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An Investigation of the Pharmacological Effects of Nao Xin Qing (NXQ), a Standardised Herbal Extract, for Ischaemic Stroke - In Vitro and In Vivo

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Declaration

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Abstract

Introduction

Stroke is one of the major causes of death and disability worldwide. Ischaemic stroke constitutes 80% of stroke incidents. Reperfusion injury following ischaemic stroke has been shown to contribute to major neurological damages. Current conventional treatments for ischaemic stroke, whether surgical or pharmaceutical, focus on restoring cerebral blood flow and preventing expansion of ischaemic damage, but have limited impact on neuron damage or restoring damaged neurological functionalities.

NXQ, a standardised, patented herbal extract derived from *Diospyros kaki L.*, offers potential therapeutic for apoplexy syndrome, including ischaemic stroke. However, NXQ's effects on ischaemic stroke are unclear. In particular, the mechanisms underlying NXQ's effects and the key active components responsible for these effects, are not fully understood.

Aims

This study aims to determine the effects of NXQ and its role in the treatment of ischaemic stroke. The study comprised of two parts.

First part to determine the effects of NXQ for ischaemic stroke by evaluating its vascular protective effects and angiogenic effects. This study further identifies the chemical profiles of the NXQ extract and determines which compound/s are responsible for the therapeutic functions for ischaemic stroke. The second part of the study aimed to evaluate the clinical effects of NXQ on ischaemic stroke patients. A study protocol for a randomised, double-blind, placebo-controlled trial was developed to facilitate future studies on the clinical efficacy and safety profile of NXQ for ischaemic stroke patients.

Methods

A chemical profile was conducted to identify the key active components of the raw extract of NXQ provided by Hutchison Whampoa Guangzhou Baiyunshan Chinese Medicine Co. These compounds are quantified, and the ratio of the key flavonoids quercetin and kaempferol were determined using HPLC-PDA. The vascular protective effects of NXQ and its key active components are evaluated in H_2O_2-induced reactive oxygen species (ROS) generation and lactate dehydrogenase leakage (LDH) leakage models in EA.hy926 cells. To evaluate the angiogenic effects of NXQ, cell proliferation and cell migration assays are conducted using EA.hy926 cells.

The results of NXQ was compared with quercetin, kaempferol, and quercetin and kaempferol combination to see which of the compounds are responsible for its vascular protective actions and whether there is a potential synergism within the kaempferol and quercetin combination at a fixed ratio identified in NXQ extract.

The angiogenic effects of NXQ were further evaluated using VRI-inhibited zebrafish Tg(fli1:EGFP) embryos and the growth of SIV vessels promoted by NXQ. A hypoxia model in zebrafish was developed to evaluate the protective effects of NXQ *in vivo*. Due to the time limitation, and technical challenges, only the results of this model development were included in this thesis. In addition, a randomised, double-blinded controlled trial protocol was developed and included in this thesis for the future study on evaluation of the clinical efficacy and safety of NXQ in ischaemic stroke.

Results

NXQ significantly attenuated H_2O_2-induced ROS generation, LDH leakage and restored cell viability. This effect was superior to that of kaempferol and quercetin alone, suggesting potential synergistic reaction between the two compounds. Additionally, NXQ significantly induced angiogenesis in VRI-inhibited Tg(fli1:EFGP) zebrafish embryos; however, NXQ did not

promote cell proliferation or migration in EA.hy926 cells. This potential re-vasculaturisation property of NXQ may be beneficial in ischaemic stroke recovery.

Conclusion

NXQ possesses vascular protective effects against H_2O_2-induced damage that may protect endothelial cells from reperfusion-induced damage. Additionally, NXQ possesses potential angiogenesis and revascularisation properties, which may contribute to its therapeutic action during ischaemic stroke recovery.

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List of Abbreviations

ACE	angiotensin-converting enzyme
ADL	activities of daily living
AF	atrial fibrillation
AGE	advanced glycation end-product
ALT	alanine aminotransferase
AMPA	α-amino-3-hydroxy-5-methul-4-isoxazole propionic acid
ANGPTL2	angiopoietin-related protein 2
ANCOVA	analysis of covariance
ANOVA	analysis of variance
AOPP	advanced oxidation protein products
APTT	active partial thromboplastin time
AST	aminotransferase
ATP	adenosine triphosphate
BBB	blood–brain barrier
BMI	body mass index
BUN	blood urea nitrogen
CANTAB	Cambridge Neurological Assessment Battery
CAT	catalase
CDR	computerised drug research
CFDA	China Food and Drug Administration
CHM	Chinese herbal medicine
CIR	cerebral ischemia reperfusion
CNED	Chinese National Essential Drug
COMPASS	Computerised Mental Performance Assessment System
COX2	Prostaglandin-endoperoxide synthase 2
C-RP	C-reactive protein
CVD	cardiovascular disease
DA	dorsal aorta
DCF	2', 7'–dichlorofluorescein
DCFDA	2',7'–dichlorofluorescein diacetate
DEDTC	sodium diethyldithiocarbamate
DEFUSE	diffusion-weighted imaging evaluation for understanding stroke evaluation
DLAV	dorsal longitudinal anastomotic vessels
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
ECG	electrocardiogram

EEG	electroencephalogram
ET	essential thrombocytosis
FASEB	Federation of American Societies for Experimental Biology
FBS	fetal bovine serum
FGF	fibroblast growth factors
FMD	flow-mediated dilation
GMP	Good Manufacturing Practices
GRADE	Grading of Recommendations Assessment, Development and Evaluation
GSH	glutathione
GSH-Px	glutathione peroxidase
HIF	hypoxia-inducible factor
HM	herbal medicine
HPLC	high-performance liquid chromatography
HPLC-PDA	high-performance liquid chromatography photodiode array detection
HS	haemorrhagic stroke
HUVEC	human umbilical vascular endothelial cells
ICAM-1	Intercellular Adhesion Molecule-1
IS	ischaemic stroke
ISV	intersegmental vessels
LDH	lactate dehydrogenase
LDL	low-density lipids
LOD	limit of detection
LOQ	limit of quantification
MAPK	mitogen-activated protein kinase
MCAO	middle cerebral artery occlusion
MMP	matrix metalloproteinases
mRNA	messenger RNA
mRS	modified Rankin Scale
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NATA	National Association of Testing Authorities
NICM	National Institute of Complementary Medicine
NIHSS	National Institute of Health Stroke Scale
NINDS	National Institute of Neurological Disorders and Stroke
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NOS	nitric oxide synthases

NSW	New South Wales
NXQ	Nao Xin Qing
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate-buffered saline
PCV	posterior cardinal vein
PGE2	prostaglandin E2
PGF	placental growth factor
PNS	Panax notoginseng saponins
PPRC	Pharmacopoeia of the People's Republic of China
PTFE	polytetrafluoroethylene
PTT	partial thromboplastin time
Q3:K4	quercetin–kaempferol 3:4 combination
qRT-PCR	real-time quantitative reverse transcription polymerase chain reaction
RCPA	Royal College of Pathologists of Australasia
RCT	randomised controlled trial
RNA	ribonucleic acid
ROS	reactive oxygen species
RSD	relative standard deviation
rtPA	(recombinant) tissue plasminogen activator
SD	standard deviation
SIV	subintestinal vessels
SOD	superoxide dismutase
SSQOL	Specific Stroke Quality of Life
TCM	traditional Chinese medicine
TCPM	traditional Chinese patent medicine
TGA	Therapeutic Goods Administration (Australia)
TNF	tumour necrosis factor
TNF-α	tumour necrosis factor-alpha
TS	thrombotic stroke
TTC	2,3,5-triphenyltetrazolium chloride
UV	ultraviolet
v/v	volume/volume
VEGF	vascular endothelial growth factor
VRI	VEGFR tyrosine kinase inhibitor II
vWF	von Willebrand factor

Chapter 1. Introduction

1.1 Background to research

Ischaemic occurs when the an artery to the brain is blocked leading to rapid development of clinical signs indicating focal or global disturbance in cerebral functionality, with symptoms lasting for 24 hours or longer, potentially resulting in death (WHO, 1988).

Ischaemic stroke is one of the leading causes of death in Australia, and more than 65% of stroke survivors are left with permanent disabilities severe enough to affect their employability (Coffey C. Edward, 2000; NSF, 2013). It is estimated that treatment of stroke costs A$5 billion each year in Australia (Cadilhac, Carter, Thrift, & Dewey, 2009; NSF, 2013). In 2017, more than 56,000 new and recurrent stroke incidences were reported in Australia. Until 2017, more than 475,000 people living with the effects of stroke and this number is expected to increase to one million by 2050. The cost is expected to increase from 1.6 – to 4.6 folds by 2051 compared with 2011 (NFS, 2017)

Conventional treatments for ischaemic stroke, both surgical and pharmaceutical, aim to restore cerebral blood flow to avoid tissue damage. Some of these agents, including anti-platelet, anti-coagulant and thrombolytic agents, act on the thrombosis or embolism by thinning the blood but usually have a limited impact on neuroprotection against reperfusion injury. It has been reported that 70% of neuron damage results from reperfusion injury rather than the ischaemic event itself (Hausenloy & Yellon, 2013). Therefore, a novel agent that can act against reperfusion-induced neuron damage is required.

Chinese herbal medicine has been used as a therapeutic agent against ischaemic stroke-related conditions for thousands of years. Its novel therapeutic principles may provide viable options for the treatment of ischaemic stroke, especially in the area of rehabilitation and prevention of future attacks.

NXQ, manufactured by Hutchison Whampoa Guangzhou Baiyunshan Chinese Medicine Co. Ltd. (HGB), is a single herbal extract of *Diospyros kaki* leaves, also known as leaf of persimmon. NXQ has been used in China as a therapeutic agent for apoplexy syndromes and ischaemic conditions such as angina, coronary artery diseases and cerebral ischaemia. Several clinical trials have conducted in China to evaluate its potential effects (which may include lowering lipids, scavenging radicals and improving blood flow improvements) on apoplexy syndromes; however, the methodology of these trials appears to be lacking in rigour and precision. To date, there has been no clinical trial evaluating the neuroprotective effects of NXQ using adapted standardised outcome measures. In addition, there is lack of preclinical data to support evaluation of NXQ's effects and the mechanisms underlying its potential cerebrovascular and neuroprotective benefits.

1.2 Aims and hypotheses

This research aims to evaluate the effects and associated risks of NXQ, a standardised, patented herbal extract, on ischaemic stroke. The research comprises four studies designed to evaluate the mechanisms underlying NXQ's effects on ischaemic stroke (including the vascular protective and angiogenic effects of NXQ) and to design a randomised clinical trial to evaluate the effectiveness and safety of NXQ in patients with ischaemic stroke.

The initial focus of this research was on undertaking a pilot randomised clinical trial of NXQ in patients with subacute ischaemic stroke supplemented by *in vivo* studies using zebrafish models to evaluate the potential neuroprotective effects of NXQ. A protocol for the clinical trial was completed, ethics approval obtained, case-report forms prepared and recruitment centres established. However, the trial sponsor eventually failed to secure Good Manufacturing Practice accreditation from the Australian Therapeutic Goods Administration

(TGA) to manufacture the trial medications needed. This led to major changes to the research plan. In this thesis, the clinical trial protocol and key supplementary documents are included (Chapter 7) as they represent a substantial amount of the work completed for this doctoral thesis.

The aims of and hypotheses for each of the sub-studies are outlined below.

Study 1. Chemical analysis of NXQ

The aim of this study is to determine the ratio of two key bioactive constituents of NXQ, kaempferol and quercetin, in the finished NXQ tablets. We hypothesised that kaempferol and quercetin can be identified and quantified.

Study 2. Vascular protective effects of NXQ

The aim of this study is to evaluate the effects of NXQ on oxidative stress-induced endothelial cellular damages. We hypothesised that the two key bioactive constituents, kaempferol and quercetin, are responsible at least in part for NXQ's therapeutic actions and that NXQ's effects were superior to those of kaempferol or quercetin alone, indicating the presence of a potential additive or synergistic effect in NXQ.

Study 3. Angiogenesis effects of NXQ

The aim of this study is to evaluate the angiogenic effects of NXQ using endothelial cells and transgenic zebrafish Tg(fli1:EGFP) embryos. We hypothesised that NXQ possesses angiogenic effects and that the two key bioactive constituents, kaempferol and quercetin, exert additive or synergistic effects at the ratio presented in NXQ tablets.

Study 4. Effectiveness and safety of NXQ for ischaemic stroke – a pilot, multicentred, randomised control trial protocol

The aim of this study is to design a randomised, double-blind, placebo-controlled trial to evaluate the effectiveness and safety of NXQ in patients with ischaemic stroke. We hypothesised that 12 weeks treatment of NXQ could improve neurological, motor and cognitive functional outcomes of ischaemic stroke patients during rehabilitation phase.

1.3 Structure of the thesis

This thesis comprises eight chapters.

Chapter One provides a brief introduction to this research, including the background to the thesis, research aims and hypothesis, and thesis structure.

Chapter Two provides a comprehensive review of the literature relevant to the thesis topic, including findings on the prevalence, aetiology, risks and pathophysiology of ischaemic stroke and current medical interventions for the management of the disease.

In Chapter Three, relevant background information, current scientific evidence and knowledge gaps associated with the use of Chinese herbal medicine for ischaemic stroke (focusing on NXQ) are summarised.

Chapter Four contains a chemical analysis of NXQ, details of the research methodology for the chemical evaluation, method validations and a presentation and discussion of results.

Chapter Five discusses the vascular protective effects of NXQ. This chapter contains background, experimental methodology, results and discussions of an *in vitro* study using EA.hy926 cells to evaluate vascular protective effects induced by H_2O_2 damage. In addition, the research protocol, model development methodology, troubleshooting, results and presentation of a hypoxia model in zebrafish are included and the results discussed.

Chapter Six discusses the angiogenic effects of NXQ. This chapter contains background, experimental methodology, results and discussion of an *in vitro* study of the angiogenic

effects of NXQ on EA.hy926 cells. In addition, the development of an angiogenesis model in transgenic zebrafish Tg(fli1:EGFP) embryos, issues and some preliminary results are presented.

Chapter Seven explores the effects of a standardised, patent herbal extract (NXQ) for ischaemic stroke through a pilot randomised controlled trial. This chapter contains a clinical trial protocol in which the background, clinical trial design, inclusion and exclusion criteria, primary and second outcome measures and assessment schedules are presented and discussed.

Chapter Eight provides a summary of the background of this research and the results of the four studies conducted. Limitations of the studies and future directions are also discussed.

Chapter 2. Atherosclerosis, Cerebral Ischaemia and Ischaemic Stroke

2.1 Introduction to ischaemic stroke

Stroke, also known as cerebrovascular accident, is the brain equivalent of a heart attack. It is a disease with multiple overlapping risk factors, primarily of vascular origin and typically complicated by ageing and lifestyle factors such as dietary habits and smoking. Stroke is defined as the rapid development of clinical signs indicating focal or global disturbance in cerebral functionality, with symptoms lasting for 24 hours or longer and potentially resulting in death with no apparent cause other than it being of a vascular origin (WHO, 1988). Stroke can be categorised into ischaemic and haemorrhagic subtypes. Ischaemic stroke (IS) is typically associated with disturbance in blood supply and generally incurs smaller lesions, while haemorrhagic stroke (HS) refers to the rupturing of blood vessels, resulting in larger lesions. IS is the more common type, accounting for approximately 80% of all cases. However, the mortality rate is generally lower in comparison to HS (Andersen, Olsen, Dehlendorff, & Kammersgaard, 2009b).

Stroke is one of the leading causes of death in Australia (after coronary heart disease) and most survivors are left with permanent disabilities. In 2017, over 475,000 Australians were living with the effects of stroke (Tursunov & Akbarkhodjaeva, 2017). According to the National Stroke Foundation (NSF), this number is expected to rise to one million by the year 2050 due to the complexity of risk factors and the increase in the aged population of Australia. Sixty-five per cent of stroke survivors suffer from a disability severe enough to decrease their employability (Coffey C. Edward, 2000; NFS, 2017; NSF, 2013), and two-thirds of these cases require assistance in daily living activities and long-term care (NSF, 2013).

The treatment of stroke in Australia costs an estimated A$5 billion dollars each year (Cadilhac et al., 2009; NSF, 2013); in 2012 alone, A$49.3 billion was spent on stroke prevention, treatment, rehabilitation and disability care (NSF, 2013). Given that the number

of disabled survivors is increasing, the total cost of stroke treatment is expected to increase proportionally (Broderick, 2004; NFS, 2017; NSF, 2013). Conventional treatments for stroke focus predominantly on IS due to its higher incidence; HS therapies are limited due to the rapid and extensive physiological damage, truncated therapeutic window and poor overall prognosis associated with HS.

The goal of acute stroke treatment in general is to increase the survival rate and reduce the dependency level of the patient (Dirnagl, Iadecola, & Moskowitz, 1999). Currently, approved treatments (both surgical and pharmaceutical) for acute ischaemic stroke aim to restore cerebral blood flow to prevent rapid tissue damage resulting from disturbed blood supply to the local tissue. Some of the pharmacological agents promoting blood flow, such as anti-platelet, anti-coagulant and thrombolytic drugs, act on thrombosis or embolisms by thinning the blood, but their value is usually time-limited and plays a minimal role in neuroprotection (Gubitz, Sandercock, & Counsell, 2008; Sandercock, Counsell, Gubitz, & Tseng, 2008; Wardlaw, Murray, Berge, & Del Zoppo, 2009). While local ischaemia does damage the affected brain tissues, it is alarming that reperfusion injury – a series of pathophysiological events after regaining blood flow – is a greater risk for damage to cerebral tissues. A significant 70% of neuro-damage is caused by reperfusion injury and only 17% is of ischaemic origin (Simon, Haj-Yehia, & Levi-Schaffer, 2000). Therefore, neuroprotection is considered an important therapeutic strategy in reducing neuron damage and dependency levels; in IS, this strategy has focused on expanding the treatment window and reducing reperfusion injury (Cheng, Al-Khoury, & Zivin, 2004; Ginsberg, 2008). However, the research on neuroprotective agents, such as glycine antagonists and calcium channel blockers, reveals discrepancies in the therapeutic benefits in animal studies and clinical trials (Cheng et al., 2004). Some researchers suggest this could be due to species-dependent differences and that

animals such as rodents may be more amenable to neuroprotection than evolutionarily higher mammals, such as humans (Dirnagl et al., 1999).

Rehabilitation is a combination of therapeutic modalities, including physiotherapy, occupational therapy, and speech and language therapy, designed to assist stroke survivors to restore functionality lost due to neurological damage (Feigenson, 1979). It is typically a long-term and continuous process and, depending on the severity of disability, the majority of survivors do not regain full functionality. In addition to rehabilitation therapies, long-term pharmaceuticals are typically prescribed for the management of vascular and thrombotic risk factors to prevent recurrence of stroke (Ringleb et al., 2007). Despite the preventative medications, there are no currently known pharmaceuticals shown to improve cognitive and functional outcomes. There is, therefore, an urgent need for alternative therapeutic options to mitigate the impact of ischaemic stroke, both by reducing the damage to the brain and improving functional outcomes.

2.2 Prevalence, aetiology and risk factors

2.2.1 Prevalence

Stroke is one of the most significant cardiovascular diseases (CVDs) leading to death, after cardiac arrest. In 2015, 442.7 million cases of CVD, accounting for 17.7 million deaths, were identified globally. Nearly half of these deaths were caused by stroke (Roth et al., 2017). Ischaemic stroke outranked haemorrhagic and other strokes, with the greatest number of cases in high-income nations such as North America. However, the highest prevalence of stroke is in the countries and islands of Oceania due to the increased metabolic and cardiovascular risks associated with the regional lifestyle (Roth et al., 2017). Such figures reflect the major risk factors associated with cardiovascular and cardiometabolic disease,

such as hyperlipidaemia, hypertension and hyperglycaemia. Managing these risk factors is regarded an important focus for preventing IS incidents.

2.2.2 Aetiology

Ischaemic stroke is caused by deprivation of oxygen and energy supply to the brain cell. It can be categorised into thrombotic and embolic ischaemic stroke (Lees, Bath, & Naylor, 2000). Thrombotic stroke (TS) is the most common type and occurs when a blood clot adhering to the vessel wall (mural occlusive thrombus) reaches a sufficient size to completely block cerebral artery blood flow. The development of an occlusive thrombus due to atherosclerosis is a major risk factor for stroke (Allen & Bayraktutan, 2008).

Atherosclerosis is a condition in which plaque builds up within an artery, causing a narrowing of the blood vessel. The formation of this plaque has shown to be associated with fatty materials such as low-density lipids (LDL) and triglycerides. However other factors such as hypertension, diabetes, smoking and ageing have also been shown to contribute to atherosclerosis (H. Adams et al., 1993; Allen & Bayraktutan, 2008). Atherosclerosis is associated with the development of athoromas during plaque formation in cerebral arteries. The first concern regarding the formation of atheromas is the narrowing of the relatively small arteries in the brain, resulting in increased blood pressure and reduced blood flow. Furthermore, should the fibrous cap of an atheroma ulcerate, the subsequent thrombogenic material released may induce thrombus formation overlying the atheroma. This can result in rapid occlusion of the artery and, hence, ischaemic stroke. In addition to ischaemic stroke, atheromas produce enzymes that enlarge the arteries over time, and this excessive widening may lead to the creation of an aneurysm, with subsequent rupture resulting in a haemorrhagic stroke (Dzau, 1994; Kher & Marsh, 2004).

In contrast, embolic strokes occur when an embolus (e.g. a blood clot, atherosclerotic plaque, fat globule or gas bubble) is carried by the bloodstream to the brain, where the larger arteries branch off into smaller vessels. The embolus reaches a point at which it can travel no further, thereby plugging a smaller cerebral artery and cutting off the blood supply to that area of the brain. Most blood clot emboli are caused by non-vascular factors such as atrial fibrillation (AF), where the two small upper chambers of the heart quiver, causing the blood to pool and form clots (H. Adams et al., 1993; Lees et al., 2000).

2.2.3 Risk factors

A number of risk factors have shown to be associated with an increased risk of ischaemic stroke. They can be stratified into modifiable and non-modifiable risk factors. Modifiable risk factors include those resulting from the environment and lifestyle choices, such as a high-fat and high- glucose diet, lack of exercise and smoking. These risks correspond to cardiometabolic risks and are typically manageable with the help of healthcare professionals, pharmaceutical treatment and continuing education. Non-modifiable risk factors encompass hereditary or natural aspects, such as ethnicity and ageing (Allen & Bayraktutan, 2008). Among these risks, complications and conditions attributable to CVD are considered as having the greatest impact for IS. For instance, cardiometabolic risk is regarded one of the most significant risks for CVD and has shown to be a potential threat to vascular health, with increased or unsustainable blood lipids and blood glucose levels. The resulting impaired metabolic function, including increased viscosity of the blood and narrowing of the arteries, stresses the vascular system and has been shown to increases the risk of developing atherosclerosis. This has been supported by studies suggesting that patients with Type 2 diabetes (T2D) are at a greater mortality risks from CVD than individual without T2D.

Furthermore, patients with impaired metabolic functions or dyslipidaemia are more susceptible to inflammation of the blood vessels and, therefore, at increased risk of both thrombosis and embolism (Barrett-Connor & Khaw, 1988; Idris, Thomson, & Sharma, 2006).

2.3 Pathophysiology

IS is typically the result of a transient or permanent reduction in cerebral blood flow to a major arterial branch within the brain caused by the occlusion of arterial blood flow either by an embolus or through local thrombosis. Brain injury following transient or permanent focal cerebral ischaemia develops from a complex series of pathophysiological events that evolve in time and space (Collins, Dobkin, & Choi, 1989; Dirnagl et al., 1999).

The brain is the centre of the nervous system and exerts critical centralised control over most of the physiological and cognitive functions of the body. As a result, brain tissue has a relatively high consumption of oxygen and glucose and depends almost exclusively on oxidative phosphorylation for energy production (Collins et al., 1989; Dirnagl et al., 1999).

2.3.1 The ischaemic core and the penumbra

During an ischaemic attack, focal impairment of cerebral blood flow restricts the delivery of oxygen and glucose, leading to severe focal hypoperfusion, and the cerebral injury continues over hours and days following the incidence. The degree and duration of the ischaemia determines the size and severity of the cerebral injuries. The region of the brain with severe hypoperfusion are referred to as the 'ischaemic core' and experience rapid and irreversible injury (Astrup, 1981; Marc Fisher & Garcia, 1996; Furlan, Marchal, Derlon, Baron, & Viader, 1996; Hossmann, 1994; Obrenovitch, 1995). The cells in the core are rapidly killed by lipolysis, proteolysis, disaggregation of the microtubules, total bioenergy failure and disruption of ion homeostasis (the functional failure of the cellular membrane of ion channels, such as

potassium (K^+), sodium (Na^+) and chloride (Cl^-) pumps, which causes the plasma membrane of neurons to depolarise). The resultant cascade of pathophysiological processes – excitotoxicity, peri-infarct depolarisation, inflammation and apoptosis – directly and/or indirectly lead to the death of neurons (Dirnagl et al., 1999; Kehrer & Smith, 1994).

A region of functionally impaired but structurally intact tissue lying between the lethally damaged core and the normal brain is referred as the ischaemic penumbra. This partially functional area has constrained blood flow but the cellular metabolism is reserved; hence, it has been associated with the neurological recovery (Baron, 1999; Markus et al., 2004; Nagesh et al., 1998; Read et al., 1999). It is also an area where ischaemic cascade triggers several deleterious mechanisms, resulting in ongoing cellular injury and infarct processes which expand the damaged core. Within hours of ischaemic onset, the penumbra may be consumed by progressive insults leading to apoptosis and necrosis. However, the penumbra can be rescued by improving the blood flow and/or interfering with the ischaemic cascade in a timely manner – usually up to 48 hours of the initial ischaemic attack (Katsura, Kristián, & Siesjö, 1994; R. Martin, Lloyd, & Cowan, 1994).

2.3.2 Cellular bioenergetic failure

Brain tissues are highly sensitive to oxidative phosphorylation for energy production. Focal hypoperfusion restrains the delivery of essential substrates such as oxygen and glucose, leading to the failure production of adenosine triphosphate (ATP) within the normal brain cell process. This leads to dysfunction of energy-dependent ion transport pumps and depolarisation of neurons and glia (Aliev, Obrenovich, Smith, & Perry, 2003).

As a consequence of the membrane depolarisation, the substantial influx of calcium (Ca2+) allows for excitatory amino acids such as glutamate to be released into the extracellular space

(Dirnagl et al., 1999). At the same time, energy-dependent processes such as presynaptic re-uptake of excitatory amino acids are impeded, further increasing the accumulation of excitatory amino acids in the extracellular space. The presence of these excitatory amino acids results in the activation of N-methyl-D-aspartate (NMDA) receptors, a specific glutamate receptor subtype known as metabotropic receptors. The combined over-activation of these receptors from accumulated extracellular excitatory amino acids contributes to the cellular Ca^{2+} overload in the neural cell (Dirnagl et al., 1999; Kehrer & Smith, 1994; Nicholls & Attwell, 1990).

Additionally, reduced oxygen availability results in anaerobic glycolysis and accumulation of lactate (Brouns et al., 2008; Nicoli et al., 2003; Schneweis et al., 2001), which has been identified as a marker of anaerobic metabolism in stroke. The anaerobic metabolism is also a possible cause of secondary damage leading to infarct expansion and poor clinical outcome, particularly in cortical infarctions (Brouns et al., 2008; Dohmen et al., 2007; Uyttenboogaart et al., 2007).

2.3.3 Excitotoxicity

Excitotoxicity refers to the secondary damage caused by the pathological activation and Ca^{2+} uptake by neurons due to abnormal release of excitatory neurotransmitters from dying cells (Choi, 1988; Meldrum, Evans, Griffiths, & Simon, 1985; Rothman & Olney, 1986). As described above, excitatory neurotransmitters, especially glutamate, accumulate in the extracellular space, resulting in overstimulation of α-amino-3-hydroxy-5-methul-4-isoxazole propionic acid (AMPA), kinate and NMDA receptors on neurons. The ensuing efflux of K^+ and influx of Na^+ and Cl^- across the cell membrane following deploarisation causes increased water permeation into the neural cells via osmosis. The oedema can negatively affect the perfusion of blood to the regions surrounding the infarct epicentre and can also have remote effects due to increased intracranial pressure, vascular compression and herniation (Marinis et al., 2010).

This increase in Ca^{2+} is thought to initiate a series of cytoplasmatic and nuclear events that result in cellular destruction and activate inflammatory responses, thereby generating free radical species that overwhelm endogenous scavenging mechanisms (Dirnagl et al., 1999; Ginsberg, 2008) and activation of Ca^{2+}-dependent enzymes. These enzymes include proteolytic enzymes that degrade cytoskeletal proteins (Z.-L. Chen & Strickland, 1997) and extracellular matrix proteins (Choi, 1988, 1995), as well as phospholipase A2, calpain, endonucleases, adenosine triphosphatase, cyclooxygenase and NO synthase type I, resulting in extensive cellular damage and generating further free radical species. These free radical species and degradative enzymes damage the cellular membrane, allowing further permeability of Ca^{2+} ions leading to acute cell death through necrosis. However, the excitotoxic mechanisms can also initiate molecular events that leads to apoptosis. In addition, the intracellular signalling pathways activated during excitotoxicity trigger the expression of the genes that initiate post-ischaemic inflammation that further contributes to the ischaemic injury (J. T. Coyle & Puttfarcken, 1993).

2.3.4 Oxidative stress

Oxidative stress occurs when the production of free radicals overpowers the endogenous scavenging capacity of the cellular anti-oxidant defences. There is considerable evidence that reactive oxygen and nitrogen molecules are important mediators of tissue injury in an acute ischaemic stroke (P. H. Chan, 2001; Cui, Holmes, Greene, & Liu, 2000; El Kossi & Zakhary, 2000; Epe, Ballmaier, Roussyn, Briviba, & Sies, 1996; Fridovich, 1978; P. J. Kelly et al., 2008; Kontos, 1985, 2001; Love, 1999; Nelson, Wei, Povlishock, Kontos, & Moskowitz, 1992; Siesjö, Agardh, & Bengtsson, 1989; Waring, 2002). Brain ischaemia generates superoxide (O_2^-), through xanthine oxidase and leakage from the mitochondrial electron transport chain. Superoxide is

the primary radical from which hydrogen peroxide (H_2O_2) is formed. Hydrogen peroxide, in turn, is the source of hydroxyl radical (OH) (Beckman & Koppenol, 1996). Nitric oxide (NO), a water- and lipid-soluble free radical produced from L-arginine by three types of NO synthases (NOS). NOS types I and III are Ca^{2+}-dependent and constitutively expressed, primarily in nerve tissues and endothelial cells respectively. Upregulation of NOS type II, a form of inducible enzyme, is mediated transcriptionally by a variety of cytokines (Masters, 1994). Ischaemia causes a surge in NOS I and III activity in neurons and vascular endothelium respectively. At a later stage, the elevated NOS type II activity occurs in a range of cells, including glia and infiltrating neutrophils (F. Zhang, Xu, & Iadecola, 1995). In the context of brain ischaemia, NOS type I and II are deleterious. However, the production of NO in blood vessels by NOS type III improves blood flow to ischaemic penumbra through several mechanisms. For instance, NOS type III can induce vessel dilation and inhibition of platelet adhesion. It also scavenges oxygen radicals and has an anti-inflammatory effect through inhibition of leukocyte adhesion to the endothelial cell. Inhibition of NOS by asymmetrical dimethylarginine may result in decreased NO bioavailability (MacAllister, Whitley, & Vallance, 1994) which is associated with vasoconstriction (Calver, Collier, & Vallance, 1993; Kielstein et al., 2006), and increasing generation of free radicals (Calver et al., 1993), platelet aggregation and leukocyte adhesion on the endothelial surfaces, a process that may in turn aggravate cerebral ischaemia.

The formation from superoxide and NO decomposes spontaneously to produce the hydroxyl radicals such as of H_2O_2 and O_2^- (Beckman, 1996; Beckman & Koppenol, 1996; Beckman, Ye, Chen, & Conger, 1996; L. L. Dugan & Choi, 1994; Fridovich, 1978; Iadecola, 1997; E. Wei & Kontos, 1990; E. P. Wei, Kontos, & Beckman, 1996). Remote effects of these hydroxyl radicals are possible, as H_2O_2 is lipid-soluble and readily crosses cell membranes while O_2^- traverses the cell membrane via the anion channel (Kontos, 2001). Hydroxyl radical is

regarded as very short lived but the most reactive oxygen radical, causes the most tissue injury (Evans & Cooke, 2004; Lennon, Martin, & Cotter, 1991; P. K. Liu et al., 1996).

The free radicals exhibit a spectrum of harmful cellular effects, including inactivation of enzymes, release of calcium ions from intracellular stores, protein denaturation, lipid peroxidation, damage to the cytoskeleton and DNA and chemotaxis (Evans & Cooke, 2004; Lennon et al., 1991; P. K. Liu et al., 1996). While mitochondrial function is impaired by free radical-mediated disruption of the inner mitochondrial membrane, oxidation of proteins that mediate electron transport, H^+ extrusion and ATP production, cytochrome C is released from mitochondria promotes a trigger for apoptosis. Given that severe oxidative stress causes cell death through necrosis, moderate oxidation can also progressively trigger tissue apoptosis, responsible for the ischaemic tissue (Marinis et al., 2010).

Besides cerebral cellular damage, oxidative stress also increases blood–brain barrier (BBB) permeability through (a) activation of matrix metalloproteinases (MMP), in particular MMP-9, a class of enzyme involved in extracellular matrix degradation (Montaner et al., 2003; G. Rosenberg, Estrada, & Dencoff, 1998), and (b) through endothelial cell damage (P. H. Chan, 2001; Kontos, 1985; Siesjö et al., 1989). Moreover, free radicals influence cerebral blood flow, as they are strong cerebral vasodilators (E. P. Wei, Kontos, Christman, DeWitt, & Povlishock, 1985) and, due to interaction between NO and superoxide, alter vascular reactivity to CO_2, thereby inducing vasoconstriction instead of vasodilation (Kontos, 2001). Additionally, oxygen radicals increase platelet aggregability (Love, 1999).

3.3.5 Blood–brain barrier (BBB) dysfunction

The BBB is a network of semipermeable membranes ensuring the protection of neuronal microenvironments. The BBB separating the blood from the cerebral spinal fluids constitutes

a barrier to the passage of cells, particles and large molecules. The BBB is formed by the brain endothelial cells and allows the passage of water, some gases and lipid-soluble molecules by passive diffusion, as well as selective transport of molecules – such as glucose and amino acids – that are crucial to neural function. The disruption of the BBB during an acute ischaemic stroke may vary from 15% to 66% depending on the severity of the stroke and the timing and methodology of therapeutic treatments (Bang et al., 2007; Hjort et al., 2008; Hornig et al., 1985; Hornig, Busse, Dorndorf, & Kaps, 1983; Kidwell et al., 2008; Latour, Kang, Ezzeddine, Chalela, & Warach, 2004; Lorberboym, Lampl, & Sadeh, 2003; Niebroj-Dobosz, Mariam, Lukasiuk, & Rafałowska, 1992; Suzuki, Kelley, Reyes-Iglesias, Alfonso, & Dietrich, 1995). The damage to the BBB has a biphasic mechanism and the damage is particularly significant after reperfusion (Belayev, Busto, Zhao, & Ginsberg, 1996; Huang, Xue, Karbalai, Buchan, & Preston, 1999; Kuroiwa, Ting, Martinez, & Klatzo, 1985). The endothelial basal lamina – a thin planar assembly of extracellular matrix protein that supports all epithelial, muscle and nerve cells outside the central nervous system, which act as semipermeable filters for macromolecules – starts to dissipate approximately two hours after the onset of ischaemia (Hamann, Okada, Fitridge, & Del Zoppo, 1995) followed by rapid increase in BBB permeability (Belayev et al., 1996). Early reperfusion may temporarily alleviate BBB alternation; however, the use of thrombolytic therapy and delayed reperfusion have been shown to be conversely exacerbate the endothelial injury (Bang et al., 2007; Hjort et al., 2008; Kastrup, Engelhorn, Beaulieu, de Crespigny, & Moseley, 1999; Kidwell et al., 2008). Loss of BBB function may lead to accumulation of bradykinin, an inflammatory mediator that causes vasodilation and drop of blood pressure (Aschner, Lum, Fletcher, & Malik, 1997; Kamiya, Katayama, Kashiwagi, & Terashi, 1993). This also affects vascular endothelial growth factor, a signalling protein that stimulates angiogenesis and triggers formation of new blood vessels (Abumiya et al., 1999).

In addition, loss of BBB functions also causes thrombin – a serine protease participant in a clotting process – to activate and initiate coagulating reactions (Okada, Copeland, Fitridge, Koziol, & Del Zoppo, 1994). Lastly, BBB activates the matrix metalloproteinases (MMPs) – a group of enzymes capable of degrading all kinds of extracellular matrix proteins – and other protease activities (A. W. Clark, Krekoski, Bou, Chapman, & Edwards, 1997; Gasche, Copin, Sugawara, Fujimura, & Chan, 2001; Gasche et al., 1999; Gidday et al., 2005; Heo, Han, & Lee, 2005; Heo et al., 1999; Hosomi et al., 2001; P. J. Kelly et al., 2008; Montaner et al., 2001; Opdenakker et al., 2001; Rosell et al., 2008; Serena et al., 2005; S. Wagner et al., 2003). As described previously in Section 3.3.4, oxidative stress initiates early injury to the BBB and may trigger the release of MMP-9 by neurons, glia and endothelial cells that damage the BBB through digestion of the endothelial basal lamina (Gasche et al., 2001; Gidday et al., 2005; Heo et al., 1999; S. Wagner et al., 2003). After the early damage to the BBB, a second phase of severe BBB injury typically occurs within 24–72 hours of infarction (Kastrup et al., 1999; Lorberboym et al., 2003). The second phase is more complicated and results in greater tissue damage through leukocyte infiltration and marked MMP-9 release from neutrophils transmigrating to the ischaemic brain (Gidday et al., 2005; Rosell et al., 2008).

Furthermore, the disruption of BBB allows leakage of blood components into the brain parenchyma, a functional tissue distinguished from connective tissue and supporting tissue. Extravasation of high molecular weight molecules, followed by water from osmosis, leads to vasogenic oedema, which contributes to secondary damage through increased intracranial tension. The extravasation of red blood cells may lead to haemorrhagic transformation of the infarct area that may turn into haemorrhagic stroke (G. J. Del Zoppo & Hallenbeck, 2000). The leaking BBB facilitates the transmigration of inflammatory cells, which has been shown to promote the post-ischaemic inflammatory response (G. J. Del Zoppo & Hallenbeck, 2000).

2.3.6 Ischaemic-induced vascular damage

A series of ischaemic events targets the microvasculature, resulting not only in cerebral tissue damage through increased endothelial cell permeability and matrix degradation, but also in loss of autoregulation, the intrinsic ability of the brain to maintain a constant blood flow despite changes in perfusion pressure. In addition to the disrupted autoregulation, vascular damage can also trigger a range of aggregating factors, forming a 'no-reflow' phenomenon that contributes further to the damage from the ischaemia.

Cerebral autoregulation is the intrinsic ability of the cerebrovascular bed to maintain a constant perfusion in spite of change in blood pressure (O. Paulson, Strandgaard, & Edvinsson, 1990; Rapela & Green, 1964). When the cerebral blood pressure falls, a series of metabolic factors (such as hypoxia), myogenic processes (such as smooth muscle relaxation) and endothelial mechanisms (such as releasing of NO), all contribute to arteriolar vasodilation to reduce the vascular resistance in attempt to maintain the blood flow (O. B. Paulson, Olesen, & Christensen, 1972; Yanagisawa et al., 1988).

The pathophysiology of impaired cerebrovascular autoregulation in acute ischaemic stroke is still controversial. However, the damage of focal and even global impairments has been demonstrated via the reperfused ischaemic brain, and ischaemia-induced endothelial damage may play a key role here. Endothelial injury reduces the release of NO (an endothelial-derived relaxing factor acting on smooth muscle for vasodilation) and prostacyclin (an effective vasodilator that inhibits platelets aggregation), and induces endothelin-1 production, which is a potent vasoconstrictor. These processes lead to increased vascular tone, which may further impair the blood flow in the ischaemic core and penumbra, negatively affecting the necessary blood flow in the ischaemic area. Endothelin-1 – which has been shown to be marked sensitive in the cerebral microvessels and is associated with

cerebral oedema – is elevated during ischaemic stroke. In addition to vasoconstriction, impaired autoregulation potentially leaves the vulnerable ischaemic penumbra unprotected against harmful blood pressure changes. Hypotension during acute ischaemic stroke is known to be detrimental to tissue, and hypertension may improve outcome after stroke in some patients but be harmful in others (Ahmed, Näsman, & Wahlgren, 2000; Cole, Drummond, Osborne, & Matsumura, 1990; Leonardi-Bee, Bath, Phillips, & Sandercock, 2002; Oliveira-Filho et al., 2003; Shin et al., 2008; Zazulia, Videen, & Powers, 2007).

The 'no-reflow' phenomenon and post-ischaemic inflammation are additional consequences of cerebral microvasculatory injury. The obstruction of the downstream microvascular bed after reperfusion of the occluded supply arteries is due to the presentation of leukocyte adhesion receptors on endothelial cells. This phenomenon is attributed to extrinsic compression from oedema, endothelial swelling and intravascular obstruction due to local activation of leukocytes, platelets and coagulations (G. Del Zoppo, 1994; G. J. Del Zoppo & Mabuchi, 2003; G. J. Del Zoppo, Schmid-Schönbein, Mori, Copeland, & Chang, 1991; Mori, Del Zoppo, Chambers, Copeland, & Arfors, 1992; Okada, Copeland, Fitridge, et al., 1994).

2.3.7 Homeostatic activation

Endothelial cell injury results in exposure of tissue factors to the blood that triggered the coagulation cascade. Subsequently, tissue factors act to convert with factor VIIa and phospholipid to convert factor IX to IXa. The main role of factor VII is to initiate the process of coagulation, in conjunction with tissue factor. Tissue factor is found on the outside of blood vessels and is only exposed during a vessel injury. Factor VIIa is a result of bonding between tissue factor and factor VII, which catalyses the conversion of factor IX to IXa, a component responsible for the 'prothrombinase' complex that converts prothrombin to thrombin

(Borensztajn, Peppelenbosch, & Spek, 2008). Thrombin cross-links the fibrin clots via cleaveation of fibrinopeptides. The fibrin molecules can aggregate, trapping platelets, clotting factors and erythrocytes to form blood clots (Green, 1998, 2006). In addition, procarboxypeptidase U, an unstable carboxypeptidase U, is formed through thrombin during the clotting process, which attenuates the fibrolysis (Bajzar, Morser, & Nesheim, 1996; Björkman, Abrahamsson, Nerme, & Mattsson, 2005; Bouma & Meijers, 2003; Hendriks, Scharpe, Sande, & Lommaert, 1989; Klement, Liao, & Bajzar, 1999; Knecht et al., 2006; Judith Leurs & Hendriks, 2005; J Leurs, Nerme, Sim, & Hendriks, 2004; Marx, Dawson, Bouma, & Meijers, 2002; Mattsson et al., 2002; Minnema et al., 1998; Nagashima et al., 2000; Schneider, Brufatto, Neill, & Nesheim, 2004; Walker & Bajzar, 2004; J. L. Willemse & Hendriks, 2007). A marked decrease in procarboxyoeotudase U activity occurs in the first 72 hours after ischaemic stroke (Heylen, Brouns, Willemse, De Deyn, & Hendriks, 2009) and in patients with poor response to thrombolytic therapy, probably reflecting the stronger activation of the procarboxypeptidase U/carboxypeptidase U pathway and thrombus propagation (Brouns et al., 2009; J. Willemse, Brouns, Heylen, De Deyn, & Hendriks, 2008).

Platelets are typically activated under conditions of ischaemia and high shear stress (Gawaz, 2004; J. A. Zeller, Tschoepe, & Kessler, 1999). The accumulation of platelets in microvessels is seen within two hours of vascular occlusion (Mark Fisher & Francis, 1990; Lip et al., 2002), releasing a variety of biochemical mediators and catalysing interactions between coagulation factors that contribute to the 'no-reflow' phenomenon, by adhering to both leukocytes and microvascular endothelial cells (J. R. Chan et al., 2000; Htun et al., 2006; J. Zeller, Lenz, Eschenfelder, Zunker, & Deuschl, 2005; Zhaozhong, Qiuping, & Jianning, 2001). Platelets also can cause temporary vasospasm via release thromboxane A2 and free radicals, which exacerbate the inflammatory cascade by releasing chemotactic mediators for

leukocyte migration (Okada, Copeland, Mori, et al., 1994; J. Zeller et al., 2005). However, whether these mechanisms are important in ischaemic stroke remains to be elucidated. The endogenous fibrinolysis in ischaemic stroke is usually outweighed by ongoing activation of coagulation cascade and platelet activation (Barber, Langhorne, Rumley, Lowe, & Stott, 2004, 2006; Fassbender et al., 1999; Feinberg, Erickson, Bruck, & Kittelson, 1996; Haapaniemi, Soinne, Syrjälä, Kaste, & Tatlisumak, 2004; J. Kelly et al., 2003; Tanne, Macko, Lin, Tilley, & Levine, 2006; Zhaozhong et al., 2001), which may affect overall homeostasis and indirectly affect the ischaemic cascade.

2.3.8 Post-ischaemic inflammation

Multiple cells are involved in post-ischaemic inflammation. First, microglia and astrocytes are activated by elevation of ROS. Astrocytes are can secrete cytokines, chemokines and the inflammatory factors and induce NO synthase (X. Che, Ye, Panga, Wu, & Yang, 2001; Y. Dong & Benveniste, 2001; Hewett et al., 1996; Yamagami et al., 1999). Microglia are the resident macrophages of the brain and play a critical role as resident immunocompetent and phagocytic cells in the central nervous system (Morioka, Kalehua, & Streit, 1993). During the ischaemia, microglia can transform into phagocytes and release a variety of cytotoxic or cytoprotective substances. Within 4–6 hours of ischaemic onset, circulating leukocytes adhere to the vessel walls and migrate into the brain, releasing more pro-inflammatory mediators leading to secondary injury of potentially salvageable tissues in the penumbra (Barone & Feuerstein, 1999; Becker, 1998; Chamorro & Hallenbeck, 2006; Danton & Dietrich, 2003; Emsley & Tyrrell, 2002; Hallenbeck, 1996; Q. Wang, Tang, & Yenari, 2007). Adhesion molecules play a pivotal role in the infiltration of leukocytes into the brain parenchyma (DeGraba, 1998). The interaction between leukocyte and vascular endothelium is mediated

by adhesion molecules such as selectins, Vascular Adhesion Molecule-1 and Intercellular Adhesion Molecule-1. Neutrophils are the first leukocyte subtype; they are significantly upregulated in gene expression and infiltrate the area of ischaemic brain tissues (Buck et al., 2008; Y. Tang et al., 2006), with infiltration of monocytes observed after several days of ischaemic onset (Buck et al., 2008; X. Che et al., 2001; Grau et al., 2001; Morioka et al., 1993; Stoll, Jander, & Schroeter, 1998; Y. Tang et al., 2006).

Cytokines are important inflammatory mediators that are produced by immune cells and resident brain cells after ischaemia (Allan & Rothwell, 2001; Feuerstein, Liu, & Barone, 1994; Rothwell & Hopkins, 1995). The most studied cytokines related to inflammation in stroke are interleukin-1, tumour necrosis factor-alpha (TNF-α), interleukin-6 and interleukin-10 (Fassbender et al., 1994; Tarkowski et al., 1995; Vila, Castillo, Dávalos, & Chamorro, 2000; Vila et al., 2003). Interleukin-1 is the main pro-inflammatory cytokine; TNF-α has pleiotropic function and may influence apoptosis or survival through different pathways (Hallenbeck, 2002). Interleukin-6, on the other hand, is thought to be a largely pro-inflammatory cytokine, but its role in ischaemic stroke is more obscure (Tarkowski et al., 1995). Increased production of pro-inflammatory protein-1a and chemokines such as fractalkine following focal ischaemia is thought to have a deleterious effect by increasing leukocyte infiltration (Stamatovic, Keep, Kunkel, & Andjelkovic, 2003). In addition to the chemotactic properties, signalling for microglia to find debris cells, chemokines are found to directly affect blood–brain barrier permeability (Dimitrijevic, Stamatovic, Keep, & Andjelkovic, 2006).

Other inflammatory cascades include upregulation of several enzymes, such as phospholipase A2, which enhances intracellular calcium levels leading to excitotoxicity reactions (as described in Section 3.3.3) and enhances the arachnoid acid cascade. The hydrolysis of these enzymes leads to release of arachidonic acid, which is metabolised to

prostaglandins or leukotrienes by cyclooxygenase and lipoxygenase respectively. Of the two forms of cyclooxygenase, type 1 is constitutively expressed in microglia and leukocytes (Schwab & Schluesener, 2003) whereas cyclooxygenase type 2 is upregulated after ischaemia and exerts toxic effects (Kawano et al., 2006; Nogawa, Zhang, Ross, & Iadecola, 1997) mainly through prostaglandins rather than ROS, although it can generate both (Manabe et al., 2004; Nogawa et al., 1997). The understanding of lipoxygenase pathways in brain ischaemia remains limited; however, leukorines known to be potent chemoattractants are believed to be implicated in BBB dysfunction, oedema and neuronal death (Rao, Hatcher, Kindy, & Dempsey, 1999; Tomimoto et al., 2002). It is worth mentioning that inflammatory cells also generate ROS and produce MMPs, inducing more damage to the ischaemic brain (Gidday et al., 2005).

2.3.9 Ischaemia-induced cell death

Ischaemic injury produces necrosis, a fulminant form of cell death associated with failure of the plasma membrane and cytotoxic oedema of both the cell and internal organelles (Johnson Jr & Deckwerth, 1993; L. J. Martin et al., 1998). When the cell dies through necrosis, more glutamate and toxins are released into the nearby environment, affecting the surrounding neurons. Meanwhile, abundant brain cells undergo apoptosis during ischaemia as consequence of a genetically regulated program that allows cells to die with minimal inflammation or release of genetic materials (Choi, 1996; Hara, Niwa, Yoshimi, & Mori, 1997; J.-M. Lee, Zipfel, & Choi, 1999; Namura et al., 1998). Several factors determine which process predominates, including the local degree of ischaemia, cell maturity, the concentration of intracellular Ca_2^+ and the cellular microenvironment (Choi, 1995; J.-M. Lee et al., 1999; Leist & Nicotera, 1998b). Activation of glutamate receptors may promote apoptosis non-specifically by inducing sufficient cellular injury to activate cellular sensors linked to the

apoptosis cascade (Choi, 1995, 1996; Leist & Nicotera, 1998a; Leist et al., 1997; Namura et al., 1998; Thornberry & Lazebnik, 1998). In addition or alternatively, early mitochondrial production of ROS (L. Dugan et al., 1995) reduction of intracellular K^+ (S. P. Yu et al., 1997) and enhancement of toxic Zn^{2+} influx (S.-J. Lee & Koh, 2010) may all trigger apoptosis. In addition, caspase-mediated apoptosis is also initiated by the release of cytochrome C, a large transmembrane protein from mitochondria during the apoptosis complex, which in turn activates caspase 3 (Green, 1998), protein-cleaving enzymes that are particularly important in modifying homeostasis and repairing proteins in a ischaemia-induced apoptosis. Necrosis is the predominant mechanism that follows acute permanent vascular occlusion in the core, whereas milder injury, particularly within the ischaemic penumbra, often results in apoptosis.

2.3.10 Cerebral reperfusion injury

The prompt restoration of the blood supply has been a priority in an attempt to reduce infarct size through salvation of the penumbra and to improve clinical outcomes in patients with ischaemic stroke (Schaller & Graf, 2004). However, reperfusion may exacerbate so-called cerebral reperfusion injury through multifactorial aetiology (G. J. Del Zoppo, VON KUMMER, & Hamann, 1998; Kidwell et al., 2001; Kuroda & Siesjö, 1997). Early recanalisation in patients with severe ischaemia also carries an increased risk of reperfusion related brain haemorrhage, which may be due to microvascular injury (Albers et al., 2006). Leukocytes appear to be responsible for reperfusion injury as they damage the endothelium (obstructing microcirculation), disrupt BBB and infiltrate the brain tissue and release cytokines, which propagate inflammation (Pan, Konstas, Bateman, Ortolano, & Pile-Spellman, 2007). It has been reported that there may be a surge in the generation of ROS during cerebral ischaemia, particularly at the onset of reperfusion. ROS – including superoxide, hydrogen peroxide and

hydroxyl radicals – are highly reactive and can break down cell membranes, damage DNA and create oxidation of proteins, amino acids and inactive specific anti-oxidant enzymes. Neurons contain abundant polyunsaturated fatty acids and produce low amounts of anti-oxidant enzymes, and are therefore are highly susceptible to ROS (Kehrer & Smith, 1994).

Here, platelets play a more potent synergistic role, enhancing leukocytes in the reperfusion injury via the 'no-reflow' phenomenon discussed in Section 3.3.7 and releasing a variety of biochemical mediators that may lead to vasospasm and exacerbation of oxidative stress as well as inflammatory cascade (Crack & Wong, 2008; J. Zeller et al., 2005; Zhaozhong et al., 2001). Additionally, breakdown of BBB and post-ischaemic hyper-perfusion may exacerbate vasogenic brain oedema and haemorrhage (G. A. Rosenberg et al., 2001), which further contribute to the neural damage.

In summary, ischaemic injury leads to a sequence of interlinked cellular injuries through multiple factors with an emphasis on vascular vulnerabilities. A therapeutic target for ischaemic stroke leads to a complex assessment of the vascular vulnerability as well as the severity of ischaemia. Due to the complex cell degradation cascade during the ischaemic attack, neuroprotection is also becoming an important critical strategy that is as yet relatively underdeveloped for minimising neuron damage. Although recent research focuses on the survival of neurons via neuroprotection and anti-thrombotic therapies, little attention has been directed to investigating the role of angiogenesis (Krupinski, Kaluza, Kumar, Kumar, & Wang, 1994).

While necrosis at the core of ischaemic infarct is irreversible, studies suggest that the penumbra may remain viable for several hours after an ischaemic event due to alternative blood supply from collateral arteries (Dirnagl et al., 1999). A study by Krupinski et al. (1994) investigating angiogenesis and ischaemic stroke suggests that adequate perfusion through

the penumbra can initiate angiogenesis. Evidence suggests that high blood vessel density is correlated with improved patient survival, independency and clinical outcome after stroke (Choo, 1993). There is also a strong correlation between the extent of spontaneous neurological recovery and the volume of penumbra that escapes infarction (Cheng et al., 2004; Dirnagl et al., 1999). Therefore, enhancing the angiogenic effect is another key aspect of improving clinical outcomes in stroke recovery.

2.4 Conventional management for ischaemic stroke

Conventional management for ischaemic stroke can be summarised into three main therapeutic strategies, prevention, general treatment and rehabilitation (Ringleb et al., 2007). Each of these therapeutic domains is broad and complicated due to the nature of stroke and vulnerabilities of brain tissues. Current pharmaceuticals for prevention and treatment display considerable overlap. Anti-thrombotic therapy is primarily employed as a treatment modality, whereas neuroprotective strategies may be beneficial in both prevention and treatment. Despite the potential benefits of neuroprotective therapies, it is currently considered a secondary modality due to the nature of ischaemic stroke and emergency priorities. Stroke rehabilitation is focused not on prevention or treatment but, rather, on recovery of motor and cognitive function and subsequent quality of life. Despite the variety of available pharmaceuticals and treatment plans, there are significant shortfalls with each of the therapeutic domains that have yet to be overcome (Patel & McMullen, 2017; Ringleb et al., 2007; van der Worp & van Gijn, 2007).

2.4.1 Anti-thrombotic therapy

General treatment usually occurs in a hospital setting after the onset of stroke. Treatment method varies depending on the severity and the time of treatment after onset. The primary

therapeutic aims are to minimise the cerebral necrosis that is usually caused by arterial occlusion or thrombosis and to prevent reoccurrence of ischaemic attack (Patel & McMullen, 2017; Ringleb et al., 2007).

Acute management, such as intravenous thrombolysis with intravenous tissue plasminogen activator (rtPA), is given within the first six hours of onset of ischaemic stroke to dissolve thrombotic embolisms (Ringleb et al., 2007). This therapy has been found to decrease post-stroke dependency. However, a review by Wardlaw et al. (Wardlaw et al., 2009) evaluating the benefit-to-death ratio suggests that thrombolysis therapy significantly increases the death rate due to a high incidence of cerebral haemorrhage.

Anti-platelet treatment is considered as a valuable primary treatment and secondary preventative modality for ischaemic stroke (Ringleb et al., 2007). However, evidence suggests the use of anti-platelet therapy could increase the risk of fatal or disabling intracranial haemorrhage (Sandercock et al., 2008). While there are numerous anti-platelet pharmaceuticals available, aspirin is the most common anti-platelet agent employed for ischaemic stroke (Mohr et al., 2001; NSF, 2013; Sandercock et al., 2008). Evidence from two large randomised, non-blinded intervention studies indicates that aspirin is safe and effective when started within 48 hours post- stroke and is beneficial for acute ischaemic stroke (Z. Chen et al., 2000; Sandercock et al., 2008).

As anti-platelet therapies such as aspirin also increase the risk of intracranial haemorrhage, studies have shown that its use in combination with thrombolysis treatment significantly increases death rate from all causes, including neurological or intracranial haemorrhage (Sandercock et al., 2008). Furthermore, studies suggest that long-term use of aspirin increases the risk of gastrointestinal bleeding and irritation and that this adverse effect is likely to be

more apparent in elderly individuals (Guazzi, Brambilla, Reina, Tumminello, & Guazzi, 2003; Silagy et al., 1993).

Anti-coagulants such as heparin and warfarin are another strategy for treatment and prevention of ischaemic stroke. This therapy is typically employed in patients with existing emboli such as deep-vein thrombosis. According to a review by Gubitz et al. (2008), anti-coagulants do not produce any significant overall short- or long-term reduction in death and disability. Despite this, anti-coagulants appear to result in fewer recurrent ischaemic stroke incidents; however, this benefit is entirely offset by a similarly-sized increase in intracranial haemorrhage (Gubitz et al., 2008; Ringleb et al., 2007).

2.4.2 Neuroprotection therapy

The primary goal of neuroprotective therapy is to salvage the ischaemic penumbra (Dirnagl et al., 1999). It has been well documented that abrupt deprivation of oxygen and glucose to neuronal tissues elicits a series of pathological cascades leading to the spread of neuronal death. However, neuroprotective treatment has yet to be implemented in conventional treatment guidelines due to a lack of solid clinical evidence (Patel & McMullen, 2017; Ringleb et al., 2007).

For the past two decades, neuroprotective agents designed to block these cascades – including glutamate receptor antagonist, calcium channel blockers, anti-inflammatory agents and free radical scavengers – have been investigated in animal models of cerebral ischaemia. Numerous agents have been found to reduce infarct size in rodents, rabbits, and primate stroke models (Gorelick et al., 1999; Osuntokun, Bademosi, Akinkugbe, Oyediran, & Carlisle, 1979) However, translation of neuroprotective benefits from laboratory experiments to clinical trials was not successful (Cheng et al., 2004; Patel & McMullen, 2017).

While blocking glutamate receptors can protect against neurological excitotoxicity, these glutamate receptor antagonists exhibit serious adverse effects, such as psychotomimesis, respiratory depression and cardiovascular dysregulation. The benefits found in clinical trials have been outweighed by these effects and long-term preventative use is discouraged (Dirnagl et al., 1999; Patel & McMullen, 2017).

Glutamate receptor antagonists such as Selfotel act on various glutamate receptor sites throughout the central nervous system (Grotta et al., 1995; Q. J. Wu & Tymianski, 2018). A phase III trial with Selfotel was terminated because of a substantial increase in the adverse event-to-benefit ratio. Eliprodil is a strong antagonist that acts on the polyamine modulator site; however phase III trials showed no efficacy upon futility analysis and the trials were stopped prematurely (Gotti et al., 1988; O'Neill, Hicks, & Ward, 1996; Patat, Molinier, Hergueta, & Brohier, 1994). Aptiganel (Cerestat, CNS 1102) is a non-competitive NMDA glutamate receptor blocker with a relatively short half-life. Although it reached phase III trial status, Aptiganel was similarly found to be ineffective for acute stroke therapy (Aronowski et al., 1994; Cohen, Hasegawa, & Fisher, 1994) due to poor tolerance of the drug and a short therapeutic window (Q. J. Wu & Tymianski, 2018).

Clinical trials employing ion channel modulators – including calcium channel antagonists, potassium channel activator and sodium channel blocker – were all terminated prematurely due to lack of sufficient benefits (Cheng et al., 2004; Patel & McMullen, 2017).

Free radical-trapping agents such as Citicoline (cytidyl diphosphocholine) were studied in preclinical and clinical trials. Citicoline is a phosphatidylcholine precursor that has membrane stabilisation properties but may also have other neuroprotective properties. Preclinical studies in animal stroke models showed that Citicoline improved neurological outcomes and reduced infarct size (D'Orlando & Sandage Jr, 1995; Schäbitz et al., 1999). However, the

clinical phase III trials did not show efficacy despite employing a variety of dosing schedules (W. Clark, Warach, Pettigrew, Gammans, & Sabounjian, 1997; W. M. Clark et al., 1999)

Other free radical-trapping agents, such as NXY-059 and magnesium sulphate, have not shown significant clinical benefits (Ringleb et al., 2007). Research into the use of thrombolysis rtPA followed by uric acid-based anti-oxidant therapy is currently in phase II study (Amaro et al., 2007). While researchers continue to investigate the potential discrepancies between the preclinical and clinical outcomes of these neuroprotective agents, pre-existing agents such as hydroxymethylglutaryl co-enzyme A reductase inhibitors, known as statins, have been recommended in the current acute ischaemic stroke management guidelines. Eighty-nine patients treated chronically with statin were evaluated under withdrawal of statin for three days and immediate administration of 20mg of atorvastatin per day. Results indicated a significant association of statin withdrawal with death rate and dependency level. This may indicate the potential neurological protection possessed by statin (Blanco et al., 2007).This was supported by a small retrospective study demonstrating that statin may potentially decrease the infarct volumes in those who suffered acute stroke (Nicholas et al., 2008). However, both studies include patients with chronic pre-treatment with statin; whether acute administration of statin provides neuroprotective benefits remains unclear.

2.4.3 Post-stroke rehabilitation

Even with optimal stroke unit care, including thrombolysis, less than one-third of patients recover fully from stroke (Hacke et al., 2004). Rehabilitation aims to assist people with disabilities to reach and maintain optimal physical, intellectual, psychological and social function. It is a fundamental goal of post-stroke treatment to increase independency and functional improvements. Current available rehabilitation modalities such as physiotherapy,

occupational therapy and nursing can help to reduce bed days for selected stroke patients (Feigenson, 1979). A meta-analysis concluded that continuous rehabilitation within the first year after discharge from hospital can reduce the risk of functional deterioration and improve activities of daily living (Legg, 2004). However, this improvement is largely associated with the quality of the rehabilitation as opposed to the duration.

