Mechanistic Study on the Antiatherogenic Effect of *Fructus Crataegi* (Shan Zha)

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A thesis submitted in partial fulfillment of the requirements for the Degree of Master of Philosophy

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August 2005

CERTIFICATE OF ORIGINALITY

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written, nor material that has been accepted for the award of any other degree or diploma, except where due acknowledgement has been made in the text.

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ABSTRACT

Cardiovascular disease (CVD) is the major source of morbidity and mortality in the developed world. Among the CVDs, atherosclerosis is the most serious because it can lead to other severe diseases like stroke, heart attack and coronary heart disease. Oxidation of low density lipoprotein is a critical factor for atherosclerosis. Shan Zha, also known as hawthorn and *Crataegus*, has long been a folk medicine in China, Europe and North America. It acts on liver, a site believed to affect the cholesterol metabolism. Apart from this, many antioxidants are found in Shan Zha. As a result, Shan Zha is believed to have an antiatherogenic effect. This study aimed to find out the mechanisms involved in the antiatherogenic effect of Shan Zha (*Fructus crataegi*), and whether the cholesterol metabolism and antioxidant mechanisms are the only mechanisms involved in the antiatherogenic effect or other mechanisms are involved as well.

In the preliminary study of this project, the percentage vasorelaxation of rat isolated aortas from the normal group was significantly higher than that of the hypercholesterolemic group ($p<0.05$) at the acetylcholine concentration greater than 100 μM and Shan Zha group showed a tendency to reduce the atherogenic effect of

the hypercholesterolemic diet. However, there is no difference in the contraction response among the groups, indicating that vasoconstriction is independent of the antiatherogenic effect.

Antioxidant is believed to be antiatherogenic, and 80 % ethanol extract was found to be the most antioxidative. Thus, the 80 % ethanol extract was used for the mechanistic studies.

Consuming 80 % ethanol Shan Zha extract for twenty-eight days significantly prevented hypertension and the loss of vascular elasticity caused by the hypercholesterolemic diet on rats. These effects are in a concentration-dependent manner. The protective effect existed without the presence of hypocholesterolemic effect. The plasma total cholesterol and low density lipoprotein of rat consuming Shan Zha was as high as those which consumed the hypercholesterolemic diet. The protective effect is independent of the increase of the antioxidative enzyme activities, as there were no significant differences in the superoxide dismutase, catalase and glutathione peroxidase activities among the groups. Furthermore, the relaxation ability was significantly lower when the endothelial cell was removed in the normal group and the group with Shan Zha consumption. However, there was no significant

difference of those with or without the endothelial cell in the hypercholesterolemia group. This indicated that the protective effect was related to the endothelium cell.

In this project, the anti-atherogenic effect was measured basing on the enhancement of the relaxation ability. However, ingredients of Shan Zha may cause vessel relaxation directly. In order to further examine whether the ingredients of Shan Zha cause vessel relaxation directly, the 80 % ethanol Shan Zha extract, chlorogenic acid and quercetin, two of the flavonoids found in Shan Zha were added instead of acetylcholine to the isolated aorta. Chlorogenic acid significantly caused vessel relaxation at 10 μM. A reliable HPLC analysis method was also developed for quantifying the chlorogenic acid and quercetin.

PUBLICATIONS ARISING FROM THE THESIS

Kwok, C.Y., Chan, S.W., Yu, H.F., 2004. Effects of Shan Zha on the cardiovascular system. Hong Kong Food Science and Technology Association 7th Anniversary Commemorative Publication. 48–51.

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ACKNOWLEDGEMENTS

Through working on this research project, I have acquired valuable and enjoyable experience. Several heroes who gave me endless support and encouragement are unforgettable.

I would like to give my deepest gratitude to Dr. Peter Yu Hoi-fu, my Chief Supervisor and Dr. Chan Shun-wan, my Co-supervisor. They continuously gave me both technical and spiritual support. Their invaluable advice and patience throughout the course helped me learn so much on this challenging and interesting project.

Special thanks to the team members of Dr. Iris Benzie from the School of Nursing, for providing chemicals, equipment and technique for the FRAP assay.

Thanks are given to the staff of State Key Laboratory of Chinese Medicine and Molecular Pharmacology, Shenzhen, China, especially to Mr. Guo De-jian, Miss Yan Li-ping, Mr Zhong Xiao-qing and Miss Gong Hui-zhen, who helped materials ordering and equipment operations.

Many thanks to all the staff and technicians in the Department of Applied Biology and Chemical Technology for their kind assistance on taking care of my experimental animals and providing the chemicals and equipment for the completion of my laboratory work.

I would also like to give my heartfelt thanks to the research team members and student helpers of Dr. Peter Yu Hoi-fu and Dr. Chan Shun-wan, as they provided a friendly and warm research environment.

Last but not least, thanks are never enough to my parents and friends, Mr. Edwin Lee Wing-lim and Mr. Jonny Li Ki-tsan, who never ceased to support me and give me solace throughout the difficult time of my study.

