Antioxidant effects of flavonoids from Ceylon green tea on stroke: a biochemical and pharmacological study

By

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"The work described in this thesis was carried out by me under the supervision of Dr. Ranil De Silva and Prof. Yi Zhun Zhu and a report on this has not been submitted in whole or in part to any university or any other institution for another Degree."

...................................

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"We certify that the above statement made by the candidate is true and that this thesis is suitable for submission to the University for the purpose of evaluation."

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LIST OF SYMBOLS / ABBREVIATIONS:

8-OHdG: 8-hydroxy-2'-deoxyguanosine

ABTS: 2,2' azinobis (3ethylbenothiazoline6sulfonic acid)

Bax: Bcl-2 associated protein X

Bcl-2: B-cell chronic lymphocytic leukaemia/lymphoma 2

BSA: Bovine serum albumin

CVD: cerebrovascular disease

Ca$$^{2+}$$: Calcium

CAT: catalase

CO: Carbon monoxide

DAB: 3, 3' diaminobenzidine tetrahydrochloride

DEPC: Diethyl pyrocarbonate

DMEM: Dulbecco’s Modified Eagle’s Media with 25mM HEPES

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

EDTA: Ethylenediaminetetraacetic acid

EGCG: epigallocatechin gallate

EGC: epigallocatechin

ECG: epicatechin gallate

EC: picatechin

EtBr: Ethidium bromide

FBS: Fetal bovine serum

GPx: glutathione peroxidase
GST: glutathione-S-transferase

GSH: Glutathione

H₂O₂: hydrogen peroxide

HCl: Hydrochloric acid

HBEC: human brain epithelial cells

HEPES: 4-(20hydroxyethyl)-1-piperazineethanesulfonic acid

HOCl: Hypochlorous acid

K: Rate constant

LDH: Lactate dehydrogenase

MI: Myocardial infarction

MIC-101TM: Modular Incubator Chamber

mRNA: Messenger ribonucleic acid

NADH: Reduced nicotinamide adenine dinucleotide

NO•: Nitric oxide

NO₂⁻: Inorganic nitrite

NMDA: N-methyl-D-aspartate

NNDPD: N, N-dimethyl-p-phenylendiammonium

ODS: oxygen derived species

PLP: Pyridoxal 5’-phosphate

Pyrogallol red: pyrogallosulphonephthalein

PBS: Phosphate-buffered Saline

PBS-Tx: Phosphate buffered saline-Triton X
PKG: Protein kinase G
PSA: Antibiotic-Antimycotic Solution
ROS: reactive oxygen species
RNA: ribonucleic acid
SAM: S-adenosyl-L-methionine
SEM: Standard error of the mean
SOD: superoxide dismutase
SNP: Sodium nitroprusside
TEAC: trolox equivalent antioxidant capacity
Tris: Tris(hydroxymethyl)-aminomethane
TCA: Trichloroacetic acid
UK: United Kingdom
UV: Ultraviolet
WHO: World Health Organization
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Most importantly, I hope that my project can offer more knowledge and information to the existing research for the scientific usage of Ceylon green tea and treatment of patients suffering from ischemic cerebral diseases like stroke. Although what I have done is definitely a minor contribution for the scientific research in the field of cerebral vascular diseases. I hope that in the future, I would like to continue research work in this field and hopefully discover more potential active ingredients from Ceylon green tea to prevent stroke.
Antioxidant effects of flavonoids from Ceylon green tea on stroke: a biochemical and pharmacological study

Sherry Shan Hong Huang

ABSTRACT

Stroke is one of the leading causes of death and long-time disability. In stroke, a reduced blood supply to the central system and the inadequate delivery oxygen to the brain results in hypoxia/ischemia. The flavonoids from Ceylon green tea (Dilmah) were extracted. In this project, an in vitro hypoxic model using Human Brain Epithelial Cells (HBEC) was studied with treatment of the tea extract before inducing hypoxia. We have tested the hypothesis that flavonoids from Ceylon green tea can reduce oxidative stress in hypoxic cells through its antioxidant properties and its ability to reduce cell death. The biochemical antioxidant tests showed that the Ceylon green tea has 68%±2.8% inhibition property of scavenging of ABTS, similar to Chinese green tea: Qian Dao (82%±1.2%) and Bi Xue Chun (80%±1.2%). The Inhibition of Pyrogallol Red Bleaching by HOCl was examined too. The results also showed that Ceylon tea (79%±4.5%) has equal inhibiting property as Chinese green tea (Qian Dao 81%±4.4% and Bi Xue Chun 83%±3.3%). Both DNA from Ceylon green tea treated hypoxic and control cells (without hypoxia) was extracted. Using GC/MS (Gas Chromatography/Mass Spectrometer), DNA base products were
measured. With flavonoids treated group showed significant lower level of total DNA base products damage (1.13±0.42 nmol/100μg DNA) when compared to hypoxia group (1.53±0.36 nmol/100μg DNA). The flavonoids were also analyzed by LC/MS (Liquid Chromatograph/Mass Spectrometer) to separate the compounds and identify the main compounds which might play an important role of antioxidant effect.

Losartan is one of the commonly used drugs with antioxidant effects to prevent further myocardial destruction. In the development of atherosclerosis, oxidation of low-density lipoprotein by free radicals is an important step. We compared Ceylon green tea (flavonoids) with it to demonstrate the antioxidant effects of Ceylon green tea.

The aim and objective of this project was thus to find out the in vitro antioxidant effects of flavonoids from Ceylon green tea on the cell viability of hypoxic human brain epithelial cells (HBEC), and measure the antioxidant enzyme activity and gene expression, including that of the proteins involved in apoptosis to compare with the Chinese green tea, as well as western drug (losartan).

Cell viability test was determined using trypan blue cell-exclusion method and lactate-dehydrogenase (LDH) assay. Both showed that flavonoids treated group in hypoxia, the cell viability was 29%±2.3% in the hypoxia control group but 41%±4.7% for flavonoids treated group and 39%±3.1% for losartan treated group. In LDH assay, flavonoids treated group had 75%±3.7% reducing of LDH release and 79%±3.5% in losartan treated group.

The flavonoids treated group significantly increased in antioxidant enzyme activity.
assays: the activity level of SOD (1.5±0.6 μmol/min/mg protein), CAT (0.61±0.06 μmol/min/mg protein), GPx (2.6±0.41 μmol/min/mg protein) and GST (6.0±2.4 μmol/min/mg protein) were significantly increased as compared with hypoxic control (0.5±0.52, 0.51±0.04, 1.2±0.35 and 3.1±1.6 μmol/min/mg protein respectively).

For the expression level of pro-apoptotic gene: Bax, Fas and Asp53, the result showed that the hypoxia cells after treatment, flavonoids treated group was significantly reduced the expression of the pro-apoptotic genes of Bax, Fas and Asp53. Meanwhile for the expression level of anti-apoptotic gene: Bcl-2, the result showed that the expression was stronger in flavonoids treated group when compared to hypoxia group. This would mean that the flavonoids from Ceylon green tea were able to reduce the amount of apoptosis after inducing hypoxia. Also, the expression levels of the pro-apoptotic genes were down-regulated and the expression of the anti-apoptotic gene was up-regulated, these would result in higher cell viability.

It also significantly reduced in immunoactivities of the protein products of BAX (1.12±0.15-fold), Fas (1.40±0.30-fold), Asp53 (1.13±0.03-fold) and Bcl-2 (0.88±0.08-fold) when compared to hypoxia control (1.55±0.25-fold, 1.66±0.20-fold, 1.52±0.15-fold and 0.61±0.13-fold respectively). These results showed that pro-apoptotic proteins Bax, Fas and Asp53 have been detected dramatically more in hypoxia group, but less detected in flavonoids treated group. Weak signal of Bcl-2 was detected in hypoxia group and positive Bcl-2 staining was detected in hypoxia with flavonoids treated group. It indicated the down-regulation of pro-apoptotic proteins and up-regulation of anti-apoptotic protein during hypoxia.
The least nuclear green fluorescence was observed in TUNEL staining assay, it indicated less apoptosis was found in flavonoids treated group as well.

The study demonstrated that frequently drink of Ceylon green tea is useful to prevent stroke.
CHAPTER 1

INTRODUCTION

1.1 Stroke

From the year 2002's report of WHO that stroke rapidly developing clinical signs of focal or global disturbance of cerebral function, with symptoms lasting 24 hours or longer or leading to death with no apparent cause other than of vascular origin. Stroke is a cerebrovascular accident (CVA) that affects 15 million worldwide every year, 10% of total deaths globally (Figure 1). Of these, five million victims die and another five million are left permanently disabled, making it the 3 most common cause of death in developed countries as seen over past quarter century (WHO 2002).

Stroke can occur in two forms, either ischemic (75%), in which a blood vessel supplying the brain is blocked, or hemorrhagic (25%), where there is bleeding into or around the brain (Figure 2). Hypertension, or high blood pressure, is a main cause of stroke. When blood pressure increases, small capillaries may be subjected to a greater force by the flowing blood. If the force exerted by the blood is too high for the one-celled thick capillary walls in the brain, the walls might give way and break, causing ischemic stroke. Stroke is defined as rapidly developing clinical signs of focal or global disturbance of cerebral function, with symptoms lasting 24 hours or longer or leading to death with no apparent cause other than of vascular origin.

The most popular classes of drugs used to prevent or treat stroke are antithrombotic
Percentages and Numbers of Deaths Worldwide (2002)
Total Deaths as 57 million

- Other Causes, 27%
- Stroke, 10%
- Coronary Heart Disease, 13%
- Cancer, 12%
- Injuries, 9%
- HIV/AIDS, 5%
- Respiratory Infections, 7%
- Pulmonary Disease, 5%

Figure 1: Percentages and numbers of deaths worldwide in year 2002

Figure 2: Ischemic Stroke and hemorrhagic stroke
like aspirin, losartan or Clopidogrel or thrombolytic as neuro-protective agents (NINDS and MOH 2003). Recently, angiotensin II receptor type antagonists showed also potential for the treatment of hypertension (Sun et al., 2003; Lu et al., 2004; Zhu et al., 2000).

So far there has been little or no emphasis on the usage of herbs and naturally occurring drugs to treat or prevent stroke, with most common treatment regimes consisting of western synthetic drugs. Nevertheless, homeopathic therapies like traditional Chinese medicine (TCM) makes use of natural products like specific herbal extracts or a combination there of to treat or prevent stroke (Loh et al., 2006 and Low et al., 2007).

The free radical scavenging effects or antioxidant capacities found in such natural products showcases their potential as neuro-protective agents, which help to prevent or alleviate the symptoms of stroke. This study aims to find out the effects of Ceylon green tea extract on stroke, and hopefully be able to benefit stroke patients and those at risk.

1.2 Oxidative Stress in Ischemic Stroke

Oxidative stress has been clearly established to contribute in ischemic damage (Figure 3) (Gilgun-Sherki et al., 2002). Unbalanced cellular production of free radicals and the ability of cells to defend against them are referred to as oxidative stress (OS).

Altered oxygen utilization and/or increased formation of reactive oxygen species (ROS) contribute to cardiovascular disease progression. ROS can be generated as a consequence of the metabolic changes that occur following the re-oxygenation of
ischemic myocardium (Tarr and Samson, 1993). But it does not mean that they cannot be formed before reperfusion when there is lack of oxygen. On the contrary, the metabolic derangement that occurs during ischemia might predispose to the formation of free radicals from residual molecular oxygen (Ferrari et al., 1998; Lefer et al., 2000; Wattanapitayakul et al., 2001). Due to their highly reactive and toxic properties, free radicals can exacerbate the degree of myocardial damage sustained by the ischemic myocardium. The contribution of oxidative stress after ischemic injury can lead to mitochondrial dysfunction, excitotoxicity, lipid peroxidation, inflammation and so forth and it is also a potential contributor to acute central nervous system (CNS) injury. Radicals can cause damage to cardinal cellular components such as lipids, proteins, and nucleic acids (e.g., DNA), leading to subsequent cell death by modes of necrosis or apoptosis. The damage can become more widespread due to weakened cellular antioxidant defense systems.

Hypoxia is one of the causes of acute central nervous system damage. Two parts of an ischemic gradient: the central ischemic zone and the peripheral zone are formed in cerebral ischemia. In the penumbra, functional alterations occur in the neurons and glial cells. Neurons are most vulnerable to hypoxia due to their dependence on the oxidative metabolism of glucose for energy. The principal of stroke is extremely complex and involve free radical-mediated toxicity, energy failure, loss of cell ion homeostasis, acidosis, increased intracellular calcium and pathological permeability of blood brain barrier (in part of the CNS injuries). This can lead to ischemic necrosis, which occurs in
the severely ischemic regions and is associated with loss of calcium and glutamate homeostasis. It can also lead to apoptosis, which is more likely to occur in the

Figure 3: Cellular mechanisms that may be involved in acute ischemia and CNS injury (Adapted from Gilgun-Sherki et al., 2002).
moderately ischemic regions, evolves more slowly, and depends on the activation of a sequence of genes (Dirnagl et al., 1999; Gennarelli, 1997 and Pulsinelli, 1992).

1.3 Formation of Reactive Oxygen Species (ROS)

Molecular oxygen plays a key role in many of the metabolic processes associated with aerobic existence. But it also leads to the formation of reactive oxygen intermediates: oxygen species that have either unpaired electrons (i.e. superoxide, hydroxyl radical) or the ability to attract electrons from other molecules (i.e. hydrogen peroxide) (Ferrari et al., 1998).

If a single electron is added to the ground-state oxygen (O₂) molecule (which is the most stable-state one), the product is the superoxide radical (O₂⁻) with only one unpaired electron. O₂⁻ is a highly reactive entity since it rapidly reacts with different metabolic enzymes as well as cations such as iron and copper (Dhalla et al., 2000). Addition of another two electrons to the peroxide ion (O₂⁻), which is formed after another electron gives O₂⁻, will form 2 O²⁻ species. Usually in biological systems the two-electron reduction product of oxygen is hydrogen peroxide (H₂O₂) that is easily diffusible within and between cells. Since the O-O bond is relatively weak, hydrogen peroxide decomposes easily to give the hydroxyl radical (OH⁻), the most reactive species that induce significant damage in the cell especially with limited diffusion capacity (Halliwell and Gutteridge, 1989). To summarize:

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6
Such intermediates react with cellular macromolecules, either damaging them directly or setting in motion a chain reaction resulting in extensive damage to cellular structures, such as membranes.

1.4 Damaging effects of Oxidative stress and Reactive Oxygen Species (ROS)

It has been reported that high oxidative stress is involved in ischemic diseases, which includes ischemic stroke (Tan et al., 2003; Ji et al. 2004; Sun et al., 2002; Sun et al., 2000), inflammation (Wang et al., 2004; Korantzopoulos et al., 2004) and neurodegenerative diseases (Emerit et al., 2004; Jellinger et al., 2003). Oxidative stress is the state in which the production of oxygen derived species (ODS) or reactive oxygen species (ROS) is not balanced by the antioxidant defense system level in the body, of which one factor leading to this imbalance is the increase in the concentration of oxygen. These ROS have the ability to attract electrons from other molecules (e.g. H$_2$O$_2$) or have unpaired electrons (O$_2^*$, OH$^*$). Their main damage to cells results from the ROS-induced alteration of macromolecules such as polyunsaturated fatty acids in membrane lipids, essential proteins and DNA (Figure 4). During ischemia, increased intracellular Ca may
activate phospholipase C and stimulate arachidonic acid metabolism, whose intermediates also generate oxygen free radicals. In the extra cellular environment, leukocytes generate large amounts of $O_2$ and $H_2O_2$ in the inflammatory response to ischemia, including ischemic stroke. These free radicals damage cellular DNA or other

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**Figure 4:** Mechanisms of cell damage by oxidative stress (Halliwell B., 1992).
macro-molecules through either direct or indirect means, which includes damaging the basal structure of cellular membranes or DNA. One of these reactions involves the conversion of guanine (a DNA base) into 8-hydroxyguanine by OH (Figure 5), quantities of which can be measured by gas chromatograph–mass spectrometry (GCMS) (Gutterridge and Halliwell, 1994).

Figure 5: Conversion of guanine into 8-OH guanine

GCMS is used widely to quantify DNA base damage and hence the level of oxidative stress by identifying and quantifying oxidized DNA base products. With higher levels indicating greater DNA damage. During ischemic stroke, higher levels of oxygen free radicals results in greater DNA damage and subsequent cellular damage in brain cells. Hence one method of cerebral protection, either as a preventative measure against stroke or during treatment, could involve the consumption of Ceylon green tea extracts.
that have shown good antioxidant activity as a complement to western drug therapy (Zhu et al., 2004). In this study, purified flavonoids extracted from Ceylon green tea were analyzed for their antioxidant scavenging properties on Pyrogallol Red Bleaching and 2,2’Azinobis(3EthylbenzThiazoline6Sulfonic Acid) (ABTS). It was also analyzed for its antioxidant capacities and its overall effectiveness on preventing DNA damage as compared to the western drug (losartan).

1.5 Antioxidant Defense System in Cells

As ROS are formed in the process of normal cellular metabolism, for this reason, aerobic cells have antioxidant defense mechanisms to protect them from oxidative stress (Facchinetti et al., 1998). The damaging effects of these particles are kept in check by an evolved system of antioxidants which can consist of small molecules as well as enzymes. Molecular free radical scavengers like glutathione (GSH), uric acid, β-carotenes (vitamin A), ascorbic acid (vitamin C) and trolox (vitamin E) can directly react with free radicals and convert them into products that are more stable, less reactive and thus less toxic (Ip et al., 2001).