Despite these benefits, cognitive deficits are common following stroke and have a significant impact on quality of life of stroke patients. There is currently no evidence supporting the efficacy of specific memory and cognitive rehabilitation (Das Nair & Lincoln, 2007). This cognitive aspect of post-stroke rehabilitation is particularly important, as cerebrovascular incidences have been shown to correlate with increased risks of severe cognitive deficits such as dementia (Ivan et al., 2004; Tatemichi et al., 1994).

In summary, current conventional management of stroke is limited as it narrowly focuses on survival principles by dissolving/preventing emboli formation. The majority of the therapeutic benefits of anti-platelets and anti-coagulants are offset by dangerous adverse effect.

While neuroprotection is important to reduce dependency rates, no standardised neuroprotective treatment is currently recommended in the clinical guidelines. Consequently, there is an urgent need to identify effective therapies that are not only safer but also can treat the underlying pathological conditions associated with stroke. Natural and traditional medicine typically retains insights for strategies in health preservations. It has made valuable contributions to health developments throughout history.

Chapter 3. Chinese Medicine for Ischaemic Stroke

3.1 Introduction

Chinese herbal medicine (CHM) is the most widely practiced form of herbalism in Asia. CHM is based on a sophisticated medical theory and practice that is distinctly different from orthodox Western medicine (Sucher, 2006). It is most appreciated for its individualistic application to cases and application in conjunction with other modalities, such as acupuncture, for debilitating diseases affecting neurological activities such as stroke (X. Zhang, Liu, & Kang, 2016).

Most traditional therapeutic formulations of CHM consist of a combination of several herbs. The combination of herbs is thought to maximise therapeutic efficacy by targeting multiple therapeutic areas and facilitating synergistic actions, and to ameliorate or prevent potential adverse effects (Bensky, Gamble, & Kaptchuk, 2004; Gong & Sucher, 1999). Orthodox drug therapy has been subject to critical analysis by the evidence-based medicine movement, and it is desirable that herbal medicine should be subject to the same kind of scrutiny for safety and efficacy (Barrett, Kiefer, & Rabago, 1999; Sucher, 2006). However, a number of issues make the evaluation of herbal medicine a very challenging task. For example, the active components of many medicinal herbs are still poorly understood; scientific data on the efficacy and mechanism of action of single herbs are lacking; rigorous quality control measures for these herbs have not been well-established; and interactions between multiple bioactive components and multiple herbs are largely unknown (E. Chan, Tan, Xin, Sudarsanam, & Johnson, 2010).

The application of CHM is not used based solely on its pharmacological principles but also on Chinese medicinal theories and principles (X. Zhang et al., 2016). A large number of CHM products included in the Chinese pharmacopoeia are based on empirical evidence rather than the best evidence in the systematic hierarchy. It is important to note that TCM has played a unique role in Chinese culture and influenced its use within the Chinese population. Chinese

medicine has been the medicinal mainstream for the Chinese people for more than 3000 years. The empirical evidence documented throughout its history influences therapeutic strategies for many diseases and ailments even today. While Western medicine dominates mainstream practices globally, Chinese medicine still plays a significant role aligned with its historical developments and clinical evidences.

Research on Chinese medicine has grown substantially over recent decades in an attempt to understand the effectiveness and efficacy of its interventions. Significant progress has been made in defining, evaluating and standardising CHM in China. An increasing number of CHM products in China have adopted standardisation and patent strategies to control the quality and efficacy of medicinal sources. These medicines have been used for a broad range of clinical indications, including some common diseases such as stroke. In recent decades, hospitals in China have adopted TCM treatment, in particular herbal medicine, for the management of stroke (Feigin, 2007; M. Liu et al., 2007).

Preclinical studies suggest that the use of herbal medicines can increase neurological recovery and enhance functional outcomes in stroke patient and, thus, may be beneficial in post-stroke rehabilitation (I. Lee, Lee, Chang, Chien, & Lin, 2005; M. Liu et al., 2007; FL Shi, Hart, Sherman, & Tegeler, 1989). According to Chen et al. (Z. Chen et al., 1997), over 66% of physicians in China incorporate some form of TCM treatment for acute management of ischaemic stroke. It is estimated that 10-20% of all healthcare services is solely provided by the TCM sector in China (Jiang, Zhang, Cao, Chan, & Lu, 2011).

Nao Xin Qing (NXQ) is a standardised and patented herbal extract from the leaves of *Diospyros kaki* that has been used for many years in China for the treatment of apoplexy syndrome, coronary artery disease and ischaemic stroke (Bei, Peng, Ma, & Xu, 2004; Bei, Zang,

et al., 2009; Y. Cai & Yang, 2001). The key bioactive components of *D. kaki* have been identified as flavonoids (particularly quercetin and kaempferol) (Bei, Peng, Ma, & Xu, 2005b). Data from previous studies have shown *D. kaki* has several therapeutic effects including anti-thrombotic, neuroprotective, haemostatic, anti-oxidative, cholesterol-lowering, and anti-hypertensive effects, thereby presenting a potential therapeutic option for both treatment and prevention of ischaemic stroke (Y. Cai & Yang, 2001; Y. Yu, Yu, & Guo, 1988). The preliminary evidence suggests there are potential therapeutic benefits of NXQ for ischaemic stroke; however, the mechanisms remain unclear.

The current chapter aims to provide an overview of the current status of CHM (both traditional and patented forms) for the treatment of ischaemic stroke, including their use, efficacy and effectiveness and safety profile. In addition, an overview of the current evidence of NXQ use for ischaemic stroke will be discussed.

3.2 Perspective of TCM

TCM originated in China over 3000 years ago and is a form of medicine that integrates philosophical concepts and empirical experiences into clinical practice (Deadman, 2006). The concept of health in TCM has been described as a holistic therapeutic intervention, in which the balance between the mind, body and spirit is involved. Disease prevention is considered in the fundamentals of TCM, emphasising body's self-healing mechanisms; when balance within the body is achieved, diseases will fade and good health be maintained. To achieve this, TCM offers a complex framework comprising numerous integrated theoretical components that describe various aspects of human physiology and pathophysiology (H.-z. Wu & Fang, 2013).

These theoretical components can be categorised into acupuncture and meridian theory (*Jing Luo*); visceral theory (*Zangfu*); and *qi*, blood, *jing* and body fluids (*Jinye*). All these theoretical approaches have yet to be sufficiently explained using biomedical medicine and modern science (Lu, Jia, Xiao, & Lu, 2004). Despite the inadequate scientific explanation of these theoretical concepts, TCM provides a comprehensive framework that allows for the integration of every possible 'symptom' and consequently, a diagnostic model that encompasses individual physiological systems (e.g. respiratory or cardiovascular) as well as the body and mind in its entirety (H.-z. Wu & Fang, 2013). While biomedical science employs the same fundamental concepts in diagnostics, the symptomology and pathological diagnoses employable in biomedicine are considerably narrower and more focused. This is one of the distinguishing characteristics of TCM; from symptomology to diagnosis to treatment, the therapeutic scope approaches the body and mind in a holistic fashion, thereby allowing individualised diagnoses and treatment for a specific set of symptoms (B. Zhu & Wang, 2011). In comparison to the more restricted perspective of biomedicine, this holistic paradigm is difficult to replicate and challenging to adequately combine with the 'gold standard' model of randomised control trials (Y. Liu, 1988; Wiseman, 1996).

3.2.1 TCM nomenclature

While it is beyond the scope of this review to discuss each aspect of TCM theory, an important distinguishing feature of TCM is the nomenclature employed to describe various physiological process or systems. The term 'meridian' and collaterals in TCM can be interpreted as the passageways of energy. Energy travelling within these passageways interconnects with viscera to maintain healthy function and life. When the flow of energy within these passageways is disturbed, illness may present (Y. Liu, 1988; Lu et al., 2004). Terms such as the

'liver', 'kidney' or 'heart' do not describe the physiological organ within the human body, but rather a system of physiological processes (Lu et al., 2004). An example of this would be the 'liver', a label that encompasses aspects of circulation and emotion. Herbal medicines used in stroke treatment are often associated with two vital components in TCM, *Qi* and 'blood'. *Qi* is typically explained as energy, a vital source that is not visible to the naked eye, while 'blood' typically refers to the biomedical perspective of blood. Thus, the TCM terminology does not equate to the common understanding of the system or the substances themselves. The terms should not be confused with the common definitions or biomedical definitions.

3.2.2 Ischaemic stroke in TCM perspective

TCM possesses its own diagnostic system and disease patterns. In the classical literature, ischaemic stroke as a disease name was not mentioned; however, clinical terms such as *Cu Zhong* and *Zhong Feng* were used to describe symptoms such as sudden collapse, weakness or numbness or paralysis of the face, arm or leg; difficulty speaking and understanding; dizziness, loss of balance or an unexplained fall. These symptoms correspond well with those of a stroke with a ischaemic or haemorrhagic cause (Tsai, 2011). The diagnosis in Chinese medicine is 'syndrome' (*Zheng*)-based. For instance, a particular syndrome (*Zheng*) is characterised by a patient's signs and symptoms, the location of the underlying 'lesion', its aetiology, and the specific relationship between the patient's resistance and the pathological agents (Lu et al., 2004). Thus, a syndrome is the concrete (physical) manifestation of a fundamental pathological process combined with the incidental aspects such as the patient's physical and psychological constitution and the physical and social environment. As such, these 'syndromes' do not, in general, correspond to the disease classification of Western medicine (Sucher, 2006). Moreover, TCM is individualised medicine, in the sense that each

patient is treated with a specific but varying combination of drugs chosen on the basis of the particular presenting syndrome (symptom complex) observed at a given point in time, rather than a nosologically defined disease.

TCM diagnostic theory was constructed based on four major diagnostic methods: observation, auscultation/olfaction, questioning and palpation. Before the advanced magnetic resonance imaging system and other advanced diagnostic tool existed, TCM practitioners were making disease judgement based on these four methods. All of these methods are equally important, as they provide important indicators to help practitioners categorise and subcategorise the cause of disease as well as evaluating the patient's current bodily constitution. This evaluation directly affects the practitioner's therapeutic strategy plan, with medicine prescribed accordingly.

With time and evolution of the TCM itself, several patterns were identified as contributing to ischaemic stroke. In the recent years, some countries with Chinese medicine regulatory boards haves systematically categorised several constitutional and pathological patterns that can lead to ischaemic stroke (Table 1).

Table 1: Clinical symptoms categorised based on Chinese medicine syndromes differentiation of stroke

Stroke category in TCM	*Main syndrome subtype*	*Main clinical Symptoms*	*Associated clinical symptoms*
Main Stroke characteristics	Wind Pattern	Paralysis of face and tongue, slurred speech, slowed reaction, dull and dark complexion, blurry vision, body paralysis	Uncontrolled drooling, somnolence, acute onset, thick tongue coating
Category 1	Phlegm pattern	Uncontrollable bowel and urination, difficulty in speech, nausea and vomiting	Difficult swallowing, stiff tongue
Category 2 (pattern translatable between I and II)	I. Blood stasis pattern	Dark dull tongue body, and purplish blue vein underneath the tongue	Chopping and knot pulse
	II. Yin deficiency pattern	Irritation, easily angry, sudden severe headache	Fixed continuous headache, numb face, sudden numbness of the tongue

Category 3	Qi deficiency pattern	Lassitude, tiredness, minimal speech	Dizziness, drowsiness, uneven pulse, dull tongue body
Category 4	Fire -heat pattern	Bitter taste, dry throat, numbness of limbs, dry mouth and lip	Red complexion, half body numbness, weakness of the limbs, vertigo

The major principle of ischaemic stroke refers to obstruction and stagnation. A study conducted by a Taiwanese research group looking at the occurrence rates of acute ischaemic stroke cases based on pattern differentiation noted that 98.4% of the cases belong to the stagnation pattern, followed by the wind pattern, phlegm pattern, deficiency pattern, heat pattern and yin deficiency with hyperactive yang pattern (Tsai, 2011). Depending on the causal factors, cases are typically further sub-categorised into more precise pattern diagnosis; in many cases, syndromes may overlap. For instance, Tsai et al. (2011) report that of the 253 studied acute ischaemic cases, 99.6 % had more than one pattern at the time of onset. On average, 30% of the studied cases had four overlapping syndromes. This indicates the complexity of the ischaemic disease itself; therefore, the therapeutic strategies are typically individualised. Nevertheless, the patterns of ischaemic stroke were mostly associated with the above six types, particularly the stagnation type. Hence, the majority of the research focuses on blood-regulating herbs such as *Panax notoginseng*, known as *Sanchi*, and *Salvia miltiorrhiza*, known as *Danshen*. Because TCM theory visualises broader aspects involving more than body physiology (for example, emotional mediators, acute or chronic, can be an aetiology themselves), the therapeutic strategies are diverse. For example, the treatment approach for ischaemic stroke typically incorporates blood regulation and stasis resolution. It is either ‘excess’ that obstructs the flow of the blood, or ‘insufficiencies’ that lead to other ischaemic changes. The blood itself in TCM can be cross-referenced with multiple body systems; in contrast to the cardiovascular system in Western medicine, in TCM, ‘heart’ and ‘liver’ is also considered to encompass metabolic systems (the spleen and stomach) and

immune systems (the kidneys and bladder). Although the treatment approach for ischaemic stroke in TCM varies between individuals, the treatment principles typically incorporate blood regulation and stasis resolution.

3.2.3 Chinese herbal medicine for ischaemic stroke

3.2.3.1Complexity of CHM and its use

Chinese herbal pharmacotherapy is as old as TCM itself (S. Yang, 1998). The Chinese *Materia Medica* has grown substantially over the last 2000 years, from a few hundred documented herbs to more than ten thousand including substances from animal, mineral and plant sources. For example, an authoritative 10-volume compendium of Chinese *Materia Medica* lists more than 11,228 plant species, 1150 animal species, and 74 minerals as sources of drugs and interactions for their use (Sucher, 2006). The official pharmacopoeia of the People's Republic of China lists information on some 530 traditional drugs, including some 460 herbal drugs, more than 40 drugs of animal origin, human hair and placenta, and some 30 minerals (hui, 2000). Individual herbal drugs can be derived from more than one species.

The drug a patient take home from the pharmacy based on a doctor's prescription is not 'fresh', but 'processed'. Herbs and other natural materials become drugs only after processing. The specific possessing procedures play an important role in Chinese medicine (K. Chen, Xu, Hao., 2003). Processing can prevent or abate adverse effects of some drugs that would be toxic in unprocessed form. It can enhance or reduce the potency of drugs and even change their therapeutic effect. Processing is also used to make drugs easier to store and prepare and to make them more palatable and more convenient to take. There are various method of processing, including cutting, cleaning, rinsing, grinding, softening, parching, roasting, steaming and boiling (K. Chen, Xu, Hao., 2003).

Most CHM formulations consist of a combination of up to 20 herbs. In fact, combination therapy is a fundamental principle of Chinese medicine. The combination of multiple herbs in complex formulations (and the presence of multiple active compounds even in single herbs) is thought to maximise therapeutic efficacy by facilitating synergistic actions (mutual reinforcement) among the herbs and ameliorating (mutual restraint) or preventing (mutual detoxification) potential adverse effects at the same time as targeting one or several pathophysiological mechanisms (mutual assistance) (Gong & Sucher, 1999). Some herbs may be weakened (mutual inhibition) in their therapeutic effects when they are combined; others may become outright poisonous or cause serious adverse effects in combination, which must be avoided (K. Chen, Xu, Hao., 2003). While herb–herb interactions have been explored since the origins of TCM, the treatment effects and efficacy continue to exhibit strong discrepancies between practitioners. Because TCM emphasises individualised therapy, on the basis of the particular presenting syndrome (symptom complex) observed at a given point in time, rather than a nosologically defined disease, standardisation becomes impossible. This creates potential safety risks, particularly with time-sensitive diseases such as stroke. Therefore, in modern times TCM therapy has been used mostly as an adjunct therapy to Western medicine, particularly with diseases requiring surgical attention. However, TCM may be able to contribute to the management of major systemic disorders if more scientific validation and development is conducted to modernise these medicines.

3.2.3.2 Current evidence on CHM for ischaemic stroke

To better validate the efficacy and effectiveness of CHM for its various clinical applications, it is important to standardise herbal medicines' resources and chemical composition. A series of scientific methods, including chemical characterisation, preclinical *in vitro* and *in vivo* testing and clinical evaluation have been commonly used in evaluation of CHM products.

Many TCM herbs that can be potentially used in stroke therapy have been investigated and some key bioactive components characterised. Chinese herbs that possess blood-regulating properties have been most investigated, as they have traditionally been used for the blood stagnation pattern, which is related to ischaemic stroke pathology. For example, *Danshen* (*Salvia miltiorrhiza*) and *Sanchi* (*Panax notoginseng*) were investigated intensively and the bioactive constituents of these herbs were found to improve microcirculation in the brain, protect against reperfusion injury, possess neuroprotective and/or anti-inflammatory properties, and inhibit apoptosis (X. Chen et al., 2008a; B Wu, M Liu, & S Zhang, 2007; Yuan, Zeng, Luo, Li, & Wu, 2008). A number of herbs or herbal formulations that have been used clinically for treating ischaemic stroke are discussed below.

3.2.3.3 Single herbs and herbal formulations for ischaemic stroke

3.2.3.3.1 *Salvia miltiorrhiza* (*Danshen*)

Danshen is one of the herbs that have been used as single extract or in complex herbal formulations either orally or intravenously for the treatment of ischaemic stroke in modern China. It is a dried root of *S. miltorrhiza Bunge* of the *Labiatae* family and contains several important bioactive components, contributing to its therapeutic effects. Tanshinone, isotanshinone and hydroxytanshinone are the important ingredients. Pharmacological studies indicate that *Danshen* can be used for dilating the cardiocerebral vessels, suppressing the aggregation of platelets, improving circulation, removing blood stasis, protecting against ischaemic reperfusion injury and enhancing the tolerance of ischaemic tissue to hypoxia (CMMCC 1998; Li 2000). *Danshen* was found to protect against cerebral injury from cerebral ischaemia reperfusion in rats. The model was designed to induce cerebral reperfusion injury by occlusion of the middle cerebral artery for two hours and then by reperfusion (X.-Y. Liang et al., 2013). The author reports that *Danshen* significantly protects against ischaemic

reperfusion injury by decreasing pro-inflammatory factors such as CRP, IL-8, IL-10, NSE and TNF-α, suggesting a strong anti-inflammatory property of the herb. The same group of researchers further demonstrated protection of cerebral damage by upregulating angiogenic mechanism TGF-B1 positive expression, which promotes blood circulation to the infarcted brain (X.-Y. Liang et al., 2013).

Furthermore, the main active components found in *Danshen*, tanshinones, have been shown to exert neuro-protective effects in rat models with transient focal cerebral ischaemia. Lam et al. (B. Lam et al., 2003) demonstrated that 16mg/kg of tanshinones reduced the infarction size by approximately 30% and significantly improved the neurological functions in MCAO-induced ischaemia stroke models in mice. The authors did not elaborate the potential mechanism of actions underlying this observation. However, it was believed that tanshinones could potentially inhibit platelet aggregation and lessen the inflammatory response by reducing both neutrophil phagocytosis and leukocyte infiltration in the infarct region (J. D. Adams, Wang, Yang, & Lien, 2006). Further, *Danshen* has found to inhibit angiotensin-converting enzyme (ACE) to lower the blood pressure and dilate arteries (Gao, Xu, Deng, & Zhang, 2004; Kang, Oh, Chung, & Lee, 2003).

Despite the preliminary evidence from preclinical studies, the efficacy and safety profile for *Danshen*'s use for acute ischaemic stroke remains inconclusive. While no clear evidence of the efficacy of *Danshen* for acute ischaemic stroke was identified, it has been widely used in both Western medicine hospitals and traditional Chinese medicine hospitals in China (Bo Wu, Ming Liu, & Shihong Zhang, 2007). *Danshen* and its patent form are considered a standard treatment in clinical practice and used as a standard control intervention in clinical trials (Bo Wu et al., 2007). A limited quality-controlled trial was conducted to evaluate the clinical efficacy and safety profile of *Danshen.* Wu et al. (2007) evaluated six randomised controlled

trials of *Danshen* for acute ischaemic stroke, with a total of 494 participants involved. Meta-analysis of these six trials showed that *Danshen* agents may improve neurological and functional outcome after acute ischaemic stroke, but this result should be interpreted with caution because of the poor methodological quality of the included trials and the small numbers of participants. Overall, the findings remain inconclusive due to methodological problems such as inadequate concealment and reporting of concealment, safety profile, and adverse events. No detailed evaluation was conducted to review the potential drug–herb interaction against standard orthodox treatments such as anti-coagulant or anti-platelet agents.

3.2.3.3.2 *Panax notoginseng (Sanchi)*

Sanchi (*Panax notoginseng*; also known by other names include *Jinbuhuan, Tianqi, Panlongqi, Xueshen, Tiansanqi* and *Shensanqi*) is a Chinese medicinal herb used for promoting blood circulation and is commonly used for the treatment of acute ischaemic stroke. *Sanchi* is the dry root of the *Araliaceae* plant, and its main active component attribute to its therapeutic function was known as *Panax notoginseng* saponins (PNS). *Sanchi* has been commercialised for patent medicine, known as Xuesetong, Xuesaitong Xueshuantong Lulutong, Zhengkangnaoming, Sanchitongshu, and Xinaotai, all of which are used for cardio- and cerebrovascular diseases (X. Chen et al., 2008a). *Sanchi* is known as an expensive and precious herb, as are *P. ginsen*g (*Renshen*) and *P. quinquefolius* (*Huaqisheng*). Many of the saponines found in *Sanchi* are similar to those in *Renshen*, but most notoginsenosides are dammarane-type tetracyclic triterpene saponins (Matsuura et al., 1983). Other saponins, such as notogisenosides R1 and R2, are found in *Sanchi* but not *Renshen* or *Huaqishen* (Ng, 2006). Apart from PNS, *Sanchi* also contains active component such as dencichine, amylose, flavanol glycosides, flavanones and naphtha (T. Wang et al., 2016). Previous studies demonstrate that

PNS is likely to dilate cerebral blood vessels, promote cerebral blood contribution (Ma, 1998; J. X. Wu, Sun, J. J., 1992), inhibit thrombosis and platelet aggregation (J. Xu, 2003), decrease serum lipid (Lv 2004), restrain atherosclerosis (A. Liu, 2005), reduce oxygen free radicals (E. Dong, Peng, LF., Gao, GL. , 1990) and inflammatory response (N. Li, Sun, J., Wang, Y., Li, XH. , 1999), relieve cerebral oedema (Yao & Li, 2002) and improve cerebral cell proliferation and decrease infarction quantity after ischaemic reperfusion damage (X. Hu, Zhou, D., Zhou, DM. , 2004; X. Hu, Zhou, DM., Zhou, D. , 2004). In addition, PNS is also reported to alleviate pain, promote calm and increase intelligence and memory (Guo, 2004; H. Yang, Zhang, J. , 2005). Although *Sanchi* has been shown to have promising effects in preliminary studies, clinical trial translation was somewhat inconclusive.

A systematic review conducted by Chen and colleague (X. Chen et al., 2008b) evaluated eight randomised controlled trials with a total of 660 cases of acute ischaemic stroke cases, administering *Sanchi* or its patent extract as an co-intervention in addition to the standardised anti-platelets agent. The total administrating dose was between 200 and 525mg per day over 14, 20 and 28 days. Subgroup analysis suggests that *Sanchi* capsules (200mg/day) given within one month of stroke onset (RR 0.22, 95% CI 0.11 to 0.45) were more efficacious in improving neurological deficit than *Sanchi* injection (200mg/day) given within 72 hours of stroke onset (RR 0.40, 95% CI 0.20 to 0.80). Further to this, the funnel plot presented asymmetrically, suggesting a high risk of bias in assessing the treatment effects. The mortality rate was extremely low in the studies, indicating that these researchers tended to choose patients with mild stroke.

3.2.3.3.3 Classical Chinese medicine formula for ischaemic stroke

In addition to *Danshen* and *Sanchi*, several formulas were commonly used for ischaemic stroke associated conditions. These include Xue Fu Zhu Yu decoction, which consists of 11

herbs: *Danggui* (*Angelica sinensis*), *Shendihuan* (*Rehmannia glutinosa Libosch*), *Taoren* (*Prunus persica L. Batsch*), *Honghua* (*Carthamus tinctorius L*), *Zhiqiao* (*Citrus aurantium L*), *Chishao* (*Paeonia lactiflora Pall*), *Chaihu* (*Bupleurum chinensis*), *Gancao* (*Glycyrrhiza uralensis Fisch*), *Jiegeng* (*Platycodon grandiflorum*), *Chuanxiong* (*Ligusticum chuanxiong*) and *Niuxi* (*Achyranthes bidentate*) (Scheid & Scheid, 2009). Xue Fu Zhu Yu decoction has been used traditionally to regulate the blood and remove stasis, and has a strong analgesic effects. It has been used in cases of coronary artery diseases, hypertension, hyperlipidaemia, atherosclerosis, epilepsy and stroke with patterns correlating to blood stasis. While it is difficult to evaluate the mechanism of action, synergism and antagonisms between the herbs of the formula, some preliminary data have demonstrated its potential pharmacological actions (Scheid & Scheid, 2009).

Song et al. (2013) used an NMR-based plasma metabolomic method to evaluate the effects of Xue Fu Zhu Yu decoction on hyperlipidemic rats, suggesting that the decoction can ameliorate hyperlipidaemia by decreasing the accumulation of ketone body (β-hydroxybutyrate) and acetyl-glycoproteins, enhancing glutathione (GSH) biosynthesis, partially reversing energy and lipid metabolism disturbance (Song et al., 2013). β-hydroxybutyrate, as a ketone body, is produced by the oxidation of fatty acids, primarily in liver. β-hydroxybutyrate is normally exported to peripheral tissues, particularly brain tissues, that have no other non-glucose-derived source of energy (Newman & Verdin, 2014). The continuous accumulation of β-hydroxybutyrate causes ketoacidosis, which can lead to a potentially life-threatening condition known as metabolic acidosis (Daum et al., 1973). GSH is the major natural anti-oxidant that can combat oxidative injury by reducing H_2O_2. Depletion of GSH leads to increased levels of ROS, causing lipid peroxidation, e.g. the oxidation of LDL to ox-LDL (oxidised low-density lipoprotein). Exposure to ox-LDL inhibits the release of NO

(nitric oxide) in endothelial cells, facilitates the production and release of endothelin (ET) and, consequently, promotes vasoconstriction and proliferation of smooth muscle cell, which are key steps in the pathogenesis of atherosclerosis (Song et al., 2013). This finding indicates that the anti-oxidative effects and lipid-lowering effects of the formula may contribute to its clinical use to treat atherosclerosis and dyslipidaemia.

Bu Yang Huan Wu decoction is another traditional formula used for post-stroke recovery. The formula consist of seven herbs: *Huangqi* (*Astragalus membranaceus Fisch*.), *Danggui wei* (*Angelica sinensis (Oliv.) Diels*), *Chishao* (*Paeonia lactiflora Pall*), *Dilong* (*Pheretima aspergillum*), *Chuanxiong* (*Ligusticum chuanxiong*), *Taoren* (*Prunus persica*) and *Honghua* (*Carthamus tinctorius L.*) (Scheid & Scheid, 2009).

Pharmacologically, the main chemical constituents of *Bu Yang Huan Wu* decoction include C-glycosyl quinochalcones, flavonoid O-glycosides, isoflavones, monoterpene glycosides, saponins, organic acids and amino acids (E. H. Liu et al., 2009). All these constituents play roles in anti-apoplexy (Jingfen et al., 2002), and some have been demonstrated to prevent injury and apoptosis of neurons (C. Deng, He, Fuyang., Tang, Yinghong., Deng, Yihui., Wan, Ming., 2000) through the reduction of glutamate and aspartic acid in brain tissue (C.-Q. Deng, Deng, Y.-H., Xiang, H.-L. , 2001).

Bu Yang Huan Wu decoction has been reported to possess neuroprotective effects in cerebral ischaemic reperfusion injury rat models (L.-D. Zhao, Wang, Jin, Zhao, & Zhang, 2012). It is suggested that the formula is involved in the inhibition of excessive release of glutamate resulting from cerebral ischaemia and the down-regulation of mGluR-1 mRNA. Cerebral ischaemia or brain injury has been shown to cause marked elevation in glutamate concentrations (Parelkar, 2008), resulting in neuronal injury or death owing to excitotoxicity (Bonde, Noraberg, Noer, & Zimmer, 2005). Sustained intracranial pressure elevations and

poor patient outcome are significantly associated with high levels of central nervous system glutamate in humans (Bullock et al., 1998). Activation of mGluR-1 promotes the post-ischaemic injury of neurons (Meli et al., 2002). Therefore, maintaining a normal extracellular glutamate concentration is a key to preventing glutamate neurotoxicity.

Further to this, *Bu Yang Huan Wu* decoction also demonstrates anti-inflammatory effects against ROS-induced oxidative stress. It is reported that the decoction modulates the gene expression of the pro-inflammatory cytokines/chemokines and suppresses the genes promoting cell death, such as IL6, CCL11, CXCL2, CXCL3, CD163, CD24a, caspase 8, Tlr4 (toll-like receptor 4), thrombospondin 1, Bmp7, Mmp8 and Mmp9 (H.-W. Wang et al., 2011).

Of particular interest, the key herb in Bu Yang Huang Wu Tang is *Huangqi* (*A. membranaceus*), which is the main source of astragalin (Shaw, Lin, & Tsai, 2012). Astragalin has been shown to involved in re-vasculature and angiogenic factor regulation (Ke, Hu, Ouyang, Dai, & Xu, 2012). Angiogenic genes such as vascular endothelial growth factor (VEGF) and its receptor Flk1 is reported to be upregulated by Bu Yang Huang Wu decoction during stroke protection in rats on days 7–14 after stroke (G. Cai et al., 2007). However, Wang et al. (2011) report various angiogenic genes were less active in *Bu Yang Huan Wu* decoction-treated mice. It has been reported that upregulation of VEGF increases BBB permeability (Vandenbroucke, Mehta, Minshall, & Malik, 2008), possibly by enhancing VEGF-mediated activation of Rac-1, which causes ROS generation (Monaghan-Benson & Burridge, 2009). In addition, the anti-VEGF receptor antagonist VGA1155 has been demonstrated to reduce infarction in rat permanent focal brain ischaemia (Chiba et al., 2008). Based on these observations, Wang et al. propose that suppression of angiogenesis by *Bu Yang Huan Wu* decoction may be due to the time point of investigated in their study, which was day one after stroke when they harvested brain RNA for transcriptome for analysis. The CI/R-induced

ischaemic response, especially BBB leakage, may have been ameliorated by *Bu Yang Huan Wu* decoction *in vivo* so that blood vessel formation was no longer active *in vivo* (H.-W. Wang et al., 2011).

While the preliminary benefits of the use of *Bu Yang Huan Wu* decoction was evident, the effects and safety profile in clinical use remain inconclusive. Hao et al. (Hao et al., 2012) reviewed nineteen randomised clinical trials including a total of 1580 individuals for the efficacy and safety of *Bu Yang Huang Wu* decoction for acute ischaemic stroke. The findings are inconclusive as methodological bias was considerable. While the effective rates of neurological deficit improvements favour *Bu Yang Huan Wu Tang*, Hao et al. (2012) report a publication bias in the funnel plot analysis. Further, all studies failed to provide randomised allocation concealments and 14 studies did not report adverse events. Therefore, more rigorous methodological trials are required to support clinical efficacy and safety of *Bu Yang Huan Wu* decoction as a routine treatment in clinical practise. Nevertheless, the decoction itself has been used for hundreds of years by Chinese medicine practitioners and is still used in cases of post-stroke management of neurological deficits and after-effects of ischaemic stroke (Chang et al., 2016).

3.2.3.4 Patented herbal medicine for ischaemic stroke

Traditional Chinese patent medicine (TCPM) has been widely used and accepted in China for several decades for the management of stroke in both Western medicine and Chinese medicine hospitals (Z. Chen et al., 1997). The Chinese National Essential Drug (CNED) 2012 edition, the Pharmacopoeia of People's Republic of China (PPRC) 2010 edition, and the China Food and Drug Administration (CFDA) list a variety of CHM preparations (mixtures of multiple herbs) that have been subjected to a relatively strict drug evaluation process and are widely

used in current clinical practice for stroke in China and these TCM preparations are defined as TCPMs. TCPMs represent some potentially important therapies in addition to the standard use of aspirin and thrombolytic and recombinant tissue plasminogen activator for the treatment of ischaemic stroke.

Clinical effectiveness and safety of TCPMs are yet to be fully investigated. One systematic review conducted by Wu et al. (2007) evaluated the efficacy and safety of 22 TCPMs listed in the CNED (2004 edition) as potential drugs for ischaemic stroke patient. Although the pooled analysis of the existing randomised and nonrandomised trials showed significant positive effects in lowering mortality rates and improving neurological impairments, highlighting the potential benefits in stroke treatment, over 97% of the included clinical trials were noted as having poor methodological quality (B Wu, M Liu, H Liu, et al., 2007). The main methodological weaknesses of these trials were lack of bias control (such as double blinding and placebo control, concealment and randomised allocation reporting) and lack of adverse event reporting.

A recent systematic review evaluated nine TCMPs, including *Danshen* agents, *Mailuoning*, *Ginkgo biloba*, *Denzhanhua*, *Acathopanax*, *Chuanxion*-type preparations, *Puerarin*, *Huangqi* and *Qing Kai Ling*, attempting using the GRADE approach to assess the outcome of the RCTs. The results showed that the quality of the scientific evidence to support these nine TCPMs ranged from low to very low, due to small sample sizes and various methodological issues in the primary studies including inadequate reporting of allocation concealment, randomisation methods, and lack of blinding and inconsistency. This highlights the need for high-quality research to validate these TCPMs before being used as standard treatment in clinical practice.

3.2.4 Summary

In short, early evidence exists to support the use of CHM products in the treatment of ischaemic stroke. However, the efficacy of these CHM interventions needs to be validated in well-designed quality-controlled trials. Standardisation of CHM products is particularly important to replicate the results of clinical trials. There is also an urgent need to understand the mechanisms underlying the observed clinical effects of these medicines. Given these medicines consist of multiple bioactive components, research is needed to understand the interactions among these herbs (synergistic effects) and their interactions with pharmaceutical medications. The latter is critical in clinical practice, as therapeutic windows for some Western pharmaceutical medicines are narrow and their effects can be either enhanced (increased toxicity) or reduced (decreased efficacy) when used together with herbal medicines with which they may interact.

3.3 Nao Xin Qing (NXQ)

Nao Xin Qing (NXQ) is a patented CHM product made from the standardised extract of *D. kaki* leaves (*D. kaki L.*). NXQ has been clinically used in China for numerous years in the treatment of apoplexy, coronary heart disease, and ischaemic stroke recovery. Previous clinical and preclinical studies have suggested that NXQ has potential therapeutic effects for cerebral atherosclerosis, transitory ischaemic syndromes, cerebral thrombogenesis, cerebral thrombosis sequelae, apoplexy sequelae, and cerebral embolism, with minimal adverse effects (Bei et al., 2007; Y. Cai & Yang, 2001).

3.3.1 NXQ bioactive profile

D. kaki is a traditional herb that has been used in Asia for hundreds of years. In 1436, it was documented in Chinese herbal *Materia Medica* (the *Dian Nan Bencao*) that the leaves of

persimmon can be used to alleviate chronic shank ulcers, which was typically a result of varicose veins in the lower limbs and traditionally caused by 'stasis of the collaterals'. In 1841, the *Bencao Zaixin* further elaborated on the use of *D. kaki L.* for chronic cough, bloody vomit, and thirst. Clinically, the application of *D. kaki L.* has been reported to stop bleeding, particularly internal bleeding due to tuberculosis, peptic ulcers, haemoptysis, uterine bleeding, haemorrhoids, and epistaxis. The use of *D. kaki L.* was not exclusive to Chinese culture; it was also used in Japan as a traditional medicine under the name of *kaki-yo* for several clinical indications, including homeostatic, diuretic, laxative, and hypotensive properties, with no appreciable side effects.

NXQ is a standardised, patented herbal extract developed by Hutchison Whampoa Guangzhou Baiyunshan Chinese Medicine Co. Ltd (HGB), China. It was listed in *China Pharmacopoeia 2015* as an approved therapeutic drug and has been active in the Chinese market for several years. The permissible clinical indications of NXQ include cerebral atherosclerosis and coronary heart diseases with particular symptoms such as shortness of breath, palpitations, dizziness, vertigo, numbness in the limbs, chest stuffiness, and chest pain. From a TCM viewpoint, NXQ is used to treat a particular Chinese medical pattern of 'blood stagnation and dampness obstruction of the collaterals that are typically related to conditions with an apoplexy origin'. NXQ is publicly accessible in China from online stores and by prescription from TCM practitioners.

Also, several patented herbal formulations include *D. kaki* leaves. These products – primarily available in mainland China – include NXQ tablets, *Shiye Su Rong* drinks, *Fu Yan Jing* capsules, *Fen Shang* pills, and *Shingshuning Pian*. They target a range of ailments including coronary artery disease, cerebral atherosclerosis, cough, proteinuria, and inflammatory gynaecological disorders. Most patented CHM products contain a mixture of herbal extracts,

however, NXQ is a product that only contains *D. kaki L.* extract. Whilst patented herbal medicines are commonly used in clinical practice throughout China, the evaluation of their efficacy, safety, and mechanisms of action remain a high priority in matching the modern standards of an evidence-based portfolio.

Among the varieties of persimmon species*, D. kaki* is one of the most investigated herbal resources in recent literature. It is traditionally used in Asian countries for alleviating multiple ailments, which can involve the fruit itself (*D. kaki*), the sepal of the fruit (*D. kaki* thund.), or the leaf of the plant (*D. kaki L.*). More than 30 potentially bioactive compounds have been isolated from the leaves of the *Diospyros* species, which can be divided into five main chemical groups: acids, biphenyls, flavonoids, polyphenols, and triterpenoids (Bei et al., 2004, 2005b). The *D. kaki L.* extract used to produce NXQ is standardised. NXQ is chemically comprised of more than 50% organic acids (including protocatcchuic acid, benzoic acid, and scopoletin) and 26.56% flavonoids, including quercetin and its glucosides (hyperin, isoquercetin, kaempferol, and astragalin) (Bei et al., 2004). Quercetin and kaempferol are known to be the two most important and abundant compounds found within NXQ. Zhang and colleagues (2019) conducted HPLC/MS evaluation of NXQ and identified eight effective constituents, including quercetin (100.0 mg/g), kaempferol (33.3 mg/g), kaempferol-3O-B-D-glucopyranoside (28.7 mg/g), kaempferol-3-O-B-galactopyranoside (76.5 mg/g), protocatechuate (66.2 mg/g), hyperoside (97.9 mg/g), myricetin (5.6 mg/g), and naringenin (0.8 mg/g), in crude drug (P. Zhang et al., 2019). Liang and colleagues, who evaluated the consistency of quercetin and kaempferol content in 10 batches of NXQ tablets using HPLC (J. Liang et al., 2012), confirmed that the two compounds were consistently detected with an average range of 3.850 – 5.578 mg/g of quercetin and 5.095 – 7.947 mg/g of kaempferol. Qin et al. further confirmed that quercetin and kaempferol are the two most abundant compounds found within the NXQ

formula responsible for the protection of cerebral infarct in cerebral ischaemic mice (Qin et al., 2011).

Flavonoids, particularly kaempferol and quercetin, are the main compounds thought to be responsible for the therapeutic action of NXQ; however, glycosides, such as astragalin, rutin, isoquercetin, and hyperin, have also been identified. Flavonoids are thought to have several pharmacological effects that benefit human health. It is reported that flavonoids possess anti-atherosclerotic effects, mainly due to their anti-oxidative properties, by preventing oxidation of low-density lipoprotein (LDL) and endothelial wall injury (Holiman, Hertog, & Katan, 1996). In addition, anti-inflammatory effects have also been reported (Hertog, Feskens, Kromhout, Hollman, & Katan, 1993; Holiman et al., 1996). Quercetin, for example, can inhibit cyclooxygenase and lipoxygenase activities, thus, diminishing the formation of inflammatory metabolites, such as arachidonic acids (Robak & Gryglewski, 1996). Other effects, such as anti-tumour, anti-thrombogenic, anti-viral, and anti-bacterial effects, have also been reported (Agrawal, 2011).

3.3.1.1 Quercetin

Quercetin (3,3',4',5,7-pentahydroxyflavone), a polyphenolic compound found abundantly in dietary resources, is known for its health benefits as a natural flavonoid. It is one of the main bioactive compounds found in NXQ that contributes to its therapeutic effects. Quercetin is potent as a dietary supplement; in fact, 10 mg of quercetin accounts for ~50% of the total flavanols in an individual's dietary intake recommendation (Kawabata, Mukai, & Ishisaka, 2015).

Research has shown that quercetin has beneficial effects on CVDs and diabetes through numerous mechanisms such as antioxidant and anti-inflammatory signalling pathways (Costa,

Garrick, Roquè, & Pellacani, 2016; Formica & Regelson, 1995). In a clinical trial, quercetin supplementation (150 mg/day; 6 weeks) reduced systolic blood pressure and serum oxidised LDL in overweight subjects with a high CVD risk phenotype (Egert et al., 2009). Similarly, quercetin reduced blood pressure, serum glucose, LDL, and total cholesterol in high-salt, diet-induced, hypertensive rats (Olaleye, Crown, Akinmoladun, & Akindahunsi, 2014). In a meta-analysis, quercetin was found to be effective in reducing blood pressure in both diastolic and systolic figures and induced superior effects on diabetic subjects (Serban et al., 2016). In addition, quercetin was found to have anti-inflammatory effects by inhibiting cyclooxygenase and lipoxygenase activities, thus, diminishing the formation of inflammatory metabolites, such as arachidonic acids (Robak & Gryglewski, 1996).

3.3.1.2 Kaempferol

Kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) is a polyphenolic compound that naturally occurs in dietary resources like tea, vegetables, and fruits. Kaempferol possesses multiple therapeutic benefits similar to quercetin, including antioxidant and anti-inflammation (A. Y. Chen & Chen, 2013; Seifried, Anderson, Fisher, & Milner, 2007). Studies have found that kaempferol decreases lipopolysaccharide (LPS)-induced tumour necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1) expression by increasing the number of macrophages that suppresses TNF-α-mediated translocation of NF-κB p65 to the nucleus (Lin et al., 2011). Kaempferol has shown some promising effects on various chronic inflammatory diseases, such as intravertebral disc degeneration, osteoarthritis, colitis, mastitis, and rheumatoid arthritis (Ren et al., 2019). It is believed that kaempferol maintains a relatively important role during chronic inflammation by inhibiting LPS-induced decreases in chondrogenic markers, such as SOX-9, collagen II, and aggrecan,

thereby, preventing tissue breakdown (Jun Zhu et al., 2017). In addition, kaempferol also reduces the IL-1B-stimulated formation of PGE2 and NO in a concentration-dependent manner and upregulates the expression of iNOS and COX-2 (H. Zhao et al., 2014). These cellular actions inhibit the NF-κB pathway and achieve an anti-arthritis effect by retarding the progression of osteoarthritis.

Furthermore, kaempferol also prevents the development of mastitis by decreasing myeloperoxidase (MPO), IL-6, and ANGPTL2 expression. Kaempferol has also shown effects in inhibiting TNF-α-induced mitogen-activated protein kinase (MAPK) activation and blocking the MAPK pathway without affecting the expression of TNF-α receptor (Xiao, Sui, Lu, & Sun, 2018).

Kaempferol has been reported for its anti-cancer effects by inhibiting angiogenesis. Li et al. reported that kaempferol inhibited the invasion of breast cancer cells by blocking protein kinase C (PKC/MAPL/activator protein-1AP-1 cascade and subsequent expression and activity of MMPs) (2017). Kaempferol can also significantly inhibit vascular endothelial growth factor (VEGF)-induced HUVEC proliferation (Xu, Zhao, Peng, Xie, & Liu, 2017). Li et al. further indicated that kaempferol reduces the expression of estrogen receptor, IGF-1, and VEGF precursor at the mRNA and protein level by inhibiting the proliferation of human uterine fibroid cells in vitro (Y. Li, Ding, & Wu, 2016).

The anti-inflammatory and anti-oxidative effects of kaempferol also benefit vascular protection. Kaempferol can inhibit pro-inflammatory response mediated by LPS or high mobility group box 1 (HMGB1) by increasing barrier integrity and inhibiting the expression of cell adhesion molecules (Kim, Ku, & Bae, 2012). Kaempferol can lower plasma LDL and reduce the risk of CVD, as well as protect cells from apoptosis (J. Che et al., 2017).

Synergism between flavanones

Besides the widely known benefits of quercetin and kaempferol, studies have suggested that interactions exist between flavonoids, which may lead to a synergistic reaction (Hidalgo, Sánchez-Moreno, & de Pascual-Teresa, 2010; Lila, 2008; Sonam & Guleria, 2017). While many believe that consuming quercetin and kaempferol individually may impart health benefits, few studies have looked into the synergistic ratio of the two compounds that may be present in a single herbal substance. Such a ratio from natural sources may be valuable for understanding the potential of additive or synergistic effects of the two bioactive compounds.

The effects of natural flavanones on human health are complex. While most studies have examined the potential bioactive compounds responsible for the effects of a natural product, few have studied the synergisms between the bioactive compounds found within the same herbal source. Quercetin and kaempferol each possess strong effects in ROS scavenging and anti-inflammatory effects, however, varied outcomes have been observed between different flavonoid compounds. Hidalgo et al. investigated the flavonoid-flavonoid interaction on antioxidant activities using a DPPH assay and found that when kaempferol was paired with myricetin, quercetin, or quercetin-3-glucoside, a significant increase in antioxidant activity was observed. While the ratio of quercetin and kaempferol used in their study was 1:1, it provided insight that flavonoids synergistically interact, which may yield improved therapeutic outcomes (2010).

In summary, synergism is observed between flavonoid interactions. However, the ratio between various compounds has yet to be determined for optimal potency.

3.3.2 Clinical evidence

Several clinical trials have been conducted in China recently assessing the clinical effects of NXQ. One study assessed NXQ as an adjunct therapy to standard dehydration, anti-hyperglycaemic and anti-lipidaemic treatment for acute ischaemic stroke in 35 Type 2 diabetic participants (B. Tang et al., 2012). The results of this study showed that NXQ positively improved blood rheology by acting on C-reactive protein (C-RP) and blood lipids. Lipids and lipoproteins modulate haemostasis by altering rheological factors. Increasing the cholesterol concentration in human erythrocyte membranes *in vitro* increases erythrocyte rigidity and blood viscosity, and loading of cholesterol into erythrocytes *in vitro* increases their surface area and decreases their deformability (Machida et al., 2010; Yagi et al., 2016). Elevation of C-RP typically indicates that pro-inflammatory factors, including tumour necrosis factor (TNF)-α and interleukin-1β, are in excessive quantities, which can be deleterious to neurons. (TNF)-α stimulates the production of fibrinogen, which affects blood rheology and viscosity (Marioni et al., 2009). The authors suggested that the active component flavonoids from *D. kaki L.* extract possess anti-ischaemic effects through promoting cerebral and cardiac blood flow and neuroprotective effects through radical scavenging and lipid lowering (Machida et al., 2010).

Another study conducted by Wang et al. (2012) assessed NXQ in combination with acute management therapy (mannitol and glycerol fructose dehydration) in comparison to acute management therapies alone in patients with cerebro-cardiac syndrome caused by acute stroke. A total of 204 participants were included; 130 suffered from ischaemic stroke, 61 from intracranial haemorrhages and 13 from sub-cranial arachnoid haemorrhages. A standard dose of NXQ was given at the acute phase in addition to acute management therapy protocols for a continuous four-week period. The results suggested that the NXQ group had superior results

in improving cardiac function recovery, including arrhythmia, changes in ST-T wave and abnormal cardiac enzymes in comparison to the control group (A. Wang et al., 2012).

Chen, Shao and Cheng (2012) conducted a clinical trial assessing NXQ as an adjunct to low-molecular weight heparin in comparison to heparin alone in 160 patients with cerebral transient ischaemic attack. Results suggested that both groups had prolonged partial thromboplastin time (PTT) and active partial thromboplastin time (APTT); however, NXQ treatment showed a significantly longer duration in both outcome measures when compared to the control group. In addition, no significant adverse events were identified.

Although few clinical studies focused directly on cerebral ischaemic stroke, a number of clinical trials were conducted assessing the benefits of NXQ for ischaemic heart disease and angina, which share a similar underlying pathological process to ischaemic stroke, namely atherosclerosis. A study conducted by Cai and Yang (2001) assessed the effects of NXQ in 60 participants (48 with atherosclerosis and 12 with coronary artery disease-induced angina). The study showed that NXQ significantly lowered blood lipids and improved symptoms such as vertigo and headaches. A recent study by Wu, Wu and Zheng (2013) comparing NXQ against a standard vasodilator in 90 patients with coronary artery disease reveals similar positive results. The authors find that NXQ treatment significantly decreased blood lipid levels and reduced cardiac stress and cardiac oxygen consumption, and improved oxygen supply to cardiac muscle through aorta dilation. In another study, clinical effects of NXQ as an adjunct treatment were compared to a standard angina treatment (including nitrates, aspirin, beta blockers, angiotensin-converting enzyme inhibitors and lipid-lowering drugs) alone in 120 elderly patients with unstable angina. The results suggested that the NXQ group had significant improvements in symptoms of angina and a reduction in blood lipid levels compared to standard treatment alone (Xiong, Tang, & Zhou, 2013).

In summary, the preliminary clinical evidence suggests that NXQ is effective for lipid reduction and may also improve cardiac blood flow as well as relieving clinical symptoms such as angina. No severe adverse events were identified in study conducted by Xiong et al. (2013). However, the methodology of these trials did not appear to be rigorous or precise and the samples in these studies were small. Of all the trials, only Tang et al. (2012) reported a randomisation method and statistical power calculation. Apart from Wang et al. (2012), no study reported the treatment duration for either intervention or control groups. Furthermore, adverse events were not reported in the studies by Cai and Yang (2001), Wu et al. (2013) and Wang et al. (2012). At present, there are no trials that have evaluated the neuroprotective effects of NXQ with standardised outcome measures.

3.3.3 Preclinical evidence

Previous research has demonstrated numerous pharmacological effects of *D. kaki L.* extract, such as improved cardiac and cerebral blood flow, anti-hypertensive, lipid-lowering and radical-salvaging capabilities (S. Cao, Zhang, Bai, Wang, & Miao, 2012). A study conducted by Xin, Feng and Yao (2007) investigated the effects of intravenous injections of *D. kaki L.* flavonoids in rabbits and found a 30–40 % increase in aortic circulation as well as significant dilation of the veins of the ears. These results were also supported by the findings of Zhang, Wang and Xiao (2004), which showed that the leaves of *D. kaki* can improve overall circulation in anaesthetised dogs and can reduce oxygen consumption of cardiac muscles. Ou, Bei and Lai (2003) found that the total flavonoid count can significantly inhibit reperfusion-induced cell apoptosis. Furthermore, Ou, Liu and Bei (2004) suggested that *D. kaki L.* can inhibit fibrin adhesion (scar tissues) on the adventitia stimulated by factors such as advanced glycation end-products (AGEs) and advanced oxidation protein products (AOPPs).

In relation to the cerebrovascular system, Cao et al. (2012) studied the fibrinolytic function in an ischaemic brain injury model and found that *D. kaki L.* flavonoids can increase tissue plasminogen activator (t-PA) which is involved in the breakdown of blood clots and reduction of plasminogen activator inhibitor-1 (PAI-1) levels, thereby improving the outcome of thrombotic-related cerebral ischaemia. Cao et al. (2012) further demonstrated that the von Willebrand Factor (vWF), a blood glycoprotein involved in haemostasis, is reduced in ischaemic brain injury rats subjected to treatment with *D. kaki L.* flavonoids. Reduction of vWF has been associated with the resolution of thrombosis during an ischaemic attack and restoration of damaged endothelial cells. Cao et al. (2012) propose that *D. kaki L.* flavonoids could reduce positive signalling of Intercellular Adhesion Molecule-1 (ICAM-1), a immunoglobulin molecule associated with the release of inflammatory factors, and thereby act against atherosclerosis and reduce sustained haemostasis.

Additionally, Bei, Li et al. (2009) propose that the potential neuroprotective effects of NXQ and *D. kaki L.* flavonoids could activate L-type Ca^{2+} channels, thus modulating intracellular Ca^{2+} level to protect neurons in the hippocampus. This action is complemented by the anti-oxidative ability of NXQ and *D. kaki L.*, which positively inhibits ROS (including hydrogen peroxide) and therefore could reduce neuron damage.

Kameda et al. (1987) first demonstrated the anti-hypertensive effects of *D. kaki L.* in hypertensive rats. The mechanism is thought be through inhibition of angiotensin-converting enzyme activity. Tan, Lin and Zhang (2009) further investigated these anti-hypertensive effects and observed an increase in plasma nitric oxide (NO) and reduced over-production of platelets by essential thrombocytosis (ET) and reduced angiotensin II in rats. The results suggested that *D. kaki L.* flavonoids have potential to enhance endogenous vasodilators and modulate endogenous vasoconstrictors.

The preliminary evidence suggests there are potential therapeutic benefits of NXQ for ischaemic stroke; however, the precise mechanism remain unclear.

3.4 Summary

In summary, over the past few decades, there has been a growing body of literature reporting on the phytochemistry and pharmacological activities of constituents isolated from *D. kaki* leaves. However, the benefits for human health are still unclear due to lack of well-designed randomised control trials. There is some available Chinese-language literature, but its content typically relates to traditional Chinese medicine with lack of scientific interpretation.

NXQ, a single standardised patent extract of *D. kaki L.*, possesses potential therapeutic benefit for ischaemic stroke. Early evidence suggests that the bioactive components of *D. kaki L.*, such as flavonoids, could benefit ischaemic conditions such as ischaemic stroke. However, the exact role and mechanisms of action of NXQ on stroke are not fully understood. Therefore, more research is needed to evaluate the potential vascular and neurological benefits of NXQ in stroke.

Chapter 4. Chemical Analysis of NXQ Extract and Key Bioactive Components

4.1 Introduction

4.1.1 Standardisation and quality control of herbal medicine

Herbal medicine (HM) in general refers to medicinal products containing botanical, animal or mineral materials obtained or processed from their original form (Loew & Kaszkin, 2002). HMs can be used in single form or in combinations. In Chinese herbal medicine (CHM), herbal preparations are typically prescribed and prepared in combination following the traditional Chinese medicine synergistic and antagonistic principles known as *Jun Chen Zuo Shi*. In contrast to chemically defined pharmaceutical drugs, HMs typically contain complex mixtures of chemicals that may have active, synergistic, complementary, antagonistic or even toxic effects. This also applies to single herbs, which contain complex mixtures of chemicals. For example, NXQ, a patented extract from *Diospyros kaki L.* contains a variety of flavonoids such as quercetin and kaemferol, glucosides such as astragalin, rutin and hyperin, and some phenolic acids including protocatechuic, benzoic, salicylic and syringic acids (K. Zhang et al., 1983).

The active components of most HMs are secondary plant metabolites, which are compounds that may not be essential for plant growth but are thought to play a role in plant defence and survival (Stamp, 2003). The amount of secondary metabolites in HMs can vary depending on growing conditions (such as soil and climate), harvest season and post-harvest treatment (Y. Liang, Xie, P. Chan, K., 2004; H. Wagner, 1999). Once ingested, some secondary plant metabolites may be metabolised into active compounds, a process called bioactivation (Fang et al., 2011). The complex chemical composition and potential interactions of bioactive components in herbal preparations create challenges in evaluating and assessing their chemical consistency and clinical efficacy.

Quality control is a critical process for standardising a medicine and ensuring batch-to-batch consistency of its active component(s). Unlike pharmaceutical medicines, which contain a simple chemical entity, HM preparations often contain a large number of bioactive components. This has made standardisation of herbal extracts a very challenging task.

Standardisation is the process whereby a medicine is prepared in a way that ensures a consistent concentration of one or more analytes (usually a bioactive compound found in high concentration) regardless of variation in the concentration of these substances in the raw material from which the extract is prepared. In HM, it is a common practice to select several representative compounds that are either unique in the herb or appear in abundance. This approach is problematic, as the compounds selected for standardisation/quality control purposes may not be consistent with those bioactive components responsible for the therapeutic effects of the herbal medicine (Ong, 2004; Yadav, 2008).

NXQ has already been previously standardised by the herbal manufacturer. In the present research, the objective was not to full development and validation of analytical methods for key bioactive compounds for quality control purposes. Instead, the aim is to identify and quantify the key bioactive components, especially quercetin and kaempferol, in the extract used for the studies described in Chapters 5 and 6 to determine the quercetin:keaempferol ratio.

4.1.2 Physical description of D. kaki leaf

D. kaki belongs to the family Ebenaceae. Native to China, where it has been cultivated for centuries and from where it spread to Korea and Japan, it was introduced into Europe in 1789 and into the USA in the 1800s. It is typically known as Japanese or Chinese persimmon and the leaves have been documented for their medicinal effects as early as the fifteenth century

in China (Xie, Xie, Xu, & Yang, 2015). The deciduous tree is 5–10 metres tall, similar in width, and has drooping branches and upright to round-headed leaves. The appearance of the leaves varies from simple elliptic-ovate to oblong or obovate, averaging 6–18 cm in length. The base of the leaf is usually broad or wedge shaped, leathery, glossy dark green above and lighter green and pubescent below. During autumn, the leaves are coloured a vivid yellow-orange and red (Celik & Ercisli, 2008; Xie et al., 2015).

D. kaki is well known by its fruit, an ovoid to slightly flattened sphere 3.5–7 cm long with orange and bright yellow colours (Xie et al., 2015).

Figure 4.1: Fresh persimmon (D. kaki) leaf (Celik & Ercisli, 2008)

Figure 4.2: Persimmon tree (Celik & Ercisli, 2008)

4.1.3 Traditional uses of D. kaki leaf

The traditional use of persimmon leaves was first recorded in the Chinese ancient medical book *Dinnan Bencao*, which was written by Lan Mao in 1436 in the Ming Dynasty. The first recorded use of persimmon leaves as medicine is for chronic ulceration of the lower legs (Lan,1436). In 1841, during the Qing Dynasty, the book of Chinese *Materia Medica* named *Bencao Zaixin* stated that persimmon leaves could be used to treat cough and haematemesis, increase salivation and slake thirst (Ye, 1933). Later it was stated in *Fei Lei Cao Yao Xing*, a medical book written in the late Qing Dynasty, that persimmon leaf could be used to treat the disease *fei zhang*, also known as lung distension in traditional Chinese medicine (Anon, 1911). It was not until the early 1990s that the effects of persimmon leaves were identified through pharmacological analysis. The report of Chinese herbal medicines, *Guangxi Standards* (published in 1990), suggests that the persimmon leaves were effective for cough, internal haemorrhage, hypertension, cerebral arteriosclerosis and coronary disease. In addition, it is also used traditionally to promote maternal health (Han, 2002). The standardised patent extract of persimmon leaf named NXQ was recorded in *Chinese Pharmacopoeia 2010* as a patented and approved TCM drug for the use of atherosclerosis (Chinese Pharmacopoeia Commission, 2012).

In Japan and Korea, persimmon leaf also has been used in health beverages for centuries (Funayama & Hikino, 1979; Sakanaka, Tachibana, & Okada, 2005). It has often been made as tea and is popular among Chinese and Japanese for its potential to reduce blood pressure, eliminate cholesterol and prevent melanin accumulation. In Japan, it was also regarded as an anti-ageing tea as it is rich in vitamin C and active nutrients (Sakanaka et al., 2005).

4.1.4 Chemical substances in D. kaki leaf

To date, numerous chemical constituents have been identified in the extract of *D. kaki. L*, including flavonoids, terpenoids, naphthoquinine, naphothol, coumarin, sterol, organic acids, fatty acids and volatile components.

4.1.4.1 Flavonoids

Flavonoids are the main compounds thought to be responsible for the therapeutic action of *D. kaki L*., with kaempferol and quercetin the main flavonoids. Glycosides such as astragalin, rutin, isoquercetin and hyperin have also been identified. The structures of different flavonoids identified in *D. kaki L.* are shown in Figure 4.3 and Table 2.

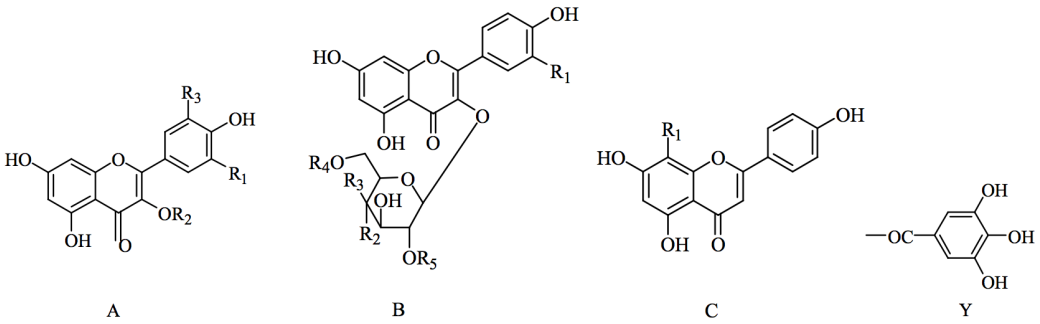


Figure 4.3: Nucleus structures and substituents of flavonoids

Table 2: Main flavonoids in Diospyros kaki L (Zhou, 2014)

	Compounds	*Nucleus structure*	*Substitutes*					*References*
			R1	R2	R3	R4	R5	
1	Rutin	A	H	Glc (4→1) Rha	OH			(G Chen, Xue, Xu, & Zhang, 2007)
2	kaempferol	A	H	H	H			(G Chen et al., 2007)
3	quercetin	A	H	H	OH			(G Chen et al., 2007)
4	annulatin	A	OH	CH3	OH			(G Chen et al., 2007)
5	isoquercitrin	A	OH	Glc	H			(G. Chen, 2003; G Chen et al., 2007; Kameda et al., 1987; Xue et al., 2011)
6	myricetin	A	OH	H	OH			(G Chen et al., 2007)

7	kaempferol-3-O-α-L-rhamnopyranoside	A	H	Rha	H			(Guang Chen, Wei, Huang, & Sun, 2009)
8	myricetin-3-O-α-L-rhamnopyranoside	A	OH	Rha	OH			(Guang Chen et al., 2009)
9	myricetin-3-O-β-D-glucopyranoside	A	OH	Glc	OH			(Guang Chen et al., 2009)
10	quercitrin	A	H	Arab	OH			(Gafner, Chapuis, Msonthi, & Hostettmann, 1987)
11	astragalin	B	H	OH	H	H	H	(Kameda et al., 1987)
12	kaempferol-3-O-(2″-galloyl) -glucoside	B	H	OH	H	H	Y	(Kameda et al., 1987; Xue et al., 2011)
13	quercetin-3-O-(2″-galloyl) -glucoside	B	OH	OH	H	H	Y	(Kameda et al., 1987; Xue et al., 2011)
14	kaempferol-3-O-β-D-galactopyranoside	B	H	H	OH	H	H	(G. Chen, 2003; Guang Chen, Lu, Wang, Yamashita, Manabe, Meng, et al., 2002; G Chen et al., 2007; Xue et al., 2011)
15	kaempferol-3-O-β-D-glucopyranoside	B	H	OH	H	H	H	(Guang Chen, Lu, Wang, Yamashita, Manabe, Meng, et al., 2002; G Chen et al., 2007; Kameda et al., 1987; Xue et al., 2011)
16	isorhamnetin-3-O-β-D-glucopyranoside	B	OCH3	H	H	H	H	(G. Chen, 2003; Guang Chen, Lu, Wang, Yamashita, Manabe, Meng, et al., 2002; Guang Chen, Xu, Wang, & Zhang, 2005)
17	quercetin-3-O-β-D-galactopyranoside	B	OH	OH	OH	H	H	(G. Chen, 2003; Guang Chen, Lu, Wang, Yamashita, Manabe, Meng, et al., 2002; G Chen et al., 2007; Gafner et al., 1987)
18	quercetin-3-O-β-D-glucopyranosyl-(6→1)-α-L rhamnopyranoside	B	OH	OH	H	Rha	H	(Guang Chen, Lu, Wang, Yamashita, Manabe, Meng, et al., 2002)
19	hyperoside	B	OH	H	OH	H	H	(Xue et al., 2011)
20	vitexin	C	Glc					(G. Chen, 2003; Guang Chen et al., 2005)
21	2″-O-rhamnosyl-vitexin	C	Glc (2→1) Rha					(G. Chen, 2003; Guang Chen et al., 2009; Guang Chen et al., 2005)
22	8-C-[α-L-rhamnopyranosyl-(1→4)]-a-D glucopyranosylapigenin	C	Glc (4→1) Rha					(Guang Chen et al., 2009)

Flavonoids are reported to have several pharmacological effects that benefit human health in a number of ways. It is reported that flavonoids possess anti-atherosclerotic effects, mainly due to their anti-oxidative properties that likely prevent oxidation of low-density lipids and endothelial wall injury, thereby ameliorating atherosclerotic changes. In addition, anti-inflammatory effects are also reported (Hertog et al., 1993; Holiman et al., 1996). Quercetin,

for example, can inhibit cyclooxygenase and lipoxygenase activities, thus diminishing the formation of inflammatory metabolites (Robak & Gryglewski, 1996). Other effects, such as anti-tumour, anti-thrombogenic, anti-viral and anti-bacterial effects, have also been reported (Agrawal, 2011).

