, as the brain tissue needs to be fixed, 4% paraformaldehyde solution is to passed through the vasculature following normal saline.

2.7.3.1 Triphenyl tetrazolium chloride staining

Efforts were made to identify the area at risk and the extent of cerebral damage following I/R injury using TTC staining. Electron transport chain which is an integral part of the inner mitochondrial membrane is made up by the pyridine nucleotide-linked dehydrogenases along with the cytochromes. TTC acts as a proton acceptor for such enzymes (Altman, 1976). Lipid-soluble formazan which is red in colour is formed when the tetrazolium salt is reduced by the enzymes. As this salt gets reduced by the enzyme present in the mitochondria, the viable tissue therefore stains deep red while the infarct brain area remains unstained. In TTC staining, since

infarct tissue volume can be quantified using the software, so it is an added benefit (Goldlust *et al*., 1996; Desai *et al.*, 2010).

Using normal saline, rat brain was perfused transcardially and immediately isolated in chilled condition. After discarding the cerebellum, the brain was transferred to –20^{o}C deep freezer. After 20 mins the frozen brain was uniformly sliced anterio-posteriorly into 2 mm thickness sections. 0.5% TTC dissolved in normal saline was then used to stain the slices and for 30 min it was incubated at 37^{o}C (Bederson *et al*., 1986, Desai *et al.*, 2010).The infarcted part of the brain remained white as it did not take up the stain whereas the normal brain tissue was stained brick red. The images of four brain slices of each animal were captured and the infarct area was calculated in the computerized image analysis system using Biovis Image plus software.

2.7.3.2 Hematoxylin and Eosin staining

Heamatoxylin and Eosin staining (H&E) is widely used to assess the morphological features of cells. Heamatoxylin is a basic dye which specifically stains nuclei, whereas eosin stains cytoplasm due to its acidic nature. Histological features such as diffused whiteness of the eosinophillic surroundings, shape alteration, cellular perikarya stainability and size and formation of vacuoles are the main features used to identify the lesioned area. Hence, H & E staining is a powerful tool to differentiate between necrotic and apoptotic cell death on the basis of cell morphology following I/R injury (Li *et al*., 1998).

In all groups, after 2/24 hr I/R injury, anesthetic ether was used to sacrifice the rats. Brain was taken out immediately in chilled medium. The cerebral hemispheres were sectioned in the coronal plane and alternate slices of 2-3 mm thickness were kept in 4% buffered paraformaldehyde for about 24 hours. Then slices were preserved in 70% alcohol. The

dehydration was done by passing the tissue through graded alcohol concentrations i.e. 30 min each in 70% alcohol, 90% alcohol, 95% alcohol, 100% alcohol then via mixture of 100% alcohol and then for 15 mins each through xylene mixture (3:1), xylene I, xylene II followed by infiltration with paraffin wax (4 h-24h) at 65ºC in Histocentre 2 workstation (Shandon).

Paraffin blocks were prepared and stored at 4^0C till sectioning. On the slides coated with poly-lysine, 4-6µm thick sections cut with a semi-automated microtome (Leica) were placed and processed (for deparaffinization) by immersing for 5 mins each in the following solvents before staining through the series of xylene and alcohol i.e. xylene I, xylene II, 100% alcohol, 70% alcohol, 50% alcohol and finally washed with tap water.

Hematoxylin stain was used to stain the brain sections for 45 seconds and washed in running water thoroughly for 5 min. Further, sections were stained with eosin for 60 seconds and processed by immersing in the following solvents.

- Acetone I (2-3 times)
- Acetone II (2-3 times)
- Acetone and Xylene (2-3 times).
- Xylene (4-5 times)

Stained sections were then mounted with DPX and examined under light microscope (X 400). Based on their morphological feature, the necrotic and apoptotic cells were identified.

2.7.3.3 Cresyl Violet Staining

To further confirm the regional distribution of cerebral damage**,** cresyl violet staining was done. The nissl substance present in the viable neurons's cell body and dendritic processes took up the CV stain. Nissl substance is primarily composed of rough endoplasmic reticulum and because of the high RNA content it is basophilic in nature and will be very sharply stained with

CV. After cellular injury, there is a loss of Nissl substance and the breakdown of myelin covering can also happen if the axon degenerates.

Normal saline was used to transcardially perfuse the rat brain, followed by paraformaldehyde (4%w/v) and taken out immediately in chilled condition and processed for paraffin embedding. Using a microtome (Leica, Germany), 4-6 μm thick sections were cut at a distance of 0.80 mm from bregma (Paxinos and Watson, 1986) and transferred on poly-L-Lysine (1:50 dilution of 0.1% w/v solution) (Sigma Aldrich, USA) coated glass slides. The sections were deparaffinized by xylene treatment and then rehydrated using the 95%, 85%, 70% and 50% graded concentrations of ethanol by sequentially immersing the slides through each one of them for 3 min each and then washed with 0.85% NaCl and phosphate buffered saline (PBS, pH 7.4) for 5 min each. The sections were then dipped in CV staining solution for few seconds, repeated 3-5 times and were air dried. Similarly, the sections were given few short exposures to n-butanol and then dipped in acetone followed by xylene. The stained sections were mounted with deoxy plasticizer xylene (DPX) and examined under light microscope (Leica, Germany) to identify the brain regions affected by I/R injury (Luna, 1968).

2.8 Statistical Analysis

One-way Analysis of Variance (ANOVA) was used to analyse the data and comparing the results obtained in different groups was done by Newman-Keuls multiple comparison test. In all the cases, the $*P<0.05$, $**P<0.01$ and $***P<0.001$ values were considered statistically significant at various level. Prism and GraphPad analysis software was used for all the statistical analyses.

2.9 Results

The results obtained at various stages of the present study have been presented here in a sequential manner to accomplish the objectives and highlight the aims. The study has been conducted on following outlines, which includes;

1. Assessment of anti oxidant activity of MLEs on rotenone induced oxidative stress in rat.

2. Further, assessment of the neuroprotective activity of MLEs in MCAO induced ischemic brain damage model in rat.

2.9.1 Effect of treatment of MLE on oxidative stress markers

The anti-oxidant activity of nine mulberry extracts has been assessed in terms of attenuation in the MDA and SOD levels in the rat brain homogenates challenged with rotenone. This was done with an aim to select potent anti-oxidant extracts for the bio-evaluation in one of the disease model eg. focal cerebral ischemia exhibiting profound oxidative stress. The results have been summarized as percentage decrease in MDA and SOD contents with each mulberry extract with respect to control values (**Table 4**).

2.9.1.1 Effect on MDA levels in brain tissue

Rotenone induced the ROS generation led to brain oxidative stress by lipid peroxidation as revealed by significant 138% increase in MDA content. The results indicate that one hour pre treatment with mulberry leaf extracts prior to rotenone challenge in general caused significant reduction in MDA levels from 13.30 to 50.49%. The maximum attenuating effect was observed with extract S-146 being 50.49% followed by 41.36% by BR-2. Further a decrease of 36.14% and 37.13% in MDA was also observed with extract AR-14 and S-1 respectively. Whereas, the

reduction in MDA by the other extracts V-1, S-13, AR-12, S-1635, TR-10 varied considerably and was less pronounced as shown in (Fig. 10).

Table 4 A comparative effect of pre treatment with MLEs (100 mg/kg p.o.) one hour prior to challenge with rotenone (75 mg/kg p.o.) on MDA and SOD levels in rat brain. (n=8-10 each).

Sl. No	Extract	% Change in brain tissue	
		MDA	SOD
1.	**Rotenone**	**138.30↑**	**189.62↑**
2.	**V-1**	**16.36↓**	**13.89↓**
3.	S-1	37.13↓	29.74↓
4.	**S-13**	**22.12↓**	**15.54↓**
5.	S-146	50.49↓	54.01↓
6.	**AR-12**	**22.54↓**	**21.38↓**
7.	AR-14	36.14↓	40.18↓
8.	**S-1635**	**29.22↓**	**8.64↓**
9.	BR-2	**41.36↓**	**34.82↓**
10.	**TR-10**	**13.30↓**	**20.78↓**

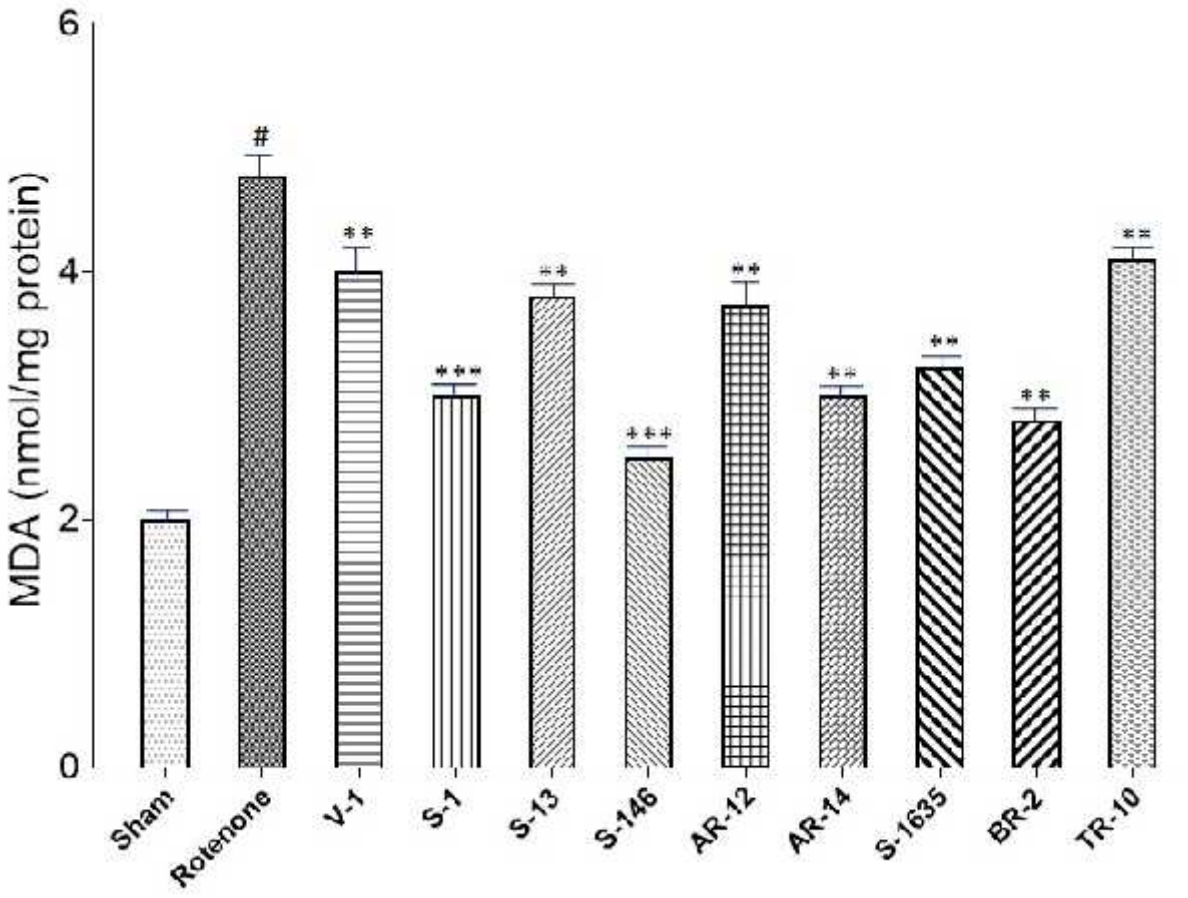


Fig. 10 Effect of MLEs (100 mg/kg p. o.) administration one hour prior to rotenone challenge on MDA levels in brain homogenate of rats.

Significance # P<0.001 vs sham, *** P<0.001, ** P<0.01 vs rotenone.

2.9.1.2 Effect on SOD levels in brain tissue

In signaling processes, cell division and lipid peroxidation, superoxide serves an important role, Lipid peroxidation, protein oxidation, and DNA damage can be initiated when the free radicals are overproduced. Owing to which cellular dysfunction and death by apoptosis or necrosis can occur. A reduction in superoxide level offers a defense against cellular damage. As the SOD levels which got elevated due to the increased oxidative stress induced by rotenone

were significantly reduced by pre treatment with various MLEs. There was significant reduction (54.01 %) in the brain of rats treated with S-146, further AR-14 treated rats showed a reduction by 40.18%. There was also significant 34.82 % and 29.74 % reduction in SOD content by BR-2 and S-1 respectively. The other mulberry varieties V-1, S-13, AR-12, S-1635, TR-10 however, had also significant effect but it was far less compared to S-146 and AR-14 (Fig. 11).

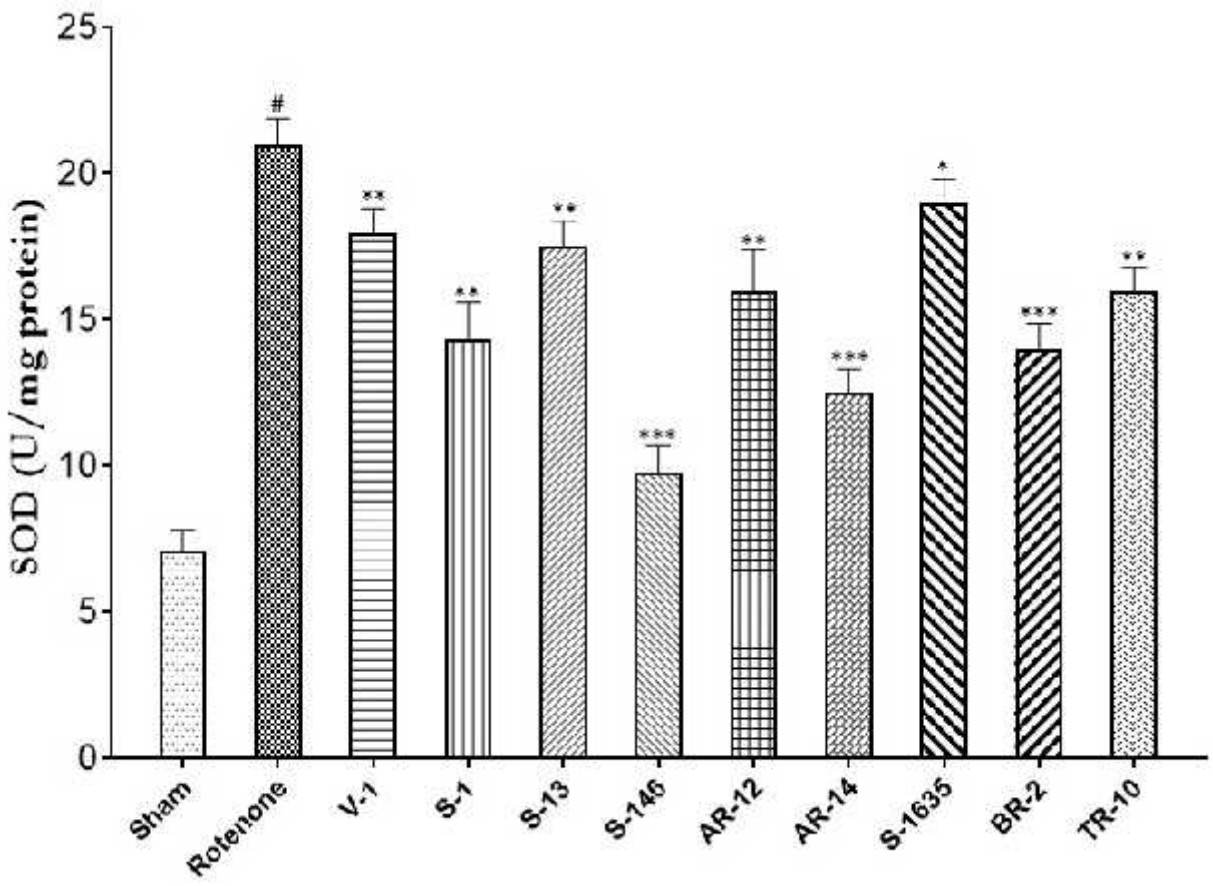


Fig. 11 Effect of MLEs (100 mg/kg p.o.) administration one hour prior to rotenone challenge on SOD levels in brain homogenate of rats. (n=8-10 each).

# P<0.001 vs sham, *** P<0.001, **P<0.01, *P<0.05 vs rotenone.

The MLE-AR-14 and MLE-S-146 were found to be potent anti oxidant in nature on rotenone induced oxidative stress model so these two extracts were taken up further to study their neuroprotective effect on ischemia/reperfusion injury induced brain damage.

2.9.2 Effect of treatment of MLE on I/R injury induced brain damage

The cerebral ischemia induced by MCAO following reperfusion of 24 h in rats was examined in the different group of animals treated with the extracts, MLE-AR-14 and MLE-S-146 to differentiate the relative neuroprotective profile. It was observed that I/R injury led varying degree of brain damage and severity of which was assessed by several parameters that includes neurological deficit, oxidative stress markers of lipid peroxidation: malondialdehyde, intracellular antioxidant (GSH) status and in addition monitoring size of cerebral infarct. Further, at microscopic level the cellular and nuclear changes were analyzed with H&E and CV staining.

A) Neuroprotective effect of MLE-AR-14

The neuroprotective effect of MLE-AR-14 was bio-evaluated in focal cerebral ischemia model. The results obtained after treatment with MLE-AR-14 on MCAO induced I/R injury are detailed below.

1 Effect of treatment on neurological deficit

A major pre-requisite for the present study was the successful occlusion of middle cerebral artery by the nylon suture. This was verified by observing ND, which is a direct consequence of ischemic (I/R) injury. The MCAO group of rats after 24 hr of reperfusion showed an average score of 7 on a 10 point scale of ND, indicating severe impairment in behavioral and motor functions due to brain damage by I/R.

One hour pre treatment with MLE-AR-14 at 50 mg oral improved ND significantly by 37% whereas, the 100 mg dose resulted further improvement by about 50% in neurological deficit scores in ischemic rats post 24 hrs of reperfusion. The standard marker, resveratrol also showed significant reduction in ND by 63% and 77% respectively with 50 mg and 100 mg doses (Fig 12).

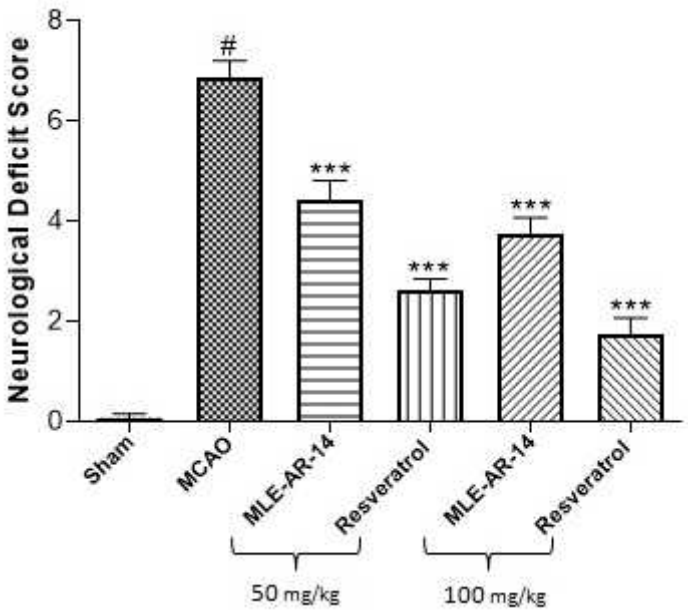


Fig. 12 The effect of 1 hr pre treatment with MLE-AR-14 and resveratrol at a dose of 50 mg/kg and 100 mg/kg p.o., on neurological deficit scores in rats. (n=8-10 animals each).

Significance #p<0.001 vs sham and ***p<0.001 vs MCAO.

The post ischemic treatment with MLE-AR-14 at a dose of 50 mg and 100 mg resulted in 40% and 57% improvement in neurological deficit scores in ischemic rats as compared to 65% and 71% observed with resveratrol with same doses. Thus the MLE-AR-14 seems to be a potent neuroprotective in preventing the brain damage caused by I/R injury. The relative marked neuroprotective effect of resveratrol may be due to its rather high doses used whereas, 50 mg /kg dose was more than adequate as it is a purified compound (Fig. 13).

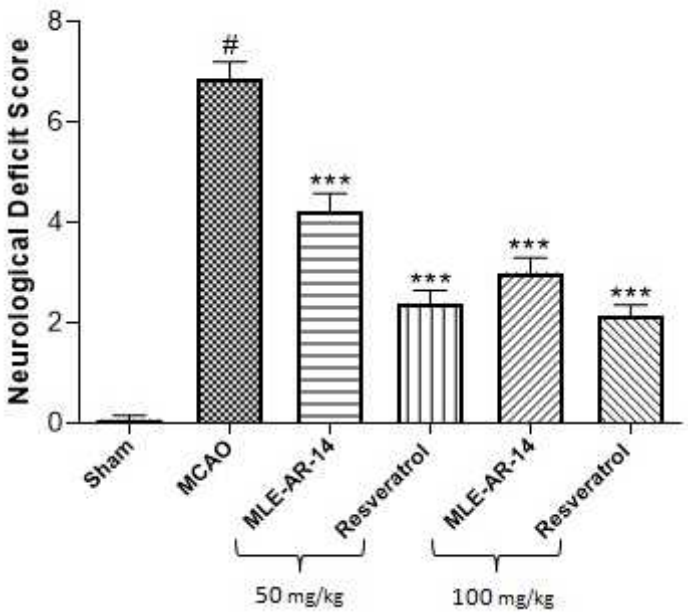


Fig. 13 The effect of 6 hr post treatment with MLE-AR-14 and resveratrol at doses of 50 mg/kg and 100 mg/kg p.o., on neurological deficit scores in rats. (n=8-10 rats each).

Significance #p<0.001 vs sham and ***p<0.001 vs MCAO.

2 Effect of MLE-AR-14 treatment on cerebral infarction

The extent of cerebral damage was assessed by the TTC, HE and CV staining.

2.1 Triphenyl tetrazolium chloride staining

TTC staining is commonly used to assess the brain damage as a result of ischemic insult. The rats showing significant ND were sacrificed and brain sections stained with TTC were used for assessing the cerebral infarct area.

The 2/24 hr I/R injury resulted in almost 65% brain infarct, as white coloured unstained mass and viable brain tissue was stained as deep red in TTC stained sections of rat brains (Fig. 14). The pre treatment with 50 mg dose of MLE-AR-14 reduced the cerebral infarct by 34%.

The increased dose of 100 mg of extract further enhanced the neuroprotection by 65% whereas, pre treatment with 50 mg and 100 mg of resveratrol resulted in reduction of brain infarction by about 55% and 76% respectively (Fig. 15).

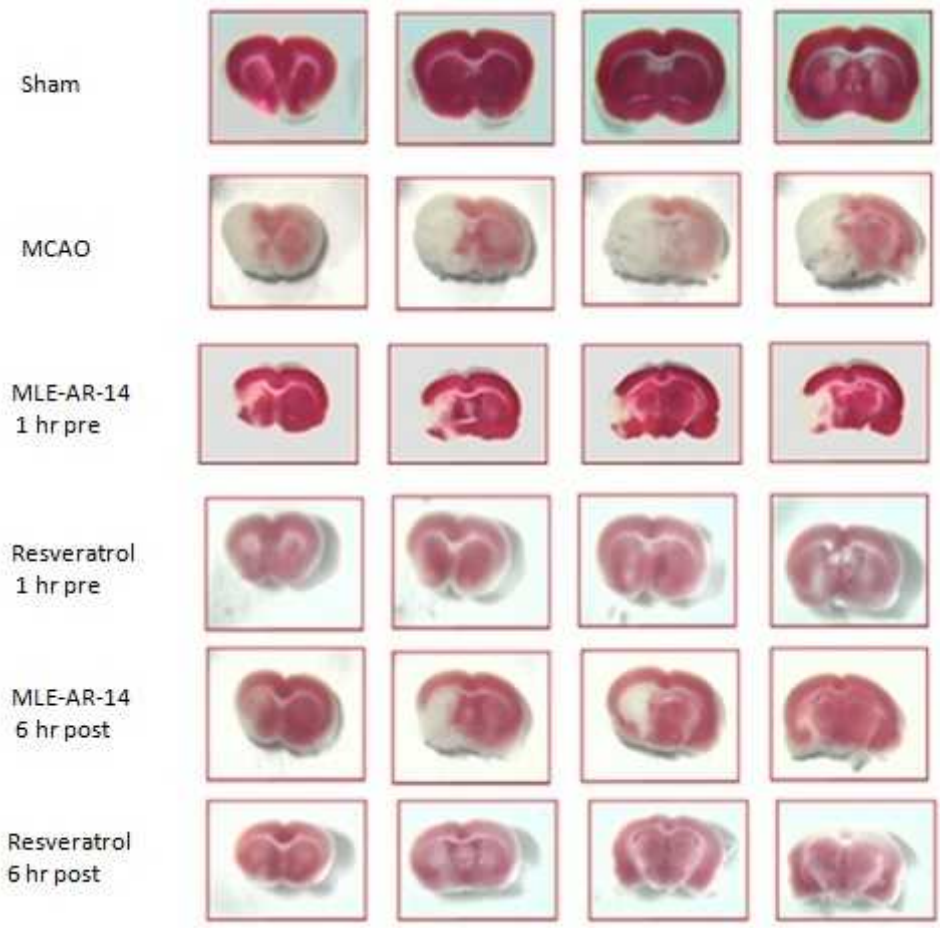


Fig. 14 TTC stained rat brain sections showing area of infarction in MCAO, Pre- and post treatment with MLE-AR-14 and resveratrol at a dose of 100 mg/kg p.o. Both MLE-AR-14 extract and resveratrol significantly reduced brain infarction on pre and post 6 hr treatment.