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LIST OF ABBREVIATIONS

AAPH	2,2´-Azobis-(2-Amidinopropane) Dihydrochloride
Abs	Absorbance
AC	Adenylyl Cyclase
ACAT	Acyl-CoA-cholesterol Acyl Transferase
Acetyl coA	Acetyl Coenzyme A
Ach	Acetylcholine
AII	Angiotensin II
ANOVA	Analysis of Variance
Apo B	Apolipoprotein
ATP	Adenosine Triphosphate
CA	Chlorogenic Acid
$CaCl_2$	Calcium Chloride
CAT	Catalase
cGMP	Guanosine 3,5-cyclic Monophosphate
CH	Cholesterol 7α-Hydroxylase
CVD	Cardiovascular Disease
DMSO	Dimethyl Sulfoxide
DPPH	1,1-Diphenyl-2-picrylhydrazyl Free Radical
EDHF	Endothelium Derived Hyperpolarizing Factor
EDRF	Endothelial Dependent Relaxation Factors
ET-1	Endothelin-I
FRAP	Ferric Reducing / Antioxidant Power
g	Gram
GADPH	Glyceraldehyde 3-Phosphate Dehydrogenase
GPx	Glutathione Peroxidase
H	Hypercholesterolemia group
HDL	High-Density Lipoprotein
HMG-CoA-R	3-Hydroxy-3-methyl glutaryl Coenzyme A Reductase
HPLC	High Performance Liquid Chromatography
hr	Hour
IL-1	Interleukin-1
IP_3	Inositol Triphosphate
l	Liter
LDL	Low-Density Lipoprotein

LDLR	LDL receptor Gene
LOOH	Lipid Hydroperoxide
MCP-1	Monocyte Chemotactic Protein 1
min	Minute
ml	Milli-liter
MLC	Myosin Light Chains
MLCK	Myosin Light Chain Kinase
mM	Milli-Molar
n	Sample Number
N	Normal group
NE	Noradrenalin
NO	Nitric Oxide
NOS	Nitric Oxide Synthese
Ox LDL	Oxidized Low-Density Lipoprotein
p	Probability
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PIP_2	Phosphatidylinositol
PL-C	Phospholipase C
PUFA	Polyunsaturated Fatty Acid
Q	Quercetin
ROS	Reactive Oxygen Species
rpm	Revolution per minute
RSD	Relative Standard Deviation
RT	Reverse Transcriptase
SD	Sprague Dawley
SEM	Standard Errors of Means
SOD	Superoxide Dismutase
SR	Sarcoplasmic Reticulum
SR %	Free Radical Scavenging Capacity
SZ+H	Shan Zha Powder Group
SA	Shan Zha 80% ethanol extract 30 mg/kg group
SB	Shan Zha 80% ethanol extract 100 mg/kg group
TC	Total Cholesterol
TNF-α	Tumor Necrosis Factor α
TPTZ	Fe^{3+}–tripyridyltriazine
v/v	Volume per Volume
VLDL	Very-Low-Density Lipoprotein

w/v	Weight per Volume
WHO	World Health Organization
µl	Micro-liter
^{o}C	Degree Celsius
%	Percent / Percentage
O_2^-	Superoxide
H_2O_2	Hydrogen Peroxide
OH・	Hydroxyl Radicals
ROO・	Peroxide
$^1O_2$	Singlet Oxygen

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1: INTRODUCTION

Maintaining a healthy cardiovascular system is important. Without a proper functioning cardiovascular system, oxygen and nutrients cannot be delivered to various parts of the body and the waste generated cannot be removed and transported out. Unfortunately, nowadays Cardiovascular Diseases (CVDs) have become one of the major killers across the world. In 2001, CVDs contributed to nearly one-third of the global deaths. Around 17 million people suffer from CVDs each year (World Health Organization (WHO) World Health Report, 2003) and this figure is increasing. The WHO estimated that by 2020 there will be nearly 25 million CVD deaths worldwide. Therefore, preventive methods for CVDs should be found out as soon as possible.

Among the CVDs, atherosclerosis, the hardening of arteries due to the presence of plaque, is the most serious because it can lead to other severe diseases such as stroke, heart attack and coronary heart disease. Atherosclerosis is the major source of morbidity and mortality in the developed world and it has claimed more lives than all types of cancer combined (Stocker and Keaney, 2004).

High plasma cholesterol level and oxidation of Low-Density Lipoprotein (LDL) by free radicals are two of the risk factors causing atherosclerosis. Substances affecting cholesterol metabolism and antioxidants are believed to have preventive effects on atherosclerosis. Chinese herbal plants such as Shan Zha, which traditionally improve digestion, increase appetite and treat dyspnea, may help prevent atherosclerosis, as it acts on stomach and liver (Zhu, 1998). It may affect the cholesterol metabolism, and plenty of antioxidants can be found in it (Zhu, 1998). However, the kind of ingredients in Shan Zha involved in the cholesterol metabolism, the kind of antioxidants involved and the mechanism for the formation of oxidized LDL are unknown. Apart from these, if Shan Zha is antiatherogenic, are there any other mechanisms involved? If the answers of the above questions can be found out, more effective methods can be developed to prevent or even cure atherosclerosis.

Western scientists and Chinese medicinal practitioners usually have different points of view in explaining the mechanism of a certain substance, which prevents / cures certain kind of diseases. In this project, the western approaches have been used to explain the function of a Chinese medicinal herb, Shan Zha, for its beneficial use in the prevention or treatment of atherosclerosis.

1.1: LITERATURE REVIEW

1.1.1: What is atherosclerosis?