Chrysontemin is another compound isolated from *D. kaki L.* (Xie et al., 2015). It has showed a moderate inhibitory effect on tyrosinase, a copper-containing enzyme that catalyses the production of melanin and other pigments from tyrosine. Chrysotemin has also been shown to inhibit tumour promoter-induced carcinogenesis and tumour metastasis (Xie et al., 2015). Other components found in *D. kaki* leaves, such as trifolin and hyperoside, demonstrate anti-fungal properties. Astragalin demonstrates anti-allergic activities and inhibits angiotensin-converting enzymes, thereby regulating blood pressure by preventing vasoconstriction (Xue et al., 2011).

4.1.4.2 Terpenoids

Triterpenoid compounds are also found in abundance in *D. kaki L*. Most of these terpenoids present in oleanane, lupine and ursan (see Figure 4.4 and Table 3). Terpenoids possess multiple pharmacological properties; for instance, ursolic acid, 19-hydroxy ursolic acid and 19, 24-dihydroxy ursolic acid isolated from *D. kaki L.* have been shown to suppress stimulus-induced superoxide generation and tyrosyl phosphorylation, which regulates the transduction of the enzymatic activity of tyrosin kinase (Guang Chen, Lu, Wang, Yamashita, Manabe, Meng, et al., 2002).

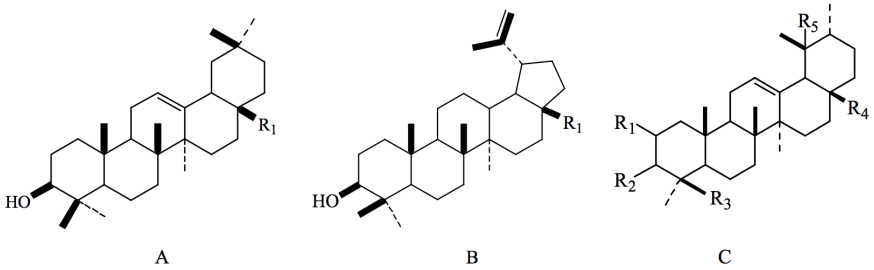


Figure 4.4: Nucleus structures of triterpenes

Table 3: Main triterpenes in Diospyros kaki leaves (Zhou, 2014)

	Compounds	*Nucleus structure*	*Substitutes*					*References*
			R1	R2	R3	R4	R5	
1	β-amyrin	A	CH3					(G. Chen, 2003; HIGA, Ogihara, & Yogi, 1998)
2	oleanolic acid	A	COOH					(G. Chen, 2003; G. Chen, Sha, Ching., 2000; HIGA et al., 1998)
3	betulin	A	CH_2OH					(HIGA et al., 1998; Uc-Cachón et al., 2013)
4	betulinaldehyde	A	CHO					(Uc-Cachón et al., 2013)
5	betulinic acid	B	COOH					(G. Chen, 2003; G. Chen, Sha, Ching., 2000; Guang Chen et al., 2005; HIGA et al., 1998)
6	lupeol	B	CH_3					(G. Chen, 2003; HIGA et al., 1998; Uc-Cachón et al., 2013)
7	α-amyrin	C	H	OH (β)	CH3	CH3	H	(G. Chen, 2003; Guang Chen, Lu, Wang, Yamashita, Manabe, Xu, et al., 2002; HIGA et al., 1998)
8	uvaol	C		OH (β)	CH3	Ch2OH	H	(G. Chen, 2003; Guang Chen, Lu, Wang, Yamashita, Manabe, Xu, et al., 2002)
9	ursolic acid	C		OH (β)	CH3	COOH	H	(G. Chen, 2003; Guang Chen, Lu, Wang, Yamashita, Manabe, Xu, et al., 2002)
10	pomolic acid	C		OH (β)	CH3	COOH	OH (α)	(G. Chen, 2003; Guang Chen, Lu, Wang, Yamashita, Manabe, Xu, et al., 2002; G. Chen, Sha, Ching., 2000)
11	19α, 24-dihydroxy ursolic acid	C		OH (β)	CH2OH	COOH	OH (α)	(G. Chen, 2003; Guang Chen, Lu, Wang, Yamashita, Manabe, Xu, et al., 2002; G. Chen, Sha, Ching., 2000)
12	barbinervic acid	C		OH (α)	CH2OH	COOH	OH (α)	(G. Chen, 2003)
13	rosamutin	C	OH (α)	OH (β)	CH3	COOGlc		(G. Chen, 2003)
14	kakisaponin A	C		OH (α)	CH2OH (β)	COOGlc	OH (α)	(G Chen et al., 2007)

4.1.4.3 Other compounds

Other compounds, such as naphthoquinone and naphthol, coumarin, sterol, organic acids, fatty acids, and volatile oils, were also identified in *D. kaki L.* extracts (Zhou, 2014) along with resins, polysaccharides, chlorophyll, carotene, kryptoanthin, cellulose, hemicelluloses, lignin, and trace elements of amino acids (Xie et al., 2015).

4.2 Aims

This study aims to evaluate the key bioactive components (especially flavonoids) of the standardised patent extracts of *D. kaki L*.

The study aims to:

— identify and quantify the key bioactive constituents of the NXQ commercial extract

— determine the ratio of quercetin and kaempferol in the extract.

4.3 Methods

4.3.1Materials

4.3.1.1 Standards availability

For an analytical method to be useful and have wide applicability, the analytes selected for quality control should be commercially available as well as being of reasonable cost. The selection of analytes is therefore a balance of commercial practicality and the desire to characterise as many analytes as possible to be more confident that the analytical results accurately reflect the functional quality of the herb.

Though the flavonoids are believed to be the key bioactive components of NXQ extract (W. Li et al., 2011), other components may also contribute to NXQ's therapeutic effects. Given the main purpose of this study was to determine the ratio of two most prominent flavonoids,

quercetin and kaempferol, in the standardised *D. kaki L.* extract, only five other bioactive compounds – for which the reference standard could be commercially obtained and analytical methods had previously been developed by Kazzem (2012) – were selected

The standards and reagents used were obtained from reputable sources with claimed purity. Kaempferol (99%), quercetin (99%), 2-furic acid (98%) and protocatechuic acid (98%) were purchased from Sigma (Sydney, Australia). Astragalin (98%), hyperin (98%) and isoquercetin (98%) were purchased from Biopurify (Chengdu, China).

4.3.1.2 Source of the D. kaki L. extract

The NXQ extract of *D. kaki L.* was supplied by Hutchison Whampoa Guangzhou Baiyunshan Chinese Medicine Co. Ltd. (China).

4.3.2 Sample stock and standards preparation

The NXQ extract was prepared in triplicate and dissolved by sonication in methanol for 60 minutes at 10mg/mL. The stock solutions of standards, quercetin, kaempferol, astragalin, isoquercetin, hyperin, protocatechuic acid and 2-furic acid were prepared to 1mg/10mL in methanol. For all in one stock of standards, a mixture standard was produced containing 10mg of each of the 7 compounds in a 100 mL methanol.

The sample and mixed standard were filtered with 0.45µm PTFE. The standard mixture was diluted to 100%, 75%, 50%, 25%, 20%, 5%, 1%, 0.1% and 0.01%.

4.3.3 HPLC system

High-performance liquid chromatography photodiode array detection (HPLC-PDA) was used to profile the phytochemical composition of the *D. kaki L.* extracts. The HPLC-PDA analysis was performed on a Shimadzu UFLC system (Shimadzu, Australia) comprising a LC-30AD pump,

SIL-30ACHT auto sampler, SPD-M20A PDA detector and DGU-20A5 inline solvent degasser. The system was controlled using Class-VP 7.4SP4 software.

4.3.4 HPLC analysis of extracts

The HPLC-PDA analysis of the extracts was performed using a Phenomenex Luna (Phenomenex, Australia) reverse-phase C18 column (46mm × 150mm I.D., 3µm) with a Phenomenex (California, USA) Security C18 guard column (20mm × 4mm, 3µm).

HPLC-PDA profiles were generated by 5µl injection. The mobile phase consisted of 0.1% (v/v) aqueous formic acid (mobile phase A) and 0.1 % (v/v) formic acid in methanol (mobile phase B). The gradient program was 16% B for 15 min with a linear increase: to 25% B at 21 min, then to 38 %B 23 minutes, then to 60% B at 35 min followed by a 7min wash at 95% B and 8 min re-equilibration at 16% B. Mobile phase flow rate was maintained at 0.8mL/min. The PDA was set to acquire absorbance data from 200 to 500nm.

4.4 Results and discussion

4.4.1 Method development

The chemical characterisation of *D. kaki L.* extract has been previously published (YIN et al., 2013; Zhou, 2014). It is outside the scope of this project to repeat the work and identify all peaks present in the NXQ extract. Instead, we focus on seven analytes, including quercetin and kaempferol, for which analytical methods were previously established in our lab. A mixture of the seven standards was composed to assess whether the known components of *D. kaki L.* were detectable in the commercial NXQ extract using the HPLC-PDA system. A method was developed to resolve all peaks of interst for quantification.

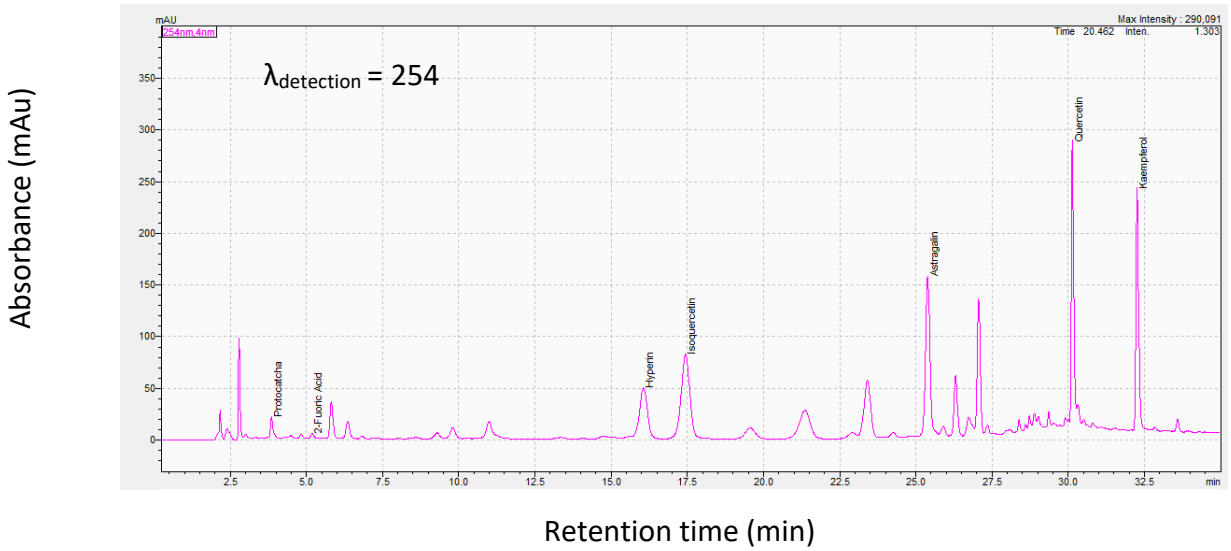


Figure 4.5: HPLC chromatogram retention times for the seven analytes analysed in NXQ at 254nm.

Figure 4.5 shows the chromatogram of the standardised patent *D. kaki L.* extract NXQ at 254nm. The two largest peaks detected was quercetin (retention time 30 min) and kaempferol (retention time 32.5 min), with other compounds also present.

Compounds have different absorption peaks (λ_{max}) and molar absorptivities (ε) at different wavelengths, depending on the chromophore in the molecule. As the patent extract of *D. kaki L.* contains different compounds with different structures, a wavelength of 254nm that detects most of the compounds was selected based on previous studies (Kazzem, 2012).

4.4.2 Accuracy

The accuracy of the analytical methods was validated by comparing the retention times and UV spectrum of the peaks in the sample to certified reference standards.

Accuracy refers to the ability of the method to produce results that are in agreement with the true values of the analyte concentration. The commonly used method to determine accuracy is by spiking the sample with known concentrations of the analyte(s) of interest. The spiking levels are usually 0 (unspiked), 50, 100 and 200 percent of the mean expected

concentration. Results from the spiked sample can be compared to the expected concentration, which indicates for both the linearity of the method and its ability to produce an accurate result. Figure 4.6 is a chromatogram of the patent standard extract of *D. kaki L.* (NXQ) overlaid with the mixed standards. Comparison of the UV spectrum of the sample and standard peaks allows the tentative identity confirmation of some peaks.

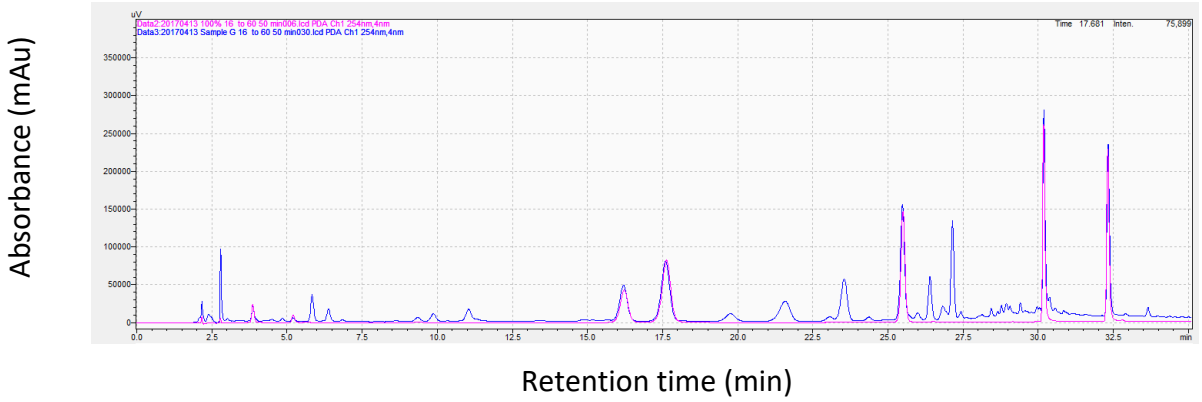


Figure 4.6: HPLC chromatogram of patent standard extract of D. kaki L. (NXQ) at 254nm. NXQ (blue), mixed standard (pink).

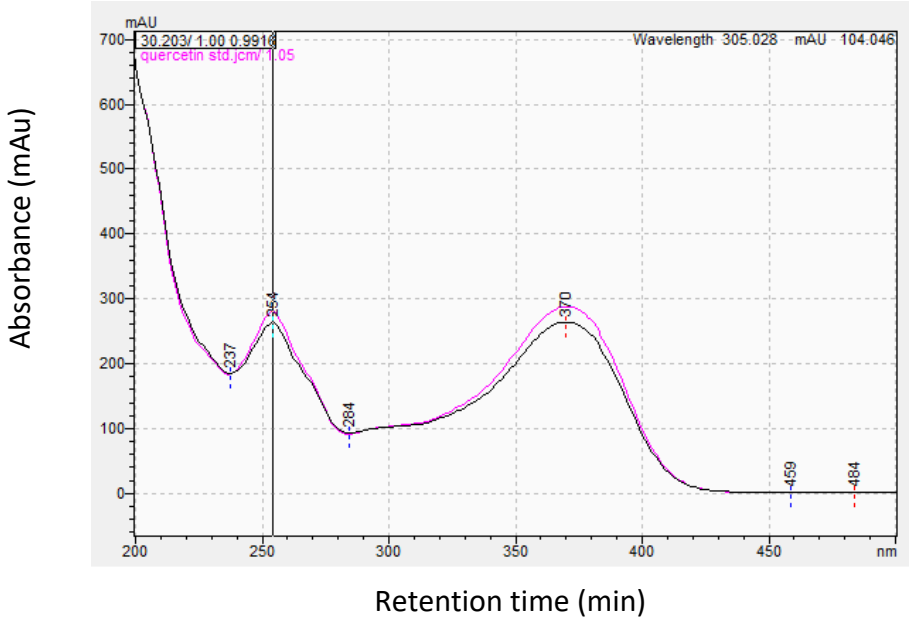


Figure 4.7: UV spectrum comparison between quercetin and peaks tentatively identified as corresponding peaks in patent standardised D. kaki extract (NXQ). The quercetin peak was Rt 30 min in the mixed standard and 30 min in NXQ (pink).

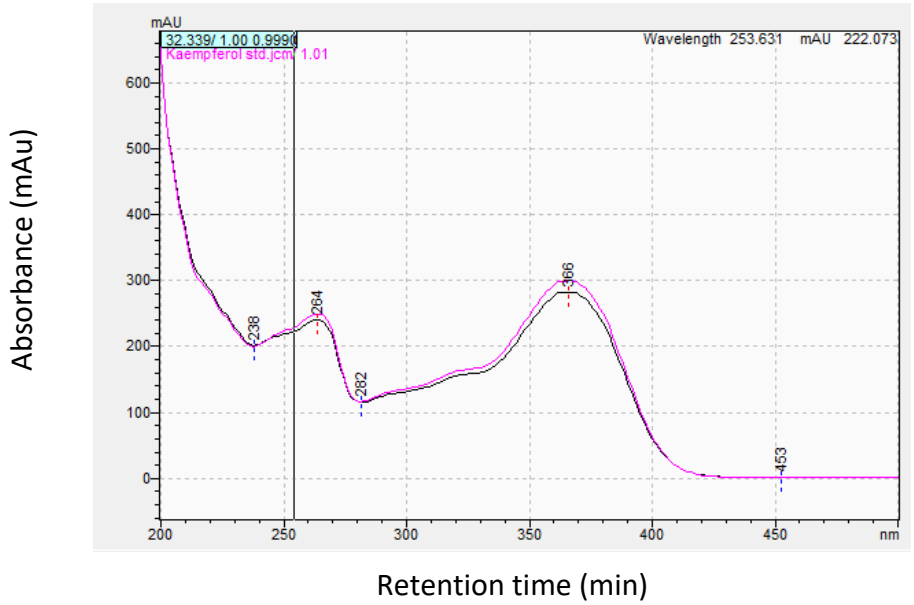


Figure 4.8: UV spectrum comparison between kaempferol and peaks tentatively identified as corresponding peaks in patent standardised D. kaki extract (NXQ). The kaempferol peak was Rt 32.3 min in the mixed standard and 32 min in NXQ (pink).

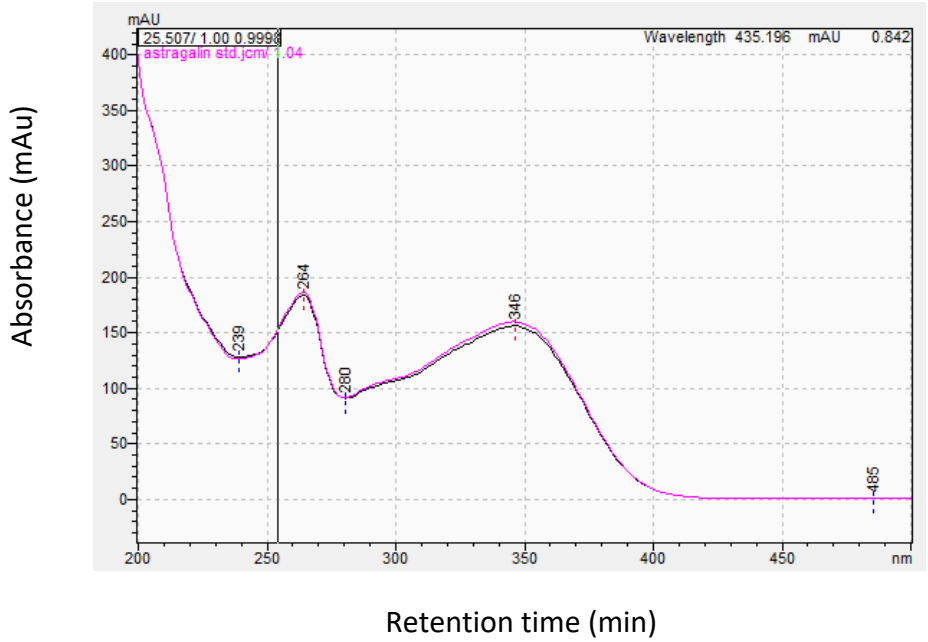


Figure 4.9: UV spectrum comparison between astragalin and peaks tentatively identified as corresponding peaks in patent standardised D. kaki extract (NXQ). The astragalin peak was Rt 25.5 min in the mixed standard and 25.5 min in NXQ (pink).

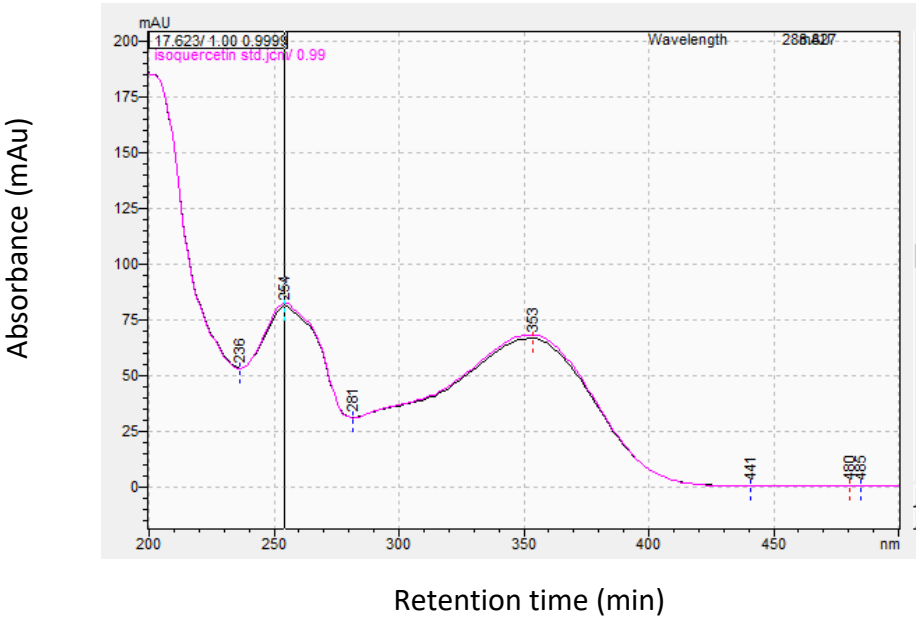


Figure 4.10: UV spectrum comparison between isoquercetin and peaks tentatively identified as corresponding peaks in patent standardised D. kaki extract (NXQ). The isoquercetin peak was Rt 17.6 min in the mixed standard and 17.6 min in NXQ (pink).

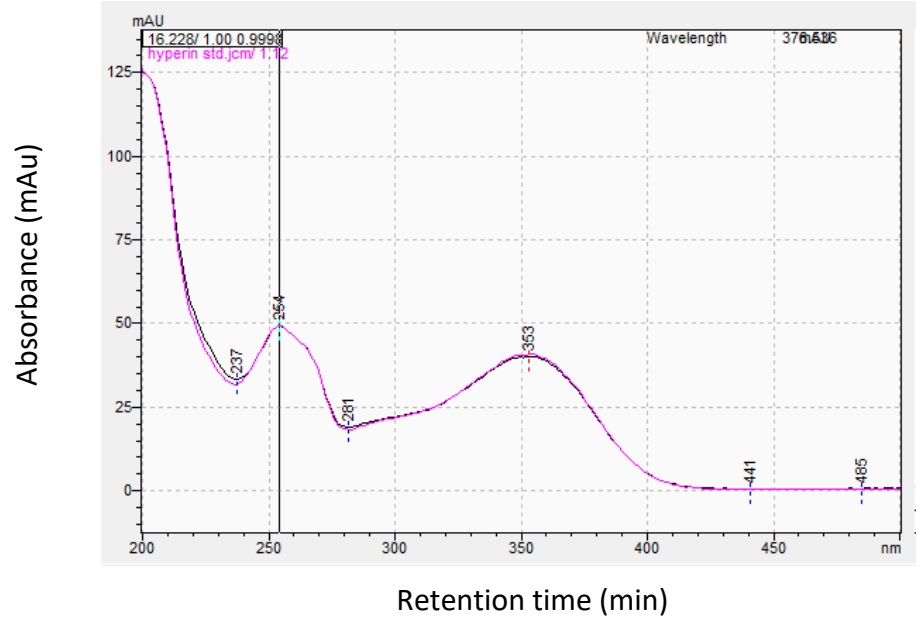


Figure 4.11: UV spectrum comparison between hyperin and peaks tentatively identified as corresponding peaks in patent standardised D. kaki extract (NXQ). The hyperin peak was Rt 16.2 min in the mixed standard and 16 min in NXQ (pink).

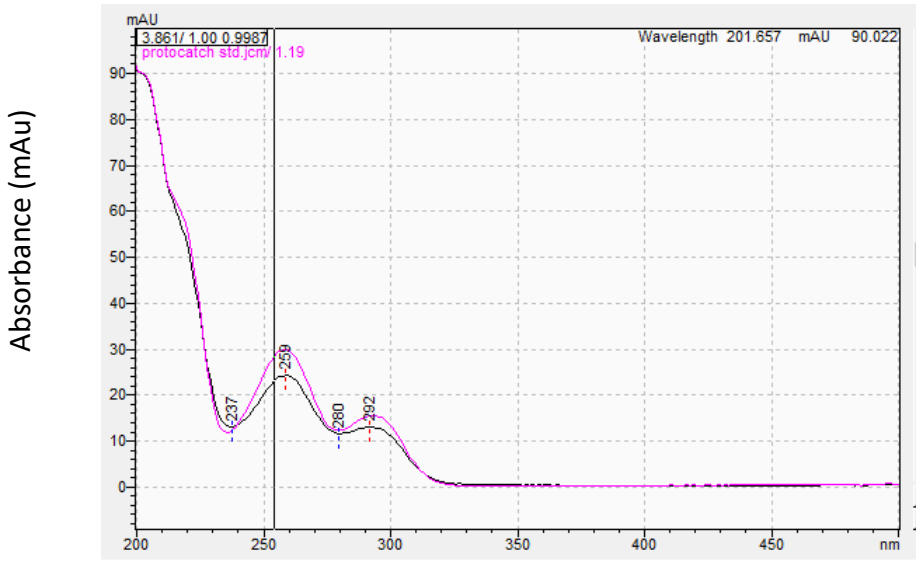


Figure 4.12: UV spectrum comparison between protocatcheuic acid and peaks tentatively identified as corresponding peaks in patent standardised *D. kaki extract (NXQ). The protocatcheuic acid peak was Rt 3.8 min in the mixed standard and 3 min in NXQ (pink).*

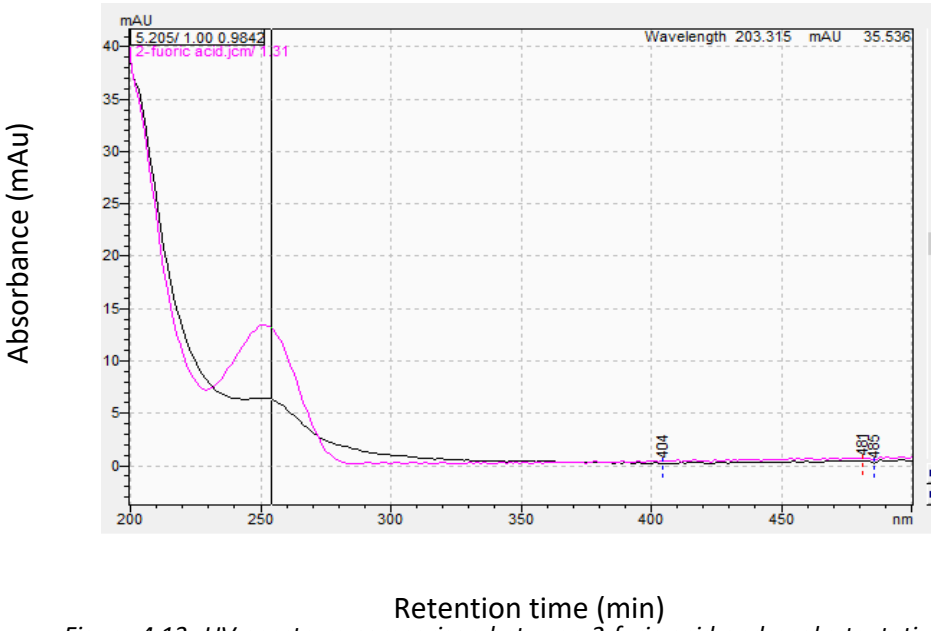


Figure 4.13: UV spectrum comparison between 2-furic acid and peaks tentatively identified as corresponding peaks in patent standardised D. kaki extract (NXQ). The 2-furic acid peak was Rt 5.2 min in the mixed standard and 5.3 min in NXQ (pink).

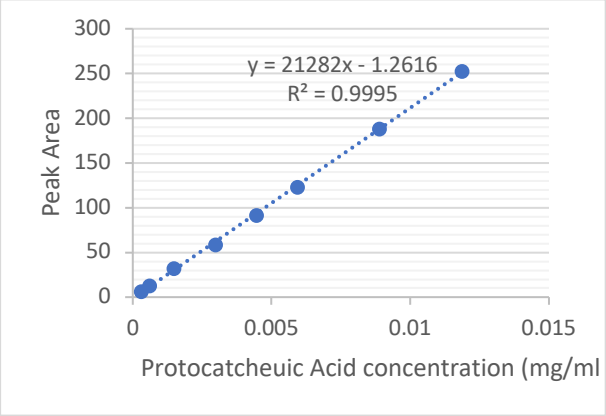
As shown in Figures 4.7–4.13, the peaks with the matching retention times to the mixed standards have similar UV spectrum. The lower concentration of compounds such as 2-furic acid and hyperin in the patent standardised *D. kaki L.* extract (NXQ) make it more difficult to

compare the UV spectrum, as at low absorbances the absorbance of the mobile phase dominates. In any case, there is a good retention time match between sample and standard peaks and a fair match between their UV spectra. The identification of the small peaks is considered tentative as some are close to detection limit.

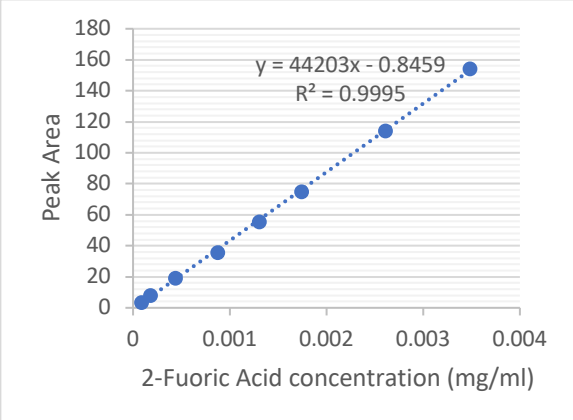
4.4.3 Linearity

Linearity is the range over which the analytical method is able to produce results that are proportional to the concentration of analyte(s) in the sample. The linearity of a standard curve is determined by a series of injections of standard solutions of different concentrations, covering the expected concentration range in the test solution. The equation fitted to the standard curve should have an intercept close to 0 and R^2 of >0.99.

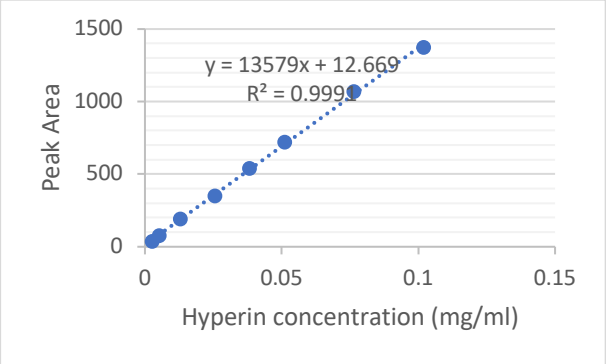
Figure 4.14 shows the linear relationship between the peak area versus concentration produced by a range of known concentration standards. The calibration curve of all tested standards was at R^2=0.999 or above, showing a good linearity for the experimental data for the analytical method.



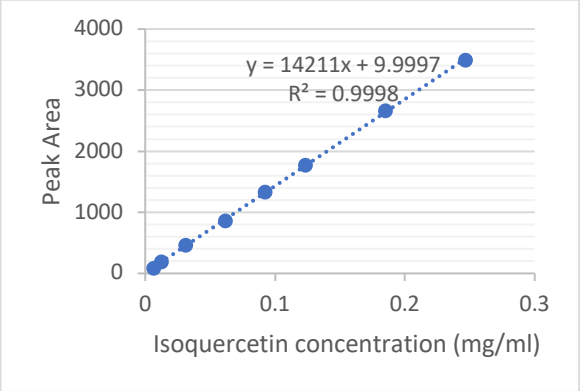
A. concentration standards curve of protocatcheuic acid. The calibration curve is linear (R^2=0.9995) (n=3).



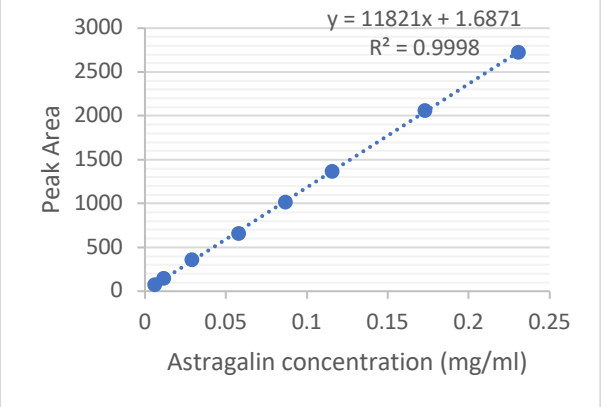
B. concentration standards curve of 2-fuoric acid. The calibration curve is linear (R^2=0.9995) (n=3).



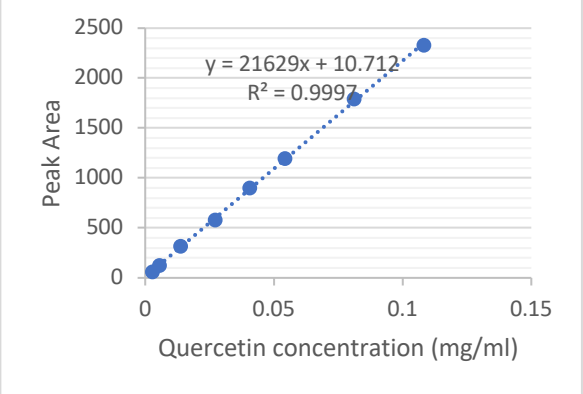
C. concentration standards curve of hyperin. The calibration curve is linear (R^2=0.9991) (n=3).



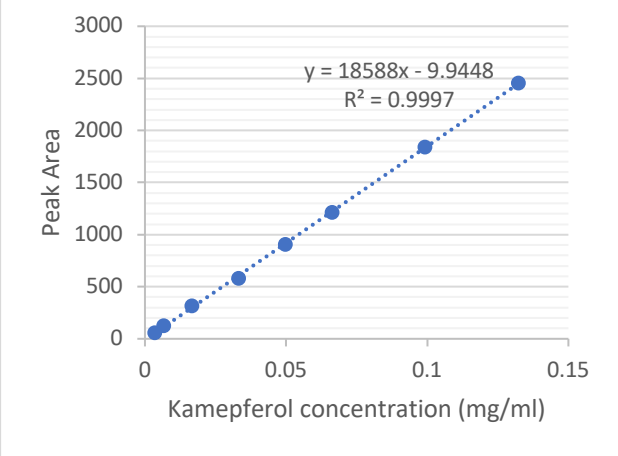
D. concentration standards curve of isoquercetin. The calibration curve is linear (R^2=0.9998) (n=3).



E. concentration standards curve of astragalin. The calibration curve is linear (R^2=0.9998) (n=3).



F. concentration standards curve of quercetin. The calibration curve is linear (R^2=0.9997) (n=3).



G. concentration standards curve of kaempferol. The calibration curve is linear (R^2=0.9997) (n=3).

Figure 4.14: Regression data for the seven analytes in the mix standard determined by HPLC.

4.4.4 Precision

Precision is the degree of agreement between a set of results produced by the same analytical method in several replicated extractions and analyses. The agreement is usually expressed and calculated from the standard deviation (SD) and relative standard deviation (RSD).

Precision is divided into two categories: repeatability and reproducibility. Repeatability is the agreement between the test results produced using the same operating conditions in a short period of time by the same analyst. This is typically referred to as intra-day precision. Reproducibility is the standard deviation between a set number of samples run using the same operating conditions but over a long period of time, two weeks or more. This is typically referred to as inter-day precision. The precision that can be expected of the analytical method depends on the concentration of the selected analyte(s), where the lower the analyte concentration, the higher the expected standard deviation.

Instrumental precision is determined by injecting a standard solution multiple times within a short period of time and determining the RSD of the peak area. The sample treatment contribution to total RSD can be estimated by subtracting the total RSD (calculated from the replicate extraction and measurement determinations) from the instrument RSD contribution. The general expectation is that the sample treatment will make the greater contribution to the total RSD.

Table 4 shows the instrumental precision and total precision. The instrumental precision of the method was determined by six repeat injections of the mixed standard. The total precision of the method was determined by preparing seven replicates of the sample. The instrumental precision was determined to be acceptable at <2 % method precision was determined to be acceptable at <5% for all analyte. As shown in Table 4, the RSD for the

instrumental precision and total precision ranged from 0.08–0.68% and 0.82–3.41%, respectively.

Table 4: Instrumental precision and total precision of the seven analytes of the mix standard

Analyte	*Instrumental Precision (RSD)%*	*Total Precision (RSD) %*
protocatcheuic acid	0.11	0.82
2-furic acid	0.16	1.40
hyperin	0.68	1.24
isoquercetin	0.37	1.20
astragalin	0.29	1.17
quercetin	0.46	3.41
kaempferol	0.08	0.85

4.4.4.1 Inter-day precision

The experiment was repeated on three separate days a week apart. Table 5 shows the SD between the results obtained on separate days. The RSD for the inter-day precision ranged from 0.5–5.7%. The inter-day RSD of <10% suggests adequate repeatability for the method.

Table 5: Regression data and precision data for the seven analytes determined in mix standard

Analyte	*Regression equation*	*R^2*	*Average mg/mL*	*SD mg/mL*	*RSD %*
Protocatcheuic acid	y=21282x - 1.2616	0.9995	0.496	0.014	2.8
2-furic acid	y=44203x - 0.8459	0.9995	0.073	0.002	3.2
Hyperin	y=13579x +12.669	0.9991	5.451	0.198	3.6
Isoquercetin	y= 14221x + 9.9997	0.9998	9.874	0.279	2.8
Astragalin	y= 11821x + 1.6871	0.9998	11.624	0.059	0.5
Quercetin	y= 21629x +10.712	0.9997	5.867	0.337	5.7
Kaempferol	y= 185888x – 9.9448	0.9997	6.969	0.073	1.0

n=3; RSD (%)=100 x SD/mean; y, peak area; x, the concentration of each compound (mg/mL); R, correlation coefficient of regression equations.

4.4.4.2 Limit of detection and limit of quantification

To determine the LOD and LOQ of the analytical method, the samples were analysed seven times on the same day. This determines the SD of the analytical method. Three times the SD gives LOD while ten times the SD gives the LOQ. The LODs and LOQs of the seven analytes ranged from 0.003–0.647 mg/g and 0.010–2.158 mg/g, respectively. The method was found to be valid and used to quantify the main bioactive constituents and quercetin and kaempferol found in NXQ (Table 6).

Table 6: Detection and quantification limits for the seven analytes determined in mix standard

Compound	*LOQ (mg/g)*	*LOD (mg/g)*
Protocatcheuic acid	0.038	0.012
2-furic acid	0.010	0.003
Hyperin	0.705	0.211
Isoquercetin	1.227	0.368
Astragalin	1.354	0.406
Quercetin	2.158	0.647
Kaempferol	0.598	0.179

LOQ: limit of quantification (10x[SD of y-intercept/mean of slope]; LOD, limit of detection (3.33 x [SD of y – intercept/mean of slope]).

4.4.4.3 Quantification of active components in NXQ

The concentration of each of the seven analytes in NXQ was determined by HPLC and the results presented in Table 7. Protocachueic acid and 2-furic acid were identified in the extract but with a very low concentration (0.58±0.01 and 0.08±0.01 mg/g respectively). The concentrations of the other five analytes including hyperin, isquercetin, astragalin, quercetin and kaempferol were found to be 5.27±0.07, 9.18±0.02, 12.93±0.08, 6.23±0.04, and 8.10±0.02 mg/g respectively. Based on these results, ratio of quercetin to kaempferol was found to be ~3:4.

Table 7: Concentration of analyte in NXQ determined by HPLC. SD calculated from seven replicates

Compound	Concentration (mg/g) ±SD
Protocatcheuic acid	0.58±0.01

2-furic acid	0.08±0.01
Hyperin	5.27±0.07
Isoquercetin	9.18±0.02
Astragalin	12.93±0.08
Quercetin	6.23±0.04
Kaempferol	8.10±0.02

4.5 Conclusion

The method was found to be valid for the quantification of the seven analytes in NXQ. The method was used to quantify the main bioactive constituents in the NXQ commercial extract and determine the ratio of kaempferol to quercetin. The ratio of quercetin to kaempferol was found to be ~3:4.

Chapter 5. Evaluation of Vascular Protective Effects of NXQ Extract

5.1 Introduction

Vascular protection is known to be an important aspect of prevention and treatment of stroke. Neural damage can be initiated by acute ischaemia and exacerbated by reperfusion injury after the ischaemic attack (Hausenloy & Yellon, 2013). Reperfusion of ischaemic tissues is often associated with microvascular injury, particularly due to increased permeability of the capillaries and arterioles leading to increased diffusion and fluid filtration across the tissues (Kalogeris, Baines, Krenz, & Korthuis, 2012). Activated endothelial cells release excessive reactive oxygen species (ROS) following the reperfusion and the imbalance results in a subsequent inflammatory response and, ultimately, cell apoptosis (Go et al., 2010; Kalogeris et al., 2012).

Living organisms produce ROS during normal and abnormal cellular metabolism. ROS are highly reactive molecules that can damage the cell structures, including carbohydrates, nucleic acids, lipids and proteins, and alter their functions (Go et al., 2010; Ray, Huang, & Tsuji, 2012). Oxidative stress refers to an increased concentration of cellular oxidant and insufficiency of oxidant-scavenging agents to combat the change in intracellular environment. Oxidative stress is known to be one of the leading mediators of tissue injury in post-stroke reperfusion due to overwhelming of the ROS and loss of combating ability, which contributes to a variety of pathological changes such as cellular necrosis and apoptosis of affected tissues (Dorweiler et al., 2007). Regulation of the reducing and oxidising states, or 'redox', is critical for cell viability, activation, proliferation and organ function (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012).

H_2O_2 is the major source of endogenous ROS responsible for oxidative stress and is mainly generated during hypoxia and ischaemic reperfusion injury (Ballinger et al., 2000). It was confirmed that H_2O_2 arose from the dismutation of superoxide generated within

mitochondria (Forman & Kennedy, 1974; Loschen, Azzi, Richter, & Flohé, 1974; Murphy, 2009). Accumulation of these ROS can lead to oxidative damage to mitochondrial proteins, membranes and DNA, impairing the ability of mitochondria to synthesis ATP and to carry out their metabolic functions (Murphy, 2009).

Pervious study has shown that low concentrations of H_2O_2 (>200μmol/L) may induce endothelial apoptosis, which makes H_2O_2 a typical agent for studies of oxidative stress injury in *in vitro* models (C. H. Coyle et al., 2006).

Previous studies have demonstrated that NXQ protects against H_2O_2-induced NG108-15 neuron cell injury (Bei et al., 2004; Bei, Peng, Ma, & Xu, 2005a). It has been shown that NXQ attenuates the reduction of activities of intracellular endogenous anti-oxidant, glutathione and glutathione peroxidase by catalasing and decreasing lactate dehydrogenase (LDH) leakage, suggesting significant protection for NG108-15 cells against H_2O_2 insults by improving redox imbalance and inhibiting apoptosis (Bei et al., 2004, 2005a). However, the role of NXQ's vascular protection property was not evaluated.

In this study, we aim to evaluate the potential vascular protective effects of NXQ against H_2O_2-induced oxidative stress in human umbilical vascular endothelial cells (HUVEC). *In vitro* studies of cell viability, ROS scavenging and LDH leakage were tested using EA.hy926 cells. In addition, we evaluated the effects of the two main bioactive components of NXQ, kaempferol and quercetin, in an attempt to evaluate their contribution to NXQ's therapeutic actions and their potential synergistic effects.

In addition to the *in vitro* model described above, we also endeavour to develop an *in vivo* hypoxia model using adult zebrafish to evaluate the neuroprotective and vascular protective effects of NXQ. The model development is based on a published protocol (X. Yu & Li, 2011); however, we have not been able to replicate the published results despite investment of

substantial efforts and time. Our results are presented and discussed below to shed light on the issues and challenges associated with this model.

5.2 Methods

5.2.1 Chemicals and reagents

The chemicals used are summarised in Table 5.1

Table 8: Chemicals and reagents used for in vitro study

Chemical	*Source*
Culture medium Dulbecco's Modified Eagle's Medium (DMEM/Ham's F12 containing 15mM HEPES and L-glutamine, with phenol red)	Life Technologies (Australia)
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich (Australia)
Fetal bovine serum (FBS)	Life Technologies (Australia)
Hydrogen Peroxide	Sigma-Aldrich (Australia)
Kaempferol	Sigma-Aldrich (Australia)
MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)	Sigma-Aldrich (Australia)
NXQ Powder extracts Diospyros kaki. L	Guangzhou Baiyunshan Pharmaceutical Co. Ltd. (PRC)
Phenol red absence culture medium (DMEM/Ham's F12 containing 15mM HEPES and L-glutamine)	Gibco™ (Australia)
Phosphate-buffered saline (PBS) tablets	Sigma-Aldrich (Australia)
Penstrep (penicillin and streptomycin)	Gibco™ (Australia)
Quercetin	Sigma-Aldrich (Australia)
Trypsin (10x) solution	Sigma-Aldrich (Australia)
VEGF receptor tyrosine kinase inhibitor II (VRI)	Calbiochem (Merck KGaA, Germany)

5.2.2 NXQ extracts stock preparation

To prepare the aqueous stock extract, the NXQ extract was weighed and dissolved in 99.9% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and mixed thoroughly with Vortex until fully dissolved. A serial dilution was made of concentrations 10, 50, 100, 250 and 500 (mg/mL). To prepare the dosing solution, the stock extract was diluted into serum-free culture mediums,

mixed thoroughly and filtered with Millex syringe filters (0.22µm pore size) to eliminate remaining precipitants.

Table 9: NXQ extract stock measurements.Final testing concentrations are labelled in data figures for comparison purpose. Volume stock dissolved in serum-free culture medium are the concentration add into 96 well micro-plate containing 200µL of serum-free medium.

NXQ stock preparation		*Final testing concentration*	*Volume stock dissolved in serum-free culture medium*
A.	250 mg/mL	500 µg/mL	4 µL of 250 mg/mL
		250 µg/mL	2 µL/mL of 250 mg/mL
B.	100 mg/mL	100 µg/mL	2 µL/mL of100 mg/mL
C.	50 mg/mL	50 µg/mL	2 µL/mL of 50mg/mL
D.	10 mg/mL	10 µg/mL	2 µL/mL of 10mg/mL

5.2.3 Quercetin and kaempferol stock preparation

Pure extracts of Q and K were purchased from Sigma-Aldrich. To prepare quercetin and kaempferol aqueous stock solution, the powdered compound was weighed based on molecular weight and dissolved in 99.9% DMSO (Sigma-Aldrich) and mixed thoroughly with Vortex until fully dissolved. A serial dilution was made of concentrations 10, 50, 100, 250 and 500 (mmol/mL). To prepare the dosing solution, the stock extract was diluted into serum-free culture mediums.

Table 10: Quercetin and kaempferol stock measurements. Final testing concentrations are labelled in data figures for comparison purpose. Volume stock dissolved in serum-free culture medium are the concentration add into 96 well micro-plate containing 200µL of serum-free medium.

Stock preparation		*Final testing concentration*	*Volume stock dissolved in serum-free culture medium*
A.	100 mmol/mL	500 µmol/mL	10 µL of 100 mmol/mL
		250 µmol/mL	5 µL/mL of 100 mmol/mL
		100 µmol/mL	2 µL/mL of 100 mmol/mL
B.	50 mmol/mL	50 µmol/mL	2 µL/mL of 50 mmol/mL
C.	10 mmol/mL	10 µmol/mL	2 µL/mL of 10 mmol/mL

5.2.4 Quercetin and kaempferol (3:4) combination stock preparation

The ratio of Q and K combination was obtained as detailed in Chapter 4. Pure extract of Q and K was purchased from Sigma-Aldrich (Australia). To prepare Q and K aqueous stock solution, the powdered compound was weighed based on the ratio 3:4 respectively. The stock solution was weighed in mg/mL at concentrations of 3, 15, 30, 75 and 150 mg/mL, which is equal to the concentrations of 10, 50, 100, 250 and 500 mmol/mL respectively. Stock solution was dissolved in 99.9% DMSO (Sigma-Aldrich) and mixed thoroughly with Vortex until fully dissolved. To prepare the dosing solution, the stock solution was diluted into serum-free culture mediums and mixed thoroughly.

Table 11: Quercetin and kaempferol (3:4) combination stock measurements. Final testing concentrations are labelled in data figures for comparison purpose. Volume stock dissolved in serum-free culture medium are the concentration add into 96 well micro-plate containing 200µL of serum-free medium.

Stock preparation		*Final testing concentration*	*Volume stock dissolved in serum-free culture medium*
A.	150 mg/mL	500 µmol/mL (150 µg/mL)	2 µL of 500 mmol/mL
B.	75 mg/mL	250 µmol/mL (75 µg/mL)	2 µL/mL of 250 mmol/mL
C.	30 mg/mL	100 µmol/mL (30 µg/mL)	2 µL/mL of 100 mmol/mL
D.	15 mg/mL	50 µmol/mL (15 µg/mL)	2 µL/mL of 50 mmol/mL
E.	3 mg/mL	10 µmol/mL (3 µg/mL)	2 µL/mL of 10 mmol/mL

5.3 In vitro cell survival bioassays – experiment methodology

5.3.1 Cell culture

Human umbilical vein cell line (EA.hy926) was kindly provided by Dr Phoebe Zhou from the NICM research institute. The cell line was cultured in DMEM Hams's F-12 containing 15 mM HEPES and L-glutamine and supplemented with 10% FBS, 100U/mL of penicillin and streptomycin (Gibco BRL, Australia). The cell line was grown in a 5% CO_2^- humidified incubator at 37°C and 95% humidity.

5.3.2 Cell viability determined by MTT dye reduction bioassays

The MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), tetrazolium reduction assay was the first homogenous cell viability assay developed for a 96-well format that regarded fitting for high throughput screenings. The MTT assay has been widely adopted and has retained its popularity in academic labs. The MTT substrate was prepared in a physiologically balanced solution, added to cells in culture, usually at a final concentration of 0.2–0.5mg/mL, and incubated for 1–4 hours. The quantity of formazan was measured by recording the changes in absorbance at 570nm using a plate-reading spectrophotometer (Riss et al., 2016).

Viable cells with active metabolism convert MTT into a purple-coloured formazan product with an absorbance maximum near 570nm. When cells die, they lose the ability to convert MTT into formazan. Hence, colour formation is a convenient marker for viable cells. The cell viability of the tested sample is calculated based on the equation of cell viability percentage equals absorbance of treated cells divided by absorbance of untreated cells (control) multiplied by 100%.

5.3.2.1 Experimental procedure

EA.hy926 cell passage numbers 26–35 were seeded on a 96-well cell culture plate with culture medium (DMEM Hams's F-12, containing 15mM HEPES and L-glutamine) and supplemented with 10% FBS, 100U/mL of penicillin and streptomycin (Gibco BRL, Australia) at a concentration of $1x10^5$ cells/mL and allowed to confluence overnight.

NXQ solution was prepared according to Sections 5.2.2 to 5.2.4 above. The cells were treated for 24 hours.

After the treatment period, 100µl of supernatant was removed and an MTT solution with a final concentration of 0.5mg/mL in PBS was added to the cells and incubated for four hours

at 37°C and 5% CO_2. After incubation, MTT solutions were removed and 50µL of DMSO (99.9%) added to dissolve the insoluble formazan crystal. The absorbance was measured at 570nm using a microplate reader (BMG Labtech Fluostar Optima, Mount Eliza, Victoria, Australia). The intensity of formazan formed in control (medium with vehicle DMSO) cells was taken as 100% cell viability. Each experiment was repeated three times.

5.3.3 Cytotoxicity induced by hydrogen peroxide (H_2O_2)

Hydrogen peroxide (H_2O_2) is a reactive oxygen metabolic by-product that serves as a key regulator for some oxidative stress-related conditions such as cancer, neurological disorders (Jenner, 2003; Lyras, Cairns, Jenner, Jenner, & Halliwell, 1997; Sayre, Smith, & Perry, 2001; Toshniwal & Zarling, 1992), atherosclerosis, hypertension, ischaemic reperfusion (Dhalla, Temsah, & Netticadan, 2000; Kašparová et al., 2005; Kerr et al., 1999; Kukreja & Hess, 1992) and metabolic disorders (Asami et al., 1997; Pick & Keisari, 1980). The oxidative burst is characterised by a sharp increase in oxygen uptake followed by the channelling of the bulk of the oxygen from a biochemical pathway initiated by the one-electron reduction of oxygen towards a biochemical pathway using nicotinamide adenine dinucleotide phosphate (NADPH or NADH), a cofactor in anabolic reaction, as the electron donor (Pick & Keisari, 1980). Also, while superoxide can be directly toxic (Fridovich, 1998), its reactivity is mostly through superoxide dismutase to H_2O_2. Therefore, H_2O_2 almost always is formed under the circumstances in which superoxide is produced, acting as an electron donor.

H_2O_2 diffuses freely into cells to produce several biochemical perturbations, including several metabolic pathways such as activation of hexose monophosphate shunt (Miller, Buettner, & Aust, 1990); introducing glycolysis and generating NADPH (Birben et al., 2012; Dupuy et al., 1991); oxidation of intracellular sulfhydryls (Ray et al., 2012); depression of

intracellular ATP (D. N. Granger, 1988); DNA damage (Ballinger et al., 2000; Halliwell & Gutteridge, 2015); loss of intracellular beta-NADH; activation of poly(ADP-ribose) polymerase; fast rise of intracellular calcium; gross perturbations to the cytoskeleton and plasma membrane; and depression in glycolytic flux (Birben et al., 2012; Sies, 2017). All of these processes occur before the loss of membrane integrity as measured by vital stains or before the loss of preloaded chromium (Sies, 2017).

5.3.3.1 Experiment procedure

To investigate the protective effect of NXQ against endothelial cell toxicity caused by H_2O_2, EA.hy926 cells were pre-treated for 30 minutes with NXQ at concentrations of 10, 50, 100, 250 and 500μg/mL, quercetin, kaempferol, or quercetin and kaempferol (3:4) combination at concentrations of 10, 50, 100, 250 and 500 mmol/mL. H_2O_2 (0.5mmol) was then dosed and incubated for 24 hours. The cell viability was measured by MTT assay as described in Section 5.3.2.1. Each experiment was repeated three times.

5.3.4 Cytotoxicity induced by ROS

ROS has been regarded as an essential source of oxidative stress leading to endothelial dysfunction. ROS are baneful to all aerobic species, and these molecules are typically generated as byproducts during the mitochondrial electron transport of aerobic respiration or by oxidoreductase enzyme and metal-catalysed oxidation (Murphy, 2009). Excess accumulation of ROS can induce endothelial cellular injury by modulating a series of intracellular signalling pathways. For example, it can directly quench the production of eNOS and upregulate the degradation of existing eNO, which onsets and exacerbates endothelium dysfunction (Förstermann, 2010).

Intracellular ROS is an important indicator in responding to cellular damage models induced by oxidative stress. In the Cellular Reactive Oxygen Species Detection Assay Kit (ab113851), a cell-permeant reagent 2',7'–dichlorofluorescein diacetate (DCFDA) (a fluorogenic dye) is used to detect ROS within the cells through a series of chemical reactions. After DCFDA is diffused into the cells, it is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidised by ROS into 2', 7'–dichlorofluorescein (DCF). DCF is a highly fluorescent compound that can be detected by fluorescence spectroscopy with maximum excitation and emission spectra of 495nm and 529nm, respectively. The amount of ROS is proportional to the DCF that is measured.

5.3.4.1 Experimental procedure

The assay was conducted based on the protocol from the cellular ROS detection assay kit manufacturer (Abcam, Australia). EA.hy 926 cells were seeded on a 96-well cell culture plate with culture medium (DMEM Hams's F-12 without phenol red), containing 15mmol HEPES and L-glutamine, and supplemented with 10% FBS, 100 U/mL of penicillin and streptomycin (Gibco BRL, Australia). Cells were seeded at $1x10^5$ cells/mL and allowed to confluence overnight. The medium was then removed and the cells were washed once with 1X assay buffer (from the kit). The cells were then stained with DCFDA (20 µmol) at 100µL per well. The plate was incubated with a staining solution for 45 min at 37°C in the dark. After incubation, the DCFDA staining was removed and the plate immediately read under a microplate reader to get the initial absorbance (A0). The cells were then washed again with PBS and tested drugs were added at concentrations 10–500 µg/mL and treated for one hour. Finally, the absorbance at the endpoint (A1) was measured in the presence of the treatments under the same microplate reader. The wavelength was set up with excitation at 455nm and

emission 535nm in fluoresce mode. The final absorbance was calculated as A1 normalised with its corresponsive A0 (A1/A0). Each experiment was repeated three times.

5.3.5 Cytotoxicity – lactate dehydrogenase leakage (LDH) measurement

Apoptosis and necrosis are the two major forms of cell death observed in normal and disease pathologies. Necrosis is a type of cell death that is morphologically characterised by swelling and rupture of intracellular organelles, leading to permeabilisation of the plasma membrane (Challa & Chan, 2010; Schweichel & Merker, 1973). Plasma membrane leakage from necrotic cells causes the release of intracellular contents into extracellular milieu, which evokes inflammatory responses and is closely associated with inflammatory diseases (Kono & Rock, 2008).

Lactate dehydrogenase (LDH) is a soluble cytosolic enzyme that presents in majority of cells and is released through damaged plasma membranes (Burd & Usategui-Gomez, 1973; F. K.-M. Chan, Moriwaki, & De Rosa, 2013). Thus, LDH leakage measurement has been widely used to evaluate the presence of damage, toxicity or necrotic tissues and cells. Released LDH in culture supernatants is quite stable and can be quantitatively measured with a 30-minute coupled enzymatic assay through a series of chemical reactions (Promega, 2016).

To detect the leakage of LDH into cell culture medium, a tetrazolium salt (iodonitrotetrazolium violet) is used in this assay. LDH released from leaky cells reacts with lactate and forms pyruvate via catalysing the oxidation of nicotinamide adenine dinucleotide (NADH), then converting the tetrazolium salt into a red formazan product (Korzeniewski & Callewaert, 1983). The amount of colour formed by formazan is proportional to the number of lysed cells (Promega, 2016). Absorbance for the optimal colour density can be measured using a microplate reader under the wavelength of 540nm.

5.3.5.1 Experimental procedure

EA.hy926 cells were pre-treated for 30 min with NXQ at various concentrations of between 10 and 500µg/mL quercetin, 10–500µg/mL kaempferol, or quercetin and kaempferol combination (3:4) at various concentrations between 10 and 500µg/mL. H_2O_2 (0.mM) was then dosed and incubated for 24 hours.

After the treatment period, the supernatants from the cells were subjected to LDH measurement using a commercial kit (Promega, Australia) according to the manufacturer's instructions. Briefly, the cells in the 96-well cell culture plate with supernatant after the treatment period were centrifuged at 250 X g for 4 min. Then 50µl of the supernatant was transferred from each test well to a fresh 96-well flat-bottom plate. Prepared CytoTox 96 substrate mix (50µl) obtained from the kit was added to each well of the new 96-well plate, containing samples transferred from the original cytotoxicity assay plate. The plate was then covered with aluminium foil to avoid light and incubated for 30 min at room temperature. Finally, 50µl of stop solution (from the kit) was added to each well, and the absorbance was monitored at 490nm.

The readings from the untreated and test wells are subtracted from the readings from the medium-only control wells. The reading from volume correction control wells are subtracted from the reading of maximum LDH release wells (H_2O_2 treatment only). Lastly, the percentage of cytotoxicity is calculated via the equation 1 (F. K.-M. Chan et al., 2013):

$$\% \text{ LDH release Cytotoxicity} = \frac{(corrected\ reading\ from\ test\ well - corrected\ reading\ from\ untreated\ well)}{(Corrected\ reading\ from\ maximum\ LDH\ release - corrected\ reading\ from\ untreated\ well)} \times 100 \quad \text{[Eq. 1]}$$

Each experiment was repeated three times.

5.3.6 Statistical analysis

All statistic comparisons were performed using GraphPad Version 5.02(US). The statistical significance was analysed by one-way ANOVA test. Data were expressed as mean±SEM. Statistical significance was regarded when P <0.05 was obtained.