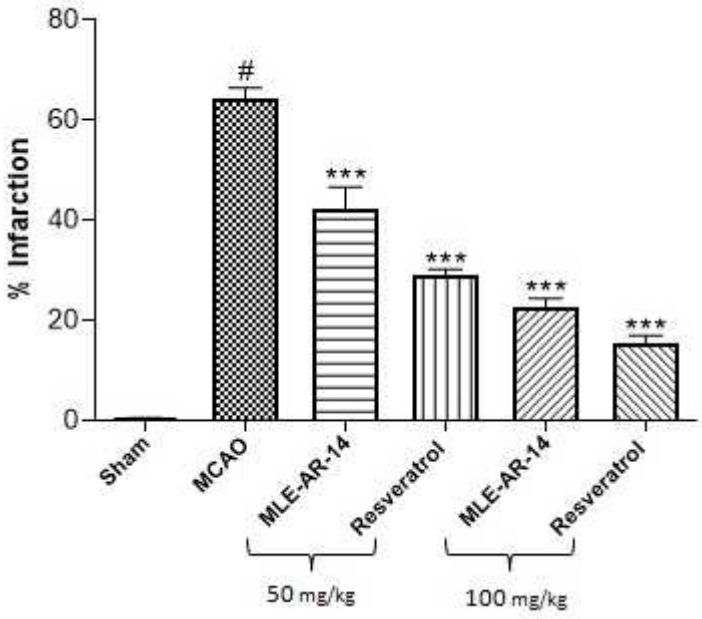


Fig. 15 The **e**ffect of 1 hr pre treatment with MLE-AR-14 and resveratrol at doses of 50 mg/kg and 100 mg/kg p.o., on brain infarction in rats. (n=8-10 animals each)

Significance #p<0.001 compared vs sham and ***p<0.001 vs MCAO

Interestingly, the extract was significantly neuroprotective even post I/R injury. The treatment with 50 mg of extract after 6 hour of I/R prevented brain damage by 28%, whereas 100 mg dose offered about 54% neuroprotection. The standard compound, resveratrol offered a reduction of 53% and 68% in cerebral infarction with doses of 50 mg and 100 mg respectively. Thus, it appears that AR-14 extract is significantly more effective after post ischemic injury (Fig. 16).

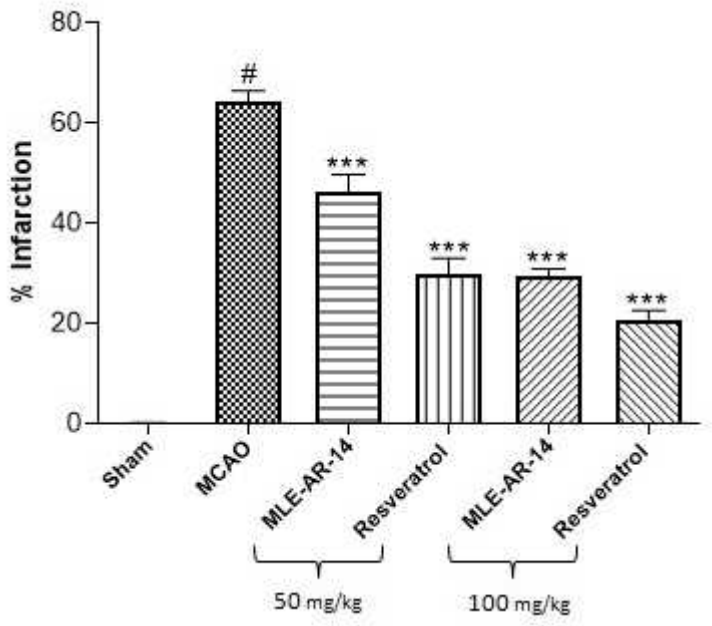


Fig. 16 The **e**ffect of treatment 6 hr post I/R with MLE-AR-14 and resveratrol at doses of 50 mg/kg and 100 mg/kg p.o., on brain infarction in rats. (n=8-10 animals each)

Significance #p<0.001 vs sham and ***p<0.001 vs MCAO

2.2 Hematoxylin and eosin staining

The histological changes observed under light microscopy after inducing 2 hour of ischemia followed by 24 hour of reperfusion showed enormous damaged cells exhibiting features of both necrosis and apoptosis. Pyknosis of the nucleus, dispersed chromatin clumps contained karyolysis, shrunken neurons, increased eosinophilic (red neurons) cytoplasm and presence of ghost neurons (nuclei lacking cellular structures) were the characteristic feature of the HE stained necrotic injury (Fig. 17).

Identification of apoptotic cells using characteristics enunciated by Kerr *et al.*, (1972) was done and the characteristic features shown were: cell surface protruberances which were detached from plasmalemma sealing, production of spherical or ovoid shaped membrane bound

apoptotic bodies (dark purple-blue stained in sections), a mark of the severity of apoptotic injury. Cells containing more than two apoptotic bodies were taken positive for apoptotic cell death. There was also evidence of pan-necrosis in the striatal and cortical regions of MCAO rats as shown by number of cavitations.

However, this cellular damage was significantly reduced in the brain sections of rats treated with MLE-AR-14 showing only a few apoptotic cells in both pre treated and post treated groups. And, relatively, reduced number of cavitations were observed in the extract and resveratrol pre treated groups. The post ischemia treated rat brains either with MLE-AR-14 or resveratrol had a very few cells with apoptotic and necrotic features in both striatal and cortical brain regions. There was no evidence of such histological abnormalities in the brain sections of sham operated rats.

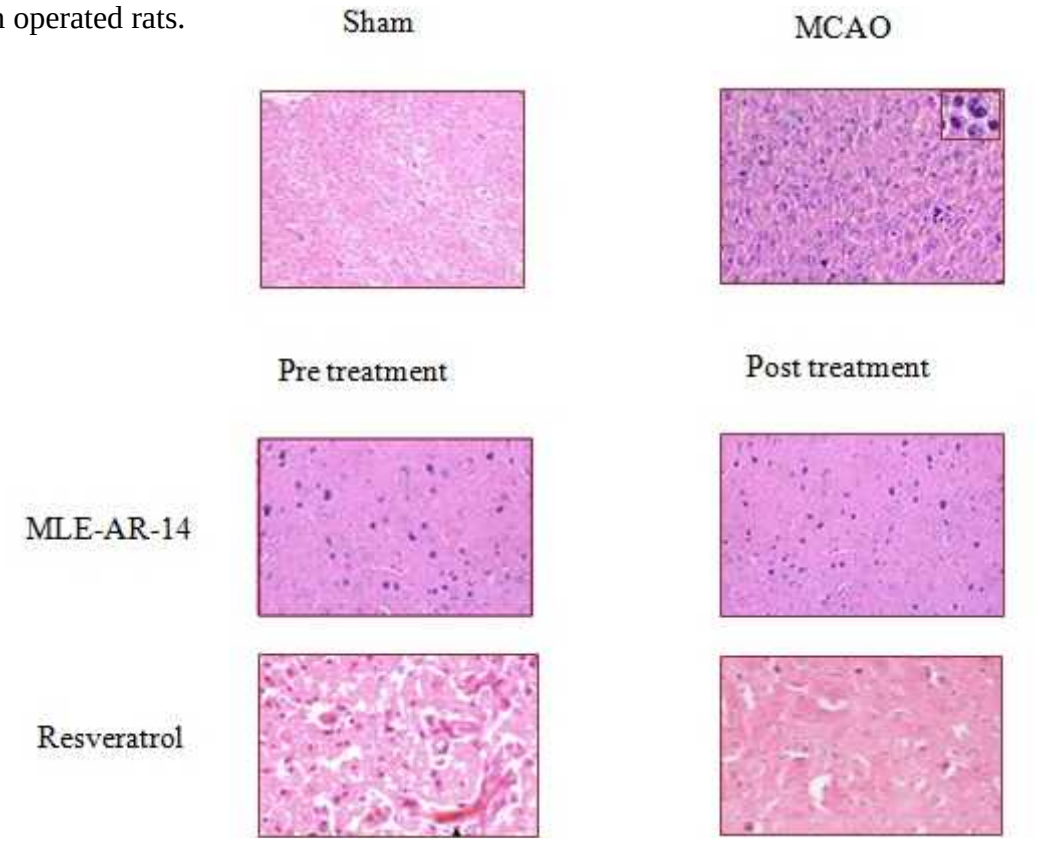


Fig. 17 Photomicrographs of HE stained 6 µm brain sections showing ischemic brain damage in MCAO, pre treated (1 hr) and post treated (6 hr post I/R) with MLE-AR-14 and resveratrol in rats at a dose of 100 mg/kg p.o.as compared to the sham operated group.

2.3 Cresyl violet staining

Cresyl violet staining also showed a significantly reduced cellular damage in both ischemic cortical and striatal sections of ischemic rats, treated with MLE-AR-14. The treatment with resveratrol was also equally effective in controlling the brain damage. The neuronal cells in the non-ischemic contralateral hemisphere were morphologically intact. Thus, these results very well corroborate with that of HE results (Fig. 18).

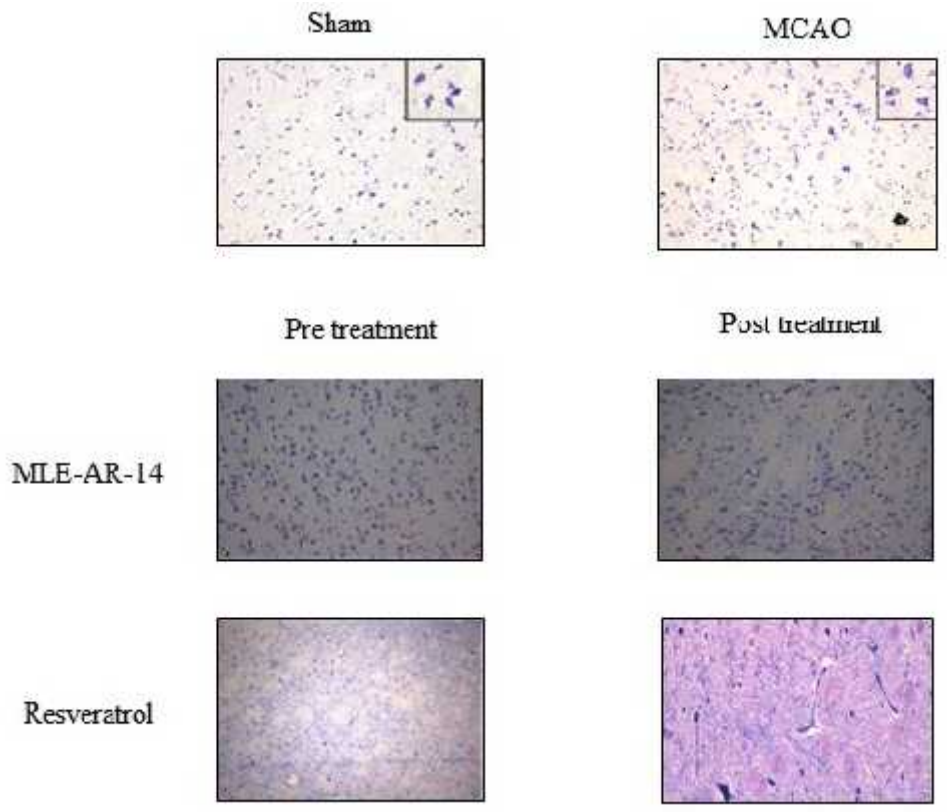


Fig. 18 The cresyl violet stained representative photomicrographs showing cellular brain damage in rats pre treated (1 hr) and post treated (6 hr post I/R) with MLE-AR-14 and resveratrol at a dose of 100 mg/kg p.o. as compared to the sham operated group.

3 Effect of MLE treatment on biochemical parameters

3.1 Effect on MDA levels in the blood

The lipid peroxidation status was measured by estimating its marker, MDA content in the serum of experimental animals. The blood MDA level was found elevated significantly by about 68% following 2/24 I/R injury. Interestingly, the MDA levels were significantly altered by both pre treatment as well as post treatment with MLE-AR-14 extract. The downward trend in MDA reduction was 30% with 100 mg dose of MLE-AR-14. The resveratrol with same dose had reducing effect by 50% on blood MDA level.

Interestingly, the MLE-AR-14 was significantly active in combating oxidative stress post reperfusion as compared to pre treatment. The extract on post treatment owing to its potent antioxidant activity was able to reduce the MDA levels in ischemic rats by about 42%. The standard drug, resveratrol was also equally effective in reducing MDA content in the blood of ischemic rats (Fig. 19).

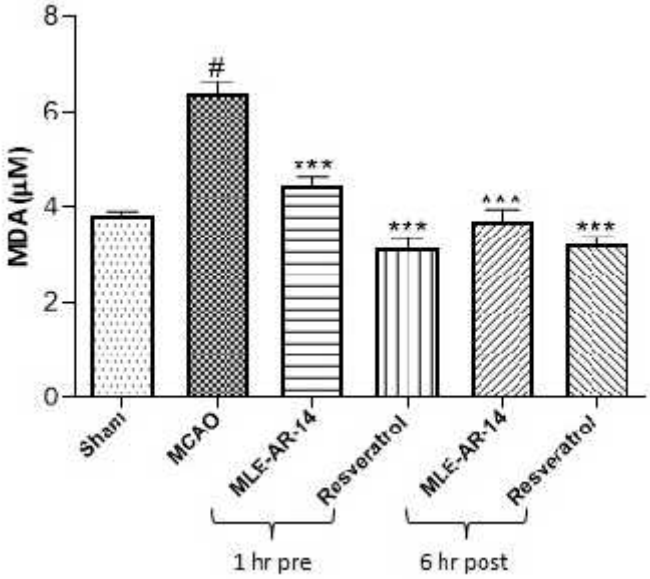


Fig. 19 The effect of 1 hr pre treatment and 6 hr post I/R treatment with MLE-AR-14 and resveratrol at a dose of 100 mg/kg p.o., on blood MDA levels in ischemic rats. (n=8-10 animals each)
Significance #p<0.001 vs sham and ***p<0.001 vs MCAO

3.2 Effect of GSH levels in the blood

To scavenge the overproduced reactive oxygen species, Glutathione being a central component in the antioxidant defense mechanism, plays an important role. It also serves as a substrate for various peroxidases. The I/R induced stress depleted the glutathione level in the blood of animals and showed an overall decrease of about 60% as compared to the sham operated rats.

MLE-AR-14 pre treatment was very effective in reverting the depletion of GSH stores following I/R injury. The 100 mg/kg p.o.dose of MLE-AR-14 tended to improve GSH level by 54 % as compared to an increase of 77% by resveratrol pre treatment with same dose. The post treatment with MLE-AR-14 also significantly prevented depletion of glutathione level by 34% (Fig. 20).

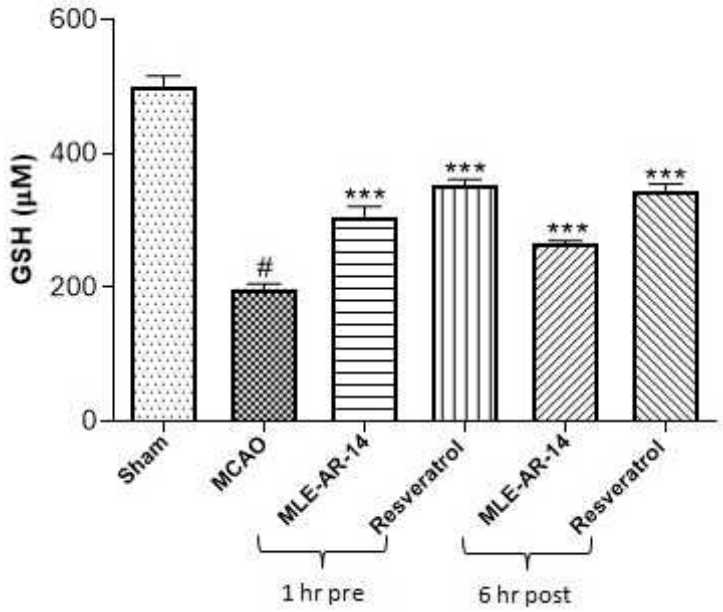


Fig. 20 The effect of 1 hr pre treatment and 6 hr post I/R treatment with MLE-AR-14 and resveratrol at a dose of 100 mg/kg p.o., on blood GSH level in ischemic rats. (n=8-10 animals each)

Significance #p<0.001 vs sham and ***p<0.001 vs MCAO

B) Neuroprotective effect of MLE-S-146

The results obtained after treatment with MLE-S-146 on MCAO induced I/R injury are detailed below. In this study, the effect of MLE-S-146 was investigated at two doses i.e. 50 and 100 mg/kg po on I/R injury induced cerebral damage. And also both pre treatment and post treatment studies were taken up.

1 Effect of treatment on neurological deficit

At the end of I/R injury, impaired sensori-motor ability was verified in all the experimental rats by differential grade of forelimb flexion, contrlateral circling, hemi-paresis and non-spontaneous movement of the animal. For ischemic rats, this deficit was severe and showed contralateral forelimb impairment, loss of postural reflex, impaired ipsilateral balancing and grip strength. Therefore, ischemic rats showed a more pronounced behavioural deficit.

The 10 point test scale recording of the sensory motor deficit of animals in different groups provides a broad suggestion on the differences in the neurological deficit . The I/R injury produced ND in control rats with an average score of 7 with 2/24 h of I/R. In the group administered with MLE-S-146 one hour prior to the ischemic insult, the ND score was conspicuously significantly reduced as compared to ischemic rats. The average ND score was about 3.8 and 2.4 in groups received with MLE-S-146 at a dose of 50 mg/kg and 100 mg/kg at 2/24 h post I/R injury respectively (Fig. 21). Further, resveratrol group showed neurological deficit score of 2.6 and 1.5 at 50 mg and 100 mg respectively. Therefore, neurological deficit was significantly attenuated in MLE-S-146 treated rats because of reduced brain damage.

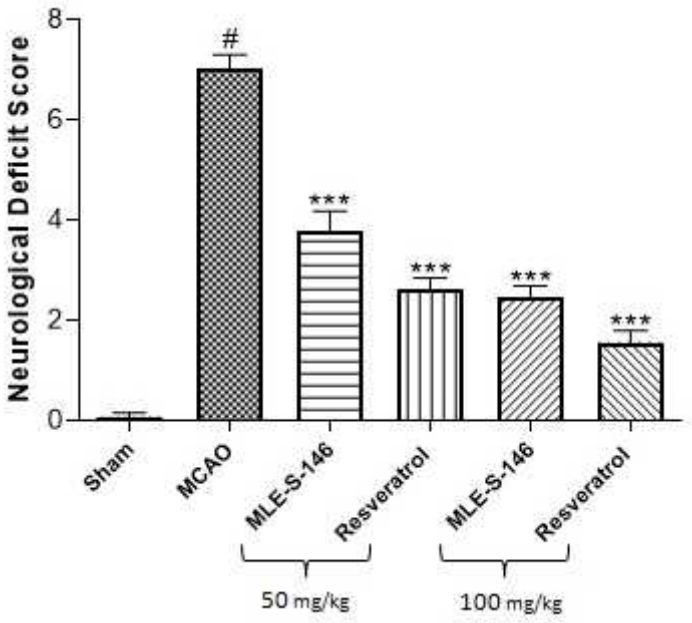


Fig. 21 The **e**ffect of 1 hr pre treatment with MLE-S-146 and resveratrol at a dose of 50 mg/kg and 100 mg/kg p.o., on neurological deficit scores in ischemic rats. (n=8-10 animals each).

Significance #p<0.001 vs sham and ***p<0.001 vs MCAO

Additionally, when the ischemic animals were administered with a dose of 50 mg and 100 mg extract, six hour post reperfusion injury, it was observed that the neurobehavioral alterations were commendable (Fig. 22). The ND which was found to be significantly increased with an average score of 7 in ischemic subjects at 2/24 hour of I/R injury. But the treatment with MLE-S-146 at 50 mg and 100 mg doses, the ND score was lowered to 3.2 and 2.0 respectively. Indeed, the groups treated with resveratrol at the same doses provided a similar decrease in neurological deficit score by 2.4 and 2.0 as well. In general, the ND was significantly (P<0.001) reduced in MLE-S-146 extract treated groups as compared to ischemic rats at 2/24 hour of I/R injury.

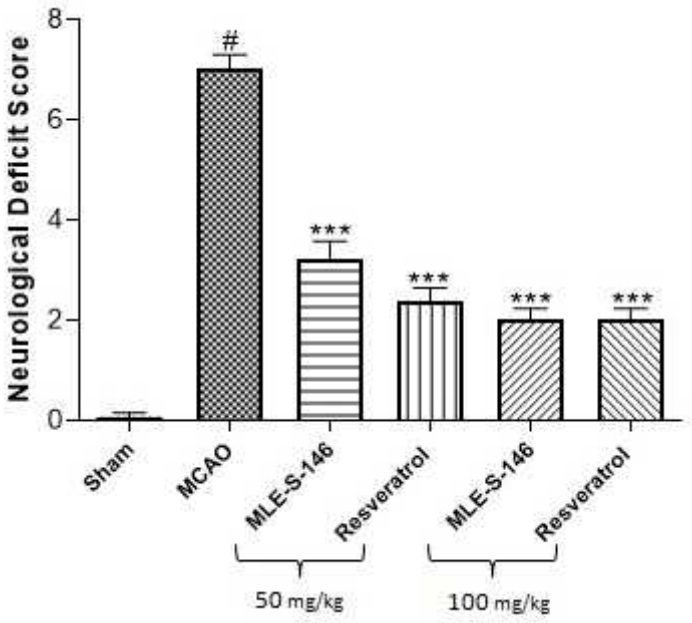


Fig. 22 The **e**ffect of 6 hr post I/R treatment with MLE-S-146 and resveratrol at a dose of 50 mg/kg and 100 mg/kg p.o., on neurological deficit scores in ischemic rats. (n=8-10 animals each).

Significance #p<0.001 vs sham and ***p<0.001 vs MCAO

2 Effect of treatment on cerebral infarction

Interestingly, the infarction correlated well with the ND score in each treatment and depicted the relative severity of ischemic stress. Extent of cerebral infarction was assessed by the TTC, HE and CV staining.

2.1 Triphenyltetrazolium chloride staining

The TTC staining in the brain slices gave a clear cut difference between the deep red coloured viable area and unstained white area depicting the damage (Fig. 23). The cerebral damage was increased in ischemic groups as compared to sham control, which showed normal viable tissue structure. The cerebral damage conspicuously showed characteristics of reduced

infarction in the group of animals administered with MLE-S-146. The pre treatment with the extract at doses of 50 mg and 100 mg proved to be effective in reducing the cerebral infarction by 40.05% and 59.32% respectively (Fig. 24). Likewise, a remarkable reduction in infarction by 55.05% and 76.14% was clearly visible in resveratrol treated rat brain with the same doses.

Similar trend was also observed in the 6 hour post treatment group as the brain tissue showed lesser infarct size. Even though, the infarct was conspicuous in brain of ischemic animals, it was pretty much reduced in the treatment groups. The reduction in infarct size in 50 mg and 100 mg of extract administered animals was about 48.14% and 64.07% respectively.Moreover, the infarction was undeniably reduced in the brain of resveratrol (50 mg 100 mg doses) treated animals by 53.48% and 67.87% respectively as compared to ischemic animals (Fig. 25).