Free radicals and reactive oxygen species can also be dealt with a set of endogenous antioxidant defense enzymes which consist of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) which serve to detoxify these aggressive moieties. Superoxide dismutases are a group of metalloenzymes that catalyze the dismutation of two molecules of superoxide anion
(O$_2^-$), to a molecule of hydrogen peroxide (H$_2$O$_2$) and a molecule of oxygen. As superoxide radicals can react with hydrogen peroxide to form singlet oxygen and hydroxyl radicals which are even more reactive than superoxide or hydrogen peroxide, it is thought that SOD should be a critical and initial component in the cellular defense against oxygen radical stress (Freeman and Crapo, 1982). Catalase enzyme is found in the cell organelle, peroxisomes. This is the site of oxidation of fatty acids with the production of the by-product hydrogen peroxide. Catalase is responsible for converting hydrogen peroxide to water and oxygen. this is important as hydrogen peroxide can convert through the iron-catalyzed Haber-Weiss reaction to the hydroxyl radical (OH) which is the most reactive species that can induce significant damage to cells by initiating lipid peroxidation, protein oxidation and DNA damage (Chan, 1996; Zhu et al., 2004). Hydrogen peroxide can be detoxified to the relatively harmless water and oxygen by catalase in the mammalian cells or in the brain by GPx. (dependent on the metal selenium for its biosynthesis and activity) at the expense of reduced glutathione (Chan, 1996). Glutathione can be regenerated from oxidized GSH by GSH reductase in the presence of NADPH. Elimination of hydrogen peroxide is therefore critical to the efficacy of SOD in reducing oxidative stress (Lee et al., 2004). In other words, SOD in the absence of catalase or glutathione peroxidase to get rid of hydrogen peroxide is of little value. In addition, glutathione-S-transferases (GST) which are selenium-independent antioxidant enzymes, can also serve similar function as glutathione peroxidases by catalyzing the reduction of cellular organic or lipid hydroperoxides and
the conjugation of electrophilic xenobiotics with GSH to produce less toxic and more water-soluble derivatives for excretion (Ip et al., 2001). These reactions are depicted as shown in Figure 6.

Figure 6: Endogenous antioxidant enzyme defense against oxidative stress. SOD as superoxide dismutase; CAT as catalase; GPx as glutathione peroxidase; GSH as reduced glutathione and GSSG as oxidized glutathione.
1.6 Apoptosis can mediate cell death after hypoxia

Many studies have shown that increased oxidative stress can induce apoptosis (Li and Jackson, 2002). Apoptosis is a genetically controlled, tightly regulated bio-molecular process employed by cells to "commit suicide" during normal organism development and also when some subset of cells pose some potential dangers to neighboring cells (Lopez-Neblina et al., 2005). The link between both oxidative stress and apoptosis is demonstrated in experiments which showed that a wide range of antioxidants can block the apoptosis that is induced directly in various cell types after exposure to oxidative stress-causing agents, thus providing evidence that ROS may be one of the factors responsible for activating apoptosis. Further evidence is provided by the inhibition of DNA fragmentation and PARP cleavage by antioxidants both of which are characteristic of apoptosis (Li and Jackson, 2002). Several mechanisms have been proposed for oxidative stress-mediated apoptosis. One of this is that DNA damage in the forms of base modifications, base free site, single and double-strand breaks and crosslinks inflicted by ROS can initiate an apoptotic response (Halliwell and Auroma, 1991) by eliciting the activation of poly-ADP-ribose transferase which is responsible for the polymerization of ADP-ribose to protein, resulting in a rapid depletion of cellular NAD and NADH pools and the collapse of ATP stores leading to apoptosis (Buttke and Sandstrom, 1994). Membrane lipid peroxidation, the result of oxidative damage has also been implicated in the activation of apoptosis. Polyunsaturated fatty acids, such as arachidonic acid or their hydroperoxides can also increase ROS-mediated DNA
fragmentation, leading to cellular death (Lopez and Nebulina, 2005). Other possible mechanisms include loss of Ca\(^{2+}\) homeostasis, alterations of certain proteins and transcription factors.

1.7 Apoptosis, Necrosis and the Mitochondria

Apoptosis is a form of cell death, which results from a precise genetically controlled program of nuclear and cytoplasmic changes with defined morphological and biochemical features. It is the major cell death pathway used to remove unwanted and harmful cells in a “clean or silent” manner during embryonic development, tissue homeostasis and immune regulation (Ellis et al., 1991). In apoptosis, cells are “neatly” engulfed by other cells, triggering no inflammation. Hallmarks of this type of cell death induce cell shrinkage while maintaining membrane integrity, extensive condensation of chromatin, DNA laddering and the formation of apoptotic bodies. Caspases, a conserved family of cystein proteases, are involved in the most of the observed morphological changes during apoptosis (Lamkanfi at al., 2002). However, there are also caspase independent apoptotic-like cell death pathways that may resemble necrosis (Leist and Jaattela, 2001; Holler at al., 2000).

Initiation of apoptosis can be form two pathways: the extrinsic apoptotic pathway starting with the aggregation of death receptors (Beyaert et al., 2002), and the intrinsic apoptotic pathway starting with the release of mitochondrial factors in response to various stimuli, such as growth factor withdrawal, UV irradiation and cytotoxic drugs.
(Festjens et al., 2004). The mitochondrial pathway amplifies the apoptotic signal in the extrinsic apoptotic pathway. It is also where the intrinsic and extrinsic pathways converge upon. Hence, the important role plays by the mitochondria and the Bcl-2 family members.

Necrosis often occurs in the circumstances of great toxic stimuli, where there is a rapid loss of plasma membrane integrity, cytoplasmic swelling, organelle breakdown, absence of DNA ladder, spillage of the intracellular contents into extra cellular environment (Fiers et al., 1999) and usually associated with inflammation (Proskuryakov et al., 2003).

In both apoptosis and necrosis, the mitochondria participate in critical signaling pathways (Green and Kroemer, 2004; Saelens et al., 2004; Kowaltowski et al., 2001). When a cell dies, there is a loss of the mitochondrial membrane potential, a crucial event occurring in the mitochondria. This loss of mitochondrial membrane potentials due to either the opening of high conductance permeability transition pores resulting in organelle swelling and membrane rupture (Kim et al., 2003; Wolter et al., 1997) or through the activation and subsequent outer mitochondrial membrane insertion of cytoplasmic pore forming Bcl-2 family proteins, such as Bcl-2-associated X protein (Bax) (Epand et al., 2002). In addition, it is also known that the exposure of the Bax protein's constitutively occluded N-terminal epitope is necessary for the mitochondrial targeting of Bax which consequently leads to the release of intra-mitochondrial proapoptotic proteins and cell death (Epand et al., 2002). Downstream of these
mitochondrial events can lead to either caspase-dependent apoptosis (involving cytochrome c) or caspase-independent mechanisms involving apoptosis inducing factor (AIF) and endonuclease G (EndoG) (Saelens et al., 2004).

1.8 Antioxidant

The term “antioxidant” is widely used but rarely defined. One suggested definition is that an antioxidant is a substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate (Halliwell, 1995).

Given the role of oxidative stress in hypoxia/ischemia-induced cell injury, ROS and free radicals targeting may serve as therapeutic strategies. Recent research have revealed that natural products may have antioxidant effects and their use could be beneficial in the treatment of a range of oxidative related diseases including ischemic diseases, inflammation and neurodegenerative diseases (Zhu et al., 2004). Generally, the natural products may alleviate oxidative stress in two aspects: by enhancing the activity of original natural antioxidants and neutralize oxygen species by non-enzymatic mechanisms, although the exact mechanisms which offer protection against oxidative stress remain to be elucidated.

In almost all instances, hypoxia is a complication of coronary atherosclerosis (Braunwald, 2003). And in the development of atherosclerosis, oxidation of low-density lipoprotein by free radicals is an important step (Sherwood, 2004). Thus drugs with
antioxidant effects are used to prevent further myocardial destruction. Angiotensin II receptor AT1 subtype antagonists (e.g. losartan) are commonly used.

1.8.1 Principles and Pharmacology of Chinese green tea

The important role of Chinese green tea and its profound influence on the health care system in Chinese communities is well recognized. In the treatment of atopic eczema that has been resistant to orthodox treatment, has created greater interest and attention on the use of Chinese green tea for treatment in Britain (Chan, 2005).

Herbs and other plants have been used as medicinal agents, firstly based on folklore and later developed on a scientific basis into single agent drugs (Lee, 2003). It has long been recognized that natural-product structures have the characteristics of high chemical diversity, biochemical specificity and other molecular properties that make them favorable as lead structures for drug discovery, and which serve to differentiate them from libraries of synthetic and combinatorial compounds (Koehn and Carter, 2005). These properties make them remarkable and relevant for drug discovery.

Chinese green tea (Figure 7) has been planted and drunk worldwide for more than a dozen centuries. It is well accepted that green tea could prevent diseases (like cancer, and heart disease) (Blumberg, 2003). Traditionally, it was thought that vitamins and microelements in green tea contributed to the health effects. However, increasing evidence indicates that antioxidant properties of green tea play a major role in producing beneficial effects. Polyphenols in green tea were reported to have potential
antioxidant effects by many groups (Zhu et al., 2004; Guo et al., 1996; Rietveld and Wiseman, 2003; Beecher 2003 and Hu et al., 2001).

Figure 7: Chinese Green Tea

Green tea contains large amounts of polyphenolic compounds (flavonoids e.g. catechins,) (Figure 8). Flavonoids have been intensively studied, with most studies focused on the medicinal properties of free radical scavenging action (Kandaswami and Middleton, 1994). The flavonoids are potent antioxidants. It was reported that the
antioxidant effect of polyphenol is 9.6 times greater than that of vitamin E (Hu et al., 2001).

Polyphenolic compounds comprise 15–35% of the dry weight of tea leaves. Catechins in green tea include epigallocatechin-3-gallate (EGCG), epicatechin-3- gallate (ECG), epigallocatechin (EGC), and epicatechin (EC) (Lambert and Yang, 2003). The
antioxidant effect of polyphenols depends on their structure e.g. 2-phenol-chromane and the position as well as number of hydroxyl groups. EGCG has the greatest potential in scavenging free radicals, followed by ECG and EC, with EGC being the weakest catechin (Hu et al., 2001).

Rice-Evans and colleagues (Rice-Evans et al., 1996) reported that up to 78% of the total antioxidant activity of green tea was accounted for by the catechins and catechi-gallate esters. Catechins in green tea have been demonstrated to protect from lipid peroxidation by epigallocatechin gallate and epicatechin gallate. Epicatechin was even 10 times more effective than vitamin E (Namiki and Osawa, 1986). Plant polyphenols can act as hydrogen donating antioxidants and singlet-oxygen quenchers. The flavonoids contain a number of phenolic hydroxyl groups attached to ring structures which form the functional part for antioxidant activity (Harborne, 1986). The polyphenolic flavonoids have the diphenylpropane (C₆C₆C₆) skeleton. The functional groups responsible for the antioxidant activity of catechin are shown in Figure 9 (Zhu et al., 2004).

![Functional groups of catechin for antioxidant activity](image)
The study of Guo (Guo et al., 1996) reported that catechin components of green tea polyphenols and inferred that active positions of EGCG, ECG, EC and EGC could be as shown in Figure 10 (Zhu et al., 2004).

**Picture 10:** Active position of catechins
They found that their active antioxidant positions were different from each other and that the ability of these compounds to protect synaptosomes from lipid peroxidation initiated by Fe$^{2+}$/Fe$^{3+}$ was dependent not only on their iron-chelating activity and free-radical scavenging activity, but also on the stability of their semiquinone free radicals.

Western medicine can provide partial prevention, but the majority of stroke survivors still have significant risk of subsequent stroke, and well-tested interventions to eliminate stroke recurrence are not available. Since reactive oxygen species are also implicated in stroke, antioxidative Chinese traditional medicines would be a reliable target of study for their potential to prevent and repair oxidative stress-related pathological conditions. However, the single use of antioxidants is usually less effective than expected (Wang et al., 2001). The therapeutic potential of Chinese green tea is attracting attention as an alternative medicine for treating diseases related to lifestyle and thus would be of advantage to consider it for treatment.

The theory and fundamental principles of Chinese green tea has a very different perspective from western drugs. They differ not only in specific diagnostic techniques and therapeutic principles, but also in its interpretation of both physiological function and pathological changes in the human body (Gong and Sucher, 2002). The mechanism of action of Chinese green tea used as composite formulae is largely unknown, however, clinical and therapeutic effects can be found in the literature. The Chinese green tea approach considers the mind and body as one entity and treats symptoms of the illness
than the cause of the disease (Chan, 1995). The guiding principle for a standard prescription should contain one herb that is non-toxic and can be used for long-term (“Master”). The rationale in this sort of combination is multifold: First, it maximizes the therapeutic efficacy by enhancing the effects of individual ingredients, which may act synergistically. Secondly, complex formulae are designed such that the different ingredients cover the variation of symptoms to be treated. Third, complex formulae may be able to ameliorate side effects linked to the toxicity of individual drugs and thus have better overall therapeutic efficiency. Precise pharmacological actions of extracts are difficult to access due to the presence of many chemical compounds, hence it would be unfair to interpret their actions by using an orthodox approach (Chan, 1995).

1.8.2 **Flavonoids from Ceylon green tea**

In the world, various types of tea are produced from the tender shoots of the tea plants (typically the bud and the first two leaves), the botanical term for the plant is *Camellia sinesis*. These teas are grouped into three main types: Black, Green and Oolong teas. The differences between them are the method of processing those tender shoots is different. The major difference of processing of green tea is that after plucking, the shoot is subjected to heat by steaming or dropping onto a hot pan. The heat can inactivate the enzymes. Thus the chemical composition is similar to the fresh tender shoots (Amarakoon, 2004). The chemical composition of tea is listed in Table 1.
Ceylon tea (Figure 11 & 12) is one of the more popular herbal supplements and has been planted and drunk world wide for a long time. Without milk and sugar, green tea does not contain significant amounts of nutrients (Amarakoon, 2004). The calorific value is almost zero and the sodium content is very low too. This makes green tea an ideal beverage for hypertensive individuals and it is also a major source of dietary polyphenols.

<table>
<thead>
<tr>
<th>Compound</th>
<th>g /100g dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cold water - soluble</strong></td>
<td></td>
</tr>
<tr>
<td>Total Polyphenols</td>
<td></td>
</tr>
<tr>
<td>Epigallocatechin gallate (EGCG)</td>
<td>9 - 13</td>
</tr>
<tr>
<td>Epigallocatechin (EGC)</td>
<td>3 - 6</td>
</tr>
<tr>
<td>Epicatechin gallate (ECG)</td>
<td>3 - 6</td>
</tr>
<tr>
<td>Epicatechin (EC)</td>
<td>1 - 3</td>
</tr>
<tr>
<td>Galloatechin (GC)</td>
<td>1 - 2</td>
</tr>
<tr>
<td>Catechin (C)</td>
<td>1 - 2</td>
</tr>
<tr>
<td>Glycosides</td>
<td>3 – 4</td>
</tr>
<tr>
<td>Leucoanthocyanins</td>
<td>2 – 4</td>
</tr>
<tr>
<td>Phenolic acids</td>
<td>4</td>
</tr>
<tr>
<td>Caffeine</td>
<td>3 – 4</td>
</tr>
<tr>
<td>Amino acids</td>
<td>4</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>4</td>
</tr>
<tr>
<td>Organic acids</td>
<td>0.5</td>
</tr>
<tr>
<td>Volatile compounds</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>Partially hot water - soluble</strong></td>
<td></td>
</tr>
<tr>
<td>Polysaccharides</td>
<td>14 – 17</td>
</tr>
<tr>
<td>Protein</td>
<td>15</td>
</tr>
<tr>
<td>Ash (inorganic material)</td>
<td>5</td>
</tr>
<tr>
<td><strong>Water - insoluble</strong></td>
<td></td>
</tr>
<tr>
<td>Cellulose</td>
<td>7</td>
</tr>
<tr>
<td>Lignin</td>
<td>6</td>
</tr>
<tr>
<td>Lipids</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 1: The chemical composition of tea
Figure 11: A tea plantation in the Sri Lankan highlands

Figure 12: A plantation of Ceylon tea
27 – 40% of water soluble chemical components in tea are polyphenols. As known, polyphenols contribute mostly to the taste of tea. It falls into the sub group “flavonoids”, which have common structural and functional features.

The green tea extract contained a range of flavanols, it could be the most potent antioxidant fraction in terms of ROS scavenging (Low et al., 2006). Chinese green tea is well-accepted that it could prevent diseases such as cancer and heart disease (Zhu et al., 2004). Ceylon green tea has the similar contents as Chinese green tea, so they should have equal antioxidant properties and this project will confirm it.

1.8.3 Losartan

Losartan (Figure 13), the formula is $C_{22}H_{23}ClN_6O$, which the systematic name is:

$$1-\left((2'-(2H\text{-}tetrazol\text{-}5\text{-}yl)\text{ biphenyl\text{-}4\text{-}yl})\text{methyl}\right)\text{-}2\text{-}butyl\text{-}4\text{-}chloro\text{-}1H\text{-}imidazol\text{-}5\text{-}yl)$$

![Figure 13: The chemical structure of Losartan](image-url)
methanol, also known by its U.S brand name Cozaar®, received approval from U.S Food and Drug Administration (FDA) in 1995. The first of a new class of antihypertensives, it works as a selective and competitive non-peptide Ang II receptor type AT₁ antagonist. It can be used alone or together with a diuretic, hydrochlorothiazide, which provides greater blood pressure-lowering effects. Losartan interacts reversibly at the AT₁ and AT₂ receptors of many tissues and has slow dissociation kinetics, having a 1000 times greater affinity for the AT₁ receptor than the AT₂ receptor (Lacy et al., 2003). Ang II is the main effector peptide of the rennin-angiotensin system in the brain which plays a crucial role in regulating blood pressure and fluid balance. The G-protein coupled receptors of Ang II are Ang II type 1 receptor (AT₁R) and Ang II type 2 receptor (AT₂R), sharing a limited homology of 34% (Triggle, 1995). AT₁R and AT₂R have dissimilar functions and distributions in the brain. AT₁R is predominant in the hypothalamus and brain stem, whereas AT₂R is concentrated in the thalamus and specific brain stem nuclei. The majority of actions ascribed to Ang II are mediated by the AT₁R, including vasoconstriction, aldosterone release, renal sodium reabsorption and cardiovascular hypertrophy (Zhu and Lee, 1999). Other actions mediated by AT₁R comprise of the enhancement of inflammation by means of macrophage activation and cell migration, smooth muscle cell proliferation and growth, as well as generation of oxygen free radicals (Schiffrin, 2002). All these effects play a role in acute ischemic events. On the other hand, the function of AT₂R is less well-defined. Stimulation of AT₂R may enhance cell differentiation, mediate
vasodilation via release of nitric oxide and cyclic guanosine monophosphate-mediated vasodilation, in which bradykinin may also be involved in effects of AT2R, as well as inhibiting cell proliferation and inflammatory responses (Schiffrin, 2002).