5. 4 Results of cellular protective effects of NXQ on EA.hy926 cells

5.4.1 Cytotoxicity of NXQ on EA.hy926 cells detected by MTT assay

Cytotoxicity of NXQ (10–1000μg/mL; n=3) on EA.hy926 cells was examined using MTT assay to determine the safe dosage range. NXQ did not show any significant cytotoxic effects up to 500 μg/mL. NXQ caused a significant reduction (~25%) in cell viability at 1000 μg/mL when compared to vehicle control. Therefore, all the subsequent experiments were conducted at doses no higher than 500 μg/mL of NXQ (Figure 5.1).

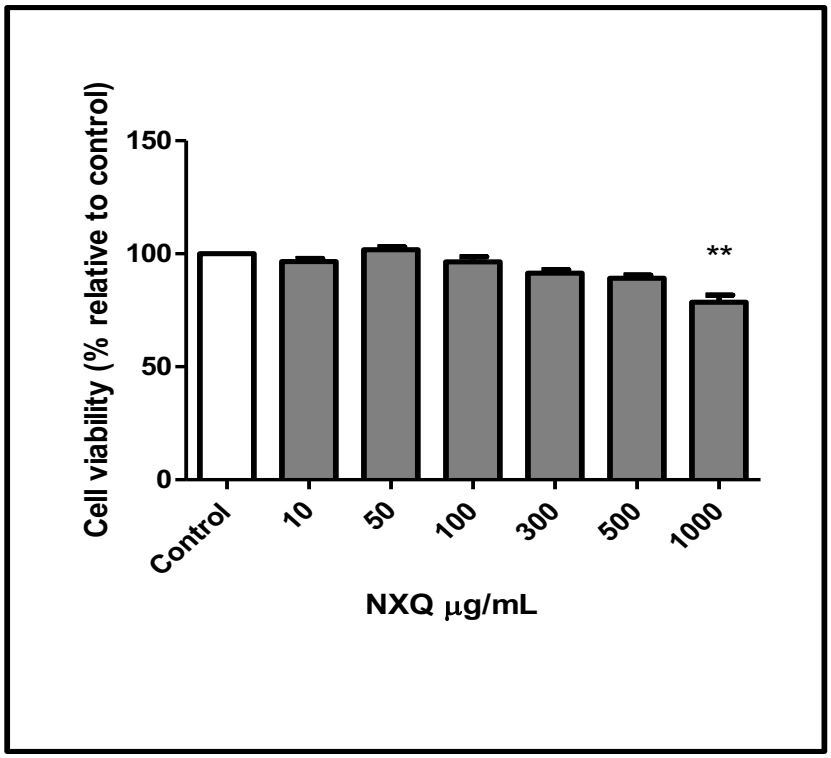


*Figure 5.1: Endothelial cell death induced by NXQ (10–1000 µg/mL). Cell viability, as determined by MTT dye reduction assay in EA.hy926 endothelial cells for 24 hrs. Cell viability was expressed as a percentage of various concentrations of NXQ pure extract compared to control (0.1% DMSO). All results were expressed as mean±SEM from three separate experiments** p<0.01 vs control.*

5.4.2 Effects of NXQ, quercetin, kaempferol and quercetin and kaempferol (3:4) combination on cell survival against H_2O_2 on EA.hy926 cells

To evaluate whether NXQ could protect against H_2O_2-induced cell damage, cells were pre-treated with NXQ for 30 minutes, then tested on H_2O_2 (0.5mM) for 24 hours; cell viability was measured by MTT assay. EA.hy926 cell viability was markedly reduced (30%) by H_2O_2 (0.5mM; 24 h) ($p<0.05$, n=3). Pre-treatment of NXQ (10–500µg/mL) protected cells against H_2O_2-induced cell death ($p<0.05$ at 50, 250 and 500 µg/mL; n=3) (Figure 5.2A).

The cell viability with pre-treatment of quercetin and kaempferol (3:4) combination followed by H_2O_2 (0.5mM) is shown in Figure 5.2D. Quercetin and kaempferol (3:4) combination also protected the EA.hy926 cell from H_2O_2-induced damage in a concentration-dependent manner ($p<0.001$; n=3).

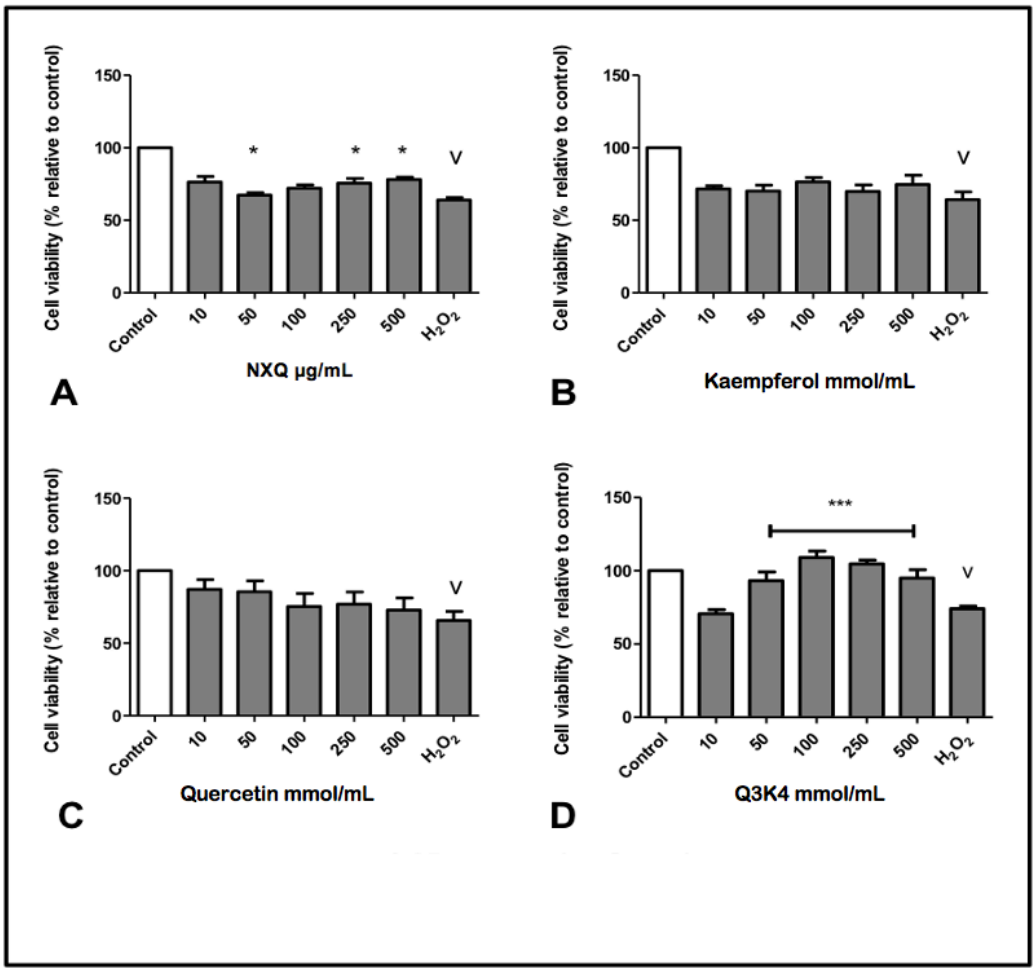


*Figure 5.2: Protective effects of NXQ on EA.hy926 cells against H_2O_2 (0.5mM)-induced cell injury by restoring reduced cell viability at various concentrations of NXQ extract, quercetin, kaempferol, and quercetin–kaempferol (3:4) combination. (A): Protective effects of NXQ on EA.hy926 cells against H_2O_2 (0.5mM) induced cell injury by restoring reduced cell viability at various concentrations of NXQ extract. Cell viability was determined by the MTT method in EA.hy926 cells for four hrs. Cell viability was expressed as a percentage vs H_2O_2 treatment only. NXQ extract at 10, 250 and 500 µg/mL showed statistical significance. All results were expressed as mean±SEM from three separate experiments. * $p<0.05$ vs H_2O_2 treatment only. (B): Protective effects of kaempferol on EA.hy926 cells against H_2O_2 (0.5mM)-induced cell injury by restoring reduced cell viability at various concentrations of kaempferol. Cell viability was determined by the MTT method in EA.hy926 cells for four hrs. Cell viability was expressed as a percentage vs H_2O_2 treatment only. (C): Protective effect of quercetin on EA.hy926 cells against H_2O_2 (0.5mM). (D): Protective effects on Q3:K4 (quercetin and kaempferol 3:4 combination) on EA.hy926 cells against H_2O_2 (0.5mM)-induced cell injury by restoring reduced cell viability at various concentration. Cell viability was determined by the MTT method in EA.hy926 cells for four hrs. Cell viability was expressed as a percentage vs H_2O_2 treatment only. Q3:K4 combinations at 50, 100, 250 and 500 mmol/mL showed statistical significance. All results were expressed as mean±SEM from three separate experiments. * $p<0.05$ compared to H_2O_2. ** $p<0.001$ vs H_2O_2 treatment only. V:$p<0.05$ compared to control. *** $p<0.001$ compared to H_2O_2.*

In addition, Figure 5.3 provides a comparison for specific concentrations between the active components and their combination in the ratio detected from NXQ. Based on the results shown in Figure 5.3, kaempferol failed to protect against H_2O_2 damage ($p>0.05$) in all

concentrations (10, 50, 100, 250 and 500mmol/mL). Interestingly, quercetin showed protection at concentration 10mmol/mL ($p<0.05$). However, at higher concentrations quercetin failed to restore cell viability (50, 100, 250 and 500mmol/mL) ($p>0.05$). This observation suggests that quercetin and kaempferol alone do not protect cell death caused by H_2O_2. In contrast, a potential effect on cell survival against H_2O_2 of the quercetin–kaempferol combination (3:4) is observed at concentrations 50, 100, 250 and 500mmol/mL. The quercetin–kaempferol combination (3:4) significantly restored cell viability against H_2O_2 damage ($p<0.05$) in comparison to quercetin and kaempferol alone at their specific concentration. This suggests that when quercetin and kaempferol are combined at a ratio of 3:4, a cellular protective effect against H_2O_2 was enhanced. These results suggest a potential synergistic or additive effect which requires further investigation.

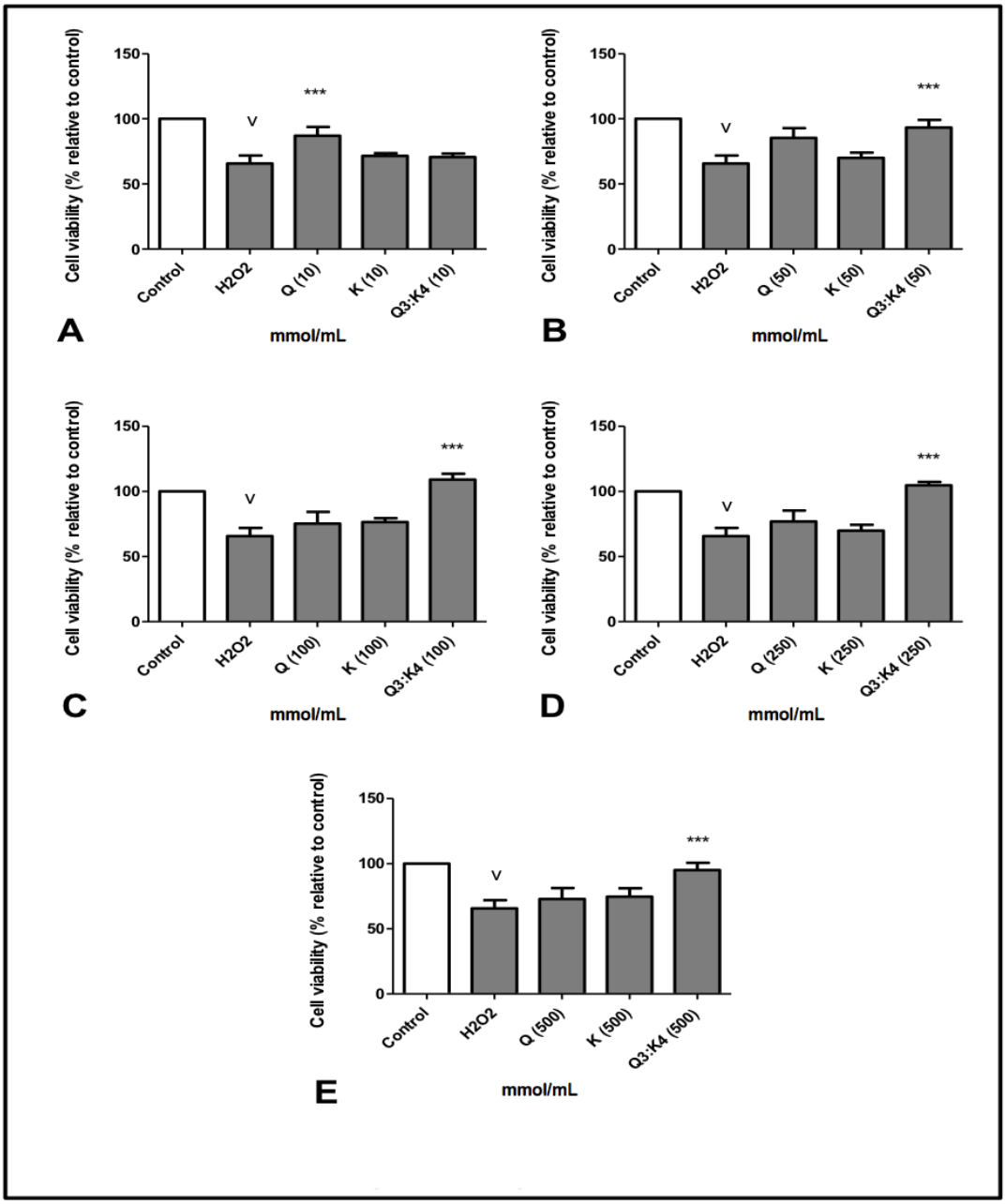


*Figure 5.3: Restoring cell viability against H_2O_2, effects determined between groups based on single concentration. Q3:K4 (quercetin–kaempferol 3:4 combination) compared with quercetin and kaempferol at concentration 10 mmol/mL (A), 50 mmol/mL (B), 100 mmol/mL(C), 250 mmollmL (D) and 500 mmol/mL (E) on EA.hy926 cells ($p<0.05$; n=3). v:$p<0.05$ compared to control. *** $p<0.001$ compared with H_2O_2.*

5.4.3 Effects of NXQ on H_2O_2-induced ROS generation in EA.hy926 cells

As shown in Figure 5.4A, EA.hy926 cells incubated with H_2O_2 exhibited a significant increase in ROS production compared to the control group ($p<0.05$; n=3). Pre-treatment of NXQ (10-

500µg/mL) significantly attenuated ROS generation by H_2O_2 ($p<0.05$: n=3) in a concentration-dependent manner.

Similarly, pre-treatment of quercetin (10–500 mmol/mL) significantly attenuated the ROS generation induced by H_2O_2 ($p<0.05$; n=3) (2.4C). The ROS attenuation effects are relatively linear between concentrations 50, 100, 250 and 500 (mmol/mL) with no significant dose dependency curve. In Figure 5.4B, pre-treatment with kaempferol showed significant attenuation of H_2O_2-induced ROS level in a concentration-dependent manner. However, a statistical significance was only observed at 250 and 500 mmol/mL ($p<0.05$; n=3). In Figure 5.4D, pre-treatment with the quercetin and kaempferol combination (10–500 µg/mL) significantly attenuated the ROS generation induced by H_2O_2 ($p<0.05$; n=3).

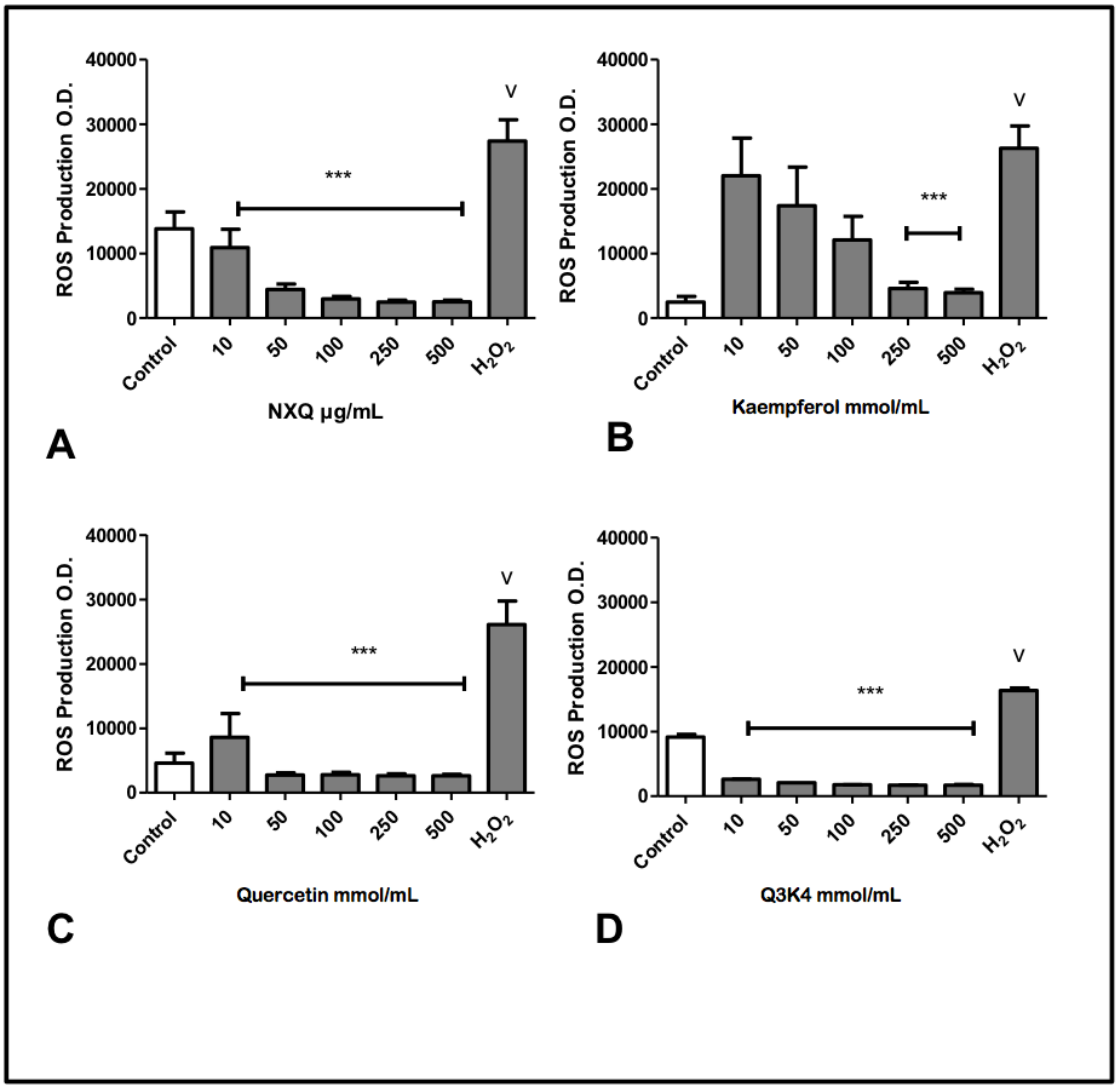


*Figure 5.4: ROS inhibition by NXQ extracts. (A), kaempferol (B), quercetin (C), and Q3:K4 (quercetin and kaempferol combination 3:4)(D) on H_2O_2 (0.5mM)-induced ROS production in EA.hy926 endothelial cells. ROS was determined using DCFDA Cellular ROS Detection Assay. ROS inhibition was expressed as O.D. compared to H_2O_2 treatment only. All results were expressed as mean±SEM from three separate experiments. V: p<0.001 compared to control. ***p<0.001 vs H_2O_2 treatment only.*

We further compared the analytes quercetin, kaempferol and their combination (3:4) for its attenuation effects on ROS induced by H_2O_2 (Figure 5.5). The quercetin–kaempferol combination shows a better attenuation of ROS generation at 100, 250 and 500 mmol/mL ($p<0.001$) compared to quercetin and kaempferol at concentrations between 100, 250 and 500 mmol/mL. Quercetin seems to be responsible for its ROS attenuation property against H_2O_2-induced ROS generation, which is significantly inhibited at concentrations 100, 250 and

500 mmol/mL. Kaempferol, in contrast, only showed inhibition of ROS generation at a higher concentration, 250 and 500 mmol/mL (Figures 5.5 D and E). The effects of the quercetin–kaempferol combination and quercetin and kaempferol alone may not show a significant difference among themselves; however, the ROS attenuation effects does seem to be more prominent in the quercetin–kaempferol combination (Figure 5.5E). While quercetin may be the main compound responsible for its ROS attenuation effects, kaempferol may also provide a protective effect against H_2O_2 at a higher concentration. The possible reason for the big inter-experimental variations on the baseline presented in Figure 5.4A and Figure 5.4 B,C&D is that the data presented in Figure 5.4A were collected at the early stage of the experimental study before further examination of individual components. The variation shown in the control bar may be due to the lack of experimental skills at the early stage of the candidature. The control baselines were relatively similar in Figure 5.4 B, C and D following improved experimental skills. Nevertheless, the data presented in Figure 5.4A provided preliminary evidence suggesting NXQ extract significantly restore cell viability by attenuating ROS.

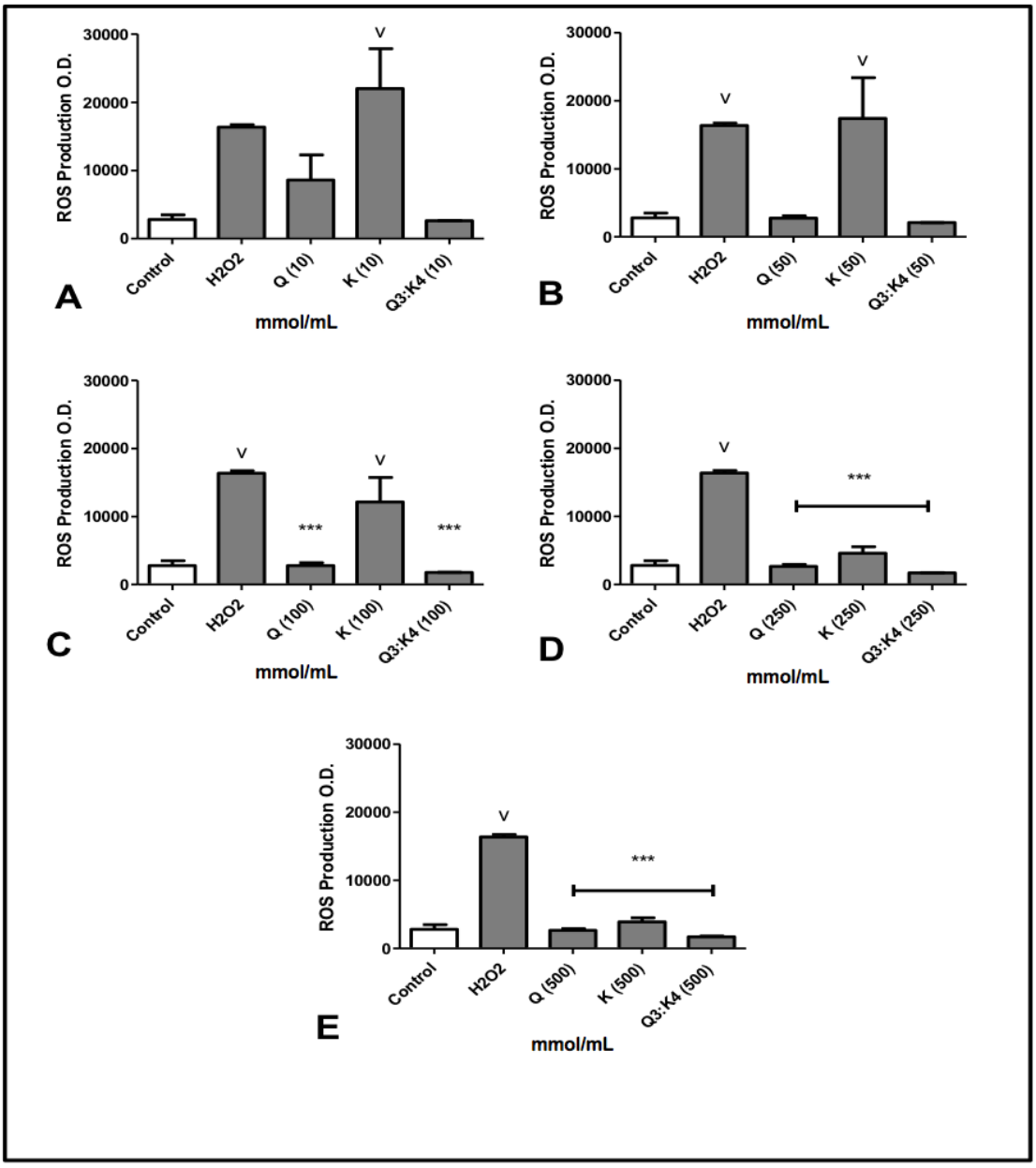


*Figure 5.5: ROS inhibition induced by H_2O_2, effects determined between groups based on single concentration. Q3:K4 (quercetin–kaempferol 3:4 combination) compared with quercetin and kaempferol at concentration 10 mmol/mL (A), 50 mmol/mL (B), 100 mmol/mL (C), 250 mmol/mL (D) and 500 mmol/mL (E) on EA.hy926 cells. v: p<0.001 compared to control. ***: p<0.001 compared to H_2O_2.*

5.4.4 Effects of NXQ on H_2O_2-induced LDH leakage in EA.hy926 Cells

As presented in Figure 5.6A, EA.hy926 cells incubated with H_2O_2 significantly increased LDH leakage compared to the control group. Cells pre-treated with NXQ (10, 50, 100, 250 and 500 μg/mL) significantly attenuated H_2O_2-induced LDH leakage in a concentration-dependent

manner ($p<0.001$, n=3) (Figure 5.6A). Similarly, in Figure 5.6B, kaempferol significantly reduced LDH leakage induced by H_2O_2 at concentrations 50–500 mmol/mL ($p<0.001$, n=3). In contrast, cells pre-treated with quercetin showed a dose-dependent manner 50 mmol/mL ($p<0.05$), 100 mmol/mL ($p<0.01$), 250 and 500 mmol/mL ($p<0.001$) in inhibiting LDH production respectively (n=3) (Figure 2.6C).

Cells pre-treated with quercetin and kaempferol (3:4) combination (10–500 mmol/mL) significantly attenuated the increased LDH leakage induced by H_2O_2 ($p<0.001$, n=3) as shown in Figure 5.6D.

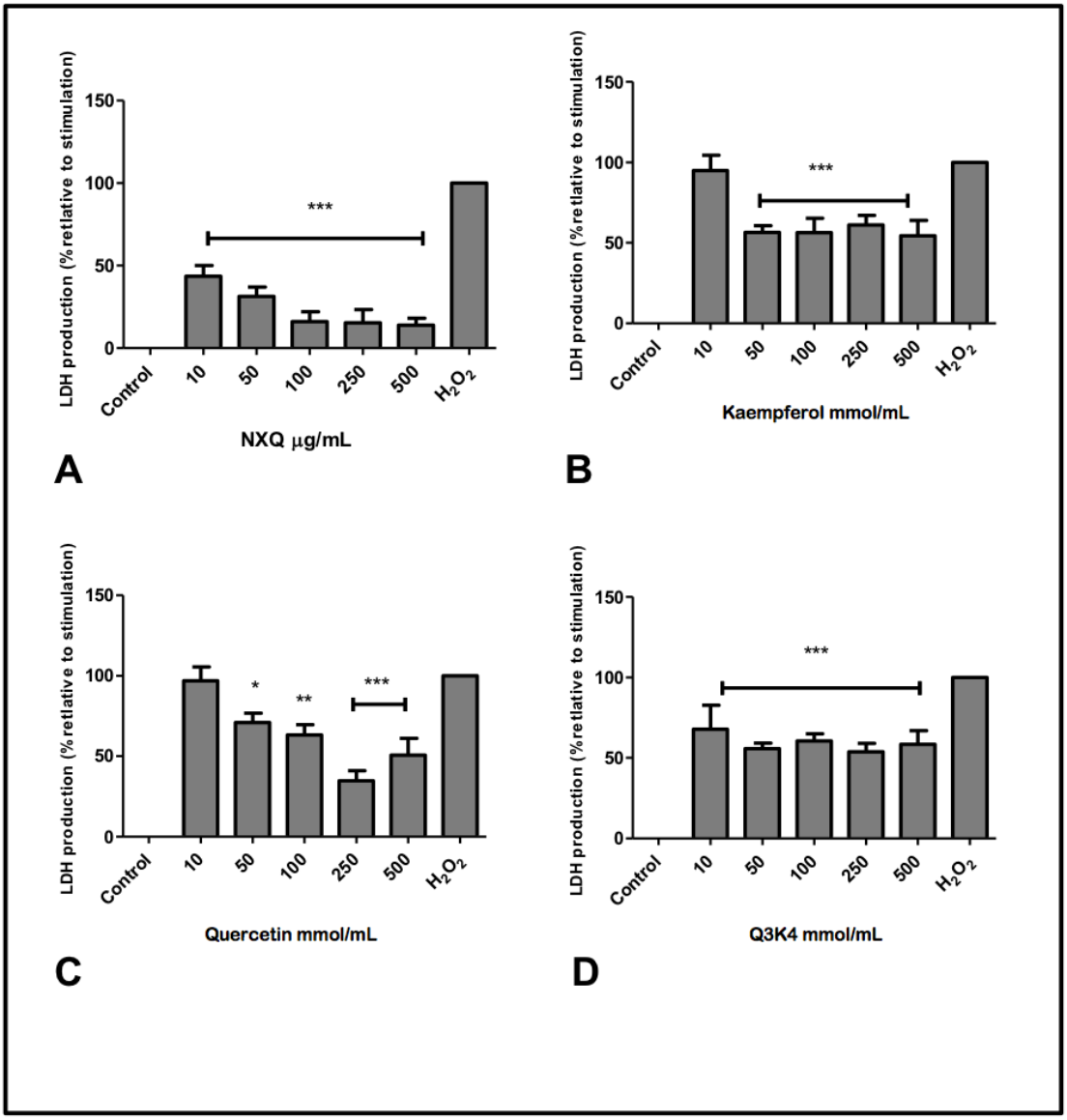


*Figure 5.6: LDH leakage induced by H_2O_2 (0.5mM) in EA.hy926 endothelial cells. LDH leakage was determined using LDH cytotoxic kit mixing the supernatant with the reaction mixture. LDH leakage was expressed as a percentage compared to H_2O_2 treatment only. NXQ with concentrations between 10 and 500 µg/mL was tested (A). Q3:K4 (quercetin–kaempferol (3:4) combination) (B) with concentrations between 10 and 500 mmol/mL were tested. Quercetin (C) and kaempferol (D) with concentrations from 10 to 500 mmol/mL were tested retrospectively. All results were expressed as mean±SEM from three separate experiments. *p<0.05, **p<0.01 and ***p<0.001 vs H_2O_2 treatment only (n=3).*

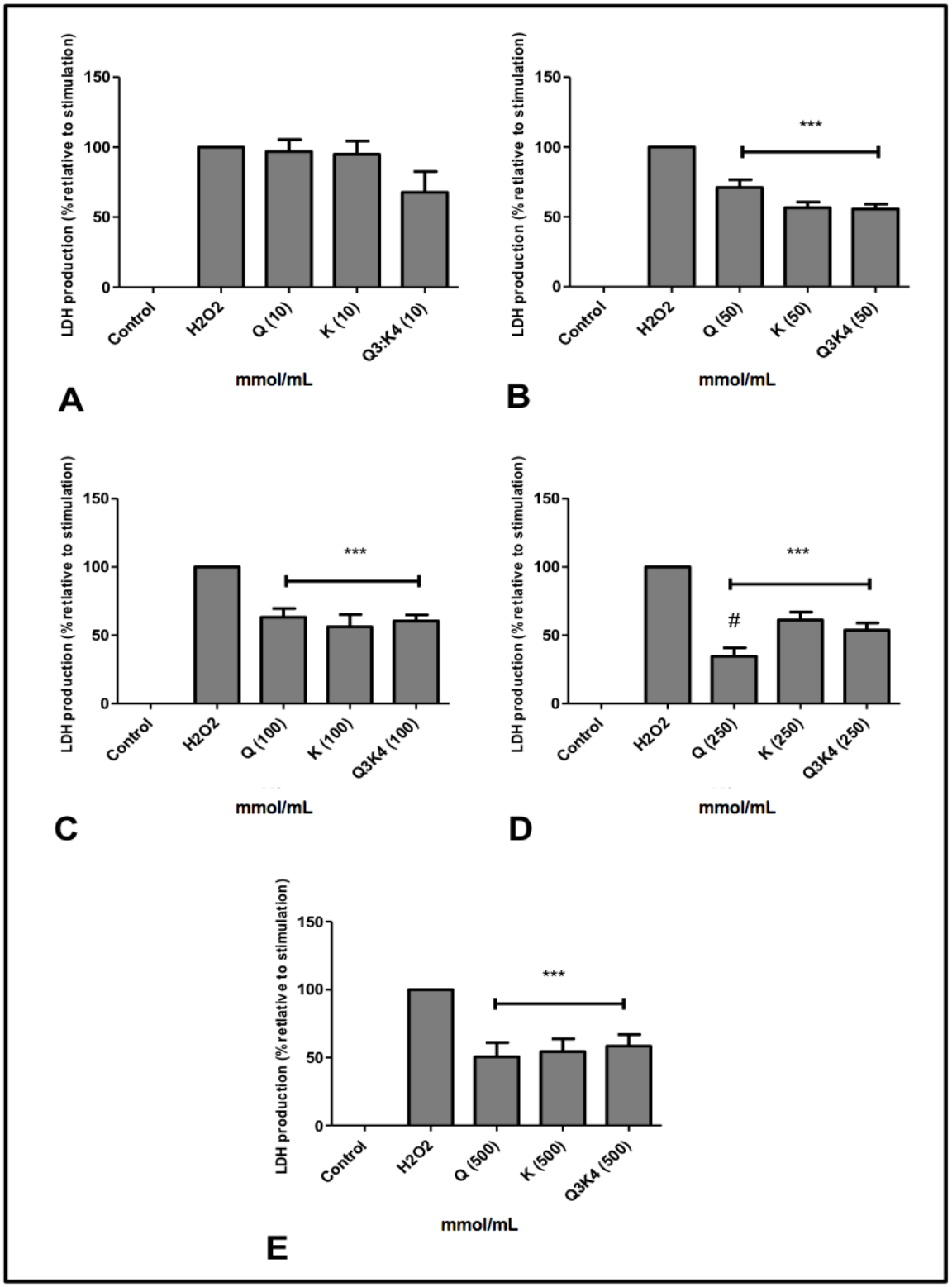


*Figure 5.7: LDH inhibition induced by H_2O_2, effects determined between groups based on single concentration. Q3:K4 (quercetin–kaempferol combination 3:4) compared with quercetin and kaempferol at concentration 10 mmol/mL (A), 50 mmol/mL (B), 100 mmol/mL (C), 250 mmol/mL (D) and 500 mmol/mL (E) on EA.hy926 cells ($p<0.001$; n=3).***: $p<0.001$ compared to H_2O_2.*

The comparison of quercetin, kaempferol and quercetin–kaempferol combination responses to the LDH attenuation induced by H_2O_2 is shown in Figure 5.7. Quercetin, kaempferol and quercetin–kaempferol (3:4) combination appear to significantly attenuate LDH leakage at concentrations of 50, 100, 250 and 500 mmol/mL. However, quercetin at 250 mmol/mL seems to have the strongest effect in attenuating LDH leakage compared to kaempferol and the quercetin–kaempferol combination. The quercetin–kaempferol combination does not appear to be better at inhibiting LDH leakage than quercetin alone.

5.5 Discussion of the protective activity of NXQ in cell survival

Endothelial dysfunction caused by elevated cerebrovascular oxidative stress is one of the main features of reperfusion injury and the consequent loss of BBB causes irreversible neural damage (Alfieri et al., 2013; Nour, Scalzo, & Liebeskind, 2012; Poon et al., 2010). Therefore, interventions that can protect endothelial cells from ROS-induced damage would be beneficial in ischaemic cerebral injuries. In the present study, we demonstrated that NXQ could protect EA.hy926 cells from oxidative stress and cell damage caused by H_2O_2.

NXQ, a standardised, patent herbal extract, has been used clinically as part of traditional Chinese medicine to improve cerebral atherosclerosis, transitory ischaemia syndromes, cerebral thrombogenesis, cerebral thrombosis sequelae, apoplexy sequelae and cerebral embolism, with minimal adverse effects (Bei et al., 2007; Y. Cai & Yang, 2001). Previous studies suggest that NXQ has potential lipid-lowering, oxidant-scavenging, anti-inflammatory and anti-hypertensive effects. It has been reported that NXQ possesses neuroprotective properties against ischaemia-related cerebral injuries (Bei et al., 2007). However, its role in vascular protection was not clearly defined. Microvascular injuries after reperfusion are regarded as the main cause of major neuron damage (Khatri, McKinney, Swenson, &

Janardhan, 2012; Nour et al., 2012). In this study, we evaluated the effects of NXQ on H_2O_2-induced microvascular injury to further understand the mechanisms underlying neuro- and cerebrovascular-protective effects of NXQ in ischaemic stroke.

First, we conducted a toxicity test of NXQ with various concentrations from 10 to 1000 μg/mL on EA.hy926 cells. We found that NXQ at concentrations over 500 μg/mL showed a toxicity effect on cell viability ($p<0.01$; $n=3$) in comparison to controls. This result was similar to that of Bei et al. (2004), who reported that NXQ markedly decreased cell viability in a dose-dependent manner when NXQ concentrations reached 500 μg/mL.

Additionally, we evaluated the protective effect of NXQ on H_2O_2-induced cell injury in EA.hy926. Our results showed that NXQ (10–500 μg/mL) significantly suppressed the elevated ROS production induced by H_2O_2. However, NXQ only restored cell viability at higher concentrations, from 250 to 500 μg/mL. This suggests NXQ is capable of protecting cells against oxidative stress by altering the ROS level, but not strong enough to protect EA.hy926 cells from apoptosis at concentrations less than 100 μg/mL.

In contrast, Bei et al. evaluated NXQ at concentrations between 2 and 30 μg/mL and reported that at 30 μg/mL NXQ significantly restored cell viability against H_2O_2 insult. However, in the current study NXQ did not show any significant protection at concentrations from 2 to 10 μg/mL. This discrepancy between the two studies may be due to the variation of cell lines and their susceptibility to NXQ treatment, and incubation time. In the latter case, Bei et al. (Bei et al., 2004) pre-treated the cells with NXQ for two hours, compared to 30 minutes in the present study, suggesting the dose and treatment time play a critical role in NXQ's effects on cell viability in response to H_2O_2 insults.

In addition, our study also showed that NXQ (10–500 μg/mL) significantly suppressed the release of LDH leakage via restoring damaged plasma permeability induced by H_2O_2 ($p<0.001$,

n=3). This result is consistent with the findings of Bei et al. In their study, NXQ attenuated LDH leakage in a dose-dependent manner even though the effective concentration range (2–30 μg/mL) was lower.

In another study, Bei et al. (Bei et al., 2005a) reported the potential neuroprotective effects of NXQ using an H_2O_2-induced oxidative stress injury model in NG108-15 cells. The results of the study confirmed that NXQ reduced elevation of malondialdehyde in cells, increased release of intracellular lactate dehydrogenase into culture medium, and increased activity of intracellular anti-oxidant enzymes including catalase (CAT), glutathione peroxidase (GSH-Px) and the amount of the intracellular anti-oxidant glutathione (GSH).

The same group of researchers further evaluated neuroprotective effects against CI/R damage, excitotoxic injury and hypoxia–reoxygen injury (Bei et al., 2007) and showed that NXQ also exhibited significant neuroprotective effects against H_2O_2-induced oxidative stress injury by improving redox balance in a concentration-dependent fashion and enhancing the expression of anti-apoptosis gene BCL2. BCL2 is a key anti-apoptosis gene and a mitochondrial protein involved in delaying neuron death and cerebral ischaemic injury through inhibiting the formation of reactive oxygen molecules, increasing ischaemic tolerance and resistance to oxidative stress and scavenging free radicals directly (Kane et al., 1993, Martinou et al., 1994; Chen & Simon, 1997).

In answering the question of whether the main bioactive compounds of NXQ, quercetin and kaempferol, play a role in the observed effect of NXQ, the current demonstrated that both quercetin and kaempferol significantly reduced elevated ROS. Quercetin, in particular, inhibited the generation of ROS-induced by H_2O_2 at all concentrations 50–500 mmol/mL ($p<0.001$, n=3), whereas kaempferol significantly inhibits the elevation of ROS only at higher concentrations (250 and 500 mmol/mL) ($p<0.001$, n=3). However, quercetin and kaempferol

alone do not improve cell viability ($p>0.05$, $n=3$) at all concentrations, suggesting that these compounds when acting alone did not protect the cell from apoptosis. NXQ, on the other hand, significantly restored cell viability at 250–500 μg/mL, suggesting possible synergistic or additive effects among the individual bioactive compounds in the mixture. To further clarify whether the two bioactive compounds have an additive or synergistic effect, we tested a combination of quercetin and kaempferol at the same ratio (3:4) detected in NXQ extract. The quercetin–kaempferol combination significantly improves cell viability at concentrations of 50, 100, 250 and 500 mmol/mL ($p<0.001$, $n=3$). This suggests that quercetin and kaempferol when used together may have a synergistic effect in restoring cell viability caused by H_2O_2.

Quercetin and kaempferol also show an ability to markedly inhibit LDH release. Quercetin most significantly inhibited LDH release at concentrations 50–500 mmol/mL in a concentration-dependent manner ($p<0.001$, $n=3$). Kaempferol, on the other hand, inhibited LDH release at concentrations 50–500 mmol/mL ($p<0.001$,$n=3$). The effects of a combination of quercetin and kaempferol at a ratio of 3:4 was also investigated. Although the combination demonstrated a significant inhibition of LDH leakage, its effect does not appears to be better than that of quercetin alone, suggesting that quercetin may play a greater role in suppressing LDH leakage in the mixture.

Soobrattee and colleagues (Soobrattee, Neergheen, Luximon-Ramma, Aruoma, & Bahorun, 2005) evaluated the anti-oxidant effects of quercetin and kaempferol and reported that kaempferol had a weaker oxidant-scavenging property compared to other flavanol aglycones such as quercetin and myricetin. The authors suggest that the low anti-oxidant activity of kaempferol could be attributed to the presence of a single hydroxyl group in the B ring which apparently makes little contribution even in the presence of the conjugated double-bond

system and the 3-OH group (Soobrattee et al., 2005). Further to this, our results showed that the combination of quercetin and kaempferol (3:4) produced a greater effect in reducing ROS elevation ($p<0.05$) than quercetin and kaempferol alone at all concentrations. This observation further supports the hypothesis that quercetin and kaempferol exert an additive or synergistic effect on cell viability at a ratio detected in NXQ extracts. This conclusion is consistent with that of Hidalgo et al. (2010), who propose that flavonoids present in a mixture can interact and that their interactions can affect the total anti-oxidant capacity of the mixture.

In their study, Hidalgo et al. report that pelargonidin-3-glucoside and kaempferol, with only one hydroxyl group in the B ring, individually possessed the lowest anti-oxidant capacity. However, when kaempferol was paired with myricetin, quercetin or quercetin-3-glucoside, a statistically significant increase in anti-oxidant activity was observed. While the ratio of the compounds in their combination was not clearly defined, the results suggest that flavonoids could interact when mixed together, leading to synergistic and/or antagonistic effects.

Put together, the results of this study demonstrate that NXQ possesses cell-protective properties against H_2O_2-induced cytotoxicity and demonstrates significant effects on restoring cell viability, reducing ROS elevation and inhibiting LDH leakage. In addition, preliminary evidence supports the synergistic/addictive interactions between the two key bioactive flavanols of NXQ, quercetin and kaempferol. Based on these results, more research is warranted to understand the nature of and mechanisms underlying the synergistic effects between quercetin and kaempferol and their potential role in the observed clinical effect of NXQ.

5.6 Development of an in vivo hypoxia model in zebrafish

In this study, we have attempted to establish a zebrafish hypoxia model to be used for the *in vivo* evaluation of the neuroprotective effect of NXQ. This model development was based on the previously published results of Yu et al. (X. Yu & Li, 2011, 2013) and Braga et al. (Braga et al., 2013). After several attempts, a model was established. However, the results from the hypoxia and normoxia groups were not significantly different, in contrast to previous reports. Hence, the test of NXQ using this model did not proceed. This section describes the model development details, the preliminary data collected, and the problems identified and potential troubleshooting strategies for future studies.

5.6.1 Zebrafish as an in vivo model for drug screening

Zebrafish (*Danio rerio*) is a freshwater fish that originated in northern India and adjacent countries. They are usually found in shallow water or streams and are a widely used vertebrate model organism in biological and biomedical research (Kari, Rodeck, & Dicker, 2007; Langheinrich, 2003). Zebrafish exhibit unique characteristics that are desirable in an animal model, including having a short reproductive cycle and being easily bred in large numbers (100–150 embryos per mating). In addition, the availability of genetically manipulated zebrafish strains, functional knockdown of specific genes by Morpholino technology and easy and low-cost maintenance provide zebrafish an advantage over other existing disease models. Furthermore, their *ex utero* development and optical clarity during embryogenesis allow for visual analysis of early developmental process and organ morphology (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995).

Zebrafish develop relative quickly, with the entire body structure plan established and all the precursor cells and tissues of the brain, eyes and heart easily visualised using light

microscopy at 24 hours post fertilisation (hpf). At 72 hpf, embryogenesis is complete, and the internal organs, including cardiovascular system, gut, liver and kidney, are fully matured by 96 hpf. This rapid development is comparable to three months of development in human embryos (Y. Sun, Fang, Xu, Lu, & Chen, 2015). In comparison to other organisms, such as fruit flies and worms, there are numerous symmetries between zebrafish and humans that make zebrafish a desirable model organism for studying complex biological processes, including generation of the nervous system, kidney, heart, hematopoietic system, and notochord (Kari et al., 2007; Kimmel et al., 1995; Parng, Seng, Semino, & McGrath, 2002).

5.6.2. Acute hypoxia-induced brain damage model in zebrafish

Recently, large-scale genetic screens have generated hundreds of zebrafish mutants, many of which show phenotypes resembling human disease states such as congenital heart disease, Alzheimer's disease and stroke. Moreover, the ectopic expression of transgenes can be used not only to study 'gain of function' phenotypes in zebrafish, but also to generate chimeric animal models to study specific human proteins such as receptors or signalling effectors (Langheinrich, 2003; Parng et al., 2002).

Currently, there are few established models to simulate the various types of ischaemic stroke. As ischaemic damage is primarily associated with deprivation of oxygen, hypoxia models were introduced as the most comparable model for the acute ischaemic condition (X. Yu & Li, 2011). A hypoxic environment was created by pumping nitrogen into a concealed container to ensure an anoxic environment where oxygen saturation is below a specified level. Zebrafish are then transferred into the chamber, triggering the onset of hypoxic brain damage. This model allows for a relatively quick induction of ischaemic stroke and is therefore a viable model for the assessment of both preventative measures (pre-treated) and therapeutic

measures (post-treated) of neuroprotection (infarct size) for an intervention. Yu and Li (2013) have further adapted this model to assess the neuroprotective effects of zinc chelation for acute ischaemic stroke. Their study was able to identify differences in infarct sizes between treatment and control groups; however, 60% of zebrafish died during hypoxic treatment before intervention was given (X. Yu & Li, 2013). Cao et al. (Z. Cao et al., 2010), who published a protocol for creating a chronic hypoxic model in zebra fish, assessed angiogenesis due to oxygen deprivation and pointed out that the oxygen saturation needed to be monitored and gradually decreased in order to reduce death rates in the fish. In this study, we aimed to develop a hypoxic model in zebrafish based on the above studies as a reliable *in vivo* model to evaluate the neuro- and cerebrovascular-protective effects of NXQ in ischaemic stroke.

6.6.2.1 Ethics statement

All animal experiments were conducted according to the ethical guidelines of Western Sydney University. The protocol was approved by Animal Ethics Committee, Western Sydney University (ethics approved protocol number A10378).

5.6.2.2 Animal

Adult zebrafish (*D. rerio*) ~6 months old were procured from the School of Medicine Zebrafish Research Facility and were kept in aquaria under a 12h light/12h dark photoperiod with half deionised water plus half dechlorinated water (made with fresh tap water with sodium thiosulfate, a dechlorinating agent, 10–15mg/L) at room temperature (X. Yu & Li, 2013).

5.6.2.3 Maintenance of zebrafish

Zebrafish were maintained in a controlled environment at a temperature of 28˚C on a 12h/12h light/dark cycle according to the zebrafish handbook (Westerfield, 1993) within the zebrafish lab facility in School of Medicine in Western Sydney University. Zebrafish were

stored in 3.5L or 5L Tecniplast tanks that complied with Australian and NSW standards, with automatic monitoring of environmental conditions such as water temperature, pH level (~pH 6.73–6.75), and dissolved oxygen saturation. Each tank housed up to a maximum of 120 fish. Adult zebrafish were fed with dry food flakes or pellets, live artemia or shell-free artemia, three times daily from Monday to Friday and once daily during weekends and public holidays in compliance with School of Medicine Zebrafish Feeding Standard Operation Procedures.

5.6.2.4 Establishment of hypoxia chamber

An hypoxic environment was created using a hypoxia chamber similar to that previously reported by Yu and Li (X. Yu & Li, 2011). The hypoxia chamber was constructed using a one-litre clear container filled with 800 mL of distilled water (Figure 5.8). A dissolved oxygen sensor (3) was placed inside the container and connected to an external readout to monitor the oxygen level of the chamber. The chamber has one port (4) which connects to a nitrogen tank, allowing for nitrogen to be perfused into the water, and a sealable lid (2) with another port (5) connecting the air space inside the chamber to the open air (1). The hypoxic environment was created by pumping pure nitrogen into the water until the dissolved oxygen level was less than 1mg/L (approximately 0.5~0.75mg/L). The time required to reach anoxia environment was recorded.

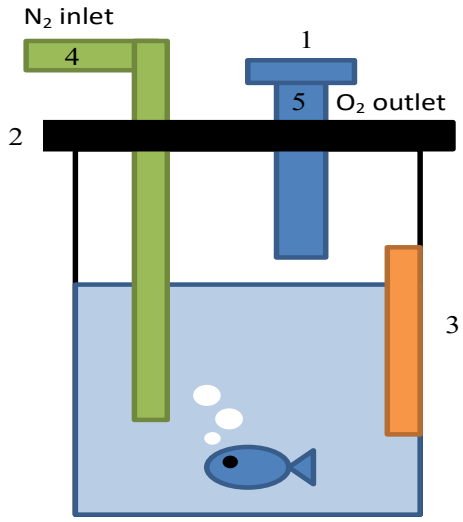


Figure 5.8: Hypoxia chamber. (1) Sealing cap for oxygen outlet port (2) Sealable lid (3) Dissolved oxygen sensor (4) Nitrogen inlet port (5) Oxygen outlet port.

5.6.2.5 Validation of hypoxia chamber and hypoxia treatment

The hypoxia model was validated to ensure the equipment and procedures produced a stable hypoxic environment similar to those previously reported. An hypoxic environment was generated by pumping pure nitrogen into the water until the desired dissolved oxygen level (<1mg/L) was reached. The chamber was repeatedly tested and oxygen levels measured in response to various rates and periods of nitrogen infusion. Once a hypoxic environment was established, a single zebrafish was transferred into the chamber. Once the lid had been resealed, nitrogen was continuously perfused for an additional period to ensure a hypoxic environment was maintained. Nitrogen was turned off and the port located on the lid was then immediately sealed. The time taken to re-establish an hypoxic environment during validation was recorded and averaged.

5.6.2.6 Measurable parameters

Several parameters were measured as the zebrafish underwent the hypoxic treatment, similar to previously reported models (Braga et al., 2013). A zebrafish was placed in the

chamber once the ideal oxygen level was achieved and was observed closely to detect the representative behaviours in response to hypoxia as described by Braga et al. (2013) (Table 12). Once the third level of behaviour sequence (i.e., maintenance of opercular beats with brief movements) was observed as the end point of global ischaemia, the time to reach this endpoint was recorded and the zebrafish was removed and transferred to a recovery chamber with oxygen level approximately 7mg/L. Death and survival rate was recorded.

For the zebrafish that survived the hypoxic treatment, duration to full recovery was assessed by recording the time between post-hypoxia treatment and fish swimming normally again in the recovery chamber. They were kept in the recovery chamber for 24 hours.

Table 12: Summary of representative behaviours reported by (Braga et al., 2013)

Behaviour sequence	*Description*
1st	Swimming at the top
2nd	Loss of posture
3rd	Maintenance of opercular beats with brief movements
4th	Death

5.6.2.7 Brain Extraction, TTC Staining and Measurement

After 24 hours of recovery time, the fish were euthanised with 8 mg/mL tricaine (ms222) (Sigma, Australia). The brain was then extracted via tissue dissection performed in cold artificial cerebral spinal fluid before staining. The average time for brain extraction was kept as minimal as possible at an average of two minutes.

Ischaemic brain damage was evaluated using 2,3,5-triphenyltetrazolium chloride (TTC) staining as described by Yu and Li (2013). The staining was performed at 24 hours after the hypoxia treatment. The entire brain was extracted immediately after dissection and

incubated in TTC solution (2% by weight) in darkness for 40 minutes. After staining, the TTC solution was discarded and the brain tissue was placed in an oven at 40°C for 4–6 hours to eliminate excess fluid. The dried brain tissue was then weighed and recorded. For dye extraction, the brain tissue was gently rinsed with two drops DMSO/ethanol (1:1) solution, and then placed individually inside a well of a 96 well plates with 100µL DMSO/ethanol (1:1) solution in darkness for extraction over six hours. The brain tissue was then removed and the supernatant of the dye was measured at 590nm with a microplate reader (Sabaeifard, Abdi-Ali, Soudi, & Dinarvand, 2014). The readings were normalised by weight and calculated for the final absorbance result. The calculation for the percentage of TTC absorbance were measured using the equation 2.

$$\% \text{ TTC absorbance} = \frac{Absorbance}{Weight\ (mg)} \text{x100} \qquad \text{[Eq. 2]}$$

5.6.3 Results

5.6.3.1 Validation of hypoxic chamber and hypoxia condition

Based on the above results, nitrogen was perfused for a minimum duration of five minutes, maintaining the oxygen level at 7mg/L according to validation averages plus 20% to ensure an anoxic environment.

5.6.3.2 Results of zebrafish undergoing hypoxia treatment

The survival rate for the hypoxia treatment was ~90%, with an average of one death in every 10 hypoxia-treated fish.

The average time to induce global ischaemia endpoint (maintenance of opercular beats with brief movements) was between 3.32 and 31.35 minutes (Table 13). The observed

recovery time (fish begin to swim after hypoxia treatment) was 11.56± 9.38 minutes on average.

Table 13: Maximum, minimum and average hypoxia and recovery time collected (n=18)

	Maximum	*Minimum*	*Average*
Hypoxia time induced global ischaemia	31.35 min	3.32 min	11.56±9.38
Recovery time until normal swimming activity observed	12.34 min	1 min	4.67±3.8

As presented in Figure 5.9, no significant difference in the TTC absorbance was observed between the hypoxia and normoxia groups (p=0.1878). The average absorbance for the hypoxia group was 35.9% for the normoxia group (control) 40.77%.

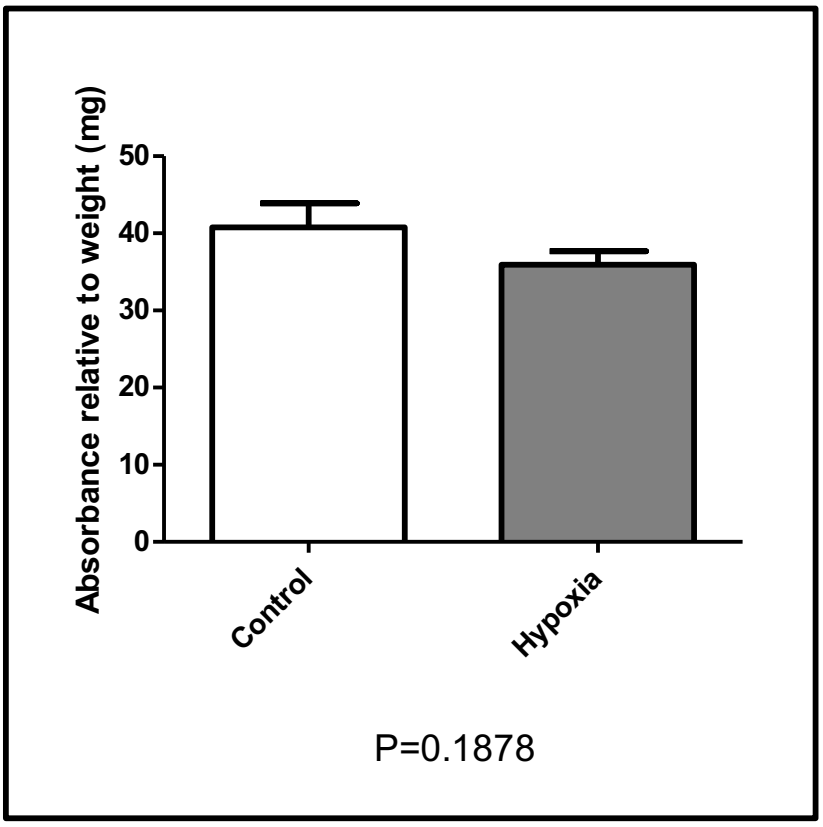


Figure 5.9: Absorbance % relative to weight (mg) of the brain sample. Control sample (n=16), hypoxia sample (n=17). Unpaired t-tests were conducted, with no significant difference obtained (p=0.1878).

5.6.4 Discussion

The model developed was not suitable for evaluation of the neuroprotective effect of NXQ as none of parameters detected a significant difference between hypoxia and normoxia groups. However, some valuable results were obtained that may shed light on further development of this potentially useful model.

Several issues were identified during the development process. The time for obtaining and preparing sliced brain tissue for staining was very long, which may lead to further tissue damage that is not purely induced by hypoxia. Initially, attempts were made to use the hypoxia end point as described by Yu and Li (X. Yu & Li, 2011, 2013), which was defined as zebrafish staying motionless for one minute. A very high fatality rate of 90% was experienced. Of a group of ten zebrafish, only one survived the hypoxia treatment, whereas Yu and Li reported a survival rate of 40%. As a result the end point was modified based on (Braga et al., 2013), and defined as maintenance of opercular beats with brief movements. This led to a significant improvement in the survival rate.

Initially the same oxygen cut-off level (0.6–0.8 mg/L) described by Yu and Li (2011) was used in the hypoxia chamber. However, this resulted in a significantly longer time to achieve the desired hypoxic condition than was reported by Yu and Li (2011). The hypoxia time needed was 3.32–31.35 min, as opposed to the 9–16 mins reported in Yu and Li's study (2011). This observation may indicate that the hypoxia tolerance of zebrafish is affected by different strain used, environment, sex, age or other factors (Arslan-Ergul & Adams, 2014; Rees, Sudradjat, & Love, 2001). Roberson et al. (2014) were the first to report that gender differences in adult fish positively affected hypoxia tolerance. In their study, hypoxic tolerance was tested in the larval stage and adulthood of fish. The authors reported that populations of fish that mounted a HIF-1 response to low O_2 during development had a higher proportion of

males as adults. Therefore, induction of the cellular hypoxia response during early development results in an improved hypoxia tolerance at the population level (Robertson et al., 2014). Further studies may be required to evaluate the potential physiological adaptability between sex in zebrafish to better design the zebrafish hypoxia model.

In addition, the absorbance extraction of TTC dye described in Yu and Li's study (2011) was not normalised by weight (eliminating water content), which introduced potential errors. In the current study, the protocol was modified by drying the brain tissue and normalising the weight of the tissue and the TTC dye absorbance.

In the current study, fish of mixed sex aged 6–18 months were used. In contrast, in Yu and Li's study (2011) fish with body size 30–49mm total length (mean: 41.56mm; median: 42.5mm) were used (X. Yu & Li, 2011, 2013). Zebrafish is a popular animal model used to study ageing and human diseases. Like humans, aged zebrafish demonstrate reduced exercise tolerance (Gilbert, Zerulla, & Tierney, 2014), degenerative changes in the spinal column (Hayes et al., 2013), and cognitive decline (Arslan-Ergul & Adams, 2014; L. Yu, Tucci, Kishi, & Zhdanova, 2006). And, like humans, differences in genetics have also been shown to contribute to differences in lifespan (Gerhard et al., 2002). Recently, mutations that affect atrial and ventricular development have been reported and age-related mitochondria have been identified (Almaida-Pagán, Lucas-Sanchez, & Tocher, 2014). A recent study by Sun et al (Y. Sun et al., 2015) evaluating the age-related functional modification and pathophysiological changes in zebrafish hearts positively identifies that impairment in cardiac performance in the older fish population and the cardiac ageing process in zebrafish are comparable to those in humans. Although a comparable study of zebrafish brains was not available, an investigation of cerebral pathophysiology and age-related difference would be useful in guiding future model development.

The underlying principle of TTC staining involves the formation of red formazan TTC products, predominantly in the mitochondrial compartment. This staining procedure enables delineation of cerebral infarct sizes, and thus reliably detects the amount of damaged brain tissue caused by the global ischaemia (Benedek et al., 2006; Popp et al., 2009). No statistical significance was detected between the hypoxia group and normoxia group, after eight separate experiments. This may be due to the fact that zebrafish have a high recovery capacity compared to other species (Braga et al., 2013; Krock, Skuli, & Simon, 2011). Similar to our result, the study conducted by Braga et al. (2013) investigated brain damage after hypoxia exposure at stage 1 to 4 and noticed complete recovery between 24 and 48 hours. Hypoxia is the principal regulator of VEGF expression, which elicits angiogenic responses to combat tissue ischaemia. It is also evident that the acute hypoxia insult can cause graduated damage to mitochondria activities (Braga et al., 2013; Murphy, 2009) at particular time-points; however, recovery was usually observed within 48 hours from introduction of the normoxic environment. Whether these reversal effects were due to the tolerance of fish to hypoxia, or to hypoxia-induced angiogenesis, was unclear.

In summary, although the model development was not successful, several valuable lessons were learned. For future development of zebrafish hypoxia models, the complexity of age-related pathophysiology and variation in sex-biased hypoxia tolerance should be considered. In addition, more sensitive parameters (such as metabolomics and/or proteomic measures) could be used to detect the differences between hypoxia and normoxia conditions. Due to time limits on this research, these new directions were not pursued.

Chapter 6. Evaluation of Pro-Angiogenic Effects of NXQ Extracts

6.1 Introduction

Angiogenesis is the morphogenic process through which new blood vessels sprout from pre-existing vessels. It plays a pivotal role in various physiological and pathological conditions and is coordinated by the endothelial cells and their niche (Kajdaniuk, Marek, Borgiel-Marek, & Kos-Kudła, 2011). Inadequate vasculature maintenance or insufficient vessel growth may lead to tissue ischaemia, while excessive vascular growth or abnormal remodelling may promote cancer, inflammatory disorders and retinopathies (Denekamp, 1993; Polverini, 1995).