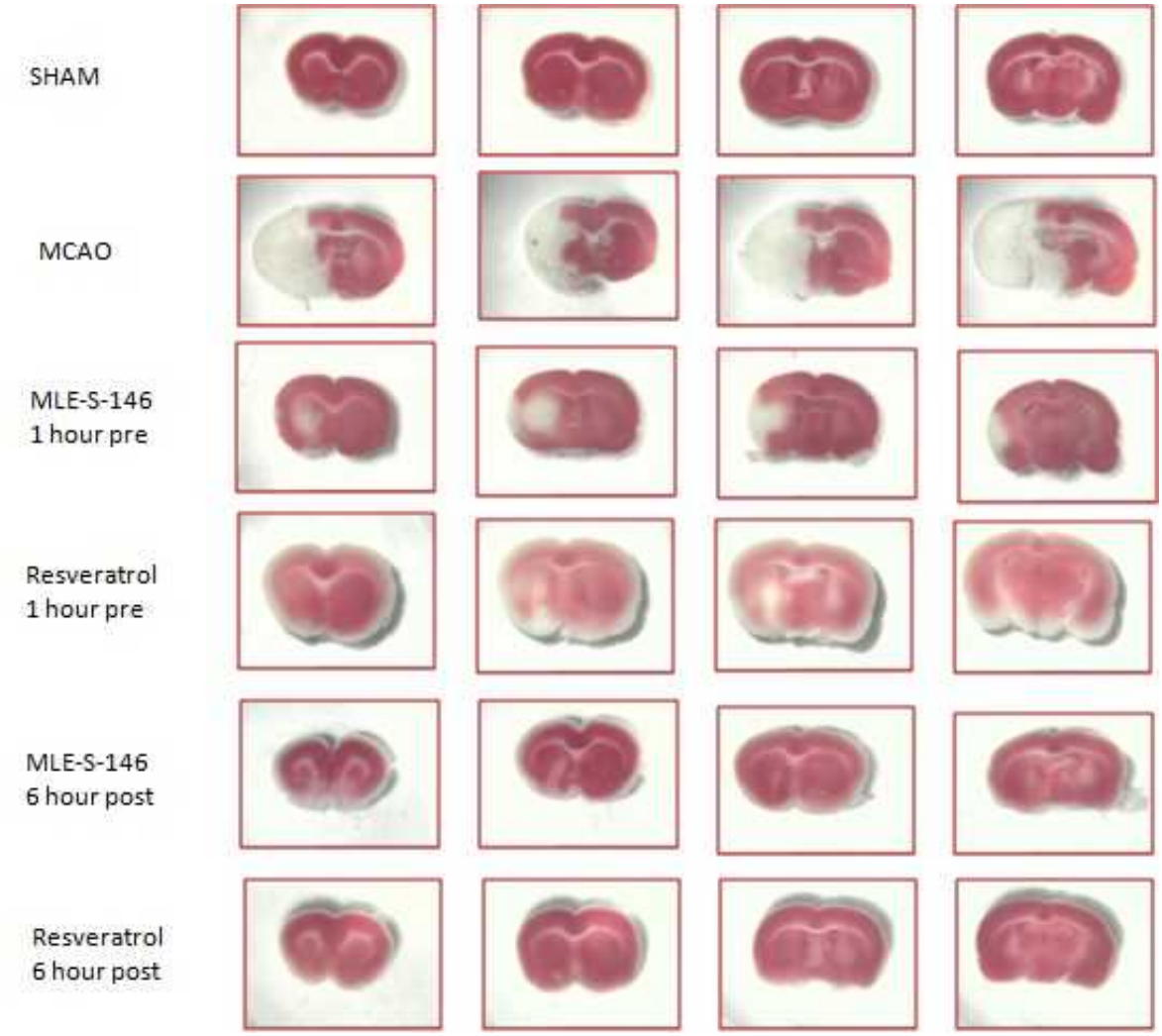


Fig. 23 TTC stained brain sections showing area of infarction in MCAO, Pre- and post treatment with MLE-S-146 and resveratrol treated rats at a dose of 100 mg/kg p.o. Both extract and resveratrol significantly reduced brain infarction at 24 hours post reperfusion.

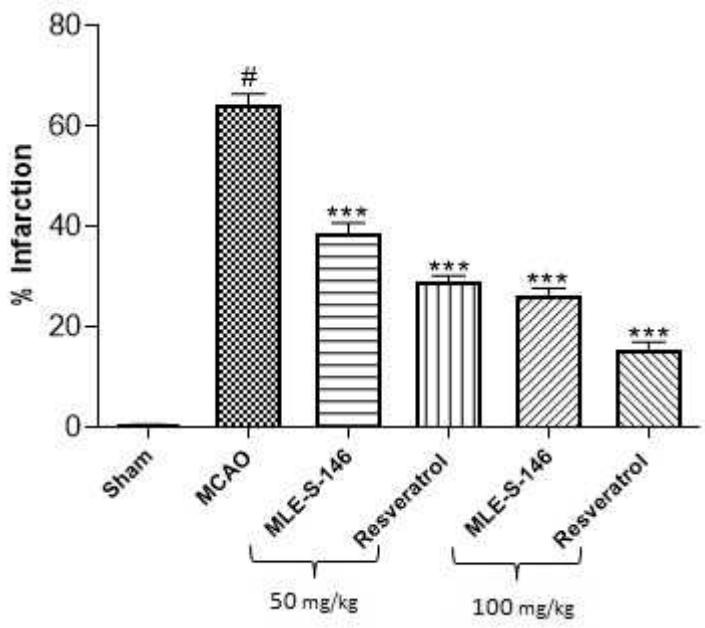


Fig. 24 The **e**ffect of 1 hr pre treatment with MLE-S-146 and resveratrol at a dose of 50 mg/kg and 100 mg/kg p.o., on brain infarction in rats. (n=8-10 animals each).

Significance #p<0.001 vs sham and ***p<0.001 vs MCAO

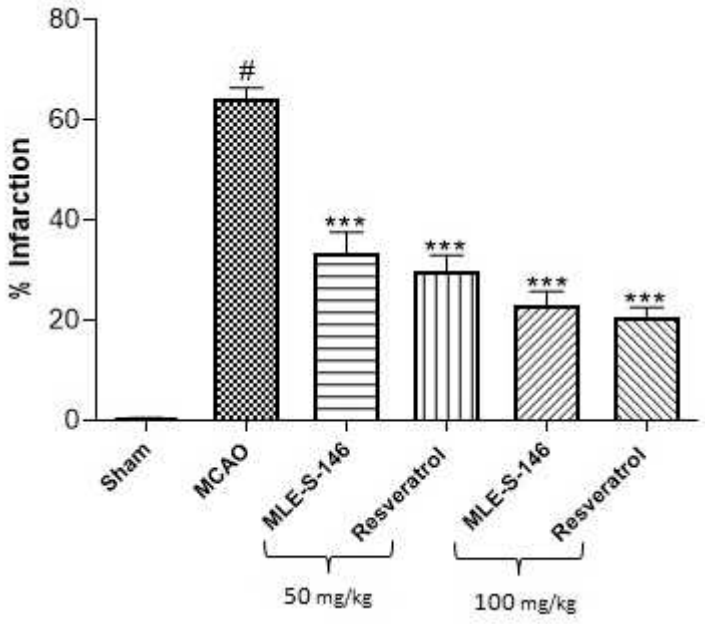


Fig. 25 The **e**ffect of 6 hr post I/R treatment with MLE-S-146 and resveratrol at a dose of 50 mg/kg and 100 mg/kg p.o., on brain infarction in ischemic rats. (n=8-10 animals each).

Significance #p<0.001 vs sham and ***p<0.001 vs MCAO

2.2 Hematoxylin and eosin staining

The cerebral I/R injury was revealed at cellular level with H&E staining. The I/R injury induced changes were more pronounced in the brain of MCAO induced animals in comparison to sham operated ones. It was observed that striatal region was greatly affected following I/R injury followed by cortex but hippocampus was not affected by ischemic insult and showed normal cellular architecture.

The cellular damage appeared immature in sham operated subjects, whereas, the cell damage showed matured changes in MCAO animals at 2/24 hr with nearly complete damage in striatal area. A very similar pattern was also observed in the cortex. Further, hippocampal region showed no cellular alterations. The ischemic injured cells exhibited features of both necrosis and apoptosis. Necrotic cells were characterized by pyknosis of nucleus, karyolysis, eosinophilia of cytoplasmic (red neurons) and ghost neurons (nuclei lacking cellular structures). Whereas, apoptotic cells were characterized by more than two vesicle shaped protuberances, called the apoptotic bodies, on the cell surface roughly spherical or ovoid in shape.

However, it was observed that the pre-treatment with mulberry leaf extract of S-146 at a dose of 100 mg/kg p.o., showed comparatively far less cellular alterations as compared to other treatments. The post treated rat brains also had very few cells showing apoptotic and necrotic features in striatal and cortical regions. There was no evidence of such histological abnormalities in the brain sections of sham operated rats (Fig. 26). The cellular damage indicated by shrunken dark cell morphology which was greater in MCAO animals but was far less in the pre treated and post treated groups with MLE. Further, reduced number of cavitations was observed in the MLE-

S-146 and also in the resveratrol treated groups, indicative of less loss of cells, which were otherwise far higher in MCAO animals in cortical and striatal area.

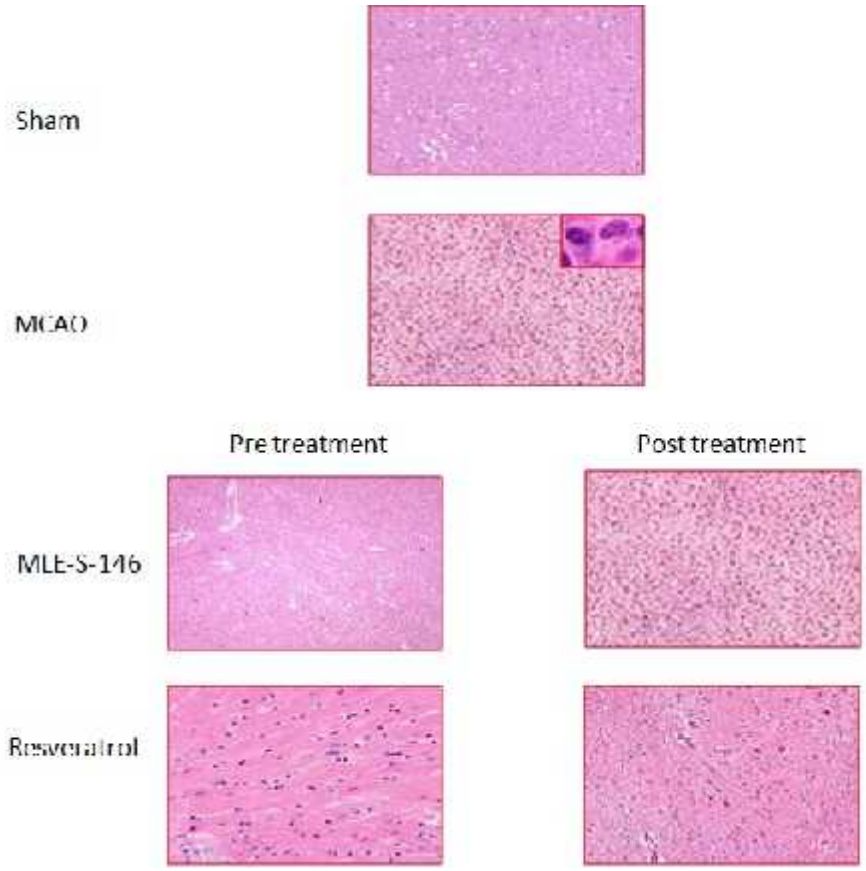


Fig. 26 Photomicrographs of HE stained 6 µm brain sections showing ischemic brain damage in MCAO, pre treated (1 hr) and post treated (6 hr post I/R) with MLE-S-146 and resveratrol in rats at a dose of 100 mg/kg poas compared to the sham operated group.

2.3 Cresyl violet staining

The morphological analysis of brain sections following CV staining also revealed degenerative cells ie. pyknosis of nuclei, disruption of eosinophilic cytoplasm in the affected

brain regions of ischemic animals as compared to the sham operated ones. Cellular changes such as edema and nuclear shrinking were also visible. The ipsilateral hemisphere was highlighted with the ischemic lesions which made easy the infarcted area to be delineated. The neuronal cells in the non-ischemic contralateral hemisphere were morphologically intact. Cells showing various stages in cytolysis could also be observed as reminants (Fig. 27).

However, significantly reduced cellular damage was observed in cortical and striatal sections of ischemic rats treated with MLE-S-146. The treatment with resveratrol was also equally effective in controlling the brain damage. Thus, these results very well suggest that the treatment with the MLE has neuroprotective effect on brain damage caused by cerebral ischemia reperfusion injury.

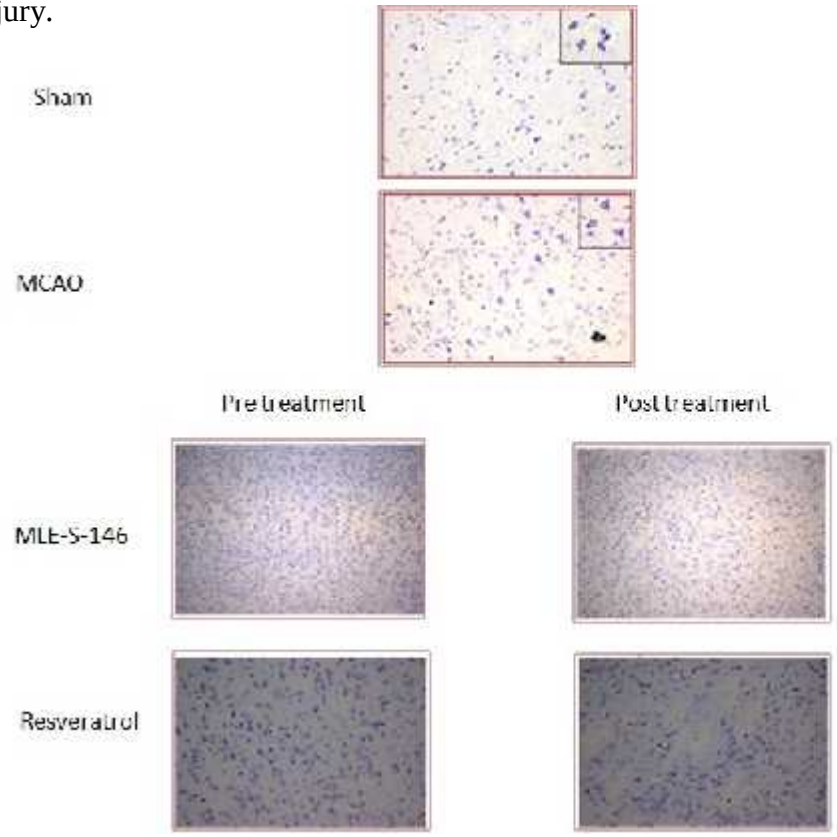


Fig. 27 The cresyl violet stained representative photomicrographs showing cellular brain damage in rats pre treated (1 hr) and post treated (6 hr post I/R) with MLE-S-146 and resveratrol at a dose of 100 mg/kg p.o. as compared to the sham operated group.

3 Effect of treatment on biochemical parameters

Lipid peroxidation's end product, MDA and GSH, a non enzymatic anti oxidant were measured at 2/24 hr I/R injury in animals subjected to treatment with MLE-S-146.

3.1 Effect on MDA levels in the blood

The MDA level estimated in the blood serum of MLE-S-146 extract, resveratrol treated and sham operated rats following ischemia/reperfusion are shown in Fig. 28. MDA level was found to be significantly increased in MCAO depicting the impact of oxidative stress on lipid peroxidation and subsequent ischemic brain damage. The increase was significantly high by over 60% at 2/24 hr I/R in MCAO animals as compared to sham operated animals.

However, the MDA level significantly decreased from the maximal level to 46.87% in the ischemic group treated with 100 mg dose of MLE-S-146 one hour prior to ischemic insult. The resveratrol with the same dose had reducing effect by 50.77% on blood MDA level.

The post treatment with MLE-S-146 at the same dose proved to be also significantly effective in ameliorating the blood MDA level in ischemic animals by 36.07%. On the other hand, owing to its potent antioxidant activity, resveratrol was able to bring down the elevated MDA levels by 50%. The oxidative stress implicated by MCAO, induced lipid peroxidation and tended to cause brain damage as is evident by the elevated MDA levels in blood. However the anti oxidant effect of the herb helped the MDA levels to significantly plunge down and thus reduced oxidative stress induced brain damage.

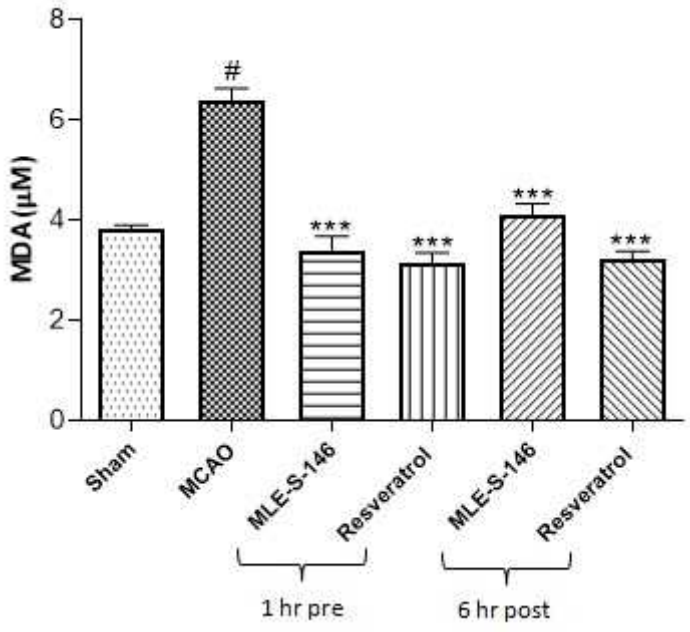


Fig. 28 The effect of 1 hr pre treatment and 6 hr post I/R treatment with MLE-S-146 and resveratrol at a dose of 100 mg/kg p.o., on MDA levels in ischemic rats. (n=8-10 animals each).

Significance #p<0.001 vs sham and ***p<0.001 vs MCAO

3.2 Effect on GSH levels in blood

Cerebral I/R injury exhibited a significant reduction in GSH levels indicative of the body's anti oxidant defence mechanism to scavenge the ROS and to serve as a substrate for various peroxidases. The I/R induced stress depleted the glutathione level in the blood of stroke animals and showed a decrease by 60.52% as compared to the sham operated rats.

The pre administration of MLE-S-146 in ischemic rats was found to have increased the glutathione level in blood by over 40%, whereas, post treatment facilitated in increasing the GSH level by 55% (Fig. 29). Similarly, the pre and post treatment by resveratrol reverted the depletion of GSH stores by 77% and 74.38% respectively.

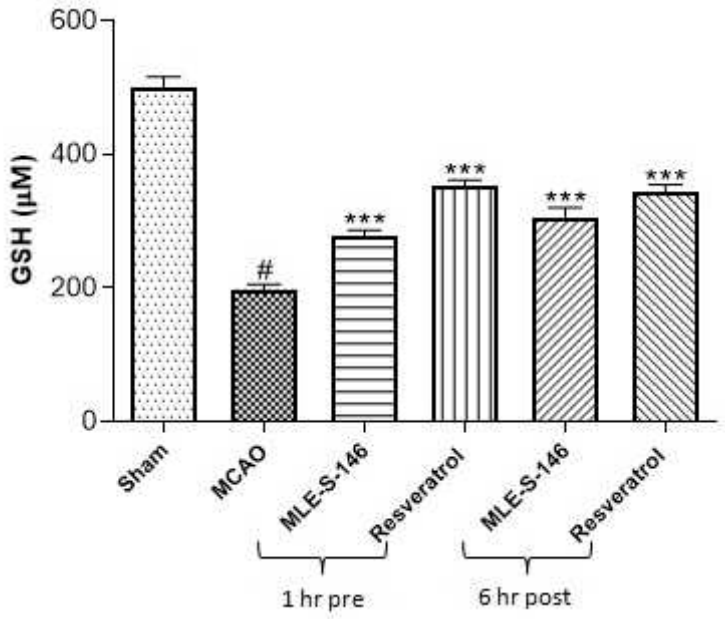


Fig. 29 The effect of 1 hr pre treatment and 6 hr post I/R treatment with MLE-S-146 and resveratrol at a dose of 100 mg/kg p.o., on GSH levels in ischemic rats. (n=8-10 animals each).

Significance #p<0.001 vs sham and ***p<0.001 vs MCAO

The broad range of antioxidant activity of the MLEs indicates the potential of the mulberry plant as a rich source of pharmaceuticals with potential to reduce oxidative stress. Therefore, these two varieties AR-14, S-146 of mulberry leaf extract seem to possess potent antioxidant activity as demonstrated in *in vivo* rotenone-induced oxidative model in rat. Also the aqueous extracts of mulberry leaf offered both preventive as well as curative anti stroke potential. The neuroprotective effect was showcased by amelioration in oxidative damage, betterment in behavioral dysfunction, reduction in cerebral infarct and reduction in morphological cell damage.

2.10 Discussion

Cerebral ischemia is third leading cause of death and the leading cause of disability all over the world. In USA, about 7,95,000 people suffer from stroke each year (Mozzafarian *et al*., 2016). Stroke is the cause of 1 in 20 deaths, making it the 3rd leading cause of death in the whole world. Stroke is a serious health concern in India as well; however, the rate of occurrence is not as clear. The estimated adjusted prevalence rate of stroke is higher in urban areas (334-424/100,000) as compared to rural areas (84-262/100,000) in India. The fatality rates owing to stroke are variable across India with the maximum fatality rate (42%) reported for Kolkata (Stroke Epidemiology and Stroke care services in India, 2013). These estimations highlight the seriousness of stroke as a disease that is gaining epidemic proportions all over the world and as well as India.

The brain is an important part of the body and is essential for regulating the central and the peripheral functions like learning and memory, motion, sight, smell, sensation etc. To maintain these functions it requires constant supply of energy (ATP). The energy required is generated by the oxidative phosphorylation of glucose and oxygen which is supplied by cerebral arteries via blood and occluding any of these arteries leads to deficiency of these fundamental metabolic substrates leading to the dysfunction of the brain cells. Thus the inability of the brain to store fundamental metabolic substrates oxygen and glucose makes it sensitive to ischemic conditions. Brain is extremely sensitive to disturbance in CBF and if this persists for long time, neuronal cells are at the thresholds of damage. To accomplish such interruptions, brain receives effective perfusion through finely distributed blood vessels and their collaterals, which manages the high metabolic demands. However, during cerebral stroke, interruption of blood supply

activates several multifaceted and overlapping mechanisms, which modulate the survival and death of brain cells (Mehta *et al*., 2007; Nakka *et al*., 2008).

Several invasive and non-invasive *in vivo* models of cerebral stroke have been reported, which simulate the clinical settings of stroke (Tamura *et al*, 1981; Buchan *et al*., 1992). The rat MCAO model has been in use for over last 40 years (Stern *et al*, 1975) and gained increasing acceptance in recent years owing to its relevance to the human clinical settings of stroke (Garcia *et al.* 1984). In 1989, Longa *et al* modified this novel and relatively noninvasive method of achieving reversible MCA occlusion by use of an intraluminal suture and demonstrated that this technique reliably produced regional infarcts. It offers the advantage of allowing reperfusion as seen in clinical situations and at the same time is non-invasive in contrast to other techniques, such as cauterization, mechanical clipping and photo-thrombotic occlusion.

The placement of the suture at the origin of the MCA obstructs blood supply to the total MCA supplying territory including the basal ganglia, which are supplied by the lenticulo-striate arteries. Unlike most cortical arteries, the MCA branches which are end-arteries do not form collaterals. As a result of which a complex sequence of events take place when the cerebral blood flow is transiently or permanently reduced. It then may result in major neurological impairment either acutely or chronically with associated sensorimotor and cognitive deficits.