Losartan has been shown that it can elevate protein levels of catalase in rats induced with myocardial infarction and that it might have antioxidant properties as its own. Thus losartan is used as a comparative study in this project.

Although angiotensin II receptor antagonists are not usually considered first-line, because of the proven effectivity and lower costs of thiazide diuretics and beta blockers, losartan may be used first-line in patients with increased cardiovascular risk.

Although Western drugs are widely used to treat ischemic disease (Zhu et al., 1998), but such drugs are often accompanied with side effects. For example losartan has common side effects including dizziness, light-headedness, blurred vision and stuffy nose etc.

Asian people still prefer to combine herbal medicines with Western treatment. There is some evidence that herbal supplements may have antioxidant effects. Our current experiment was designed to study the cerebral protective effects of Ceylon green tea extract in preventing cell survival after hypoxia. Thus in this experiment, losartan was selected as positive control to compare with Ceylon green tea for their effects on HBEC cells, antioxidant levels and immunoreactivity of the protein products after hypoxia.
1.9 Non-enzymatic Antioxidants

1.9.1 Ascorbic Acid (Vitamin C)

Ascorbic acid, also named Vitamin C (Figure 14), is a well-documented antioxidant without any known adverse effects, except that in very high concentrations it might function as a pro-oxidant in the presence of Fe, giving rise to the formation of dehydroascorbate radical, an innocent product. Ascorbic acid, presenting in plasma of healthy humans at concentrations of 50-200 μM, is a scavenger of HOCl and of O$_2^·$. Although it is unable to scavenge the peroxyl radical in the lipid phase, it can help to regenerate any vitamin E that is oxidized in plasma lipoproteins (Halliwell et al., 1989; Das et al., 1994).

![Figure 14: The chemical structure of Vitamin C](image)

Figure 14: The chemical structure of Vitamin C
1.9.2 Trolox (Vitamin E)

Trolox, which called as vitamin E (Figure 15), the chemical name: α-Tocopherol is a lipid-soluble antioxidant and stabilizer of cell membranes. Among four tocopherols, which are known to date α-tocopherol is the most biologically-important. Being hydrophobic, it will tend to concentrate in the interior of membranes, so it effectively prevents lipid oxidation. Their major antioxidant action under most conditions is to react with lipid peroxyl and alkoxyl radicals, donating labile hydrogen to them and so terminating the chain reaction of peroxidation by scavenging chain-propagating radicals. But a high concentration of α-tocopherol can become harmful by functioning as a prooxidant (Das et al., 1994; Halliwell et al., 1989).

![The chemical structure of Vitamin E](image)

**Figure 15:** The chemical structure of Vitamin E
Recent studies have demonstrated the activation of extrinsic and intrinsic pathways of caspase-mediated cell death as well as the activation of caspase-independent signaling pathway of apoptosis following cerebral ischemia. Cerebral ischemia results in changes in gene expression and protein synthesis. Gene expression is under the control of inducible transcription factors, which forms the links between extracellular signals and the initiation of intracellular genomic and metabolic events that are involved in survival or death of neurons (Dai et al., 1999). The extrinsic pathway, also known as the cell-death receptor pathway, is initiated when the Fas receptor binds to the Fas ligand (FasL). The complex of trimers of aggregated Fas and the adaptor protein Fas-associated death domain (FADD) constitutes the death-inducing signaling complex. FADD contains death-effector domains (DED) which binds to complementary DEDs in procaspase-8, resulting in the close positioning of procaspase-8 molecules which in turn causes their autolytic cleavage and activation. Activated caspase-8 later activates downstream caspases mostly by cleaving the BH3-only protein, Bid, to free truncated Bid which generates the release of cytochrome c from the mitochondria (Love, 2003). It has been shown that Fas and FasL are elevated following focal ischemia, and mice expressing nonfunctional (mutated) Fas show greatly reduced focal ischemia.

The intrinsic (mitochondrial) pathway following focal ischemia is activated when Bax translocates and competes with Bcl-2 and other Bcl-2 family members in the mitochondrial membrane, which is coincidental with the release of cytochrome c into the cytosol. Cytochrome c binds to apoptotic-protease-activating factor 1 (Apaf-1) and
ATP and later recruits and cleaves pro-caspase 9 in the apoptosome. Caspase-9, together with caspase-8 further activates caspase-3, among other caspases, which in turn cleave a number of important substrates, such as the DNA-repairing enzyme poly (ADP-ribose) polymerase (PARP). The mitochondrial release of several other factors, such as second mitochondrial activator of caspases/direct IAP binding protein with low pl (Smac/Diablo) binds to and neutralizes the effects of the X-linked inhibitor of apoptosis (XIAP). Bax, together with BH3-only proteins such as Noxa and Puma, are transcriptional targets of p53. Currently, the relationship between p53, oxidative stress, DNA damage and neuronal loss is still poorly understood.

The Bcl-2 family, by its regulation of both caspase-independent and caspase-dependent cell death pathways, plays an indispensable role in apoptosis. The anti-apoptosis gene, Bcl-xL, belongs to the class of anti-death Bcl-2 family members that contains four conserved motifs termed Bcl-2 homology (BH) domains, most include a hydrophobic C-terminal anchor segment by means of which they attach to the endoplasmic reticulum, nuclear envelope or outer mitochondrial membrane. On the other hand, Bcl-xS and Bax belongs to the class of multi-domain pro-death Bcl-2 family members that posses at least BH 1 and BH 2 domains. There is a third class of Bcl-2 family members termed the BH3-only pro-death subgroup consisting of genes such as Bim and Bad. The Bcl-x gene has two isoforms, Bcl-xL and Bcl-xS which are produced by alternative splicing. Bcl-xL consists of 233 amino acids while Bxl-xS lacks an internal 63 amino acid region that comprises the BH1 and BH2 domains. Out of the four conserved BH domains in
the Bcl-2 family, BH1 and BH2 domains are most likely involved in pro-survival activity while BH3 is related to apoptotic cell death (Adams and Cory, 1998). Bcl-xL is highly homologous to Bcl-2 is expressed in the embryonic and mature nervous system at rather elevated levels. Its expression is elevated in immature neurons as they migrate away from the mitotically active ventricular zone, remains highly expressed in mature neurons in the adult brain and is localized to the outer mitochondrial membrane. On the contrary, Bcl-xS is pro-apoptotic and is present at low levels in the mammalian nervous system. The mechanism of Bcl-xS protein-mediated apoptosis is controversial, with the postulation that Bcl-xS proteins competitively bind to the same downstream regulators of apoptosis as Bcl-2 such as the interleukin-1β converting enzyme proteins or that Bcl-xS itself counteracts pro-survival activity by being an active effector of cell death since it possesses a hydrophobic C-terminal (Ng et al., 2001). On the other hand, the anti-apoptotic properties of Bcl-xL present itself in many cellular paradigms and involves the activation of the intrinsic, mitochondrial-dependent, apoptotic death pathway. Bcl-xL may heterodimerize or hetero-oligomerize with Bax to inhibit Bax function (Akhtar et al., 2004). Also, Bcl-xL can play a role in calcium homeostasis, act as antioxidants by the increased scavenging of mitochondrial ROS directly or by upregulating other ROS scavengers such as antioxidant enzymes and thiol compounds. Bcl-xL proteins are located in higher amounts at the mitochondrial permeability transition pore sites, thus can directly alter mitochondrial membrane permeability resulting in the inhibition the release of apoptogenic factors, such as cytochrome c and
Smac/Diablo from the intermembrane space into the cytoplasm. Bcl-xL has also been shown to play a role in mitochondrial respiratory function, since Bcl-xL overexpression prevented a decrease in state III respiration and mediated the increase in state IV respiration in astrocytes (Ouyang and Giffard, 2004). The importance of Bcl-xL for neuronal survival has been demonstrated by gene targeting. Mice that were homozygously deficient for Bcl-xL died on approximately embryonic day 13, and widespread apoptotic cell death was shown to occur in postmitotic immature neurons of the developing brain, spinal cord and dorsal root ganglia. Furthermore, cultured neurons from Bcl-xL \(-/-\) mice show a higher level of apoptosis as compared to those from Bcl-xL \(+/-\) or \(+/+\) mice (Shinoura et al., 2000). The caspase-independent cell death pathway of apoptosis is initiated when apoptosis-inducing factor translocates to the mitochondria and the nucleus after cerebral ischemia, leading to peripheral chromatin condensation and large-scale DNA strands (Ferrer and Planas, 2003).

Two major strategies are employed in the treatment of acute ischemic stroke – the vascular approach, in which the ischemic insult is limited by early reperfusion; and the cellular approach, whereby there is interference with the pathobiochemical cascade that results in ischemic neuronal damage. One necessary requirement for either of these approaches is the presence of functionally damaged but viable and potentially salvageable tissue. The time window for effective treatment is rather short for the vascular approach, while it is of longer duration for the cellular approach, especially for the anti-apoptotic and anti-inflammatory methods (Heiss et al., 1999). Stroke therapies
targeted at the ischemic core (whereby neurons die swiftly as a result of oxygen starvation) need to be fast and efficient in reversing the blockage of blood supply and being able to raise the blood flow above the critical threshold before cells become permanently damaged. On the other hand, the ischemic penumbra is deemed as the most promising target for stroke therapies as the therapeutic window is prolonged for several hours and because this area can be defined by functional neuroimaging modalities. Sufficient reperfusion before irreversible cell damage at the ischemic penumbra, as well as added neuro-protective agents aimed at different steps in the pathobiochemical cascade could help prevent or alleviate secondary ischemic cell damage (Heiss et al., 1999). As such, many current neuroprotective strategies have been targeted at molecules that are able to intervene with apoptotic mechanisms in the penumbra where ATP levels are sufficient to allow energy-dependent apoptosis to take place. Scientists at Celgene Corporation (San Diego, CA) experimented with a c-Jun N-terminal kinase inhibitor and showed that the number of TdT-mediated dUTP nick end labeling (TUNEL)-positive cells, an indicator of the number of apoptotic cells was reduced (Harbeck 2002). Transactivator domain (TAT)-fusion proteins that have transmembrane passage capabilities, such as fusion proteins containing the anti-apoptotic molecule Bcl-2, showed a substantial reduction in cerebral infarction when administered intraperitoneally following focal transient ischemia. Another anti-apoptotic protein, Survivin, an inhibitor of caspase-1, promotes cell proliferation in vitro (Onteniente et al., 2003). Such anti-apoptotic drugs are promising in the field of stroke therapy, yet, since
these protein molecules are able to penetrate any cell type, the risk of cancer induction or the dysregulation of the immune system raises a whole host of new concerns. As such, the exploration of different classes of drugs that employ different mechanisms of apoptosis interference will further our understanding in stroke therapy.

1.10 Objective

Positive conclusions were derived from various groups in the study of tea compounds on experimental stroke. Ceylon green tea extract is being investigated to determine if they might have better pharmacological effects than losartan. The properties of Ceylon green tea extract for the neuro-protective effects after hypoxia was investigated. We used losartan, for a comparative study of physiological and hemodynamic effects with Ceylon green tea extract. Further, we sought to elucidate possible molecular mechanisms of Ceylon green tea extract. mRNA expression and localization, a potent initiator of angiogenesis, will be studied to provide more information in neuro-protection after hypoxia.
CHAPTER 2

LITERATURE REVIEW

2.1 Background of traditional Chinese medicine and its use in Stroke Therapy

In traditional Chinese medicine (TCM), the human body is considered as a whole. The background of TCM is founded on the principles of “Yin-Yang”, which explores the relationship between pathogenic factors and the patient, “Zang” and “Fu” organs (the internal organs of the human body) represented by the “Five Elements”, wood, fire, earth, metal and water, which correspond to the organs lung, spleen, kidney, heart and liver (Gong, 1999). TCM draws attention to the importance of identifying dynamic changes in symptoms that are observed in the disease duration, thus allowing distinctive treatments to be administered at different phases of the disease. The treatment principle of TCM is to harmonize the balance of “Yin” and “Yang” by regulating body constituents, such as “qi” and blood among the five-organ network (Ip et al., 2001).

The TCM viewpoint of stroke is one that differs from the modern scientific belief in that the TCM equivalent of a stroke is a syndrome called “wind stroke” which involves five pathogenic factors including “external wind”, “stagnation”, “fire”, “liver wind” and “phlegm”. Currently, there are more than 100 TCM used in stroke therapy in China, with some of their therapeutic effects in stroke being proven in recent clinical studies.

In TCM, drugs for stroke therapy are grouped generally into four categories according to their primary mode of action. The first class of drugs would be those that are based
on TCM principals as described above and are hence more obscure when utilized in modern pharmacological research. The second group of drugs would be the anti-inflammatory drugs while the third class of drug would be the anti-thrombotic drugs, which promote blood circulation by clearing blocked blood vessels. The fourth class of drugs is the neuro-protective drugs, in which the neuro-protective effects attributed to glutamate receptor antagonists, intervention of neurotoxic free radicals still remains to be investigated (Gong, 1999).

2.2 Antioxidants and their role in Ischemic Injury

The non-enzymatic antioxidant system (low molecular weight antioxidants) consists of indirectly acting oxidants such as chelating agents as well as many directly acting antioxidants, such as free radical scavengers like glutathione (GSH), uric acid, beta-carotenes (vitamin A), ascorbic acid (vitamin C) and tolox (vitamin E). The enzymatic antioxidant system include superoxide dismutase, catalase, peroxidase and some supporting enzymes. Similarly to other neuro-protectants, antioxidants must be able to cross the blood brain barrier and be given as early as possible and within the “neuroprotective window” for tissue rescue to be possible. In addition, the antioxidants must also fit the precise oxidative stress physiology, such as the type of ROS involved, the place of generation as well as the severity of damage (Gilgun-Sherki et al., 2002). It has been shown that during cerebral ischemia, plasma and cerebral tissue levels of antioxidants such as GSH are decreased, as a reflection of elevated oxidative stress.
Several antioxidants, such as dietary flavanoid rutin, micronutrient selenium and garlic oil, have been demonstrated to have neuro-protective effects against cerebral injury (Gupta et al., 2003). Vitamin E, Chinese green tea extract, and red wine / resveratrol, are antioxidants which have been shown to protect against cerebral ischemia (Ikeda et al., 2003).

2.3 Losartan and the roles of AT1R and AT2R in cerebral ischemia

The role of AT2R in the induction of apoptosis is currently controversial. Grammatopoulos (Grammatopoulos et al., 2002) showed that blockage of AT1R in mouse cortical neurons in culture resulted in a total inhibition of sodium azide-induced apoptosis. This meant that the activation of AT2R completely inhibited sodium-azide induced apoptosis, while activation of AT1R only inhibited the apoptosis by 47%. These findings suggested that AT2R were largely responsible for angiotensin protection of culture mouse neurons after hypoxic injury. In addition, AT1R is able to inhibit $K^+$ currents and AT2R is able to activate $K^+$ currents by means of the delayed rectifier $K^+$ channel in hippocampal cultures. The modulation of $K^+$ currents by angiotensin could also be involved in its ability to protect neurons from sodium azide-induced hypoxia. In addition to the hypothesis that AT2R could considerably contribute to the observed advantageous effects of AT1R antagonists such as lowering the expression of c-Fos and c-Jun proteins in the brain, long-term administration of AT1R antagonists has been reported to prevent the occurrence of stroke in stroke-prone SHR and salt-loaded Dahl
salt-sensitive rats (Dai et al., 1999). On the contrary, the pro-apoptotic effect of AT2R has been demonstrated by Makino (Makino et al., 1996), Zhu (Zhu et al., 2000) and Kagiyama (Kagiyama et al., 2003), showing that AT2R was upregulated and correlated with apoptosis after cerebral ischemia. Another major finding by Sato (Sato et al., 2000) showed that losartan provided cardioprotection by both bradykinin-dependent and bradykinin-independent pathways since losartan-mediated decrease in cardiomyocyte apoptosis was totally abolished by HOE 140, a bradykinin B₂ receptor blocker.
CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Green tea

3.1.1.1 Ceylon green tea

Dilmah™ Ceylon green tea was provided by Tea Research Institute, Talawakelle, Sri Lanka.

3.1.1.2 Chinese green tea

Chinese green tea, Qian Dao® (Qian Dao Hu tea Manufacturing Co.) and Bie Xue Chun® (Jiang Shu green tea Co. Ltd) which used for this project, was chosen randomly and purchased from the supermarket from China.

3.1.2 Losartan

Losartan is an angiotensin II receptor antagonist drug used mainly to treat high blood pressure (hypertension). Losartan was the first angiotensin II receptor antagonist to be marketed. It is purchased from Merck & Co.

3.1.3 In vitro Antioxidant test

3.1.3.1 ABTS assay

ABTS: 2,2’azinobis (3ethylbenothiazoline6sulfonic acid) from Sigma
Trolox: from Sigma

Ascorbic acid: from Sigma

### 3.1.3.2 Pyrogallol Red assay

Pyrogallol red: pyrogallolsulphonephthalein from Sigma

HOCl: Hypochlorous acid from Honeywell Riedel-de-Haen (Seelze, Germany)

### 3.1.4 Measurement of DNA damage

DNA base standards and heavy labeled standards:

<table>
<thead>
<tr>
<th>Standard</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-azathymine</td>
<td>Sigma</td>
</tr>
<tr>
<td>2,6-diaminopurine</td>
<td>Sigma</td>
</tr>
<tr>
<td>5-Hydroxy 5-Methyl Hydantoin</td>
<td>Sigma</td>
</tr>
<tr>
<td>5-(Hydroxymethyl) uracil</td>
<td>Sigma</td>
</tr>
<tr>
<td>5-Hydroxy Cytosine</td>
<td>Sigma</td>
</tr>
<tr>
<td>Thymine Glycol</td>
<td>Sigma</td>
</tr>
<tr>
<td>Xanthine</td>
<td>Sigma</td>
</tr>
<tr>
<td>FAPy Adenine</td>
<td>Sigma</td>
</tr>
<tr>
<td>FAPy Guanine</td>
<td>Sigma</td>
</tr>
<tr>
<td>8-Hydroxy Adenine</td>
<td>Sigma</td>
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<tr>
<td>8-Hydroxy Guanine</td>
<td>Sigma</td>
</tr>
<tr>
<td>5-Hydroxy 5-Methyl Hydantoin 2-^{13}C</td>
<td>CIL*</td>
</tr>
<tr>
<td>5-(HydroxyMethyl) Uracil, 4,5-^{13}C_{2}-5',5'-d_{2}^{13}C</td>
<td>CIL</td>
</tr>
<tr>
<td>5-Hydroxy Cytosine2-^{13}C_{1,3}-^{15}N_{2}^{13}C</td>
<td>CIL</td>
</tr>
<tr>
<td>FAPy Adenine-formyl-^{13}C-diamino-^{15}N_{2}^{13}C</td>
<td>CIL</td>
</tr>
<tr>
<td>FAPy Guanine-formyl-^{13}C-4-amino-5-amino-^{15}N_{2}</td>
<td>CIL</td>
</tr>
<tr>
<td>8-Hydroxy Adenine-8-^{13}C-6,9-diamino-^{15}N_{2}^{13}C</td>
<td>CIL</td>
</tr>
<tr>
<td>8-Hydroxy Guanine-8-^{13}C-7,9-^{15}N_{2}^{13}C</td>
<td>CIL</td>
</tr>
</tbody>
</table>

*CIL: Cambridge Isotope Laboratories
3.1.5 Cell Culture

3.1.5.1 Cell line

HBEC: Human Brain Epithelial Cells: were purchased from American Type Culture Collection; Manassas, VA (ATCC).