Atherosclerosis is a kind of CVD. In atherosclerosis, plaque builds up on the inner walls of arteries, and causes the narrowing of the lumen, which affects the transport of nutrients in the body. The word “Atherosclerosis” originates from Greek and it can be divided into two components (American Heart Association, 2004; Blankenhorn and Kramsch, 1989):

Athero: paste-morphologic wall thickening due to the formation of plaque

Sclerosis: hardness-functional stiffening

A picture of an artery suffering from atherosclerosis is shown in Fig. 1.

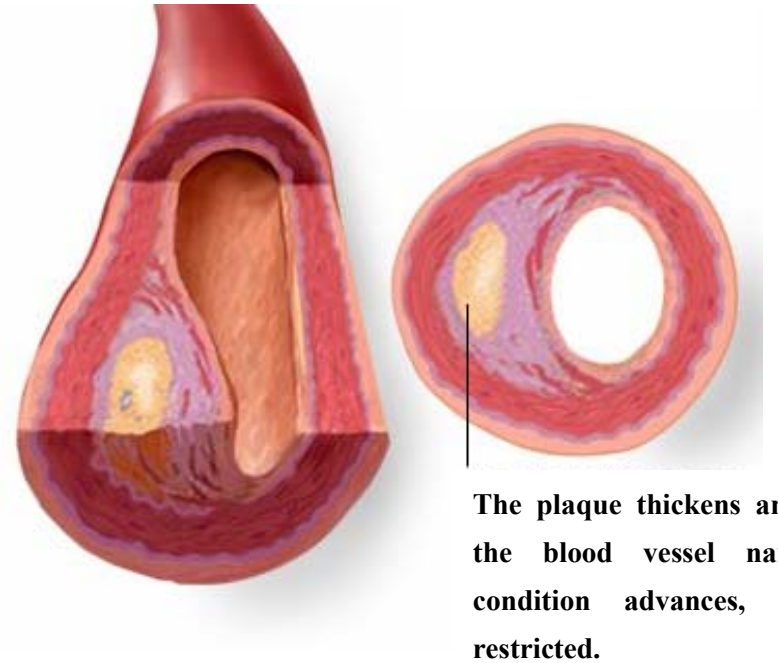


Fig. 1: Section of artery suffering from atherosclerosis (U.S. Food and Drug Administration, 2004).

1.1.2: Serious effects of atherosclerosis

Atherosclerosis is the target disease to be studied, because it is the most dangerous CVD, which can lead to other serious diseases like stroke, heart attack and gangrene of body parts. Preventing people from suffering atherosclerosis is also preventing them from suffering other kinds of diseases. The formation of stroke, heart attack and gangrene of body parts depends on the blood vessel that is blocked. Blocking a blood vessel that feeds the brain will cause stroke. Blocking a blood vessel that feeds the heart will cause heart attack. If the blood supply to legs is reduced, walking will become difficult and gangrene will be developed eventually.

1.1.3: Development of atherosclerosis

In order to find out the ways to prevent atherosclerosis, the mechanism of atherosclerosis should be understood. 4 phases are involved in the process of atherosclerosis (Betteridge *et al*., 1999). They are:

1. Endothelial dysfunction
2. Formation of fatty-streak
3. Formation of advanced and complicated lesion
4. Formation of unstable fibrous plaques

Endothelial dysfunction

In the branches or at the turning points of blood vessels, the pressure and the distraction of blood are greater than those of the straight-flow vessels. Macrophage and T-cells grow easily because of the presence of the specific molecules found on the endothelium that are responsible for the adherence, migration and accumulation of monocytes and T-cells. Upon stimulation, the endothelial permeability to lipoprotein and other plasma constituents increases. Lipoproteins accumulate in the intima will be oxidized. The Oxidized Low-Density Lipoprotein (Ox-LDL) activates

endothelial cells to express Monocyte Chemotactic Protein 1 (MCP-1), which attracts monocytes from the vessel lumen to go into the subendothelial space (Navab *et al*., 1991). The Ox-LDL also plays an important role in promoting the differentiation of monocytes (a kind of leukocyte) into macrophages (Steinberg *et al*., 1989). After Ox-LDL promotes the differentiation of monocytes into macrophages, the macrophages release a variety of chemicals, including cytokines. Among these cytokines, Tumor Necrosis Factor α (TNF-α) and Interleukin-1 (IL-1) activate endothelial cells to express adhesion molecules that bind monocytes (Fig. 2). Up-regulation of leukocyte adhesion molecules also occurs, making them available for recruitment into the subendothelial space by MCP-1 (Nathan, 1987). These events affect the function of releasing Nitric Oxide (NO), a blood vessel relaxing factor, in endothelial cells.

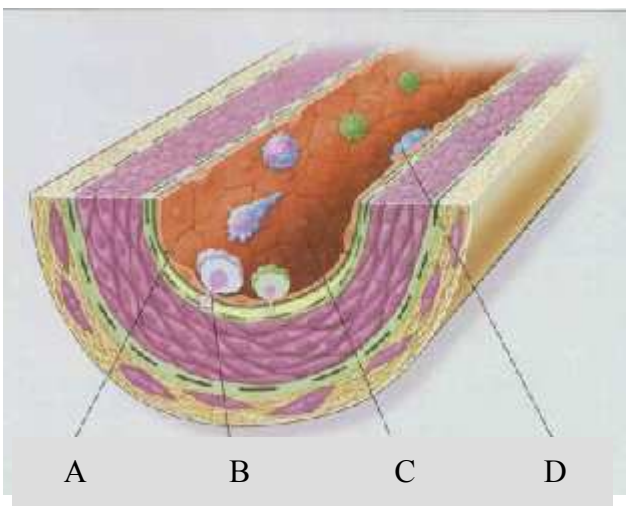


Fig. 2: Endothelial dysfunction in atherosclerosis: Endothelial permeability (A), Leukocyte migration (B), Endothelial adhesion (C) and Leukocyte Adhesion (D). (Ross, 1999).