Recombinant tissue plasminogen activator remains the only FDA approved treatment available to treat ischemic episodes; this treatment has to be administered within a narrow time window of 3 hours from the occurrence of ischemia (Hacke *et al.*, 2008). Initiating reperfusion of ischemic tissue restores the blood flow and therefore makes availability of glucose and oxygen. However, reperfusion of ischemic tissue is not without serious unwanted consequences. These

include a surge in the production of free radicals, which leads to the paradoxical 'reperfusion injury' (Jean *et al*., 1998).

The tissue injury after cerebral stroke is caused due to multiple overlapping mechanisms. A decrease of ATP in the affected brain region leads to failure of ion pumps, causing glutamate excitotoxicity and thus further depleting ATP. Energy failure precipitates necrosis in the immediate area and cessation in activity but continued viability in the surrounding 'penumbral' tissue that is thought to be salvageable. The penumbral tissue suffers from oxidative stress as well as acute and delayed inflammation that may spread and compromise more cerebral tissue. The multiple mechanisms involved in the pathophysiology of ischemia imply that potential drug candidates that target specific pathological pathways are unlikely to be effective as treatment alone. Also, generation of oxidative stress as a part of reperfusion injury means that neutralizing ROS by direct quenching and by elevating the endogenous anti-oxidant capacity is likely to be effective as a treatment measure.

The system of traditional medicine in India, the Ayurveda, has been practiced for thousands of years. It includes hundreds of herbal preparations recommended for a variety of ailments. Many of these have antioxidant and anti-inflammatory properties that make them potentially relevant to ischemic pathology. Although such preparations from natural products may hold promise, they have not been seriously considered as treatment options by the medical world at large. One of the reasons may be the absence of rigorous research and lack of knowledge regarding the mode of action, optimal dosage, and possible adverse effects (Gladstone *et al*., 2002). A recent report has surveyed hundreds of stroke patients from June 2010 to December 2010 in the Stroke Units of Christian Medical College, Ludhiana, and Sree

Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, India (Pandian *et al*., 2014). The survey found that 15 percent of the patients were using herbal medications.

Thus there is a need for exploring alternative medicines as these may offer a range of benefits from neuroprotection to palliative effects and also aid in long term recovery during rehabilitation of stroke patients. It was with this hope that the studies that constitute this doctoral thesis were designed and performed. The present investigation was therefore undertaken to investigate the neuroprotective effect of herbs mulberry and curcuma in cerebral ischemia and reperfusion injury.

Mulberry extracts from parts of mulberry have been used in systems of traditional medicine such as traditional Chinese medicine and Ayurveda for hundreds of years based on belief that the plant has important medicinal properties. There are several reports in pre-clinical research showing the benefits of mulberry-derived crude extracts on many indicators of disease in diabetes (Kim *et al*., 2011), neurotoxicity (Niidome *et al*., 2007), inflammation (Park *et al*., 2013) and atherosclerosis (Chan *et al*., 2013). Mulberry leaves are rich in polyphenols, especially quercetin glycosides and chlorogenic acid (Katsube *et al*., 2009). These polyphenols can serve as potent antioxidants (Katsube *et al*., 2009).

In the present study, the aqueous extract of mulberry leaf effectively reduced blood levels of malonedialdehyde, a marker of lipid peroxidation while elevating the level of glutathione, a small molecule endogenous antioxidant. This effect is likely due to the antioxidant effect of polyphenols. Different mulberry leaf extracts have been shown to contain 28-55mg/g of polyphenols. The polyphenolic content of these leaf extracts has also been positively correlated

with radical scavenging activity (Kim *et al*., 2014). Like many polyphenols, quercetin and chlorogenic acid are extensively metabolized in the body (Olthof *et al*., 2003). Polyphenols that are absorbed are extensively conjugated hence these are not metabolized by the gut microflora (Scalbert *et al*., 2002) and can have antioxidant activity comparable to the parent polyphenol (Villano *et al*., 2005). Thus, not only the polyphenols in the mulberry but also their metabolites may be responsible for the antioxidant effect observed in mulberry leaf extract treated rats. These agents can directly combine with and neutralize free radicals, which reduces oxidative damage and the ensuing reduced MDA levels in the brain is a direct measure of redox state in treated animals. The increase in glutathione level can be due to firstly, a decrease in free radicals resulting in reduced utilization (oxidation) of glutathione; secondly, the glutathione synthesis pathway can be triggered leading to more glutathione production. Thus, by combating oxidative stress, MLE clearly limited a primary pathological mechanism after stroke.

The reduction in gross tissue injury seen after cerebral ischemia-reperfusion by TTC staining in the brain sections of rats treated with mulberry leaf extract corresponds to decrease in indicators of cellular injury in brain sections stained with HE or cresyl violet stains. Mulberry leaf extract has been reported to reduce nitric oxide production, prostaglandin E2 expression, and inflammatory cytokine expression (probably by decreasing NF-κB activity (Park *et al*.,2013). Mulberry also reduces leukocyte migration (Chen *et al*., 2013). These phenomena are important in increasing brain injury. Also, in addition to their antioxidant effect, several metabolites of polyphenols can reduce inflammation (Larrosa *et al.,* 2009). Mulberry leaf extract reduces inflammation in a rat immobilization-induced stress model (Lee *et al.,* 2007). It also decrease obesity-induced inflammation in obese mice (Lim *et al*., 2013) and in LPS induced inflammation in rat (Ou *et al*., 2013). These and other studies provide strong preclinical evidence regarding the

anti-inflammatory effects of mulberry extracts which may be one of the key mechanism of reducing injury after cerebral ischemia-reperfusion.

The extracts of mulberry leaf used in the experiments that constitute this thesis have neuroprotective effect in the transient cerebral ischemia model through similar mechanisms. They also share a polyphenol moiety in their structure. Polyphenols are bioactive components present in food that have a myriad of beneficial effects. One of the most widely reported effects is as an anti-oxidant. The variety of polyphenols discovered in mulberry leaf are gallocatechin, rutin, naringenin, quercetin, isoquercitin, astragalin etc. as reported by Yang et al. (2011) and Lee et al. (2012). The neuroprotection observed in ischemic rats treated with MLE is likely the end point that results from the pleiotropic actions of polyphenols (Yang *et al*., 2011; Lee *et al*., 2012). The free radical scavenging, increased expression of anti-oxidant enzymes and chelation of metal ions like Fe^{+2} leads to a decrease in oxidative stress. Interestingly, oxidative stress can regulate inflammation, moreover, redox sensitive genes that are upregulated as a result of increase in oxidative stress by the activity of transcription factors such as NF-κB can lead to increased expression of pro-inflammatory cytokines and trigger inflammation (Crack and Wong, 2008). Therefore, polyphenol rich mulberry leaf extract can reduce post-ischemic inflammation by decreasing oxidative stress. The cumulative effect of a reduction in oxidative stress, inflammation and excitotoxicity is to limit brain tissue damage in stroke.

Therefore, after analyzing the results obtained in this study, we conclude that as neuroprotective agent, mulberry leaf extract may be an exemplary contender in pharmaceutical research for therapeutic or prophylactic treatment of ischemic stroke.

CHAPTER III

STUDIES ON NEUROPROTECTIVE EFFECT OF CURCUMA LONGA EXTRACTS

3 Introduction

Curcuma longa also known as turmeric is the rhizomatous perennial herb distributed throughout tropical and subtropical regions of the world. *Curcuma longa* is a relative of ginger and is mostly grown in the tropical regions of Southern Asia. It is a perennial plant with a growth of 5 - 6 feet height. It has trumpet-shaped, dull yellow flowers. It has tuberous roots which are oblong and palmate in shape and has a brown outer appearance with deep orange inside. Leaves are smooth surfaced, lanceolate shaped, tapering at each end and are about 2 feet long. It is uniformly green coloured and petiolated which are centrally placed, erect, spikey and oblong in shape. Bracteolae surround a bunch of three or five flowers which are dull yellow in colour (Fig. 30). Worldwide, Curcuma has been identified in great numbers which can be categorized into 133 species. Curcuma has a broad range of growing conditions as it can grow in diverse tropical conditions with a temperature range of 20-35ºC, with 1500 mm or more of annual rainfall. It can be grown in rain fed or irrigated conditions with well-drained sandy or clay loam soils, with a pH range of 4.5-7.5 with good organic status. For various medicinal formulations, curcuma is used and most of them have local names which are common.

Fig. 30 The *Curcuma longa* plantation

Rhizomes are the most gathered part of this plant and it is done annually. These rhizomes can be used in the following season for development of new plantation. When these rhizomes are dry, they have a pointed or tapering ends with cylindrical appearance nearly an inch in diameter. They are yellowish externally and internally deep orange or reddish brown in colour, with parallel rings which are transverse, marked with shining points. (Fig.31). They are generally boiled and then dried, turning into the familiar yellow powder. It has an odour which is fragrant but peculiar. It has a bitterish and slightly acrid taste similar to that of a ginger. The rhizomes get matured when they are kept underground beneath the foliage in the ground. The roots, or rhizomes and bulbs, are used in medicine and food.

Fig. 31 The rhizomes of *Curcuma longa*

Curcuma grows in many tropical locations. Interestingly, India is the largest producer of the world's curcuma crop and the largest consumer too as it consumes 80% of it. Indian curcuma with its inherent qualities is considered the best in the world as it has curcumin, an important bioactive compound in high content. The most important trading center for curcum and also the world's largest producer is Erode, in Tamil Nadu followed by Sangli, a city of Maharashtra.

*Curcuma longa's m*ore than 100 components have been isolated from. There is the presence of coloring agents called curcuminoids in curcuma. Turmerone, a volatile oil is the main component of the root. The natural antioxidants, curcumin demethoxycurcumin, 5'-methoxycurcumin, and dihydrocurcumin are the main constituents of Curcuminoids (Selvam *et al*., 1995; Ruby *et al*., 1995). In a standard form, curcuma contains >9% moisture, 5–6.6% curcumin, <0.5% by weight extraneous matter, <3% mould and <3.5% volatile oils (d-α-phellandrene, d-sabinene, cinol, borneol, zingiberene, and sesquiterpenes) (Ohshiro *et al*., 1990). The components responsible for the aroma of curcuma are turmerone, AR-turmerone, and zingiberene. The ukonans along with cholesterol, stigmasterole, 2-hydroxymethyl anthraquinone and β-sitosterole are the four new polysaccharides reported to be contained in the rhizome (Kapoor 1990; Kirtikar and Basu 1993). Nutritional analysis showed that curcuma contains total fat, saturated fat, calcium, phosphorous, sodium, potassium, iron, thiamine, riboflavin, niacin, ascorbic acid, total carbohydrates, dietary fiber, sugars and protein (Balakrishnan 2007). Turmeric is also a good source of the Ω-3 fatty acid and α-linolenic acid (Goud *et al*., 1993).

3.1 Background of the study

Dating back to nearly 4000 years, one can find the mention of Curcuma as a medicine. Not only as a principle spice, but also as religious ceremonial component, the curcuma is used in southeast Asian countries. Because of its brilliant yellow color, curcuma is also known as "Indian saffron." Prior to its use in medicine, it was used as a dye. Since this herb is very well believed and found to be an antiseptic and antibiotic agent, it can be used in disinfecting scrapes, burns and cuts. It may be helpful for people aiming to lose weight as it aids in fat metabolism. The Chinese have long used curcuma as an effective treatment for depression. In folk medicine, curcuma has been used in therapeutic preparations over the centuries in different parts of the world. Turmeric is thought to have many medicinal properties in Ayurvedic practices. It is used

to boost up the overall energy of the body, aiding in dispelling of worms, relief from gas formation, regulation in menstrual disorders, gallstone removal, improvement in digestion, and also in relieving pain due to arthritis. Apart from the Ayurvedic applications, Indians used to make use of turmeric as a blood purifier and remedy for skin condition improvement. Superfluous hair on the body can also be removed by it as in some parts of India women use it in the form of a paste. To have a healthy glowing skin, and also to ward off harmful bacteria from the body, in some parts of India, Pakistan and Bangladesh, Curcuma paste is applied to the skin of the bride and groom before marriage. Several sunscreens also have the base of Curcuma. Several multinational companies are involved in making face creams based on turmeric. Various diseases, including cough, hepatic disorders, sinusitis, biliary disorders, diabetic wounds, anorexia, rheumatism, jaundice, menstrual difficulties flatulence, hematuria and hemorrhage are treated traditionally by this wonder herb. Curcuma longa can also be applied topically in poultices to relieve pain and inflammation (Leung, 1980).

Chronic anterior uveitis, skin cancer, small pox, wound healing, rheumatoid arthritis, chicken pox, conjunctivitis, urinary tract infections, and liver ailments were treated using this herbal medine (Dixit *et al*., 1988). It is also used for digestive disorders; to reduce flatus, jaundice, menstrual difficulties, and colic; for abdominal pain and distension (Bundy *et al*., 2004); and for dyspeptic conditions including loss of appetite, postprandial feelings of fullness, and liver and gallbladder complaints. It has anti-inflammatory, choleretic, antimicrobial, and carminative actions (Mills and Bone 2000). In Ayurvedic medicine, turmeric is a well-documented treatment for various respiratory conditions (e.g., asthma, bronchial hyperactivity, and allergy), as well as for liver disorders, anorexia, rheumatism, diabetic wounds, runny nose, cough, and sinusitis (Araujo and Leon 2001). In traditional Chinese medicine, it is used to treat diseases associated with abdominal pain (Aggarwal, *et al*., 2004). Turmeric has been used to

treat sprains and swelling from ancient times, as prescribed by Ayurveda, (Araujo and Leon 2001). Turmeric is considered to be a better digestive and a carminative in both Ayurvedic and traditional Chinese medicine. To expel phlegm Unani practitioners make use of turmeric. They also use to open blood vessels in order to improve blood circulation.

For the last few decades, extensive work has been done to establish the biological activities and pharmacological actions of curcuma and its extracts. Today, it is considered potentially beneficial in treating or reducing symptoms associated with a wide range of health conditions. There are substantial data supporting the anti-bacterial, anti-tumour, reno-protective and cardioprotective, activities of curcumin. Over the last few decades, more than 3000 studies have been done by the researchers demonstrating curcumin as an antibacterial, antiviral, anti-inflammatory, antioxidant, pro-apoptotic, antiproliferative, antifungal and anti-atherosclerotic agent in treating against psoriasis,neurodegenerative diseases multiple sclerosis, nephrotoxicity, arthritis, cardiovascular disease, allergy, diabetes, inflammatory bowel disease,AIDS and lung fibrosis (Aggarwal and Harikumar, 2009; Sharma *et al*., 2005; Aggarwal *et al*., 2003; Shishodia *et al.*, 2005).

Much research is being conducted right now into the possibility of this herb being used to prevent and treat different types of cancer. Curcumin has been earlier found to induce cell-cycle arrest and apoptosis in a variety of cancer cell lines grown in culture (Duvoix *et al.*, 2005; Moos *et al*., 2004). In various models, turmeric has been reported to exhibit activity against the development of skin cancer (Villaseñlor *et al.*, 2002), breast cancer (Deshpande *et al.*, 1998), oral cancer (Azuine and Bhide 1992a), and stomach cancer (Azuine and Bhide 1992b). It prevents carcinogenesis at various steps, including inhibiting mutation (Polasa *et al*., 1991), detoxifying carcinogens (Thapliyal *et al*., 2001), decreasing cell proliferation, and inducing apoptosis of tumor cells (Garg *et al*., 2008). Animal tumors induced by Dalton's lymphoma can

be prevented by Turmeric extract (Kuttan *et al*., 1985). Several cell-signaling pathways gets inhibited by the use of turmeric and this is the mechanism by which curcumin reduces apoptosis.

Furthermore curcuma raises interest as an agent of potential use in therapy of many diseases (not only cancer) with an inflammation constituent including cardiovascular diseases, Alzheimer's disease, rheumatoid arthritis and metabolic syndrome. Although, in 2011, the U.S. National Institutes of Health has registered 61 worldwide clinical trials to study the use of dietary curcuma for a variety of clinical disorders, a plethora of studies using animal and cell line models have also been undertaken to elucidate the molecular mechanisms and biological effects of curcuma. Curcuma shows anticoagulant activity by inhibiting collagen and adrenaline induced platelet aggregation in vitro as well as *in vivo* in rat thoracic aorta (Srivastava *et al*., 1985). Curcumin also inhibits human sperm motility and has the potential for the development of a novel intravaginal contraceptive (Rithaporn *et al.*, 2003). Curcumin prevents galactose induced cataract formation at very low doses (Suryanarayana *et al*., 2003). Both turmeric and curcumin decrease blood sugar level in alloxan-induced diabetes in rat (Arun and Nalini, 2002). Curcumin also decreases advanced glycation end products induced complications in diabetes mellitus (Sajithlal *et al.*, 1998). Curcuma extract also tend to possess antibacterial activity (Bhavani Shankar *et al.*, 1979), antifungal activity (Jayaprakasha *et al.*, 2001), *antiviral* activity (Hergenhahn *et al*., 2002) and anti protozoan activity (Rasmussen *et al.*, 2000).

In vitro studies have shown that Curcuma has the ability to inhibit amyloid beta oligomer formation (Yang *et al*., 2005). When injected peripherally, curcumin was found to cross the blood brain barrier in an animal model of Alzheimer's disease (Yang *et al.*, 2005). In animal models of Alzheimer's disease, dietary curcumin has decreased biomarkers of inflammation and oxidative damage, amyloid plaque burden in the brain, and amyloid beta-induced memory deficits (Lim *et al*., 2001; Frautschy *et al.*, 2001; Pan *et al.*, 2008; Sreejayan and Rao,1996).

Turmeric is also helpful in reducing bad cholesterol in the blood stream. Bad cholesterol is one of the primary causes of heart attacks and blocked arteries. Its inhibition of ADP-epinephrine and collagen induced platelet aggregation (Srivastava *et al*., 1986); protection against thrombotic challenge (Srivastava *et al*., 1985); inhibition of platelet aggregation, metabolic disorders and hyperlipidemia (Khanna *et al*., 1984) makes it useful to prevent cardiovascular disorders like ischemic heart attacks, myocardial infarction etc. As relayed by a report, the potential neuroprotective actions of this substance were discovered during a screening of its potential to protect against the adverse effects from high doses of alcohol, which revealed positive results (Ringman *et al.*, 2005). Since then, research groups have begun to earnestly study and zero in on the neuroprotective property of curcumin. It has been hailed as a champion for its ability to protect the brain. Several studies have indicated that curcumin has protective effects against cerebral ischemia in rats and gerbils. These effects could be attributed to the antioxidant activity of the curcuma.

The antioxidant activity of curcuma was reported as early as 1975. It acts as a scavenger of oxygen free radicals. It can protect haemoglobin from oxidation. *In vitro* studies have shown that curcumin from curcuma can significantly inhibit the generation of ROS like superoxide anions, H_2O_2 and nitrite radical generation by activated macrophages which play an important role in inflammation. Curcumin's ability in lowering the production of ROS has been proven in *in vivo* studies also. The derivatives of curcumin, demethoxycurcumin and bis-demethoxycurcumin also have antioxidant effect. Curcumin exerts powerful inhibitory effect against H_2O_2 induced damage in human keratinocytes and fibroblasts (Phan *et al*., 2001). It also decreased lipid peroxidation in rat liver microsomes, erythrocyte membranes and brain homogenates (Reddy *et al*., 1994). This is brought about by maintaining the activities of antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase. Since ROS

have been implicated in development of various pathological conditions (Bandyopadhyay *et al*., 1999; Halliwell *et al.*, 1990), curcuma has the potential to control these diseases through its potent antioxidant activity. The antioxidant mechanism of curcumin is attributed to its unique conjugated structure which includes two methoxylated phenols and an enol form of β-diketone. The structure shows typical radical-trapping ability as a chain breaking antioxidant. In another study, Masuda *et al.*, (2001) proposed that the mechanism involves oxidative coupling reaction at the 3'position of the curcumin with the oxidizable polyunsaturated lipid linoleate.

It was reported in a study that curcumin ameliorates cerebral ischemia/reperfusion injury by preventing ONOO− mediated blood–brain barrier damage (Jiang *et al.*, 2007). In a different study it was hinted that high levels of NO generated by NOS isoforms are partially responsible for exacerbating the neuronal damage induced by middle cerebral artery occlusion (Dohare *et al*., 2008). The neuroprotective activity of curcumin as illustrated from these studies can hold it as a promising agent not only for the treatment of cerebral stroke, but also for the treatment of other disorders.

Curcuma has been used in ayurvedic medicine since ancient times, with various biological applications. Although some work has been done on the possible medicinal applications, a few studies for drug-development have been carried out as yet. Although the crude extract has numerous medicinal applications, clinical applications can be made only after extensive research on its bioactivity, mechanism of action, pharmacotherapeutics and toxicity studies. However, as curcuma is now available in pure form, which shows a wide spectrum of biological activities, it would be easier to develop new drugs from this compound after extensive studies on its mechanism of action and pharmacological effects. Recent years have seen an increased enthusiasm in treating various diseases with natural products. Curcuma is a rich in highly promising natural antioxidant compounds having a wide spectrum of biological functions

so it is expected that curcuma may find application as a novel drug in the near future to control various diseases, including stroke.

3.2 Materials used

Chemicals, Biochemicals, Instruments used

Table 5 The list of chemicals and biochemical used in the experiments.

Sl. No.	Chemical/Biochemical	Manufacturer
1.	2,3,5-Triphenyl tetrazolium chloride (TTC)	Sigma
2.	2-Thiobarbituric acid (TBA)	Sigma
3.	5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB)	Sigma
4.	5-Sulphosalicylic acid	Qualigens
5.	Absolute alcohol	Qualigens
6.	Acetone	Qualigens
7.	Anaesthetic ether	TKM Pharma
8.	Avidin –conjugated FITC	Santa Cruz
9.	Benzene	Merck
10.	Calcium chloride	Sigma
11.	Chloral hydrate	Sigma
12.	Cresyl violet	Sigma
13.	DAPI	Sigma
14.	Dimethyl sulfoxide (DMSO)	Sigma
15.	Disodium hydrogen phosphate (Na_2HPO_4)	Sigma
16.	DPX mountant	Fluka Biochemika
17.	Dead End Colorimetric TUNEL Kit	Promega
18.	Eosin	HiMedia
19.	Ethylene diamine tetra acetic acid (EDTA)	Sigma

20.	Fenton Reagent	San Jose Scientific
21.	Formaldehyde	Merck
22.	Glutathione	SRL
23.	Gum accacia	HL Pharma
24.	Hematoxylin	HiMedia
25.	Hydrochloric acid	Merck
26.	Liquid paraffin	Merck
27.	Magnesium chloride ($MgCl_2$)	USB TM Chemicals
28.	Magnesium sulphate ($MgSO_4$)	Qualigens
29.	Mounting medium UltraCruz	Santa Cruz
30.	OCT embedding matrix	Leica
31.	Paraffin wax	SD fine chemicals
32.	Paraformaldehyde	Merck
33.	Poly-L-lysine	Sigma
34.	Potassium chloride	Qualigens
35.	Potassium dihydrogen phosphate(KH2PO4)	Sigma
36.	Resveratrol	Sigma
37.	Sodium carbonate (Na_2CO_3)	Merck
38.	Sodium chloride (NaCl)	Merck
39.	Sodium citrate	SRL
40.	Sodium hydrogen carbonate (NaHCO3)	SD fine chemicals
41.	Sodium phosphate	Sigma
42.	Sucrose	Sigma
43.	Tetra methoxy propane (TMP)	Sigma
44.	Trichloro acetic acid (TCA)	Sigma
45.	Xylene	Rankem

Table 6 The list of instruments used in the experiments.

Sl. No.	Instruments	Manufacturer
1.	Cryostat	Microm GmbH, Germany
2.	Histocentre 2 workstation	Shandon
3.	Image Acquisition System (Biovis Image Plus)	Expert Vision Labs Pvt Ltd, India
4.	Incubator (Fine PCR Combi SV12)	Fine PCR, Korea
5.	Lambda 35 UV/VIS spectrophotometer	Perkin Elmer, Germany
6.	Light/Fluorescent Microscope (Leica DM5000B)	Leica Microsystem, Germany
7.	Magnetic stirrer	Genie, Bangalore
8.	Microtome (Leica RM2255)	Leica Microsystem, Germany
9.	Incubator (Fine PCR Combi SV12)	Fine PCR, Korea
10.	pH meter	Systronics, India
11.	Polytron Homogenizer (PT 3100)	Kinematica AG, Switzerland
12.	Table top Centrifuge (Sigma 3K30)	Sigma, Germany
13.	Ultracentrifuge	Beckmann, USA
14.	Vortex Mixer	Spinix, Tarsons, India
15.	Water Bath (Julabo SW22)	Julabo, Germany
16.	Weighing Balances (Precisa)	Toledo, Germany

3.3 Preparation of *Curcuma longa* root extract

The *Curcuma longa* samples from different geographical locations were chemically analysed and they were found same. Therefore, a source of sample was identified and supply was monitored and subjected to extraction and evaluation.