3.1.5.2 Cell culture reagent

PBS: 10 x Phosphate-buffered Saline from Media preparation Unit, National University Medical Institutes (NUMI)

PSA: Antibiotic-Antimycotic Solution from Gibco (USA)

DMEM: Dulbecco’s Modified Eagle’s Media with 25mM HEPES from Sigma

FBS: Fetal bovine serum from HyClone (Logan, Utah, USA)

trypsin-EDTA solution from Invitrogen (USA).

3.1.5.3 Antibodies

HRP/DAB kit (Lab Vision Corporation, CA, USA)

Polyclonal rabbit anti- Fas antibody (Santa Cruz, CA, USA)

polyclonal rabbit anti-Bax antibody (Santa Cruz, CA, USA)

polyclonal rabbit anti- Asp53 antibody (Santa Cruz, CA, USA)

polyclonal rabbit anti-Bcl-2 antibody (Santa Cruz, CA, USA)
### 3.1.6 General Chemicals

<table>
<thead>
<tr>
<th>Chemical name</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium Chloride</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>CH₃COONa</td>
<td>Sodium acetate</td>
</tr>
<tr>
<td>C₂H₃NaO₃</td>
<td>Sodium lactate</td>
</tr>
<tr>
<td>K₂S₂O₈</td>
<td>Potassium Persulfate</td>
</tr>
<tr>
<td>K₃H₂PO₄</td>
<td>di-Potassium hydrogen phosphate</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>(CH₃)₂CHOH</td>
<td>Isopropyl alcohol</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehydes-3-phosphatase</td>
</tr>
<tr>
<td>CHCl₃/C₁₀H₂₃OOH</td>
<td>Chloroform / isoamyl alcohol (24:1)</td>
</tr>
<tr>
<td>C₆H₅OH</td>
<td>Phenol</td>
</tr>
<tr>
<td>Trypsin</td>
<td>Digestive protein</td>
</tr>
<tr>
<td>dye solution</td>
<td>Tetrazolium salt</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutathione</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>BH</td>
<td>t-Butyl Hydroperoxide</td>
</tr>
<tr>
<td>propidium iodide</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>C₁₄H₂₂O(C₂H₄O)ₙ</td>
<td>Triton X-100</td>
</tr>
<tr>
<td>C₂H₅OH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>CH₂O₂</td>
<td>Formic acid</td>
</tr>
<tr>
<td>BSTFA+1% TMCS with 1% Trimethylchlorosilane</td>
<td>PIERCE</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>CH₃CH₂SH</td>
<td>Ethaneethiol</td>
</tr>
<tr>
<td>agarose gel</td>
<td>Electrophoresis</td>
</tr>
<tr>
<td>green loading dye</td>
<td>Bromphenol blue and Ethidium</td>
</tr>
<tr>
<td>DNA ladder</td>
<td>Promega, USA</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione reduced form</td>
</tr>
<tr>
<td>GSSH</td>
<td>Glutathione Reductase</td>
</tr>
</tbody>
</table>
3.1.7 **Instruments and Equipments**

Following instruments and equipments were used for the current project as listed:

<table>
<thead>
<tr>
<th>Name</th>
<th>Model</th>
<th>Brand</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV / visible spectrophotometer</td>
<td>DU640B</td>
<td>Beckman, USA</td>
</tr>
<tr>
<td>Freeze dryer</td>
<td>Advantage</td>
<td>Virtis, USA</td>
</tr>
<tr>
<td>Dri-Block</td>
<td>DB-3D</td>
<td>Techne, UK</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>5810R</td>
<td>Eppendorf, Germany</td>
</tr>
<tr>
<td>Gas Chromatography / Mass Spectrometer</td>
<td>6890/5973</td>
<td>Agilent, USA</td>
</tr>
<tr>
<td>Plate reader</td>
<td>Sunrise</td>
<td>TECAN,</td>
</tr>
<tr>
<td>Fluorescent Microscope</td>
<td>Microplate</td>
<td>Switzerland</td>
</tr>
<tr>
<td>Reverse Transcription–Polymerase Chain</td>
<td>GeneAmp® PCR</td>
<td>Applied</td>
</tr>
<tr>
<td>Reaction</td>
<td>Microsystem</td>
<td>Leica, USA</td>
</tr>
<tr>
<td></td>
<td>9700</td>
<td></td>
</tr>
</tbody>
</table>

### 3.2 METHODS

#### 3.2.1 Preparation of Ceylon green tea extract

Briefly, 100g raw Ceylon green tea was extracted twice by boiling in 400ml distilled water for 20 minutes. The liquid was freeze-dried into a powder according to the standard procedure after frozen overnight at -80°C (Sun *et al.*, 2004). Approximately 20g of freeze-dried powder was obtained from 100g of raw Ceylon green tea. For the experiment, 20g of powder was dissolved in 1L of distilled water and used for all the assays.
3.2.2 Separation and isolation of flavonoids by HPLC

To isolate the main polyphenolic compounds which play important roles for anti-oxidative effects from flavonoids, the above tea extract was injected to HPLC (High-performance liquid chromatography). The sample to be analyzed is introduced in small volume to the stream of mobile phase and is retarded by specific chemical or physical interactions with the stationary phase as it traverses the length of the column. The analysis was accomplished with a Zorbax SB-18 column (150 mm × 2.1 mm I.D.) at room temperature. The injection volume was 5μl. 0.1% of Formic acid and methanol system was used as the mobile phase in gradient mode as follows: 0.1% of Formic acid: 0-30 minutes, 80-20%; methanol: 0–30 minutes, 20–80%. The flow-rate of the mobile phase was 0.3 ml/min. The effluents were monitored at 280 nm by a photodiode array detector.

After identifying the main compounds, the Drug Extraction system (Figure 16) was used to collect those important compounds, the flowchart of extraction of flavonoids is shown in Figure 17.
Figure 16: Herbal extraction system

1Kg Ceylon green tea + 10L water, boil at 100°C For 30 mins

Filter the supernatant + 10L water again and boil at 100°C For 30 mins (repeat this step 1 more time)

Concentrate the filtered Supernatant at 65°C till no more water condensed

Extract active compounds

Dry the powder

Packing and γ-Sterilization

Figure 17: Flowchart of Flavonoids extraction from Ceylon green tea
3.2.3 *In Vitro* Antioxidant Test

3.2.3.1 Inhibition of ABTS Assay

2,2'-azinobis (3-ethylbenothiazoline-6-sulfonic acid) (ABTS) quantity can be determined spectrophotometrically at 734nm. 10μl of 10mg/ml of Ceylon green tea or Chinese green tea (Qian Dao & Bi Xue Chun green tea) was mixed with 990μl of ABTS reagent and incubated for 3 minutes before the absorbance was measured at 734nm.

Firstly, 0.03311g ABTS was dissolved in 9.8ml H₂O to get A) 7mM ABTS; secondly 0.03311g K₂S₂O₈ was dissolved in 5ml H₂O to get B) 2.45mM K₂S₂O₈; then 0.2ml of B) was transferred to A) to obtain the stock ABTS solution C). Lastly, 1ml of C) was added to 79ml of PBS buffer and the pH value of the solution was 7.4.

For the standards, 0.0176g ascorbic acid was dissolved in 100ml H₂O to obtain 1mM ascorbic acid solution and 0.005g trolox was dissolved in 10ml H₂O to obtain 2mM trolox, then diluted with water (1: 2) to obtain final concentration of 1mM.

Absorbance was measured at 734nm, 3 minutes after initial mixing. Firstly, abundance of 2ml of H₂O was measured as blank, secondly 2ml of ABTS⁺ solution alone was measured as control, then 5 or 10μl (to obtain 2 different final concentrations: 1 and 10μg/ml) of 1mM ascorbic acid or trolox was added in 1995 or 1990μl of ABTS⁺ solution as a standard and measure the absorbance, followed 5 or 10μl of Ceylon green tea or Chinese green tea was added in 1995 or 1990μl of ABTS⁺ solution as the samples and 5 or 10μl of water was added in 1990μl of ABTS⁺ solution as a sample
blank and the Absorbance were measured. All the samples were done duplicated.

Trolox was used as a standard and the percentage trolox equivalent antioxidant capacity
(TEAC) were calculated (Whiteman et al., 2002) before the antioxidant capacities were
expressed as ascorbic acid equivalent values (Long and Halliwell, 2001; Re et al., 1999).

The result was calculated as:

\[
\text{TEAC} = \frac{\text{Abs}_{\text{ABTS}} - \text{Abs}_{\text{SB}}}{\text{Abs}_{\text{ABTS}} - \text{Abs}_{\text{std}}}
\]

- \( \text{Abs}_{\text{ABTS}} \) = Absorbance of ABTS solution
- \( \text{Abs}_{\text{SB}} \) = Absorbance of Sample blank
- \( \text{Abs}_{\text{S}} \) = Absorbance of Sample

3.2.3.2 Inhibition of Pyrogallol Red Bleaching by Hypochlorous Acid (HOCl)

Firstly, 0.004g was dissolved in 10ml 100mM phosphate buffer with pH 7.4 to obtain
1mM PR stock solution and then 10 times diluted with phosphate buffer to obtain
100μM PR working solution. Secondly the Absorbance of HOCl was checked to know
its concentration: 10μl HOCl was added in 990μl H₂O and mixed in a microcuvette,
read by UV Spectrophotometer at 290nm. All the samples were done duplicated.

\[
\text{Concentration} = \left( \frac{\text{Abs}}{350} \right) \times \text{dilution factor}
\]

if Abs= 0.2941 Concentration = \( \left( \frac{1.225}{350} \right) \times 100 = 0.350 \text{M} = 350 \text{mM} \)

Volume to take for 1mM = \( \frac{1000}{350} \text{mM} = 2.86 \mu l \)

So for 125μM HOCl, volume needed is \( 2.86 \mu l / 8 = 0.3575 \mu l \)

Absorbance was measured by UV at 542nm by UV Spectrophotometer. 1ml of H₂O was
read as blank; 250μl 100μM PR and 750μl buffer were mixed and read as control, the
Absorbance should be around 1; then 100μl 1mM PR and 900μl buffer were mixed; 100μl 1mM PR solution and 800μl buffer and 100μl ascorbic acid or 100μl Ceylon green tea solution or Chinese green tea solution (Qian Dao & Bi Xue Chun green tea to get 2 different final concentrations: 100μg/ml and 1mg/ml) were mixed. Duplicate for each sample, then left them at room temperature for 10 minutes, followed by adding 0.3575μl HCl in each tube, mixed well, then dilute them 1:4 with buffer (250μl sample + 750μl buffer mixed in a microcuvette), measured by UV at 542nm. The decreases in absorbance were expressed as percentage inhibition as described previously (Goto et al., 1996). The inhibition value was calculated as:

\[
\text{Inhibition Value} = \frac{(\text{Abs}_S - \text{Abs}_SB)}{(\text{Abs}_{PR} - \text{Abs}_SB)} \times 100\%
\]

- \text{Abs}_S = \text{Absorbance of Sample}
- \text{Abs}_SB = \text{Absorbance of Sample blank}
- \text{Abs}_{PR} = \text{Absorbance of PR solution}

### 3.2.4 Evaluation of DNA Damage using GC/MS

An \textit{in vitro} hypoxic model using Human Brain Epithelial Cells (HBEC) was studied with the treatment of flavonoids. We have tested the hypothesis that flavonoids from Ceylon green tea would act as potential therapeutic ingredients to reduce oxidative stress in hypoxic cells through its antioxidant properties and its ability to reduce ischemic cerebral cellular death.
3.2.4.1 DNA Extraction from cell homogenate

DNA was extracted from HBEC collected from the different treatment groups of flavonoid, control with/without hypoxia using the phenol-chloroform method.

Add 1ml extraction buffer which containing 0.1M NaCl, 10mM KCl, 10mM MgCl₂, 10mM Tris-base, 10mM EDTA and 0.5% SDS at pH 8.0 to large plate of cells (150mm D) containing at least 10million cells and mix well by hand. Add 15units of RNAase A and 10units of RNAase T1 to remove contaminating and incubated at 37°C for 60 minutes. Then digest contaminating protein with 30 units of proteinase K (approx 0.1 mg/ml) at 50°C for 2 hours. Followed by adding saturated phenol (1:2 v/v) vortexed and centrifuged at 4000g for 10 minutes. Phenol layer was removed and Phenol/Chloroform/isoamyl alcohol (25: 24:1) was added to homogenate (1:2 v/v), vortexed and centrifuged at 4000g for 10 minutes. Carefully the upper layer (containing DNA) was removed and 3M sodium acetate with pH 6.0 (1:10 v/v) was added. Followed by adding 2.5 volume of 100% ice-cold ethanol, DNA was precipitated and left for over night to maximize the precipitation. The next day the tube was centrifuged at 2000g for 10 minutes after that discard the solvent and the pellet was washed twice with 70% ethanol (ice-cold). Finally the pellet was dissolved in 200µl H₂O. DNA concentration was measured by spectrophotometer at 260nm.

50µg/ml DNA = Absorbance of 1 at 260nm

- \( \frac{A_{260}}{A_{280}} = 1.8 \) = good quality DNA
- \( \frac{A_{260}}{A_{280}} > 2 \) = poor quality DNA (possible RNA contamination)
- \( \frac{A_{260}}{A_{280}} < 1.2 \) = too much protein
3.2.4.2 Measurement of DNA damage using GC/MS

The DNA samples were then freezing dried, hydrolyzed, derivatized and finally analyzed by GC/MS.

3.2.4.3 Acid Hydrolysis

Release of normal and modified bases from DNA is achieved by cleaving glycosidic bonds between bases and sugar moieties during acidic hydrolysis. Overnight freeze dried DNA samples (100μg) containing the internal standards 6-azathymine and 2,6-diaminopurine (plus the heavy isotope standards available in the lab (0.5nmol)) were hydrolyzed by addition of 0.5ml 60%v/v cold formic acid and heating at 150°C for 45 minutes in an evacuated, sealed hydrolysis tube. Samples were cooled and lyophilized together with a set of calibration standards (for compounds that we do not have heavy labeled isotopic standards for) which were prepared as mixtures of all the DNA base damage products to be measured (content range 0.02-1.0 nmol).

3.2.4.4 Derivatisation

After acid hydrolysis of DNA, GC-MS requires a derivatisation procedure in order to convert the polar nucleosides/bases and internal standards to volatile, thermally stable derivatives which possess characteristic mass spectra. Trimethylsilylation is the most common derivatisation reaction used. A mixture of acetonitrile/ethanethiol (3:1) was freshly prepared. Samples and calibration standards were derivatized in poly (tetrafluoroethylene)-capped glass vials after purging with nitrogen. 60μl of a BSTFA
(+1% TMCS) and 15μl acetonitrile/ethanethiol mixture was added and mixed well. Samples were derivatized at 23°C for 2 hours.

3.2.4.5 GC-MS Analysis

GC-MS analysis is essentially as described by Spencer and Jenner (Spencer et al., 2000; Jenner et al., 1998). Derivatized samples were analyzed by Agilent 5973 mass selective detector interfaced with Agilent 6890 gas chromatograph and equipped with an automatic sampler and a computer workstation. The injection port and the GC/MS interface were kept at 250°C and 290°C, respectively. Separations were carried out on a fused silica capillary column (12m x 0.2mm i.d.) coated with cross-linked 5% phenylmethylsiloxane (film thickness 0.33μm), 99.999% Helium was the carrier gas with a flow rate of 1ml/min. Derivatized samples (1μl) were injected into the GC injection port using a split ratio of 8:1. Column temperature was increased from 125°C to 175°C at 8°C/min after 2min at 125°C, then from 175°C to 220°C at 30°C/min and finally from 220°C to 290°C at 40°C/min and held at 290°C for 1min. Selected-ion monitoring was performed using the electron-ionization mode at 70eV with the ion source maintained at 230°C. Quantitation of modified bases was achieved by relating the peak area of the compound with the internal standard peak.

Concentration of DNA base product = \( \frac{A_s}{A_{std}} \times 0.5\text{nmol} \times \frac{1}{K} \times \frac{1000}{100\mu g} \)

- \( A_s \) = peak area of compound in the sample
- \( A_{std} \) = peak area of internal standard
In mass spectrometer, a stable isotope-labeled analog of an analyte can be used as an internal standard. 0.5nmol heavy-isotope labeled standards were added in DNA bases before sample were hydrolyzed in order to performing accurate quantification.