Formation of fatty-streak

After oxidation of LDL, macrophage forms and engulfs the Ox-LDL at the intima. As feedback system is not applied in the engulfment of oxidized LDL, macrophage will engulf more and more Ox-LDL. After engulfing a large amount of Ox-LDL, the macrophage enlarges and the enlarged macrophage is called “Foam cell” (Steinberg *et al*., 1989). Foam cell, T cell and smooth muscle cells grow towards the lumen, and fatty-streak is formed. This non-smooth lumen makes the blood flow more difficult, causing the blood cells and platelets to accumulate more easily. Monocyte and macrophage keep on getting into the intima. To make the matter worse, under the stimulation of foam cell, smooth muscle cells proliferate through internal elastic lamina to endothelial cell. The artery becomes narrow.

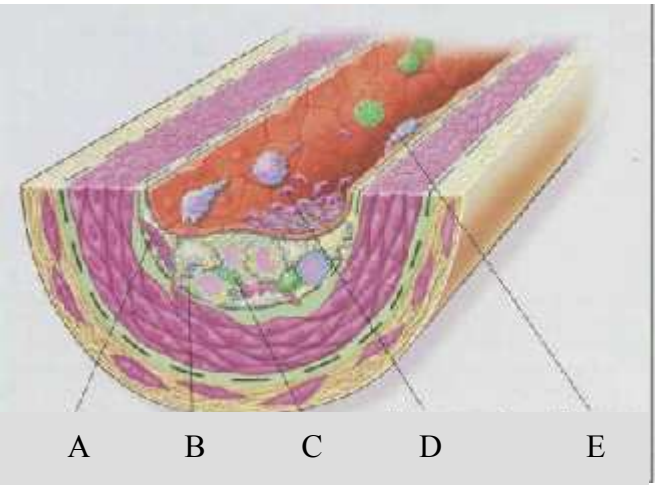


Fig. 3: Hypercholesterolemiaty-streak formation in atherosclerosis: Smooth muscle migration (A), Foam cell formation (B), T cell activation (C), Adherence and aggregation of platelets (D) and Adherence and entry of leukocytes (E) (Ross, 1999).

Formation of advanced and complicated lesion

Those macrophages, which engulfed the oxidized LDL, die in the intima and apoptosis occurs under the influence of interferon. These materials and the lipoprotein fragment form a necrotic core. At the same time, a fibrous cap, which is composed of out-growing smooth muscle cells and collagen, is formed. The fibrous cap is used for covering the necrotic core and separating it from the lumen (Fig. 4).

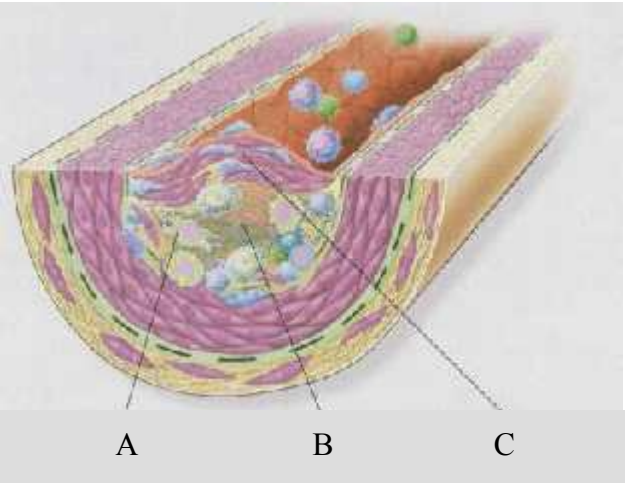


Fig. 4: Formation of an advanced, complicated lesion of atherosclerosis: Macrophage accumulation (A) Necrotic core formation (B) and Fibrous cap formation (C) (Ross, 1999).

Formation of unstable fibrous plaques

Foam cells, smooth muscle cells, LDL-cholesterol, other lipoprotein, influxed calcium and other substances in blood mix together to form plaque (U.S. Food and Drug Administration, 2004). The lumen becomes narrower because of the accumulation of plaques. If this situation continues, macrophage will release enzyme to denature certain kinds of protein. This makes the fibrous cap thinner, and loses its function of separation of the plaque. The plaque will then leak and thrombus will form (Fig. 5). The thrombus flows via the circulation system to all parts of the body and causes serious damage. If the thrombus forms in the coronary artery, coronary heart disease develops; if the thrombus flows to the brain, stroke occurs.