Rhizomes were freshly removed, cleaned and washed with water. After slicing it was dried for one week in the sun. In a hot air oven it was again dried for six hours at 50˚c. After proper drying, the dried rhizomes were broken into small bits. From these coarse broken bits, 100gm was transferred carefully into a glass bottle to which 100% ethanol (about 5 litre) was added. The

bottle was gradually shaken to mix up the materials and placed in the dark at room temperature. The extract sample was filtrated first by using muslin cloth and then by Whatman No.1 filter paper. In a dark glass bottle, the clear solution was collected. In order to obtain high concentration of the sample, further process had to be done by using rotary evaporator (Revathy *et al*., 2011). The bath temperature was set at 70^{o}C and the rotation at 90 rpm. After completion of extraction, the crude dried extract, the final product which was dark orange in colour was collected and transferred into dark brown glass bottle till further use. The extract was re-dissolved in double distilled water in desired concentration prior to use in all the experiments. The final dissolved extract is denominated as *Curcuma longa* extract (CLE) in the text.

3.4 Experimental protocol

Adult male Sprague-Dawley (SD) rats were procured from the National Laboratory Animal Centre of CSIR-Central Drug Research Institute (CDRI), Lucknow, India and used throughout the study. After necessary approval of Institutional Animal Ethical Committee (IAEC Approval no. **86/08/Pharmacology/IAEC/Renew 03(70/09))**, all experimental procedures and laboratory animal handling were carried out carefully in strict accordance. Throughout the study rats were allowed food and water *ad libitum*.

The animal groupings and the number of rats in each group were according to the type of experiment for each single dose of extract used in varying studies.

Study of neuroprotective activity of CLE

Adult male Sprague Dawley rats, weighing 260±20g, were used in the I/R experiments. Five different experimental groups consisting of 8-10 animals each were taken for the study. Group one and two were sham operated and MCAO induced animals respectively. Group three

and four animals were administered CLE orally one hour prior to induction of ischemia at a dose of 150 mg/kg and 300mg/kg respectively. Group five and six animals were administered with CLE, 6 hr post reperfusion at the dose of 150 and 300 respectively.

At the end of 24 hr of reperfusion, rats were subjected to behavioral studies to assess the neurological deficit scores in all the groups. Rats showing a neurological score of >6 were selected, euthanized and, biochemical and histochemical studies were performed for assessment of brain injury.

3.5 Focal cerebral ischemia model

Neuroprotective activity of the extracts was assessed using focal cerebral ischemia model which is described as follows:

A modification of the intraluminal technique (Longa E.Z. *et al*., 1989) was used for inducing occlusion in the middle cerebral artery (MCA) to generate focal cerebral ischemia model. The animals were anesthetized with chloral hydrate (300 mg/kg i.p., Sigma-Aldrich). Surgical table which was thermoregulated was used during the surgery to maintain the animal's body temperature. In the neck region, a midline incision was made and through which the left common carotid artery (CCA) was exposed. The external and internal carotid arteries (ECA and ICA) revealed by tracing the CCA anteriorly. Through a small nick in the ECA, with a rounded tip a 4.5 cm long 3-0 nylon filament (Ethicon, Johnsons & Johnsons Ltd. Mumbai) was introduced into the internal carotid artery and it was gently advanced forward till a resistance was felt indicative of its blocking the origin of MCA. Reperfusion was allowed by retracting the filament after 2 hr. to allow. Reperfusion continued till 24 hr after which the rats were sacrificed. A similar surgical procedure was performed in the sham operated rats except the suture

insertion. • As the animals recovered from anaesthesia, they were kept for regaining the righting reflex. Then they were transferred into cages and placed in an experimental animal room and the temperature was maintained at 25±0.1ºC. Animals were provided food and water ad libitum and were closely monitored for any gross behavioural disturbances. Rats showing tremor and seizures were not included in the studies. After the 2/24 hour of ischemia/reperfusion, rats were examined for neurologic deficit. Thereafter, re-anaesthesized with ether and about 3.5ml of blood was taken from retro orbital plexus to determine the levels of MDA and GSH in blood. Finally the brains were dissected out and to assess the extent of brain damage and cerebral infarct area, the brain was horizontally cut with 2 mm thickness and stained with TTC.

3.6 Assessment of cerebral ischemia/reperfusion induced injury

The brain damage following I/R was assessed using various parameters, which included gross behavioral, biochemical and histological studies.

3.6.1 Neurobehavioral studies

Sensorimotor functions are compromised as a consequence of cerebral ischemia reperfusion (I/R). By scoring the neurological deficit (ND) on a ten point scale, neurobehavioral assessment was performed to confirm proper MCAO occlusion and also cerebral damage severity can be determined by following the method of Longa *et al*. (1989) and Bederson *et al*. (1986).

ND scoring was done on the following scaling:

(i) Failure to fully extend left forepaw	Flexion	*1
(ii) Movement in circles in a clockwise, contralateral direction	Circling	*2
(iii) Failure to scale a vertical grid due to loss of grip	Hemiparesis	*3
(iv) No spontaneous movement observed	Non-spontaneity	*4

The sum of all points corresponding to the above characters following reperfusion was recorded as the ND for each animal. Thus, an impact of ischemic injury is indicated by enhancement in the behavioral deficit scores. After careful assessment of each rat, the scores obtained on neurobehavior were averaged as neurological deficit scores for each experimental group. The neurological deficit score was also used as an inclusion/exclusion criterion and I/R rats showing no sign of neurological deficits were excluded from the study.

3.6.2 Biochemical estimations

Excessive production of free radicals is a consequence of I/R injury that contributes to ischemic damage. Free radicals directly oxidize various macromolecules as well as activate or directly participate in ischemic cascades. The markers of oxidative stress, malonedialdehyde and glutathione were estimated biochemically.

3.6.2.1 Estimation of malonedialdehyde in serum

As a byproduct of lipid peroxidation, MDA is a three carbon dialdehyde which is highly reactive and is often measured as an index of lipid peroxidation. Thiobarbituric acid reactive substances (TBARS) were quantified using the method established by Colado *et al*. (1997) and used as a measure of MDA in the sample. 300 µl of 30% trichloroacetic acid, 150 µl of 5 M HCl and 300 µl of 2% (w/v) thiobarbituric acid in 0.5 M NaOH and ultrapure water was added to the 500µl serum obtained from blood collected from MCAO rats, vortexed and incubated at 90°C in a water bath for 15 min. The resulting pink suspension was centrifuged at 13000g for 10 min. The absorbance of the clear, pink supernatant was determined spectrophotometrically at 532nm. The readings were interposed in the standard graph prepared using different concentrations of tetramethoxy propane (TMP) and the values were expressed as nmol/mg protein.

3.6.2.2 Estimation of glutathione in plasma

Found in high concentration in brain, GSH is a major intracellular antioxidant and involved in protection against oxidative damage caused by ROS. GSH peroxidase catalyzes the GSH-dependent reduction of H_2O_2. The glutathione is oxidized, and therefore, 'consumed' in the process.

In the present study, GSH was estimated on the basis of quantifying a yellow chromogen formed by its reaction with 5, 5'-dithio- bis(2-nitrobenzoic acid) (DTNB) (Anderson M.E., 1985). 5% sulphosalicylic acid, 0.1M phosphate buffer (pH7.0), DTNB and ultrapure water were added to the serum samples. After incubation at 37°C for 15 min., the intensity of the yellow colour was quantified at 412nm in a spectrophotometer and the values thus obtained were interpolated in the standard plot for GSH to obtain GSH values in the samples expressed as nmol per mg protein.

3.6.3 Histological studies

Transcardiac Perfusion of the rat- The skin on the ventral aspect of body was incised and the peritonium was carefully dissected out. The thoracic cage was exposed so that there is easy access to the heart. During perfusion, to ensure that the fluid spreads throughout the body blood vessels should be kept intact. The cardiac perfusion was done using chilled normal saline through the left ventricle's apex region. A small incision was made with a fine scissors in the left auricle, to serve as an outlet. For histological staining, as the brain tissue needs to be fixed, 4% paraformaldehyde solution is to passed through the vasculature following normal saline.

3.6.3.1 Triphenyl tetrazolium chloride staining

Efforts were made to identify the area at risk and the extent of cerebral damage following I/R injury using TTC staining. Electron transport chain which is an integral part of the inner

mitochondrial membrane is made up by the pyridine nucleotide-linked dehydrogenases along with the cytochromes. TTC acts as a proton acceptor for such enzymes (Altman, 1976). Lipid-soluble formazan which is red in colour is formed when the tetrazolium salt is reduced by the enzymes. As this salt gets reduced by the enzyme present in the mitochondria, the viable tissue therefore stains deep red while the infarct brain area remains unstained. In TTC staining, since infarct tissue volume can be quantified using the software, so it is an added benefit (Goldlust *et al*., 1996; Desai *et al.,* 2010).

The rat brain was removed after perfusion. After freezing it for a few minutes to facilitate sectioning, the brain was sliced into approximately 2 mm thick sections. The slices were stained with 0.5% TTC dissolved in normal saline and then incubated in at 37^{o}C for 30 min (till the tissue assumed red/dark pink colour) Bederson *et al*., 1986, Desai *et al.,* 2010). The stained brain sections were analyzed using Biovis image software.

3.6.3.2 Hematoxylin and Eosin staining

Heamatoxylin and Eosin staining (H&E) is widely used to assess the morphological features of cells. Heamatoxylin is a basic dye which specifically stains nuclei, whereas eosin stains cytoplasm due to its acidic nature. Histological features such as diffused whiteness of the eosinophillic surroundings, shape alteration, cellular perikarya stainability and size and formation of vacuoles are the main features used to identify the lesioned area. Hence, H & E staining is a powerful tool to differentiate between necrotic and apoptotic cell death on the basis of cell morphology following I/R injury (Li *et al*., 1998).

In all groups, rats were sacrificed by using anesthetic ether after 2/24 hr I/R injury. Brain was taken out immediately in chilled medium. The cerebral hemispheres were sectioned in the coronal plane and alternate slices of 2-3 mm thickness were kept in 4% buffered

paraformaldehyde for about 24 hours. Then slices were preserved in 70% alcohol. The dehydration was done by passing the tissue through graded alcohol concentrations i.e. 30 min each in 70% alcohol, 90% alcohol, 95% alcohol, 100% alcohol then via mixture of 100% alcohol and then for 15 mins each through xylene mixture (3:1), xylene I, xylene II followed by infiltration with paraffin wax (4 h-24h) at 65ºC in Histocentre 2 workstation (Shandon).Paraffin blocks were prepared and stored at 4^0C till sectioning. The sections of 4-6µm were then cut with a semi-automated microtome (Leica) and placed on poly-lysine coated slides and processed (for deparaffinization) by immersing in the following solvents before staining through the series of xylene and alcohol i.e. xylene I (5 min), xylene II (5 min), 100% alcohol (5 min), 70% alcohol (5 min), 50% alcohol (5 min) and finally washed with tap water.

Brain sections were stained with hematoxylin stain for 45 seconds and washed in running water thoroughly for 5 min. Further, sections were stained with eosin for 60 seconds and processed by immersing in the following solvents.

- Acetone I (2-3 times)
- Acetone II (2-3 times)
- Acetone and Xylene (2-3 times).
- Xylene (4-5 times)

Stained sections were then mounted with DPX and examined under light microscope (X 400). Based on their morphological feature, the necrotic and apoptotic cells were identified.

3.6.3.3 Cresyl Violet Staining

To further confirm the regional distribution of cerebral damage**,** cresyl violet staining was done.CV stains the nissl substance present in the cell body and dendritic processes of the viable neurons. Primarily rough endoplasmic reticulum is the main constituent of Nissl substance and because of the high RNA content it is basophilic in nature and will be very sharply stained with

CV. After cellular injury, there is a loss of Nissl substance and the breakdown of myelin covering can also happen if the axon degenerates.

With normal saline the brain of rats was transcardially perfused followed by paraformaldehyde (4%w/v) and taken out immediately in chilled condition and processed for paraffin embedding. Using a microtome (Leica, Germany), 4-6 µm thick brain sections were cut at a distance of 0.80 mm from bregma (Paxinos and Watson, 1986) and transferred on poly-L-Lysine coated glass slides (1:50 dilution of 0.1% w/v solution) (Sigma Aldrich, USA). The sections were deparaffinized by xylene treatment and then rehydrated using the 95%, 85%, 70% and 50% graded concentrations of ethanol by sequentially immersing the slides through each one of them for 3 min each and then washed with 0.85% NaCl and phosphate buffered saline (PBS, pH 7.4) for 5 min each. The sections were then dipped in CV staining solution for few seconds, repeated 3-5 times and were air dried. Similarly, the sections were given few short exposures to n-butanol and then dipped in acetone followed by xylene. The stained sections were mounted with deoxy plasticizer xylene (DPX) and examined under light microscope (Leica, Germany) to identify the brain regions affected by I/R injury (Luna, 1968).

3.6.3.4 *in situ* labeling of fragmented DNA

Terminal deoxy nucleotidyl transferase (TdT)-mediated dUTP nick-end-labeling (TUNEL) Staining *i*s a well recognized technique employed for the identification of DNA fragmentation, a hallmark of apoptosis. It utilizes the enzyme TdT to catalyze addition of labeled dUTP to free 3'-OH ends of DNA fragments, which can then be assessed using a suitable detection method such as microscopy.

Fragmented DNA labeling was performed using the Dead End Colorimetric TUNEL system (Promega Corporation, Madison, USA) following the protocol with slight modifications

which provided with the kit. The brain sections were immersed in PBS for 5 min. In 4% paraformaldehyde the tissue was fixed for 15 mins followed by 5 min washing twice in PBS and then permeabilized for 5 min using 20µg/ml proteinase K. The sections were then washed in PBS, refixed in paraformaldehyde for 5 min and washed twice for 5 min each with PBS. After equilibrating for 15 min in equilibration buffer, in TDT-Mix (biotin-16-dUTP and terminal deoxy nucleotidyl transferase) prepared in equilibration buffer, the sections were incubated and covered with plastic cover slips for homogeneous distribution of solution. By washing the sections for 15 minutes in 2X SSC, the reaction was terminated post incubation for 60 minutes at 37°C,. Subsequently, the sections were washed twice in PBS for 5 minutes each. The biotinylateddUTP was then labeled with avidin-conjugated FITC (Santa Cruz, 1:200) by incubating in dark for 45 min and then sections were washed thrice with PBS to remove unbound fluorescent conjugate. The sections were mounted with cover slips in an aqueous mounting medium (Ultracruz mounting medium) containing anti-fade agent and DAPI to stain the nuclei. For negative controls, incubation of sections was done in absence of enzyme. The normal nuclei had relatively insignificant numbers of DNA 3'-OH ends, and hence, did not stain with this technique. Cells exhibiting necrotic morphology, containing detectable concentration of DNA ends, showed a more diffuse labeling as compared with apoptotic cells. Using a fluorescent microscope, DNA fragmentation was scanned in different areas of the brain and TUNEL positive cells were recorded after averaging the number of cells in minimum 5 fields per sections with IM50 software (Leica Microsystems).

3.7 Statistical Analysis

One-way Analysis of Variance (ANOVA) was used to analyse the data and comparing the results obtained in different groups was done by Newman-Keuls multiple comparison test. In all the cases, the $*P<0.05$, $**P<0.01$ and $***P<0.001$ values were considered statistically

significant at various level. Prism and GraphPad analysis software was used for all the statistical analyses.

3.8 Results

Effect of treatment of CLE on of I/R injury induced brain damage

The neuroprotective effect of CLE was bio-evaluated in focal cerebral ischemia model. The results obtained with CLE on MCAO induced I/R injury are detailed below:

3.8.1 Effect of treatment on neurological deficit

In all the experimental rats, the neurological deficits were scored at the end of 2/24 hr ischemia/reperfusion period. Impairment in the sensori-motor ability in experimental animals was observed and these animals demonstrated varying degrees of fore limb flexion, contralateral circling, hemi-paresis of the body and non-spontaneous movement. For MCAO rats, the deficit was pronounced showing contralateral forelimb impairment, loss of postural reflex, problem in balance and grip strength on the contralateral side.

The ND test scale used provides a general indication of neurological differences between the two groups studied. The I/R injury in MCAO rats produced ND at 2/24 hr I/R injury with an average score of 6 out of 10. The pretreatment of CLE at 300 mg/kg p.o. one hour prior to ischemic insult resulted in 60% improvement in neurological deficit in MCAO rats. There was a similar trend of equal magnitude of reversal of ND (55%) with pretreatment with 150 mg/kg p.o. dose as observed post 24 hrs of I/R injury (Fig. 32).

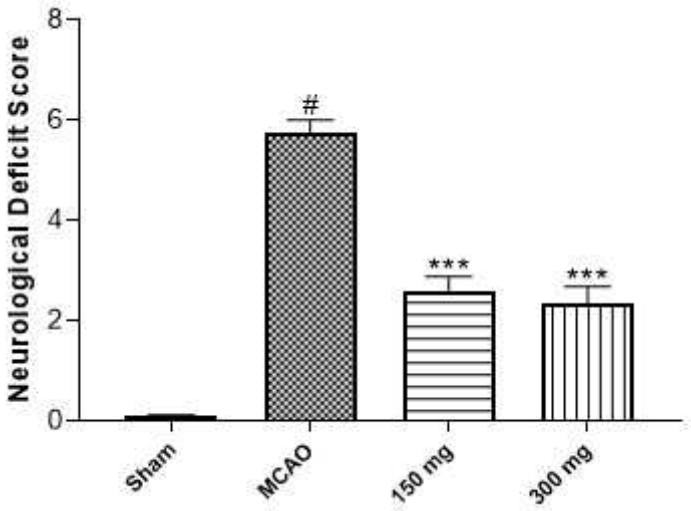


Fig. 32 The **e**ffect of 1 hr post treatment with CLE at doses of 150 mg/kg and 300 mg/kg p.o., on neurological deficit scores in ischemic rats. (n=8-10 animals each)

Significance #p<0.001 vs sham and ***p<0.001 vs MCAO

3.8.2 Effect of treatment on cerebral infarction

The extent of cerebral damage was assessed by the TTC, HE and CV staining.

3.8.2.1 Triphenyltetrazolium chloride staining

TTC staining is a well-established methodological option to inspect the severity of brain damage in terms of infarction. Red-colored, lipid-soluble formazan is formed as the TTC gets reduced in functioning mitochondria. Hence this stainining can be used as a marker to delineate the dead area from the live ones. Here the infarcted tissue without viable mitochondria remains unstained whereas as rest of the viable live cells takes up the stain due to the formazon formation in the mitochondria.

TTC staining is commonly used to assess the brain damage that ensues as a result of ischemic insult The rats showing significant ND were sacrificed and brain sections stained with TTC were used for assessing the cerebral infarct area. The 2/24 hr I/R injury resulted in almost

65.15% brain infarct, as white mass in TTC stained sections of rat brains (Fig. 33). A dose of 150mg CLE, was able to illicit a significant reduction of 42.69% and higher dose of 300mg showed maximum reduction in infarct area, by 68.32% (Fig. 34).

Fig. 33 TTC stained brain sections showing area of infarction in MCAO rats, CLE pretreated (150 mg and 300 mg) ischemic rats.

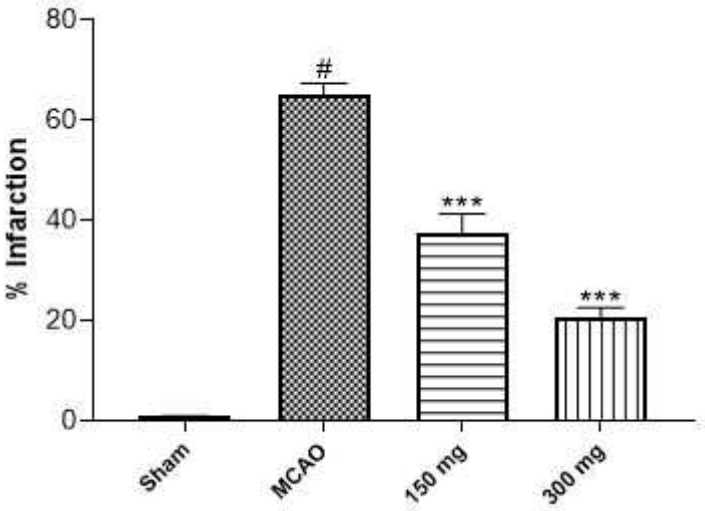


Fig.34 Effect of pretreatment with CLE (150mg and 300 mg) one hour prior to ischemia insult on cerebral infarction in rats following 2/24 hr I/R injury. (n=8-10 animals each)

Significance #p<0.001 vs sham and ***p<0.001 vs MCAO

3.8.2.2 Hematoxylin and Eosin staining

The cerebral I/R injury revealed the histological changes exhibiting features of both necrosis and apoptosis at 2 hr of Ischemia followed by 24 hr of reperfusion. Darkly stained pyknotic nuclei, dispersed chromatin clumps contained karyolysis, shrunken neurons, increased eosinophilic (red neurons) cytoplasm and presence of ghost neurons (nuclei lacking cellular structures) were the characteristic feature of the HE stained necrotic injury. It was observed that striatal region was greatly affected following I/R injury followed by cortex but hippocampus was not affected by ischemic insult and showed normal cellular morphology.

However, this cellular damage was significantly reduced in the brain sections of rats pre-treated with CLE at a dose of 300 mg/kg p.o. showing only a few apoptotic cells in both the striatal and cortical regions. But a relatively some cavitations were observed in the striatal and cortical brain regions along with necrotic neurons in the rats pre treatedwith CLE at a dose of

150 mg/kg p.o, though these alterations were comparatively far less as compared to seen in non treated rats. There was no evidence of such histological abnormalities in the brain sections of sham operated rats (Fig. 35).

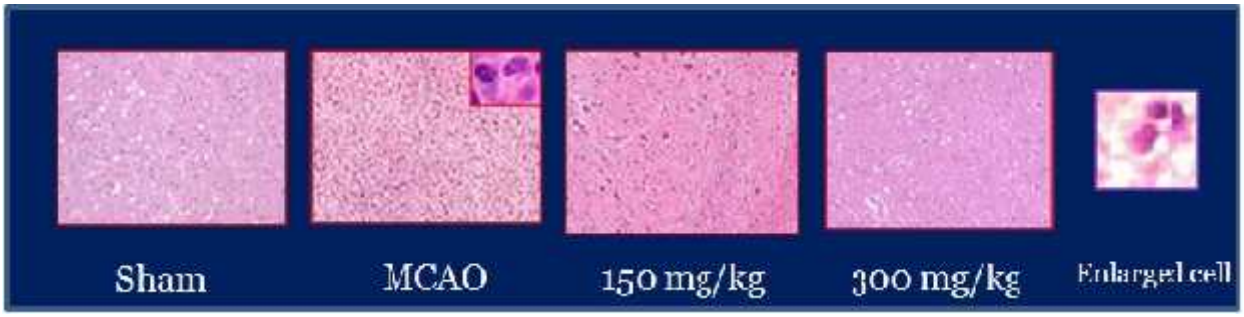


Fig. 35 Photomicrographs of HE stained 6 µm brain sections showing ischemic brain damage in MCAO, pre treated (1 hr) with CLE in rats at doses of 150 mg and 300 mg/kg p.o.as compared to the sham operated group.

3.8.2.3 Cresyl violet staining

Basically CV stains the ribosomal RNA, which is present in the rough endoplasmic reticulum giving the cytoplasm a mottled appearance. There is the characteristic presence of 'basophilic neurons', which are also called dark neurons possessing characteristics similar to amphophilic staining and cells appear to be shrunken which is monomorphic. In the brain sections of ischemic animals, different stages of degeneration can be detected. In animals which were sham operated and in MCAO induced animals where the brain region is non-affected following ischemia, intact cells could be found. After 2 hr of severe ischemia, morphological features of the cell could be visualized as atypical. Due to condensation of cytoplasm and karyoplasms, these cells exhibited a particular triangular shape with dark staining. A clear demarcation of infarcted areas was obvious and the affected tissue was brightened with the

pronounced Nissl stain. From the rough endoplasmic reticulum, as the ribosomes get dissociated because of which the neurons which are getting degenerated gets a pale appearance in a Nissl-stained tissue.

