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

By

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

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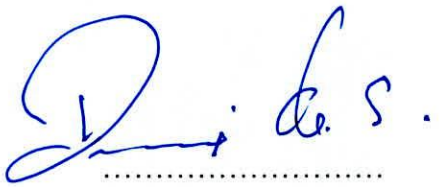

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LIST OF SYMBOLS / ABBREVIATIONS:

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

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

Bax: Bcl-2 associated protein X

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

BSA: Bovine serum albumin

CVD: cerebrovascular disease

Ca^{2+}: Calcium

CAT: catalase

CO: Carbon monoxide

DAB: 3, 3' diaminutesobenzidine tetrahydrochloride

DEPC: Diethyl pyrocarbonate

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

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

EDTA: Ethylenediaminetetraacetic acid

EGCG: epigallocatechin gallate

EGC: epigallocatechin

ECG: epicatechin gallate

EC: picatechin

EtBr: Ethidium bromide

FBS: Fetal bovine serum

GPx: glutathione peroxidase

GST: glutathione-S-transferase

GSH: Glutathione

H_2O_2: hydrogen peroxide

HCl: Hydrochloric acid

HBEC: human brain epithelial cells

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

HOCl: Hypochlorous acid

***K*:** Rate constant

LDH: Lactate dehydrogenase

MI: Myocardial infarction

MIC-101TM: Modular Incubator Chamber

mRNA: Messenger ribonucleic acid

NADH: Reduced nicotinamide adenine dinucleotide

NO•: Nitric oxide

NO_2-: Inorganic nitrite

NMDA: N-methyl-D-aspartate

NNDPD: N, N-dimethyl-p-phenylendiammonium

ODS: oxygen derived species

PLP: Pyridoxal 5'-phosphate

Pyrogallol red: pyrogallolsulphonephthalein

PBS: Phosphate-buffered Saline

PBS-Tx: Phosphate buffered saline-Triton X

PKG: Protein kinase G

PSA: Antibiotic-Antimycotic Solution

ROS: reactive oxygen species

RNA: ribonucleic acid

SAM: S-adenosyl-L-methionine

SEM: Standard error of the mean

SOD: superoxide dismutase

SNP: Sodium nitroprusside

TEAC: trolox equivalent antioxidant capacity

Tris: Tris(hydroxymethyl)-aminomethane

TCA: Trichloroacetic acid

UK: United Kingdom

UV: Ultraviolet

WHO: World Health Organization

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Most importantly, I hope that my project can offer more knowledge and information to the existing research for the scientific usage of Ceylon green tea and treatment of patients suffering from ischemic cerebral diseases like stroke. Although what I have done is definitely a minor contribution for the scientific research in the field of cerebral vascular diseases. I hope that in the future, I would like to continue research work in this field and hopefully discover more potential active ingredients from Ceylon green tea to prevent stroke.

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

Sherry Shan Hong Huang

ABSTRACT

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

measured. With flavonoids treated group showed significant lower level of total DNA base products damage (1.13±0.42nmol/100μg DNA) when compared to hypoxia group (1.53±0.36 nmol/100μg DNA). The flavonoids were also analyzed by LC/MS (Liquid Chromatograph/Mass Spectrometer) to separate the compounds and identify the main compounds which might play an important role of antioxidant effect.

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

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

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

The flavonoids treated group significantly increased in antioxidant enzyme activity

assays: the activity level of SOD (1.5±0.6μmol/min/mg protein), CAT (0.61±0.06μmol/min/mg protein), GPx (2.6±0.41μmol/min/mg protein) and GST (6.0±2.4μmol/min/mg protein) were significantly increased as compared with hypoxic control (0.5±0.52, 0.51±0.04, 1.2±0.35 and 3.1±1.6μmol/min/mg protein respectively). For the expression level of pro-apoptotic gene: Bax, Fas and Asp53, the result showed that the hypoxia cells after treatment, flavonoids treated group was significantly reduced the expression of the pro-apoptotic genes of Bax, Fas and Asp53. Meanwhile for the expression level of anti-apoptotic gene: Bcl-2, the result showed that the expression was stronger in flavonoids treated group when compared to hypoxia group. This would mean that the flavonoids from Ceylon green tea were able to reduce the amount of apoptosis after inducing hypoxia. Also, the expression levels of the pro-apoptotic genes were down-regulated and the expression of the anti-apoptotic gene was up-regulated, these would result in higher cell viability.

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

The least nuclear green fluorescence was observed in TUNEL staining assay, it indicated less apoptosis was found in flavonoids treated group as well.

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