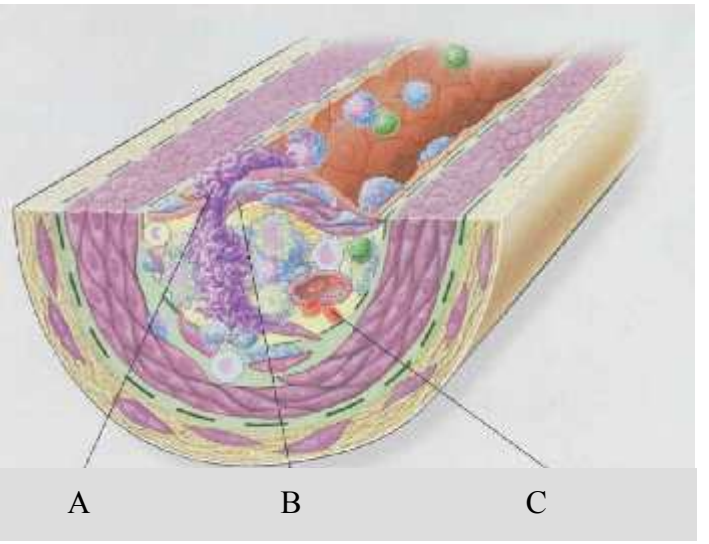


Fig. 5: Unstable fibrous plaques in atherosclerosis: Plaque rupture (A), Thinning of fibrous cap (B) and Hemorrhage from plaque microvessels (C) (Ross, 1999).

1.1.4: Vascular elasticity

In atherosclerosis, “sclerosis” means the hardening of blood vessel. The inability to relax and contract at the same time leads to sclerosis.

1.1.4.1: Relaxation

There are two types of vasodilatations, namely endothelial dependent relaxation and endothelial independent relaxation.

Endothelial dependent relaxation

The Endothelial Dependent Relaxation Factors (EDRF) include prostachlin, Endothelium Derived Hyperpolarizing Factor (EDHF) and NO (Mitani and Kimura, 2003). Among them, NO is the most studied one. The regulation and effect of NO is summarized in Fig. 6. Briefly, upon stimulation by various agonists e.g. acetylcholine (Moyna and Thompson, 2004), the concentration of intracellular Ca^{2+} increases, which causes the opening of the Ca^{2+}-dependent K^+ channel and increase of K^+ efflux. The increase of K^+ efflux causes hyperpolarization of the endothelial cells, providing a driving force for transmembrane Ca^{2+} influx into the cells, which in turn triggers the NO production from L-arginine with the presence of nitric oxide synthase (NOS). The released NO stimulates soluble guanylate cyclase in smooth muscle cells, leading to an increase in guanosine 3',5'-cyclic monophosphate (cGMP) and finally to vasodilation (Moyna and Thompson, 2004; Jen *et al*., 2002).

Endothelial independent relaxation

The endothelial independent relaxation stimulates the smooth muscle directly, without the involvement of endothelial cells. The detailed mechanism of the

endothelium-dependent and endothelium-independent vasorelaxation is shown in Fig. 6.

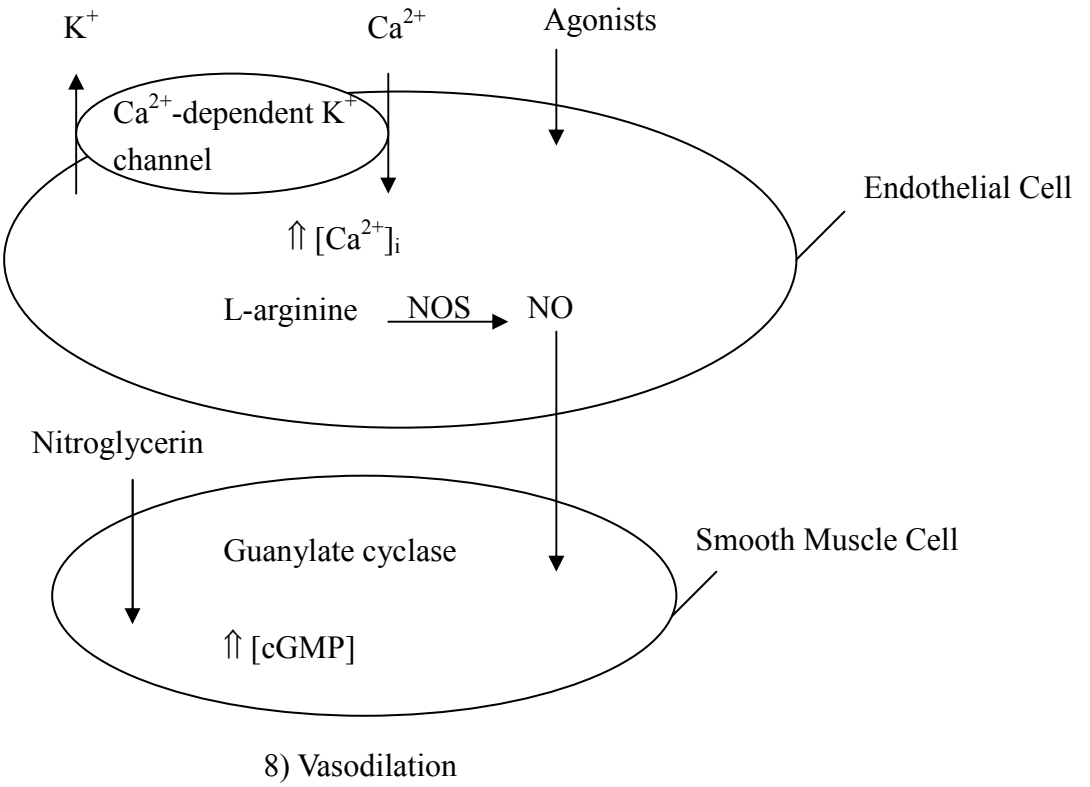


Fig. 6: Detailed mechanism of endothelium-dependent and endothelium-independent vasorelaxation.