Therefore, the present study has evaluated the neuronal cell damage after 24h of I/R injury on paraffin embedded brain sections. CV staining showed that both ischemic striatum and cortex were less damaged in the brain sections of rats treated with CLE 300 mg/kg po. Whereas, the damage was visible in the striatal region of 150mg/kg p.o. The neuronal cells in the non-ischemic contralateral hemisphere were morphologically intact. Thus, these results also clearly indicate the massive neuronal cell damage and were consistent with that of HE staining results (Fig. 36).

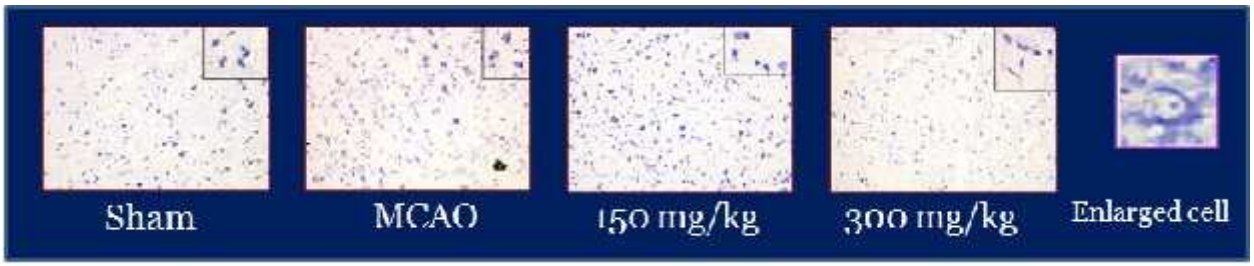


Fig. 36 The cresyl violet stained representative photomicrographs showing cellular brain damage MCAO, pre treated (1 hr) with CLE in rats at doses of 150 mg and 300 mg/kg p.o.as compared to the sham operated group.

3.8.2.4 TUNEL staining

There were no TUNEL positive cells in any of the the brain areas on the contralateral side. TUNEL positive cells are maximum in the striatum and cortex at 2/24 I/R injury. TUNEL positive cells labels fragmented DNA as a result of necrosis so a moderate increase in the TUNEL positive cells is observed at 2/24 hr of I/R which is the time point at

which rapid cell death termed as necrosis takes place and therefore the fragmented DNA is labelled with dUTP-FITC. The TUNEL positive cells was double stained with DAPI which revealed typical apoptotic morphology but when there was no ATP to carry out this programmed genetic mechanism the cells later died of necrosis, the major event of cell death.

The hippocampus showed no evidence of TUNEL positive cells. TUNEL was employed for the detection of DNA fragmentation and apoptotic bodies in the cells. Consistent with the results obtained with H&E and CV staining, which indicated significant morphological alterations and massive neuronal loss, TUNEL staining was performed. Apoptotic DNA fragmentation which was induced by the I/R injury was closely observed in the cortex and striatal brain regions to make a evaluation on the mode of cell death. Non-ischemic contralateral side was excluded from observing TUNEL positive cells.

Both striatum and cortex region of the affected ischemic or the ipsilateral side exhibited intensely stainined TUNEL positive cells. Apoptotic features like nuclear condensation and formation of apoptotic bodies could be observed in morphology of some TUNEL positive cells. Double staining was done using nuclear stain DAPI, to confirm TUNEL positive staining. The striatal region showed maximum number of TUNEL positive cells as compared to cortical region.

The appearance of apoptotic cell death following I/R injury as revealed by the TUNEL-positive cells in striatum was significantly reduced by CLE pretreatment at 300mg/kg. A moderate decrease in the TUNEL positive cells was also observed in rats, which were treated with CLE at a dose of 150 mg/kg (Fig.37).

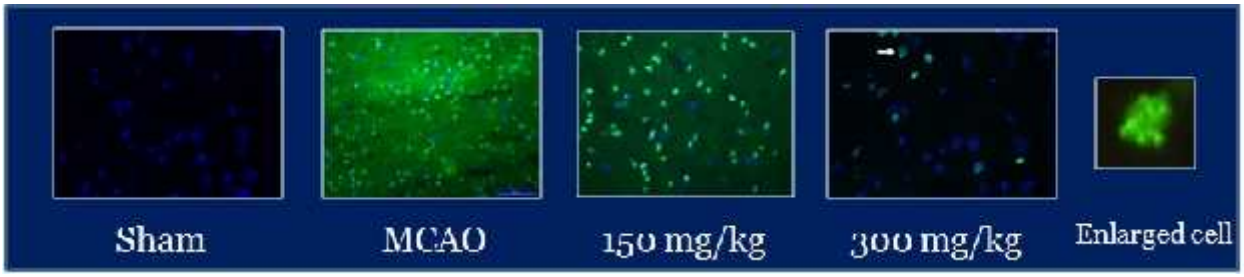


Fig. 37 Representative photomicrographs showing the TUNEL positive staining (green) in the striatal brain region following 2h/24h of I/R injury in rats pretreated with CLE (one hour prior to ischemic insult) at different doses as compared to the sham operated group. Nuclei were counterstained with DAPI (blue).

3.8.3 Effect of treatment on biochemical parameters

The effect of CLE in MCAO induced animals on oxidative stress markers, MDA and GSH was evaluated and the results are as follows:

3.8.3.1 Effect of treatment on MDA levels

The level of lipid peroxidation was measured by estimating its marker MDA, content in the serum of experimental animals. The blood MDA level, a marker of oxidative stress, increased significantly (74%) post 2 I/24 R post ischemic injury indicating high lipid peroxidation due to ROS accumulation. However, the blood MDA level in the group of ischemic rats pretreated with CLE at 150 mg/kg p.o. was reduced by 37.66%. A further downward trend in MDA reduction by 53.04% was observed at a higher dose of CLE ie 300mg/kg p o (Fig 38).

A significant increase in MDA level in serum of MCAO rats suggests the impact of oxidative stress on lipid peroxidation and subsequent ischemic brain damage. Whereas, the CLF

pretreatment significantly reversed the levels of MDA indicating the potential role of CLF in intervening the oxidative mechanisms resulting due to cerebral I/R injury.

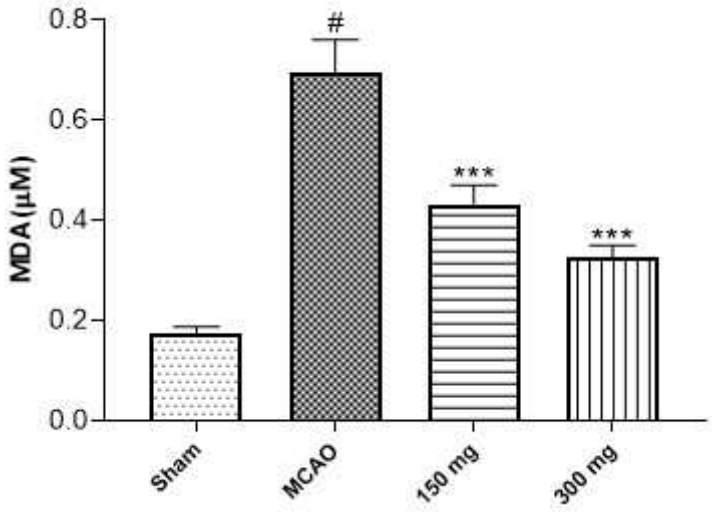


Fig.38 Effect of pretreatment with CLE (150 mg and 300 mg) one hour prior to ischemia insult on blood MDA levels in rats following 2/24 hr I/R injury. (n=8-10 animals each)

Significance #p<0.001 vs sham and ***p<0.001 vs MCAO

3.8.3.2 Effect of treatment on GSH levels

Glutathione is a central component in the antioxidant defense mechanism, acting directly to scavenge reactive oxygen species and also as a substrate for various peroxidases. The I/R induced stress depleted the glutathione level in the blood of affected animals and showed overall decrease of about 32% as compared to the sham. On the other hand, CLE pre treatment averted the depletion of GSH stores following I /R injury. There was 24.48% improvement in the GSH level by 150 mg/kg p.o. of CLE treatment. However, the dose of 500 mg/kg p o tended to further improve GSH level by 51.94% (Fig. 39).

These observations suggest that I/R induced injury leads to depletion of endogenous free radical scavengers, due to combating of oxidative stress. But the CLE pretreatment prevented oxidative stress resulting into up- regulation of glutathione stores.

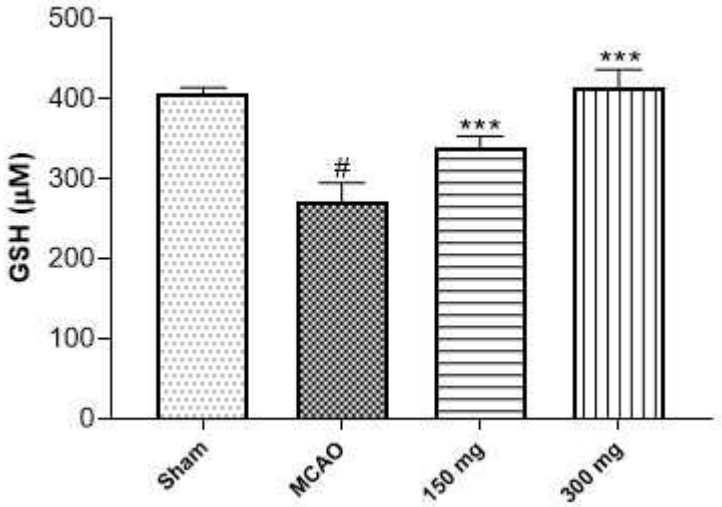


Fig. 39 Effect of pretreatment with CLE (150 mg and 300 mg) one hour prior to ischemia insult on blood GSH levels in rats following 2/24 hr I/R injury. (n=8-10 animals each)

Significance #p<0.001 vs sham and ***p<0.001 vs MCAO

3.9 Discussion

Stroke is a cerebrovascular accident that occurs due to substantially reduced cerebral blood flow. Cerebral stroke, which can result from interruption in blood supply to the brain even for a short duration, underlines the vulnerability of the central nervous system to glucose and oxygen deficiency. Such an interruption in blood supply can occur due to presence of a thrombus or an embolus clogging the blood vessel (ischemic stroke) or due to bleeding (haemorrhagic stroke). Stroke is the third leading cause of human death and the leading cause of disability worldwide as its symptoms are general and easily misdiagnosed and no suitable treatment is

commonly available. The only FDA approved drug, tissue plasminogen activator, is to be administered within three hours of onset of stroke.

Ischemic brain injury involves a variety of mechanisms. Moreover, prolonged ischemic episodes result in irreversible tissue damage. As the levels of GSH and Vitamins E and C,all of which are endogenous antioxidants are very low in the brain, it is more prone to oxidative damage. Not only this being the reason for its vulnerability for oxidative damage, brain has abundant content of lipid and high consumption rate of oxygen. After ischemic reperfusion (I/R) injury, oxidative stress involvement is at its height leading to neuronal loss due to over production of ROS such as hydrogen peroxide, superoxide, peroxynitrite radical and hydroxyl radical. In cerebral ischemia, secondary cell death is generally due to apoptosis or other related phenomena. Neuronal death in cerebral ischemia has been found to be involved due to activation of caspases including caspase-3, the up-regulation.

The other herb of interest is *Curcuma longa* or turmeric whose rhizome has the presence of a curcuminoid called Curcumin or 1,7-bis (4-hydroxy-3-methoxyphenyl)-1E,6E-heptadiene-3,5-dione. The use of curcuma is very common in India as a household/traditional medicine for minor ailments, as a spice in food preparations as well as in religious and cultural ceremonies. From time immemorial, curcumin has attracted interest because of its hepatoprotective, anti-mutagenic, antimicrobial, chemoprotective, anti-inflammatory, anti-parasitic, anticancer and antiviral activities (Jayaprakasha *et al*., 2005; Anand *et al*., 2008). Anti-oxidant activities depicting therapeutic properties of Curcumin was also seen in many studies (Dutta *et al*., 2005; Suryanarayana *et al*., 2007). It has been shown to be effective in models of cell cultures and small animals.

The pathological and biochemical features such as oxidative stress, inflammatory responses, neurobehavioural changes characteristic of human stroke can be very well duplicated in animals induced with MCAO to create the model of focal cerebral ischemia. And efforts had been made to screen the naturally occurring neuroprotecive materials. According to the focal cerebral ischemic stroke model induced by the occlusion of middle cerebral artery, the affected areas are caudate, putamen, parietal cortex, neocortex and entorhinal cortex (Lipton, 1999). Therefore, the occlusion of MCA induces both sensory and motor impairments. Patients with brain injuries are often demonstrating neurological sequelae in the form of functional behavioral deficits which are aso very common in animal models of cerebral ischemia. The severity of injury and functional deficit can be represented in the form of sensorimotor dysfunction showcased by the animals following experimental focal cerebral ischemia. Release of excessive ROS during ischemic reperfusion injury, plays a crucial role in cellular damage and death. Hence the biochemical mechanism involved during cerebral ischemia and reperfusion can be assessed by measuring ROS. The levels of lipid peroxides in the blood, plasma, and brain tissues and TBARS (MDA), indicative of lipid peroxidatio could be augmented during ischemic stroke due to the elevated levels of free radicals.

In this thesis, the neuroprotective potential of CLE was explored in a MCAO model of transient focal ischemia in rats. It had anti-oxidative and neuroprotective effect after ischemia-reperfusion injury. The present study utilized various parameters of sensori-motor functions such as flexion, circling, hemiparesis, and non-spontaneity at 2/24 I/R injury in SD rats leading to a profound impairment in the sensorimotor functionand but the treatment with CLE showed a decrease in the ND score at 2h/24h. The recovery may also be due to restoration of CBF and ATP. The biochemical, cellular, and molecular basis for this type of neurobehavioral pattern has

been analysed in this study to further understand the stroke-induced damage and recovery. A significant infarction was seen in the striatal region as well as cortical region at 2/24 I /R injury suggesting that the occlusion of the MCA has damaged the striatum and cortex which is more prone to insult because it receives direct supply from the MCA and its colateral. The CLE treatment however reduced the brain damage suggesting its neuroprotective mechanisms.

An increase in lipid peroxidation (MDA) results from an overproduction of free radicals or from loss of efficacy of the scavenging systems (GSH depletion) at 2h/24h of I/R injury. Our studies showed significant increase in the level of TBARS in blood plasma and depletion of GSH levels at 24 hours of reperfusion and is well correlated with ND score suggesting an increased load of free radicals during I/R injury. And the treatment with CLE helped in reducing the elevated MDA levels and attenuating the GSH levels thereby depicting its antioxidative property. The cellular alteration revealed by H&E and CV staining revealed that cellular damage following I/R injury may proceed through necrosis and or apoptosis. This ultimately may have contributed differently to the infarction in the striatal and the cortical region although vascular distribution in these regions is also important in damage progression and maturation. However neuroprotective effect was evdent in the group treated with CLE as the cellular damage in was found to be far less.

The anti-oxidant properties of curcumin have been well documented. Several studies have also indicated that curcumin has protective effects against cerebral ischemia in rats and gerbils (Thiyagarajan and Sharma, 2004; Ghoneim *et al*., 2002; Wang *et al*., 2005). Curcumin present in Curcuma extract is detectable within 30 minutes of oral administration and the levels are maintained till 4 hours in rat plasma with 1% bioavailability (Yang *et al*., 2007). Curcumin is anti-oxidative in streptozotocin-induced retinal oxidative stress (Kowluru and Kanwar, 2007),

cyclosporine-induced kidney dysfunction (Tirkey *et al.,* 2005), parkinson's disease model (Rajeshwari, 2006) and in traumatic brain injury model (Wu et al., 2006). In a model of transient global ischemia in the gerbil, oral and intraperitoneal curcumin treatment decreased lipid peroxidation, cell death and improved behavioral recovery (Wang et al., 2005). Jagatha *et al*.(2008), investigated the effect of curcumin on GSH depletion *in vitro* and *in vivo* using a neuronal culture and mouse model of GSH depletion. They found that curcumin was able to restore the cellular GSH pool, demonstrating the ability to protect cells against oxidative stress. They also found that the treatment leads to significant protection against protein oxidation and preservation of mitochondrial complex I activity which normally is impaired due to GSH depletion. Curcumin has also been proven as a powerful scavenger of superoxide anions, hydroxyl radicals, nitrogen dioxide and hydrogen donor, and therby exhibiting antioxidant activity directly and indirectly (Lim *et al*., 2005; Biswas *et al*., 2005).

Curcumin administration at 100 and 300mg/kg i.p. after 30 minutes of MCAO resulted in reduced infarction at 22 hours post reperfusion and attenuated oxidative stress (Thyagrajan and Sharma, 2004). These results are in agreement with the findings obtained by us. These anti-oxidant effects of curcuma are due to its polyphenolic structure. Indeed, the anti-oxidant activity of curcumin has been attributed to its phenolic ring (Priyadarsini et al., 2003). However, curcumin analogues devoid of the phenolic ring have also been reported to exert anti-oxidant activity (Weber et al., 2005), which suggests that the phenol ring is not the only mediator of this effect and that it is not indispensable for this activity. Curcumin is also reported to have anti-inflammatory action by inhibiting NF-κB regulated proteins such as the expression of iNOS, lipoxygenase, cyclooxygenase-2, and cytokines such as CXCL-1 and 2, TNF-α and monocyte chemo attractant protein-1 (Bachmeier et al., 2008; Chen et al., 2008; Chainani et al., 2003;

MachovaUrdzikova et al., 2015). Disruption of blood brain barrier is an important pathological event in cerebral ischemia-reperfusion injury. The increase in the permeability of blood brain barrier is caused by increased ROS generation and subsequent inflammation (Rosenberg, 2012) and curcumin treatment has been shown to alleviate blood brain barrier damage after cerebral stroke (Jiang et al., 2007). Similarly, excitotoxicity, which is a major contributor to ischemic injury, is also decreased by curcumin (Braidy et al., 2010). Thus modulation of these target mechanisms may also be important for the neuroprotection observed with curcumin treatment.

Similarly, resveratrol, which also possess anti-oxidative and neuroprotective effects after stroke, is a naturally occurring polyphenol. Therefore, these polyphenols may be the neuroprotective components in the extracts. One important consideration for any candidate molecule for neuroprotection is its ability to cross the blood brain barrier. Information regarding the ability of these polyphenols to cross the blood brain barrier is not very clear, although curcumin has been located in brain tissue (Garcia et al., 2007). Even though some molecules may not be able to enter the brain under normal conditions, the increase in blood brain barrier permeability brought about by ischemic injury may also allow them access to brain parenchyma. Moreover, there may also be systemic effects that result in lesser recruitment of monocytes and neutrophils at the site of injury and these alterations facilitate the passage of drug molecules into brain.

The neuroprotection observed in ischemic rats treated with CLE is likely due to the presence of polyphenols (Yang et al., 2011; Lee et al., 2012). The free radical scavenging, increased expression of anti-oxidant enzymes and chelation of metal ions like Fe^{+2} leads to a decrease in oxidative stress. Interestingly, oxidative stress can regulate inflammation, moreover, redox sensitive genes that are upregulated as a result of increase in oxidative stress by the

activity of transcription factors such as NF-κB can lead to increased expression of pro-inflammatory cytokines and trigger inflammation (Crack and Wong, 2008). Therefore, polyphenol rich mulberry leaf extract and CLE can reduce post-ischemic inflammation by decreasing oxidative stress. The cumulative effect of a reduction in oxidative stress, inflammation and excitotoxicity is to limit brain tissue damage in stroke.

The studies in this thesis show that treatment of ischemic rats with mulberry leaf extract MLE-AR-14, MLE-S-146 and CLE decreased the oxidative stress and afforded neuroprotection. These effects are observed with both pretreatment and post treatment dosage schedule indicating the potential of these herbal preparations in attenuating cerebral ischemic injury. The proposed neuroprotective effect of MLE and CLE has been hypothesized in the figure 40.

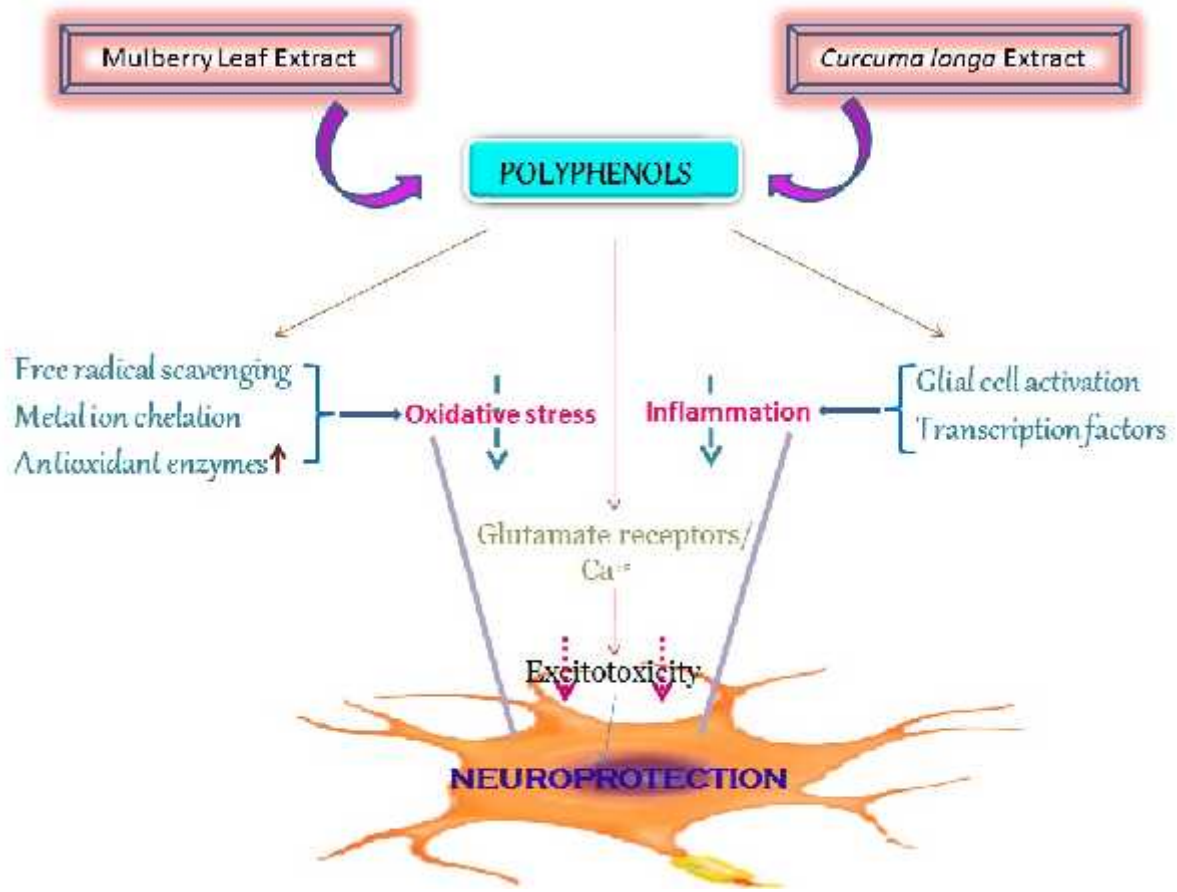


Fig. 40 A proposed possible mechanism of neuroprotection in ischemic rats treated with MLE and CLE.

Thus the results clearly demonstrate potent preventive and curative effects of MLE and CLE in cerebral stroke. These attenuate the redox status owing to reperfusion injury. Therefore, these appear to be promising neuroprotective candidate drugs for stroke which after further advanced studies in animals may be clinically exploited in humans.

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PUBLICATIONS

Neuroprotective profile of mulberry leaf extract in focal cerebral ischemia model in rats

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ABSTRACT

The cerebral ischemia causes severe degree of oxidative stress as a result of reperfusion injury. An attempt has been made to analyze the preventive and curative potential of mulberry leaf extract, in focal cerebral ischemia model in rat, as it is rich source of novel chemicals possessing antioxidant and neuroprotective properties. The aqueous extract (MLE-AR-14) pre-treatment one hour prior to ischemia significantly (65%) prevented the ischemic cerebral damage. It also tended to revert the behavioral deficit caused due to brain damage. The biochemical markers of oxidative stress MDA and GSH were also significantly altered towards basal levels. The most important and exciting finding is the effectiveness of MLE-AR-14 administration after six hours post cerebral injury. The extract at a dose of 100 mg/kg p.o. caused reduction in cerebral infarct by 54% indicating its curative potential in cerebral stroke. The oxidative end product MDA was reduced by 42 % and the level of GSH was elevated by 54% on post-treatment.