Angiogenesis is considered an essential physiological process for maintaining blood vessel homeostasis by facilitating nutrient transportation, as well as promoting tissue growth and repair (Potente, Gerhardt, & Carmeliet, 2011). In ischaemia, the injured tissue triggers an angiogenic response that helps the blood flow adjacent to the ischaemic core in order to rebuild the blood vessel and restore the delivery of oxygen. Lack of angiogenesis can complicate recovery from ischaemic conditions such as myocardial infarction and ischaemic stroke, which are associated with inadequate blood supply (Potente et al., 2011). Presently, therapeutic angiogenesis is a novel approach to improving the outcome of ischaemic vascular diseases (Losordo & Dimmeler, 2004a). In such conditions, the therapeutic goal is to stimulate angiogenesis, or allow generation of neovascularisation to improve perfusion, deliver survival factors to sites of tissue repair, mobilise regenerative stem cell populations and, ultimately, restore form and function to the tissue (Losordo & Dimmeler, 2004b). Early clinical evidence evaluating the effects of angiogenesis and vasculogenesis on ischaemic diseases with cell-based therapy has shown promising results. For example, in a clinical study, bone marrow-derived mononuclear cells were infused into patients 5–9 days after acute myocardial infarction and shown to enhance regional infarct region perfusion when compared to ten nonrandomised control patients, as assessed by thallium scintigraphy. Moreover, stroke

volume, end-systolic volume and contractility indices were improved after cell therapy (Yousef et al., 2009).

The angiogenesis process typically consists of six major steps: (1) vasodilation of the parental vessels, reducing endothelial cell contact; (2) degradation of the basement membrane by a variety of proteolytic enzymes; (3) migration and proliferation of endothelial cells at the spearhead of new vessels; (4) production of capillary lumen and formation of tube-like structures; (5) basement membrane synthesis; and (6) recruitment of vascular smooth muscle cells. The sequence of the molecular events resulting in angiogenesis requires precise fine-tuning of multiple signalling pathways, cell–cell and cell–matrix interactions. In these events, chemical stimulants such as vascular endothelial growth factor (VEGF), fibroblast growth factors (FGF) and various angiogenic proteins such as integrins and prostaglandins typically play an initiating role (Jain, 2003; Kelm et al., 2005).

Therapeutic angiogenesis aims to induce, augment and control the host angiogenic response in order to revascularise ischaemic tissues, and often involves delivery of growth factors or stem/progenitor cells. Growth factors may be delivered in the form of proteins or genes encoding target proteins. The premise behind this approach is to apply well-studied growth factors such as VEGF to ischaemic tissues to guide angiogenic cellular and tissue behaviour. VEGF and bFGF are the two most well-studied growth factors and have reached human clinical trials (Deveza, Choi, & Yang, 2012). VEGF is the most important regulator of physiological angiogenesis during growth and healing in response to hypoxia (Carmeliet & Jain, 2011; Losordo & Dimmeler, 2004a). VEGF is upregulated 30-fold by hypoxia-inducible transcription factor, which is more than any other inducible angiogenic factors (Deveza et al., 2012). To date, there have been several preclinical and clinical studies evaluating the safety and efficacy of growth factors for inducing angiogenesis in ischaemic disease in relation to

heart, brain and peripheral arteries. Numerous clinical trials applying gene therapy have mostly used adenovirus or plasmids to deliver VEGF or bFGF (Gupta, Tongers, & Losordo, 2009). Early phase 1 and 2 human clinical trials support the safety of angiogenic gene delivery; however, efficacy has not yet been conclusively demonstrated. Moreover, one phase III trial investigating patients with critical limb ischaemia who received FGF-1 expressing plasmid failed to demonstrate benefits (Chu & Wang, 2012). Another phase III trial utilising an adenoviral vector to express FGF-4 in patients with angina also revealed no difference from the placebo group (Henry et al., 2007). The inconsistency of the trial outcomes may be caused by differences in patient selection criteria and assessment methods (Chu & Wang, 2012; Deveza et al., 2012). Despite of the complex mechanisms of neovascularisation, neurovascular remodelling is a key component of recovery after stroke. It is increasingly recognised that without vascular support, neuronal plasticity cannot be achieved (Navaratna, Guo, Arai, & Lo, 2009). Over the past few years, complex mechanisms are beginning to be dissected that underlie these endogenous responses in damaged brain parenchyma. As we understand more of these pathways, we may design better therapies to augment angiogenesis for promoting recovery. While augmentation of angiogenesis pathways is not the aim of this study, VEGF remains the prototypical growth factor involved (Navaratna et al., 2009). Given that traditional herbal medicines are usually less invasive than conventional pharmaceutical treatment, they might provide the optimal approach for promoting angiogenesis in the brain.

6.1.1 Vascular endothelial growth factor (VEGF)

VEGF is one of the major mediators for angiogenesis process. VEGF is a major regulator of neovascularisation under physiological and pathological conditions (Gerber et al., 1999) and

is produced in five homodimeric isoforms (VEGF121, VEGF145, VEGF165, VEGF189 and VEGF206). The various isoforms of VEGF differ in their expression levels and localisation. They are involved in (a) the formation of immature vasculature (with VEGF receptor 2 (VEGFR-2) mediated signalling in angioblasts resulting in formation of the dorsal aorta and the cardinal vein; (Yancopoulos et al., 2000); (b) induction of migration and proliferation of endothelial cells (Conway, Collen, & Carmeliet, 2001); (c) vessel dilation and sprouting in the presence of angioprotein-2 (Tsigkos, Koutsilieris, & Papapetropoulos, 2003); (d) stabalisation of immature vasculature (VEGF-induced platelet-derived growth factor secretion of endothelial cells facilitates recruitment of mural cells; (Blau & Banfi, 2001); (e) sequestration of angioprotein-2 which destabilises (Tsigkos et al., 2003); (f) suppression of apoptosis (Folkman, 2003); (g) branching, remodelling and pruning of vasculature (protease-mediated release of matrix-sequestrated VEGF; (Peirce & Skalak, 2003); and (h) vessel specialisation (arterial growth promoted by VEGF–VEGFR2 neurophilin-1 signalling). Studies indicate that VEGF action goes beyond vascularisation and may be involved in neurogenesis (Jin et al., 2002) as well as growth- and survival-modulation of chondrocytes, a cell that secretes the matrix of cartilage and becomes embedded in it (Schipani et al., 2001).

Several control circuits are at work to balance VEGF action. For example, in response to hypoxia, activation of hypoxia-inducible factor (HIF-)1 alpha results in induction of angiogenic factors such as VEGF which bind to their receptors on endothelial cells, leading to their activation (Gapizov et al., 2018). Another inducible factor is endostatin, a matrix-associated protease-mediated cleavage product of collagen XVIII that inhibits the VEGF-induced moblisation of endothelial cells (Gapizov et al., 2018).

In recent years, the potential of angiogenesis as a therapeutic strategy for stroke recovery has been intensively studied (Navaratna et al., 2009). Many traditionally used herbal sources

contain potential pro-angiogenic substances that are usable in apoplexy conditions. For instance, *Astragalus*, *Panax notoginseng* (P. Chan, Thomas, & Tomlinson, 2002) and *Angelica sinensis* (J. Sun, Tan, Huang, Whiteman, & Zhu, 2002) have been used clinically for ischaemic heart diseases. NXQ has been clinically used for apoplexy diseases such as angina, ischaemic heart diseases, ischaemic stroke and atherosclerosis (Y. Cai & Yang, 2001). We hypothesise that the clinically observed beneficial effect of NXQ is at least partly mediated via its pro-angiogenic properties. Hence, the aim of this chapter is to evaluate the angiogenic effect of NXQ using two cultured endothelial cell *in vitro* models and an *in vivo* model of angiogenesis using zebrafish.

6.1.2 Evaluation of angiogenesis effects in in vitro bioassays

Angiogenesis effects are commonly studied *in vitro* using vascular endothelial cells based on three essential characteristics of angiogenesis: cell proliferation, cell migration and tube formation (Staton, Reed, & Brown, 2009). Typically, endothelial cells start to proliferate before migration. At last, the cells outgrow, and form new, tubular structure with the assistance of adhesion molecules such as integrins, cadherins or selectins (Bischoff, 1997; Fan, Yeh, Leung, Yue, & Wong, 2006). The three bioassays described below are the most commonly used models to investigate the effects of pro-angiogenic and anti-angiogenic agents. Each of these assays mimics one of the major processes of angiogenesis.

6.1.2.1 Cell proliferation assay

Crystal violet staining was applied to determine the rate of cell proliferation. Crystal violet is frequently used as a histological stain for classifying bacteria based on Gram's method, first published in 1954 (Bartholomew & Finkelstein, 1954). It is used also in cellular models to determine the number of cells, as crystal violet can bond to the DNA of both adherent and

non-adherent cells (Kueng, Silber, & Eppenberger, 1989). The density cell number based on the colour stained to the cell can then be quantified with a microplate reader with the wavelength at 590nm (Gillies, Didier, & Denton, 1986). The absorbance of the density is directly proportional to the total number of cells detected.

In this study, we evaluated whether NXQ could induce cell proliferation using cultured human endothelial cell, EA.hy926, using the crystal violate assay. In brief, the cells were pre-treated with testing compounds and compared with a vehicle (0.1% DMSO) and a positive control (VEGF 50 μg/mL). The vehicle control group was considered as the normal cell growth rate with a maximum cell number (100%). VEGF was used here as a positive control for model validity on proliferation. The % of proliferation was calculated using the following equation.

$$\% \text{ of proliferation in relation to vehicle control} = \frac{Absorbance\ of\ sample\ treated\ cells}{Absorbance\ of\ control\ cells} \times 100\% \quad \text{[Eq. 3]}$$

6.1.2.2 Cell migration (wound healing) assay

Wound scratch assay is a simple and well-developed method for measuring the cell migration activity *in vitro* (C.-C. Liang, Park, & Guan, 2007). The measurement was obtained based on observing the gap-closing activity of the cells, which was either promoted or inhibited in the tested samples (Yarrow, Perlman, Westwood, & Mitchison, 2004). The change in gap area caused by the test compound can be observed under a microscope and recorded by a camera. Computer software such as Image J or Tscratch is frequently used to quantify and compare the open area of the cells before and after treatment period (Gebäck, Schulz, Koumoutsakos, & Detmar, 2009; Poujade et al., 2007).

6.1.3 Evaluation of angiogenesis effects in zebrafish model

While *in vitro* assays are cheaper and simpler models that could provide basic information on the angiogenic properties and potential signalling mechanisms of drugs and bioactive compounds, it is not uncommon for *in vitro* studies to fail to translate effectively to a complete physiological system. Therefore, *in vivo* models are essential for the evaluation of new drugs and herbs.

Aside from the numerous established applications of zebrafish and zebrafish embryos, they are also used to study angiogenesis, apoptosis, toxicity responses and preclinical drug screening (Parng et al., 2002). Transgenic zebrafish and their embryos are commonly employed in studies assessing vascular changes in angiogenesis and vasculogenesis (Langheinrich, 2003; Lawson & Weinstein, 2002). Tg(fli1:EGFP) is a common transgenic zebrafish model in which enhanced green fluorescent proteins are expressed in all endothelial cells of the vasculature, allowing for vascular changes to be detectable through fluorescent microscopy (Higashijima, Hotta, & Okamoto, 2000). Numerous studies have utilised this advantage to investigate vascular modifications of zebrafish embryos in both pathological conditions and pharmacologically induced changes due to the relatively quick embryonic development phase. For instance, Lam et al. (2008) successfully identified angiogenic changes in subintestinal vessels (SIV) of Tg(fli1:EGFP) embryos induced by a herbal extract of *A. sinensis*. Hong et al. (2009) adopted the same method to assess the angiogenic effects in zebrafish embryos of saponin extract from the herb *P. notoginseng*. Lam et al. (2008) and Hong et al. (2009) were both able to identify changes in embryo SIV at 72 hpf under fluorescent microscopy in three separated-dose variants of the herbal extracts when compared with positive and negative controls. Both studies observed gross vascular modifications using imaging software by comparing overall vasculature changes with controls;

however, modifications to the vasculature could not be quantified as no distinction could be made between normal physiological vasculogenesis and drug-induced angiogenesis.

Addressing this issue, Tran et al. (2007) demonstrated an automated, quantitative screening assay for angiogenic compounds in zebrafish embryo models. The assay-adapted software could differentiate the normal vasculogenesis from drug-induced angiogenesis by identifying the width of arteries, the fluorescent intensity of endothelial cell body, and the outgrowth from the cell body. These features allowed for quantification and in-depth interpretation of vascular changes which were induced chemically rather than by normal progressive development.

In summary, zebrafish provide a series of advantages as a model of study due to their rapid development, optical transparency, high number of offspring and straightforward strategies for forward and reverse genetic manipulation. Furthermore, the early development of a cardiovascular system in the transparent zebrafish embryo and larva translates into a unique opportunity for direct observation of blood flow and the development of the system's related organs in both wild type and transgenic fish, without the need for complex instrumentation. Lastly, genetic studies have revealed conservation of the molecular pathways between fish and mammals, making research in vascular biology in teleosts directly translatable into potentially relevant information for human health.

6.1.3.1 Vascular development in transgenic zebrafish

Transgenic technology has enhanced the inherent *in vivo* imaging capabilities that zebrafish may offer the investigator. Though vessels and blood flow can easily be visualised with a simple dissecting scope, it was with introduction of tissue-specific expression of fluorescent protein that vascular and blood development could be examined in great detail. Confocal

microscopy and time-lapse imaging can both be carried out with live specimens, which allows detailed morphogenetic movements and cell shape changes to be followed directly.

Thus, vascular development has been described in great detail, both from the anatomical and cellular point of view and with comprehensive examination of the molecular player involved (Gore, Monzo, Cha, Pan, & Weinstein, 2012; Schuermann, Helker, & Herzog, 2014).

The development of the vascular anatomy of the zebrafish has been extensively described and has demonstrates a high similarity to that of other vertebrates. Many of the studies of vascular development have used molecular tracers during the early embryonic stages of zebrafish. One such strategy is the injection of fluorescent microspheres and their detection after lumenisation and anastomosis of the vascular network is complete. This strategy has also been used to compare the development of blood and lymphatic vasculature in zebrafish (Ellertsdóttir et al., 2010; Gore et al., 2012; Isogai, Horiguchi, & Weinstein, 2001).

A remarkable feature of zebrafish compared to other vertebrates is that they rely on passive oxygen diffusion during the early embryonic stages, rather than oxygen perfusion, as the completion of vascular development takes place after hatching. Moreover, the generation and characterisation of zebrafish mutants has shown that embryos are able to sustain normal development even in absence of a functional vascular system or in the absence of blood. This attribute has made the analysis of late phenotypes related to circulatory system malformations possible (Chávez, Aedo, Fierro, Allende, & Egaña, 2016; Isogai, Lawson, Torrealday, Horiguchi, & Weinstein, 2003; Stainier, Weinstein, Detrich, Zon, & Fishman, 1995).

6.1.3.2 Zebrafish angiogenic pre-inhibition model by VEGF receptor tyrosine kinase inhibitor II (VRI)

Angiogenesis is one of the main focuses of vascular regeneration research; models for this type of vascular development and growth have been developed. In the embryo, the

intersegmental vessels form by angiogenic sprouting from the dorsal aorta and have been the target of studies using drugs or genetic perturbations. Angiogenic sprouting is inducible by recombinant VEGF (Q.-q. Xin et al., 2016; H. Zhao, Huang, & Lin, 2016).

VEGF receptor tyrosine kinase inhibitor II (VRI) is a small molecule that strongly inhibits the kinase activity of VEGF receptors 1 and 2 (Furet et al., 2003). Visual analysis of zebrafish embryos from 24 hpf to 36 hpf, VRI can completely inhibit development of intersegmental blood vessels (ISV) (B.-r. Yang et al., 2016). After removing VRI by washing, these blood vessels remain largely inhibited until 72 hpf. During this period, pro-angiogenic compounds can be added to determine whether they induce more recovery of ISV, an indicator of angiogenesis, compared to a black control. VRI has been used as a valid angiogenesis-recovering model for evaluation of pro-angiogenic activities *in vivo* (H. Zhao et al., 2016).

6.2 Angiogenesis in vitro bioassays (material and method)

6.2.1 Chemical and reagent

The chemicals used are summarised in Table 14 below:

Table 14: Chemical and reagent used for angiogenesis in vitro bioassays

Chemical	Company and Country
Crystal Violet	Sigma-Aldrich (Australia)
Cultrex Basement Membrane Extract (BME)	Trevigen (US)
Culture medium Dulbecco's Modified Eagle's Medium (DMEM/Ham's F12 containing 15mM HEPES and L-glutamine, with phenol red)	Life Technologies (Australia)
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich (Australia)
Fetal bovine serum (FBS)	Life Technologies (Australia)
Hydrogen Peroxide	Sigma-Aldrich (Australia)
Kaempferol	Sigma-Aldrich (Australia)
MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)	Sigma-Aldrich (Australia)
NXQ Powder extracts Diospyros kaki. L	Guangzhou Baiyunshan Pharmaceutical Co. Ltd. (PRC)
Phenol red absence culture medium (DMEM/Ham's F12 containing 15mM HEPES and L-glutamine)	Gibco™ (Australia)
Phosphate-buffered saline (PBS) tablets	Sigma-Aldrich (Australia)

Penstrep (penicillin and streptomycin)	Gibco™ (Australia)
Quercetin	Sigma-Aldrich (Australia)
Trypsin (10x) solution	Sigma-Aldrich (Australia)
VEGF receptor tyrosine kinase inhibitor Ⅱ (VRI)	Calbiochem (Merck KGaA, Germany)

6.2.1.1 NXQ

The herbal extract preparation was described in the previous chapter (Chapter 5 Section 5.2.2, *NXQ Extracts Stock Preparation*).

6.2.1.2 Quercetin

The stock preparation was described in the previous chapter (Chapter 5 Section 5.2.3, *Quercetin and Kaempferol Stock Preparation*).

6.2.1.3 Kaempferol

The stock preparation was described in the previous chapter (Chapter 5 Section 5.2.3, *Quercetin and Kaempferol Stock Preparation*).

6.2.1.4 Quercetin and kaempferol combination

The stock preparation was described in the previous chapter (Chapter 5 Section 5.2.4, *Quercetin and Kaempferol (3:4) Combination Stock Preparation*).

6.2.3 Cell line and culture conditions

EA.hy926 cells were used for the angiogenic *in vitro* experiments. The cells were cultured as described in Chapter 5 Section 5.3.1, *Cell Culture*.

6.2.4 Crystal violet staining for cell proliferation assay

6.2.4.1 Experimental procedure

1 x 10^4 cells per well were seeded in a 96-well plate for 2 h, and then treated with samples (final dimethyl sulfoxide (DMSO) concentration 0.1%) in a serum-free medium. After a 72-h

incubation period, the cell supernatant was fixed with 3% buffered paraformaldehyde for 20 min. The cells were then stained with 0.2% crystal violet in 20% methanol for 5 min. The excess crystal violet stain was rinsed 4–5 times with distilled water and air-dried. Glacial acetic acid (33%) in Milli-Q water was added to extract the crystal violet stain. Finally, the optical density was measured using a microplate reader (BMG Labtech Fluostar Optima, Mount Eliza, Victoria, Australia) at the wavelength of 595nm (A. Martin & Clynes, 1993).

6.254 Cell migration (scratch wound healing) assay

6.2.5.1 experimental procedure

The wound scratch assay procedure was performed based on Gebäck's protocol (Gebäck et al., 2009; C.-C. Liang et al., 2007). EA.hy926 cells were seeded at the concentration of 1 x 10^5 EA.hy926 cells per well on a 24-well plate and growth allowed until 80–90% confluence. Then the cell monolayer was scratched using a 1000µL pipette tip and rinsed gently with phosphate-buffered saline (PBS) to remove cell debris. Cross-shaped gaps were scratched in each well and these were instantly centre-imaged at 4 × magnification, using a Motic AE20 microscope and a Tucsen ISH500 digital camera with maximum contrast. The photo was viewed with ISCapture software, recorded as 0 h, and the cells were served with serum-free medium and incubation for 2 h for starvation. Tested samples were then administered to the cells and incubated for 24 h. The wound of the same well was photographed again and recorded at 24 h. The percentages of cell-free areas were then compared between 0 and 24 h, and the data were analysed using TScratch software (Gebäck et al., 2009; C.-C. Liang et al., 2007). The percentage of wound healing were calculated using the equation:

$$\text{Percentage of wound healing} = \frac{Area(baseline) - Area\ (24hr)}{Area\ (baseline)} \text{ x } 100\% \qquad \text{[Eq. 4]}$$

6.3 Western Blotting

6.3.1 Background

This study aimed to explore the mechanistic insight of NXQ for its observed effects on prevention of endothelial cell apoptosis and proliferation.

Endothelial nitric oxide synthase (eNOS) is the nitric oxide synthase (NOS) isoform that produces endothelium-derived NO (Heiss, Rodriguez-Mateos, & Kelm, 2015). eNOS is found primarily in the endothelium of cerebral blood vessels. Nitric oxide generated by eNOS is crucial for vascular function, homeostasis prevent the cells from sheer stress (Fulton et al., 1999; Jinqiang Zhu, Song, Li, & Fan, 2016). Treatment of endothelial cells with vascular endothelial growth factor or insulin stimulates the production of NO by a PI3K-akt dependent mechanism (C.-J. Li, Elsasser, & Kahl, 2009).

The Akt proto-oncogene is an important regulator of the various cellular process, including glucose metabolism and cell survival. Activation of receptor tyrosine kinases and G-protein-coupled receptors, and stimulation of cells by mechanical forces, can lead to the phosphorylation and activation of Akt (Fulton et al., 1999). Akt is a key mediator of eNOS activation and accounted for eNOS upregulation in vascular endothelial cells (C.-J. Li et al., 2009).

The mechanistic insight of NXQ on endothelial cell protection and proliferation were unclear, and we hypothesis that it may be mediated via Akt-eNOS pathway.

6.3.2 Experimental procedure

Cells were seeded in 6-well plates at a density of 1.0 x 10^6 cells/well and allowed to attach for 24 hours. The cells were treated with vehicle, NXQ (10 and 50 μg/mL) or VEGF (100 ng/mL) for 24 hours. After the treatment, the cells were homogenised and lysed in a RIPA buffer (Thermo Scientific, USA) in the presence of protease inhibitors (Roche Applied Science, USA) and PhosSTOP (Sigma, USA) to obtain protein extracts. Protein concentrations were determined using the BSA protein assay kit (Pierce, USA). Samples (30 μg of protein per lane) were loaded onto a mini-PROTEAN TGXTM precast electrophoresis gel (BioRad, USA). After

electrophoresis (110 V, 90 min), the separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes using iBlot 2 gel transfer system (Thermofisher, USA). Non-specific sites were blocked with 5% non-fat dry milk in PBSt for 60 min, and the blots were then incubated with anti-Akt, 1:1,000 (Cell Signalling, USA), anti-phospho-Akt, 1:1,000 (Cell Signalling, USA), anti-eNOS, 1:1,000 (Cell Signalling, USA) and anti-phospho-eNOS, 1:1,000 (Cell Signalling, USA) in PBSt overnight at 4 °C. Anti-rabbit HRP conjugated IgG, 1:1000 (DakoCytomation, Denmark) in PBSt (60 min, room temperature) was used to detect the binding of its correspondent antibody. The protein expression was detected with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, USA) and quantified by ImageJ software (NIH, USA).

6.4 Angiogenesis effects of NXQ in zebrafish embryos

The experiments were conducted in collaboration with Institute of Chinese Medical Sciences, Macau University, where animals, maintenance of zebrafish and ethics approval complied with the University's standard operations procedure and ethical guidelines.

6.4.1 Ethics statement

All animal experiments were conducted according to the ethical guidelines of Institute of Chinese Medical Sciences, University of Macau. The protocol was approved by Animal Ethics Committee Western Sydney University (ethics approved protocol number A10378).

6.4.2 Animals

Transgenic zebrafish Tg(fli1:EGFP) embryos were used in this study. The embryos were generated by natural pair-wise mating with 3–12-month-old zebrafish. Breeding boxes were

placed inside the tanks with light. Four to five pairs of zebrafish were set up in each tank for each mating and were left undisturbed for 15–30 minutes. Embryos were transferred using a fine fishing net into separate containers and maintained in embryo water (5g of instant ocean salt in 25L distilled water) at 28˚C. Embryos were then removed from their chorions at 19 hpf immediately prior to any treatment (Westerfield, 1995).

6.4.3 Maintenance of zebrafish

Zebrafish were maintained in a controlled environment at 28˚C on a 12/12h light/dark cycle according to the zebrafish handbook (Westerfield, 1995). Zebrafish were stored in 3.5L or 5L Tecniplast tanks with automatic monitoring of environmental conditions such as water temperature, pH level and dissolved oxygen saturation. Each tank housed up to a maximum of ten fish. Adult zebrafish were fed with dry food flakes or pellets, live artemia or shell-free artemia, three times daily from Monday to Friday and once daily during weekends and public holidays in compliance with Zebrafish Feeding Standard Operation Procedures.

6.4.4 NXQ solution and control

The NXQ extract was prepared by dissolving it in an aqueous solution of 10% DMSO with double-distilled water. This was then administered to the embryos by diluting the NXQ 10% to 0.1% DMSO in the embryos' container water. An embryo container with 0.1% DMSO was used as negative control and microinjection of VEGF was used as positive control. VEGF was injected either into the yolk ball or into the perivitelline space between the yolk and periderm. These two sites for injection were chosen because proteins in the yolk are often taken up by the embryo, and the second location is in the path of venous return; therefore, after injection, proteins end up in the circulation of the embryo.

6.4.5 Experimental design and assessment

Embryos were treated with the test agent 27 hpf for 24 h. Embryos were divided into five groups (n= 15 per group) as shown below:

Group 1: NXQ extract low dose (25 µg/mL)

Group 2: NXQ extract medium dose (50 µg/mL),

Group 3: NXQ extract medium high dose (100 µg/mL)

Group 4: NXQ extract high dose (300 µg/mL)

Group 4: negative control (VRI 300 ng/mL)

Group 5: Vehicle control (0.1% DMSO).

6.4.5.1 Embryo collection

Zebrafish were kept separately in 14/10 h light/dark cycle water circulation system. Zebrafish embryos were generated by natural pair-wise mating (3–12 months old) and were raised at 28.5°C in embryo water. Healthy embryo collection was performed according to the previous methods (J. Y. Tang et al., 2010).

Embryos were removed from their chorions at 24 hpf immediately prior to VRI treatment by incubating with 1 mg/mL protease at room temperature for 3 min. Dechorionated embryos were then treated with VRI and transferred into a 24-well microplate by a siphon tube for drug treatment (10 embryos per well).

6.4.5.2 Drug Treatment/compound addition

VRI was used to establish vascular insufficiency model, which is an pyridinyl-anthranilamide compound that displays anti-angiogenic properties and strongly inhibits the kinase activities of VEGF receptors 1 and 2. The 24 hpf zebrafish were treated with 300ng/mL VRI for 3 h and then distributed into 24-well plate (10 embryos per well). VRI was washed out and replaced

with NXQ of different concentrations (25, 50, 100 and 300 μg/mL). Embryos treated with embryo water containing 0.1% DMSO only served as vehicle control. Zebrafish embryos were raised in incubators at 28°C for 24 h before morphological observation.

6.4.5.3 Morphological observation and angiogenesis quantifying in zebrafish

Zebrafish embryos were placed on glass slices and observed for viability and morphological changes with a fluorescence microscope (Olympus IX81 Motorised Inverted Microscope, Japan) equipped with a digital camera (DP controller, Soft Imaging System, Olympus, Germany). Images were analysed with Axiovision 4.2 and Adobe Photoshop 7.0. Intersegmental vessels (ISVs) observed sprouting and elongating from dorsal aorta (DA) or posterior cardinal vein (PCVs) to dorsal longitudinal anastomotic vessels (DLAVs) were defined as intactness in the control group. In the VRI-only treatment group and the drug treatment groups, some of the ISVs observed sprouting from DA or PCVs but not forming completed ISVs were defined as defective blood vessels. The number of intact and defective ISVs in each zebrafish embryo was counted.

6.4.5.4 Quantifying angiogenesis in zebrafish

To evaluate the effects of NXQ treatment on angiogenesis, the intact and defective ISVs sprouting were counted. Images were analysed with Adobe Photoshop 7.0. The data (number of intersegmental vessels and branching arteries) were exported as a summary log to Microsoft Excel.

6.4.6 Statistical analysis

Data were analysed with one-way ANOVA followed by Turkey's multiple comparison test. A chart was made with GraphPad Prism 5.0 software (San Diego, CA). All relevant data were

presented as mean±standard error of mean (x–±SEM). Differences were considered significant at $p<0.05$.

6.5 Results

6.5.1 Effects of NXQ extract on EA.hy926 endothelial cell proliferation

As shown in Figure 6.1, NXQ and kaempferol did not significantly increase cell proliferation in comparison to either DMSO (0.1%)-treated control or VEGF (50ng/mL)-treated control ($P>0.05$). VEGF significantly increased cell proliferation when compared to vehicle control ($p<0.001$) ($n=3$) (Figure 6.1). In contrast, quercetin at concentrations of 100, 250 and 500 mmol/mL significantly inhibited the cell proliferation compared to control ($P>0.05$). Similarly, Q3:K4 at concentration 500 mmol/mL significantly inhibited the cell proliferation of EA.hy926 cells compared to control ($p<0.05$), suggesting a possible antagonist effect between kaempferol and quercetin on cell proliferation (Figure 6.1).

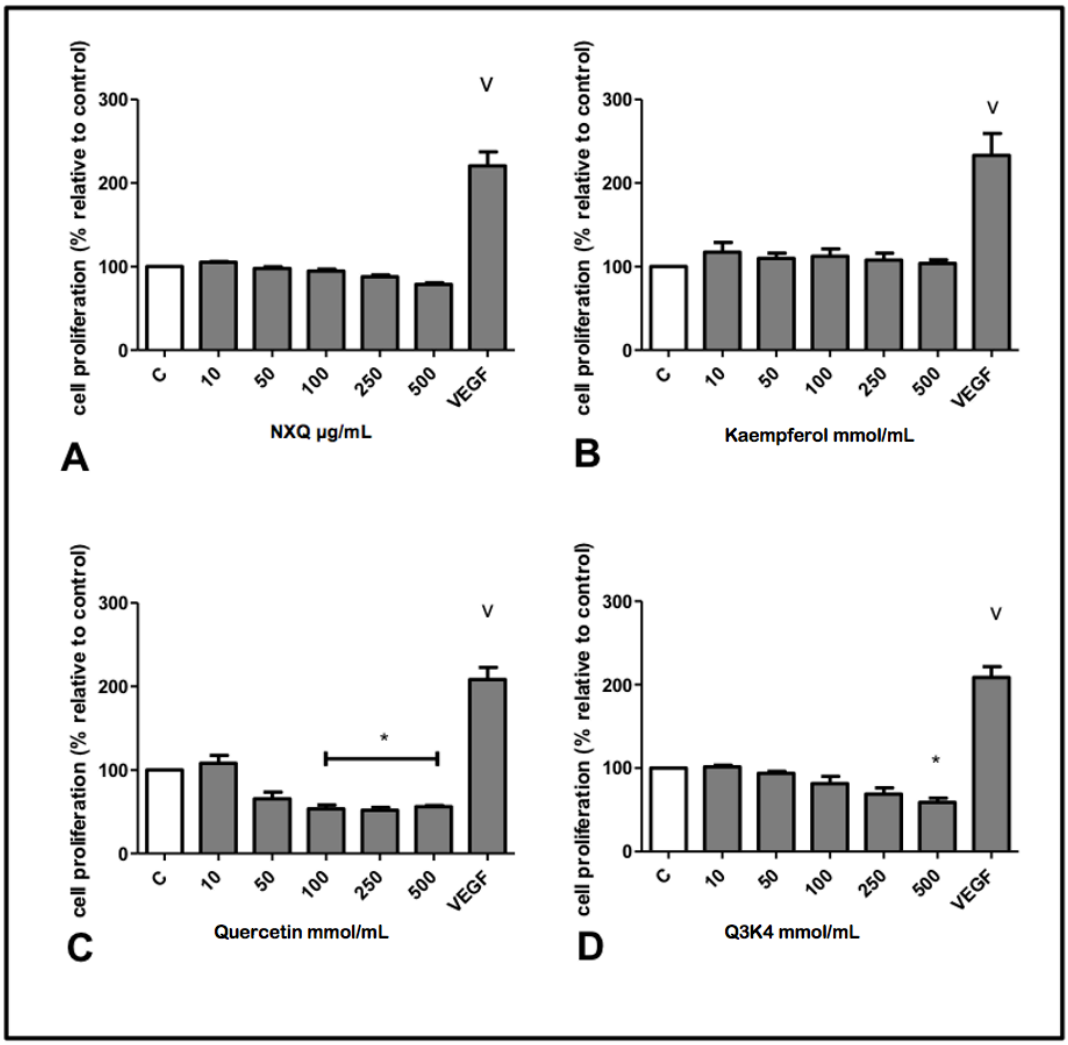


*Figure 6.1 Effects of NXQ (A), kaempferol (B), quercetin (C) and Q3:K4 combination (D) aqueous extract on cell proliferation. V=p<0.01 (n=3) VEGF stimulation (positive control) compared with vehicle control (0.1% DMSO); *=p<0.05 (n=3) compared to vehicle control.*

6.5.2 Effects of NXQ extract on EA.hy926 endothelial cell migration

As shown in Figure 6.2, NXQ did not significantly induce cell migration in EA.hy926 cells in comparison to vehicle control (p>0.05) (6.2A). Similar results were observed in kaempferol, quercetin and Q3:K4 combination.

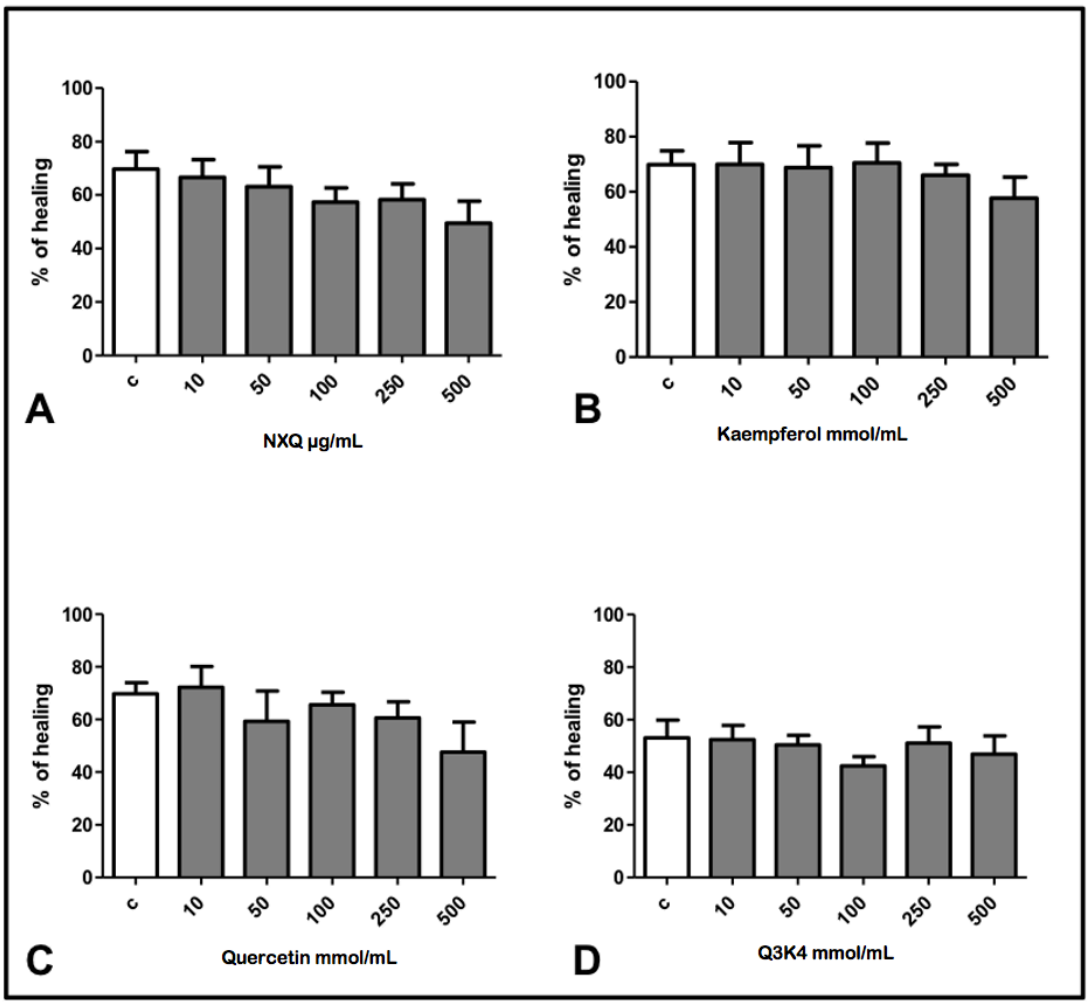


Figure 6.2: Cell migration (relative to control) concentration curve for NXQ (A), kaempferol (B), quercetin (C) and Q3:K4 combination (D) aqueous extract on cell proliferation assay (p>0.05, n=3).

6.5.3 Mechanisms and Signalling pathways

Activity of the Akt/eNOS signalling pathway were evaluated using Western blotting with the result expressed as p-Akt/t-Akt and p-eNOS/t-eNOS ratio. As shown in Figure 6.3, VEGF (100 µg/mL) treatment increased both the p-Akt/t-Akt ratios in the EA.hy926 cells but not in p-eNOS/t-eNOS. NXQ treatments (10 and 50 µg/mL) caused a modest increase in p-Akt/t-Akt ratios in a concentration-dependent manner in the EA.hy926 cells. However, these changes did not reach statistical significance (n=3).

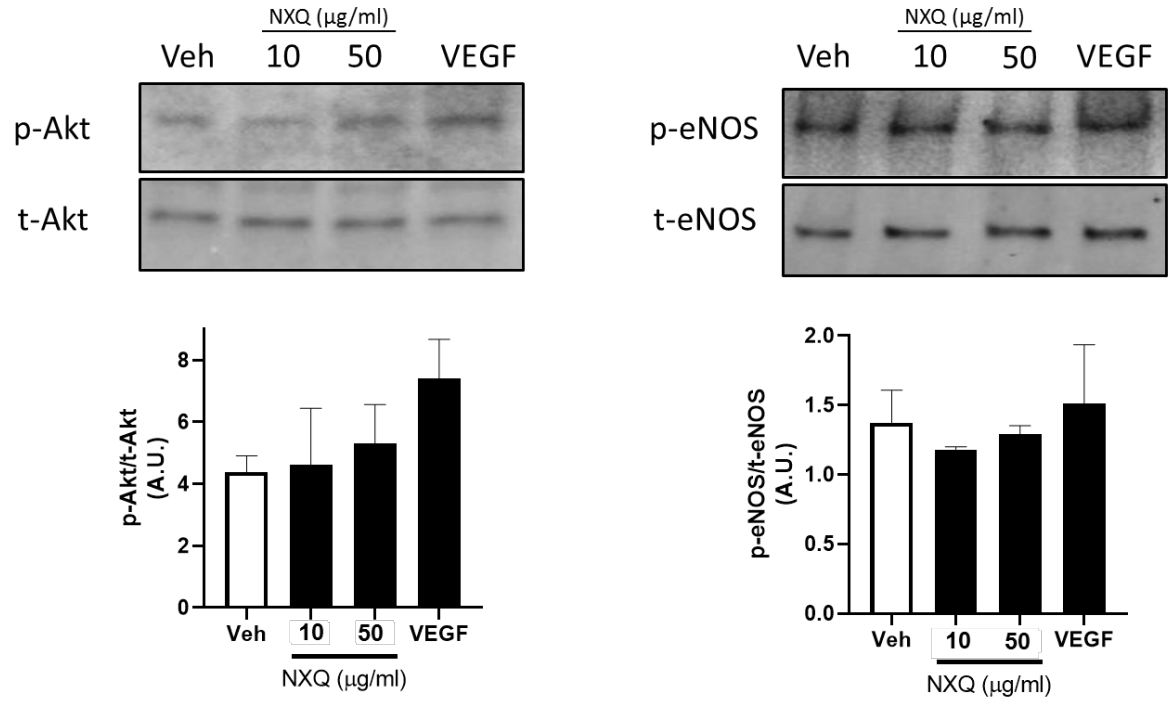


Figure 6.3: Western blot analysis of expression of Akt/eNOS signalling pathway. NXQ 10 and 50 µg/mL showing modest increase in p-Akt/t-Akt ratios but these changes did not reach statistical significance (p>0.5, n=3). NXQ 10 and 50 µg/mL showing no increase on p-eNOS.t-eNOS compare to vehicle control (p>0.5,n=3).

6.5.4 Effects of NXQ extract on VEGF inhibited zebrafish embryos

VRI (500ng/mL) significantly inhibited VEGF-stimulated SIV growth when compared to the vehicle control. All the NXQ extract dosages tested in the current study (25, 50, 100 and 300 µg/mL) significantly increased ISV vessel growing against VRI ($p<0.05$). However, NXQ at 100µg/mL presented as the most effective dose ($p<0.01$) (Figure 6.3).

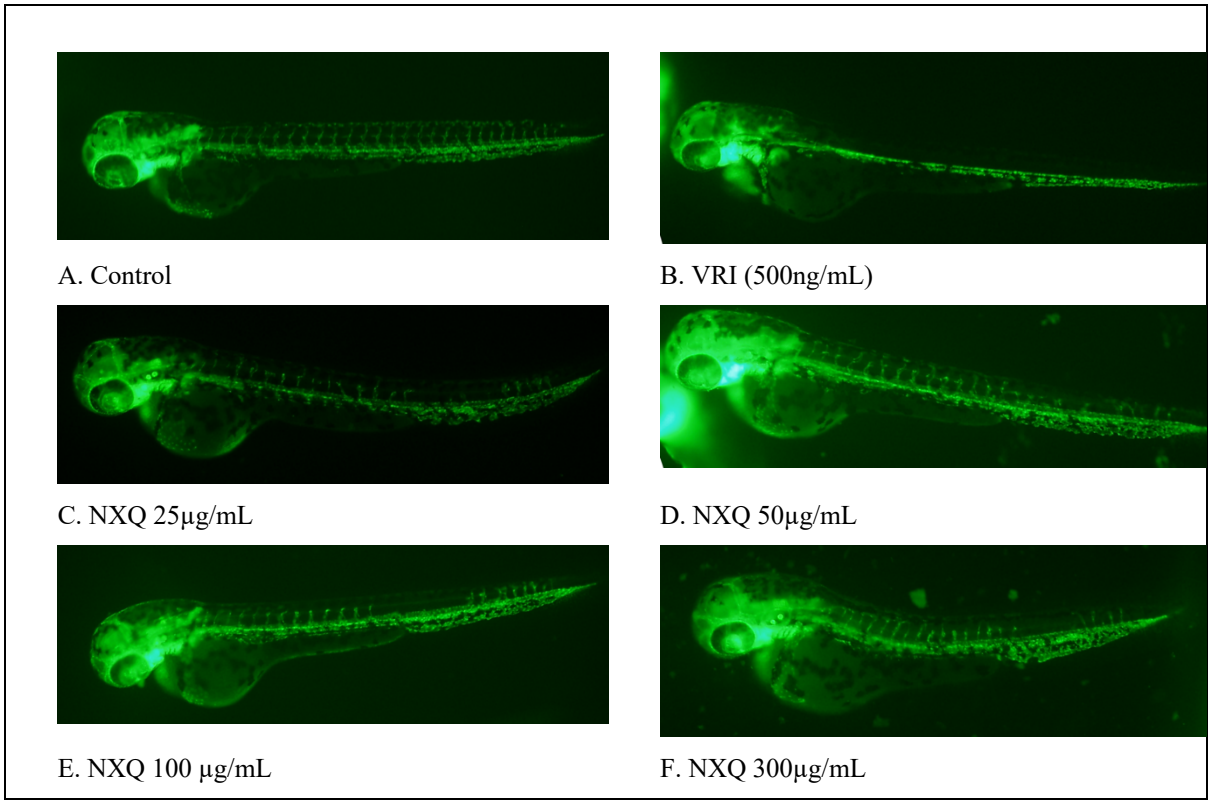


Figure 6.4: Treatment and control groups of Tg(fli1:EGFP) zebrafish larvae observed under co-focal microscope. SIV vessels were observed and counted for statistical analysis. (A) SIV vessels of vehicle control (DMSO 0.1%) zebrafish larvae (B) negative control by VRI (500ng/mL) zebrafish larvae (C) NXQ treated 25µg/mL (D) NXQ treated 50µg/mL (E) NXQ treated100µg/mL and (F) NXQ treated 300µg/mL.

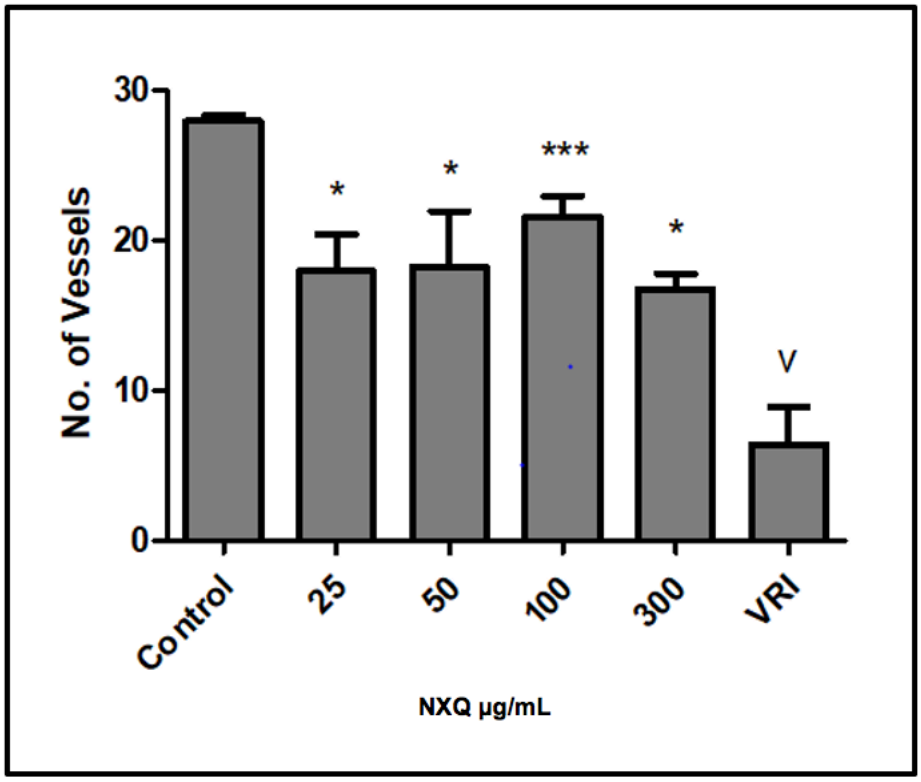


*Figure 6.5: Angiogenic vessel growth on zebrafish Tg(fli1:EGFP) embryos. V: p<0.001 (n=8) VRI inhibition of vessel growth compare to vehicle control (DMSO0.1%); *p<0.05 (n=8) compared to VRI; ***=p<0.001 (n=8) compared to VRI. Statistics were conducted using one-way ANOVA (GraphPad) Prism 5.*

6.6 Discussion

NXQ, a standardised, patent herbal extract, has been used clinically as part of traditional Chinese medicine to treat cerebral atherosclerosis, transitory ischaemia syndromes, cerebral thrombogenesis, cerebral thrombosis sequelae, apoplexy sequelae and cerebral embolism, with minimal adverse effects (Bei et al., 2007; Y. Cai & Yang, 2001). Previous studies suggest that NXQ possess potential effects on lipid-lowering, oxidant-scavenging, anti-inflammatory and anti-hypertensive actions. It has been reported that NXQ can improve cardiac and cerebral ischaemia associated symptoms by improving blood flow. While these studies provided some evidence to support the use of NXQ for the management of cerebral ischaemia, its role in improving cerebral blood flow was not clearly identified. No previous studies were conducted to evaluate the angiogenic property of NXQ and its role on vasculatures of cerebral ischaemia. This study evaluated the angiogenic effects of NXQ, and its two major bioactive compounds, kaempferol and quercetin, using EA.hy926 cells and Tg(fli1:EGFP) zebrafish embryos. NXQ, kaempferol and quercetin and Q3:K4 combination tested under normal cellular conditions produced no observations of any significant changes in EA.hy926 cell proliferation and migration. Quercetin, however, showed a significant inhibition of cell proliferation at concentrations 100, 250 and 500 mmol/mL. The results were similar to those of a study by Zhao et al. (2014), who reported that quercetin exerts anti-angiogenic activity through regulation of VEGFR-2 activation. VEGFR-2 is the most biologically important receptor for VEGF. It regulates endothelial cell proliferation, migration, differentiation, tube formation and angiogenesis (Nakatsu et al., 2003). Zhao et al. (2014) investigated how quercetin affected VEGFR-2 protein expression in HUVECs using qRT-PCR analysis and

identified that quercetin significantly inhibited the expression of VEGFR-2 at concentrations of 50, 100 and 200 mmol/mL.

As in to the current study, Donnini et al. (2006) suggest that neither quercetin nor its glucuronide exerted any effects on endothelial cell proliferation. It has been suggested that quercetin metabolites appear to exert divergent actions on cell proliferation and migration depending on the presence of VEGF. Under VEGF stimulation, quercetin and its glucuronide metabolites, but not the sulphate conjugate, exhibit an inhibitory effect on cell migration and proliferation (Donnini et al., 2006).

In addition, kaempferol has also been reported to suppress cell proliferation, migration and tube formation of HUVECs in high-glucose condition, PI3K expression and Erk1/2, Src and Ak1 activation (Tjwa, Luttun, Autiero, & Carmeliet, 2003; Xu et al., 2017). Xu et al. (2017) therefore conclude that the anti-angiogenic effects of kaempferol may be mediated via suppressing mRNA and protein expression levels of VEGF and placental growth factor (PGF). In contrast, kaempferol did not induce cell proliferation in the current study. However, significant inhibition of cell proliferation or migration was not observed. It may be that kaempferol suppressed VEGF expression only by high glucose-induced VEGF and PGF stimulation. In addition, Liang et al. (2015) compared the effects of kaempferol in two cell lines, HUVECs and HEK-293 kidney cells, using cell viability assay and showed that kaempferol reduced HUVEC viability in a dose-dependent manner: 87% cell viability at 20µM and 52% cell viability at 80µM. However, the reduction of HEK-293 cells was relatively insensitive to kaempferol. At 80µM, the viability of HEK-293 cells was 93% while HUVECs displayed a viability of 52%, suggesting that kaempferol is more selective in inhibiting endothelial cells (F. Liang et al., 2015). The divergence of these results from those of Liang et al. may be due to the variety of tested kaempferol analyte. Liang and colleague were using fractions of extract

from *D. versipellis* and isolated kaempferol in specific fraction DYVE-D3, whereas the current study used pure bioactive compound purchased from Sigma at 97% purity.

The effects of kaempferol and quercetin on inhibition of angiogenesis factor were also demonstrated in zebrafish models in several independent studies. Quercetin was found to significantly reduce the SIV vessels of Tg(fli1:EGFP) zebrafish embryos at concentrations of 50, 100 and 200μM. SIV branch points were reduced in a dose-dependent manner by 26% and 58% following treatment with 100 and 200μM quercetin respectively, compared to the control (0.1% DMSO) group (D. Zhao et al., 2014). Kaempferol, on the other hand, also demonstrated anti-angiogenesis effect in zebrafish embryos and larvae (F. Liang et al., 2015; Xu et al., 2017). Liang et al. analysed kaempferol compounds using Tg(kdrl:GFP)zn1 zebrafish and observed significant inhibition of ISV vessels. While studies demonstrating inhibitory effects of kaempferol compounds in zebrafish embryos and larvae are prominent, the variation in type of transgenic zebrafish may influence the outcome (F. Liang et al., 2015).

Interestingly, although NXQ failed to induce proliferation and migration in Ea.hy926 cells, it significantly induced angiogenesis in the *in vivo* experiments using zebrafish embryos. NXQ extracts (25–300 μg/mL) significantly reversed the VRI-inhibited SIV growth in Tg(fli1:EGFP)y1 zebrafish. Although this observation was contradicted to our *in vitro* observations, that may be due to the complexity of animal metabolism and conjugations of active components including quercetin. This observation was similar to (Donnini et al., 2006) and it is possible that quercetin contents are conjugated and exert pro-angiogenic effects that need to be activated during metabolism in animals. NXQ significantly induced new vessel formation in zebrafish embryos treated with VRI.

In addition to this, other potential active components within NXQ may also contribute to the angiogenic effects when treated against VRI zebrafish embryos. The most abundant active

constituent identified after kaempferol and quercetin is astragalin. Astragalin has been shown to be a potential pro-angiogenic compound in several studies (Fan et al., 2006; Tu, Shao, Ren, Chen, & Yao, 2014; L. Zhang, Yang, Wang, & Gao, 2011). Although the effect of astragalin was not evaluated in the current study, it is logical to suggest that the pro-angiogenic effect of NXQ extract in zebrafish is at least partly mediated by astragalin. Future study is required to confirm the role and contribution of astragalin to inducing angiogenesis.

We have further assessed the potential mechanisms of action underlying the cellular protective effects and angiogenic effect of NXQ targeting the Akt/eNOS pathways. The Akt-eNOS signalling pathway has been implicated in the regulation of cell survival and proliferation (Fei Shi et al., 2012). Akt, or protein kinase B, a serine/threonine protein kinase that is recruited to the membrane by its binding to PI3K-produced phosphoinositide. Activation of Akt pathways corresponds to the reduction of cell apoptosis and enhances cell proliferation. We examined whether NXQ promotes proliferation and migration of the endothelial cells via the PI3K-Akt pathway. Our results showed that NXQ (10 and 50 μg/mL) caused a modest increase in p-Akt/t-Akt ratios, in a concentration-dependent manner. However, these changes did not reach statistical significance (n=3). These results suggest that the NXQ-induced cell survival and proliferation may be partially mediated by the p-akt pathway. In addition, we also evaluated the cell-protective effects of NXQ via eNOS signalling pathway. eNOS is responsible for cell homeostasis and prevents endothelial cell damage by increase NO production. However, no significance was observed on NXQ (10 and 50 μg/mL) compared to vehicle control (n=3). We anticipated other signalling pathways might be involved such as MAPK, p38, or GSK. Due to the limitation of time of this candidature, we have only evaluated the Akt and eNOS pathways, further evaluation of additional pathways will be conducted in the near future for publication purposes.

6.7 Conclusion

Overall, we observed that NXQ exerted angiogenic effects at various concentrations (25–300μg/mL) in VRI treated zebrafish embryos. NXQ and its top two bioactive components failed to induce proliferation and migration in the current *in vitro* assays, suggesting a complex conjugating reaction of NXQ and its active constituents. Overall, this study provided insights into the potential angiogenic effects of NXQ treatment, and future studies on the pro-angiogenic effects and its metabolic conjugation are suggested.

Chapter 7. A Randomised, Double-Blind, Placebo-Controlled Trial to Evaluate the Effectiveness and Safety of Nao Xin Qing (NXQ), a Standardised Herbal Medicine Formula in Patients with Ischaemic Stroke – Experiment Protocol

7.1 Background

Stroke, also known as cerebral-vascular accident. It is a disease with multiple overlapping risk factors, primarily of vascular origin and typically complicated by ageing and lifestyle factors such as dietary habits and smoking. Stroke is one of the leading cause of death in Australia after coronary heart disease, and most survivors are left with permanent disabilities. In 2012, over 420,000 Australians were living with the effects of stroke; 75% of them suffered from a disability severe enough to decrease their employability (Coffey C. Edward, 2000; NSF, 2013) and two-thirds require assistance in daily living activities and long-term care (NSF, 2013). This number is expected to rise to 709,000 by the year 2032 due to the complexity of risk factors and the increase in the aged population of Australia (NSF, 2013).

Management of stroke, including prevention and rehabilitation, cost A$49.3 billion in 2012 (Cadilhac et al., 2009; NSF, 2013). Given that the number of disabled survivors is increasing, the cost of stroke treatment is expected to increase proportionally (Broderick, 2004).

Stroke is the rapid development of clinical signs indicating focal or global disturbance in cerebral functionality, with symptoms lasting for 24 hours or longer and potentially resulting in death with no apparent cause other than it being of a vascular origin (WHO, 1988). Ischaemic stroke (IS) is typically associated with disturbance in blood supply and generally incurs smaller lesions. Haemorrhagic stroke (HS) refers to the rupturing of blood vessels, resulting in larger lesions. IS is the more common type, accounting for approximately 80% of all cases. However, the IS mortality rate is generally lower in comparison to HS (Andersen, Olsen, Dehlendorff, & Kammersgaard, 2009a). Stroke treatment is predominantly focused on IS due to its higher incidence and potential therapeutic outcomes, whereas HS therapies are limited due to the rapid and extensive physiological damage, truncated therapeutic window and poor overall prognosis.

Ischaemic stroke is caused by deprivation of oxygen and energy supply to the brain cells. It can be sub-categorised into thrombotic and embolic ischaemic stroke (Lees et al., 2000). Thrombotic stroke (TS) is the most common type and occurs when a blood clot adhering to the vessel wall (mural occlusive thrombus) reaches a sufficient size to completely block cerebral artery blood flow. The development of an occlusive thrombus due to atherosclerosis is a major risk factor for stroke (Allen & Bayraktutan, 2008). Atherosclerosis is a condition in which plaque builds up within an artery causing a narrowing of the blood vessel. The formation of this plaque is associated with fatty materials such as low-density lipids (LDL) and triglycerides; however, other factors such, as hypertension, diabetes, smoking and ageing, have also been shown to contribute to atherosclerosis (H. Adams et al., 1993; Allen & Bayraktutan, 2008). Atherosclerosis is a serious risk factor due to the development of atheromas during plaque formation in the cerebral arteries. The first concern regarding formation of atheromas is the narrowing of the relatively small arteries in the brain, resulting in increased blood pressure and reduced blood flow. Additionally, should the fibrous cap of an atheroma ulcerate, the subsequent thrombogenic material released may induce thrombus formation overlying the atheroma. This can result in rapid occlusion of the artery and, hence, ischaemic stroke. In addition to ischaemic stroke, atheromas produce enzymes that enlarge the arteries over time, and this excessive widening may lead to the creation of an aneurysm with subsequent rupturing resulting in a haemorrhagic stroke (Dzau, 1994; Kher & Marsh, 2004).

In contrast, embolic strokes occur when an embolus (e.g., a blood clot, atherosclerotic plaque, fat globule or gas bubble) is carried by the bloodstream to the brain, where the larger arteries branch off into smaller vessels. The embolus reaches a point at which it can travel no further, thereby plugging a small cerebral artery and cutting off the blood supply to that area

of the brain. Most blood clot emboli are caused by non-vascular factors such as atrial fibrillation (AF), in which the two small upper chambers of the heart quiver, causing the blood to pool and form clots (H. Adams et al., 1993; Lees et al., 2000).

A number of risk factors have been associated with an increased risk of ischaemic stroke. They can be stratified into modifiable and non-modifiable risk factors. Modifiable risk factors include those resulting from the environment and lifestyle choices such as a high-fat and high-glucose diet, lack of exercise and smoking. These risks are typically manageable with the help of healthcare professionals, pharmaceutical treatment and continuing education. Non-modifiable risk factors encompass hereditary or natural features such as ethnicity and ageing (Allen & Bayraktutan, 2008).

Ischaemic stroke is typically the result of a transient or permanent reduction in cerebral blood flow to a major arterial branch within the brain caused by the occlusion of arterial blood flow, either by an embolus or local thrombosis. Brain injury following the transient or permanent focal cerebral ischaemia develops from a complex series of pathophysiological events (Dirnagl et al., 1999).

The brain is the centre of the nervous system and exerts critical centralised control over most of the physiological and cognitive functions of the body. As a result, brain tissue requires relatively high levels of oxygen and glucose and depends almost exclusively on oxidative phosphorylation for energy production (Dirnagl et al., 1999).

Focal impairment of cerebral blood flow restricts the delivery of oxygen and glucose, leading to a functional failure of cellular membrane ion channels such as potassium, sodium and chloride pumps, causing the plasma membrane of neurons to depolarise. The resultant cascade of pathophysiological processes – such as excitotoxicity, peri-infarct depolarisation,

inflammation and apoptosis –directly or indirectly lead to the death of neurons (Dirnagl et al., 1999; Kehrer & Smith, 1994).

The ensuing efflux of K^+ and influx of Na^+ and Cl^- across the cell membrane following depolarisation causes increased water permeation into the neural cells via osmosis. The oedema can negatively affect the perfusion of blood to the regions surrounding the infarct epicentre and can also have remote effects due to increased intracranial pressure, vascular compression and herniation.

As a further consequence of the membrane depolarisation, the substantial influx of Ca^{2+} allows for excitatory amino acids to be released into the extracellular space (Dirnagl et al., 1999). At the same time, energy-dependent processes such as presynaptic re-uptake of excitatory amino acids are impeded, further increasing the accumulation of excitatory amino acids such as glutamate in the extracellular space. The presence of these excitatory amino acids results in the activation of N-methyl-D-aspartate (NMDA) receptors and a specific glutamate receptor subtype known as metabotropic receptors. The combined over-activation of these receptors from accumulated extracellular excitatory amino acids contributes to the cellular Ca^{2+} overload in the neural cell (Dirnagl et al., 1999; Kehrer & Smith, 1994).

This increase in Ca^{2+} is thought to initiate a series of cytoplasmatic and nuclear events that result in cellular destruction and activate inflammatory responses, thereby generating free radical species that overwhelm endogenous scavenging mechanisms (Dirnagl et al., 1999; Ginsberg, 2008). These free radical species damage the cellular membrane, allowing further influx of Ca^{2+} ions. Of these free radicals, reactive oxidation species (ROS) serve as important signalling molecules that trigger inflammation and apoptosis (J. T. Coyle & Puttfarcken, 1993).

Studies show a strong correlation between neurodegenerative diseases, including stroke and Alzheimer's disease, and ROS (J. T. Coyle & Puttfarcken, 1993). It has been reported that

there may be a surge in the generation of ROS during cerebral ischaemia, particularly at the onset of reperfusion. ROS including superoxide, hydrogen peroxide and hydroxyl radicals are highly reactive and can break down cell membranes, damage DNA, create oxidation on proteins and amino acids and inactive specific anti-oxidant enzymes. Neurons, in particular, consist of abundant polyunsaturated fatty acids and produce low amounts of anti-oxidant enzymes; they are, therefore, highly susceptible to ROS (Kehrer & Smith, 1994). Moreover, cerebral ischaemia can lead to increased level of excitatory amino acids, such as glutamate, which generate more ROS and enhance cellular destruction (van der Worp & van Gijn, 2007). As these effects are considerably more pronounced in tissue areas surrounding the focal point of the infarct, neuroprotection is therefore a critical yet relatively undeveloped therapeutic strategy for minimising neuron damage in ischaemic stroke patients.