1.1.4.2: Contraction

An increase in free intracellular calcium, whether the increased flux of calcium into the cell is through calcium channels or by the release of calcium from internal stores (e.g., sarcoplasmic reticulum; SR), causes vasoconstriction. The free calcium binds to a special calcium binding protein called calmodulin. Calcium-calmodulin

activates Myosin Light Chain Kinase (MLCK), an enzyme that is capable of phosphorylating Myosin Light Chains (MLC) in the presence of Adenosine Triphosphate (ATP). MLC phosphorylation leads to cross-bridge formation between the myosin heads and the actin filaments, and hence, the smooth muscle contracts (Klabunde, 2004). The mechanism of vasoconstriction is shown in Fig. 7.

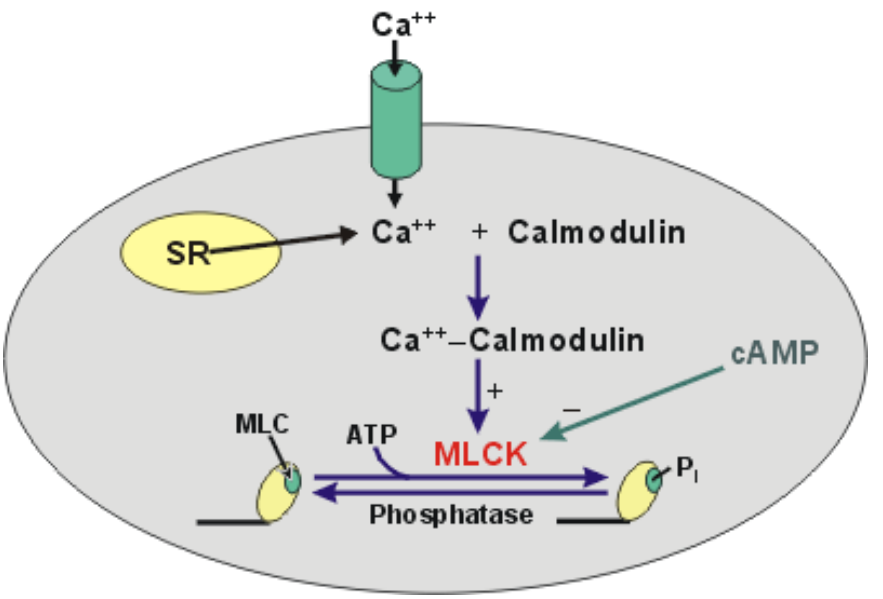


Fig. 7: Mechanism of vasoconstriction (Klabunde, 2004).

Three different mechanisms of vasoconstriction will be described here: 1) phosphatidylinositol pathway, 2) G-protein-coupled pathway, and 3) nitric oxide-cGMP pathway.

Phosphatidylinositol pathway

Noradrenalin (NE) acting via alpha1-adrenoceptors, Angiotensin II (AII) acting via AII receptors, and Endothelin-I (ET-1) acting through ETA receptors activate Phospholipase C (PL-C) causing the formation of Inositol Triphosphate (IP_3) from Phosphatidylinositol (PIP_2). The IP_3 then stimulates the Sarcoplasmic Reticulum (SR) to release calcium (Fig. 8).

G-protein-coupled pathway

The G-protein coupled pathway either stimulates (via Gs protein) or inhibits (via Gi protein) Adenylyl Cyclase (AC) that catalyzes the formation of cAMP. The mechanism for contraction is the cAMP inhibition of MLCK. This decreases MLC phosphorylation, thereby decreasing the interactions between actin and myosin. Drugs which increase cAMP cause vasodilation (Fig 8).

Nitric oxide-cGMP pathway

Briefly, increase in NO activates guanylyl cyclase, causing an increased

formation of cGMP and vasodilation. cGMP activates a cGMP-dependent protein kinase, and inhibits calcium entry into the smooth muscle. It activates K^+ channels and decreases IP_3 level (Fig. 8).