Thus, it seems that MLE-AR-14 has a potent neuroprotective profile and also has modulatory effect on cellular and biomolecular processes in preventing redox status to arrest apoptotic and necrotic processes underlying focal cerebral ischemia, as evidenced by histological parameters. The post-ischemic efficacy of the extract suggests that it may be clinically exploited in cerebral stroke.

Key words: Mulberry, Cerebral ischemia, stroke, neuroprotection, antioxidants.

1. INTRODUCTION

Cerebral stroke is a clinical syndrome characterized by abrupt loss of brain functions in humans due to blood clots, plaque formation in the blood vessels or hemorrhage in the brain that impairs blood supply to the brain. The rapid depletion of oxygen and glucose results in widespread cellular damage in the vicinity of the affected brain area. Usually an ischemic episode is followed by spontaneous reperfusion and serious adverse effects may arise due to re-oxygenation, leading to overproduction of reactive oxygen species (ROS) and leading to oxidative stress in the affected brain regions[1-3]. The oxidative stress is an important factor in brain damage during post-ischemic reperfusion. Therefore, antioxidants may act as a major defense by preventing or attenuating the damage caused by free radicals. Numerous antioxidants are neuroprotective in stroke models and significant evidence is available supporting the role of dietary antioxidants and polyphenolic compounds from botanical sources like green tea extract, *Ginkgo biloba* extract and resveratrol to combat ischemia-reperfusion induced oxidative stress[4]. Several plant candidates like *Morus alba*, *Withania somnifera*, *Centella asiatica*, *Tinospora cordifolia* and *Convolvulus pluricaulis* are best sources of potential therapeutic agents[5].

Mulberry, a plant belonging to a family of Moracaea, is predominantly grown in eastern, southern and southeastern Asia. The extracts of mulberry leaves, stems, root bark and fruits have been used for various ailments in the traditional system of medicine in Asian countries[6-8]. The plant is reported to contain a number of biologically active phytoconstituents viz. tannins, phytosterols, sitosterols, saponins, triterpenes, flavanoids, benzofuran derivatives, morusimic acid, anthocyanins, anthroquinones, glycosides and oleanolic acid as the main active principles[9]. The hypoglycemic and hypotensive effect of mulberry extracts has been attributed to the presence of phenolic mulberrofurans found in the plant[10]. The antioxidant and anti-apoptotic potential of mulberry extract is due to the free radical scavenging property of the flavonoids present there in, which consequently leads to the regulation of nitric oxide generation, Bcl-2 and Bax proteins, mitochondrial membrane depolarization and caspase-3 activation[11]. The mulberry-leaf extract is effective in suppressing the progression of atherosclerosis by inhibiting the oxidation of LDL-cholesterol, which is a major factor in the development of atherosclerotic plaque[12]. The study by Naowaboot and colleagues, 2009, showed that white mulberry leaf extract helps to restore the vascular reactivity, besides producing antihyperglycemic, antioxidant and antiglycation effects in chronic diabetic rats[13]. The clinical studies in humans have also shown mulberry leaf powder's potential to decrease serum triglyceride, LDL, and CRP levels in mild dyslipidemic patients without causing any adverse reactions[14].

The plant leaf extract has been used in some studies to explore its neuroprotective effect. Further, the accumulation of gamma-aminobutyric acid (GABA) in mulberry leaf as a result of the anaerobic treatment

enhances the neuroprotective effect against cerebral ischemia[15]. Recently, it was found that anthocyanin, a flavonoid pigment in mulberry fruit, offered protection against cerebral ischemia[16]. In another study, it was demonstrated that the antioxidative flavanoid, Quercetin-3-O-β-D-glucopyranoside from mulberry leaf significantly decreased certain stroke parameters like hemoglobin, strokin, cortexin, frontalin, temporalin, parietalin, occipitalin, brain venticulin, hemorraghic clot in rabbits[17]. Further, Cynidin-3-0-beta-D-glucopyranoside isolated from mulberry fruit also offered neuroprotective effect *in vivo* using a transient focal cerebral ischemia model of brain injury[18]. Hence, presence of novel chemicals in mulberry having antioxidant, dyslipidemic, antihypertensive with potent neuroprotective properties prompted us to analyse the preventive as well as curative anti-stroke activity of mulberry leaf extracts using middle cerebral artery occlusion (MCAO) model of focal cerebral ischemia in rats.

2. MATERIALS AND METHODS

Preparation of aqueous leaf extract of Mulberry: Fresh leaves from the mulberry variety AR-14 was obtained from mulberry plantation in Babasaheb Bhimrao Ambedkar University campus, Lucknow. The leaves were processed and powdered using standard methods. The resulting powder was then passed through an 80-mesh sieve and stored in a sealed aluminum pouch at 4 °C, till further use.

The mulberry leaf powder about 2 grams was soaked in 200 ml of boiling water for 20 minutes on a rotating shaker (200 rpm). The mixture was cooled at room temperature then filtered through Whatman No.1 filter paper and lyophilized. The freeze-dried solid leaf extract, MLE-AR-14 was stored in plastic tubes at −20 °C to protect from light. The solid extract was dissolved in double distilled water prior to use in all the experiments.

Experimental animals: Adult male Sprague Dawley rats, weighing 260±20 g, were used in the present study. The animals were procured from the National Laboratory Animal Centre of CSIR- Central Drug Research Institute, Lucknow. All animal experiments were performed strictly in compliance with the guidelines for the care and use of animals after necessary approval of the Institutional Animal Ethical Committee of the institute. Rats were allowed food and water *ad libitum* and housed in the experimental room in the animal house under proper laboratory condition.

Induction of Middle Cerebral Artery Occlusion: Focal cerebral ischemia was induced by occluding the middle cerebral artery (MCA) using the modified method of the intraluminal technique routinely used in the lab[19,20]. Briefly, the animals were anesthetized with chloral hydrate (300 mg/kg i.p). Then left common carotid artery (CCA) was exposed through the midline incision in the neck region and was traced rostrally to expose the external carotid artery (ECA) and internal carotid artery (ICA). A 4.5 cm long 3-0 mono filament (Ethicon, Johnsons & Johnsons Ltd., Mumbai) with rounded tip was gently introduced into the internal carotid artery through a small nick in the ECA and gently advanced about 2 cm from the ICA origin till a mild resistance was felt. The protocol of 2 hours ischemia and 24 hour reperfusion period (2/24 I/R) was used for developing cerebral stroke. The monofilament was retracted after 2 hours and allowed reperfusion for 24 hours. In sham operated rats, a similar surgical procedure was performed except for the insertion of monofilament. Post-surgery, the experimental animals were returned to cages with free access to food and water.

Experimental groups and treatment schedule: A different set of eight experimental groups were made consisting of 6-10 rats in each group. It includes sham, placebo (gum accacia), MLE-AR-14 (50 and 100 mg/kg p.o.) and resveratrol (50 and 100 mg/kg p.o.) as standard agent. The focal cerebral ischemia/ reperfusion injury of (2/24hrs) was used throughout the study. The schedule of treatment for preventive effect includes treatment with test substance or standard compound 1 hour prior to ischemia and for curative effect, 6 hour post reperfusion injury.

Assessment of Ischemia/Reperfusion injury: The ischemic brain damage was characterized by neurobehavioural as well as by biochemical and histological parameters.

Neurobehavioral studies: Rats were examined for neurologic deficits (ND) post 2/24 h I/R injury. The neurobehavioral deficits were scored on a 10 point scale by evaluating four parameters[19, 20]. The deficit is symbolized in the form of neurobehavioral incoordination and characterized by flexion (1 point), contra-lateral circling (2 points), hemiparesis (3 points) and non-spontaneity (4 points). Thus, an impact of ischemic injury is indicated by enhancement in the behavioral deficit scores. The neurobehavioral scores obtained after careful assessment of each animal were averaged as neurological deficit scores for each experimental group. The neurological deficit was also used as an inclusion/exclusion criterion and I/R rats showing no sign of neurological deficits were excluded from the study.

Biochemical Studies:

Malonedialdehyde estimation in blood: The malondialdehyde (MDA) an important biomarker of lipid peroxidation was measured in blood serum by the method of Colado, 1997[21]. It is based on its reaction

with thio-barbituric acid (TBA). Two molecules of TBA react stoichiometrically with one molecule of MDA to form a pink pigment that has absorption at 532nm. Thiobarbituric acid reactive substances (TBARS) were and used as a measure of MDA in the sample.

Rats were anaesthetized with anaesthetic ether and about 3ml blood was withdrawn from the retro-orbital plexus using the fine glass capillary tube. Trichloroacetic acid, 30% followed by 5N HCl, 2% 2-thiobarbituric acid and triple distilled water were added to the serum obtained after the samples were spun down at 13,000 rpm for 10 min at 25^0 C addition of. Vigorous vortexing was done after every step. Post incubation at 90^0C for 15 min in the water-bath, samples were then spun down at 3000 rpm for 10 minutes to settle down the particulate material. The adsorbance of the clear, pink supernatant was determined spectrophotometrically at 532nm. The readings were interposed in the standard graph for determining the MDA contents for each samples tested.

Glutathione estimation in blood: Glutathione (GSH) is a major intracellular antioxidant found in high concentration in the brain. Hydrogen peroxide is a potent oxidizing agent that is reduced by glutathione in a reaction catalyzed by glutathione peroxidase. GSH was estimated in blood by the 5, 5'dithiobis-(2-nitrobenzoic acid) (DTNB)-glutathione reductase coupled assay essentially as described by Anderson, 1985[22].

Blood was withdrawn from the retro-orbital plexus of the anaesthesized rats using a glass capillary, an anticoagulant, sodium citrate was added. Blood aliquots taken in different tubes were added with 6% acetic acid and 10% sulphosalysilic acid. Vigorous vortexing was done after every step. The plasma obtained after spinning the sample at 13,000 rpm for 10 min at 25^0 C, phosphate buffer, triple distilled water and DTNB was added followed by vortexing. After incubation at 37°C for 15 min., the intensity of the yellow coloured chromogen formed by reaction of GSH with DTNB was quantified at 412nm in a spectrophotometer and the values thus obtained were interpolated in the standard plot for GSH.

Histological studies:

TTC Staining: Brain damage following cerebral ischemia/reperfusion injury was visualized with 0.5 % 2, 3, 5 triphenyl tetrazolium chloride (TTC) staining[23]. TTC stains the live mitochondria of the cell, which appears bright red in colour. The dead or infarct tissue remains unstained by this dye owing to lack of oxygen and appears white.

The brains were rapidly dissected out after trans-cardiac perfusion with saline. The brain tissue was sectioned into 2mm thick serial coronal sections. All the brain slices were incubated for 20 min in a 0.5% solution of TTC at 37°C. The stained sections were digitally photographed and the infarct tissue of each brain section was traced and quantified by computerized image analysis software (Biovis Image Plus). The infarct area of each brain section was summed up and averaged and this was divided by the pooled average of ipsilateral brain area to obtain the percentage of brain infarction.

Hematoxylin and Eosin (HE) Staining: HE staining is a powerful tool to differentiate brain damage in the form of apoptotic and necrotic cell death on the basis of alteration in cellular morphology following I/R injury (24). The rats after 2/24 hours of I/R injury were subjected to neurobehavioral assessment prior to euthanizing them by anesthetic ether. This was followed by trans-cardiac perfusion with normal saline followed by 4% paraformaldehyde, thereafter the brain was immediately taken out in chilled medium. The cerebral hemispheres were sectioned in the coronal plane and processed for paraffin embedding. Sections of 4-6 μm thickness were cut using a microtome (Leica, Germany) and were transferred on poly-L-Lysine coated glass slides. The sections were deparaffinized by xylene treatment and rehydrated sequentially by immersing the slides through graded concentrations of ethanol (95%, 85%, 70% and 50%) for 3 minutes each and then washed with saline and phosphate buffered saline (PBS, pH 7.4) for 5 minutes each. The slides were dried and processed for HE staining for morphometric analysis under light microscope (Leica, Wetzlar, Germany) at 40X magnification. A minimum of 10 microscopic fields were examined of each slide to obtain a true picture of cellular architecture of affected brain tissue.

Cresyl Violet staining: To further confirm the regional distribution of cerebral damage, cresyl violet (CV) staining was also done. CV acetate solution is used to stain Nissl substance in the cell body and dendritic processes of the viable neurons. The Nissl substance (rough endoplasmic reticulum) appears dark blue due to the staining of ribosomal RNA, giving the cytoplasm a mottled appearance.

Basically the preparation of brain section slide is same as described previously under HE staining section. The sections were then dipped in CV staining solution for few seconds and air dried. Similarly, the sections were given few short exposures to n-butanol and then dipped in acetone followed by xylene. The stained sections were mounted with DPX and examined under light microscope (Leica, Germany) to assess the brain parts affected by I/R injury (25).

Statistical Analysis: The comparisons of results among different groups were made using the One-way Analysis of Variance (ANOVA) followed by Newman-Keuls

multiple comparison test. In all the cases, the *P<0.05, **P<0.01 and ***P<0.001 were considered statistically significant at various level. All statistical analyses were conducted using Prism and GraphPad analysis software.

3. RESULTS

Assessment of Neuroprotective profile: The neuroprotective effect was assessed by improvement in neurobehavioral deficit and cerebral infarct size. The biochemical indices GSH level was up regulated and MDA level was deregulated in treated rats with mulberry extract.

Neurological Deficit: A major prerequisite for the present study was the successful occlusion of middle cerebral artery by the nylon monofilament. This was verified by observing ND, which is a direct consequence of ischemic (I/R) injury. The MCAO group of rats after 24 hour of reperfusion showed a score of 7 on a 10 point scale of ND, indicating severe impairment in behavioral and motor functioning due to brain damage by I/R.

One hour pre-treatment with MLE-AR-14 at 50 mg improved ND by 37% whereas, the 100 mg dose resulted in significant improvement by about 50% in neurological deficit scores in ischemic rats post 24 hrs of reperfusion. The standard marker, resveratrol also showed significant reduction in ND by 63% and 77% respectively with 50 mg and 100 mg doses (Fig. 1a).

The post-treatment of MLE-AR-14 at a dose of 50 mg and 100 mg resulted in 40% and 57% improvement in neurological deficit scores in ischemic rats as compared to 65% and 71% observed with post-treatment with resveratrol with same dose. Thus the MLE-AR-14 was quite neuroprotective in preventing the brain damage caused by I/R injury. The relative marked effect of antioxidant resveratrol may be due to high dose of standard used whereas, 50 mg /dose was adequate as it is purified compound (Fig. 1b).

Cerebral infarction: TTC staining is commonly used to assess the brain damage as a result of ischemic insult. The rats showing significant ND were sacrificed and brain sections stained with TTC were used for assessing the cerebral infarct area.

The 2/24 hr I/R injury resulted in almost 65% brain infarct, as white mass in TTC stained sections of rat brains (Fig. 2a). The pre-treatment with 50 mg dose of MLE-AR-14 reduced the cerebral infarct by 34%. The increased dose of 100 mg of extract further enhanced the neuroprotection by 65% whereas, pre-treatment with 50 mg and 100 mg of resveratrol resulted in reduction of brain infarction by about 55% and 76% respectively.

Interestingly, the extract was significantly active even post I/R injury. The treatment with 50 mg of extract after 6 hour of I/R prevented brain damage by 28% whereas, 100 mg dose offered about 54% neuroprotection. The standard compound, resveratrol offered a reduction of 53% and 68% in cerebral infarction at a dose of 50 mg and 100 mg respectively. Thus, it appears that extract is significantly effective even after post ischemic injury (Fig. 2b).

Malondialdehyde levels in blood: The level of lipid peroxidation was measured by estimating its marker, MDA content in the serum of experimental animals. The blood MDA level was found elevated significantly by about 68% following 2/24 I/R injury. Interestingly, the MDA levels were significantly altered by both pre-treatment as well as post-treatment with MLE-AR-14 extract. The downward trend in MDA reduction was 30% with 100 mg dose of MLE-AR-14. The resveratrol pre-treatment with same dose had reducing effect by 50% on blood MDA level.

Interestingly, the MLE-AR-14 was significantly active in combating oxidative stress post reperfusion as compared to pre-treatment. The extract on post-treatment owing to its potent antioxidant activity was able to reduce the MDA levels in ischemic rats by about 42%. The standard drug, resveratrol when post-treated proved slightly more effective in reducing MDA content in the blood of ischemic rats (Fig. 3a).

Glutathione levels in blood: Glutathione is a central component in the antioxidant defense mechanism, acting directly to scavenge reactive oxygen species and also as a substrate for various peroxidases. The I/R induced stress depleted the glutathione level in the blood of stroke animals and showed overall decrease of about 60% as compared to the sham operated rats.

MLE-AR-14 pre-treatment was very effective in averting the depletion of GSH stores following I/R injury. The 100 mg/kg p.o.dose of MLE-AR-14 tended to improve GSH level by 54 % as compared to an increase of 77% by resveratrol pre-treatment with same dose. The post-treatment with the MLE-AR-14 also significantly prevented depletion of glutathione level by 34% (Fig. 3b).

Hematoxylin and Eosin staining: The histological changes observed under light microscopy after inducing 2 hour of ischemia followed by 24 hour of reperfusion showed enormous damaged cells exhibiting features of both necrosis and apoptosis. Necrotic injury stained with HE was characterized by nuclear pyknosis, karyolysis which contained dispersed chromatin clumps, associated with shrunken neurons, increased cytoplasmic eosinophilia (red neurons) and nuclei lacking cellular structures known as ghost neurons (Fig. 4).

Apoptotic cells were identified using characteristics enunciated by Kerr, (1972)[26], and these

were characterized by protruberances on the cell surface separated with plasmalemma sealing, which produced membrane bound apoptotic bodies of roughly spherical or ovoid shape. The severity of apoptotic injury was visible in sections as rounded or oval apoptotic bodies, typically dark purple-blue masses. Cells containing more than two apoptotic bodies were taken positive for apoptotic changes. There was also evidence of pan-necrosis in the striatal and cortical regions of MCAO induced rats as shown by cavitations.

However, this cellular damage was significantly reduced in the brain sections of rats treated with MLE-AR-14 showing only a few apoptotic cells in both pre-treated and post-treated groups. And, relatively, similar reduced number of cavitations were observed in the resveratrol pre-treated group. The post treated rat brains either with MLE-AR-14 or resveratrol had very few cells showing apoptotic and necrotic features in both striatal and cortical brain regions. There was no evidence of such histological abnormalities in the brain sections of sham operated rats.

Cresyl-violet staining: Cresyl-violet staining showed a significantly reduced cellular damage in both ischemic cortical and striatal sections of ischemic rats, treated with MLE-AR-14. The treatment with resveratrol was also equally effective in controlling the brain damage. The neuronal cells in the non-ischemic contralateral hemisphere were morphologically intact. Thus, these results very well corroborate with that of HE results (Fig. 5).

DISCUSSION

Cerebral stroke is characterized by sudden reduction of blood flow to affected part of the brain due to blockade of blood vessels or owing to hemorrhage. This results in deprivation of oxygen and glucose leading to breakdown of metabolic machinery in the affected part of the brain. The cerebral ischemia activates various overlapping cascades responsible for brain damage. The important ones are excitotoxicity, oxidative stress, intracellular signaling, inflammation, unfolded protein response and altered gene expression, and these mechanisms modulate cell survival and damage[1-3].

The aqueous mulberry leaf extract produced significant preventive as well as curative anti stroke effects in focal cerebral ischemia model of rat. The most significant finding is that it offered neuroprotection even six hours post reperfusion injury. The results have also demonstrated that, it has also significantly improved the neurobehavioral deficits and significantly reduced the cerebral infarct area. Thus, the antioxidant defense mechanism of MLE-AR-14 seems to be quite effective in reducing the overall deleterious effects of cerebral ischemia. Moreover, HE and cresyl violet staining of affected brain tissue also showed that MLE-AR-14 significantly protected cellular damage leading to neuroprotection.

Mulberry has been widely used in Chinese medicine since ancient times[27]. The pharmacological properties of the plant may be attributed to the presence of high amounts of polyphenolic compounds and secondary metabolites. Mulberry leaf extract contains many known polyphenolic compounds including rutin, quercetin, glucupyranoide, aastragalin, kaempferol etc., which exerts potent antioxidant activity[29-32]. Therefore, it is reasonable to believe that presence of such polyphenolic compounds in mulberry leaf extract would exert potent antioxidant activity to prevent the ischemic brain damage[28].

One of the most important factors responsible for brain damage induced by cerebral ischemia is oxidative stress caused by the reactive oxygen species (ROS) generated during hypoxia and reperfusion. During oxidative stress, rapid overproduction of free radicals overwhelms the detoxification and scavenging capacity of cellular antioxidant enzymes resulting in a severe and immediate damage to cellular proteins, DNA and lipids leading to brain dysfunction and cell death[33,34]. In order to protect tissues against the devastating consequences of ROS, all cells exhibit defense mechanisms that involve SOD, catalase, glutathione reductase and glutathione peroxidase[35]. These antioxidant enzymes vitamin E, glutathione, superoxide dismutase and catalase are free radical scavengers[36].

With respect to stroke patient recovery, the most common neurological sequelae in patients with brain injuries and in animal models of cerebral ischemia are functional behavioral deficits. The degree of sensorimotor dysfunction is an important indicator of severity of brain injury. A considerably severe neurobehavioral deficit was demonstrated in rats following MCAO. The results indicate that MLE-AR-14 treatment significantly improved neurobehavioral deficits when administered orally one hour prior to ischemic insult or six hour post reperfusion. The improvement in ND is indicative of the neuroprotective activity of the extract.

The level of MDA, an oxidative stress marker and a byproduct of lipid peroxidation was found to be elevated after focal cerebral I/R injury. This was indicative of oxidative stress exerted during focal cerebral ischemic injury in rats. Resveratrol treatment significantly reversed the increased MDA levels. Similar effect was exhibited by the MLE-AR-14 extract. Therefore, attenuating effect of MLE-AR-14 on MDA may be attributed to free radical scavenging activity of the extract as well as to its protective effect

against lipid peroxidation in cellular membranes and DNA damage due to free radicals.

Glutathione, an important endogenous antioxidant, prevents damage to important cellular components caused by reactive oxygen species. A decreased GSH level is considered to be indicative of oxidative stress. In this study, GSH level in the MCAO rats was significantly reduced compared to sham group. The GSH level of the resveratrol treated groups was significantly higher than the MCAO groups. Likewise, MLE-AR-14 also proved to be quite effective in ameliorating the level of GSH in ischemic rats. These results indicated that MLE-AR-14 remarkably attenuated neuronal oxidative stress. This antioxidant effect may be attributed to the presence of high amounts of quercetin[37] oxyresveratrol, 5,7-dihydroxycoumarin 7-methyl ether, cyanidin 3-glucoside[38], cyaniding 3-rutinoside[39] , Mulberroside A[40] etc. in the mulberry extract.

Neurons die under ischemic conditions when delivery of oxygen and glucose are not sufficient to meet their metabolic demands for a long period. The histologically defined infarct area in the brain indicates the end point of ischemic damage[41]. However, treatment with MLE-AR-14 as well as resveratrol proved to be effective in decreasing the brain infarct size as displayed by TTC staining.

Histopathological studies also reveal that the ischemic core region undergo irreversible process i.e. necrosis giving rise to morphological injury like development of eosinphilic cytoplasm and shrunken nuclei in more than 80% of neurons after vascular occlusion[42]. However, the ischemic penumbra, the brain areas situated at a larger distance to the occluded vessel exhibit apoptosis with only mild ischemic damage with a possibility of reversal of the ischemic cellular damages. The MCAO induced rats in our study, showed a high extent of cellular ischemic damage in the apoptotic region. However, the damage was reverted by the administration of MLE-AR-14 as revealed by HE and Cresyl violet staining.