Although recent research focuses on the survival of neurons via neuroprotection and anti-thrombotic therapies, little attention has been directed to the role of angiogenesis (Krupinski et al., 1994). During an ischaemic attack, the brain region of low perfusion (ischaemic), where cells have lost their membrane potential, is known as the core; this core is surrounded by an area called the penumbra, where intermediate perfusion prevails and cells depolarise intermittently (peri-infarct depolarisation). By definition, the penumbra is an area in which ischaemic tissue is potentially destined for infarction but is not yet irreversibly injured and is, therefore, the primary target of acute therapy (Dirnagl et al., 1999).

While necrosis at the core of ischaemic infarct is irreversible, studies have suggested that the penumbra may remain viable for several hours after an ischaemic event due to alternative blood supply from collateral arteries (Dirnagl et al., 1999). A study by Krupinski et al. (1994) investigating angiogenesis and ischaemic stroke suggests that adequate perfusion through the penumbra can initiate angiogenesis. Evidence suggests that high blood vessel density is

correlated with improved patient survival, independency and clinical outcome after stroke (Choo, 1993). There is also a strong correlation between the extent of spontaneous neurological recovery and the volume of penumbra that escapes infarction (Cheng et al., 2004; Dirnagl et al., 1999). Therefore, enhancing the angiogenic effect is another key aspect of improving clinical outcomes in stroke recovery.

7.2 Therapeutic options for ischaemic stroke

The goal of acute stroke treatment is to increase the survival rate and reduce the dependency level of the patient (Dirnagl et al., 1999). The current pharmaceutical treatments for ischaemic stroke, including anti-thrombotic therapies, are predominantly time-sensitive and have minimal neuropreotective effects (Ringleb et al., 2007). These anti-thrombotic therapies, such as anti-platelet agents, anti-coagulants and thrombolytic drugs, typically act on thrombosis or embolisms by thinning the blood; however, there is a relatively high risk of haemorrhagic incidents (Gubitz et al., 2008; Sandercock et al., 2008; Wardlaw et al., 2009). Neuroprotection is considered an important therapeutic strategy in reducing neuron damage and dependency levels and, in IS, has been suggested to expand the treatment window and reduce reperfusion injury (Cheng et al., 2004; Ginsberg, 2008). However, research into neuroprotective agents, such as glycine antagonists and calcium channel blockers, has shown discrepancies in the therapeutic benefits between animal studies and clinical trials (Cheng et al., 2004). Some researchers suggest this could be due to species-dependent differences and that animals such as rodents may be more amenable to neuroprotection than evolutionarily higher mammals such as humans (Dirnagl et al., 1999).

Rehabilitation is a combination of therapeutic modalities, including physiotherapy, occupational therapy, speech and language therapy, designed to assist stroke survivors and restore functionality lost due to neurological damage (Feigenson, 1979). In addition to rehabilitation therapies, long-term pharmaceuticals are typically prescribed for the management of vascular and thrombotic risk factors to prevent recurrence of stroke (Ringleb et al., 2007). Aside from these preventative medications, currently there are no known pharmaceuticals available for the improvement of cognitive and functional outcomes. There is an urgent need for alternative therapeutic options to improve ischaemic stroke outcomes by both reducing the damage to the brain and improving functional outcomes.

Traditional Chinese medicine (TCM) originated over 3000 years ago in China and is a form of traditional medicine that has integrated philosophical concepts and empirical evidence into clinical practice (Deadman, 2006). TCM comprises numerous treatment modalities, including herbal medicine, acupuncture, massage therapies, nutritional therapies and mind–body exercise regimes such as *qigong* (Feigin, 2007; M. Liu et al., 2007).

Preclinical studies have suggested that the use of herbal medicines can increase neurological recovery and enhance functional outcomes in stroke patients and, thus, may be beneficial in post-stroke rehabilitation (I. Lee et al., 2005; M. Liu et al., 2007; FL Shi et al., 1989).

Nao Xin Qing (NXQ) is a standardised and patented herbal extract from the leaves of *Diospyros kaki* that has been used for numerous years in China for the treatment of apoplexy syndrome, coronary artery disease and ischaemic stroke (Bei et al., 2004; Bei, Zang, et al., 2009; Y. Cai & Yang, 2001). The key bioactive components of *D. kaki L.* have been identified as flavonoids (e.g., quercetin and kaempferol) (Bei et al., 2005b). Data from previous studies

have shown *D. kaki L.* offers several therapeutic effects, including anti-thrombotic, neuroprotective, haemostatic, anti-oxidative, cholesterol-lowering, and anti-hypertensive effects, thereby presenting itself as a potential therapeutic option for both treatment and prevention of ischaemic stroke (Y. Cai & Yang, 2001; Y. Yu et al., 1988).

The herbs used for IS are documented in classical TCM literature for use in blood regulation and stasis resolution. These herbal agents, including *Danshen*, *Chuanxiong* and *Sanchi*, have been investigated and the bioactive constituents of these herbs have been found to improve microcirculation in the brain, protect against reperfusion injury, possess neuroprotective and/or anti-inflammatory properties, and inhibit apoptosis (X. Chen et al., 2008a; B Wu, M Liu, & S Zhang, 2007; Yuan et al., 2008).

For instance, preclinical pharmacological studies indicate that *Danshen* agents can be used for dilating cardiocerebral vessels, suppressing the aggregation of platelets, improving circulation, removing blood stasis, protecting against ischaemic reperfusion injury and enhancing the tolerance of ischaemic tissue to hypoxia (J. L. Li, Liu, H. , 2000; B Wu, M Liu, & S Zhang, 2007).

Furthermore, a *Chuanxiong* preparation was found to be effective in prohibiting platelet aggregation and preventing thrombosis by maintaining the balance of thromboxane A2/prostacyclin I2 (TXA2/PGI2) in the blood and increasing the activity of intrinsic superoxide dismutase (SOD) (Ge, 1994). Additional studies also found that *Chuanxiong* could improve brain microcirculation through prevention of thrombus formation and platelet aggregation as well as the reduction of blood viscosity, thereby highlighting that it may have significant beneficial effects in the treatment of ischaemic stroke (H. J. Sun, Wu, W., Chen, H.G., Mi, H.Q., 2002; J. Wang, Shi, Y. M., Zhen, H. M., Liu, Z. R., Li, X. X., Ding, G. H., 1993; Q. H. Wei, Zhou, D.

W., Wang, W., 1994; H. Xu, Shi, D. Z., Guan, C. Y., 2003; J. Y. Zhang, Yang, R. P., Zhu, J. M., 2001).

Despite this, the clinical effectiveness and safety of these interventions are yet to be confirmed. One systematic review conducted by Wu et al. (2007) evaluates the efficacy and safety of 22 traditional Chinese patent medicines (TCPMs) listed in the Chinese National Essential Drug list (2004) as potential drugs for ischaemic stroke patient. The pooled analysis of randomised and nonrandomised trials showed significant positive effects in lowering mortality rates and reducing neurological impairment for the majority of TCPM treatments (21 out of 22). However, over 97% of the included clinical trials were noted as having poor methodological quality (B Wu, M Liu, H Liu, et al., 2007). The main methodological weakness of these trials is lack of bias control, such as double blinding and placebo control. While there are numerous issues surrounding the methodology of the clinical trials assessing TCM herbal substances for stroke, the existing evidence has highlighted its potential therapeutic benefits.

7.3 Nao Xin Qing

Nao Xin Qing (NXQ) is an approved, patented TCM product made from the standardised extract of *Diospyros kaki* leaves (*D. kaki L.*). NXQ has been used clinically in China for many years in the treatment of apoplexy and coronary heart disease and to enhance recovery from ischaemic stroke. Previous studies, both clinical and preclinical, have suggested that the patent extract has potential therapeutic effects for cerebral atherosclerosis, transitory ischaemia syndromes, cerebral thrombogenesis, cerebral thrombosis sequelae, apoplexy sequelae and cerebral embolism, with minimal adverse effects (Bei et al., 2007; Y. Cai & Yang, 2001).

7.3.1 Current evidence of NXQ

Laboratory studies have quantified more than 30 potentially bioactive compounds isolated from the leaves of *Diospyros* species. These can be divided into five main chemical groups: acids, biphenyls, flavonoids, polyphenols and triterpenoids (Bei et al., 2004, 2005b). Among these chemical compounds, flavonoids were considered to be the key therapeutic components responsible for the observed pharmacological and clinical effects due to their multiple biological properties, including anti-oxidant, anti-allergenic, anti-inflammatory, and vasodilatory actions (Bei et al., 2004; S. Cao et al., 2012).

Previous research has demonstrated numerous pharmacological effects of *D. kaki L.* extract, such as improved cardiac and cerebral blood flow and anti-hypertensive, lipid-lowering and radical-scavenging capabilities (S. Cao et al., 2012). A study conducted by Xin, Feng and Yao (2007) investigated the effects of intravenous injections of *D. kaki L*. flavonoids in rabbits and found a 30–40% improvement in aortic circulation as well as significant dilation in the veins of the ears. These results were supported by Zhang, Wang and Xiao (2004), who suggest that the leaves of *D. kaki* can improve overall circulation in anaesthetised dogs and can reduce oxygen consumption of cardiac muscles. Ou, Bei and Lai (2003) found that the total flavonoid count can significantly inhibit reperfusion-induced cell apoptosis. Ou, Liu and Bei (2004) also suggest that *D. kaki L.* can inhibit fibrin adhesion (scar tissues) on the adventitia stimulated by factors such as advanced glycation end-products (AGEs) and advanced oxidation protein products (AOPP).

In relation to the cerebrovascular system, Cao et al. (2012) studied the fibrinolytic function in an ischaemic brain injury model and suggest that *D. kaki L.* flavonoids can increase tissue plasminogen activator (rtPA), which is involved in the breakdown of blood clots and reduction of plasminogen activator inhibitor-1 (PAI-1) levels, thereby improving the outcome of

thrombotic-related cerebral ischaemia. Cao et al. (2012) further demonstrate that von Willebrand Factor (vWF), a blood glycoprotein involved in haemostasis, is reduced in ischaemic brain injury rats subjected to *D. kaki L.* flavonoids. Reduction of vWF has been associated with the resolution of thrombosis during an ischaemic attack and restoration of damaged endothelial cells. Cao et al. (2012) propose that *D. kaki L.* flavonoids could reduce positive signalling of Intercellular Adhesion Molecule 1 (ICAM-1), an immunoglobulin molecule associated with the release of inflammatory factors, and thereby act against atherosclerosis and reduce haemostasis.

Further, Bei, Li et al. (2009) propose that the potential neuroprotective effects of NXQ and *D. kaki L*. flavonoids could activate L-type Ca^{2+} channels, thus modulating intracellular Ca^{2+} levels to protect neurons in the hippocampus. This action is complemented by the anti-oxidative ability of NXQ and *D. kaki L.,* which positively inhibits ROS (including hydrogen peroxide) thereby reducing neuron damage.

Kameda et al. (1987) first demonstrated the anti-hypertensive effects of *D. kaki L.* in hypertensive rats. The mechanism of action is thought be through inhibition of angiotensin-converting enzyme activity. Tan, Lin and Zhang (2009) further investigated these anti-hypertensive effects and observed an increase in plasma nitric oxide (NO) and reduced over-production of platelets by essential thrombocytosis (ET) and reduced angiotensin II in rats. The results suggest that *D. kaki L*. flavonoids have the potential to enhance endogenous vasodilators and modulate endogenous vasoconstrictors.

Several clinical trials assessing the clinical effects of NXQ have been conducted in China recently. One study assessed NXQ as an additional therapy to standard dehydration, anti-hyperglycaemic and anti-lipidaemic treatment for acute ischaemic stroke in 35 Type 2

diabetic participants (B. Tang et al., 2012).This study suggests that NXQ positively improves blood rheology by acting on C-reactive protein and blood lipids. Tang et al. (2012) imply that the underlying mechanism of action is due to the active component flavonoids from *D. kaki L.* extract having anti-ischaemic effects on cerebral and cardiac blood flow, neuroprotective effects through free radical scavenging and anti-atheroscleroma effects by lowering blood lipids.

Another study conducted by Wang et al. (2012) assesses NXQ in combination with acute management therapy (mannitol and glycerol fructose dehydration) in comparison to acute management therapies alone in patients with cerebro-cardiac syndrome caused by acute stroke. A total of 204 participants were included;130 patients suffered from ischaemic stroke, 61 from intracranial haemorrhages and 13 from sub-cranial arachnoid haemorrhages. A standard dose of NXQ was given at the acute phase in addition to acute management therapy protocols for a continuous four-week period. The results suggest that the NXQ group had superior outcomes in improving cardiac function recovery including arrhythmia, changes in ST-T wave and abnormal cardiac enzyme production (A. Wang et al., 2012).

Chen, Shao and Cheng (2012) conducted a clinical trial assessing NXQ as an adjunct to low-molecular weight heparin in comparison to heparin alone on 160 cerebral transient ischaemic attack patients. Results suggested that both groups had prolonged partial thromboplastin time (PTT) and active partial thromboplastin time (APTT); however, NXQ showed significantly longer duration in both outcome measures when compared to controls. In addition, no significant adverse events were identified (Y. Chen et al., 2012).

Although limited clinical studies have been conducted on cerebral ischaemic stroke, a number of clinical trials were conducted assessing the effects of NXQ for ischaemic heart disease and angina, which share a similar underlying pathological process with ischaemic

stroke – atherosclerosis. A study conducted by Cai and Yang (2001) assesses the pre- and post-effects of NXQ in 48 atherosclerosis cases and 12 coronary artery disease-induced angina cases. The study concludes that NXQ can significantly lower blood lipids and improve symptoms such as vertigo and headaches (Y. Cai & Yang, 2001). Furthermore, a recent study by Wu, Wu and Zheng (2013) evaluates 90 patients with coronary artery disease and angina, comparing NXQ against a standard vasodilator, and shows similar positive results. The authors found statistically significant results in lipid levels and suggested that the significant reduction of cardiac stress and cardiac oxygen consumption, and improvements in oxygen supply to cardiac muscle, occurred through aorta dilation (D. Wu et al., 2013). This is supported by the findings of Xiong, Tang and Zhou (2013), who assess the clinical effects of standard angina treatment – including nitrates, aspirin, beta blockers, angiotensin-converting enzyme inhibitors and lipid-lowering drugs – compared to standard treatment combined with NXQ in 120 elderly patients with unstable angina. The results suggest that the NXQ combination group experienced significant improvements in symptoms of angina and lower blood lipid levels (Xiong et al., 2013).

In summary, the preliminary clinical evidence suggests NXQ is effective for lipid reduction and may also improve cardiac blood flow as well as relieving clinical symptoms such as angina. No severe adverse event was identified when used in conjunction with anti-coagulant. However, the methodology of these trials did not appear to be rigorous and precise. Of all the trials, only Tang et al. (2012) reported a randomisation method, and statistical power across all the studies was relatively low due to the small sample sizes. Apart from Wang et al. (2012), no study reported the treatment duration of either intervention or control. Furthermore, adverse events were not reported in the studies by Cai and Yang (2001), Wu et al. (2013) and Wang et al. (2012). Despite the sample sizes and debatable methodological quality, the

clinical observations emphasise the potential benefits of NXQ for ischaemic stroke. To date, there have been no trials evaluating the neuroprotective effects of NXQ with adapted standardised outcome measures. While there is sparse clinical evidence available, there is a considerable body of preclinical evidence that provides a preliminary foundation for further clinical assessment into the potential use of NXQ in stroke.

7.4 Research hypothesis

The aim of this study is to assess the clinical effectiveness and safety profile of NXQ for ischaemic stroke patients undergoing rehabilitation. The hypothesis of this study is that a 12-week intervention of NXQ will improve neurological, motor and cognitive functional outcomes for ischaemic stroke patients during the rehabilitation phase.

7.4.1 Study design and trial group

This study is a parallel group, double-blinded, randomised, placebo-controlled clinical trial to assess a 12-week intervention of NXQ treatment during the rehabilitation of patients suffering from ischaemic stroke. Potential participants are randomised to either intervention or placebo groups.

Participants in the intervention group will receive NXQ tablet (4 tablets, tid) over a period of 12 weeks. Participants in the placebo control group will receive a placebo (4 tablets, tid) dose equivalent to the intervention group over a period of 12 weeks. Primary outcome measures include the National Institute of Health Stroke Scale (NIHSS) and the Specific Stroke Quality of Life (SSQOL) and will be assessed at baseline and at weeks 6 and 12, and at a follow-up assessment performed at week 16 of the intervention. Secondary outcome measures will be performed at baseline, week 6 and week 12 of the treatment period. Blood rheology and coagulation assessment will be performed at week 2 of the intervention as part of safety

measures. At week 36, a long-term efficacy follow-up will be conducted assessing adverse events, all-causes mortality and reoccurrence of stroke (Table 15).

The study will be conducted at the Stroke Care Unit, Hornsby Ku-ring-gai Hospital; additional sites may be included at a later date. All study procedures will be conducted in full compliance with good clinical practice guidelines and with the approval of the University of Western Sydney and Hornsby Ku-ring-gai Hospital Human Research Ethics Committees before trial commencement. Upon receiving ethical approval, this study will also be registered with the Australian New Zealand Clinical Trials Registry (www.anzctr.org.au).

7.4.2. Participant selection and enrolment

7.4.2.1 Identifying participants

Potential participants who meet all inclusion and exclusion criteria will be recruited from the Hornsby Ku-ring-gai Hospital. These participants will be identified through physician referral and poster advertisements placed in the Hornsby Ku-ring-gai Hospital and stroke care units. Additional participants will be identified through advertisements through the Stroke Care Unit of Hornsby Ku-ring-gai Hospital and University of Western Sydney websites.

7.4.2.2 Consenting participants

Once participants are identified, there will be a brief telephone interview to provide preliminary details of the study and to confirm eligibility under basic inclusion/exclusion criteria and access to the test site for screening, intervention and testing sessions.

Once confirmed, a suitable day and time for a screening session will be advised to potential participants. Following this, a participant information sheet and an appointment letter detailing the arranged time and date, location and any additional information to be brought with them will be sent via email and/or post.

At the screening session, participants will be fully briefed and provided with an information sheet in the event they have not received it prior to the screening session. Participants will then be required to provide informed written consent before the screening procedure begins and will be invited and encouraged to ask questions throughout the recruitment procedure and while the study is in progress.

7.4.2.3 Screening for eligibility

Participants will undergo physical examination at screening by a trained medical staff and NIHSS scores will be assessed by a physician. Full blood tests will follow to assess liver and renal function as well as lipids profile. The blood test results will be used as the baseline assessment. If no abnormalities are discovered during the questionnaire or physical examination, the participant will be tentatively enrolled and asked to complete a training session on the COMPASS test battery. During this training session, a researcher will be present to answer any questions as well as to correct any mistakes. The results generated from this screening session will not be included in data analysis.

7.4.2.4 Ineligible and non-recruited participants

Participants deemed ineligible for the study will be notified via email or telephone. They will be informed that they do not meet the criteria outlined for the study.

7.4.3 Inclusion criteria and exclusion criteria

7.4.3.1 Inclusion criteria

— Meets standard criteria for atherosclerotic ischaemic stroke.

— Disease onset between 2 weeks and 3 months post-stroke.

— NIHSS score equal or greater than 5 and equal to or less than 25.

— Age between 40 and 80.

— Initial onset or recurrent onset but full recovery with mRS score of 0 to 1.

— Informed consent obtained.

7.4.3.2 Exclusion criteria

— Presents with cerebral haemorrhagic history within the past 6 months.

— Clinical assessment concludes the cause of ischaemic stroke is brain tumour, trauma, parasites, metabolic deficits, rheumatic heart disease, coronary artery disease or its arterial fibrillation complications.

— Combined with other complications affecting neural and motor function assessment, including lameness, osteoarthritis, rheumatoid arthritis and gout.

— Severe pulmonary and cardiovascular hepatic (ALT, AST>2 times normal range) and renal dysfunction (BUN>1.2 times normal range, Cr>normal range).

— Pregnancy and breast feeding.

— Disability patients by law (blindness, deafness, cognitive deficits, metal deficits, physical deficits).

— Known allergy to the medication ingredients or allergic body type.

— Participated in other clinical trial in the past 3 months.

— Consuming any other supplements or herbal medicine relevant to stroke treatment.

7.4.4 Concomitant medication provision

1. Standard anti-platelets/anti-coagulant treatment can be used as required.
2. Foundation treatment: Bayaspirin Enteric-coated, oral administration of 1 tablet tid (0.1g/tablet). If the patients have allergies or adverse reactions to Bayaspirin Enteric-

coated, they can use Clopidogrel as replacement. However, Bayaspirin and Clopidogrel cannot be used at concurrently.

3. Medication for relevant complications (hypertensive, hyperlipidaemic, hyperglycaemic and antibiotics) can be used as normal.
4. All medications and treatment procedures applied during the trial should be recorded in detail including medication type, dosage, method, and treatment duration for adverse event reporting and assisting in statistical analysis.
5. All treatment procedures and medications used during the follow-up period (note: protocol does not specify follow-up period duration) for both ischaemic stroke and unrelated complications should be recorded and include disease name, medication, dosage and mode of administration for adverse event reporting and assisting in statistical analysis.

7.4.5 Intervention and treatment allocation

Participants in the intervention group will receive NXQ tablets (4 tablets, tid) over a period of 12 weeks. Intervention: NXQ tablets, weight 0.41g per tablet, oral administration of 4 tablets tid.

Participants in the placebo control group will receive placebo (4 tablets, tid) dose equivalent to intervention group over a period of 12 weeks. Placebo Control: NXQ placebo tablets, weight 0.41g per tablet, oral administration of 4 tablets tid.

7.4.6 Sample size and randomisation

This trial is a pilot study to evaluate the effects of NXQ in comparison to placebo in patients with ischaemic stroke during rehabilitation period. The target sample size is 30 participants per group; with a 20% dropout boundary, the target will be 72 participants. Previous clinical

trial conducted using NXQ were not evaluating the outcome measures relevant to ischaemic stroke. According to Connelly (2008), extant literature suggests that a pilot study sample should be 10% of the sample projected for the larger parent study. However, Hertzog (2008) cautions that this s not a simple or straight forward issue to resolve because these types of studies are influenced by many factors. Nevertheless, Isaac and Michael (1995) suggested 10 to 30 participants are typically considered for a pilot study to examine the practicality and feasibility of the methods.

Once the patients have been recruited, they will be randomly allocated to the NXQ intervention group or placebo control group by means of a computer randomisation package.

Randomisation will be conducted by a NICM Research Program Coordinator external to the research team. The research team will be concealed to the randomisation process. The NICM Research Program Coordinator will prepare individually assigned and sealed envelopes containing the participant's group allocation, which will only be opened once the participant is confirmed to be eligible for the study. Participants will also be stratified based on disease type and treatment received.

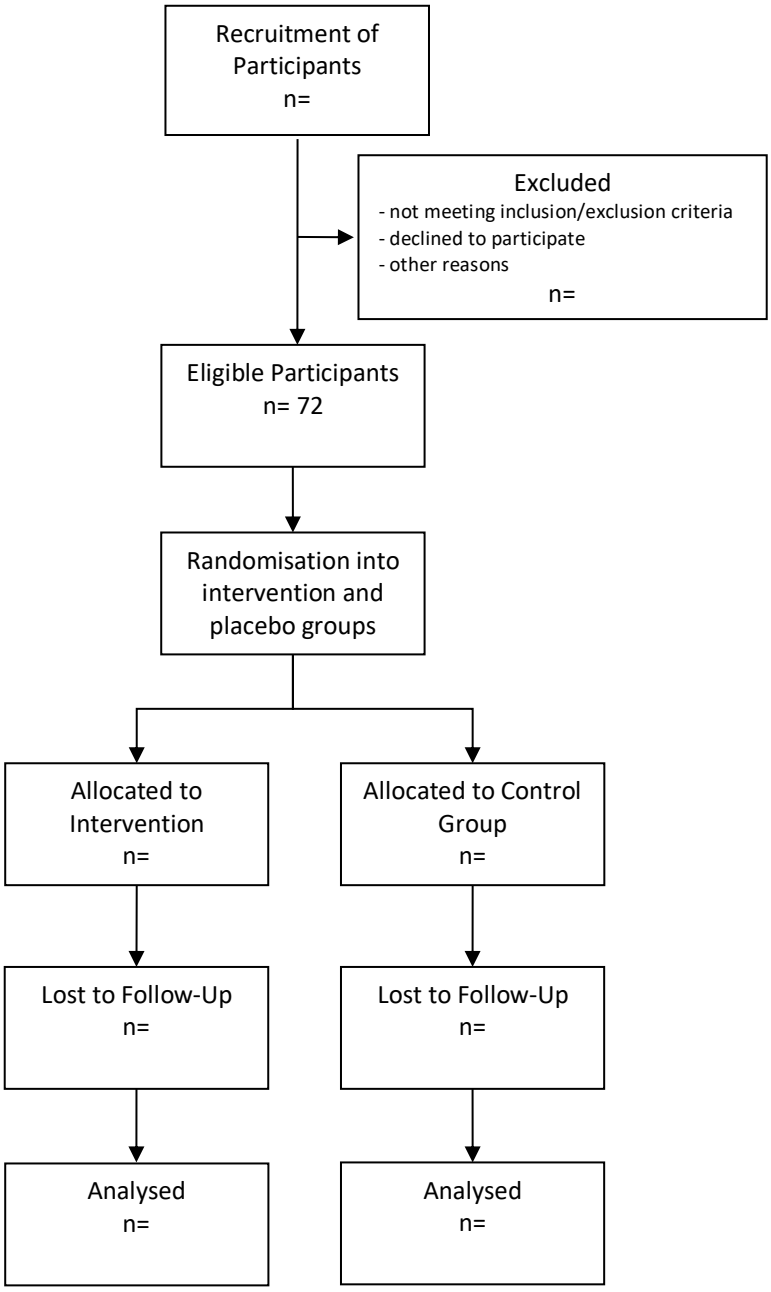


Figure 7.4: Schematic trial protocol.

7.4.7 Withdrawal procedures

Participants have the right to withdraw at any point in the study at their own request. In the event of allergic reaction or a severe adverse reaction, participants will undergo a medical examination and be withdrawn from the study. If disease prognosis worsens during the trial period, participants will undergo medical examination and be withdrawn from the study. In

the event that participants do not follow the instruction protocols – including poor compliance (<80% and >120%), abstaining from alcohol and caffeine prior to testing sessions, abstaining from illicit drug use and other exclusionary medications – investigators reserve the right to withdraw participants on the basis that these actions will contaminate the results. Should this occur, participants will be notified via email or telephone that they have been withdrawn from the study.

Whether participants are replaced will be determined based on the number of participants who choose, or for other reasons are forced, to withdraw. Should the need for additional participants arise, additional recruitment will be identical to the initial recruitment procedure.

7.4.8 Study assessments

7.4.9.1 Primary outcome measures

7.4.9.1.1 National Institute of Health Stroke Scale (NIHSS)

The National Institute of Health Stroke Scale (NIHSS) is a graded neurological examination rating speech and language, cognition, visual field deficits, motor and sensory impairments, and ataxia and has become a standard part of the clinical assessments used in many recent interventional trials. This assessed by neurologist or trained physician based on 11 sections scoring accordingly between 0 to 2, 0 to 3 and 0 to 4 of each of the sections (Berger et al., 1999; Fischer et al., 2005).

Post-treatment score of each section ≤ 0–1 indicates good clinical recovery; scores ≥ 0–1 indicate poor clinical recovery. Appropriate statistical methods will be used to analyse the comparison of good vs poor clinical recovery ratio between intervention and control groups.

A total score ≥16 forecasts a high probability of death or severe disability, whereas a score ≤6 forecasts a good recovery.

7.4.9.1.2 Stroke-Specific Quality of Life Scale (SSQOL)

The SSQOL is a more comprehensive questionnaire than the 36-item Short Form Health Survey (SF-36). For example, the SF-36 and the Euro Quality of Life questionnaires are commonly used in stroke trials but do not assess language, hand function, cognition or vision (Muus, Williams, & Ringsberg, 2007; Williams, Weinberger, Harris, & Biller, 1999).

SSQOL scales have been shown to be more valid and reliable in measuring stroke-specific health-related quality of life which is moderately responsive to changes in most domains during the first three months after stroke. A study conducted by Williams, Weinberger, Harris, Clark and Biller (1999) suggests that the SSQOL is superior to common generic health-related quality of life measures.

The SSQOL includes 12 domains of assessment and covers a broader range of functions typically affected by stroke. These areas include mobility, energy, upper extremity function, work and productivity, mood, self-care, social roles, family roles, vision, language, thinking and personality.

7.4.9.2 Secondary outcome measures

7.4.9.2.1 Modified Rankin Scale (mRS)

The modified Rankin Scale (mRS) is a commonly used scale for measuring degree of global disability or dependency in daily activities caused by stroke and other neurological impairments. The scale ranges from 0 to 5, from perfect health without symptoms to severe disability (Sulter, Steen, & De Keyser, 1999). While mRS is widely applied for evaluating stroke patient outcomes, it is also widely used as valid end point in randomised clinical trials assessing new treatment paradigms (Banks & Marotta, 2007).

The primary investigator will be trained in the use of mRS. Scores will be recorded according to patients' degree of disability and dependence.

7.4.9.2.2 Barthel Scale

The Barthel Scale is a widely used ordinal scale measuring performance in activities of daily living (ADL), including feeding, bathing, grooming, dressing, toilet use, mobility and ascending/descending stairs (C. Granger, Dewis, Peters, Sherwood, & Barrett, 1979).

The Barthel index is one of the first contributions to the functional status literature and it represents occupational therapists' lengthy period of inclusion of functional mobility and ADL measurement within their scope of practice. It has been used extensively to monitor functional changes in individuals receiving in-patient rehabilitation, mainly in predicting the functional outcomes related to stroke (Collin, Wade, Davies, & Horne, 1988).

The Barthel Scale will be assessed by asking relevant question and rating responses from 0 to 100. A score of 100 represents independence; scores between 75 and 95 represent mild dependency; scores between 50 and 70 represent moderate dependency; scores between 25 and 45 represent severe dependency; and scores between 0 and 25 suggest total dependency. Responses will be sought from a patient's direct carer if the patient is highly dependent.

7.4.9.2.3 Lipid profile, blood rheology, coagulation index and inflammatory marker.

Patients will be sent to a Douglass Hanly Moir Pathology laboratory (multiple locations across metropolitan Sydney and regional NSW) for blood sampling and pathology testing. Assessment areas for lipid profiles include triglycerides, total serum cholesterol, high-density lipids and low-density lipids. Douglass Hanly Moir Pathology and all its laboratories are fully accredited with the National Association of Testing Authorities (NATA) and registered under the accreditation scheme of the Royal College of Pathologists of Australasia (RCPA).

A copy of the pathology results will be inserted into the participant file during the study and stored in a locked filing cabinet in a secure and lockable room, accessed only by the trial research staff. The email copy of results can only be accessed by secure password known only

to the Chief Investigator and Principal Researcher. Upon completion of the trial, the pathology results will be securely and separately stored from the coded participant files, as the pathology results will be identifiable. These paper files will remain securely stored.

7.4.9.2.4 COMPASS computer test battery

The Computerised Mental Performance Assessment System (COMPASS) features a number of computerised cognitive tasks designed to test various aspects of mental function. The COMPASS test battery employs similar tasks to those found in the Computerised Drug Research (CDR) and Cambridge Neurological Assessment Battery (CANTAB) test batteries, both of which have been used extensively in cognitive studies (Robbins et al., 1994; Sahakian & Owen, 1992; Wesnes, Ward, McGinty, & Petrini, 2000). The COMPASS test battery can be customised with a range of computerised tests. Alternative forms of each task are to be presented during each testing session and data will be automatically captured by the COMPASS software.

7.4.9.2.4.1 Rapid visual information processing (RVIP)

A series of digits from 1 to 9 will be presented sequentially, with a stimulus presentation time of 500ms. Participants are required to press the spacebar whenever they detect three consecutive even or three consecutive odd digits. The tasks will last for 5 minutes and included a total of 40 targets, i.e. runs of three consecutive odd/even digit series. The outcome measures will be measured in averaged response time and percentage of correct responses.

7.4.9.2.4.2 Wisconsin card sorting test

This task will present four static cards at the top of the screen with each card being different based on the four variables of colour, shape, number of shapes and arrangement of shapes.

Below these four cards, a new card will be presented to the participant. This card will be randomised based on the abovementioned four variables. The participant is required to match this card to one of the four static cards at the top of the screen based on the abovementioned variables. Participants will not be given instructions as to which variable has been chosen as the key variable to which they should match. Periodically, the test will randomly switch the key variable; again, participants will not be notified when this occurs. The outcome measure will be measured in number of correct and incorrect responses, average reaction time and accuracy per category shift.

7.4.9.2.4.3 4 Choice reaction time (4CRT)

This task employs an on-screen representation of the four directional arrows found on a standard keyboard. One of the four arrows will light up and participants will be instructed to press the corresponding directional key with the index finger of their dominant hand as quickly and as accurately as possible. There will be 48 trials (12 per choice) presented with a varying inter-stimulus interval of 1–3 seconds. The outcome measure will be measured in averaged response time and percentage of correct response.

7.4.9.2.4.3.5 N-Back

The participant will be presented with a series of letters one at a time. The participant will be required to decide if the current letter is the same as the letter shown three letters earlier. To reduce visual recognition strategies, a mix of upper and lower case letters are used. Participants are required to press the 'Z' key for NO and the 'M' key for YES. The outcome measure will be measured in averaged response time and percentage of correct response.

7.4.9.2.4.3.6 Word presentation, immediate word recall, delayed word recall and word recognition

In word presentation, participants will be presented with a list of ten words to be remembered. These will be presented on the monitor for one second at a rate of one per

second. Immediately after presentation, participants will be given one minute to recall as many of the ten words as possible. The outcome measures to be measured are number of correct responses.

At the end of the COMPASS session, participants will be given one minute to recall as many of the ten words from the word presentation as possible. The outcome measures will be measured in number of correct responses. Following this delayed word recall task, participants will be presented with the original ten words from the word presentation task as well as ten distracter words. Participants are required to decide whether the word being presented is from the original ten or not. Participants are required to press the 'Z' key for NO and the 'M' key for YES. The outcome measures will be measured in correct responses.

7.4.9.2.5 Ultrasound – flow-mediated dilation (FMD)

High-resolution B-mode ultrasonography will be performed using a 13Mhz linear-array transducer on a GE Vivid I ultrasound system to determine brachial artery diameter and change due to flow-mediated dilation (both dependent and independent). Doppler ultrasound will be employed to determine peak blood flow velocity and shear rate when combined with additional diameter data.

Pre-processing configurations will be held constant during all examinations. The image should clearly show vascular boundaries in the longitudinal plane allowing for visualisation of the double lines of Pignoli and distinguishable demarcated boundaries for precise diameter measurement (≤0.05mm) by automated edge-detection software.

7.4.9.2.6 Dependent endothelial function

With the subject lying supine, a blood pressure cuff will be attached below the imaged brachial artery and inflated to 250mmHg to ensure complete arterial occlusion. Prior to cuff inflation, baseline vessel diameter and blood flow velocities will be captured. The cuff will

remain inflated for a period of 5 minutes and once the cuff is deflated, measurements will be conducted using a Doppler beam-vessel angle of ≤60° that will allow for an estimation of blood flow velocity. Continued measurements for peak arterial diameter will be conducted over a period of at least 180 seconds post-cuff deflation.

Subjects will be fasted and have avoided exercise, caffeine, alcohol, drugs, stimulants and medications for a consistent period of time (at least 6 h) to minimise the effect of these confounding factors. The assessments should be conducted after a standardised period of time following any necessary medication and a careful history of medication use and timing should be collected. Furthermore, assessments are to be conducted at similar times of day in a quiet (preferably darkened), temperature-controlled thermoneutral room after the subject has been resting quietly for 5 minutes.

7.4.9.2.6 Safety measures

7.4.9.2.6.1 Adverse events and severity criteria

The presence and frequency of adverse events will be recorded at each assessment session on a paper-based form. Participants will be encouraged to note any adverse events during the trial period between the assessment time-points and to contact the researchers immediately. For classification of adverse events, refer to Appendix B.

7.4.9.2.6.2 General medical assessment

General medical information, including body temperature, heart rate, breathing and blood pressure, will be measured and recorded at each assessment visit.

7.4.9.2.6.3 Blood Test, LFT and RFT

Routine blood, urine and faeces tests, liver function (ALT, AST, TBIL, γ-GT, ALP), kidney function (BUN, Cr) and electrocardiogram (ECG) will be assessed at each assessment in week 2, week 6 and week 12 of the treatment period.

7.4.9.2.6.4 Long-term efficacy assessment

Participant will be followed up by phone at week 16 (4 weeks post-treatment) and week 36 (24 weeks post-treatment) during the trial period. Any incidents – including death, recurrence of ischaemic stroke, cerebral-vascular incidents and cardiovascular incidents – will be recorded and the reasons analysed.

7.4.10 Data collection and statistics

7.4.10.1 Treatment duration

A 12-week treatment duration and a 24-week follow-up period.

Table 15: Data collection timeline

Exam			Screening	Baseline	Wk2	Wk 6	Wk 12	Wk16	Wk 36
Screening	Medical History		•						
	Physical Exam		•						
Primary	NIHSS			•		•	•	•	
	SSQOL			•		•	•	•	
Secondary	MRS			•		•	•	•	
	Barthel Scale			•		•	•	•	
	Lipids Profile						•		
	Blood rheology			•	•	•	•		
	Coagulation Study			•	•	•	•		
	Blood Pressure			•		•	•		
	Inflammatory marker	C-RP		•		•	•		
		TNF-a		•			•		
	COMPASS			•			•		
	Ultrasound			•			•		
Safety	Adverse event			•	•	•	•	•	•
	Full blood count		•			•	•		
	LFT; RFT		•		•	•	•		
Long-term efficacy	Death; Reoccurrence of IS; incidents of CardioVD or CerebroVD							•	•

Data will be collected using proprietary software employed by equipment manufacturers for the COMPASS and Vivid I. Data from COMPASS will be exported from an Excel spreadsheet

directly to an SPSS database. Data from the Vivid I system, pathology results and stroke outcome assessments will be entered into or collected by Evado Clinical Trial Software during the course of the trial and exported to a SPSS database for analysis.

Analysis will take the form of ANCOVA of each variable at post-treatment while controlling for baseline measurements and other possible confounders such as age, gender, ethnicity and BMI. Only participants with complete datasets on the relevant variables will be included in the analysis. Results will be considered statistically significant when $p<0.05$.

Chapter 8. Summary and Conclusion

Ischaemic stroke is responsible for 80% of stroke incidence (Andersen et al., 2009b). Patients who suffer ischaemic stroke may be left with permanent neurological and motor dysfunctions and increased risks of vascular dementia (Desmond et al., 2000). Current conventional treatment for ischaemic stroke, whether surgical or pharmaceutical, is focused on restoring cerebral blood flow and preventing expansion of ischaemic damage (Prabhakaran, Ruff, & Bernstein, 2015). However, the restoration of blood flow typically induces reperfusion injury, which contributes to major neuron damage additional to that of ischaemic origin (Hausenloy & Yellon, 2013). Currently, no therapeutic options are available to prevent neuron damage associated with reperfusion injury; therefore, patients who suffer ischaemic stroke are at great risk of motor and neurological deficits and increased risk of developing vascular dementia.

NXQ, a standardised, patented herbal extract derived from *Diospyros kaki. L.*, has been used as therapeutic agent in China for apoplexy syndromes, atherosclerosis, cardiovascular diseases such as angina, and cerebral ischaemia (Bei et al., 2007; Y. Cai & Yang, 2001). Preclinical research demonstrates that NXQ has neuroprotective effects against H_2O_2-induced NG108 neuron cells and reduces cerebral infarction in MCAO transient focal cerebral ischaemic rats (Bei et al., 2007). However, its role in vascular protection and angiogenic vascularisation has not been not evaluated. Quercetin and kaempferol are reported to be the main bioactive components of *D. kaki. L.* responsible for the therapeutic effects of NXQ (Bei et al., 2007). However, no studies have been conducted to evaluate how these individual components contribute to the therapeutic effects of NXQ, or whether they interact to generate synergistic effects. Moreover, the efficacy and safety of NXQ in the management of

ischaemic stroke has not been evaluated in rigorous clinical trials (Y. Cai & Yang, 2001; Y. Chen et al., 2012; B. Tang et al., 2012; A. Wang et al., 2012; D. Wu et al., 2013).

This doctoral project contains four components. Three experimental studies were conducted to determine the ratio of quercetin and kaempferol in NXQ extracts and to evaluate NXQ's anti-oxidant and pro-angiogenesis properties. In the last study, a randomised clinical trial protocol has been developed to evaluate the effectiveness and safety of NXQ.

In the first study, the analytical methods previously developed for seven key analytes of NXQ, including quercetin and kaempferol, were re-established and briefly validated. The seven analytes were identified and quantified in NXQ extracts and the ratio of quercetin and kaempferol was determined in NXQ extract (3:4).

The second study demonstrated that NXQ significantly attenuated H_2O_2-induced ROS generation and LDH leakage and restored cell viability. Further to this, a combination of quercetin and kaempferol in the ratio of 3:4 significantly restored cell viability when compared to quercetin and kaempferol used alone, suggesting a potential synergistic or additive interaction. In addition, quercetin appeared to possess a greater anti-oxidant effect than kaempferol. A combination of two components produced a greater anti-oxidant effect than that used alone, further demonstrating potential synergistic/additive interactions between the two components. This series of studies also attempted to develop a zebrafish hypoxia model to evaluate the *in vivo* neuroprotective effects of NXQ. However, we were unable to successfully develop this model due to several methodological issues. Some preliminary results were presented and discussed.

In the third study, it was demonstrated that NXQ significantly induced angiogenesis in VRI-inhibited Tg(fli1:EFGP) zebrafish embryos. However, NXQ did not promote cell proliferation or migration in EA.hy926 endothelial cells.

In the last study, a protocol for a randomised, double-blind, placebo-controlled trial was developed. This pilot clinical trial protocol aims to evaluate the safety profile and clinical effects of NXQ on neurological and cognitive functions in patients suffering from ischaemic stroke. Ethics approval was obtained and recruitment centres established. This study provides a solid foundation for the conduct of this important trial in the future.

In summary, it has been demonstrated that NXQ possesses vascular protection effects against H_2O_2-induced oxidative stress and may play a role in post-stroke revascularisation via angiogenesis. It has also been demonstrated that a quercetin and kaempferol combination at a ratio similar to that in the NXQ extract has superior effects against H_2O_2-induced EA.hy926 cell damage compared to the constituents alone, suggesting a potential synergistic or additive effect.

The current study provides preliminary evidence to support NXQ's role in vascular protection and angiogenesis. However, several limitations were identified in these studies. All these studies were conducted in an *in vitro* setting, and *in vivo* evaluations of these findings are needed. Only two of the key bioactive components of NXQ were studied and more research is needed to evaluate interactions among other components. In addition, more rigorous synergistic models should be used to determine and quantify the synergistic effects. For safety reasons, the clinical trial protocol was designed to evaluate the clinical effects of NXQ during the rehabilitation phase of ischaemic stroke. However, reperfusion injuries typically develop from the moment of regained blood flow until several days post-reperfusion. It is, therefore, desirable to consider the timing of administration of NXQ in future trials to evaluate its effects on restoring and preventing neuron damage induced by reperfusion.

In conclusion, these studies have provided some useful *in vitro* data to deepen our understanding of mechanisms underlying NXQ's effects in ischaemic stroke. Future studies

have been planned, including further development of the hypoxia model in zebrafish using metabolomics and/or proteomic methods and initiation of the clinical trial.

Bibliography

Abumiya, T., Lucero, J., Heo, J. H., Tagaya, M., Koziol, J. A., Copeland, B. R., & Del Zoppo, G. J. (1999). Activated microvessels express vascular endothelial growth factor and integrin αvβ3 during focal cerebral ischemia. *Journal of Cerebral Blood Flow & Metabolism, 19*(9), 1038-1050.

Adams, H., Bendixen, B. H., Kappelle, L. J., Biller, J., Love, B. B., Gordon, D. L., & Marsh, E. r. (1993). Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment. *Stroke, 24*(1), 35-41.

Adams, J. D., Wang, R., Yang, J., & Lien, E. J. (2006). Preclinical and clinical examinations of Salvia miltiorrhiza and its tanshinones in ischemic conditions. *Chinese medicine, 1*(1), 3.

Agrawal, A. (2011). Pharmacological activities of flavonoids: a review. *International journal of pharmaceutical sciences and nanotechnology, 4*(2), 1394-1398.

Ahmed, N., Näsman, P., & Wahlgren, N. G. (2000). Effect of intravenous nimodipine on blood pressure and outcome after acute stroke. *Stroke, 31*(6), 1250-1255.

Albers, G. W., Thijs, V. N., Wechsler, L., Kemp, S., Schlaug, G., Skalabrin, E., . . . Shuaib, A. (2006). Magnetic resonance imaging profiles predict clinical response to early reperfusion: the diffusion and perfusion imaging evaluation for understanding stroke evolution (DEFUSE) study. *Annals of neurology, 60*(5), 508-517.

Alfieri, A., Srivastava, S., Siow, R. C., Cash, D., Modo, M., Duchen, M. R., . . . Mann, G. E. (2013). Sulforaphane preconditioning of the Nrf2/HO-1 defense pathway protects the cerebral vasculature against blood–brain barrier disruption and neurological deficits in stroke. *Free Radical Biology and Medicine, 65*, 1012-1022.

Aliev, G., Obrenovich, M. E., Smith, M. A., & Perry, G. (2003). Hypoperfusion, mitochondria failure, oxidative stress, and Alzheimer disease. *BioMed Research International, 2003*(3), 162-163.

Allan, S. M., & Rothwell, N. J. (2001). Cytokines and acute neurodegeneration. *Nature Reviews Neuroscience, 2*(10), 734.

Allen, C. L., & Bayraktutan, U. (2008). Risk factors for ischaemic stroke. *International journal of Stroke, 3*(2), 105-116.

Almaida-Pagán, P. F., Lucas-Sanchez, A., & Tocher, D. R. (2014). Changes in mitochondrial membrane composition and oxidative status during rapid growth, maturation and aging in zebrafish, Danio rerio. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 1841*(7), 1003-1011.

Amaro, S., Soy, D., Obach, V., Cervera, Á., Planas, A. M., & Chamorro, Á. (2007). A pilot study of dual treatment with recombinant tissue plasminogen activator and uric acid in acute ischemic stroke. *Stroke, 38*(7), 2173-2175.

Andersen, K. K., Olsen, T. S., Dehlendorff, C., & Kammersgaard, L. P. (2009a). Hemorrhagic and Ischemic Strokes Compared Stroke Severity, Mortality, and Risk Factors. *Stroke, 40*(6), 2068-2072.

Andersen, K. K., Olsen, T. S., Dehlendorff, C., & Kammersgaard, L. P. (2009b). Hemorrhagic and ischemic strokes compared: stroke severity, mortality, and risk factors. *Stroke, 40*(6), 2068-2072.

Aronowski, J., Ostrow, P., Samways, E., Strong, R., Zivin, J., & Grotta, J. (1994). Graded bioassay for demonstration of brain rescue from experimental acute ischemia in rats. *Stroke, 25*(11), 2235-2240.

Arslan-Ergul, A., & Adams, M. M. (2014). Gene expression changes in aging zebrafish (Danio rerio) brains are sexually dimorphic. *BMC neuroscience, 15*(1), 29.

Asami, S., Manabe, H., Miyake, J., Tsurudome, Y., Hirano, T., Yamaguchi, R., . . . Kasai, H. (1997). Cigarette smoking induces an increase in oxidative DNA damage, 8-hydroxydeoxyguanosine, in a central site of the human lung. *Carcinogenesis, 18*(9), 1763-1766.

Aschner, J. L., Lum, H., Fletcher, P. W., & Malik, A. B. (1997). Bradykinin-and thrombin-induced increases in endothelial permeability occur independently of phospholipase C but require protein kinase C activation. *Journal of cellular physiology, 173*(3), 387-396.

Astrup, J. (1981). Thresholds in cerebral ischemia: the ischemic penumbra. *Stroke, 12*, 723-725.

Bajzar, L., Morser, J., & Nesheim, M. (1996). TAFI, or plasma procarboxypeptidase B, couples the coagulation and fibrinolytic cascades through the thrombin-thrombomodulin complex. *Journal of Biological Chemistry, 271*(28), 16603-16608.

Ballinger, S. W., Patterson, C., Yan, C.-N., Doan, R., Burow, D. L., Young, C. G., . . . Freeman, B. A. (2000). Hydrogen peroxide–and peroxynitrite-induced mitochondrial DNA damage and dysfunction in vascular endothelial and smooth muscle cells. *Circulation research, 86*(9), 960-966.

Bang, O. Y., Buck, B. H., Saver, J. L., Alger, J. R., Yoon, S. R., Starkman, S., . . . Sanossian, N. (2007). Prediction of hemorrhagic transformation after recanalization therapy using T2*-permeability magnetic resonance imaging. *Annals of neurology, 62*(2), 170-176.

Banks, J. L., & Marotta, C. A. (2007). Outcomes validity and reliability of the modified Rankin scale: Implications for stroke clinical trials a literature review and synthesis. *Stroke, 38*(3), 1091-1096.

Barber, M., Langhorne, P., Rumley, A., Lowe, G. D., & Stott, D. J. (2004). Hemostatic function and progressing ischemic stroke: D-dimer predicts early clinical progression. *Stroke, 35*(6), 1421-1425.

Barber, M., Langhorne, P., Rumley, A., Lowe, G. D., & Stott, D. J. (2006). D-dimer predicts early clinical progression in ischemic stroke: confirmation using routine clinical assays. *Stroke, 37*(4), 1113-1115.

Baron, J.-C. (1999). Mapping the ischaemic penumbra with PET: implications for acute stroke treatment. *Cerebrovascular diseases, 9*(4), 193-201.

Barone, F. C., & Feuerstein, G. Z. (1999). Inflammatory mediators and stroke: new opportunities for novel therapeutics. *Journal of Cerebral Blood Flow & Metabolism, 19*(8), 819-834.

Barrett, B., Kiefer, D., & Rabago, D. (1999). Assessing the risks and benefits of herbal medicine: an overview of scientific evidence. *Alternative Therapies in Health and Medicine, 5*(4), 40.

Barrett-Connor, E., & Khaw, K.-T. (1988). Diabetes mellitus: an independent risk factor for stroke? *American journal of epidemiology, 128*(1), 116-123.

Bartholomew, J., & Finkelstein, H. (1954). Crystal violet binding capacity and the Gram reaction of bacterial cells. *Journal of bacteriology, 67*(6), 689.

Becker, K. J. (1998). Inflammation and acute stroke. *Current opinion in neurology, 11*(1), 45-49.

Beckman, J. S. (1996). Oxidative damage and tyrosine nitration from peroxynitrite. *Chemical research in toxicology, 9*(5), 836-844.

Beckman, J. S., & Koppenol, W. H. (1996). Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *American Journal of Physiology-Cell Physiology, 271*(5), C1424-C1437.

Beckman, J. S., Ye, Y. Z., Chen, J., & Conger, K. A. (1996). The interactions of nitric oxide with oxygen radicals and scavengers in cerebral ischemic injury. *Advances in neurology, 71*, 339.

Bei, W., Li, C., Wang, D., Hu, D., Peng, W., & Xu, A. (2009). Effect of Naoxinqing and its flavonoid components on the L-type Ca2+ channel current in the cultured hippocampal pyramidal neurons of rats. *Journal of Guangdong Pharmaceutical College, 25*(3), 304-309.

Bei, W., Peng, W., Ma, Y., & Xu, A. (2004). NaoXinQing, an anti-stroke herbal medicine, reduces hydrogen peroxide-induced injury in NG108-15 cells. *Neuroscience letters, 363*(3), 262-265.

Bei, W., Peng, W., Ma, Y., & Xu, A. (2005a). Flavonoids from the leaves of Diospyros kaki reduce hydrogen peroxide-induced injury of NG108-15 cells. *Life sciences, 76*(17), 1975-1988.

Bei, W., Peng, W., Ma, Y., & Xu, A. (2005b). Flavonoids from the leaves of< i> Diospyros kaki</i> reduce hydrogen peroxide-induced injury of NG108-15 cells. *Life sciences, 76*(17), 1975-1988.

Bei, W., Peng, W., Zang, L., Xie, Z., Hu, D., & Xu, A. (2007). Neuroprotective effects of a standardized extract of Diospyros kaki leaves on MCAO transient focal cerebral ischemic rats and cultured neurons injured by glutamate or hypoxia. *Planta medica, 73*(07), 636-643.

Bei, W., Zang, L., Guo, J., Peng, W., Xu, A., Good, D. A., . . . Zhu, X. (2009). Neuroprotective effects of a standardized flavonoid extract from Diospyros kaki leaves. *Journal of ethnopharmacology, 126*(1), 134-142.

Belayev, L., Busto, R., Zhao, W., & Ginsberg, M. D. (1996). Quantitative evaluation of blood-brain barrier permeability following middle cerebral artery occlusion in rats. *Brain research, 739*(1-2), 88-96.

Bensky, D., Gamble, A., & Kaptchuk, T. J. (2004). *Chinese herbal medicine: materia medica* (Vol. 3): Eastland Press Seattle.

Berger, K., Weltermann, B., Kolominsky-Rabas, P., Meves, S., Heuschmann, P., Böhner, J., . . . Büttner, T. (1999). The reliability of stroke scales. The german version of NIHSS, ESS and Rankin scales]. *Fortschritte der Neurologie-Psychiatrie, 67*(2), 81.

Birben, E., Sahiner, U. M., Sackesen, C., Erzurum, S., & Kalayci, O. (2012). Oxidative stress and antioxidant defense. *World Allergy Organization Journal, 5*(1), 9.

Bischoff, J. (1997). Cell adhesion and angiogenesis. *The Journal of clinical investigation, 99*(3), 373-376.

Björkman, J.-A. E., Abrahamsson, T. I., Nerme, V. K., & Mattsson, C. J. (2005). Inhibition of carboxypeptidase U (TAFIa) activity improves rt-PA induced thrombolysis in a dog model of coronary artery thrombosis. *Thrombosis research, 116*(6), 519-524.

Blanco, M., Nombela, F., Castellanos, M., Rodriguez-Yanez, M., Garcia-Gil, M., Leira, R., . . . Moro, M. (2007). Statin treatment withdrawal in ischemic stroke: a controlled randomized study. *Neurology, 69*(9), 904-910.

Blau, H. M., & Banfi, A. (2001). The well-tempered vessel. *Nature medicine, 7*(5), 532.

Bonde, C., Noraberg, J., Noer, H., & Zimmer, J. (2005). Ionotropic glutamate receptors and glutamate transporters are involved in necrotic neuronal cell death induced by oxygen–glucose deprivation of hippocampal slice cultures. *Neuroscience, 136*(3), 779-794.

Borensztajn, K., Peppelenbosch, M. P., & Spek, C. A. (2008). Factor Xa: at the crossroads between coagulation and signaling in physiology and disease. *Trends in molecular medicine, 14*(10), 429-440.

Bouma, B., & Meijers, J. (2003). Thrombin-activatable fibrinolysis inhibitor (TAFI, plasma procarboxypeptidase B, procarboxypeptidase R, procarboxypeptidase U). *Journal of Thrombosis and Haemostasis, 1*(7), 1566-1574.

Braga, M. M., Rico, E. P., Córdova, S. D., Pinto, C. B., Blaser, R. E., Dias, R. D., . . . Souza, D. O. (2013). Evaluation of spontaneous recovery of behavioral and brain injury profiles in zebrafish after hypoxia. *Behavioural brain research, 253*, 145-151.

Broderick, J. P. (2004). William M. Feinberg Lecture: Stroke Therapy in the Year 2025 Burden, Breakthroughs, and Barriers to Progress. *Stroke, 35*(1), 205-211.

Brouns, R., Heylen, E., Sheorajpanday, R., Willemse, J. L., Kunnen, J., De Surgeloose, D., . . . De Deyn, P. P. (2009). Carboxypeptidase U (TAFIa) decreases the efficacy of thrombolytic therapy in ischemic stroke patients. *Clinical neurology and neurosurgery, 111*(2), 165-170.

Brouns, R., Sheorajpanday, R., Wauters, A., De Surgeloose, D., Mariën, P., & De Deyn, P. P. (2008). Evaluation of lactate as a marker of metabolic stress and cause of secondary damage in acute ischemic stroke or TIA. *Clinica chimica acta, 397*(1-2), 27-31.

Buck, B. H., Liebeskind, D. S., Saver, J. L., Bang, O. Y., Yun, S. W., Starkman, S., . . . Salamon, N. (2008). Early neutrophilia is associated with volume of ischemic tissue in acute stroke. *Stroke, 39*(2), 355-360.

Bullock, R., Zauner, A., Woodward, J. J., Myseros, J., Choi, S. C., Ward, J. D., . . . Young, H. F. (1998). Factors affecting excitatory amino acid release following severe human head injury. *Journal of neurosurgery, 89*(4), 507-518.

Burd, J., & Usategui-Gomez, M. (1973). A colorimetric assay for serum lactate dehydrogenase. *Clinica Chimica Acta, 46*(3), 223-227.

Cadilhac, D. A., Carter, R., Thrift, A. G., & Dewey, H. M. (2009). Estimating the Long-Term Costs Of Ischemic and Hemorrhagic Stroke for Australia New Evidence Derived From the North East Melbourne Stroke Incidence Study (NEMESIS). *Stroke, 40*(3), 915-921.

Cai, G., Liu, B., Liu, W., Tan, X., Rong, J., Chen, X., . . . Shen, J. (2007). Buyang Huanwu Decoction can improve recovery of neurological function, reduce infarction volume, stimulate neural proliferation and modulate VEGF and Flk1 expressions in transient focal cerebral ischaemic rat brains. *Journal of ethnopharmacology, 113*(2), 292-299.

Cai, Y., & Yang, S. (2001). Effect of Naoxinqing Tablet for cerebral atherosclerosis and angina pectoris of coronary heart disease: an observation of 60 cases. *Tradit. Chin. Drug Res. Clin. Pharmacol, 12*(6), 414-416.

Calver, A., Collier, J., & Vallance, P. (1993). Nitric oxide and the control of human vascular tone in health and disease. *The European journal of medicine, 2*(1), 48-53.

Cao, S., Zhang, X., Bai, M., Wang, H., & Miao, M. (2012). Exploration on the Pharmacological Effects of the Total Flavonoids of Persimmon and Its Clinical Application. *China Journal of Chinese Medicine, 27*(171), 987-989.

Cao, Z., Jensen, L. D., Rouhi, P., Hosaka, K., Länne, T., Steffensen, J. F., . . . Cao, Y. (2010). Hypoxia-induced retinopathy model in adult zebrafish. *Nature protocols, 5*(12), 1903-1910.

Carmeliet, P., & Jain, R. K. (2011). Molecular mechanisms and clinical applications of angiogenesis. *Nature, 473*(7347), 298.

Celik, A., & Ercisli, S. (2008). Persimmon cv. Hachiya (Diospyros kaki Thunb.) fruit: some physical, chemical and nutritional properties. *International Journal of Food Sciences and Nutrition, 59*(7-8), 599-606.

Challa, S., & Chan, F. K.-M. (2010). Going up in flames: necrotic cell injury and inflammatory diseases. *Cellular and molecular life sciences, 67*(19), 3241-3253.

Chamorro, Á., & Hallenbeck, J. (2006). The harms and benefits of inflammatory and immune responses in vascular disease. *Stroke, 37*(2), 291-293.

Chan, E., Tan, M., Xin, J., Sudarsanam, S., & Johnson, D. E. (2010). Interactions between traditional Chinese medicines and Western. *Curr Opin Drug Discov Devel, 13*(1), 50-65.

Chan, F. K.-M., Moriwaki, K., & De Rosa, M. J. (2013). Detection of necrosis by release of lactate dehydrogenase activity *Immune Homeostasis* (pp. 65-70): Springer.

Chan, J. R., Böger, R. H., Bode-Böger, S. M., Tangphao, O., Tsao, P. S., Blaschke, T. F., & Cooke, J. P. (2000). Asymmetric dimethylarginine increases mononuclear cell adhesiveness in hypercholesterolemic humans. *Arteriosclerosis, thrombosis, and vascular biology, 20*(4), 1040-1046.

Chan, P., Thomas, G. N., & Tomlinson, B. (2002). Protective effects of trilinolein extrated from Panax notoginseng against cardiovascular disease. *Acta Pharmacologica Sinica, 23*(12), 1157-1162.

Chan, P. H. (2001). Reactive oxygen radicals in signaling and damage in the ischemic brain. *Journal of Cerebral Blood Flow & Metabolism, 21*(1), 2-14.

Chang, C.-C., Lee, Y.-C., Lin, C.-C., Chang, C.-H., Chiu, C.-D., Chou, L.-W., . . . Yen, H.-R. (2016). Characteristics of traditional Chinese medicine usage in patients with stroke in Taiwan: a nationwide population-based study. *Journal of ethnopharmacology, 186*, 311-321.

Chávez, M. N., Aedo, G., Fierro, F. A., Allende, M. L., & Egaña, J. T. (2016). Zebrafish as an emerging model organism to study angiogenesis in development and regeneration. *Frontiers in physiology, 7*, 56.

Che, J., Liang, B., Zhang, Y., Wang, Y., Tang, J., & Shi, G. (2017). Kaempferol alleviates ox-LDL-induced apoptosis by up-regulation of autophagy via inhibiting PI3K/Akt/mTOR pathway in human endothelial cells. *Cardiovascular Pathology, 31*, 57-62.

Che, X., Ye, W., Panga, L., Wu, D.-C., & Yang, G.-Y. (2001). Monocyte chemoattractant protein-1 expressed in neurons and astrocytes during focal ischemia in mice. *Brain research, 902*(2), 171-177.

Chen, A. Y., & Chen, Y. C. (2013). A review of the dietary flavonoid, kaempferol on human health and cancer chemoprevention. *Food chemistry, 138*(4), 2099-2107.

Chen, G. (2003). *Chemical constituents and the biaoactivities of Diospyros Kaki Leaf.* (Doctor dissertation), Shenyang pharmacetical university Shenyang.

Chen, G., Lu, H., Wang, C., Yamashita, K., Manabe, M., Meng, Z., . . . Kodama, H. (2002). Effect of five flavonoid compounds isolated from leaves of Diospyros kaki on stimulus-induced superoxide generation and tyrosyl phosphorylation of proteins in human neutrophils. *Clinica chimica acta, 326*(1-2), 169-175.

Chen, G., Lu, H., Wang, C., Yamashita, K., Manabe, M., Xu, S., & Kodama, H. (2002). Effect of five triterpenoid compounds isolated from leaves of Diospyros kaki on stimulus-induced superoxide generation and tyrosyl phosphorylation in human polymorphonuclear leukocytes. *Clinica chimica acta, 320*(1-2), 11-16.

Chen, G., Sha, Ching. (2000). Chemical analysis of Persimmon (Ⅰ). *China Phamaco chemisty journal, 10*(4), 298-299.