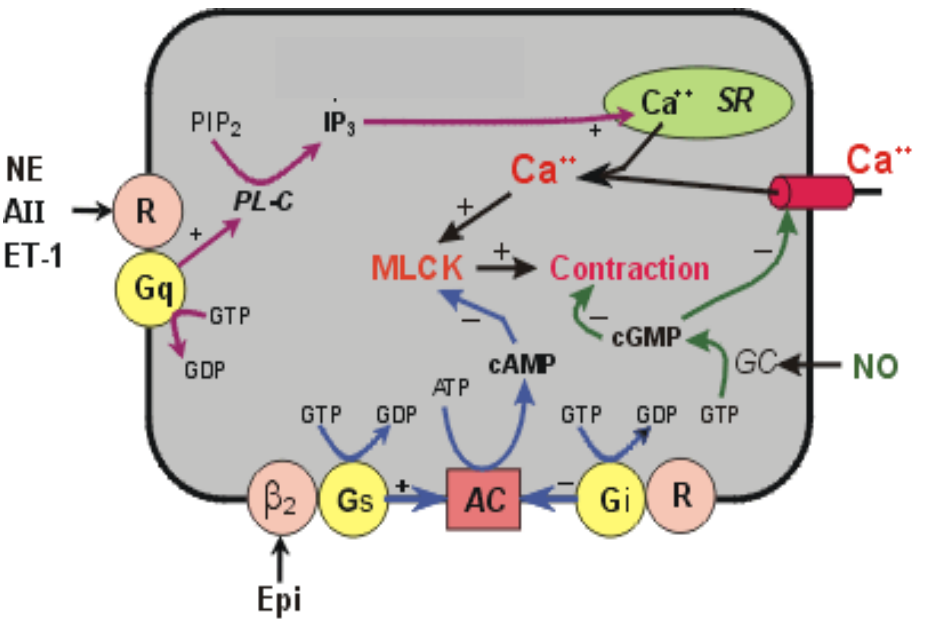


Fig. 8 : Mechanism of increasing calcium ion concentration in vasoconstriction (Klabunde, 2004).

1.1.5: Life-style related risk factors for atherosclerosis

According to the deduced mechanism of atherosclerosis, it is known that the initiation of atherosclerosis is due to the oxidation of LDL. Cholesterol is a major component of LDL and free radical starts the lipid peroxidation of LDL, so cholesterol and free radical are two main risk factors for atherosclerosis, and these two factors are life-style related.

Fast food usually has high cholesterol content. If too much fast food is consumed, it will cause hypercholesterolemia. There are plenty of data, both on human and animal, to support that hypercholesterolemic diet causes atherosclerosis (Beitz and Mest, 1991; Nistor *et al*., 1987).

Cigarette smoking is found to be associated with endothelial dysfunction (Sabha *et al*., 2002; Drexler and Hornig, 1999). There are a lot of free radicals in a cigarette, which cause oxidative stress. LDL will be oxidized which finally leads to atherosclerosis (Joshua *et al*., 2004).

1.1.5.1: Cholesterol metabolism

Since cholesterol is the major component of LDL, the metabolism of cholesterol is related to the amount of LDL present in the circulation, which can be oxidized to cause the formation of atherosclerosis.

Cholesterol can be synthesized in liver by two ways as shown in Fig. 9. Firstly it can be synthesized from Acetyl Coenzyme A (acetyl CoA), which is transformed to β-Hydroxy-β-methylglutaryl-CoA, and together with hepatic

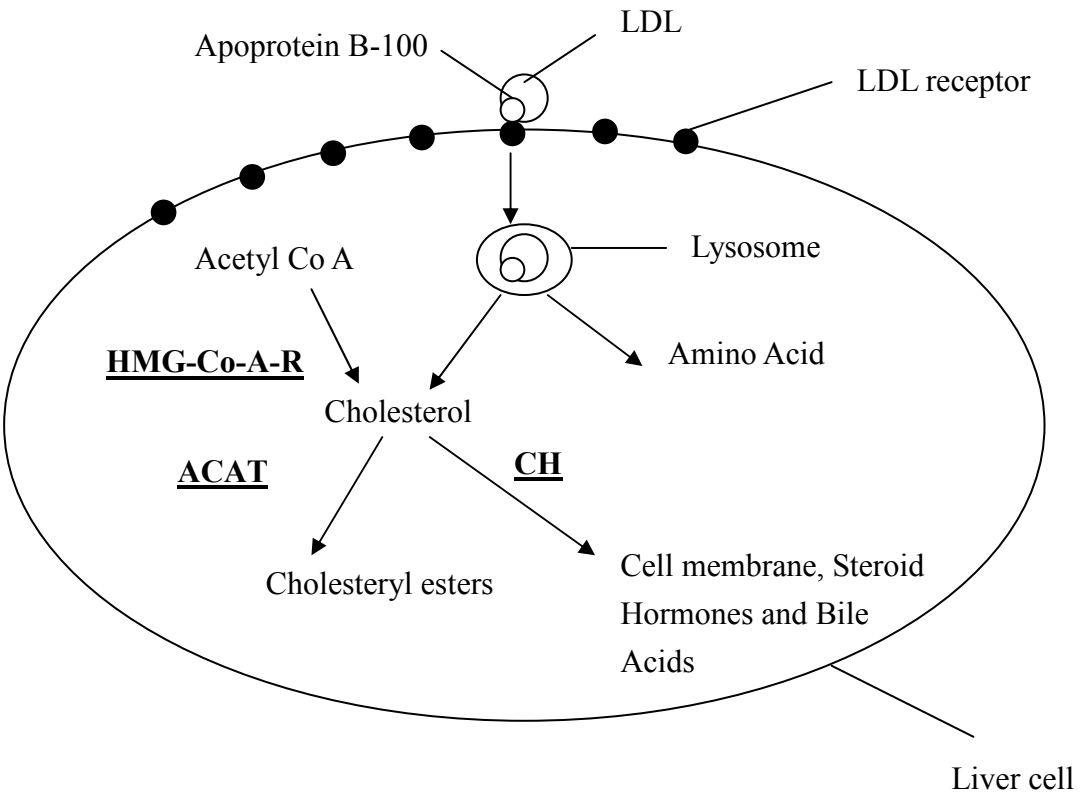
3-Hydroxy-3-Methyl Glutaryl Coenzyme A Reductase (HMG-CoA-R) to form mevalonate, which will soon be transformed to cholesterol in the presence of lanosterol 14α-demethylase. The other way is cholesterol re-collected from LDL. Cholesterol in LDL can be separated to free cholesterol by redirecting the LDL to liver with the recognition of the apoprotein B-100 on the LDL by the LDL receptor on the liver cell. The LDL is then degraded by lysosome and cholesterol is regenerated.