Concentration of DNA base product = \( \frac{A_s}{A_{std}} \times 0.5\text{nmol} \times \frac{1000}{100\text{ug}} \)
- \( A_s \) = peak area of compound in the sample
- \( A_{std} \) = peak area of labeled standard

3.2.5 Cell cultures

Human Brain Epithelial Cells (HBEC) was first seeded into T25 flask. After approximately 80% confluence had been achieved, the cells were subsequently transferred to T75 flask in DMEM supplemented with 10% FBS and 1% PSA. The procedure involved was that the cells were detached with 1ml of 0.25% Trypsin-EDTA, and then 3ml of DMEM was added to prevent the cells from dying. The cells were then subjected to centrifugation at 1000 rpm for 5 minutes at 23°C. After the centrifugation, the pellet was re suspended with 1ml of DMEM. The cells are then placed into a T75 flask and topped up with 14ml of DMEM. After 3 days, approximately 1x 10^5 cells per 35mm well, were then ready for seeding into 6 well cell culture plates (NUNC). The cells were then pre incubated at 37°C in humidified air enriched with 5% CO₂ for 12 hours. After 12 hours, the cells were washed with phosphate buffered saline (PBS). Immediately, the medium was changed to serum free DMEM. The purpose of serum
free DMEM was to deprive the cells of any nutrients required so as to detect the positive results achieved by the addition of respective drugs. Subsequently, the drugs were added into the respective wells (Table 2).

<table>
<thead>
<tr>
<th>Group number</th>
<th>Treatment groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normoxic control</td>
</tr>
<tr>
<td>2</td>
<td>Hypoxic control</td>
</tr>
<tr>
<td>3</td>
<td>10μl of 10mg/ml flavonoids</td>
</tr>
<tr>
<td>4</td>
<td>10μl of 100μM losartan</td>
</tr>
</tbody>
</table>

Table 2: Groups for cell culture

3.2.6 Simulated hypoxia-induced ischemic model

The hypoxia-induced ischemic model is achieved via the modular incubator chamber System (Billups-Rothenberg, USA) as previously described (Namiki et al., 1995). As shown in Figure 18, the chamber uses surface type seal whereby all parts of the O-ring are compressed uniformly by a stainless steel ring clamp. This structure of the Modular Incubator Chamber is such that it guarantees little gas flow resistance and uniform infusion of gas. This is attained by having semi spherical top and bottom as well as cylindrical walls all around. Furthermore, to ensure that there is no leakage via cracks during any point in the experiment, the material utilized for the chamber is high quality.
polycarbonate.

The procedure for the creation of a hypoxic environment is as follows: Firstly, the 6 well plates were placed in the chamber. Secondly, attach the flow meter to unit. The chamber allows for simulation of hypoxia-induced ischemic model by providing reliable airtight seal and fast gas exchange. A non-fluctuating hypoxic environment is easily attained. Flush for several minutes with desired gas mixture by opening both the inlet and outlet ports. Then close the outlet port, to allow the infusion of the desired gas for 10 minutes to ensure a uniform low O₂ environment. Seal the Chamber and place at 37°C in the cell incubator. The gas utilized is a gas mixture consisting of 95% nitrogen, 4.5% carbon dioxide and 0.5% oxygen. Furthermore, it must be noted that the pressure cannot exceed 2 PSI as it will rupture the chamber under intense pressure. The 12-hour hypoxia treatment was chosen because from pilot studies, it was indicated that this was the optimum time span to achieve hypoxic conditions. This time period was based on cell viability results as well as other tests obtained from varying the exposure length in the pilot studies (data not shown).

3.2.7 Pre-treatments and treatment groups

The oxygenated cells in the control group (Group I) were incubated with serum free DMEM at 37°C in humidified air enriched with 5% CO₂ for 12 hours. As for the cells subjected to hypoxic conditions (group 3 & 4), after the addition of serum free DMEM, appropriate doses of the drugs were applied (Table 2), they are incubated for one hour and then subjected to hypoxic conditions. For the selection of the effective doses for the
respective drugs, the final concentration for flavonoids was chosen to be 0.1mg/ml. As for losartan, the final concentration was 1μM. The reason for the choice of the above final concentration was based on pilot studies (data not shown) from observation of the cell morphology of HBEC. The cell morphology for normoxic control cells (Figure 19) was that the cells were spindle shaped and well defined. The cells when treated with the effective doses also exhibited similar morphology as the normoxic control cells. On the contrary, for cells exposed to hypoxic conditions (Figure 20), the cells would be rounded up in shape and some cells might be floating in the medium. This situation is detected if the chosen concentration of drug were not effective.

Cells for experiments were grown in standard six-well plate. Stock solutions of 100μM losartan, 10mg/ml of flavonoids were prepared by dissolving the drugs in autoclaved distilled water. Distilled water was pipetted into each well for normoxic control and hypoxic control groups; losartan was pipetted into each well for the losartan treated group and flavonoids was pipetted into each well for Ceylon tea treated group to get a final concentration of 1μM of losartan and 0.1mg/ml of flavonoids.
Figure 18: Modular Incubator Chamber (MIC-101TM) for hypoxia

Figure 19: Cell morphology of HBEC in normoxic conditions. The cells are spindle shaped, well characterized and attached to the flask.

Figure 20: Cell morphology of HBEC in hypoxic conditions. The cells are rounded up, not attached to the flask and some are floating in the medium.
3.2.8 Evaluation of cell viability

3.2.8.1 Trypan Blue-Exclusion Viable Cell Counting

The principle of the trypan-blue exclusion assay is to make use of the uncompromised membranes in healthy cells exclude the entry of stains or dyes into the cytosol. Trypan-blue is a negatively charged chromophore, it does not interact with cells unless the cells membrane is damaged. Those damaged or dead cells are stained blue in color while the viable cells would exclude trypan blue and are not stained blue.

The cells which seeded in the 6-well plate were detached by removing the medium firstly and incubated with 1ml of trypsin for 2-3 minutes. After incubation the action of trypsin is inhibited by adding 3ml medium and pipetting it up and down to ensure they are well mixed. Transfer 1ml of the solution into an Eppendorf tube and mixed thoroughly with 1ml of 0.4% trypan blue. 9μl of this mixture is then placed into a haemocytometer for cell counting. This is done by placing the pipette onto the edge of one of the 2 chambers of a haemocytometer which with a cover-slip on the top. The mixture is then drawn into the empty chamber by capillary action and to make sure that no bubbles are formed. Under the microscope, the number of unstained cells lying within the border of each of the four corner squares of the camber counted. It is repeated for the other camber of the haemocytometer.

As the concentration of cells has been diluted by a factor of 2 (when 1ml of cells were mixed with 1ml of trypan-blue) and the 4 corner squares each represents a volume of 1
x $10^4$ ml, the total number of unstained cells in the 4 corner squares were added up and divided by 4 to find the average value which would represent the average number of unstained cells in $1 \times 10^4$ ml of the mixture. The actual cell concentration in the well would be twice the average number of unstained cells in $1 \times 10^4$ ml of the mixture. The formula of cell concentration per ml of medium is: average number of unstained cells in $1 \times 10^4$ ml of the mixture x $2 \times 10^4$ ml. Thus, the subsequent cell concentration per ml is determined as follows:

$$\text{Viable cells per ml} = \text{average unstained cells per square} \times \text{dilution factor of } 2 \times 10^4$$

For assessment of cell viability, both stained and unstained cells in the square were counted also. Cell viability, expressed as % trypan blue exclusion was calculated as follows:

$$\text{Cell viability} = \left[ \frac{I}{(I + II)} \right] \times 100\%$$

- $I$ = average number of unstained cells
- $II$ = average number of stained cells

3.2.8.2 Lactate Dehydrogenase (LDH) assay

The LDH assay measures the injury or damage inflicted on the cells by the state of the membrane integrity. This method was described by Legrand et al., 1992 and Decker et al., 1988, using TOX-7 LDH based *in vitro* toxicology assay kit (Sigma, St Louis, MO, USA). The states of injury inflicted on the cells under hypoxia condition are measured
by the level of lactate dehydrogenase enzyme into the medium that the cells were grown in. The lactate dehydrogenase enzyme level has been released in the extracellular medium. As LDH is formed in the cytosol, detection of LDH in the extracellular medium would indicate that the membrane, which acts as a selective barrier for substances entering and exiting the cells, has been disrupted and thus a higher level of LDH released indicate a reduction in cell viability. The assay is carried on based on the reduction of NAD to NADH as lactate is converted to pyruvate by LDH. NADH is utilized in the stoichiometric conversion of a tetrazolium dye formazan (Figure 21). The colored compound is then measured spectrophotometrically under the microplate reader at 490nm wavelength with 690nm wavelength as the background (Nachlas et al., 1960).

Figure 21: The conversion of NAD to NADH via LDH released from hypoxic cells. NADH was then used in the conversion of the tetrazolium dye to a colored formazan derivative for spectrophotometer measurement.
After the pre-treatments, the cell-free culture medium from each well was extracted and placed in Eppendorf tubes. It was wrapped in aluminum foil and incubated at room temperature for 30 mins. In a 96-well microplate, 10μl of dye solution (Tetrazolium salt) (Sigma) was added, 10μl of sodium lactate and NAD as a substrate and 10μl of diaphorase (enzyme) were added too. 60μl of medium was added to the well, 9μl HCl was added to stop the reaction. Measurement of the formazan formed is then measured spectrophotometrically at 490nm with a background wavelength at 690nm. The blank and control were also assayed in the same way.

After the results were obtained, the LDH levels measured from each of the treatment groups (Group 3 & 4) were then compared with respect to the baseline LDH level in the control group (Group 1). As for the prediction of the protective effects of the different treatments, the LDH level for each treatment group was expressed with respect to the LDH level in the hypoxia group (Group 2). % Cytotoxicity was calculated based on:

\[
\% \text{ Cytotoxicity} = \frac{\text{Abs}_s - \text{Abs}_c}{\text{Abs}_m - \text{Abs}_c} \times 100
\]

- \(\text{Abs}_s\) = Absorbance of Sample cells
- \(\text{Abs}_c\) = Absorbance of Control cells
- \(\text{Abs}_m\) = Absorbance of Maximum LDH release sample

3.2.9 Total RNA isolation

3.2.9.1 Homogenization and phase separation

After removing the culture medium from 6-well plate, 1ml of the TRIzol Reagent
(Invitrogen, CA, USA) was then added to each well to lyse the cells. Then, with a cell scraper, the cells were being detached via a circular scraping motion, and transferred into a fresh tube. The cell lysate was then incubated at room temperature for 5 minutes to allow the nucleoprotein complexes to dissociate completely. Next for phase separation, 0.2ml of chloroform was added to the cell lysate followed by shaking the tubes for 15 seconds for the formation of a homogenous mixture. The tubes were then incubated at room temperature for 2 minutes. After which, the tubes were centrifuged at 12,500g for 5 minutes at 4°C. RNA should be found exclusively in the upper aqueous layer which was colorless. After the phase separation, RNA precipitation was carried out.

3.2.9.2 RNA precipitation and quantitation

0.5ml of Isopropyl alcohol was added to the clean tube which contained extracted RNA to precipitate RNA, mix well and incubated at room temperature for 10 minutes. Then the tube was centrifuged at 12000g at 4°C for 10 minutes. Before centrifugation, the RNA precipitate which is often invisible forms a gel-like pellet on the side and bottom of the tube. After centrifugation, the supernatant was discarded. The pellet was washed with 1ml of 75% ethanol and mixed by hand gently, then centrifuged at 7500g at 4°C for 5 minutes. After that, the supernatant was discarded and the RNA pellet was air-dried for 15minutes. The RNA pellet was re-dissolved in 30μl of deionized water and incubated at 55°C until the pellet no longer become visible.
The concentration RNA in the sample can be determined by measuring the absorbance at 260nm. The total RNA extraction was diluted 100 times with water and measured by UV spectrometer. The purity of the RNA was assessed based on the ratio of absorbance at 260nm/absorbance at 280nm. A value of 1.5 to 2 is considered as pure.

3.2.10 Reverse Transcription –Polymerase chain reaction (RT-PCR)

This is used to quantify the expression level of the specific genes of interest by measuring the abundance of the specific mRNA transcript, glyceraldehydes-3-phosphatase dehydrogenase (GAPDH) was considered as an internal standard gene.

The following pre-PCR thermal conditions were used for all the RNA, consisting of reverse transcription at 50°C for 30 minutes, followed by activation of the HotStart DNA polymerase at 95°C for 15 minutes. Before carrying out the RTPCR, a master mix was prepared. Table 3 shows the composition of the master mix.

The PCR cycling conditions used were as follows: A reverse transcription step at 50°C for 30 minutes, activation of HotStarTaq DNA polymerase at 94°C for 15mins and GAPDH at 25 cycles of 94°C for 1minute, 60°C for 1minute and 72°C for 1minute; CAT, GPx and p53 at 35 cycles of 94°C for 45 seconds, 55°C for 1minute and 72°C for 1minute; Bax, Bcl-2, Fas and GST at 30 cycles of 94°C for 45 seconds, 56°C for 1minute and 72°C for 1minute and SOD at 25 cycles of 94°C for 45 seconds, 50°C for 1minute and 72°C for 1minute. A final 10 minutes extension at 72°C was performed.
after the thermal cycling. The number of cycles was 30 cycles. The primer sequences used for the cDNAs of GAPDH, CSE and HO-1 genes are listed in Table 4.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume to be added</th>
</tr>
</thead>
<tbody>
<tr>
<td>QIAGEN OneStep RT-PCR</td>
<td>5x buffer 2.5μl</td>
</tr>
<tr>
<td>QIAGEN OneStep RT-PCR dNTP mix</td>
<td>0.5μl</td>
</tr>
<tr>
<td>Anti sense primer</td>
<td>0.75μl</td>
</tr>
<tr>
<td>Sense primer</td>
<td>0.75μl</td>
</tr>
<tr>
<td>QIAGEN OneStep RT-PCR Enzyme Mix</td>
<td>0.5μl</td>
</tr>
<tr>
<td>Template RNA</td>
<td>Vary according to quantification</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>XX - top up to 12.5μl</td>
</tr>
<tr>
<td><strong>Total volume of mixture</strong></td>
<td><strong>12.5μl</strong></td>
</tr>
</tbody>
</table>

**Table 3:** RT-PCR constituents

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences</th>
<th>Base pairs &amp; Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH-Sense</td>
<td>5'-CATGGTCTACATGTTCCAGT-3'</td>
<td>House keeping</td>
</tr>
<tr>
<td>GAPDH-Antisense</td>
<td>5'-GGCTAAGCAGTTGTTGGTGTC-3'</td>
<td>349pb</td>
</tr>
<tr>
<td>Bax-Sense</td>
<td>5'-GCAGGGAGGATGGCTGGGGAGAGA-3'</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>Bax-Antisense</td>
<td>5'-TCCAGACAAGCAGCCGCTCAGC-3'</td>
<td>352pb</td>
</tr>
<tr>
<td>Fas-Sense</td>
<td>5'-ACCATGAGAACATCTTGCTGCC-3'</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>Fas -Antisense</td>
<td>5'-TCCCTGCTCATGATGTCTACC-3'</td>
<td>249pb</td>
</tr>
<tr>
<td>Asp53-Sense</td>
<td>5'-GGACTAGCATTGTCTTGTCAAGC-3'</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>Asp53-Antisense</td>
<td>5'-ATGTTCCGAGAGCTGAATGAGG-3'</td>
<td>270pb</td>
</tr>
<tr>
<td>Bcl-2-Sense</td>
<td>5'-CCGGGAGATCGATGGAAGTA-3'</td>
<td>Anti-Apoptosis</td>
</tr>
<tr>
<td>Bcl-2-Antisense</td>
<td>5'-CATATTTGTGTTGGGCGATGCT-3'</td>
<td>280pb</td>
</tr>
</tbody>
</table>

**Table 4:** Oligonucleotide sequences of primers used for RT-PCR
3.2.10.1 Agarose Gel Electrophoresis

After PCR, the PCR products were then electrophoresed on 1.5% agarose gel (Bio-Rad). 1.5μl of ethidium bromide (EtBr) was added, so that the bands would be fluorescently labeled for the ease of manipulation of results subsequently. 10μl of the PCR products were then removed from each of the reaction tubes and mixed thoroughly with 2μl of 6x green loading dye (Promega, USA). Before loading on the samples, 10μl of 100 bp DNA ladder (Promega, USA) was mixed well with 2μl of the 6x green loading dye. Finally, the DNA ladder, as well as the sample, were all loaded into the wells. In order to run the gel, the MultiGenius Bioimaging system (Syngene, UK) was utilized and set to a constant voltage of 125 volts and for 55 minutes.

The gel was viewed under the option of ‘UV Transilluminatesation’. The software program used for this machine was ‘Quantity One’. In order to quantify the amount of mRNA, the band intensity was then analyzed via the ChemiDoc XRS (Bio-Rad) under the option of ‘Volume INT*mm2’. To normalize for differences in total RNA added into the respective tubes, the constitutively expressed GAPDH was selected as a reference for the total cellular RNA loaded respectively for each sample. Subsequently, the amount of RNA used for each reaction was then adjusted accordingly to ensure the house keeping gene band intensities were constant, with a permissible difference of ±20%. Finally, the results were then expressed relative to the equivalent intensity of the housekeeping gene, GAPDH, from the same RNA sample.
3.2.11 Antioxidant Enzyme Activity Assay

Cells were detached and removed from the bottom of T-75 flasks by adding 1ml of trypsin and incubated at 37°C for 2-3 minutes. 3ml of medium was then added to stop the action of trypsin. The mixture was pipetted up and down repeatedly to separate and prevent clumping of the cells. Then transferred to a clean 15ml centrifuge tube and centrifuged at 1000g for 5 minutes at room temperature to get the cell pellet. 5ml of phosphate buffer, pH 7.4, was added to the cell pellet and homogenized by using a Polytron homogenizer. The homogenate were mixed into a tube and centrifuged at 1000g at 4°C for 10 minutes.