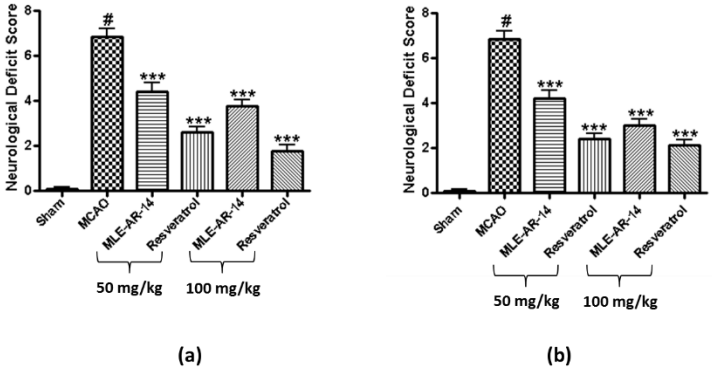

Figure.1.The effect of (a)1 hr pre-treatment and (b) 6 hr post-treatment with MLE-AR-14 and resveratrol at a dose of 50 mg/kg and 100 mg/kg p.o., on neurological deficit scores in rats. #p＜0.001 compared with the sham and ***p＜0.001 compared with MCAO. (n=8-10 animals each)

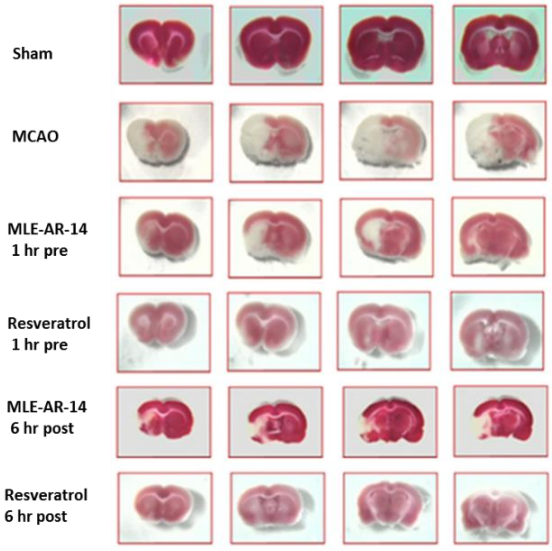

Figure.2a.TTC stained brain sections showing area of infarction in MCAO, Pre- and post-treatment with MLE-AR-14 and resveratrol rats. Both extract and resveratrol significantly reduced brain infarction at 24 hours post reperfusion

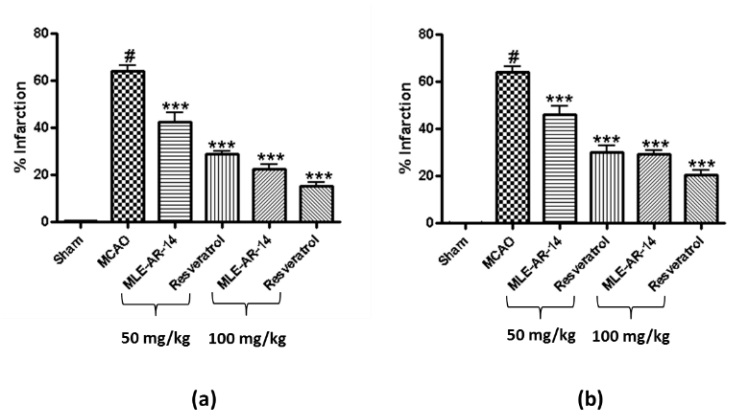

Fig.2b.The effect of (a)1 hr pre-treatment and (b) 6 hr post-treatment with MLE-AR-14 and resveratrol at a dose of 50 mg/kg and 100 mg/kg p.o., on brain infarction in rats. #p<0.001 compared with the sham and ***p<0.001 compared with MCAO. (n=8-10 animals each)

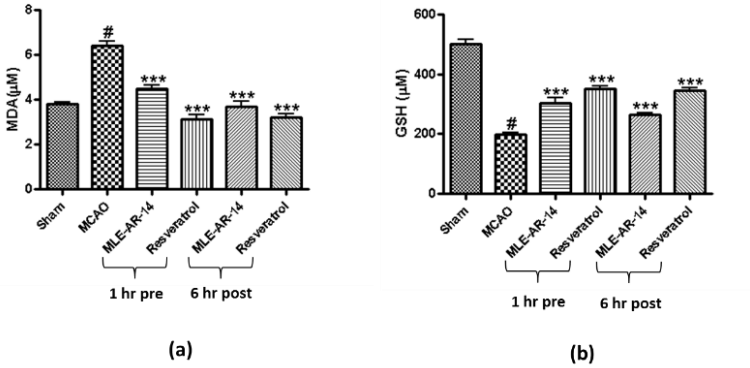

Fig.3.The effect of 1 hr pre-treatment and 6 hr post treatment with MLE-AR-14 and resveratrol at a dose of 100 mg/kg p.o., on (a) MDA level and (b) GSH level in rats. #p<0.001 compared with the sham and ***p<0.001 compared with MCAO. (n=8-10 animals each)

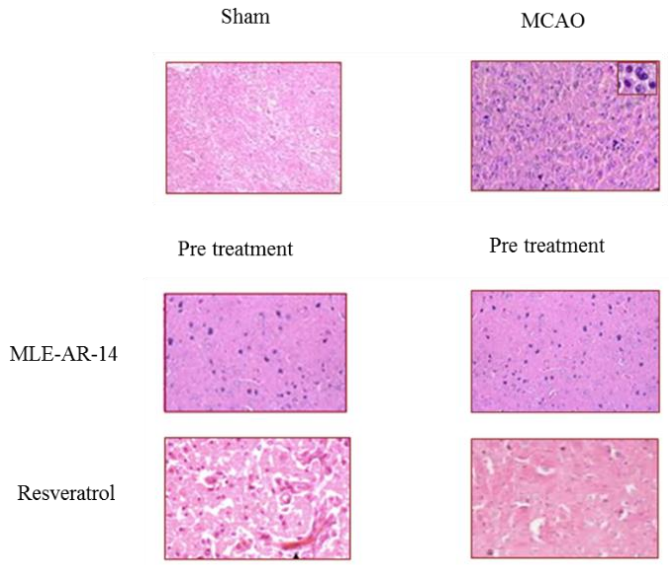

Fig.4.Photomicrographs of HE stained 6 µm brain sections showing ischemic brain damaged area of MCAO treated, pre-treated (1 hour) and post-treated (6 hour) with MLE-AR-14 and resveratrol in rats at a dose of 100 mg/kg p.o.as compared to the sham operated group

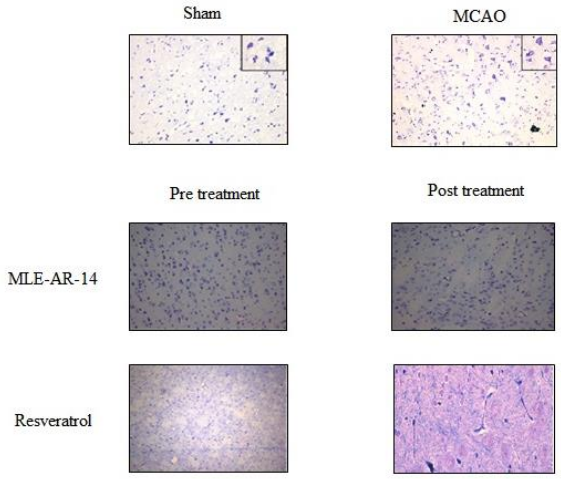

Fig.5.The cresyl violet stained representative photomicrographs showing the brain damaged area of rats pre-treated (1 hour) and post treated (6 hour) with resveratrol and MLE-AR-14 at a dose of 100 mg/kg p.o. as compared to the sham operated group

4. CONCLUSIONS

Mulberry plant is rich source of chemicals with potent anti-oxidant activity and has been traditionally used for various ailments. Therefore, it was selected to investigate its neuroprotective effect in focal cerebral ischemia. The aqueous mulberry leaf extract offered both preventive as well as curative anti stroke potential. The neuroprotective effect was showcaused by amelioration in oxidative damage, betterment in behavioral dysfunction, reduction in cerebral infarct. The neuroprotective effect of MLE-AR-14 may be attributed owing to its potent antioxidant activity.

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Anti-Oxidant activity of various leaf extracts of mulberry species in rotenone induced oxidative stress model of rat

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ABSTRACT

The oxidative stress is an important cause of a number of diseases in humans, which can be prevented or reduced by timely intervention of anti-oxidants. Rotenone induced oxidative stress model of rat has been used to assess the anti-oxidant profile of nine freshly prepared aqueous extracts of mulberry leaf. The results suggest that pre-treatment with four varieties S-146, AR-14, BR-2 and S-1 have shown highly significant anti-oxidant activity by attenuating both malondialdehyde (50.49%, 36.14%, 41.36%, 37.13%) and superoxide dismutase (54.01%, 40.18%, 34.82%, 29.74%) levels in the brain of experimental animals. The remaining extracts were relatively less active. The most significant ones AR-14 and S-146 are being tried in focal cerebral ischemia model of rat to assess their neuroprotective (anti-oxidant) activity. The potent mulberry extracts can be used as a dietary supplement to suppress the oxidative stress.

KEY WORDS: Anti-oxidant, MDA, SOD, Rotenone, Mulberry.

1. INTRODUCTION

Oxidative stress is produced as a result of imbalance between highly reactive oxygen species (ROS) produced in the cell and the ability of endogenous anti-oxidant system to scavenge them. The cellular free radicals (hydroxyl (OH·), superoxide (O_2^{-}·) and nitric monoxide (NO·) can interact with macromolecules, membrane lipids, proteins, enzymes and DNA, causing cell damage. This oxidative damage can result in the development of a wide range of ailments such as atherosclerosis, cancer, diabetes, rheumatoid arthritis, chronic inflammation, cardiovascular diseases, stroke, aging and other degenerative diseases in humans(Kathy, 2003;Maritim, 2003).In normal physiological conditions, the free radicals are immediately consumed by the body's naturally produced antioxidants like superoxide dismutase, catalase and peroxidase enzymes. However, when the level of free radicals becomes quite high in the body, the endogenous antioxidant system is unable to neutralize the excess of free radicals produced. At the same time, antioxidants, such as glutathione, vitamin E, vitamin C, also help in regulating the ROS generated.

Diet is a major source of antioxidants, and medicinal herbs are rich source of antioxidants. Therefore, consumption of fruits and vegetables lower the risk of several diseases, caused by oxidative stress (Willett, 2002). Their antioxidant activity is owing to the presence of phytochemicals, such as polyphenols, carotenoids and vitamin E and C. The substantial evidence is available supporting the role of dietary antioxidants and polyphenolic compounds from botanical sources like green tea extract, G*inkgo biloba* extract and red wine/resveratrol to combat oxidative stress (Bridi, 2001). These antioxidants act as a major defense against free radical-mediated toxicity. Hence, antioxidants as supplements are now being looked upon as persuasive therapeutics against oxidative stress and related damage. Based on the growing interest in mulberry due to its nutritional value, and biological activities, it was decided to study the *in vivo*anti-oxidant activity of mulberry leaf extracts.

Mulberry belongs to the family *moraceae*, is a perennial herb with highly branching shoot systems. It is one of the herbs which are used in medicine since ancient times due to its chemical novelty and pharmacological functions. The plant is reported to contain the phytoconstituents as tannins, phytosterols, saponins, triterpenes, flavanoids, morusimic acid, anthocyanins, anthroquinones, glycosides and oleanolic acid as the main active constituents (Nomura, 1983).The medicinal uses of the plant reported so far include analgesic, antiasthmatic, antirheumatic, antitussive, astringent, diaphoretic, diuretic, emollient and expectorant, hypotensive and as brain tonic (Fukai, 1985).It was reported that higher amount of quercetin in the leaves of mulberry isresponsible for reduction of oxidation process (Enkhmaa, 2005; Chen and Li, 2007).Hypoglycemic activity of dried leaves of mulberry hasbeen alsoreported (Lemus,1999). The ethanolicas well as the aqueous extract of mulberry leaves contains oxyresveratrol and 5,7-dihydroxycoumarin 7-methyl ether which scavenge superoxide and have antioxidant potential (Oh,2002).The plant leaf extract has also been used in various studies to prove its neuroprotective effect as well (Tong, 2006).

Therefore, a search for novel natural resource with anti-oxidant activity is of particular interest. The study is aimed to evaluate the anti-oxidant property of aqueous mulberry leaf extracts using rotenone induced oxidative stress model in rats.

2. MATERIALS AND METHODS

Animals: Adult male Sprague Dawley rats, 200±10g, were used in the experiments. The animals were procured from the National Laboratory Animal Centre, CSIR-Central Drug Research Institute, Lucknow. All animal experiments

were done strictly in compliance with the guidelines for care and use of animals after necessary approval of the Institutional Animal Ethical Committee. Rats were allowed food and water *ad libitum* throughout the experiment.

Preparation of aqueous leaf extracts: Fresh mulberry leaves of both (*Morusalba* and *Morusindica*) nine varieties-V-1, S-1, S-13, S-146, AR-12, AR-14, S-1635, BR-2 and TR-10, were obtained from mulberry plantation in Babasaheb Bhimrao Ambedkar University, Lucknow. The leaves were cleaned, dried and grinded into a fine powder. The resulting powder was then passed through an 80-mesh sieve and the powder was kept in a sealed aluminum foil at 4 °C, till further use.

The aqueous extract of powder was prepared following the standard method (Katsube 2006). Briefly, 2 g of mulberry leaves powder was soaked in 200 ml of boiling water for 20 minutes. The mixture was cooled at room temperature before being filtered through Whatman no.1 filter paper and then lyophilized. The freeze-dried solid extract was transferred in plastic tubes and storedat −20 °Cto protect from light. The extract was re-dissolved in double distilled water in desired concentration prior to use in all the experiments.

Rotenone induced oxidative stress model: Rotenone, a phyto-toxin obtained from roots of *Derris sp* belonging to *Leguminosae* family is a broad-spectrum pesticide and is generally used to induce oxidative stress. Rotenone interferes with the electron transport chain in mitochondria thus affecting the ATP synthesis. It causes neurotoxicity by inhibiting the oxidation of NADH to NAD, which blocks the oxidation of substrates such as glutamate, α-ketoglutarate and pyruvate, thereby generating ROS and other free radicals leading to various adverse effects in cellular physiology (Uversky, 2004).

Treatment schedule: Eleven experimental groups comprising of 8-10 male rats were used in the study. Group I wastaken as sham. Animals of Group II were subjected to oxidative stress by rotenone treatment alone. Rotenone was dissolved in DMSO and administered orally at a dose of 75 mg/kg to induce oxidative stress model in rats. The group III to XI animals received pretreatment with nine varieties of mulberry leaf extracts (100mg/kg, p.o.)Each one hour prior to challenge with same dose of rotenone. Animals were sacrificed after one hour post rotenone challenge and brain tissue was gently removed in chilled ice-cold conditions for further studies.

Estimation of oxidative stress markers: Malondialdehyde (MDA) and Superoxide Dismutase (SOD) were monitored in the brain homogenates of experimental animals as *in vivo* biomarker for oxidative stress. The procedure of estimating both the biomarker is detailed below.

Malondialdehyde estimation in brain: Biological specimens contain a mixture of thiobarbituric acid reactive substances (TBARS), including lipid hydro-peroxides and aldehydes, which gets elevated as a result of oxidative stress. Since, brain tissue is rich in lipid content hence vulnerable to lipid peroxidation. TBARS return to normal level over a period of time, depending upon the presence of anti-oxidants. In practice, TBARS are expressed in terms of malondialdehyde (MDA) equivalents. As an important biomarker of lipid peroxidation, The MDA level was estimated using the standard protocol (Okhawa 1979) as an important biomarker of lipid peroxidation. It is determined based on its reaction with two molecules of TBA with one molecule of MDA and measured in acidic solution at 532 to 535 nm. The MDA concentration (nmol/mg protein) in the samples was extrapolated from the standard curveobtained by plotting the optical density of the standard MDA concentrations.

Superoxide Dismutase estimation in brain: SOD was estimated using the standard method (Fridovich and McCord, 1969). The SOD activity is based on the inhibition of auto-oxidation of epinephrine. One unit is equal to the amount of the enzyme required to inhibit the auto-oxidation of epinephrine by 50%.

Statistical analysis: Data was analyzed by one-way analysis of variance (ANOVA).Newman – Keuls multiple comparison test was performed for comparison among different groups. The P value $*P<0.05$, $**P<0.01$ and $***P<0.001$ were considered statistically significant.

3. RESULTS

The anti-oxidant activity of nine mulberry extracts has been monitored in terms of attenuation in the MDA and SOD levels in the brain homogenates of rats challenged with rotenone. This was done to select potent anti-oxidant extracts for the bio-evaluation in the disease models exhibiting profound oxidative stress. The results have been summarized as percentage decrease in MDA and SOD contents with each test group (Table 1).

Effect on MDA levels: Rotenone induced the ROS generation led to brain oxidative stress by lipid peroxidation as revealed by significant increase138% in MDA content. The results indicate that one hour pre-treatment with mulberry leaf extracts prior to rotenone challenge in general caused significant reduction in MDA levels from 13.30 to 50.49%. The maximum reducing effect was observed with extractS-146which attenuated the MDA levels by 50.49% followed by 41.36% by BR-2. Further the decrease of 36.14% and 37.13% in MDA was also observed with extract AR-14 and S-1 respectively. Whereas, the reduction in MDAby the other extracts V-1, S-13, AR-12, S-1635, TR-10 varied considerably and was less pronounced (Fig.1).

Effect on SOD levels: Superoxide serves animportant role in signaling processes, cell division and lipid peroxidation. However, when overproduced, the free radical can even initiate lipid peroxidation, protein oxidation, and DNA damage, leading to cell dysfunction and death by apoptosis or necrosis. A reduction in superoxide level

offers a defense against these cellular damages. As the SOD levels which got elevated due to the increased oxidative stress induced by rotenone were significantly reduced bypre-treatment with mulberry leaf extracts. There wassignificant reduction (54.01 %) in the brain of rats treated with S-146, further AR-14 treated rats showed a reduction by 40.18%. There was also significant 34.82 % and 29.74 % reduction in SOD content by BR-2 and S-1 respectively. The other mulberry varieties V-1, S-13, AR-12, S-1635, TR-10 however, had also significant effect but it was far less compared to S-146 and AR-14(Fig.2).

DISCUSSION

Table.1. A comparative effect of pre-treatment with mulberry leaf extracts (100 mg/kg p.o.) one hour prior to challenge with rotenone (75 mg/kg p.o.) on MDA and SOD levels in rat brain.(n=8-10 each).

Extract	% Change in brain tissue	
	MDA	**SOD**
Rotenone	138.30↑	189.62↑
V-1	16.36↓	13.89↓
S-1	**37.13↓**	**29.74↓**
S-13	22.12↓	15.54↓
S-146	**50.49↓**	**54.01↓**
AR-12	22.54↓	21.38↓
AR-14	**36.14↓**	**40.18↓**
S-1635	29.22↓	8.64↓
BR-2	**41.36↓**	**34.82↓**
TR-10	13.30↓	20.78↓

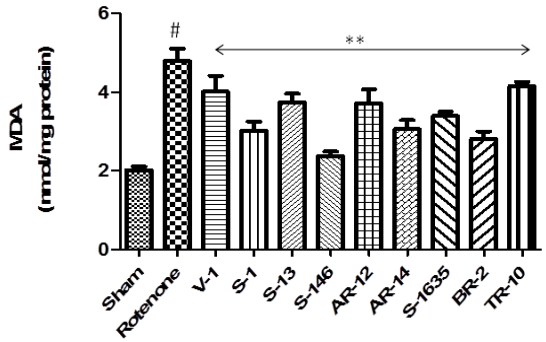

Figure.1. Effect of pretreatment with mulberry leaf extracts (100 mg/kg p. o.) one hour prior to rotenone challenge on MDA levels in brain homogenate of rats. Significance
 # P<0.001 vssham, *** P<0.001 vsrotenone

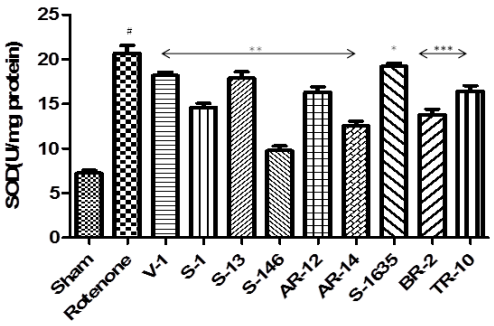

Figure.2. Effect of pretreatment with mulberry leaf extracts (100 mg/kg p.o.) one hour prior to rotenone challenge on SOD levels in brain homogenate of rats. (n=8-10 each).Significance # P<0.001 vssham,* P<0.001,**P<0.01, *P<0.05vsrotenone**

It is now well recognized that anti-oxidants can be therapeutically used to counter cellular damage caused by oxidative stress as well as these can also successfully scavenge free radicals to prevent further damage. The free radicals are chemical species produced in the body which contains one or more unpaired electrons and therefore are highly unstable and cause damage to other molecules by extracting electrons from them to stabilize themselves. The reactive oxygen species (ROS) are formed '*in vivo*'such as superoxide anion radical (O_2^-) and hydrogen peroxide, which produces hydroxyl radicals. The latter is highly reactive and mainly responsible for oxidative stress. The defense against the radicals is provided by a number of antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT). SOD converts $O_2^-$to H_2O_2 and GPx and CAT convert H_2O_2toH_2O. The enzymatic anti-oxidants along with other non-enzymatic anti-oxidants such as ascorbic acid, vitamin-E and glutathione tend also reduce levels of ROS. But when there is an imbalance due to overproduction of free radicals induced by exposure to external oxidant substances or a decrease in anti-oxidant defenses, the oxidative stress results causing damage to lipid, protein and DNA which eventually leads to cell death either via apoptosis or necrosis. Oxidative stress is associated with number of diseases including diabetes, inflammation, arthritis, dyslipidemia, cardiovascular diseases including cerebral ischemia (Mehta, 2008, Raghubir, 2010).

Antioxidants play an important role in protecting damage caused by oxidative stress. Plants having phenolic compounds are reported to possess antioxidant properties. The present study was designed to investigate the antioxidant properties of the mulberry leaf extract which contains phenolics, flavonoids, glycosides, saponins, vitamin, and minerals. The available literature indicates that different parts of the plant have been used to treat a variety of diseases. The plant has found useful in the treatment of a number of diseases as reported earlier (Katsube,

2006; Kang, 2006) Moreover, it has also been reported to possess several pharmacological activities such as antioxidative, antiinflammtory (Kim, 1998) and antihyperlipidemic (Kim, 2001) as well.

The pharmacological activities may be due to the presence of polyphenolic compounds in the plant mainly the anthocyanins. The anthocyanins are considered to bevery good antioxidants and their high activity can be attributed to their peculiarstructure, having the oxonium ion in the C ring (van Acker, 1996).Recent study has shown that anthocyaninsand the other phenolics possess high antioxidant activity (Linghong, 2012) which maybe well compared with the activity of the established antioxidants α-tocopherol (Kähkönen, 2003).

Our study with mulberry leaf extracts had displayed anti-oxidant property against rotenone induced oxidative stress.It is interesting to find that some of the extracts exhibited highly significant anti-oxidant activity as evidenced by reduced level of MDA and SOD in the brain tissue of rats challenged with oxidative marker, rotenone. Our results indicated that aqueous mulberry leaf extract exhibited strong antioxidant property this is confirmatory with the results obtained by Naowaboot, 2009.

The broad range of antioxidant activity of these extracts indicates the potential of the mulberry plant as a rich source of pharmaceuticals with potential to reduce oxidative stress. Therefore, these two varieties AR-14, S-146 of mulberry leaf extract seem to possess potent antioxidant activity in *in vivo* rotenone-induced oxidative model in rat. Hence, this plant could serve as an effective free radical scavenger which may be further used to reduce the oxidative stress induced by certain diseases. We have found the significant neuroprotective activity of one of the extracts, MLE-AR-14 in focal cerebral ischemia model of rats(Samuel, 2016).Further, the neuroprotective efficacy of the extract S-146 is being investigated in focal cerebral ischemia model in rats because the cerebral ischemia is known to cause severe oxidative stress (Mehta , 2008, Raghubir, 2010).

4. CONCLUSION

Thus the study has provided a good lead about the use of selected extracts for overall reducing oxidative stress associated with number of diseases. The extracts with potent anti-ischemic activity can be used as neuroprotectants. Hence the mulberry leaves can be safely used as food supplement and even can be therapeutically exploited.

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