Chen, G., Wei, S.-H., Huang, J., & Sun, J. (2009). A novel C-glycosylflavone from the leaves of Diospyros kaki. *Journal of Asian natural products research, 11*(6), 503-507.
Chen, G., Xu, S.-X., Wang, H.-Z., & Zhang, R.-Q. (2005). Note: Kakispyrol, a new biphenyl derivative from the leaves of Diospyros kaki. *Journal of Asian natural products research, 7*(3), 265-268.
Chen, G., Xue, J., Xu, S.-X., & Zhang, R.-Q. (2007). Chemical constituents of the leaves of Diospyros kaki and their cytotoxic effects. *Journal of Asian natural products research, 9*(4), 347-353.
Chen, K., Xu, Hao. (2003). The integration of traditional Chinese medicine and Western medicine. *European Review, 11*(2), 225-235.
Chen, X., Zhou, M., Li, Q., Yang, J., Zhang, Y., Zhang, D., . . . He, L. (2008a). Sanchi for acute ischaemic stroke. *Cochrane Database Syst Rev, 4*.
Chen, X., Zhou, M., Li, Q., Yang, J., Zhang, Y., Zhang, D., . . . He, L. (2008b). Sanchi for acute ischaemic stroke. *Cochrane Database of Systematic Reviews*(4).
Chen, Y., Shao, L., & Cheng, Z. (2012). Effects of Low-molecular weight heparins calcium combined with Naoxinqingpian in treatment of transient ischemic attack. *China Modern Doctor, 50*(14), 75-76.
Chen, Z., Sandercock, P., Pan, H., Counsell, C., Collins, R., Liu, L., . . . Peto, R. (2000). Indications for early aspirin use in acute ischemic stroke a combined analysis of 40 000 randomized patients from the Chinese Acute Stroke Trial and the International Stroke Trial. *Stroke, 31*(6), 1240-1249.
Chen, Z., Sandercock, P., Xie, J.-X., Peto, R., Collins, R., & Liu, L.-S. (1997). Hospital management of acute ischemic stroke in China. *Journal of Stroke and Cerebrovascular Diseases, 6*(5), 361-367.
Chen, Z.-L., & Strickland, S. (1997). Neuronal death in the hippocampus is promoted by plasmin-catalyzed degradation of laminin. *Cell, 91*(7), 917-925.
Cheng, Y. D., Al-Khoury, L., & Zivin, J. A. (2004). Neuroprotection for ischemic stroke: two decades of success and failure. *NeuroRx, 1*(1), 36-45.
Chiba, Y., Sasayama, T., Miyake, S., Koyama, J., Kondoh, T., Hosoda, K., & Kohmura, E. (2008). Anti-VEGF receptor antagonist (VGA1155) reduces infarction in rat permanent focal brain ischemia. *Kobe J Med Sci, 54*(2), E136-146.
Choi, D. W. (1988). Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. *Trends in neurosciences, 11*(10), 465-469.
Choi, D. W. (1995). Calcium: still center-stage in hypoxic-ischemic neuronal death. *Trends in neurosciences, 18*(2), 58-60.
Choi, D. W. (1996). Ischemia-induced neuronal apoptosis. *Current opinion in neurobiology, 6*(5), 667-672.
Choo, V. (1993). CONFERENCE: Healing with recovery of function. *The Lancet, 342*(8872), 673.
Chu, H., & Wang, Y. (2012). Therapeutic angiogenesis: controlled delivery of angiogenic factors. *Therapeutic delivery, 3*(6), 693-714.
Clark, A. W., Krekoski, C. A., Bou, S.-S., Chapman, K. R., & Edwards, D. R. (1997). Increased gelatinase A (MMP-2) and gelatinase B (MMP-9) activities in human brain after focal ischemia. *Neuroscience letters, 238*(1-2), 53-56.
Clark, W., Warach, S., Pettigrew, L., Gammans, R., & Sabounjian, L. (1997). A randomized dose-response trial of citicoline in acute ischemic stroke patients. *Neurology, 49*(3), 671-678.

Clark, W. M., Williams, B. J., Selzer, K. A., Zweifler, R. M., Sabounjian, L. A., & Gammans, R. E. (1999). A randomized efficacy trial of citicoline in patients with acute ischemic stroke. *Stroke, 30*(12), 2592-2597.

Coffey C. Edward, C. J. L., Starkstein Sergio, Robinson Robert (2000). *Stroke - the American Psychiatric Press Textbook of Geriatric Neuropsychiatry* (Second ed.). Washington DC: American Psychiatric Press.

Cohen, R. A., Hasegawa, Y., & Fisher, M. (1994). Effects of a novel NMDA receptor antagonist on experimental stroke quantitatively assessed by spectral EEG and infarct volume. *Neurological research, 16*(6).

Cole, D. J., Drummond, J. C., Osborne, T. N., & Matsumura, J. (1990). Hypertension and hemodilution during cerebral ischemia reduce brain injury and edema. *American Journal of Physiology-Heart and Circulatory Physiology, 259*(1), H211-H217.

Collin, C., Wade, D., Davies, S., & Horne, V. (1988). The Barthel ADL Index: a reliability study. *Disability & Rehabilitation, 10*(2), 61-63.

Collins, R. C., Dobkin, B. H., & Choi, D. W. (1989). Selective vulnerability of the brain: new insights into the pathophysiology of stroke. *Annals of internal medicine, 110*(12), 992-1000.

Conway, E. M., Collen, D., & Carmeliet, P. (2001). Molecular mechanisms of blood vessel growth. *Cardiovascular research, 49*(3), 507-521.

Costa, L. G., Garrick, J. M., Roquè, P. J., & Pellacani, C. (2016). Mechanisms of neuroprotection by quercetin: counteracting oxidative stress and more. *Oxidative medicine and cellular longevity, 2016*.

Coyle, C. H., Martinez, L. J., Coleman, M. C., Spitz, D. R., Weintraub, N. L., & Kader, K. N. (2006). Mechanisms of H2O2-induced oxidative stress in endothelial cells. *Free Radical Biology and Medicine, 40*(12), 2206-2213.

Coyle, J. T., & Puttfarcken, P. (1993). Oxidative stress, glutamate, and neurodegenerative disorders. *Science, 262*(5134), 689-695.

Crack, P. J., & Wong, C. H. (2008). Modulation of neuro-inflammation and vascular response by oxidative stress following cerebral ischemia-reperfusion injury. *Current medicinal chemistry, 15*(1), 1-14.

Cui, J., Holmes, E. H., Greene, T. G., & Liu, P. K. (2000). Oxidative DNA damage precedes DNA fragmentation after experimental stroke in rat brain. *The FASEB Journal, 14*(7), 955-967.

D'Orlando, K., & Sandage Jr, B. W. (1995). Citicoline (CDP-choline): mechanisms of action and effects in ischemic brain injury. *Neurological research, 17*(4), 281.

Danton, G. H., & Dietrich, W. D. (2003). Inflammatory mechanisms after ischemia and stroke. *Journal of Neuropathology & Experimental Neurology, 62*(2), 127-136.

Das Nair, R., & Lincoln, N. (2007). Cognitive rehabilitation for memory deficits following stroke. *Cochrane Database of Systematic Reviews, 3*.

Daum, R. S., Scriver, C. R., Mamer, O. A., Delvin, E., Lamm, P., & Goldman, H. (1973). An inherited disorder of isoleucine catabolism causing accumulation of α-methylacetoacetate and α-methyl-β-hydroxybutyrate, and intermittent metabolic acidosis. *Pediatric research, 7*(3), 149.

Deadman, P., Al-Khafaji, M., Baker, K. (2006). (2006). *A Manual of Acupuncture.* Hove: Eastland Press.

DeGraba, T. J. (1998). The role of inflammation after acute stroke Utility of pursuing anti-adhesion molecule therapy. *Neurology, 51*(3 Suppl 3), S62-S68.

Del Zoppo, G. (1994). Microvascular changes during cerebral ischemia and reperfusion. *Cerebrovascular and brain metabolism reviews, 6*(1), 47-96.

Del Zoppo, G. J., & Hallenbeck, J. M. (2000). Advances in the vascular pathophysiology of ischemic stroke. *Thrombosis research, 98*(3), 73-81.
Del Zoppo, G. J., & Mabuchi, T. (2003). Cerebral microvessel responses to focal ischemia. *Journal of Cerebral Blood Flow & Metabolism, 23*(8), 879-894.
Del Zoppo, G. J., Schmid-Schönbein, G. W., Mori, E., Copeland, B. R., & Chang, C.-M. (1991). Polymorphonuclear leukocytes occlude capillaries following middle cerebral artery occlusion and reperfusion in baboons. *Stroke, 22*(10), 1276-1283.
Del Zoppo, G. J., VON KUMMER, R., & Hamann, G. F. (1998). Ischaemic damage of brain microvessels: inherent risks for thrombolytic treatment in stroke: BMJ Publishing Group Ltd.
Denekamp, J. (1993). Angiogenesis, neovascular proliferation and vascular pathophysiology as targets for cancer therapy. *The British Journal of Radiology, 66*(783), 181-196.
Deng, C., He, Fuyang., Tang, Yinghong., Deng, Yihui., Wan, Ming. (2000). Effect of Buyang Huanwu Decoction and its available composition combination on ultrastructure of hippocampal gyrus CA~ 1 area after cerebral ischemia reperfusion in gerbils. *Pharmacology and Clinics of Chinese Materia Medica, 16*(3), 7-11.
Deng, C.-Q., Deng, Y.-H., Xiang, H.-L. . (2001). Effects of Bu Yang Huan Wu Decoction and its active fraction on excitatory amino acid of brain tissue after cerebral ischemia and reperfusion in gerbils. . *Chinese Journal of Experimental Traditional Medical Formulae, 7*, 24-27.
Desmond, D. W., Moroney, J. T., Paik, M. C., Sano, M., Mohr, J. P., Aboumatar, S., . . . Remien, R. H. (2000). Frequency and clinical determinants of dementia after ischemic stroke. *Neurology, 54*(5), 1124-1131.
Deveza, L., Choi, J., & Yang, F. (2012). Therapeutic angiogenesis for treating cardiovascular diseases. *Theranostics, 2*(8), 801.
Dhalla, N. S., Temsah, R. M., & Netticadan, T. (2000). Role of oxidative stress in cardiovascular diseases. *Journal of hypertension, 18*(6), 655-673.
Dimitrijevic, O. B., Stamatovic, S. M., Keep, R. F., & Andjelkovic, A. V. (2006). Effects of the chemokine CCL2 on blood–brain barrier permeability during ischemia–reperfusion injury. *Journal of Cerebral Blood Flow & Metabolism, 26*(6), 797-810.
Dirnagl, U., Iadecola, C., & Moskowitz, M. A. (1999). Pathobiology of ischaemic stroke: an integrated view. *Trends in neurosciences, 22*(9), 391-397.
Dohmen, C., Bosche, B., Graf, R., Reithmeier, T., Ernestus, R.-I., Brinker, G., . . . Heiss, W.-D. (2007). Identification and clinical impact of impaired cerebrovascular autoregulation in patients with malignant middle cerebral artery infarction. *Stroke, 38*(1), 56-61.
Dong, E., Peng, LF., Gao, GL. . (1990). Effect of sanchi on rats LPO and SOD. *Chinese Traditional and Herbal Drugs, 21*(4), 26-27.
Dong, Y., & Benveniste, E. N. (2001). Immune function of astrocytes. *Glia, 36*(2), 180-190.
Donnini, S., Finetti, F., Lusini, L., Morbidelli, L., Cheynier, V., Barron, D., . . . Ziche, M. (2006). Divergent effects of quercetin conjugates on angiogenesis. *British journal of nutrition, 95*(5), 1016-1023.
Dorweiler, B., Pruefer, D., Andrasi, T. B., Maksan, S. M., Schmiedt, W., Neufang, A., & Vahl, C. F. (2007). Ischemia-reperfusion injury. *European Journal of Trauma and Emergency Surgery, 33*(6), 600-612.
Dugan, L., Sensi, S., Canzoniero, L., Handran, S., Rothman, S., Lin, T.-S., . . . Choi, D. (1995). Mitochondrial production of reactive oxygen species in cortical neurons following exposure to N-methyl-D-aspartate. *Journal of Neuroscience, 15*(10), 6377-6388.

Dugan, L. L., & Choi, D. W. (1994). Excitotoxicity, free radicals, and cell membrane changes. *Annals of neurology, 35*(S1).

Dupuy, C., Virion, A., Ohayon, R., Kaniewski, J., Deme, D., & Pommier, J. (1991). Mechanism of hydrogen peroxide formation catalyzed by NADPH oxidase in thyroid plasma membrane. *Journal of Biological Chemistry, 266*(6), 3739-3743.

Dzau, V. J. (1994). Pathobiology of atherosclerosis and plaque complications. *American heart journal, 128*(6), 1300-1304.

Egert, S., Bosy-Westphal, A., Seiberl, J., Kürbitz, C., Settler, U., Plachta-Danielzik, S., . . . Rimbach, G. (2009). Quercetin reduces systolic blood pressure and plasma oxidised low-density lipoprotein concentrations in overweight subjects with a high-cardiovascular disease risk phenotype: a double-blinded, placebo-controlled cross-over study. *British Journal of Nutrition, 102*(7), 1065-1074.

El Kossi, M. M. H., & Zakhary, M. M. (2000). Oxidative stress in the context of acute cerebrovascular stroke. *Stroke, 31*(8), 1889-1892.

Ellertsdóttir, E., Lenard, A., Blum, Y., Krudewig, A., Herwig, L., Affolter, M., & Belting, H.-G. (2010). Vascular morphogenesis in the zebrafish embryo. *Developmental biology, 341*(1), 56-65.

Emsley, H. C., & Tyrrell, P. J. (2002). Inflammation and infection in clinical stroke. *Journal of Cerebral Blood Flow & Metabolism, 22*(12), 1399-1419.

Epe, B., Ballmaier, D., Roussyn, I., Briviba, K., & Sies, H. (1996). DNA damage by peroxynitrite characterized with DNA repair enzymes. *Nucleic acids research, 24*(21), 4105-4110.

Evans, M. D., & Cooke, M. S. (2004). Factors contributing to the outcome of oxidative damage to nucleic acids. *Bioessays, 26*(5), 533-542.

Fan, T.-P., Yeh, J.-C., Leung, K. W., Yue, P. Y., & Wong, R. N. (2006). Angiogenesis: from plants to blood vessels. *Trends in Pharmacological Sciences, 27*(6), 297-309.

Fang, Z. Z., Zhang, Y. Y., Wang, X. L., Cao, Y. F., Huo, H., & Yang, L. (2011). Bioactivation of herbal constituents: simple alerts in the complex system. *Expert Opin Drug Metab Toxicol, 7*(8), 989-1007. doi:10.1517/17425255.2011.586335

Fassbender, K., Dempfle, C.-E., Mielke, O., Schwartz, A., Daffertshofer, M., Eschenfelder, C., . . . Hennerici, M. (1999). Changes in coagulation and fibrinolysis markers in acute ischemic stroke treated with recombinant tissue plasminogen activator. *Stroke, 30*(10), 2101-2104.

Fassbender, K., Rossol, S., Kammer, T., Daffertshofer, M., Wirth, S., Dollman, M., & Hennerici, M. (1994). Proinflammatory cytokines in serum of patients with acute cerebral ischemia: kinetics of secretion and relation to the extent of brain damage and outcome of disease. *Journal of the Neurological Sciences, 122*(2), 135-139.

Feigenson, J. (1979). Stroke rehabilitation: effectiveness, benefits, and cost. Some practical considerations. *Stroke, 10*(1), 1-4.

Feigin, V. L. (2007). Herbal Medicine in Stroke Does It Have a Future? *Stroke, 38*(6), 1734-1736.

Feinberg, W. M., Erickson, L. P., Bruck, D., & Kittelson, J. (1996). Hemostatic markers in acute ischemic stroke: association with stroke type, severity, and outcome. *Stroke, 27*(8), 1296-1300.

Feuerstein, G., Liu, T., & Barone, F. (1994). Cytokines, inflammation, and brain injury: role of tumor necrosis factor-alpha. *Cerebrovascular and brain metabolism reviews, 6*(4), 341-360.

Fischer, U., Arnold, M., Nedeltchev, K., Brekenfeld, C., Ballinari, P., Remonda, L., . . . Mattle, H. P. (2005). NIHSS score and arteriographic findings in acute ischemic stroke. *Stroke, 36*(10), 2121-2125.

Fisher, M., & Francis, R. (1990). Altered coagulation in cerebral ischemia platelet, thrombin, and plasmin activity. *Archives of neurology, 47*(10), 1075-1079.

Fisher, M., & Garcia, J. H. (1996). Evolving stroke and the ischemic penumbra. *Neurology, 47*(4), 884-888.

Folkman, J. (2003). *Angiogenesis and apoptosis.* Paper presented at the Seminars in cancer biology.

Forman, H. J., & Kennedy, J. A. (1974). Role of superoxide radical in mitochondrial dehydrogenase reactions. *Biochemical and biophysical research communications, 60*(3), 1044-1050.

Formica, J., & Regelson, W. (1995). Review of the biology of quercetin and related bioflavonoids. *Food and chemical toxicology, 33*(12), 1061-1080.

Förstermann, U. (2010). Nitric oxide and oxidative stress in vascular disease. *Pflügers Archiv-European Journal of Physiology, 459*(6), 923-939.

Fridovich, I. (1978). The biology of oxygen radicals. *Science, 201*(4359), 875-880.

Fridovich, I. (1998). Oxygen toxicity: a radical explanation. *Journal of Experimental Biology, 201*(8), 1203-1209.

Fulton, D., Gratton, J.-P., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., . . . Sessa, W. C. (1999). Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature, 399*(6736), 597-601.

Funayama, S., & Hikino, H. (1979). Hypotensive principles of Diospyros kaki leaves. *Chemical and Pharmaceutical Bulletin, 27*(11), 2865-2868.

Furet, P., Bold, G., Hofmann, F., Manley, P., Meyer, T., & Altmann, K.-H. (2003). Identification of a new chemical class of potent angiogenesis inhibitors based on conformational considerations and database searching. *Bioorganic & medicinal chemistry letters, 13*(18), 2967-2971.

Furlan, M., Marchal, G., Derlon, J. M., Baron, J. C., & Viader, F. (1996). Spontaneous neurological recovery after stroke and the fate of the ischemic penumbra. *Annals of neurology, 40*(2), 216-226.

Gafner, F., Chapuis, J.-C., Msonthi, J. D., & Hostettmann, K. (1987). Cytotoxic naphthoquinones, molluscicidal saponins and flavonols from Diospyros zombensis. *Phytochemistry, 26*(9), 2501-2503.

Gao, X., Xu, D., Deng, Y., & Zhang, Y. (2004). Screening of angiotensin converting enzyme inhibitors from Salvia miltiorrhizae. *Zhongguo Zhong yao za zhi= Zhongguo zhongyao zazhi= China journal of Chinese materia medica, 29*(4), 359-362.

Gapizov, S. S., Petrovskaya, L., Shingarova, L., Svirschevskaya, E., Dolgikh, D., & Kirpichnikov, M. (2018). The Effect of TNF and VEGF on the Properties of Ea. hy926 Endothelial Cells in a Model of Multi-Cellular Spheroids. *Acta naturae, 10*(1), 34.

Gasche, Y., Copin, J.-C., Sugawara, T., Fujimura, M., & Chan, P. H. (2001). Matrix metalloproteinase inhibition prevents oxidative stress-associated blood–brain barrier disruption after transient focal cerebral ischemia. *Journal of Cerebral Blood Flow & Metabolism, 21*(12), 1393-1400.

Gasche, Y., Fujimura, M., Morita-Fujimura, Y., Copin, J.-C., Kawase, M., Massengale, J., & Chan, P. H. (1999). Early appearance of activated matrix metalloproteinase-9 after focal cerebral ischemia in mice: a possible role in blood—brain barrier dysfunction. *Journal of Cerebral Blood Flow & Metabolism, 19*(9), 1020-1028.

Gawaz, M. (2004). Role of platelets in coronary thrombosis and reperfusion of ischemic myocardium. *Cardiovascular research, 61*(3), 498-511.

Ge, J. Y., Zhang, Z. L. (1994). Study of pharmacology and new developments of chuanxiong. . *Chinese Journal of Integrated Traditional Chinese and Western Medicine 1994, 14*(10), 638–640.

Gebäck, T., Schulz, M. M. P., Koumoutsakos, P., & Detmar, M. (2009). TScratch: a novel and simple software tool for automated analysis of monolayer wound healing assays: Short Technical Reports. *Biotechniques, 46*(4), 265-274.

Gerber, H.-P., Hillan, K. J., Ryan, A. M., Kowalski, J., Keller, G.-A., Rangell, L., . . . Ferrara, N. (1999). VEGF is required for growth and survival in neonatal mice. *Development, 126*(6), 1149-1159.

Gerhard, G. S., Kauffman, E. J., Wang, X., Stewart, R., Moore, J. L., Kasales, C. J., . . . Cheng, K. C. (2002). Life spans and senescent phenotypes in two strains of Zebrafish (Danio rerio). *Experimental Gerontology, 37*(8-9), 1055-1068.

Gidday, J. M., Gasche, Y. G., Copin, J.-C., Shah, A. R., Perez, R. S., Shapiro, S. D., . . . Park, T. S. (2005). Leukocyte-derived matrix metalloproteinase-9 mediates blood-brain barrier breakdown and is proinflammatory after transient focal cerebral ischemia. *American Journal of Physiology-Heart and Circulatory Physiology, 289*(2), H558-H568.

Gilbert, M. J., Zerulla, T. C., & Tierney, K. B. (2014). Zebrafish (Danio rerio) as a model for the study of aging and exercise: physical ability and trainability decrease with age. *Experimental Gerontology, 50*, 106-113.

Gillies, R., Didier, N., & Denton, M. (1986). Determination of cell number in monolayer cultures. *Analytical biochemistry, 159*(1), 109-113.

Ginsberg, M. D. (2008). Neuroprotection for ischemic stroke: past, present and future. *Neuropharmacology, 55*(3), 363-389.

Go, Y.-M., Park, H., Koval, M., Orr, M., Reed, M., Liang, Y., . . . Jones, D. P. (2010). A key role for mitochondria in endothelial signaling by plasma cysteine/cystine redox potential. *Free Radical Biology and Medicine, 48*(2), 275-283.

Gong, X., & Sucher, N. J. (1999). Stroke therapy in traditional Chinese medicine (TCM): prospects for drug discovery and development. *Trends in pharmacological sciences, 20*(5), 191-196.

Gore, A. V., Monzo, K., Cha, Y. R., Pan, W., & Weinstein, B. M. (2012). Vascular development in the zebrafish. *Cold Spring Harbor perspectives in medicine*, a006684.

Gorelick, P. B., Sacco, R. L., Smith, D. B., Alberts, M., Mustone-Alexander, L., Rader, D., . . . Brass, L. M. (1999). Prevention of a first stroke. *JAMA: the journal of the American Medical Association, 281*(12), 1112-1120.

Gotti, B., Duverger, D., Bertin, J., Carter, C., Dupont, R., Frost, J., . . . Scatton, B. (1988). Ifenprodil and SL 82.0715 as cerebral anti-ischemic agents. I. Evidence for efficacy in models of focal cerebral ischemia. *Journal of Pharmacology and Experimental Therapeutics, 247*(3), 1211-1221.

Granger, C., Dewis, L., Peters, N., Sherwood, C., & Barrett, J. (1979). Stroke rehabilitation: analysis of repeated Barthel index measures. *Archives of physical medicine and rehabilitation, 60*(1), 14-17.

Granger, D. N. (1988). Role of xanthine oxidase and granulocytes in ischemia-reperfusion injury. *American Journal of Physiology-Heart and Circulatory Physiology, 255*(6), H1269-H1275.

Grau, A. J., Reis, A., Buggle, F., Al-Khalaf, A., Werle, E., Valois, N., . . . Grond-Ginsbach, C. (2001). Monocyte function and plasma levels of interleukin-8 in acute ischemic stroke. *Journal of the Neurological Sciences, 192*(1), 41-47.

Green, D. R. (1998). Apoptotic pathways: the roads to ruin. *Cell, 94*(6), 695-698.

Green, D. R. (2006). Coagulation Cascade. *Hemodial Int, 10*(Suppl 2), S2-4.

Grotta, J., Clark, W., Coull, B., Pettigrew, L. C., Mackay, B., Goldstein, L. B., . . . LaRue, L. (1995). Safety and Tolerability of the Glutamate Antagonist CGS 19755 (Selfotel) in Patients With Acute Ischemic Stroke Results of a Phase IIa Randomized Trial. *Stroke, 26*(4), 602-605.

Guazzi, M., Brambilla, R., Reina, G., Tumminello, G., & Guazzi, M. D. (2003). Aspirin-angiotensin-converting enzyme inhibitor coadministration and mortality in patients with heart failure: a dose-related adverse effect of aspirin. *Archives of internal medicine, 163*(13), 1574.

Gubitz, G., Sandercock, P., & Counsell, C. (2008). Anticoagulants for acute ischaemic stroke. *The Cochrane Library*.

Guo, C., Wu, JX., Li, RX. . (2004). Effects of panax notoginseng saponins on the concentrations of neurotransmitters in brain of Alzheimer's disease model mouse. . *Chinese Journal of Clinical Pharmacy 2004;13(3):150-2., 13*(3), 150-152.

Gupta, R., Tongers, J. r., & Losordo, D. W. (2009). Human studies of angiogenic gene therapy. *Circulation research, 105*(8), 724-736.

Haapaniemi, E., Soinne, L., Syrjälä, M., Kaste, M., & Tatlisumak, T. (2004). Serial changes in fibrinolysis and coagulation activation markers in acute and convalescent phase of ischemic stroke. *Acta neurologica scandinavica, 110*(4), 242-247.

Hacke, W., Donnan, G., Fieschi, C., Kaste, M., Von Kummer, R., Broderick, J., . . . Haley Jr, E. (2004). Association of outcome with early stroke treatment: pooled analysis of ATLANTIS, ECASS, and NINDS rt-PA stroke trials. *Lancet, 363*(9411), 768-774.

Hallenbeck, J. M. (1996). Significance of the inflammatory response in brain ischemia *Mechanisms of Secondary Brain Damage in Cerebral Ischemia and Trauma* (pp. 27-31): Springer.

Hallenbeck, J. M. (2002). The many faces of tumor necrosis factor in stroke. *Nature medicine, 8*(12), 1363.

Halliwell, B., & Gutteridge, J. M. (2015). *Free radicals in biology and medicine*: Oxford University Press, USA.

Hamann, G. F., Okada, Y., Fitridge, R., & Del Zoppo, G. J. (1995). Microvascular basal lamina antigens disappear during cerebral ischemia and reperfusion. *Stroke, 26*(11), 2120-2126.

Hao, C.-z., Wu, F., Shen, J., Lu, L., Fu, D.-l., Liao, W.-j., & Zheng, G.-q. (2012). Clinical efficacy and safety of buyang huanwu decoction for acute ischemic stroke: a systematic review and meta-analysis of 19 randomized controlled trials. *Evidence-Based Complementary and Alternative Medicine, 2012*.

Hara, A., Niwa, M., Yoshimi, N., & Mori, H. (1997). Apoptotic cell death in vulnerable subpopulation of cerebellar granule cells. *Acta neuropathologica, 94*(5), 517-518.

Hausenloy, D. J., & Yellon, D. M. (2013). Myocardial ischemia-reperfusion injury: a neglected therapeutic target. *The Journal of clinical investigation, 123*(1), 92-100.

Hayes, A. J., Reynolds, S., Nowell, M. A., Meakin, L. B., Habicher, J., Ledin, J., . . . Hammond, C. L. (2013). Spinal deformity in aged zebrafish is accompanied by degenerative changes to their vertebrae that resemble osteoarthritis. *PloS one, 8*(9), e75787.

Heiss, C., Rodriguez-Mateos, A., & Kelm, M. (2015). Central role of eNOS in the maintenance of endothelial homeostasis. *Antioxidants & redox signaling, 22*(14), 1230-1242.

Hendriks, D., Scharpe, S., Sande, M. v., & Lommaert, M. (1989). Characterisation of a carboxypeptidase in human serum distinct from carboxypeptidase N. *Clinical Chemistry and Laboratory Medicine, 27*(5), 277-286.

Henry, T. D., Grines, C. L., Watkins, M. W., Dib, N., Barbeau, G., Moreadith, R., . . . Engler, R. L. (2007). Effects of Ad5FGF-4 in patients with angina: an analysis of pooled data from the AGENT-3 and AGENT-4 trials. *Journal of the American College of Cardiology, 50*(11), 1038-1046.

Heo, J. H., Han, S. W., & Lee, S. K. (2005). Free radicals as triggers of brain edema formation after stroke. *Free Radical Biology and Medicine, 39*(1), 51-70.

Heo, J. H., Lucero, J., Abumiya, T., Koziol, J. A., Copeland, B. R., & Del Zoppo, G. J. (1999). Matrix metalloproteinases increase very early during experimental focal cerebral ischemia. *Journal of Cerebral Blood Flow & Metabolism, 19*(6), 624-633.

Hertog, M. G., Feskens, E. J., Kromhout, D., Hollman, P., & Katan, M. (1993). Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *The lancet, 342*(8878), 1007-1011.

Hewett, S. J., Misko, T. P., Keeling, R. M., Behrens, M. M., Choi, D. W., & Cross, A. H. (1996). Murine encephalitogenic lymphoid cells induce nitric oxide synthase in primary astrocytes. *Journal of neuroimmunology, 64*(2), 201-208.

Heylen, E., Brouns, R., Willemse, J., De Deyn, P., & Hendriks, D. (2009). Procarboxypeptidase U (tafi) consumption in acute ischemic stroke correlates with stroke severity, evolution and outcome. *Journal of Thrombosis and Haemostasis, 7*, 394-395.

Hidalgo, M., Sánchez-Moreno, C., & de Pascual-Teresa, S. (2010). Flavonoid–flavonoid interaction and its effect on their antioxidant activity. *Food chemistry, 121*(3), 691-696.

HIGA, M., Ogihara, K., & Yogi, S. (1998). Bioactive naphthoquinone derivatives from Diospyros maritima Blume. *Chemical and pharmaceutical bulletin, 46*(8), 1189-1193.

Higashijima, S.-i., Hotta, Y., & Okamoto, H. (2000). Visualization of cranial motor neurons in live transgenic zebrafish expressing green fluorescent protein under the control of the islet-1 promoter/enhancer. *The Journal of neuroscience, 20*(1), 206-218.

Hjort, N., Wu, O., Ashkanian, M., Sølling, C., Mouridsen, K., Christensen, S., . . . Østergaard, L. (2008). MRI detection of early blood-brain barrier disruption: parenchymal enhancement predicts focal hemorrhagic transformation after thrombolysis. *Stroke, 39*(3), 1025-1028.

Holiman, P. C., Hertog, M. G., & Katan, M. B. (1996). Analysis and health effects of flavonoids. *Food chemistry, 57*(1), 43-46.

Hong, S. J., Wan, J. B., Zhang, Y., Hu, G., Lin, H. C., Seto, S. W., . . . Lee, S. M. Y. (2009). Angiogenic effect of saponin extract from Panax notoginseng on HUVECs in vitro and zebrafish in vivo. *phytotherapy research, 23*(5), 677-686.

Hornig, C., Busse, O., Buettner, T., Dorndorf, W., Agnoli, A., & Akengin, Z. (1985). CT contrast enhancement on brain scans and blood-CSF barrier disturbances in cerebral ischemic infarction. *Stroke, 16*(2), 268-273.

Hornig, C., Busse, O., Dorndorf, W., & Kaps, M. (1983). Changes in CSF blood-brain barrier parameters in ischaemic cerebral infarction. *Journal of neurology, 229*(1), 11-16.

Hosomi, N., Lucero, J., Heo, J. H., Koziol, J. A., Copeland, B. R., & Del Zoppo, G. J. (2001). Rapid differential endogenous plasminogen activator expression after acute middle cerebral artery occlusion. *Stroke, 32*(6), 1341-1348.

Hossmann, K. A. (1994). Viability thresholds and the penumbra of focal ischemia. *Annals of neurology, 36*(4), 557-565.

Htun, P., Fateh-Moghadam, S., Tomandl, B., Handschu, R., Klinger, K., Stellos, K., . . . Gawaz, M. (2006). Course of platelet activation and platelet-leukocyte interaction in cerebrovascular ischemia. *Stroke, 37*(9), 2283-2287.

Hu, X., Zhou, D., Zhou, DM. . (2004). Protective effects of PTS on cerebral ischemia-reperfusion injury in rats. *Journal of Apoplexy and Nervous Diseases, 21*(4), 354-356.

Hu, X., Zhou, DM., Zhou, D. . (2004). Effect of PTS on expression of cell proliferation following focal cerebral ischemia-reperfusion in rats. *West China Medical Journal, 19*(3), 458-459.

Huang, Z. G., Xue, D., Karbalai, H., Buchan, A. M., & Preston, E. (1999). Biphasic opening of the blood-brain barrier following transient focal ischemia: effects of hypothermia. *Canadian journal of neurological sciences, 26*(4), 298-304.

hui, Z. R. G. w. s. b. y. d. w. y. (2000). *Pharmacopoeia of the People's Republic of China* (Vol. 1): Chemical Industry Press.

Iadecola, C. (1997). Bright and dark sides of nitric oxide in ischemic brain injury. *Trends in neurosciences, 20*(3), 132-139.

Idris, I., Thomson, G., & Sharma, J. (2006). Diabetes mellitus and stroke. *International journal of clinical practice, 60*(1), 48-56.

Isogai, S., Horiguchi, M., & Weinstein, B. M. (2001). The vascular anatomy of the developing zebrafish: an atlas of embryonic and early larval development. *Developmental biology, 230*(2), 278-301.

Isogai, S., Lawson, N. D., Torrealday, S., Horiguchi, M., & Weinstein, B. M. (2003). Angiogenic network formation in the developing vertebrate trunk. *Development, 130*(21), 5281-5290.

Ivan, C. S., Seshadri, S., Beiser, A., Au, R., Kase, C. S., Kelly-Hayes, M., & Wolf, P. A. (2004). Dementia After Stroke The Framingham Study. *Stroke, 35*(6), 1264-1268.

Jain, R. K. (2003). Molecular regulation of vessel maturation. *Nature medicine, 9*(6), 685.

Jenner, P. (2003). Oxidative stress in Parkinson's disease. *Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society, 53*(S3), S26-S38.

Jiang, M., Zhang, C., Cao, H., Chan, K., & Lu, A. (2011). The role of Chinese medicine in the treatment of chronic diseases in China. *Planta medica, 77*(09), 873-881.

Jin, K., Zhu, Y., Sun, Y., Mao, X. O., Xie, L., & Greenberg, D. A. (2002). Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. *Proceedings of the National Academy of Sciences, 99*(18), 11946-11950.

Jingfen, L., Chenxu, L., Gulinuer, M., Tingfeng, L., Pengfei, T., Junjie, Y., & Shaoqing, C. (2002). Effects of BYHW decoction and its effective constituents on the fluidity of the cell membrane in a stroke-modeled rat brain. *Journal of Chinese Pharmaceutical Sciences, 11*(4), 132-136.

Johnson Jr, E., & Deckwerth, T. (1993). Molecular mechanisms of developmental neuronal death. *Annual review of neuroscience, 16*(1), 31-46.

Kajdaniuk, D., Marek, B., Borgiel-Marek, H., & Kos-Kudła, B. (2011). Vascular endothelial growth factor (VEGF)—part 1: in physiology and pathophysiology. *Endokrynologia Polska, 62*(5), 444-455.

Kalogeris, T., Baines, C. P., Krenz, M., & Korthuis, R. J. (2012). Cell biology of ischemia/reperfusion injury *International review of cell and molecular biology* (Vol. 298, pp. 229-317): Elsevier.

Kameda, K., Takaku, T., Okuda, H., Kimura, Y., Okuda, T., Hatano, T., . . . Arichi, S. (1987). Inhibitory effects of various flavonoids isolated from leaves of persimmon on angiotensin-converting enzyme activity. *Journal of natural products, 50*(4), 680-683.

Kamiya, T., Katayama, Y., Kashiwagi, F., & Terashi, A. (1993). The role of bradykinin in mediating ischemic brain edema in rats. *Stroke, 24*(4), 571-575.

Kang, D. G., Oh, H., Chung, H. T., & Lee, H. S. (2003). Inhibition of angiotensin converting enzyme by lithospermic acid B isolated from Radix Salviae miltiorrhiza Bunge. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives, 17*(8), 917-920.

Kari, G., Rodeck, U., & Dicker, A. P. (2007). Zebrafish: an emerging model system for human disease and drug discovery. *Clinical Pharmacology & Therapeutics, 82*(1), 70-80.

Kašparová, S., Brezová, V., Valko, M., Horecký, J., Mlynárik, V., Liptaj, T., . . . Dobrota, D. (2005). Study of the oxidative stress in a rat model of chronic brain hypoperfusion. *Neurochemistry international, 46*(8), 601-611.

Kastrup, A., Engelhorn, T., Beaulieu, C., de Crespigny, A., & Moseley, M. E. (1999). Dynamics of cerebral injury, perfusion, and blood-brain barrier changes after temporary and permanent middle cerebral artery occlusion in the rat. *Journal of the Neurological Sciences, 166*(2), 91-99.

Katsura, K.-i., Kristián, T., & Siesjö, B. K. (1994). Energy metabolism, ion homeostasis, and cell damage in the brain: Portland Press Limited.

Kawabata, K., Mukai, R., & Ishisaka, A. (2015). Quercetin and related polyphenols: new insights and implications for their bioactivity and bioavailability. *Food & function, 6*(5), 1399-1417.

Kawano, T., Anrather, J., Zhou, P., Park, L., Wang, G., Frys, K. A., . . . Iadecola, C. (2006). Prostaglandin E 2 EP1 receptors: downstream effectors of COX-2 neurotoxicity. *Nature medicine, 12*(2), 225.

Kazzem, M. (2012). *Chromatographic analysis of selected analytes found in the commercially prepared pill of Diospyros Kaki leaves.* (Bachelor of Medical Science (Honours)), University of Western Sydney, Australia.

Ke, M., Hu, X.-Q., Ouyang, J., Dai, B., & Xu, Y. (2012). The effect of astragalin on the VEGF production of cultured Müller cells under high glucose conditions. *Bio-medical materials and engineering, 22*(1-3), 113-119.

Kehrer, J., & Smith, C. (1994). Free radicals in biology: sources, reactivities, and roles in the etiology of human diseases. *Natural antioxidants in human health and disease. Academic Press, San Diego*, 25-62.

Kelly, J., Rudd, A., Lewis, R. R., Parmar, K., Moody, A., & Hunt, B. J. (2003). The relationship between acute ischaemic stroke and plasma D-dimer levels in patients developing neither venous thromboembolism nor major intercurrent illness. *Blood coagulation & fibrinolysis, 14*(7), 639-645.

Kelly, P. J., Morrow, J. D., Ning, M., Koroshetz, W., Lo, E. H., Terry, E., . . . Stevenson, E. (2008). Oxidative stress and matrix metalloproteinase-9 in acute ischemic stroke: the Biomarker Evaluation for Antioxidant Therapies in Stroke (BEAT-Stroke) study. *Stroke, 39*(1), 100-104.

Kelm, J. M., Sanchez-Bustamante, C. D., Ehler, E., Hoerstrup, S. P., Djonov, V., Ittner, L., & Fussenegger, M. (2005). VEGF profiling and angiogenesis in human microtissues. *Journal of biotechnology, 118*(2), 213-229.

Kerr, S., Brosnan, M. J., McIntyre, M., Reid, J. L., Dominiczak, A. F., & Hamilton, C. A. (1999). Superoxide anion production is increased in a model of genetic hypertension: role of the endothelium. *Hypertension, 33*(6), 1353-1358.

Khatri, R., McKinney, A. M., Swenson, B., & Janardhan, V. (2012). Blood–brain barrier, reperfusion injury, and hemorrhagic transformation in acute ischemic stroke. *Neurology, 79*(13 Supplement 1), S52-S57.

Kher, N., & Marsh, J. D. (2004). *Pathobiology of atherosclerosis-a brief review.* Paper presented at the Seminars in thrombosis and hemostasis.

Kidwell, C. S., Latour, L., Saver, J. L., Alger, J. R., Starkman, S., Duckwiler, G., . . . Warach, S. (2008). Thrombolytic toxicity: blood brain barrier disruption in human ischemic stroke. *Cerebrovascular diseases, 25*(4), 338-343.

Kidwell, C. S., Saver, J., Mattiello, J., Starkman, S., Vinuela, F., Duckwiler, G., . . . Villablanca, J. (2001). Diffusion-perfusion MRI characterization of post-recanalization hyperperfusion in humans. *Neurology, 57*(11), 2015-2021.

Kielstein, J. T., Donnerstag, F., Gasper, S., Menne, J., Kielstein, A., Martens-Lobenhoffer, J., . . . Bode-Böger, S. M. (2006). ADMA increases arterial stiffness and decreases cerebral blood flow in humans. *Stroke, 37*(8), 2024-2029.

Kim, T. H., Ku, S.-K., & Bae, J.-S. (2012). Inhibitory effects of kaempferol-3-O-sophoroside on HMGB1-mediated proinflammatory responses. *Food and chemical toxicology, 50*(3-4), 1118-1123.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., & Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Developmental dynamics, 203*(3), 253-310.

Klement, P., Liao, P., & Bajzar, L. (1999). A novel approach to arterial thrombolysis. *Blood, 94*(8), 2735-2743.

Knecht, W., Willemse, J., Stenhamre, H., Andersson, M., Berntsson, P., Furebring, C., . . . Hendriks, D. (2006). Limited mutagenesis increases the stability of human carboxypeptidase U (TAFIa) and demonstrates the importance of CPU stability over proCPU concentration in down-regulating fibrinolysis. *The FEBS journal, 273*(4), 778-792.

Kono, H., & Rock, K. L. (2008). How dying cells alert the immune system to danger. *Nature Reviews Immunology, 8*(4), 279.

Kontos, H. A. (1985). George E. Brown memorial lecture. Oxygen radicals in cerebral vascular injury. *Circulation Research, 57*(4), 508-516.

Kontos, H. A. (2001). Oxygen radicals in cerebral ischemia: the 2001 Willis lecture. *Stroke, 32*(11), 2712-2716.

Korzeniewski, C., & Callewaert, D. M. (1983). An enzyme-release assay for natural cytotoxicity. *Journal of immunological methods, 64*(3), 313-320.

Krock, B. L., Skuli, N., & Simon, M. C. (2011). Hypoxia-induced angiogenesis: good and evil. *Genes & cancer, 2*(12), 1117-1133.

Krupinski, J., Kaluza, J., Kumar, P., Kumar, S., & Wang, J. M. (1994). Role of angiogenesis in patients with cerebral ischemic stroke. *Stroke, 25*(9), 1794-1798.

Kueng, W., Silber, E., & Eppenberger, U. (1989). Quantification of cells cultured on 96-well plates. *Analytical biochemistry, 182*(1), 16-19.

Kukreja, R. C., & Hess, M. L. (1992). The oxygen free radical system: from equations through membrane-protein interactions to cardiovascular injury and protection. *Cardiovascular Research, 26*(7), 641-655.
Kuroda, S., & Siesjö, B. (1997). Reperfusion damage following focal ischemia: pathophysiology and therapeutic windows. *Clinical Neuroscience (New York, NY), 4*(4), 199-212.
Kuroiwa, T., Ting, P., Martinez, H., & Klatzo, I. (1985). The biphasic opening of the blood-brain barrier to proteins following temporary middle cerebral artery occlusion. *Acta neuropathologica, 68*(2), 122-129.
Lam, B., Lo, A., Sun, X., Luo, H., Chung, S., & Sucher, N. (2003). Neuroprotective effects of tanshinones in transient focal cerebral ischemia in mice. *Phytomedicine, 10*(4), 286-291.
Lam, H. W., Lin, H. C., Lao, S. C., Gao, J. L., Hong, S. J., Leong, C. W., . . . Wang, Y. T. (2008). The angiogenic effects of Angelica sinensis extract on HUVEC in vitro and zebrafish in vivo. *Journal of cellular biochemistry, 103*(1), 195-211.
Langheinrich, U. (2003). Zebrafish: a new model on the pharmaceutical catwalk. *Bioessays, 25*(9), 904-912.
Latour, L. L., Kang, D. W., Ezzeddine, M. A., Chalela, J. A., & Warach, S. (2004). Early blood–brain barrier disruption in human focal brain ischemia. *Annals of neurology, 56*(4), 468-477.
Lawson, N. D., & Weinstein, B. M. (2002). In vivo imaging of embryonic vascular development using transgenic zebrafish. *Developmental biology, 248*(2), 307-318.
Lee, I., Lee, C., Chang, C., Chien, C., & Lin, M. (2005). Sheng mai san, a Chinese herbal medicine, protects against renal ischaemic injury during heat stroke in the rat. *Clinical and experimental pharmacology and physiology, 32*(9), 742-748.
Lee, J.-M., Zipfel, G. J., & Choi, D. W. (1999). The changing landscape of ischaemic brain injury mechanisms. *Nature, 399*(Supplementary), A7.
Lee, S.-J., & Koh, J.-Y. (2010). Roles of zinc and metallothionein-3 in oxidative stress-induced lysosomal dysfunction, cell death, and autophagy in neurons and astrocytes. *Molecular brain, 3*(1), 30.
Lees, K. R., Bath, P. M., & Naylor, A. R. (2000). ABC of arterial and venous disease: secondary prevention of transient ischaemic attack and stroke. *BMJ: British Medical Journal, 320*(7240), 991.
Legg, L. (2004). Rehabilitation therapy services for stroke patients living at home: systematic review of randomised trials. *The Lancet*.
Leist, M., & Nicotera, P. (1998a). Apoptosis, excitotoxicity, and neuropathology. *Experimental cell research, 239*(2), 183-201.
Leist, M., & Nicotera, P. (1998b). Calcium and neuronal death *Reviews of Physiology Biochemistry and Pharmacology, Volume 132* (pp. 79-125): Springer.
Leist, M., Volbracht, C., KÃ¼hnle, S., Fava, E., Ferrando-May, E., & Nicotera, P. (1997). Caspase-mediated apoptosis in neuronal excitotoxicity triggered by nitric oxide. *Molecular Medicine, 3*(11), 750.
Lennon, S., Martin, S., & Cotter, T. (1991). Dose-dependent induction of apoptosis in human tumour cell lines by widely diverging stimuli. *Cell proliferation, 24*(2), 203-214.
Leonardi-Bee, J., Bath, P. M., Phillips, S. J., & Sandercock, P. A. (2002). Blood pressure and clinical outcomes in the International Stroke Trial. *Stroke, 33*(5), 1315-1320.

Leurs, J., & Hendriks, D. (2005). Carboxypeptidase U (TAFIa): a metallocarboxypeptidase with a distinct role in haemostasis and a possible risk factor for thrombotic disease. *Thrombosis and haemostasis, 93*(03), 471-487.

Leurs, J., Nerme, V., Sim, Y., & Hendriks, D. (2004). Carboxypeptidase U (TAFIa) prevents lysis from proceeding into the propagation phase through a threshold-dependent mechanism. *Journal of Thrombosis and Haemostasis, 2*(3), 416-423.

Li, C.-J., Elsasser, T. H., & Kahl, S. (2009). AKT/eNOS signaling module functions as a potential feedback loop in the growth hormone signaling pathway. *Journal of molecular signaling, 4*(1), 1-13.

Li, J. L., Liu, H. . (2000). Compound Dan Shen Injection for noncardiocerebrovascular diseases. *Research of Traditional Chinese Medicine, 16*(1), 57-58.

Li, N., Sun, J., Wang, Y., Li, XH. . (1999). Panax notoginseng saponins and dextran for cerebral infarction. *Chinese Journal of New Drugs and Clinical Remedies, 18*(6), 411-412.

Li, S., Yan, T., Deng, R., Jiang, X., Xiong, H., Wang, Y., . . . Zhu, Y. (2017). Low dose of kaempferol suppresses the migration and invasion of triple-negative breast cancer cells by downregulating the activities of RhoA and Rac1. *OncoTargets and therapy, 10*, 4809.

Li, W., Yi, S., Wang, Z., Chen, S., Xin, S., Xie, J., & Zhao, C. (2011). Self-nanoemulsifying drug delivery system of persimmon leaf extract: Optimization and bioavailability studies. *International Journal of Pharmaceutics*.

Li, Y., Ding, Z., & Wu, C. (2016). Mechanistic study of the inhibitory effect of kaempferol on uterine fibroids in vitro. *Medical science monitor: international medical journal of experimental and clinical research, 22*, 4803.

Liang, C.-C., Park, A. Y., & Guan, J.-L. (2007). In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nature protocols, 2*(2), 329.

Liang, F., Han, Y., Gao, H., Xin, S., Chen, S., Wang, N., . . . Yao, X. (2015). Kaempferol identified by zebrafish assay and fine fractionations strategy from Dysosma versipellis inhibits angiogenesis through VEGF and FGF pathways. *Scientific reports, 5*, 14468.

Liang, J., Luo, J., Yin, R., Li, Y., Wu, R., & Wang, D. (2012). Simultaneous Determination of Quercetin and Kaempferol in Naoxinqing Tablets by HPLC. *Pharm. Today, 8*.

Liang, X.-Y., Li, H.-N., Yang, X.-Y., Zhou, W.-Y., Niu, J.-G., & Chen, B.-D. (2013). Effect of Danshen aqueous extract on serum hs-CRP, IL-8, IL-10, TNF-α levels, and IL-10 mRNA, TNF-α mRNA expression levels, cerebral TGF-β1 positive expression level and its neuroprotective mechanisms in CIR rats. *Molecular biology reports, 40*(4), 3419-3427.

Liang, Y., Xie, P. Chan, K. (2004). Quality control of herbal medicines. *Journal of Chromatography B, 512*, 53-70.

Lin, M.-K., Yu, Y.-L., Chen, K.-C., Chang, W.-T., Lee, M.-S., Yang, M.-J., . . . Chu, C.-L. (2011). Kaempferol from Semen cuscutae attenuates the immune function of dendritic cells. *Immunobiology, 216*(10), 1103-1109.

Lip, G., Blann, A., Farooqi, I., Zarifis, J., Sagar, G., & Beevers, D. (2002). Sequential alterations in haemorheology, endothelial dysfunction, platelet activation and thrombogenesis in relation to prognosis following acute stroke: The West Birmingham Stroke Project. *Blood Coagulation & Fibrinolysis, 13*(4), 339-347.

Liu, A. (2005). Observation on curative effect of xuesaitong for acute cerebral infarction. *Modern Journal of Integrated Traditional Chinese and Western Medicine, 14*(1), 47.

Liu, E. H., Qi, L. W., Peng, Y. B., Cheng, X. L., Wu, Q., Li, P., & Li, C. Y. (2009). Rapid separation and identification of 54 major constituents in Buyang Huanwu decoction by ultra-fast HPLC system coupled with DAD-TOF/MS. *Biomedical Chromatography, 23*(8), 828-842.

Liu, M., Wu, B., Wang, W.-Z., Lee, L.-M., Zhang, S.-H., & Kong, L.-Z. (2007). Stroke in China: epidemiology, prevention, and management strategies. *The Lancet Neurology, 6*(5), 456-464.

Liu, P. K., Hsu, C. Y., Dizdaroglu, M., Floyd, R. A., Kow, Y. W., Karakaya, A., . . . Cui, J.-K. (1996). Damage, repair, and mutagenesis in nuclear genes after mouse forebrain ischemia–reperfusion. *Journal of Neuroscience, 16*(21), 6795-6806.

Liu, Y. (Ed.) (1988). *The Essential Book of Traditional Chinese Medicine (Vol. 1).* (Vol. 1). New York: Columbia University Press.

Loew, D., & Kaszkin, M. (2002). Approaching the problem of bioequivalence of herbal medicinal products. *Phytother Res, 16*(8), 705-711. doi:10.1002/ptr.1248

Lorberboym, M., Lampl, Y., & Sadeh, M. (2003). Correlation of 99mTc-DTPA SPECT of the blood–brain barrier with neurologic outcome after acute stroke. *Journal of Nuclear Medicine, 44*(12), 1898-1904.

Loschen, G., Azzi, A., Richter, C., & Flohé, L. (1974). Superoxide radicals as precursors of mitochondrial hydrogen peroxide. *FEBS letters, 42*(1), 68-72.

Losordo, D. W., & Dimmeler, S. (2004a). Therapeutic angiogenesis and vasculogenesis for ischemic disease: part I: angiogenic cytokines. *Circulation, 109*(21), 2487-2491.

Losordo, D. W., & Dimmeler, S. (2004b). Therapeutic angiogenesis and vasculogenesis for ischemic disease: part II: cell-based therapies. *Circulation, 109*(22), 2692-2697.

Love, S. (1999). Oxidative stress in brain ischemia. *Brain pathology, 9*(1), 119-131.

Lu, A.-P., Jia, H.-W., Xiao, C., & Lu, Q.-P. (2004). Theory of traditional Chinese medicine and therapeutic method of diseases. *World Journal of Gastroenterology: WJG, 10*(13), 1854.

Lv , P., Chen, HF. (2004). Experimental study of sanchi leaf saponins on reducing serum lipid. . *Chinese Journal of Biochemical Pharmaceutics, 25*(4), 235-236.

Lyras, L., Cairns, N. J., Jenner, A., Jenner, P., & Halliwell, B. (1997). An assessment of oxidative damage to proteins, lipids, and DNA in brain from patients with Alzheimer's disease. *Journal of neurochemistry, 68*(5), 2061-2069.

Ma, L. Y., Wang, C. L., Zhang, Q. (1998). Influence of panax notoginseng saponins on cerebral blood supply and metabolism. *Chinese Pharmacological Bulletin, 14*(1), 27-29.

MacAllister, R. J., Whitley, G. S. J., & Vallance, P. (1994). Effects of guanidino and uremic compounds on nitric oxide pathways. *Kidney international, 45*(3), 737-742.

Machida, T., Sumino, H., Fukushima, M., Kotajima, N., Amagai, H., & Murakami, M. (2010). Blood rheology and the low-density lipoprotein cholesterol/high-density lipoprotein cholesterol ratio in dyslipidaemic and normolipidaemic subjects. *Journal of International Medical Research, 38*(6), 1975-1984.

Manabe, Y., Anrather, J., Kawano, T., Niwa, K., Zhou, P., Ross, M. E., & Iadecola, C. (2004). Prostanoids, not reactive oxygen species, mediate COX-2–dependent neurotoxicity. *Annals of neurology, 55*(5), 668-675.

Marinis, A., Argyra, E., Lykoudis, P., Brestas, P., Theodoraki, K., Polymeneas, G., . . . Voros, D. (2010). Ischemia as a possible effect of increased intra-abdominal pressure on central nervous system cytokines, lactate and perfusion pressures. *Critical Care, 14*(2), R31.

Marioni, R. E., Stewart, M. C., Murray, G. D., Deary, I. J., Fowkes, F. G. R., Lowe, G. D., . . . Price, J. F. (2009). Peripheral levels of fibrinogen, C-reactive protein, and plasma viscosity predict future cognitive decline in individuals without dementia. *Psychosomatic medicine, 71*(8), 901.

Markus, R., Reutens, D., Kazui, S., Read, S., Wright, P., Pearce, D., . . . Donnan, G. (2004). Hypoxic tissue in ischaemic stroke: persistence and clinical consequences of spontaneous survival. *Brain, 127*(6), 1427-1436.

Martin, A., & Clynes, M. (1993). Comparison of 5 microplate colorimetric assays forin vitro cytotoxicity testing and cell proliferation assays. *Cytotechnology, 11*(1), 49-58.

Martin, L. J., Al-Abdulla, N. A., Brambrink, A. M., Kirsch, J. R., Sieber, F. E., & Portera-Cailliau, C. (1998). Neurodegeneration in excitotoxicity, global cerebral ischemia, and target deprivation: a perspective on the contributions of apoptosis and necrosis. *Brain research bulletin, 46*(4), 281-309.

Martin, R., Lloyd, H., & Cowan, A. (1994). The early events of oxygen and glucose deprivation: setting the scene for neuronal death? *Trends in neurosciences, 17*(6), 251-257.

Marx, P. F., Dawson, P. E., Bouma, B. N., & Meijers, J. C. (2002). Plasmin-mediated activation and inactivation of thrombin-activatable fibrinolysis inhibitor. *Biochemistry, 41*(21), 6688-6696.

Masters, B. S. S. (1994). Nitric oxide synthases: why so complex? *Annual review of nutrition, 14*(1), 131-145.

Matsuura, H., Kasai, R., Tanaka, O., SARUWATARI, Y., Fuwa, T., & ZHOU, J. (1983). Further studies on dammarane-saponins of Sanchi-Ginseng. *Chemical and pharmaceutical bulletin, 31*(7), 2281-2287.

Mattsson, C., Björkman, J., Abrahamsson, T., Nerme, V., Schatteman, K., Leurs, J., . . . Hendriks, D. (2002). Local proCPU (TAFI) activation during thrombolytic treatment in a dog model of coronary artery thrombosis can be inhibited with a direct, small molecule thrombin inhibitor (melagatran). *Thrombosis and haemostasis, 88*(04), 557-562.

Meldrum, B., Evans, M., Griffiths, T., & Simon, R. (1985). Ischaemic brain damage: the role of excitatory activity and of calcium entry. *British journal of anaesthesia, 57*(1), 44-46.

Meli, E., Picca, R., Attucci, S., Cozzi, A., Peruginelli, F., Moroni, F., & Pellegrini-Giampietro, D. E. (2002). Activation of mGlu1 but not mGlu5 metabotropic glutamate receptors contributes to postischemic neuronal injury in vitro and in vivo. *Pharmacology Biochemistry and Behavior, 73*(2), 439-446.

Miller, D. M., Buettner, G. R., & Aust, S. D. (1990). Transition metals as catalysts of "autoxidation" reactions. *Free Radical Biology and Medicine, 8*(1), 95-108.

Minnema, M. C., Friederich, P. W., Levi, M., Von Dem Borne, P., Mosnier, L. O., Meijers, J., . . . ten Cate, H. (1998). Enhancement of rabbit jugular vein thrombolysis by neutralization of factor XI. In vivo evidence for a role of factor XI as an anti-fibrinolytic factor. *The Journal of clinical investigation, 101*(1), 10-14.

Mohr, J., Thompson, J., Lazar, R., Levin, B., Sacco, R., Furie, K., . . . Adams Jr, H. (2001). A comparison of warfarin and aspirin for the prevention of recurrent ischemic stroke. *New England Journal of Medicine, 345*(20), 1444-1451.

Monaghan-Benson, E., & Burridge, K. (2009). The regulation of VEGF-induced microvascular permeability requires Rac and ROS. *Journal of Biological Chemistry*, jbc. M109. 009894.

Montaner, J., Alvarez-Sabín, J., Molina, C., Anglés, A., Abilleira, S., Arenillas, J., . . . Monasterio, J. (2001). Matrix metalloproteinase expression after human cardioembolic stroke: temporal profile and relation to neurological impairment. *Stroke, 32*(8), 1759-1766.

Montaner, J., Molina, C. A., Monasterio, J., Abilleira, S., Arenillas, J. F., Ribó, M., . . . Alvarez-Sabín, J. (2003). Matrix metalloproteinase-9 pretreatment level predicts intracranial hemorrhagic complications after thrombolysis in human stroke. *Circulation, 107*(4), 598-603.

Mori, E., Del Zoppo, G. J., Chambers, J. D., Copeland, B. R., & Arfors, K. E. (1992). Inhibition of polymorphonuclear leukocyte adherence suppresses no-reflow after focal cerebral ischemia in baboons. *Stroke, 23*(5), 712-718.

Morioka, T., Kalehua, A. N., & Streit, W. J. (1993). Characterization of microglial reaction after middle cerebral artery occlusion in rat brain. *Journal of Comparative Neurology, 327*(1), 123-132.

Murphy, M. P. (2009). How mitochondria produce reactive oxygen species. *Biochemical journal, 417*(1), 1-13.

Muus, I., Williams, L. S., & Ringsberg, K. C. (2007). Validation of the Stroke Specific Quality of Life Scale (SS-QOL): test of reliability and validity of the Danish version (SS-QOL-DK). *Clinical Rehabilitation, 21*(7), 620-627.

Nagashima, M., Werner, M., Wang, M., Zhao, L., Light, D. R., Pagila, R., . . . Verhallen, P. (2000). An Inhibitor of Activated Thrombin-Activatable Fibrinolysis Inhibitor Potentiates Tissue-Type Plasminogen Activator-Induced Thrombolysis in a Rabbit Jugular Vein Thrombolysis Model☆. *Thrombosis research, 98*(4), 333-342.

Nagesh, V., Welch, K., Windham, J., Patel, S., Levine, S., Hearshen, D., . . . Soltanian-Zadeh, H. (1998). Time course of ADCw changes in ischemic stroke: beyond the human eye! *Stroke, 29*(9), 1778-1782.

Namura, S., Zhu, J., Fink, K., Endres, M., Srinivasan, A., Tomaselli, K. J., . . . Moskowitz, M. A. (1998). Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. *Journal of Neuroscience, 18*(10), 3659-3668.

Navaratna, D., Guo, S., Arai, K., & Lo, E. H. (2009). Mechanisms and targets for angiogenic therapy after stroke. *Cell adhesion & migration, 3*(2), 216-223.

Nelson, C. W., Wei, E. P., Povlishock, J. T., Kontos, H. A., & Moskowitz, M. A. (1992). Oxygen radicals in cerebral ischemia. *American Journal of Physiology-Heart and Circulatory Physiology, 263*(5), H1356-H1362.

Newman, J. C., & Verdin, E. (2014). β-hydroxybutyrate: much more than a metabolite. *Diabetes research and clinical practice, 106*(2), 173-181.

NFS. (2017). Deloitte Access Economics - No Postcode untouched - Stroke in Australia. *National Stroke Foundation Australia*.

Ng, T. (2006). Pharmacological activity of sanchi ginseng (Panax notoginseng). *Journal of Pharmacy and Pharmacology, 58*(8), 1007-1019.

Nicholas, J., Swearingen, C., Thomas, J., Rumboldt, Z., Tumminello, P., & Patel, S. (2008). The effect of statin pretreatment on infarct volume in ischemic stroke. *Neuroepidemiology, 31*(1), 48-56.

Nicholls, D., & Attwell, D. (1990). The release and uptake of excitatory amino acids. *Trends in pharmacological sciences, 11*(11), 462-468.

Nicoli, F., Lefur, Y., Denis, B., Ranjeva, J., Confort-Gouny, S., & Cozzone, P. (2003). Metabolic counterpart of decreased apparent diffusion coefficient during hyperacute ischemic stroke: a brain proton magnetic resonance spectroscopic imaging study. *Stroke, 34*(7), e82-e87.