Carefully transfer the primary supernatant into a clean tube and it was divided into two parts (A & B) for different enzyme tests. One part of supernatant (A) would be used for SOD and CAT activity assays. These tubes were stored at -80°C deep freezer. Supernatant (B), which would be used for GST and GPx activity assays, was further centrifuged at 2300g at 4°C for 10 minutes. After centrifugation, the supernatant was transferred to an ultracentrifuge tube and spin at 100,000g at 0°C for 1 hour using the ultra centrifuge machine (Beckman L8-70; Beckman Instruments Inc, Fullerton, CA, USA). The supernatant was poured out and aliquot into several Eppendorf tubes for GST, GPx and total protein assay. The procedure was simplified as shown in Figure 22:
Cultured HBEC cells from at least two 15cm flasks

\[ \downarrow \]
Homogenate obtained by suspension and re-suspension, mixing well

\[ \downarrow \]
Primary supernatant

\[ \downarrow \]
Supernatant divided into 2 parts

\[ \begin{align*}
\text{Supernatant} & \quad \text{Supernatant} \\
\downarrow & \quad \downarrow \\
\text{Centrifuged at 2300g at 4°C for 10 min} & \quad \text{Centrifuged at 100,000g at 0°C for 90 min} \\
\downarrow & \\
\text{Supernatant I} & \quad \text{Supernatant II} \\
1:40 & 1:20 \\
\text{For} & \quad \text{For} \\
\text{Protein Assay} & \quad \text{CAT Assay} \\
\text{SOD Assay} & \quad \text{GPx Assay} \\
& \quad \text{GST Assay}
\end{align*} \]

Figure 22: Preparation of cultured cell to Antioxidant Enzyme Activity Assay

### 3.2.11.1 Superoxide Dismutase Enzyme (SOD) Activity Test

SOD activity in the sample is assessed by using an improved method of Marklund and Marklund as stated by Ji (Ji et al., 2003). This method is based on the principle that SOD inhibits the auto-oxidation of pyrogallol (1, 2, 3-Trihydroxybenzene) by catalyzing the breakdown of superoxide. The function of all SODs remains the same - they catalyze \( \text{O}_2^- \) reduction to \( \text{H}_2\text{O}_2 \):
In alkaline solution, pyrogallol auto-oxidize rapidly to form several intermediate products, causing the solution to appear yellow-brown then green after a few minutes and finally a yellow color after a few hours. The rate of auto-oxidation of pyrogallol was first determined using a standard solution of 980μl of 0.1M Tris-HCl, 0.5mM EDTA, pH 8.0 and 20μl of 10mM pyrogallol solution in 10mM HCl. The rate of auto-oxidation is measured by measuring the change of optical density with a wavelength at 420nm for 1 minute. After this, system solution mixtures of 1000μl were prepared using varying amounts of the sample (Supernatant diluted 10 times with phosphate buffer, pH 8.0) ranging from 10-15μl and top up with Tris-HCl in cuvettes. The mixtures were incubated at room temperature for 10 mins before adding 20μl of 10mM Pyrogallol. The rate of oxidation of pyrogallol in the presence of 10-50μl of sample is followed by measuring the change in optical density at 420nm for 1 minute. The concentration of supernatant at which 50% inhibition of auto-oxidation occur is determined from the plot of the change in optical density per minute at 420nm versus volume of sample. The amount of SOD enzyme producing 50% inhibition is defined as one unit of enzyme activity (unit / mg of protein). The concentration of the antioxidant enzymes in the samples is expressed in term of μmol per minute per mg of protein.
3.2.11.2 Catalase Enzyme (CAT) Activity Test

The activity of catalase is measured by using the method of Aebi (1984) as stated by Ji (Ji et al., 2003). This method measures the rate of decomposition, catalyzed by catalase, of hydrogen peroxide ($\text{H}_2\text{O}_2$) into oxygen and water. The principle of this reaction is expressed as follows:

$$\text{CAT} \quad \frac{2 \text{H}_2\text{O}_2}{\rightarrow} \frac{2 \text{H}_2\text{O} + \text{O}_2}{\text{2 H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2}$$

As hydrogen peroxide absorbs light at 240nm, the solution which contained hydrogen peroxide was measured spectrophotometrically with a wavelength at 240nm. The rate of decomposition is obtained by calculating the change in optical density.

50µl of 0.1M Tris-HCl, 0.5mM EDTA at pH 8.0 and 940µl of H$_2$O were added into a Quartz cuvette and mixed by pipetting up and down and left in room temperature for 10 minutes incubation. After incubation, then it was placed in the Spectrophotometer (Beckman) and the rate of decomposition of hydrogen peroxide was measured at 240nm for 1 minute. This was a blank, used as a control. For the sample, the steps are similar except that 940µl of H$_2$O$_2$ was added instead of H$_2$O and 10µl of sample (diluted 10 times with phosphate buffer: 100µl of sample with 900µl of buffer) was added to the quartz cuvette mixed by covering with a parafilm and inverting up and down for a few times. Triplicates of the samples were carried out. The average value of the rate of decomposition of hydrogen peroxide is obtained. 1µmol of hydrogen peroxide
decomposed 1 minute is expressed as 1μmol/min/mg protein of enzyme activity.

3.2.11.3 Glutathione-S-Transferase Enzyme (GST) Activity Test

The activity of the enzyme of GST is measured by using the method of Habig and Pabst (1974) as stated by Ji (Ji et al., 2003). This is based on the fact the GST catalyzes the conjugation of GSH to 1-chloro-2, 4-dinitrobenzene (CNDB) by the –SH group of glutathione to form CNDB-S-glutathione which absorbs light at 340nm. The activity of glutathione-S-Transferase was measured by measuring the rate of increase of the CNDB-S-glutathione conjugate. The principle of this method is that GST catalyzes the reaction of CDNB with the -SH group of glutathione:

\[
\text{CDNB} + \text{GSH} \xrightarrow{\text{GST}} \text{CDNB-S-glutathione}
\]

960μl of H2O; 50mM of glutathione reduced form of GSH; 20μl of 50mM CNDB in ethanol were added in a cuvette, mixing well by pipetting up and down and incubating at room temperature for 3 minutes before measuring the change in optical density of CNDB-S-glutathione at a wavelength at 340nm. This was a blank, used as a control. For the sample, the steps are similar except that 960μl of 0.1M phosphate buffer, pH 6.5 was added instead of H2O and 10μl of 10 times diluted sample was added after incubation and the rate of change of optical density was measured at 340nm for 1 minute. 1 unit of GST is expressed as the amount of enzyme necessary to conjugate of
1 μmol CNDB with 1 μmol GSH per minute per mg protein.

3.2.11.4 Glutathione Peroxidase Enzyme (GPx) Activity Test

The activity of the enzyme of GPx is measured by using the method of Buetler (1984) as stated by Ji (Ji et al., 2003). The activity of the GPx enzyme is accessed by the rate of formation of GSSH. This is reflected by the detection and measurement of the formation of NADP+, which is formed by the glutathione reductase catalyzed reaction of GSSH. Oxidation of NADPH to NADP+ is measured with a wavelength at 340nm. Activity of GPx is defined as μmol/min/mg protein. The above reactions are summarized as follows:

\[
\text{GPx} \quad 2\text{GSH} + \text{R-O-O-H} \rightarrow \text{GSSG} + \text{H}_2\text{O} + \text{R-OH}
\]

760μl of 1.0M Tris-HCl, 0.5mM EDTA buffer, pH 8.0; 20μl of 1.0M GSH (glutathione reduced form); 100μl GSSH Glutathione Reductase (10unit/ml); 100μl of 2.4mM NADPH (20mg/ml) and 10μl of 10 times diluted sample were added into a cuvette, mixing well and incubated at room temperature for 10 minutes. After incubation, 10μl of 7mM t-Butyl Hydroperoxide was added and mixed well. Immediately placed the cuvette into the Spectrophotometer and measured at 340nm to get the rate of decrease of NADPH. The cuvette which consisted of 1ml of H₂O was measured as a blank.
3.2.11.5 Total Protein assay

The total amount of protein content in each sample is quantified by using the Bradford method. Bovine Serum Albumin standard of 1mg/ml was prepared by dissolving 20mg of BSA in 20ml of phosphate buffer. The BSA standard was then diluted 10 times to obtain a concentration of 0.1mg/ml BSA working standard. Total protein standard was prepared by adding 20μl, 40μl, 60μl and 80μl of the BSA working standard into 980μl, 960μl, 940μl and 920μl of phosphate buffer respectively. The final standard concentrations of Bovine Serum Albumin were: 2μg/ml, 4μg/ml, 6μg/ml and 8μg/ml. A standard curve would be then calibrated. 200μl of Bio-Rad dye reagent is added to all the tubes and mixed well for 5 minutes incubation at room temperature. The total protein in each sample is obtained by measuring the optical density with a wavelength at 595nm.

5μl of sample (supernatant) was added into the cuvette which contained 995μl of phosphate buffer. 200μl of the Bio-Rad dye reagent was added into the cuvette and mixed well. The protein contented sample was measured at 595nm.

3.2.12 Immunohistochemical Staining

3.2.12.1 Sample preparation

To prepare for immunostaining of cells, cover slips were placed into the 6-well plates with autoclaved tweezers. The cells were seeded onto the cover slips and confluence was attained after 24 hours. After which, the cells were treated with appropriate drug
dosages similar to the addition of drugs as before. The cells were fixed with 4% paraformaldehyde for 25 minutes at 4°C. Subsequent steps for immunostaining were carried out in the wells.

3.2.12.2 Antibody Staining

Immunohistochemical staining (Immunostaining) is a method (Loh et al., 2006) to show the expression level regulation of interested genes.

The purpose of immunostaining was that it permitted the visualization of antigens via the sequential application of a specific primary antibody to the antigen, with subsequent binding of secondary antibody to the primary antibody, as well as formation of an enzyme complex and a chromogenic substrate with interposed washing steps. After this chromogenic enzymatic activation, a visible reaction product was detected at the antigen site. A ready to use UltraVision Detection System Anti-Polyvalent, HRP/DAB kit (Lab Vision Corporation, CA, USA) was purchased for the antibody staining. The cover slips in the wells were first washed with 0.2% 1x phosphate buffered saline-Triton X (0.2% PBS-Tx) for five minutes. Then the cover slips were incubated with hydrogen peroxide block for 5 minutes at room temperature. The role of hydrogen peroxide was to block endogenous peroxidase activity which could mask any positive staining of the specific antigens. After blocking, the cover slips were washed twice in 0.2% PBS-Tx, five minutes each time. Subsequently, Ultra V Block was used to incubate the cover slips for 5 minutes. This step was required for suppressing any possible nonspecific background
staining. Then the cover slips were rinsed in 0.2% PBS-Tx. After which, primary antibody was added. The following primary antibodies were used: polyclonal rabbit anti-CSE antibody (manufactured from our laboratory) and polyclonal rabbit anti-HO-1 antibody (sc-10789, Santa Cruz, CA, USA). The polyclonal rabbit anti-CSE antibody was used for incubation of the specimens for 2 hours. Whereas the polyclonal rabbit anti-HO-1 antibody was used for incubation of the specimens for 3 hours. The dilutions of the antibody were done according to manufacturer’s instruction and the antibodies were diluted with 0.2% PBS-Tx. Extra caution was needed to prevent the cover slips from drying up and this is done by continual addition of the primary antibodies. After incubation with the primary antibody for the optimized times, the cover slips were washed with 1x phosphate buffered saline (1x PBS) for 4 times, 5 minutes each. After the successful incubation of the primary antibody, the specimens were incubated with Biotinylated Goat Anti-Polyvalent for 2 hours. Similarly, continual addition of the secondary antibody was needed to prevent complete drying up of the slides. After the optimal incubation duration with secondary antibody, the specimens were then washed with 1x PBS 4 times, 5 minutes each. Then, the cover slips were incubated with streptavidin peroxidase for 10 minutes, which conjugated with biotin found on the secondary antibody. Subsequently, the specimens were washed in 1x PBS 4 times, 5 minutes each. After which, 3, 3’ diaminobenzidine tetrahydrochloride (DAB) working solution was used for detection of specific antibody, secondary antibody and streptavidin-enzyme complex. This colorimetric detection was performed with a
working mixture of one drop of liquid DAB in 1ml of chromogen solution. The slides were rinsed immediately in 1x PBS for 4 times once a brown tinge was detected.

3.2.12.3 Hematoxylin staining

The purpose of the hematoxylin staining was that it stained nuclei blue via the binding of a mordant dye complex to nucleic acids and histone proteins of the heterochromatin. This allows for the detailed observation of the morphology of the specimens. After the brown tinge was detected, hematoxylin was added to the wells for attachment to the cover slips. This is an essential step for counter staining. To prevent strong background staining, the specimens were washed immediately with tap water to remove traces of hematoxylin. After counter staining, the next step involved was dehydration. This is implemented by addition of gradual increasing concentrations of ethanol. Firstly, with 70% ethanol for one minute, then 80% ethanol for one minute, then 100% ethanol for 2 times, one minute each. Lastly, addition of xylene 2 times, one minute each.

3.2.12.4 Mounting of Slides

The last step is indispensable for the permanent storage of specimens. The specimens were mounted by a drop of mounting medium Permount (Fisher Scientific International, PA, USA). Subsequently the specimens were then mounted to polysinecoated slides. After the slides were dried overnight in the hood, they were viewed and photographed using a fluorescent Leica® microscope (Leica Microsystems, IL, USA).
3.2.13 TUNEL (Terminal deoxynucleotidyl Transferase mediated dUTP-Fluorescein Nickend Labeling) Staining

By detecting DNA fragmentation in programmed cell death to detect apoptotic cells, this technique is used for the assay follow the protocol which provided from the TUNEL System. A DeadEnd™ Fluorometric TUNEL System (G3250, Promega, USA) kit was chosen for detection of apoptosis-induced nuclear DNA fragmentation via fluorescence microscopy. This assay was performed as described previously in Loh (Loh et al., 2006). Slides were viewed and photographed using a fluorescent Leica microscope using standard fluorescence filter set to view the green fluorescence of fluorescence at 520 ± 20nm while red DAPI was viewed at 620nm.

Cells were grown on microscope slides placed in each well in a six-well plate until 70-80% confluency and then subjected to the various treatments of drug incubation and hypoxia. After treatment, the slides were removed from the wells and placed onto glass slides and fixed into position by applying mounting medium on the four corners or edges to prevent them from falling off. Then the cells were fixed on the sliders by immersing them in freshly prepared 4% paraformaldehyde in PBS in a Coplin jar for 25 minutes at 4°C. The slides were then washed by immersing them in fresh PBS at room temperature for 5 minutes and repeat it. The cells were pre-metabolized by immersing the slides in 0.2% Triton X-100 solution in PBS for 5 minutes and then rinsed by immersing in fresh PBS for 5 minutes at room temperature, repeat it again. Excess liquid were removed by tapping the slides and the cells were then equilibrated in 100µl
of Equilibration buffer at room temperature for 5-10 minutes. During this period, the Nucleotide mix was thawed on ice and the incubation buffer, which consisted 45μl of Equilibration buffer, 5μl of Nucleotide mix and 1μl of the rTdT enzyme per standard, 50μl reaction for an area not larger than 5cm², was prepared for a volume required for the total number of reactions. The incubation buffer was kept on ice and protected from light. The equilibrated area was then blotted with tissue paper and 50μl of rTdT incubation buffer was added to 5cm² of cells. The slides were placed onto paper towel soaked with water and incubated for 1 hour at 37°C in the dark to allow the tailing reaction to occur, be very careful that the cells didn’t over dried. Then immersing the slides into Coplin jars filled with 40ml of 20 x 10 times diluted SSC with deionized water at room temperature for 15 minutes to terminate the reaction. After that the slides were washed by immersing in fresh PBS for 5 minutes at room temperature for a total of 3 washes to remove unincorporated fluorescence 12-dUTP. The samples were then stained by immersing the slides in Coplin jars in 40ml of propidium iodide solution freshly diluted to 1μg/ml in PBS for 15 minutes at room temperature in the dark. After staining, the slides were washed using deionized water for 5 minutes for a total of 3 washes. Coverslips were then placed on the dried samples and sealed with nail polish on the sides and edges. Samples were analyzed by using under a fluorescence microscope with a standard fluorescence filter set at 520±20nm to view the green fluorescence and at > 620nm to view the red fluorescence of propidium iodide. Slides could be stored overnight at 4°C in the dark.
3.2.14 Data Analysis and Graph Presentation

In experiments where there was only single comparison, that is, one treatment to control, the Student's t-Test was used to analyze the data.

All relevant data collected were expressed as means ± SEM. As for the statistical analysis, it was achieved using the unpaired Student's t test. The calculation of mean, standard deviation, standard error of the mean (SEM) and the Student's t-Test (using 2-tail and unequal variance or samples) was done using Microsoft Office Excel 2003. The R software (R Development Core Team 2005) was used to analyze the data by plotting box plots, one-way anova analysis, and if anova test indicated significant results, a $p$ value of less than 0.05 indicated that the result was significant.

In general, graphs were plotted using the mean with Y error representing the SEM of the sample and '*' denoting significant data is less than 0.05 compared with vehicle or hypoxic group; '**' denoting significant data is less than 0.01 compared with vehicle or hypoxic group.
4.1 Biochemical Assays:

Anti-oxidant test *in vitro*:

4.1.1 Inhibition of ABTS Assay

All the green tea extracts showed significantly antioxidant effect results. The Ceylon green tea (final concentration of 10μg/ml) had 68±2.8% scavenging effect for ABTS⁺ that of ascorbic acid (100%) as well as trolox, which was taken as a standard in this experiment. Its average TEAC value was similar as Chinese green tea (Qian Dao green tea, 82±1.2% and Bi Xue Chun green tea, 80±1.4%) which are well known strong antioxidant. Of course it has slightly lower (52±1.2%) scavenging effect for ABTS⁺ if the final concentration of Ceylon green tea was 1μg/ml, same for Qian Dao green tea (61±4.1%) and Bi Xue Chun green tea (55±1.1%) (Plate 1).
Plate 1: Effects of ascorbic acid and various green tea extracts on TEAC values expressed as ascorbic acid equivalents (ABTS test). Two concentrations: 1μg/ml and 10μg/ml were tested. Results shown are mean ± S.D. of at least three independent experiments performed in duplicate.
4.1.2 Inhibition of Pyrogallol Red Bleaching by HOCl

The scavenging effects of Ceylon green tea extract on HOCl as determined by pyrogallol red bleaching assay showed that the Ceylon green tea had an equivalent percentage inhibition (79%±4.5%) as that for Chinese green tea (Qian Dao green tea, 81%±4.4% and Bi Xue Chun green tea, 83%±3.3%) when the concentration was 1mg/ml. Ascorbic acid was used as a positive control. With the final concentration of 100μg/ml of Ceylon green tea, it had slightly lower (28%±3.1%) inhibition effect, same for Qian Dao green tea (56%±4.3%) and Bi Xue Chun green tea (45%±2.1%) (Plate 2).
Plate 2: Effects of ascorbic acid and various green tea extracts on the inhibition of pyrogallol red (PR) bleaching by hypochlorous acid (HOCl). Samples were tested at a final concentration of 100µg/ml and 1mg/ml. Results shown are mean ± S.D. of at least three independent experiments performed in duplicate.
4.2 Evaluation of DNA Damage using GC/MS

From analysis of DNA damage using gas chromatography-mass spectrometry, the hypoxia with flavonoids treated group showed a significantly lower (p<0.005) quantity for the total oxidized DNA bases (1.13±0.42) when compared to Vehicle (1.53±0.36, respectively) (Table 5). 8-OH Guanine, as a DNA damage biomarker, showed that its level in flavonoids treated group (0.12±0.03) is lower than in vehicle group (0.23± 0.04) (p<0.05).