Niebroj-Dobosz, I., Mariam, A., Lukasiuk, M., & Rafałowska, J. (1992). Blood-cerebrospinal fluid barrier integrity in cerebral infarction. *Neuropatologia polska, 30*(1), 29-33.
Nogawa, S., Zhang, F., Ross, M. E., & Iadecola, C. (1997). Cyclo-oxygenase-2 gene expression in neurons contributes to ischemic brain damage. *Journal of Neuroscience, 17*(8), 2746-2755.
Nour, M., Scalzo, F., & Liebeskind, D. S. (2012). Ischemia-reperfusion injury in stroke. *Interventional neurology, 1*(3-4), 185-199.
NSF. (2013). *The economic impact of stroke in Australia*. Retrieved from http://strokefoundation.com.au/site/media/Final-Deloitte-Stroke-Report-14-Mar-13.pdf
O'Neill, M. J., Hicks, C., & Ward, M. (1996). Neuroprotective effects of 7-nitroindazole in the gerbil model of global cerebral ischaemia. *European journal of pharmacology, 310*(2), 115-122.
Obrenovitch, T. (1995). The ischaemic penumbra: twenty years on. *Cerebrovascular and brain metabolism reviews, 7*(4), 297-323.
Okada, Y., Copeland, B. R., Fitridge, R., Koziol, J. A., & Del Zoppo, G. J. (1994). Fibrin contributes to microvascular obstructions and parenchymal changes during early focal cerebral ischemia and reperfusion. *Stroke, 25*(9), 1847-1853.
Okada, Y., Copeland, B. R., Mori, E., Tung, M.-M., Thomas, W. S., & del Zoppo, G. J. (1994). P-selectin and intercellular adhesion molecule-1 expression after focal brain ischemia and reperfusion. *Stroke, 25*(1), 202-211.
Olaleye, M., Crown, O., Akinmoladun, A., & Akindahunsi, A. (2014). Rutin and quercetin show greater efficacy than nifedipin in ameliorating hemodynamic, redox, and metabolite imbalances in sodium chloride-induced hypertensive rats. *Human & experimental toxicology, 33*(6), 602-608.
Oliveira-Filho, J., Silva, S., Trabuco, C., Pedreira, B., Sousa, E., & Bacellar, A. (2003). Detrimental effect of blood pressure reduction in the first 24 hours of acute stroke onset. *Neurology, 61*(8), 1047-1051.
Ong, E. S. (2004). Extraction methods and chemical standardization of botanicals and herbal preparations. *Journal of Chromatography B, 812*, 23-33.
Opdenakker, G., Van den Steen, P. E., Dubois, B., Nelissen, I., Van Coillie, E., Masure, S., . . . Van Damme, J. (2001). Gelatinase B functions as regulator and effector in leukocyte biology. *Journal of leukocyte biology, 69*(6), 851-859.
Osuntokun, B., Bademosi, O., Akinkugbe, O., Oyediran, A., & Carlisle, R. (1979). Incidence of stroke in an African city: Results from the Stroke Registry at Ibadan, Nigeria, 1973-1975. *Stroke, 10*(2), 205-207.
Ou, Y., Bei, W., & Lai, W. (2003). The effects of Diospyros Kaki leaves flavonoids on reperfusion injury in cardiac muscles on mice. *First Military Medicine University Journal, 23*(7), 680-682.
Ou, Y., Liu, S., & Bei, W. (2004). The effects of flavonoids of *Diospyros Kaki* leaves on vascular adventitial fibroblast proliferation induced by oxidative proteins. *Chinese Herbal Med, 27*(3), 186-188.
Pan, J., Konstas, A.-A., Bateman, B., Ortolano, G. A., & Pile-Spellman, J. (2007). Reperfusion injury following cerebral ischemia: pathophysiology, MR imaging, and potential therapies. *Neuroradiology, 49*(2), 93-102.
Parelkar, N. K., Wang, John Q. (2008). Upregulation of metabotropic glutamate receptor 8 mRNA expression in the rat forebrain after repeated amphetamine administration. *Neuroscience letters, 433*(3), 250-254.

Parng, C., Seng, W. L., Semino, C., & McGrath, P. (2002). Zebrafish: a preclinical model for drug screening. *Assay and drug development technologies, 1*(1), 41-48.
Patat, A., Molinier, P., Hergueta, T., & Brohier, S. (1994). Lack of amnestic, psychotomimetic or impairing effect on psychomotor performance of eliprodil, a new NMDA antagonist. *International clinical psychopharmacology*.
Patel, R. A., & McMullen, P. W. (2017). Neuroprotection in the treatment of acute ischemic stroke. *Progress in cardiovascular diseases, 59*(6), 542-548.
Paulson, O., Strandgaard, S., & Edvinsson, L. (1990). Cerebral autoregulation. *Cerebrovascular and brain metabolism reviews, 2*(2), 161-192.
Paulson, O. B., Olesen, J., & Christensen, M. S. (1972). Restoration of autoregulation of cerebral blood flow by hypocapnia. *Neurology, 22*(3), 286-286.
Peirce, S. M., & Skalak, T. C. (2003). Microvascular remodeling: a complex continuum spanning angiogenesis to arteriogenesis. *Microcirculation, 10*(1), 99-111.
Pick, E., & Keisari, Y. (1980). A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. *Journal of immunological methods, 38*(1-2), 161-170.
Polverini, P. J. (1995). The pathophysiology of angiogenesis. *Critical Reviews in Oral Biology & Medicine, 6*(3), 230-247.
Poon, C. C. W., Seto, S. W., Au, A. L. S., Zhang, Q., Li, R. W. S., Lee, W. Y. W., . . . Ngai, S. M. (2010). Mitochondrial monoamine oxidase-A-mediated hydrogen peroxide generation enhances 5-hydroxytryptamine-induced contraction of rat basilar artery. *British journal of pharmacology, 161*(5), 1086-1098.
Potente, M., Gerhardt, H., & Carmeliet, P. (2011). Basic and therapeutic aspects of angiogenesis. *Cell, 146*(6), 873-887.
Poujade, M., Grasland-Mongrain, E., Hertzog, A., Jouanneau, J., Chavrier, P., Ladoux, B., . . . Silberzan, P. (2007). Collective migration of an epithelial monolayer in response to a model wound. *Proceedings of the National Academy of Sciences, 104*(41), 15988-15993.
Prabhakaran, S., Ruff, I., & Bernstein, R. A. (2015). Acute stroke intervention: a systematic review. *Jama, 313*(14), 1451-1462.
Qin, R., Zang, Z., Zhao, Z., Xu, X., & Xu, X. (2011). The protective effects of Naoxinqing on experimental cerebral ischemia [J]. *Pharmacology and Clinics of Chinese Materia Medica, 3*.
Rao, A. M., Hatcher, J., Kindy, M., & Dempsey, R. (1999). Arachidonic acid and leukotriene C 4: role in transient cerebral ischemia of gerbils. *Neurochemical research, 24*(10), 1225-1232.
Rapela, C. E., & Green, H. D. (1964). Autoregulation of canine cerebral blood flow. *Circulation Research, 15*, SUPPL: 205-212.
Ray, P. D., Huang, B.-W., & Tsuji, Y. (2012). Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cellular signalling, 24*(5), 981-990.
Read, S., Hirano, T., Abbot, D., Sachinidis, J., Tochon-danguy, H., Chan, J., . . . Mckay, W. (1999). Identifying Hypoxic Tissue After Acute Ischemic Stroke Using Pet and F-fluoromisonidazole. *Stroke: A Journal of Cerebral Circulation, 30*(3), 701.
Rees, B. B., Sudradjat, F. A., & Love, J. W. (2001). Acclimation to hypoxia increases survival time of zebrafish, Danio rerio, during lethal hypoxia. *Journal of Experimental Zoology, 289*(4), 266-272.
Ren, J., Lu, Y., Qian, Y., Chen, B., Wu, T., & Ji, G. (2019). Recent progress regarding kaempferol for the treatment of various diseases. *Experimental and therapeutic medicine, 18*(4), 2759-2776.

Ringleb, P., Bousser, M., Ford, G., Bath, P., Brainin, M., Caso, V., . . . Csiba, L. (2007). Guidelines for management of ischaemic stroke and transient ischaemic attack 2008. *Cerebrovascular diseases (Basel, Switzerland), 25*(5), 457-507.

Riss, T. L., Moravec, R. A., Niles, A. L., Duellman, S., Benink, H. A., Worzella, T. J., & Minor, L. (2016). Cell viability assays.

Robak, J., & Gryglewski, R. (1996). Bioactivity of flavonoids. *Polish Journal of Pharmacology, 48*(6), 555-564.

Robbins, T., James, M., Owen, A., Sahakian, B., McInnes, L., & Rabbitt, P. (1994). Cambridge Neuropsychological Test Automated Battery (CANTAB): a factor analytic study of a large sample of normal elderly volunteers. *Dementia and Geriatric Cognitive Disorders, 5*(5), 266-281.

Robertson, C. E., Wright, P. A., Köblitz, L., & Bernier, N. J. (2014). Hypoxia-inducible factor-1 mediates adaptive developmental plasticity of hypoxia tolerance in zebrafish, Danio rerio. *Proceedings of the Royal Society of London B: Biological Sciences, 281*(1786), 20140637.

Rosell, A., Cuadrado, E., Ortega-Aznar, A., Hernández-Guillamon, M., Lo, E. H., & Montaner, J. (2008). MMP-9–positive neutrophil infiltration is associated to blood–brain barrier breakdown and basal lamina type iv collagen degradation during hemorrhagic transformation after human ischemic stroke. *Stroke, 39*(4), 1121-1126.

Rosenberg, G., Estrada, E., & Dencoff, J. (1998). Matrix metalloproteinases and TIMPs are associated with blood-brain barrier opening after reperfusion in rat brain. *Stroke, 29*(10), 2189-2195.

Rosenberg, G. A., Cunningham, L. A., Wallace, J., Alexander, S., Estrada, E. Y., Grossetete, M., . . . Gearing, A. (2001). Immunohistochemistry of matrix metalloproteinases in reperfusion injury to rat brain: activation of MMP-9 linked to stromelysin-1 and microglia in cell cultures. *Brain research, 893*(1-2), 104-112.

Roth, G. A., Johnson, C., Abajobir, A., Abd-Allah, F., Abera, S. F., Abyu, G., . . . Alam, K. (2017). Global, regional, and national burden of cardiovascular diseases for 10 causes, 1990 to 2015. *Journal of the American College of Cardiology*, 23715.

Rothman, S. M., & Olney, J. W. (1986). Glutamate and the pathophysiology of hypoxic–ischemic brain damage. *Annals of neurology, 19*(2), 105-111.

Rothwell, N. J., & Hopkins, S. J. (1995). Cytokines and the nervous system II: actions and mechanisms of action. *Trends in neurosciences, 18*(3), 130-136.

Sabaeifard, P., Abdi-Ali, A., Soudi, M. R., & Dinarvand, R. (2014). Optimization of tetrazolium salt assay for Pseudomonas aeruginosa biofilm using microtiter plate method. *Journal of microbiological methods, 105*, 134-140.

Sahakian, B., & Owen, A. (1992). Computerized assessment in neuropsychiatry using CANTAB: discussion paper. *Journal of the Royal Society of Medicine, 85*(7), 399.

Sakanaka, S., Tachibana, Y., & Okada, Y. (2005). Preparation and antioxidant properties of extracts of Japanese persimmon leaf tea (kakinoha-cha). *Food chemistry, 89*(4), 569-575.

Sandercock, P., Counsell, C., Gubitz, G. J., & Tseng, M.-C. (2008). Antiplatelet therapy for acute ischaemic stroke. *Cochrane Database Syst Rev, 3*.

Sayre, L. M., Smith, M. A., & Perry, G. (2001). Chemistry and biochemistry of oxidative stress in neurodegenerative disease. *Current medicinal chemistry, 8*(7), 721-738.

Schäbitz, W. R., Li, F., Irie, K., Sandage, B. W., Locke, K. W., & Fisher, M. (1999). Synergistic effects of a combination of low-dose basic fibroblast growth factor and citicoline after temporary experimental focal ischemia. *Stroke, 30*(2), 427-432.

Schaller, B., & Graf, R. (2004). Cerebral ischemia and reperfusion: the pathophysiologic concept as a basis for clinical therapy. *Journal of Cerebral Blood Flow & Metabolism, 24*(4), 351-371.
Scheid, V., & Scheid, V. (2009). *Chinese herbal medicine: formulas & strategies*: Eastland press Seattle.
Schipani, E., Ryan, H. E., Didrickson, S., Kobayashi, T., Knight, M., & Johnson, R. S. (2001). Hypoxia in cartilage: HIF-1α is essential for chondrocyte growth arrest and survival. *Genes & development, 15*(21), 2865-2876.
Schneider, M., Brufatto, N., Neill, E., & Nesheim, M. (2004). Activated thrombin-activatable fibrinolysis inhibitor reduces the ability of high molecular weight fibrin degradation products to protect plasmin from antiplasmin. *Journal of Biological Chemistry, 279*(14), 13340-13345.
Schneweis, S., Grond, M., Staub, F., Brinker, G., Neveling, M., Dohmen, C., . . . Heiss, W.-D. (2001). Predictive value of neurochemical monitoring in large middle cerebral artery infarction. *Stroke, 32*(8), 1863-1867.
Schuermann, A., Helker, C. S., & Herzog, W. (2014). *Angiogenesis in zebrafish.* Paper presented at the Seminars in cell & developmental biology.
Schwab, J. M., & Schluesener, H. J. (2003). Cyclooxygenases and central nervous system inflammation: conceptual neglect of cyclooxygenase 1. *Archives of neurology, 60*(4), 630-632.
Schweichel, J. U., & Merker, H. J. (1973). The morphology of various types of cell death in prenatal tissues. *Teratology, 7*(3), 253-266.
Seifried, H. E., Anderson, D. E., Fisher, E. I., & Milner, J. A. (2007). A review of the interaction among dietary antioxidants and reactive oxygen species. *The Journal of nutritional biochemistry, 18*(9), 567-579.
Serban, M. C., Sahebkar, A., Zanchetti, A., Mikhailidis, D. P., Howard, G., Antal, D., . . . Muntner, P. (2016). Effects of quercetin on blood pressure: a systematic review and meta-analysis of randomized controlled trials. *Journal of the American Heart Association, 5*(7), e002713.
Serena, J., Blanco, M., Castellanos, M., Silva, Y., Vivancos, J., Moro, M. A., . . . Dávalos, A. (2005). The prediction of malignant cerebral infarction by molecular brain barrier disruption markers. *Stroke, 36*(9), 1921-1926.
Shaw, L.-H., Lin, L.-C., & Tsai, T.-H. (2012). HPLC–MS/MS analysis of a traditional Chinese medical formulation of Bu-Yang-Huan-Wu-Tang and its pharmacokinetics after oral administration to rats. *PLoS One, 7*(8), e43848.
Shi, F., Hart, R. G., Sherman, D. G., & Tegeler, C. H. (1989). Stroke in the People's Republic of China. *Stroke, 20*(11), 1581-1585.
Shi, F., Wang, Y.-C., Zhao, T.-Z., Zhang, S., Du, T.-Y., Yang, C.-B., . . . Sun, X.-Q. (2012). Effects of simulated microgravity on human umbilical vein endothelial cell angiogenesis and role of the PI3K-Akt-eNOS signal pathway. *PloS one, 7*(7), e40365.
Shin, H. K., Nishimura, M., Jones, P. B., Ay, H., Boas, D. A., Moskowitz, M. A., & Ayata, C. (2008). Mild induced hypertension improves blood flow and oxygen metabolism in transient focal cerebral ischemia. *Stroke, 39*(5), 1548-1555.
Sies, H. (2017). Hydrogen peroxide as a central redox signaling molecule in physiological oxidative stress: oxidative eustress. *Redox biology, 11*, 613-619.
Siesjö, B., Agardh, C.-D., & Bengtsson, F. (1989). Free radicals and brain damage. *Cerebrovascular and brain metabolism reviews, 1*(3), 165-211.

Silagy, C. A., McNeil, J. J., Donnan, G. A., Tonkin, A. M., Worsam, B., & Campion, K. (1993). Adverse effects of low-dose aspirin in a healthy elderly population. *Clinical Pharmacology & Therapeutics, 54*(1), 84-89.

Simon, H.-U., Haj-Yehia, A., & Levi-Schaffer, F. (2000). Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis, 5*(5), 415-418.

Song, X., Wang, J., Wang, P., Tian, N., Yang, M., & Kong, L. (2013). 1H NMR-based metabolomics approach to evaluate the effect of Xue-Fu-Zhu-Yu decoction on hyperlipidemia rats induced by high-fat diet. *Journal of pharmaceutical and biomedical analysis, 78*, 202-210.

Soobrattee, M. A., Neergheen, V. S., Luximon-Ramma, A., Aruoma, O. I., & Bahorun, T. (2005). Phenolics as potential antioxidant therapeutic agents: mechanism and actions. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 579*(1), 200-213.

Stainier, D., Weinstein, B. M., Detrich, H. r., Zon, L. I., & Fishman, M. C. (1995). Cloche, an early acting zebrafish gene, is required by both the endothelial and hematopoietic lineages. *Development, 121*(10), 3141-3150.

Stamatovic, S. M., Keep, R. F., Kunkel, S. L., & Andjelkovic, A. V. (2003). Potential role of MCP-1 in endothelial cell tight junctionopening': signaling via Rho and Rho kinase. *Journal of cell science, 116*(22), 4615-4628.

Stamp, N. (2003). Out of the quagmire of plant defense hypotheses. *Q Rev Biol, 78*(1), 23-55.

Staton, C. A., Reed, M. W., & Brown, N. J. (2009). A critical analysis of current in vitro and in vivo angiogenesis assays. *International journal of experimental pathology, 90*(3), 195-221.

Stoll, G., Jander, S., & Schroeter, M. (1998). Inflammation and glial responses in ischemic brain lesions. *Progress in neurobiology, 56*(2), 149-171.

Sucher, N. J. (2006). Insights from molecular investigations of traditional Chinese herbal stroke medicines: implications for neuroprotective epilepsy therapy. *Epilepsy & Behavior, 8*(2), 350-362.

Sulter, G., Steen, C., & De Keyser, J. (1999). Use of the Barthel index and modified Rankin scale in acute stroke trials. *Stroke, 30*(8), 1538-1541.

Sun, H. J., Wu, W., Chen, H.G., Mi, H.Q. (2002). The pharmacokinetics study of ligustrazine. . *Traditional Chinese Drug Research & Clinical Pharmacology & Therapeutics, 13*(1), 61-63.

Sun, J., Tan, B. K., Huang, S.-H., Whiteman, M., & Zhu, Y.-Z. (2002). Effects of natural products on ischemic heart diseases and cardiovascular system. *Acta Pharmacologica Sinica, 23*(12), 1142-1151.

Sun, Y., Fang, Y., Xu, X., Lu, G., & Chen, Z. (2015). Evidence of an association between age-related functional modifications and pathophysiological changes in zebrafish heart. *Gerontology, 61*(5), 435-447.

Suzuki, S., Kelley, R. E., Reyes-Iglesias, Y., Alfonso, V. M., & Dietrich, W. D. (1995). Cerebrospinal fluid and peripheral white blood cell response to acute cerebral ischemia. *Southern medical journal, 88*(8), 819-824.

Tan, F., Lin, X., & Zhang, X. (2009). The anti-hypertensive effects and phamacokenetics of flavonoids of *Diospyros kaki* leaves *Guangxi Med. J., 16*(3), 313.

Tang, B., Liang, Z., Zheng, W., Jiang, Y., Wu, H., & Cheng, B. (2012). NXQ for the treatment of diabetic indcued ischaemic stroke - 35 cases. *Global Traditional Chinese Medicine, 5*(8), 616-619. doi:10.3969/j.issn.1674-1749.2012.08.018

Tang, J. Y., Li, S., Li, Z. H., Zhang, Z. J., Hu, G., Cheang, L. C. V., . . . Chan, S. W. (2010). Calycosin promotes angiogenesis involving estrogen receptor and mitogen-activated protein kinase (MAPK) signaling pathway in zebrafish and HUVEC. *PloS one, 5*(7), e11822.

Tang, Y., Xu, H., Du, X. L., Lit, L., Walker, W., Lu, A., . . . Pancioli, A. (2006). Gene expression in blood changes rapidly in neutrophils and monocytes after ischemic stroke in humans: a microarray study. *Journal of Cerebral Blood Flow & Metabolism, 26*(8), 1089-1102.

Tanne, D., Macko, R. F., Lin, Y., Tilley, B. C., & Levine, S. R. (2006). Hemostatic activation and outcome after recombinant tissue plasminogen activator therapy for acute ischemic stroke. *Stroke, 37*(7), 1798-1804.

Tarkowski, E., Rosengren, L., Blomstrand, C., Wikkelsö, C., Jensen, C., Ekholm, S., & Tarkowski, A. (1995). Early intrathecal production of interleukin-6 predicts the size of brain lesion in stroke. *Stroke, 26*(8), 1393-1398.

Tatemichi, T., Paik, M., Bagiella, E., Desmond, D., Stern, Y., Sano, M., . . . Mayeux, R. (1994). Risk of dementia after stroke in a hospitalized cohort Results of a longitudinal study. *Neurology, 44*(10), 1885-1885.

Thornberry, N. A., & Lazebnik, Y. (1998). Caspases: enemies within. *Science, 281*(5381), 1312-1316.

Tjwa, M., Luttun, A., Autiero, M., & Carmeliet, P. (2003). VEGF and PlGF: two pleiotropic growth factors with distinct roles in development and homeostasis. *Cell and tissue research, 314*(1), 5-14.

Tomimoto, H., Shibata, M., Ihara, M., Akiguchi, I., Ohtani, R., & Budka, H. (2002). A comparative study on the expression of cyclooxygenase and 5-lipoxygenase during cerebral ischemia in humans. *Acta neuropathologica, 104*(6), 601-607.

Toshniwal, P. K., & Zarling, E. J. (1992). Evidence for increased lipid peroxidation in multiple sclerosis. *Neurochemical research, 17*(2), 205-207.

Tran, T. C., Sneed, B., Haider, J., Blavo, D., White, A., Aiyejorun, T., . . . Dingledine, R. (2007). Automated, quantitative screening assay for antiangiogenic compounds using transgenic zebrafish. *Cancer research, 67*(23), 11386-11392.

Tsai, F.-K., Lin, Chien-Hsiung., Chang, Yeu-Jhy., Ryu, Shan-Jin., Lee, Tsong-Hai., Chen, Chun-Hsien., Chang, Her-Kun. (2011). ANALYSIS OF TCM SYNDROMES OF ISCHEMIC STROKE PATIENTS AT ACUTE STAGE. *J Chin Med, 22*(1,2), 95-105.

Tsigkos, S., Koutsilieris, M., & Papapetropoulos, A. (2003). Angiopoietins in angiogenesis and beyond. *Expert opinion on investigational drugs, 12*(6), 933-941.

Tu, S., Shao, A., Ren, L., Chen, T., & Yao, D. (2014). Angiogenesis effect of Astragalus polysaccharide combined with endothelial progenitor cells therapy in diabetic male rat following experimental hind limb ischemia. *Chinese medical journal, 127*(11), 2121-2128.

Tursunov, D., & Akbarkhodjaeva, Z. (2017). Epidemiological condition of stroke in the world. *Journal of the Neurological Sciences, 381*, 1115.

Uc-Cachón, A., Molina-Salinas, G., Said-Fernández, S., Méndez-González, M., Cáceres-Farfán, M., & Borges-Argáez, R. (2013). A new dimeric naphthoquinone from Diospyros anisandra. *Natural product research, 27*(13), 1174-1178.

Uyttenboogaart, M., Koch, M. W., Stewart, R. E., Vroomen, P. C., Luijckx, G.-J., & De Keyser, J. (2007). Moderate hyperglycaemia is associated with favourable outcome in acute lacunar stroke. *Brain, 130*(6), 1626-1630.

van der Worp, H. B., & van Gijn, J. (2007). Acute ischemic stroke. *New England Journal of Medicine, 357*(6), 572-579.

Vandenbroucke, E., Mehta, D., Minshall, R., & Malik, A. B. (2008). Regulation of endothelial junctional permeability. *Annals of the new York Academy of Sciences, 1123*(1), 134-145.

Vila, N., Castillo, J., Dávalos, A., & Chamorro, Á. (2000). Proinflammatory cytokines and early neurological worsening in ischemic stroke. *Stroke, 31*(10), 2325-2329.

Vila, N., Castillo, J., Dávalos, A., Esteve, A., Planas, A. M., & Chamorro, Á. (2003). Levels of anti-inflammatory cytokines and neurological worsening in acute ischemic stroke. *Stroke, 34*(3), 671-675.

Wagner, H. (1999). Phytomedicine research in Germany. *Environmental Health Prespectives, 107*, 779-781.

Wagner, S., Nagel, S., Kluge, B., Schwab, S., Heiland, S., Koziol, J., . . . Hacke, W. (2003). Topographically graded postischemic presence of metalloproteinases is inhibited by hypothermia. *Brain research, 984*(1-2), 63-75.

Walker, J. B., & Bajzar, L. (2004). The intrinsic threshold of the fibrinolytic system is modulated by basic carboxypeptidases, but the magnitude of the antifibrinolytic effect of activated thrombin-activable fibrinolysis inhibitor is masked by its instability. *Journal of Biological Chemistry, 279*(27), 27896-27904.

Wang, A., Ding, X., & Wang, H. (2012). Clinical study on NXQ for cerebro-cardiac syndrome induced by ischaemic stroke. . *China Prac Med, 7*(4), 169-171.

Wang, H.-W., Liou, K.-T., Wang, Y.-H., Lu, C.-K., Lin, Y.-L., Lee, I.-J., . . . Lin, H.-J. (2011). Deciphering the neuroprotective mechanisms of Bu-yang Huan-wu decoction by an integrative neurofunctional and genomic approach in ischemic stroke mice. *Journal of ethnopharmacology, 138*(1), 22-33.

Wang, J., Shi, Y. M., Zhen, H. M., Liu, Z. R., Li, X. X., Ding, G. H. (1993). The experimental study of hemodynamic parameters about chuanxiong's effects on cerebrovascular disease. *Chinese Journal of Integrated Traditional and Western Medicine, 13*(7), 417-419.

Wang, Q., Tang, X. N., & Yenari, M. A. (2007). The inflammatory response in stroke. *Journal of neuroimmunology, 184*(1), 53-68.

Wang, T., Guo, R., Zhou, G., Zhou, X., Kou, Z., Sui, F., . . . Wang, Z. (2016). Traditional uses, botany, phytochemistry, pharmacology and toxicology of Panax notoginseng (Burk.) FH Chen: a review. *Journal of ethnopharmacology, 188*, 234-258.

Wardlaw, J. M., Murray, V., Berge, E., & Del Zoppo, G. J. (2009). Thrombolysis for acute ischaemic stroke. *Cochrane Database Syst Rev, 4*(CD000213).

Waring, W. (2002). Uric acid: an important antioxidant in acute ischaemic stroke. *Qjm, 95*(10), 691-693.

Wei, E., & Kontos, H. (1990). H2O2 and endothelium-dependent cerebral arteriolar dilation. Implications for the identity of endothelium-derived relaxing factor generated by acetylcholine. *Hypertension, 16*(2), 162-169.

Wei, E. P., Kontos, H. A., & Beckman, J. S. (1996). Mechanisms of cerebral vasodilation by superoxide, hydrogen peroxide, and peroxynitrite. *American Journal of Physiology-Heart and Circulatory Physiology, 271*(3), H1262-H1266.

Wei, E. P., Kontos, H. A., Christman, C. W., DeWitt, D. S., & Povlishock, J. T. (1985). Superoxide generation and reversal of acetylcholine-induced cerebral arteriolar dilation after acute hypertension. *Circulation Research, 57*(5), 781-787.

Wei, Q. H., Zhou, D. W., Wang, W. (1994). The pharmacology and effects of chuanxiong.

. *Foreign Medicine: Botanic Department*

, *9*(4), 164-166.
Wesnes, K., Ward, T., McGinty, A., & Petrini, O. (2000). The memory enhancing effects of a Ginkgo biloba/Panax ginseng combination in healthy middle-aged volunteers. *Psychopharmacology, 152*(4), 353-361.
Westerfield, M. (1995). *The zebrafish book: a guide for the laboratory use of zebrafish (Brachydanio rerio)*: University of Oregon press.
Willemse, J., Brouns, R., Heylen, E., De Deyn, P., & Hendriks, D. (2008). Carboxypeptidase U (TAFIa) activity is induced in vivo in ischemic stroke patients receiving thrombolytic therapy. *Journal of Thrombosis and Haemostasis, 6*(1), 200-202.
Willemse, J. L., & Hendriks, D. F. (2007). A role for procarboxypepidase U (TAFI) in thrombosis. *Frontiers in bioscience: a journal and virtual library, 12*, 1973-1987.
Williams, L. S., Weinberger, M., Harris, L. E., & Biller, J. (1999). Measuring quality of life in a way that is meaningful to stroke patients. *Neurology, 53*(8), 1839-1839.
Williams, L. S., Weinberger, M., Harris, L. E., Clark, D. O., & Biller, J. (1999). Development of a stroke-specific quality of life scale. *Stroke, 30*(7), 1362-1369.
Wiseman, N. (Ed.) (1996). *Fundamentals of Chinese Medicine.* Brookline: Paradigm Publications.
Wu, B., Liu, M., Liu, H., Li, W., Tan, S., Zhang, S., & Fang, Y. (2007). Meta-analysis of traditional Chinese patent medicine for ischemic stroke. *Stroke, 38*(6), 1973-1979.
Wu, B., Liu, M., & Zhang, S. (2007). Dan Shen agents for acute ischaemic stroke. *Cochrane Database Syst Rev, 2*.
Wu, B., Liu, M., & Zhang, S. (2007). Dan Shen agents for acute ischaemic stroke. *Cochrane Database of Systematic Reviews*(2).
Wu, D., Wu, B., & Zheng, Z. (2013). Naoqinxing Tablet for the treatment of coronary heart disease and angina : a clinical obeservation on 90 cases *Guide of China Medicine, 11*(10), 274-275.
Wu, H.-z., & Fang, Z. (2013). *Fundamentals of Traditional Chinese Medicine* (Vol. 1): World Scientific.
Wu, J. X., Sun, J. J. (1992). Comparison of panax notoginseng saponins, virapamil and norepinephrine on the effection to the cerebral circulation of rat and rabbit. *Acta Pharmacologica Sinica 13*(6), 520-523.
Wu, Q. J., & Tymianski, M. (2018). Targeting NMDA receptors in stroke: new hope in neuroprotection. *Molecular brain, 11*(1), 15.
Xiao, H.-B., Sui, G.-G., Lu, X.-Y., & Sun, Z.-L. (2018). Kaempferol modulates Angiopoietin-like protein 2 expression to lessen the mastitis in mice. *Pharmacological Reports, 70*(3), 439-445.
Xie, C., Xie, Z., Xu, X., & Yang, D. (2015). Persimmon (Diospyros kaki L.) leaves: a review on traditional uses, phytochemistry and pharmacological properties. *Journal of ethnopharmacology, 163*, 229-240.
Xin, N., Feng, J., & Yao, P. (2007). Pharmacological effects of Extract of *Diospyros Kaki* Leaves *Chinese Medicine Journal, 35*(2), 49-51.
Xin, Q.-q., Yang, B.-r., Zhou, H.-f., Wang, Y., Yi, B.-w., Cong, W.-h., . . . Chen, K.-j. (2016). Paeoniflorin promotes angiogenesis in a vascular insufficiency model of zebrafish in vivo and in human umbilical vein endothelial cells in vitro. *Chinese journal of integrative medicine*, 1-8.
Xiong, Y., Tang, X., & Zhou, S. (2013). Combined treatment of Western and Eastern Medicine for unstable angina in elderly: a clinical analysis. *China External Medical Research, 11*(12), 36-37.

Xu, H., Shi, D. Z., Guan, C. Y. (2003). Clinical application and pharmacological action of ligustrazine. *Chinese Journal of Integrated Traditional and Western Medicine, 23*(5), 376-379.
Xu, J. (2003). Clinical observation of xuesaitong for acute cerebral infarction. . *Journal of North China Coal Medical College, 5*(1), 66.
Xu, X., Zhao, C., Peng, Q., Xie, P., & Liu, Q. (2017). Kaempferol inhibited VEGF and PGF expression and in vitro angiogenesis of HRECs under diabetic-like environment. *Brazilian Journal of Medical and Biological Research, 50*(3).
Xue, Y.-L., Miyakawa, T., Hayashi, Y., Okamoto, K., Hu, F., Mitani, N., . . . Tanokura, M. (2011). Isolation and tyrosinase inhibitory effects of polyphenols from the leaves of persimmon, Diospyros kaki. *Journal of agricultural and food chemistry, 59*(11), 6011-6017.
Yadav, N. P., Dixit, V. K. (2008). Recent approaches in Herbal drug standardisation. *International Journal of Intagrative Biology, 2*, 195-203.
Yagi, H., Sumino, H., Aoki, T., Tsunekawa, K., Araki, O., Kimura, T., . . . Murakami, M. (2016). Impaired blood rheology is associated with endothelial dysfunction in patients with coronary risk factors. *Clinical hemorheology and microcirculation, 62*(2), 139-150.
Yamagami, S., Tamura, M., Hayashi, M., Endo, N., Tanabe, H., Katsuura, Y., & Komoriya, K. (1999). Differential production of MCP-1 and cytokine-induced neutrophil chemoattractant in the ischemic brain after transient focal ischemia in rats. *Journal of leukocyte biology, 65*(6), 744-749.
Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., . . . Masaki, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *nature, 332*(6163), 411.
Yancopoulos, G. D., Davis, S., Gale, N. W., Rudge, J. S., Wiegand, S. J., & Holash, J. (2000). Vascular-specific growth factors and blood vessel formation. *Nature, 407*(6801), 242.
Yang, B.-r., Hong, S.-j., Lee, S. M.-Y., Cong, W.-h., Wan, J.-b., Zhang, Z.-r., . . . Lin, Z.-x. (2016). Pro-angiogenic activity of notoginsenoside R1 in human umbilical vein endothelial cells in vitro and in a chemical-induced blood vessel loss model of zebrafish in vivo. *Chinese journal of integrative medicine, 22*(6), 420-429.
Yang, H., Zhang, J. . (2005). Clinical observation on xuesaitong injection for 38 cases of acute cerebral infarction. *Journal of Practical Medical Techniques, 12*(6), 1445-1446.
Yang, S. (1998). *The divine farmer's materia medica: a translation of the Shen Nong Ben Cao Jing*: Blue Poppy Enterprises, Inc.
Yao, X.-H., & Li, X.-J. (2002). Protective effects and its mechanism of panaxatriol saponins isolated from Panax notoginseng on cerebral ischemia. *Zhongguo Zhong yao za zhi= Zhongguo zhongyao zazhi= China journal of Chinese materia medica, 27*(5), 371-373.
Yarrow, J. C., Perlman, Z. E., Westwood, N. J., & Mitchison, T. J. (2004). A high-throughput cell migration assay using scratch wound healing, a comparison of image-based readout methods. *BMC biotechnology, 4*(1), 21.
YIN, R., WANG, D., LUO, J., LI, Y., LIANG, J., & LI, C. (2013). Study on HPLC-DAD Fingerprint of Naoxinqing Tablets. *Traditional Chinese Drug Research and Clinical Pharmacology, 1*, 021.
Yousef, M., Schannwell, C. M., Köstering, M., Zeus, T., Brehm, M., & Strauer, B. E. (2009). The BALANCE Study: clinical benefit and long-term outcome after intracoronary

autologous bone marrow cell transplantation in patients with acute myocardial infarction. *Journal of the American College of Cardiology, 53*(24), 2262-2269.
Yu, L., Tucci, V., Kishi, S., & Zhdanova, I. V. (2006). Cognitive aging in zebrafish. *PloS one, 1*(1), e14.
Yu, S. P., Yeh, C.-H., Sensi, S. L., Gwag, B. J., Canzoniero, L. M., Farhangrazi, Z. S., . . . Choi, D. W. (1997). Mediation of neuronal apoptosis by enhancement of outward potassium current. *Science, 278*(5335), 114-117.
Yu, X., & Li, Y. V. (2011). Zebrafish as an alternative model for hypoxic-ischemic brain damage. *International journal of physiology, pathophysiology and pharmacology, 3*(2), 88.
Yu, X., & Li, Y. V. (2013). Neuroprotective Effect of Zinc Chelator DEDTC in a Zebrafish (Danio rerio) Model of Hypoxic Brain Injury. *Zebrafish, 10*(1), 30-35.
Yu, Y., Yu, Z., & Guo, J. (1988). The experimental and clinical studies of NaoXin Ning in the treatment of ischemic cerebral vascular disease. *Med. J. Chin. PLA, 13*, 30-31.
Yuan, Y., Zeng, X., Luo, Y., Li, Z., & Wu, T. (2008). Chuanxiong-type preparations for acute ischemic stroke. *Cochrane Database Syst Rev, 4*.
Zazulia, A. R., Videen, T. O., & Powers, W. J. (2007). Symptomatic autoregulatory failure in acute ischemic stroke. *Neurology, 68*(5), 389-390.
Zeller, J., Lenz, A., Eschenfelder, C., Zunker, P., & Deuschl, G. (2005). Platelet-leukocyte interaction and platelet activation in acute stroke with and without preceding infection. *Arteriosclerosis, thrombosis, and vascular biology, 25*(7), 1519-1523.
Zeller, J. A., Tschoepe, D., & Kessler, C. (1999). Circulating platelets show increased activation in patients with acute cerebral ischemia. *Thrombosis and haemostasis, 81*(03), 373-377.
Zhang, F., Xu, S., & Iadecola, C. (1995). Time dependence of effect of nitric oxide synthase inhibition on cerebral ischemic damage. *Journal of Cerebral Blood Flow & Metabolism, 15*(4), 595-601.
Zhang, J. Y., Yang, R. P., Zhu, J. M. (2001). The clinical use and evaluation about ligustrazine injection.

. *Chinese Journal of Clinical Pharmacy, 10*(4), 268–270.
Zhang, K., Wu, J., Liang, C., Zhou, F., Liang, P., Wen, J., & Ma, Y. (1983). Studies on the chemical constituents of persimmon (Diospyros kaki) leaves (I). *Chin Tradit Herb Drugs, 14*(2), 4-6.
Zhang, L., Yang, Y., Wang, Y., & Gao, X. (2011). Astragalus membranaceus extract promotes neovascularisation by VEGF pathway in rat model of ischemic injury. *Die Pharmazie-An International Journal of Pharmaceutical Sciences, 66*(2), 144-150.
Zhang, P., Chen, L., Wang, X., Chen, J., Xu, S., Ye, L., & Yao, Y. (2019). Simultaneous Determination of Night Effective Constituents and Correlation Analysis of Multiconstituents and Antiplatelet Aggregation Bioactivity In Vitro in Chuanxiong Rhizoma Subjected to Different Decoction Times. *Journal of Analytical Methods in Chemistry, 2019*.
Zhang, X., Liu, X.-T., & Kang, D.-Y. (2016). Traditional Chinese patent medicine for acute ischemic stroke: An overview of systematic reviews based on the GRADE approach. *Medicine, 95*(12).
Zhang, Y., Wang, L., & Xiao, F. (2004). Progress of Pharmacological effects research of *Diospyros Kaki* Leaves. *Hebei Medical College Journal, 21*(3), 39-41.

Zhao, D., Qin, C., Fan, X., Li, Y., & Gu, B. (2014). Inhibitory effects of quercetin on angiogenesis in larval zebrafish and human umbilical vein endothelial cells. *European journal of pharmacology, 723*, 360-367.

Zhao, H., Huang, H., & Lin, S. (2016). Chemical approaches to angiogenesis in development and regeneration *Methods in cell biology* (Vol. 134, pp. 369-376): Elsevier.

Zhao, H., Zhang, T., Xia, C., Shi, L., Wang, S., Zheng, X., . . . Zhang, B. (2014). Berberine ameliorates cartilage degeneration in interleukin-1β-stimulated rat chondrocytes and in a rat model of osteoarthritis via Akt signalling. *Journal of cellular and molecular medicine, 18*(2), 283-292.

Zhao, L.-D., Wang, J.-H., Jin, G.-R., Zhao, Y., & Zhang, H.-J. (2012). Neuroprotective effect of Buyang Huanwu decoction against focal cerebral ischemia/reperfusion injury in rats–time window and mechanism. *Journal of ethnopharmacology, 140*(2), 339-344.

Zhaozhong, Z., Qiuping, X., & Jianning, S. (2001). Effects and mechanisms of triacetylshikimic acid on platelet adhesion to neutrophils induced by thrombin and reperfusion after focal cerebral ischemia in rats. *Acta pharmacologica Sinica, 22*(8), 679-684.

Zhou, X.-t., Wang, Li-li., Han, Lu., Zhang, Tie-jun.,Liu, Fang. (2014). Research progress on chemical constituents and pharmacological effects of Diospyros kaki leaves *Chinese Traditional and Herbal Drugs, 45*(21), 3195-3203.

Zhu, B., & Wang, H. (2011). *Diagnostics of traditional Chinese medicine*: Singing Dragon.

Zhu, J., Song, W., Li, L., & Fan, X. (2016). Endothelial nitric oxide synthase: a potential therapeutic target for cerebrovascular diseases. *Molecular brain, 9*(1), 1-8.

Zhu, J., Tang, H., Zhang, Z., Zhang, Y., Qiu, C., Zhang, L., . . . Li, F. (2017). Kaempferol slows intervertebral disc degeneration by modifying LPS-induced osteogenesis/adipogenesis imbalance and inflammation response in BMSCs. *International immunopharmacology, 43*, 236-242.

Appendix A. Experiment Protocol – Participant Information sheet

Project title

A Randomised, Double-Blind, Placebo Controlled Trial to Evaluate the Effectiveness and Safety of Nao Xin Qing (NXQ), a Standardised Herbal Medicine Formula in Patients with Ischaemic Stroke.

Who is carrying out the study?

You are invited to participate in a study conducted by Miss Yu-Ting Sun, a PhD candidate from the National Institute of Complementary Medicine (NICM) and the University of Western Sydney (UWS). This research will form a part of a PhD degree at the University of Western Sydney under the supervision of Associate Professor Dennis Chang, Professor Alan Bensoussan, NICM, UWS and Professor Hasan Kiat, School of Advanced Medicine, Macquarie University. Amongst the primary investigators of this study is a stroke rehabilitation specialist Dr. Alan Lam, MBBS (Syd), FAFRM from Mt Wilga Private Hospital and Lady Davidson Private Hospital.

What is the study about?

The purpose is to investigate the clinical effects and safety profile of NXQ for patients with ischaemic stroke to determine the merit of future research into this treatment. You will be selected as a possible participant in this study if you are diagnosed with ischaemic stroke within the past 6 months, currently undergoing rehabilitation, and between 40 to 80 years of age.

Ischaemic stroke accounts for more than 80% of all stroke incidences and has substantial disability rates. The currently available pharmaceuticals for the treatment and prevention of ischaemic stroke such as anti-thrombotics possesses relatively high risks of side effects such as bleeding while only having a low therapeutic impact on rehabilitation. NXQ has been used

over the past 10 years in China for ischaemic condition such as coronary artery disease and ischaemic stroke. Preclinical studies have revealed that NXQ has a number of therapeutic effects including neuroprotective, anti- inflammatory, anti-hypertensive, anti-cholesterol and anti-thrombotic. This study aims to assess whether NXQ can improve neurological functionalities and quality of life of ischaemic stroke patients during their rehabilitation phase.

What does the study involve?

This trial runs for a total period of 36 weeks including a 12 week intervention and a 24 week follow up. You will be required to attend a total of 6 clinical visits and will be randomly allocated in a ratio of 1:1 manner to one of the two treatment groups (active treatment of NXQ or placebo). The dose regimen will involve the oral ingestion of 4 tablets, 3 times a day. The outcome measure will be collected in the form of Case Record Files (CRF), blood samples, medical measurements and questionnaires.

Initial screening: Prior to the commencement of study, you will be asked to attend an initial medical screening session. Before beginning the session, detailed information about the study will be provided to you and have any of your questions answered. If you wish to continue, you will be requested to sign a consent form for this trial before commencing of the medical screening session. The screening session is organised to clarify whether you are eligible for the study which will involve a medical questionnaire, a medical examination on neurological function conducted by a physician/neurologist, a questionnaire on the status of disability, and a blood pathology test. You will then be given a short practise session on the computer testing system used to measure your memory and cognitive functions. You will then be randomized into either active treatment group or placebo group.

First week of the Study: On the first day (Day 1), you will be required to attend the at Mt Wilga Private Hospital or Lady Davidson Private Hospital for approximately 2 hours for the assessment session before treatment intervention is given. These assessments include a questionnaire on quality of life (Stroke Specific Quality of Life); a short answer questions on status of disability - Modified Rankin Scale; a short answer questions on daily living activities - Barthel index; a blood pathology test; a computer based test battery on your memory and cognitive function and ultrasound to assess flow medial dilation. After the baseline assessment you will be given either trial medication or a placebo and you are required to take 4 tablets, three times a day, continuously for 12 weeks. A diary will be given to you for your to record any missing dose or additional medication used.

Week 2 of the study: You are required to visit the hospital where you were recruited for this study or a designated pathology lab for a blood sample test.

Week 6 and Week 12 of the study: You are required to visit the hospital where you were recruited for a mid-treatment assessment (week 6) and a post-treatment assessment (week 12). The assessments will be the same as your baseline assessment as well as a neurological function tests performed by a physician or neurologist.

Follow up sessions: You are required to attend this last clinical visit at week 16 of the study for a follow up assessment. This assessment will be a neurological function test (NIHSS) performed by the physician, a questionnaire on quality of life, a questionnaire on the status of disability and a questionnaire on daily living activities. Upon the completion of this visit, a follow up call will be made on week 36 to assess long term efficacy of the intervention.

How much time will the study take?

You will be asked to attend 6 clinical visits in total. These sessions Includes a screening, baseline, week 2, mid-treatment (week 6), post-treatment (week 12) and a follow up session (week 16). Each assessment session will be approximately 1 2 hours.

Will the study benefit me?

This study aims to further medical knowledge and may improve future treatment for ischaemic stroke, however it may not directly benefit you if you are randomly allocate into placebo group. If you are randomly allocated to the active treatment group you may experience temporary improvements in neurological functions, quality of life and reduction of vascular risk factor as a result of this intervention; however, no long term improvements are known at this stage.

Your participation of the current study will provide important data on whether NXQ tablets have the potential to improve post-stroke neurological functionality, improvement of quality of life and has implication for the vascular risk factors management of recurrence of ischaemic stroke.

Will the study have any risks?

During the initial screening session, participants may feel some discomfort during the blood test and may experience minor bruising. Participants may also feel some discomfort while having blood pressure measurements taken. All blood tests will be performed by a trained professional to ensure all discomfort are minimised.

NXQ has been listed and approved by the Therapeutic Goods Administration for human therapeutic consumption. NXQ has been using clinically for the treatment of coronary heart

disease and stroke more than 10 years. Some minor adverse events such as abdominal bloating, abdominal pain and diarrhoea has been reported, but these symptoms are alleviated when stop administration of the intervention medication. No major side effects are reported. To minimise the risk, all adverse events will be recorded and monitored throughout the trial. Potential participants will undergo safety tests including full blood counts, liver function test, renal function test, blood rheology and coagulation index before intervention, 2 weeks into intervention, 6 weeks into intervention and at week 12 (post-treatment) to monitor the safety index. Participant is encouraged to contact the primary investigator or the chief investigator at any time during the trial if any adverse events are experienced.

How much is this study being paid for?

You will be reimbursed with $100 (approximately $17 per visit) to compensate for travel related expenses incurred during your attendance to study visits at the completion of the trial. If you withdraw from the study prematurely, the compensation will be calculated on a pro rata basis.

Will anyone else know the results? How will the result be disseminated'?

All aspects of the study, including results, will be confidential and only the researchers will have access to information on participants. Data will be de-identified when published as thesis, journal articles and presentation in the conferences.

Can I withdraw feom the study?

Participation is entirely voluntary. You are not obliged to participate and if you do participate you can withdraw at any time without giving any reason. Whatever your decision, it will not affect your medical treatment or your relationship with the research team and their affiliations.

Can I tell other people about the study?

Yes, you can tell other people about the study by providing them with the chief investigator's and primary investigator's contact details. They can contact the chief investigator or primary investigator to discuss their participation in the research project and obtain an information sheet.

What if I require further information?

When you have read this information, Miss Yu-Ting Sun the primary investigator will discuss it with you further and answer any questions you may have. If you would like to know more at any stage, please feel free to contact Miss Yu-Ting Sun on (02) 4620 3278 or 0468 672 521 or Associate Professor Dennis Chang on (02) 46203310 or 0404453682

What if I have a complaint?

This study has been approved by the University of Western Sydney Human Research Ethics Committee. The Approval number is [to be inserted after approval granted by HREC].

If you have any complaints or reservations about the ethical conduct of this research, you may contact Ethics Committee through the Office of Research Services on Tel +612 4736 0229 Fax +61 2 4736 0013 or email humanethics@uws.edu.au.

Any issues you raise will be treated in confidence and investigated fully, and you will be informed of the outcome. If you agree to participate in this study, you may be asked to sign the Participant Consent Form.

The information sheet is for the participant to keep and the consent form is retained by the researcher.

Appendix B. Experiment Protocol – Adverse Events

The Investigator is responsible for the detection and documentation of events meeting the criteria and definitions detailed below.

Full details of contraindications and side effects that have been reported following administration of the trial Investigational Product can be found in the Investigator's Brochure.

Participants should be instructed to contact their Investigator at any time after consenting to join the trial if any symptoms develop. All reported adverse events (AEs) that occur after joining the trial must be recorded in detail in the CRF. In the case of an AE, the Investigator should initiate the appropriate treatment according to their medical judgment. Participants with AEs present at the last visit must be followed up until resolution of the event.

Definitions

An **adverse event** (AE) is any untoward medical event affecting a clinical trial participant. An AE can be any unfavourable and unintended sign (including an abnormal laboratory finding), symptom, or disease (new or exacerbated) temporally associated with the use of a medicinal product, whether or not considered related to the medicinal product. Each initial AE will be considered for severity, causality or expectedness and may be reclassified as a serious event or reaction based on prevailing circumstances.

An **adverse reaction** (AR) is where it is suspected that an AE has been caused by a reaction to a trial drug

A **serious adverse event** (SAE), **serious adverse reaction** (SAR) or **suspected unexpected serious adverse reaction** (SUSAR) is any AE, AR or UAR that at any dose:

- results in death;
- is life threatening (i.e. the subject was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe);
- requires hospitalisation or prolongation of existing hospitalisation;
- results in persistent or significant disability or incapacity;
- is a congenital anomaly or birth defect.

Note: Hospitalisations for treatment planned prior to randomisation and hospitalisation for elective treatment of a pre-existing condition will not be considered as an AE. Complications occurring during such hospitalisation will be AEs.

Detecting AEs and SAEs

All AEs and SAEs must be recorded from the time a participant consents to join the study until the last study visit.

The Investigator and designated study personnel will monitor each subject for adverse events during the study. All adverse events reported between consent and final follow-up will be recorded in the case report form (CRF). The investigator or designee will ask the subject non-leading questions in an effort to detect adverse events eg:

"**How are you feeling?"**

or

"Since you were last asked, have you felt unwell or different from usual?"

In addition, subjects should be encouraged to spontaneously report any unusual feelings or sensations.

Participants should also be asked if they have been admitted to hospital, had any accidents, used any new medicines or changed concomitant medication regimens. If there is any doubt as to whether a clinical observation is an AE, the event should be recorded.

Recording AEs and SAEs

Depending on severity, when an AE/SAE occurs, it is the responsibility of the Investigator to review all documentation (e.g. hospital notes, laboratory and diagnostic reports) related to the event. The Investigator should then record all relevant information in the CRF and on the SAE form.

Information to be collected includes dose, type of event, onset date, Investigator assessment of severity and causality, date of resolution as well as treatment required, investigations needed and outcome.

Evaluation of AEs and SAEs

Seriousness, causality, severity and expectedness should be evaluated as though the participant is taking active drug. Cases that are considered serious, possibly, probably or definitely related to drug and unexpected (i.e. SUSARs) are likely to be un-blinded.

Assessment of Seriousness

The Investigator should make an assessment of seriousness as defined in Section 10.1.

Assessment of Causality

The Investigator must make an assessment of whether the AE/SAE is likely to be related to treatment according to the following definitions:

Unrelated: where an event is not considered to be related to the study drug.

Possibly: although a relationship to the study drug cannot be completely ruled out, the nature of the event, the underlying disease, concomitant medication or temporal relationship make other explanations possible.

Probably: the temporal relationship and absence of a more likely explanation suggest the event could be related to the study drug.

Definitely: The known effects of the study drug or its therapeutic class, or based on challenge testing, suggest that study drug is the most likely cause.

All AEs/SAEs judged as having a reasonable suspected causal relationship (e.g. possibly, probably, definitely) to the study drug will be considered as ARs/SARs. All AEs/SAEs judged as being related (e.g. possibly, probably, definitely) to an interaction between the study drug and another drug will also be considered to be ARs/SAR.

Alternative causes such as natural history of the underlying disease, concomitant therapy, other risk factors and the temporal relationship of the event to the treatment should be considered. The blind should not be broken for the purpose of making this assessment.

Assessment of Severity

The Investigator should make an assessment of severity for each AE/SAE and record this on the CRF according to one of the following categories:

Mild: an event that is easily tolerated by the participant, causing minimal discomfort and not interfering with every day activities.

Moderate: an event that is sufficiently discomforting to interfere with normal everyday activities.

Severe: an event that prevents normal everyday activities.

Note: the term 'severe', used to describe the intensity, should not be confused with 'serious' which is a regulatory definition based on participant/event outcome or action criteria. For example, a headache may be severe but not serious, while a minor stroke is serious but may not be severe.

Assessment of Expectedness

If an event is judged to be an AR/SAR, the evaluation of expectedness should be made based on knowledge of the reaction and the relevant product information documented in the Investigator's Brochure.

Reporting SAEs/SARs/SUSARs

Once the Investigator becomes aware that an SAE has occurred in a study participant, they must report the information to the Pharmacovigilance Sponsor within 24 hours of becoming aware of the event. The SAE form must be completed as thoroughly as possible with all available details of the event, signed by the Investigator or designee. If all the required information is not available at the time of reporting, the Investigator must ensure that any missing information is provided as soon as this becomes available. It should be indicated on the report that this information is follow-up information of a previously reported event.

The SAE report must provide an assessment of causality and expectedness at the time of the initial report to the Pharmacovigilance Sponsor according to Sections 10.4.2, Assessment of Causality and 10.4.4, Assessment of Expectedness.

Follow Up Procedures

After initially recording an AE or recording and reporting an SAE, the Investigator is required to follow each participant until resolution. Follow up information on an SAE should be reported to the Pharmacovigilance Sponsor.

Unless otherwise stated in the protocol, AEs and SAEs should be followed up until resolution or death of the trial subject.

Appendix C. Experiment Protocol – Monitoring and Quality Assurance

Project Management Group

The trial will be coordinated by a Project Management Group, consisting of Chief Investigators, the Principal Investigator and supervisors, an associate researcher and coordinating research officer.

Study Monitoring

A trial committee consisting of the chief and principal investigators, co-supervisors and a NICM Research Program Coordinator who is external to the research team will be formed to monitor this research project.

This committee will attend regular scheduled meetings to provide updates on the study progression as well as addressing any issues regarding the conduct and progression of the project. This committee will also ensure that the study is being conducted in accordance to Good Clinical Practice (GCP) guidelines.

Risk Assessment

Potential Risks

The risk of harm to participants will be minimal as NXQ has been proven safe with minimal adverse events. NXQ has been listed and approved for human consumption by the Therapeutic Goods Authority (TGA). Toxicity test of NXQ has been conducted on rats including acute toxicity and long-term toxicity. No adverse reaction was reported on maximum administration dose (350 times of the adult's clinical dose) was identified for acute toxicity test and basic safety administration dose was identified at 16time of the adult's clinical dose in long-term toxicity study.

The formula has previously been used in clinical trials on patients with apoplexy syndrome such as ischaemic stroke and coronary artery diseases and no major side effect was reported. A recent ongoing clinical trial conducted in China on NXQ for ischaemic stroke has 74 participants completed to date identified 1 participant with decreased in platelet counts which may be affected by NXQ but no other adverse events were identified. The clinical data

from the past 10 years on NXQ for apoplexy syndrome has identified a total of 21 minor adverse events which may be related to the use of NXQ. The reported adverse events are nausea, abdominal bloating, abdominal pain and diarrhoea, which are alleviated after stop administration of the medication.

Participants may feel some discomfort during FMD ultrasound assessment (which involves blood pressure measurements). During pre-, mid-, post- testing and safety measurement sessions participants may also feel discomfort when blood samples are taken and may have minor bruising as a result.

Minimising Risk

To minimise the risk of adverse events from the use of NXQ, potential participants will undergo safety tests including full blood counts, liver function test, renal function test, blood rheology and coagulation index at baseline, 2 weeks, 6 weeks and week 12 (post-treatment) to monitor the safety index.

The risk to participants are minimal as all procedures will be carried out by an trained researcher and blood samples taken by trained venipuncturist at the register and reputable Douglas Hanly Moir Pathology Laboratory.

Participants are encouraged to inform the principle investigators as soon as any adverse event is noticed where the investigator will report to the trained physician/ neurologists and chief investigator to evaluate the severity of the adverse event in accordance to the guideline.

Blood samples may produce mild pain however all samples will be taken from participants by trained and qualified nurses and couriered to Sydney Adventist Hospital Pathology Laboratories (San Pathology) where pathology testing will be conducted.

All Adverse Events (AE) will be recorded and signed off by the principal research. Serious Adverse Events (SAE) will be immediately reported to local Human Research Ethics Committees in accordance with the guidelines.

Given the increasing burden of cerebro-vascular disease, particularly when viewed as a chronic disease, the importance of improving quality of life and reducing recurrences, the minor discomfort consenting participants may experience is justified given the potential utility of this intervention.

Appendix E. Experiment Protocol – Good Clinical Practice Module

Ethical Conduct of the Study

This study will be carried out according to the Declaration of Helsinki, the NHMRC National Statement on Ethical Conduct in Research Involving Humans (1999) and the Notes for Guidance on Good Clinical Practice as adopted by the Australian Therapeutic Goods Administration (2000) (CPMP/ICH/135/95) and the ICH GCP Guidelines.

The protocol and related documents will be submitted for review by the Human Research Ethics Committee (HREC) and written approval received before the study can commence.

The amount of blood to be sampled in the study is not considered to be excessive in healthy adult subjects.

Regulatory Compliance of the Study

The study will not commence until a Clinical Trial Authorisation (CTA) is obtained from the appropriate Regulatory Authority. The protocol and study conduct will comply with the Medicines for Human Use (Clinical Trials) Regulations 2004, and any relevant amendments.

Investigators Responsibilities

The Investigator is responsible for the overall conduct of the study at the site and compliance with the protocol and any protocol amendments. In accordance with the principles of GCP, the following areas listed in this section are also the responsibility of the Investigator. Responsibilities may be delegated to an appropriate member of study site staff. Delegated tasks must be documented on a Delegation Log and signed by all those named on the list.

Informed Consent

The Investigator will obtain written informed consent from each participant prior to participation in the study, in accordance with International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Good Clinical Practice (GCP) Guidelines, Declaration of Helsinki 2000, and any local regulatory requirements

The decision of a participant to participate in clinical research is voluntary and should be based on a clear understanding of what is involved.

Participants will receive adequate oral and written information; Participant Information and Informed Consent Forms will be provided. The oral explanation to the participant should be performed by the Investigator or designated person, and will cover all the elements specified in the Participant Information Sheet/Informed Consent.

The participant must be given sufficient time to consider the information provided. The participant may withdraw their consent to participate at any time without loss of benefits to which they otherwise would be entitled.

The participant should be informed and agree to their medical records being inspected by regulatory authorities but understand that their name will not be disclosed outside the hospital.

The Investigator or delegated member of the trial team and the participant will sign and date the Informed Consent Form(s) to confirm that consent has been obtained. The participant will receive a copy of this document and a copy filed in the Investigator Site File (ISF).

Subject Reimbursement

All enrolled participants will be reimbursed $100 for their travel/parking costs.

Notification of Primary Care Physician

With the consent of the volunteer, it is the Investigator's responsibility to notify the primary care physician of the subject's participation in the study, provided that such a physician can be identified for the subject and this notification is relevant for the particular circumstances of the trial. Where relevant, a letter will be sent to the physician stating the nature of the study, treatments, expected benefits or adverse events and concomitant drugs to be avoided. A copy shall be retained by the study site for verification by the Study Monitor.

Investigator Indemnification

The study is being conducted subject to the 'Guidelines for Compensation for Injury Resulting from Participation in a Company-sponsored Clinical Trial' published by the Medicines

Australia. University of Western Sydney will reimburse subjects for costs of medical care that occur as a result of complications directly related to participation in this study.

Study Site Staff

The Investigator must be familiar with the Investigational Product, protocol and the study requirements. It is the Investigator's responsibility to ensure that all staff assisting with the study is adequately informed about the investigational product, protocol and their trial related duties.

Data Recording

The Investigator is responsible for the quality of the data recorded in the CRF.

Confidentiality

All laboratory specimens, evaluation forms, reports, and other records must be identified in a manner designed to maintain participant confidentiality. All records must be kept in a secure storage area with limited access. Clinical information will not be released without the written permission of the participant, except as necessary for monitoring and auditing by the Sponsor, its designee, Regulatory Authorities, or the HREC. The Investigator and study site staff involved with this study may not disclose or use for any purpose other than performance of the study, any data, record, or other unpublished, confidential information disclosed to those individuals for the purpose of the study. Prior written agreement from the Sponsor or its designee must be obtained for the disclosure of any said confidential information to other parties.

Data Protection

All Investigators and study site staff involved with this study must comply with the requirements of the appropriate Data Protection or Privacy Act with regard to the collection, storage, processing and disclosure of personal information and will uphold the Act's core principles. Access to collated participant data will be restricted to those clinicians treating the participants.

Computers used to collate the data will have limited access measures via user names and passwords.

Published results will not contain any personal data that could allow identification of individual participants.

Appendix D. Experiment Protocol – Study Administrative Procedures

Protocol Amendments

Any changes in research activity, except those necessary to remove an apparent, immediate hazard to the participant, must be reviewed and approved by the Chief Investigator. Amendments to the protocol must be submitted in writing to the appropriate HREC, for approval prior to participants being enrolled into an amended protocol.

Protocol Violations and Deviations

The Investigator should not implement any deviation from the protocol without agreement from the Chief Investigator and appropriate HREC approval except where necessary to eliminate an immediate hazard to trial participants.

In the event that an Investigator needs to deviate from the protocol, the nature of and reasons for the deviation should be recorded in the CRF. If this necessitates a subsequent protocol amendment, this should be submitted to the HREC for review and approval if appropriate.

Study Record Retention

The Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) annotated with TGA comments requires study documentation to be retained for a minimum of 15 years. Participant files and other essential documents (study protocol, signed informed consent forms, IP dispensing logs, correspondence, CRFs, source documents and other documents pertaining to the conduct of the study) must be kept for the maximum period permitted by the research institute in accordance with these requirements.

Should the Investigator wish to assign the study documentation to another party or move to another location, the trial Coordinating Centre/ Study Sponsor should be notified.

End of Study

The end of study is defined as the last participant's last visit.

The Investigators and/or the trial steering committee have the right at any time to terminate the study for clinical or administrative reasons.

A summary report of the study will be provided to the HREC at the end of the study.

Reporting, Publications and Notification of Results

Authorship Policy

Ownership of the data arising from this study resides with the study team. On completion of the study, the study data will be analysed and tabulated, and a clinical study report will be prepared.

Publication

The clinical study report will be used for publication and presentation at scientific meetings. Investigators have the right to publish orally or in writing the results of the study.

Summaries of results will also be made available to Investigators for dissemination within their clinics (where appropriate and according to their discretion).