<table>
<thead>
<tr>
<th>DNA base Products</th>
<th>Non-hypoxia</th>
<th>Hypoxia (Vehicle)</th>
<th>Hypoxia+ flavonoids treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-OH, Me Hydantoin</td>
<td>0.12±0.16</td>
<td>0.13±0.19</td>
<td>0.13±0.06</td>
</tr>
<tr>
<td>5-Formyl Uracil</td>
<td>0.17±0.05</td>
<td>0.98±0.01</td>
<td>0.89±0.65</td>
</tr>
<tr>
<td>5-OH Uracil</td>
<td>0.03±0.00</td>
<td>0.05±0.00</td>
<td>0.05±0.03</td>
</tr>
<tr>
<td>5-(OH, Me) Uracil</td>
<td>0.04±0.01</td>
<td>0.08±0.02</td>
<td>0.05±0.03</td>
</tr>
<tr>
<td>5-OH Cytosine</td>
<td>0.04±0.01</td>
<td>0.24±0.01</td>
<td>0.14±0.02*</td>
</tr>
<tr>
<td>FAPy Adenine</td>
<td>0.08±0.07</td>
<td>0.16±0.05</td>
<td>0.12±0.04</td>
</tr>
<tr>
<td>8-OH Adenine</td>
<td>0.06±0.02</td>
<td>0.49±0.02</td>
<td>0.09±0.02*</td>
</tr>
<tr>
<td>2-OH Adenine</td>
<td>0.04±0.02</td>
<td>0.06±0.01</td>
<td>0.02±0.02*</td>
</tr>
<tr>
<td>FAPy Guanine</td>
<td>0.03±0.02</td>
<td>0.08±0.01</td>
<td>0.02±0.02*</td>
</tr>
<tr>
<td>8-OH Guanine</td>
<td>0.07±0.06</td>
<td>0.23±0.04</td>
<td>0.12±0.03*</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>0.68±0.32</strong></td>
<td><strong>1.53±0.36</strong></td>
<td><strong>1.13±0.42</strong>*</td>
</tr>
</tbody>
</table>

Table 5: Oxidized DNA base products were analyzed and quantified by GC/MS and Vehicle group was set as positive control. Results are expressed as mean ± S.D. of 3 determinations performed in triplicate and expressed as nmol/mg DNA. *p<0.05.
4.3 The finger printing of flavonoids from Ceylon green tea obtained by HPLC

The Ceylon green tea sample were analyzed by HPLC. HPLC chromatograms of flavonoids and its peak fractions were shown in Plate 3. The mean compounds (peak 1, 2, 3, 4, 5, 6 and 7) referred to the standards, they are: Epigallocatechin (EGC), Catechin (C), Epicatechin (EC), Caffeine (CA), Epigallocatechin gallate (EGCG), Epicatechin gallate (ECG) and Catechin gallate (CG).
Plate 3: HPLC chromatograms of Ceylon green tea sample and peak fractions (1–4), Column: Zorbax SB-18 (150 mm × 2.1 mm I.D.); Injection volume: 5 μl; mobile phase: 0.1% formic acid-methanol (methanol: 0–30 min, 20–80%; 0.1% formic acid: 0–30 min, 80-20%); flow rate: 0.3 ml/min; detection wavelength: 280 nm.
4.4 Cell viability

As shown in Plate 4 & Plate 5, after being subjected to 12 hours hypoxia, the trypan-blue exclusion was slightly reduced. Both flavonoids and losartan combined treated group reduced significantly cells death in the hypoxic condition.

The higher percentage LDH release was significantly higher in HBEC in the hypoxic group than in the normoxic group. Flavonoids and losartan treated groups could reduce significantly LDH release in the hypoxic condition. Combined flavonoids and losartan treated group had significant lower LDH release in the hypoxic condition.

4.4.1 Trypan Blue Exclusion Assay

Assessment of cell viability using trypan blue was performed, and the results obtained (Plate 4) are: 41%±4.7% viability in flavonoids treated, 39%±3.1% viability in losartan treated compared to 29%±2.3% in the hypoxia control group consistently. However, the effects have synergized when flavonoids and losartan combined together (67%±10.2%).
Plate 4: Cells viability measurement using trypan blue exclusion of the normoxia, hypoxia, flavonoids treated, losartan treated and combined flavonoids and losartan treated groups with hypoxic conditions. * $p<0.05$, ** $p<0.01$. 
4.4.2 Lactate Dehydrogenase Assay

Similar observations were made for the LDH assay where flavonoids treated group had 75%±3.7% reducing of LDH release and 79%±3.5% in losartan treated group in hypoxia control (Plate 5). Same as Typan blue assay, the reducing of LDH release is more effective when flavonoids and losartan combined together (48%±5.2%).

![Graph showing LDH release comparison]

**Plate 5:** Percentage of LDH release in normoxia, hypoxia, flavonoids treated, losartan treated and combined flavonoids and losartan treated groups with hypoxic conditions. *p<0.05; **p<0.01.
Both of these two assays demonstrated that the hypoxic stimulus is sufficiently damaging enough to inflict injury to HBEC, resulting in significantly lower cell viability.

4.5 Antioxidant enzyme activity Assays

In addition to find the effects of hypoxia and Ceylon green tea extract treatments on antioxidant enzyme at mRNA transcript levels, the activity levels of the anti-oxidant enzymes were measured. It has been proposed that anti-oxidant changes reflect an altered redox balance in several pathological states (Halliwell and Gutteridge, 1995). This would mean that anti-oxidants would be consumed in their reaction with free radicals. Therefore the measurement of antioxidant concentration or activity can give us an idea of the state of oxidative stress in the cells.

4.5.1 SOD (Superoxide dismutase enzyme activity)

SOD activity was shown significant decreased in the hypoxic control after 12 hours hypoxia (Plate 6). This is an indication of increased oxidative stress most likely. Both flavonoids and losartan pre-treated HBEC groups show the levels of SOD activities were higher (1.5±0.6µmol/min/mg protein and 1.7±0.38µmol/min/mg protein) than the hypoxic control group (0.5±0.02µmol/min/mg protein). This might be explained that the possess of superoxide scavenging properties such as endogenous SOD activity could be spared under oxidative stress.
Plate 6: Measurement of SOD activity in HBEC in normoxic control, hypoxic control, flavonoids treated and losartan treated groups in hypoxia. Values are expressed as mean ± S.D. *p<0.05.
4.5.2 CAT (Catalase enzyme activity)

The activity of catalase has been reported in a number of studies in several cell types in response to hypoxia (Costa, 1990; Plateel et al., 1999). In this study, it also shows the significant reduce in untreated HBEC after hypoxia (0.51±0.04μmol/min/mg protein) and the slightly increase of the activity of catalase after hypoxia in flavonoids pre-treated (0.61±0.06μmol/min/mg protein) and losartan pre-treated (0.63±0.07μmol/min/mg protein) groups (Plate 7).

Plate 7: Measurement of CAT activity in HBEC in normoxic control, hypoxic control, flavonoids treated and losartan treated groups in hypoxia. Values are expressed as mean ± S.D.
4.5.3 GPx (Glutathione peroxidase enzyme activity)

The activity level of glutathione peroxidase enzyme in HBEC after hypoxia (1.2±0.35\(\mu\)mol/min/mg protein) is significantly reduced when compare to the normoxic group. Both flavonoids (2.6±0.41\(\mu\)mol/min/mg protein) and losartan (4.2±0.81\(\mu\)mol/min/mg protein) pre-treated groups increased the level of GPx activity when compare to the hypoxic group (Plate 8).

Plate 8: Measurement of GPx activity in HBEC in normoxic control, hypoxic control, flavonoids treated and losartan treated groups in hypoxia. Values are expressed as mean ± S.D. *\(p<0.05\).
4.5.4 GST (Glutathione-S-Transferase enzyme activity)

The activity level of GST in HBEC after hypoxia shows significantly decreased (3.1±1.6μmol/min/mg protein) and both pre-treated with flavonoids (6.0±2.4μmol/min/mg protein) and losartan (6.7±1.5μmol/min/mg protein) groups show some increase after hypoxia (Plate 9).

Plate 9: Measurement of GST activity in HBEC in normoxic control, hypoxic control, flavonoids treated and losartan treated groups in hypoxia. Values are expressed as mean ± S.D. *p<0.05.
4.6 Gene expression of proteins involved in regulating apoptosis

Many studies reported that hypoxia and increase in oxidative stress can activate apoptosis, the genetically encoded program that cells employ to “commit suicide” when injured or damaged cells pose a threat to the surrounding cells. Exposure of various cell types to oxidative stress-causing agents can directly induce apoptosis. A wide range of antioxidants play a role in the reduction in cell viability to block the apoptotic pathway or to extent after hypoxia with the treatment. Gene expression of the pro-apoptotic proteins: Bax, Asp53, Fas and anti-apoptotic protein: Bcl-2 was measured by RT-PCR. The relative amount of genes of interest was measured using Syngene™.

As there are four different groups of treatment conditions, the results were presented on the same gel picture to allow comparison (Plate 10). The normal control standard (GAPDH) against Bax, Asp53, Fas and Bcl-2 were analyzed in each group.

4.6.1 Expression level of Bax

Bax, as a classic pro-apoptotic protein, its expression was weak in the normoxic group but it was over-expressed in the hypoxic group (1.55±0.25-fold, Plate 10 lane B and Plate 11) as compared to normoxic group (1-fold, Plate 10 lane A and Plate 11). Both flavonoids and losartan treated groups had slightly down regulated the gene expression of Bax at 1.12±0.15-fold and 1.20±0.3-fold in hypoxic group (Plate 10 lane C & D and Plate 11). The results were statistically significant, comparing the two drug treated groups with the hypoxic group.
The down-regulation of Bax after inducing hypoxia is only marginally significant when compared against the normoxic group. This would mean that the level of Bax in the flavonoids treated group is close to the level of Bax in the normoxic groups. This is a good sign as it indicates that the flavonoids is able to down-regulate the Bax expression in the hypoxic cells similar to normoxic level.
Plate 10: Gene expression level of Bax, Fas Asp53 and Bcl-2 and standardization of GAPDH. Both flavonoids and losartan treated groups were performed under hypoxic conditions. mRNA levels of Bax, Fas, Asp53 and Bcl-2 were normalized to the amount of GAPDH for flavonoids and losartan treated group respectively.

Lane A: normoxic group,
B: Hypoxic group,
C: flavonoids treated group,
D: Losartan treated group.
Plate 11: Effects of hypoxia and flavonoids treated and losartan treated groups with hypoxia on the gene expression levels of Bax. Data are expressed as % fold difference of normoxic control and are mean ± S.D. *p<0.05.
4.6.2 Expression level of Fas

Fas was weakly expressed in the normoxic group. It was detected significantly over-expressed (1.66±0.2-fold, Plate 10 lane B and Plate 12) in hypoxia as compared to the normoxic control (1-fold, Plate 10 lane A and Plate 12). Both flavonoids and losartan treated groups had slightly down regulated the Fas expression in hypoxia cells at 1.40±0.3-fold and 1.35±0.2-fold respectively (Plate 10 lane C & D and Plate 12).

Plate 12: Effects of hypoxia and flavonoids treated and losartan treated groups with hypoxia on the gene expression levels of Fas. Data are expressed as % fold difference of normoxic control and are mean ± S.D.
4.6.3 Expression level of Asp53

Asp53 expression is also relatively weak in the normoxic group but significantly increased in the hypoxia group (1.52±0.15-fold, Plate 10 lane B and Plate 13) as compared to the normoxic control (1-fold, Plate 10 lane A and Plate 13). Flavonoids and losartan treated groups had significantly reduced the Asp53 expression in hypoxia cells at 1.13±0.03-fold and 1.10±0.12-fold respectively (Plate 10 lane C & D and Plate 13).

Plate 13: Effects of hypoxia and flavonoids treated and losartan treated groups with hypoxia on the gene expression levels of Asp53. Data are expressed as % fold difference of normoxic control and are mean ± S.D. *p<0.05.
4.6.4 Expression level of Bcl-2

The level of Bcl-2 expression in drug treated group is marginally significant. This could mean that both flavonoids and losartan are very efficient such that it could up-regulate the level of Bcl-2 in the hypoxic cell. Its expression level was reduced in the hypoxic group (0.61±0.13-fold, Plate 10 lane B and Plate 14) as compared to the normoxic control (1-fold, Plate 10 lane A and Plate 14). Both flavonoids and losartan treated groups had significantly increased the Bcl-2 expression levels in hypoxia cells at 0.88±0.08-fold and 0.89±0.18-fold respectively (Plate 10 lane C & D and Plate 14).

The expression of the anti-apoptotic gene Bcl-2 was up-regulated, hence increasing the chances of cell survival. These would result in higher cell viability, and is consistent with the cell viability tests.
Plate 14: Effects of hypoxia and flavonoids treated and losartan treated groups with hypoxia on the gene expression levels of Bcl-2. Data are expressed as % fold difference of normoxic control and are mean ± S.D. *p<0.05.
4.6.5 Ratio of Bc1-2 and Bax in each treatment group

As known, Bcl-2 is a classic anti-apoptotic protein while Bax is a classic pro-apoptotic protein so the Bcl-2/Bax ratio is very useful in cases of determination of the status of the cells (Loh et al., 2006). It is obviously shown (Plate 15) that the flavonoids extract treated group shows lower apoptosis potential, i.e higher cell survival than in hypoxic control. It was observed that flavonoids treated group has a Bcl-2/Bax ratio of 79%±20% and losartan treated group has a ratio of 74%±12%. The ratio for hypoxia group is 40%±11%. This could imply two things: either flavonoids has lower capability in up-regulating the expression of anti-apoptotic protein Bcl-2 (or similarly down-regulating the expression of pro-apoptotic protein Bax) and hence leading to a state where both Bax and Bcl-2 were in equal activity (in this sense the cell is neither in the pro or anti-apoptotic state), or that the flavonoids was very efficient such that it was able to bring the cells to about 80% of normoxic state.

The gene expression results showed that the hypoxia cells after treatment, both flavonoids and losartan were able to significantly reduce the expression of the pro-apoptotic genes of Bax and Asp53. This would mean that the flavonoids was able to reduce the amount of apoptosis after inducing hypoxia. Also, the expression of the anti-apoptotic protein Bcl-2 was up-regulated, hence increasing the chances of cell survival.
Plate 15: Effects of flavonoids treated and losartan treated groups on the state of cell via Bcl-2/Bax ratio. Hypoxic group has the lowest ratio whereas flavonoids treated and losartan treated groups have 80% and 77% of the normoxic group. Data are expressed as % of normoxic control and are mean ± S.D. *p<0.05.
4.7 Immunohistochemical Staining result

Pro-apoptotic proteins Bax, Fas and Asp53 have been dramatically increased in the hypoxic group as compared to the normoxic group. The situation of increased pro-apoptotic proteins in hypoxic group is consistent with the increased levels of pro-apoptotic gene expression in hypoxic group. Furthermore, these pro-apoptotic proteins could only be detected in hypoxic group and not for normoxic group, indicating up-regulation of these protein during hypoxia as compared to undetectable levels of these proteins under normal conditions. It shows that the apoptosis are much more reduced in both flavonoids and losartan treated groups. No positive staining of any protein product of targeted gene was observed in the negative control from each treatment group (Plate 16 I). No Bax staining was observed to be in the normoxic group, strongest signal of Bax, where the positive staining was depicted by arrows, was observed to be in the hypoxic group. Weak positive staining of Bax was observed in hypoxia with flavonoids and losartan treated group (Plate 16 II). Similarly, the result shows the same as for Asp53 (Plate 16 IV). No Fas staining was observed in the normoxic control. Stronger signal of Fas was observed in hypoxic group. Weaker positive staining of Fas was observed in hypoxia with flavonoids and losartan treated group (Plate 16 III).

Weak signal of Bcl-2 (arrow) was detected in hypoxia group. Positive Bcl-2 staining was also observed in hypoxia with flavonoids and losartan treated group (Plate 16V).
<table>
<thead>
<tr>
<th>Non-hypoxia</th>
<th>Hypoxia</th>
<th>Flavonoids</th>
<th>Losartan</th>
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</thead>
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<tr>
<td><img src="image1.jpg" alt="Image" /></td>
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</tr>
</tbody>
</table>

**Plate 16:** Light photomicrographs of HBECs after antibody (Bax, Fas Asp53 or Bcl-2) staining.

- **I**) negative control,  
- **II**) Bax,  
- **III**) Fas,  
- **IV**) Asp53,  
- **V**) Bcl-2

In the following treatment groups:

- 1) normoxic group,  
- 2) hypoxic group,  
- 3) flavonoids treated group  
- 4) Losartan treated group

**Magnification:**  
\[ \text{bar} = 20\mu\text{m} \]
4.8 TUNEL staining (Terminal deoxynucleotidyl Transferase mediated dUTP-Fluorescein Nickend Labeling) result

TUNEL staining was used to identify the apoptotic cells. Fragmented DNA of apoptotic cells is catalytically incorporated fluorescein-12-dUTP at 3'-OH DNA ends using the Terminal Deoxynucleotidyl Transferase. The fluorescein-12-dUTP-labeled DNA is then detected by fluorescence microscopy. The strongest nuclear green fluorescence was observed in the hypoxic group (Plate 17 B). This can be correlated with the up-regulation of Bax, Fas and Asp53 and the down-regulation of Bcl-2 as shown in gene expression as well as the highest immunoreactivity of pro-apoptotic proteins in hypoxia groups observed in the immunohistochemical staining. was used to identify the apoptotic cells. Fragmented DNA of apoptotic cells is catalytically incorporated fluorescein-12-dUTP at 3'-OH DNA ends using the Terminal Deoxynucleotidyl Transferase. The fluorescein-12-dUTP-labeled DNA is then detected by fluorescence microscopy. The strongest nuclear green fluorescence was observed in the hypoxic group taking place in the hypoxic group of cells. Less nuclear green fluorescence was observed in the flavonoids treated group (Plate 17 C) and losartan treated group (Plate 17 D) when compared to hypoxic group. The ability to reduce the gene mRNA levels of pro-apoptotic genes, as well as the lowered expression of pro-apoptotic proteins in immunostaining together with its reduced green fluorescence from the TUNEL assay all indicates the therapeutic potential after inducing hypoxia. Meanwhile there is no apoptosis (green signal) found in the normoxic group (Plate 17 A).
Plate 17:  Apoptotic staining in HBEC inducing hypoxia for each treatment group. Apoptotic cells exhibited strong, nuclear green fluorescence (arrows). Treatment Groups are:
A) Normoxic control group,
B) Hypoxic control,
C) Hypoxia with 0.1mg/ml flavonoids treated group,
D) Hypoxia with 1μM Losartan treated group

Magnification:   -   = 20μm
CHAPTER 5

DISCUSSION

Cerebral ischemia results in different degrees of neuronal death depending on how acute the insult is. The most severely damaged cells may undergo necrosis, while in the ischemic penumbra, there is delayed neuronal death. Many studies have demonstrated that this delayed cell death is attributed to apoptosis and rational treatment of stroke has likewise focused on protecting this area of cells. As such, the characterization and analysis of genes involved in the apoptotic pathway after cerebral ischemia with drug treatment may shed light on new potential loci for therapeutic intervention.

More importantly, Ceylon green tea was found to have anti-oxidative effects where their scavenging effects on hypochlorous acid (HOCl) and 2,2'-Azino-bis (3-Ethylbenz-Thiazoline-6-Sulfonic Acid) (ABTS \( ^{+} \)) were similar to Chinese green tea. As many cerebrovascular diseases, including stroke, are suspected to be cause by an imbalance of free radicals and anti-oxidants, this implies that Ceylon green tea could be useful to counter this unbalance and potentially prevent stroke occurrence or improvement of neurological outcomes.

5.1 Effect on in vitro Antioxidant Tests

The results of ABTS \(^{+}\) assay and Pyrogallol Red bleaching demonstrate that all the green teas show in vitro their antioxidant capacities to certain degrees. Flavonoids from
Ceylon green tea, its antioxidant activity was shown as strong as Chinese green tea, this project evidenced many ways to prove its antioxidant properties. Chinese green tea was known contain large amounts of polyphenollic compounds called flavonoids like catechins. Previously, the health benefits of green tea in preventing diseases like heart disease and cancer were attributed to its vitamins and microelements, but more evidence is showing that these beneficial effects were largely due to its antioxidant properties as its polyphenols have been found to have potential antioxidant effects (Dan and Andrew, 1993; Guo et al., 1993; Rietveld and Wiseman, 2003; Beecher, 2003; Lambert and Yang, 2003). Catechins found include epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epigallocatechin (EGC) and epicatechin (EC) (Beecher, 2003), with EGC being the weakest catechin in terms of antioxidant capacity, followed by EC and ECG, and EGCG having the greatest (Li et al., 2003). Polyphenols have also shown an antioxidant effect 9.6 times greater than vitamin E and part of their antioxidant activity includes donating hydrogen atoms and quenching O$_2$. This antioxidant effect of polyphenols was attributed to their structure e.g. number of hydroxyl groups present, presence of 2-phenolchromane, etc (Li et al., 2003). Since the main active ingredients of flavonoids have been shown to be strong antioxidants, it was not surprising therefore that the antioxidant capacity was found for the in vitro antioxidant tests in this study.

Ceylon green tea was found to have strong anti-oxidant effects in in vitro study. Our results demonstrated that, like losartan, Ceylon green tea had similar cerebral protective effects after hypoxia. However, the mechanisms behind are different. Losartan blocks
the active vasoconstriction effects of angiotensin II AT receptor while Ceylon green tea mainly works through its antioxidant properties.

5.2 Effect on DNA damage

The results obtained in this study suggested that flavonoids from Ceylon green tea had some degree of DNA base protection from oxidation in the event of hypoxia, which could probably be attributed to their anti-oxidant properties whether in scavenging free radicals or in preventing their formation. Nevertheless, a more in depth study could be undertaken to find out the other mechanisms by which they could prevent DNA base damage.

It was found in this study that hypoxic + flavonoids treated group appear to be more preventative of DNA damage in that it has significantly lower quantity of total DNA base products as compared to the non-hypoxia group. What the results suggest was that the antioxidant activity of flavonoids prevents DNA damage in different bases under different conditions, adenine under non-hypoxia condition and guanine under hypoxia condition. Nevertheless for the non-hypoxia group, there is already a significantly lower quantity of FAPy Guanine as compared to the hypoxia group even without treatment under non-hypoxia condition. Taking this into consideration with the result from the flavonoids treatment group, it is clear that flavonoids could have effect in preventing DNA damage under hypoxia condition, and that flavonoids was useful in this aspect more as a cerebral protective agent, perhaps as part of a prevention regime instead.
5.3 Effect on cell viability and antioxidant enzyme activities

Ischemic cells would undergo necrosis. Cells died by apoptosis or necrosis could not be regenerated because neurons are irreplaceable. Thus finding out how to reduce and prevent neuronal deaths is important.

Pretreatment with either flavonoids or losartan increased significantly the cell viability of hypoxic HBEC. The experimental results thus suggest that Ceylon green tea and losartan could also exert protective effects in brain cells.

A number of studies reporting decreased activity levels of superoxide dismutase, catalase, glutathione peroxidase and Glutathione S-Transferase during hypoxia in vivo rats (Costa, 1990) and in several aerobic cell types subjected to hypoxia (Jackson et al., 1996; Kirshenbaum and Singal, 1992; Robin et al., 1984). Reduced enzyme activities might suggest an increased level of oxidative stress and free radicals induced by hypoxia. This could reduce greatly of endogenous antioxidant enzymes and lower cell viability. As we know that ROS and free radicals productions is often increased during hypoxia or ischemia. This can be accompanied by the depletion of natural antioxidants like SOD, CAT, GST and GPx (Zhu et al., 2004). Analyzing flavonoids effects on the activity levels of endogenous free radical scavenging enzymes SOD, CAT, GST and GPx in treatment groups, can investigate if the increasing of cell viability after flavonoids and losartan treated group are attributed to their antioxidant effects.

The result shown that the activity levels for all the four antioxidant enzymes were significantly increased after hypoxia. This observation was consistent with which are
important antioxidative enzymes found in the body as a natural defense against oxygen free radicals. This suggested that flavonoids could have a strong effect on directly scavenging for oxygen free radicals and by increasing the activity of SOD, CAT, GST and GPx. Significant increase of the activity of level SOD, CAT, GST and GPx in hypoxia HBEC, might as suggested: account for the saving of the activity of endogenous SOD under oxidative stress. As the function of these enzymes are to remove hydrogen peroxide by converting it to oxygen and water, the increase in activity in these enzymes may reflect a decrease in oxidative stress in flavonoids treated group. This might also be related to the potent effects of flavonoids to inhibit lipid peroxidation in vitro, which inhibit the production of hydroperoxides and reduce oxidative stress.

However, since a certain degree of damage to brain cells during ischemic stroke is due to the increased production of oxygen free radicals, cerebral protection could be helped by the consumption of Ceylon green tea actively in a form of prevention of stroke or antioxidant therapy which could complement a regime of western drugs during treatment for stroke.

5.4 Effect on apoptotic gene expression

To investigate if apoptosis play a role for the observed reduced cell viability after hypoxia, mRNA expression of the proteins involved in regulating apoptosis were measured using RT-PCR.

The experimental results showed that hypoxia do not appear to cause significant
differences in Fas mRNA expressions. The level expressed in HBEC pretreated with flavonoids were only slight decrease from the hypoxia group which was higher than normoxic group.

In healthy individuals, Fas ligand (FasL) and Fas are mainly restricted to the immuno tissues and to sites of immune privilege such as the eye and the testes where they can trigger apoptosis of invading immune cells (Mehmet, 2001). Fas expression was detected in the normoxic group and significantly higher in the hypoxic group in this experiment. The function of this death-inducing receptor in the HBEC however is not clear.

In this study, Bax and Asp53 level were significantly decreased in the flavonoids and losartan treated groups. Up-regulated Asp53, which induce DNA damage and block cell arrest from the G1 to the S phase to allow for DNA repair, can also mediate the activation of apoptosis. This down-regulation of Asp53 gene expression correlated with data for cell viability in HBEC cells pretreated with both flavonoids and losartan are significantly higher than the hypoxic group.

The result also showed that Bcl-2 mRNA expression was significantly down-regulated in the hypoxic group to the normoxic group. This might suggest that the 12 hours hypoxia incubation has inflicted some injury such as the expression level of Bcl-2 is decreased with the subsequent increase in mitochondrial membrane permeability and the release of cytochrome-C to activate caspases. This significant down-regulation of the anti-apoptotic Bcl-2 might explain in part the reduced cell viability in hypoxic group.
The result shown that over-expression of Bcl-2 can suppress superoxide production and also prevent the release of cytochrome C and the subsequent caspase-mediated apoptosis cascade (Chan, 2001). The study (Chan, 1996) reported that in vivo experiments have also that over-expression of Bcl-2 in the brains of transgenic mice protects the brain from ischemic injury. This suggests that higher level of Bcl-2 might play a role for the decreasing the level of apoptosis which correlate with high cell viability than the hypoxia.

The basis of survival by cells is dependent on the levels of the pro and anti-apoptotic proteins expressed by their genes. Bax, a member of the Bcl-2 family, is able to heteromerize with anti-apoptotic protein Bcl-2 to sequester its anti-apoptotic function, thereby initiating the intrinsic pathway of apoptosis. Thus, during flavonoids pre-treatment, when the level of Bcl-2 has been up-regulated, there will be more Bcl-2 available than what could be sequestered by the Bax. Hence, Bcl-2 would then be able to maintain the stability of the outer mitochondrial membrane and prevent the release of pro-apoptotic components from the mitochondria, which in turn would prevent cell death.

The ability of flavonoids to decrease pro-apoptotic genes and increase anti-apoptotic genes is attributed to its anti-oxidant effects, since all its constituents have been shown individually to possess anti-oxidant capabilities. During cerebral ischemia, the perturbed mitochondrial metabolism and inflammatory reaction to injury results in superoxide production. Superoxide was able to cause oxidative damage of iron/sulfate clusters of
aconitase, an essential enzyme in the tricarboxylic acid cycle. In addition, superoxide was also able to react with nitric oxide to form peroxynitrite as well as participate in the iron-catalyzed Haber-Weiss reaction resulting in the conversion of hydrogen peroxide to hydroxyl radical. Consequently, hydroxyl radical, peroxynitrite, peroxynitrite-derived products such as hydroxyl radicals, carboxyl radicals and nitrogen dioxide were all able to react with and cause damage to lipids (lipid peroxidation), proteins and DNA. The brain possesses defenses against superoxide including dietary free-radical scavengers, such as ascorbate and α-tocopherol, endogenous tripeptide glutathione and enzymatic antioxidants. Enzymatic antioxidants were able to dismutate superoxide to hydrogen peroxide, which was further converted to water or dismutation into water and oxygen. Nitric oxide overproduction also occurs via the glutamatergic-mediated increase in intracellular calcium concentration and subsequent calmodulin-dependent upregulation of nitric oxide synthase. This huge increase in nitric oxide causes nitrosative damage by independent nitrosylation of protein heme sites or via reaction products with oxygen or other nitrogen oxides. In mammalian cells, flavohemoglobin-like activity is present to metabolize nitric oxide (Warner et al., 2004). As a result of oxidative stress, it has been reported that endogenous antioxidant enzymes such as superoxide dismutase, catalase, glutathione S-transferase and glutathione peroxidase and others decrease during ischemia (Ip et al., 2001). As such, the administration of anti-oxidant compounds that are able to directly scavenge free radicals or enhance the activity of endogenous antioxidants will greatly decrease free-radical induced damage, especially lipid
peroxidation which has been implicated as a major step in brain damage progression after ischemia.

By measuring the apoptosis-related gene expression, this study aimed to show the direct effects of flavonoids in mediating apoptosis, which was a crucial factor in determining the fate of cells in the ischemic penumbra. In order to further prove that the anti-apoptotic effect of flavonoids is precisely credited to its anti-oxidant activity, supplementary tests that analyzes the scavenging activity of flavonoids would bridge the link between the anti-oxidant activity and apoptosis.

The ratio of Bcl-2 and Bax indicates the ability of flavonoids on interfering with apoptosis, thereby potentially reducing the cell death after inducing hypoxia. Thus, the increasing ratio of Bcl-2/Bax towards anti-apoptotic situation further demonstrated that it had a promising therapeutic potential for ischemia.

5.5 Effect on immunohistochemical and TUNEL staining

One of the most important biomarker of apoptosis in many cells is nuclear DNA fragmentation. The TUNEL system is designed to detect and stain apoptotic cells via this biomarker. In this study, fragmented DNA of apoptotic cells is catalytically incorporated fluorescein-12-dUTP at 3’-OH DNA ends using the Terminal Deoxynucleotidyl Transferase (TdT). Cells undergoing or have undergone apoptosis have their DNA cleaved into fragments which are able to incorporate fluorescence-dUTP into their 3’-OH ends through the catalytic action of the TdT
enzyme. This polymeric tail can be visualized using the fluorescence microscopy and presence of apoptotic cells detected. The strongest nuclear green fluorescence was observed in the hypoxic group. The green fluorescence detect the fluorescence and thus represent the DNA fragments onto which it is bound to and the red fluorescence represent the cytoplasm stained by the propium iodide. Yellow regions represent sites of cytoplasm and DNA fragmentation overlap. This can be correlated with the up-regulation of Bax, Fas and Asp53 and the down-regulation of Bcl-2 as shown in gene expression as well as the highest immunoreactivity of pro-apoptotic proteins in hypoxia groups observed in the immunohistochemical staining.

This indicated dramatic morphological changes and intense cell death taking place in the hypoxic group of cells. Less nuclear green fluorescence was observed in flavonoids and losartan treated groups when compared to hypoxic group. The ability of flavonoids to reduce the gene mRNA levels of pro-apoptotic genes, as well as the lowered expression of pro-apoptotic proteins in immunostaining together with its reduced green fluorescence from the TUNEL assay all indicated the therapeutic potential of flavonoids treated after inducing hypoxia.

The results showed also that the 12-hour hypoxia induced apoptosis in HBEC. The degree of apoptosis, as observed by the intensity of the green and yellow fluorescence, was greatest in the hypoxic group and hypoxic HBEC pre-treated with flavonoids and losartan show reduced extent of DNA fragmentation, suggesting lower level of apoptosis which correlated with cell viability data.
Losartan is commonly used drug as part of a preventive regime for stroke and cardiovascular diseases by lowering blood pressure. It is an angiotensin subtype I (AT1) receptor antagonist (Zhu et al., 2000) and is very effective in lowering blood pressure, the mechanism for which involves an ACE inhibitor. ACE is an enzyme in the body which converts angiotensin I to angiotensin II. This angiotensin II then acts on the AT1 and AT2 receptors in the body to narrow peripheral blood vessels and to stimulate the production of aldosterone, a corticoid hormone which leads to the retention of salt and water by the kidneys, increasing the amount of body fluid and subsequently increasing the blood pressure. Therefore, by inhibiting ACE, losartan could effectively lead to reduction of blood pressure.
Tea, as the most consumed beverage after water, has a great impact on balanced nutrition. It is easily available almost everywhere in the world. It is a natural food, processed and prepared without additives and preservatives. It has been time tested by populations and civilizations over millennia. In general nothing but good is said about tea.

Our results demonstrated that, Ceylon green tea is found to have strong anti-oxidant effects in our *in vitro* study. Both have similar cerebral protective effects after hypoxia. However, the mechanisms behind are different. Losartan blocks the active vasoconstriction effects of angiotensin II AT receptor while Ceylon green tea, as well as other Chinese green tea, mainly work through its antioxidant properties.

A greater understanding about the antioxidant activities of losartan could be done with more antioxidant assays, e.g. assessing the amount of lipid peroxidation in treatment groups. However, since a certain degree of damage to brain cells during hypoxia is due to the increased production of oxygen free radicals, cerebral protection could be helped by the consumption of losartan or its constituent herbs as well as Ceylon green tea or other green tea as supplements in a form of antioxidant therapy which could complement a regime of western drugs during treatment for stroke.

An alternative method that could be used instead is the reduction of tetrazolium salts in
an assay called the MTT Cell Proliferation Assay, which can evaluate a cell population's response to external factors: an increase in cell growth, no effect, or a decrease in growth due to necrosis or apoptosis. The yellow tetrazolium salt (MTT) is reduced in metabolically active cells to form insoluble purple formazan crystals, which are then solubilized by the addition of a detergent. The color can then be quantified by spectrophotometric means. Since a linear relationship between viable cell number and absorbance can established, a more accurate result of the percentage cell viability can be obtained.

Though Ceylon green tea shows significant down-regulation of pro-apoptotic genes (Bax and Asp53) and up-regulation of anti-apoptotic genes (Bcl-2), less apoptosis was observed in TUNEL staining assay and hence is therapeutically promising, but the underlying molecular mechanisms of Ceylon green tea need to be further elucidated. The mechanisms of apoptosis and necrosis are complex and involve many factors besides free radical induced damage, so although the anti-oxidant properties of flavonoids tested may play a part is reducing cell death, a more in depth study could be conducted before a conclusion can be made on the mechanism responsible for the flavonoids' protective effects.

Future studies can also be done to isolate the individual active compounds in Ceylon green tea that contribute to their antioxidant properties.

This study showed that Ceylon green tea is able to display cerebral protective effects on HBEC cells through the various experiments done on cell viability, antioxidant
enzyme's activity tests, gene expression, immunohistochemical staining as well as fluorescent TUNEL staining. This is definitely contributed to the acceptance of the frequently drink of Ceylon green tea in helping to prevent cerebral disease.
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