The cholesterol produced is consumed in two ways as shown in Fig. 9. Through the action of Cholesterol 7α-Hydroxylase (CH), cholesterol is converted to bile acid, and other products like cell membrane and steroid hormone. Excess cholesterol will be transformed to cholesterol ester with the help of Acyl-CoA-Cholesterol Acyl Transferase (ACAT) for absorption and storage.

The free cholesterol and cholesterol ester will be packed together with other substances like phospholipids, triacylglycerols and proteins into mainly LDL (Krichevsky, 1986). The LDL will then go via the blood stream and be received by tissues with the corresponding receptor, like the artery. The more the cholesterol, the more the LDL will be formed. There will be a higher chance of oxidation, thus

causing endothelial dysfunction and finally atherosclerosis.

In order to regulate the cholesterol metabolism and reduce the LDL content, 4 possible methods can be applied. They involve a decrease in cholesterol synthesis, the activation of the LDL receptors, the inhibition of cholesterol absorption and the conversion of cholesterol to bile acids.



HMG-CoA-R: 3-Hydroxy-3-methyl glutaryl coenzyme A reductase
CH: Cholesterol 7α-hydroxylase
ACAT: Acyl CoA:cholesterol acyltransferase

Fig. 9: Cholesterol metabolism.

1.1.5.2: Free radical

A free radical is defined as a molecule that contains an unpaired electron (Olinescu and Smith, 2002). The most harmful effect of free radicals in atherosclerosis is the lipid-peroxidation of LDL. Attack of the reactive radicals on lipoproteins starts lipid peroxidation (Halliwell and Gutteridge, 1999), in which a free radical attacks the Polyunsaturated Fatty Acid (PUFA) carried by LDL. Initiation of lipid peroxidation is caused by the attack of any species that has sufficient reactivity to remove a hydrogen atom from a PUFA (Halliwell and Gutteridge, 1999). Since hydrogen atom in principle is a free radical with a single unpaired electron, its removal leaves behind an unpaired electron on the carbon atom to which it was originally attached. The carbon-centered radical is stabilized by a molecular rearrangement to form diene conjugates, followed by the reaction with oxygen to give a peroxyl radical. Peroxyl radicals are capable of abstracting a hydrogen atom from another adjacent fatty acid side-chain to form a Lipid Hydroperoxide (LOOH). They can also combine with each other or attack membrane proteins. When a peroxyl radical abstracts a hydrogen atom from the fatty acid, the new carbon-centered radical can react with oxygen to form another peroxyl radical, and so the propagation of the chain reaction of lipid peroxidation continues

(Halliwell and Gutteridge, 1999). The peroxidation of membrane lipid may lead to the enhancement of membrane permeability (Elliot and Koliwad, 1995) and the lipid peroxidation products may lead to endothelial injury (Cohrane, 1991). These two effects contribute to endothelial dysfunction. Linoleic acid is a kind of PUFA, which can illustrate the lipid peroxidation (Fig. 10).

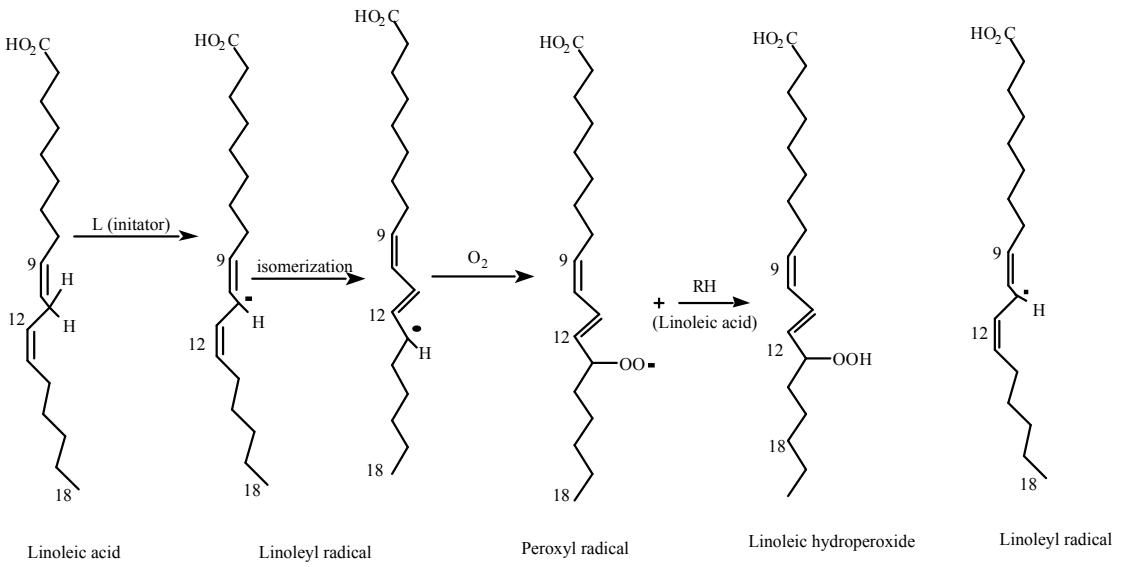


Fig. 10: Mechanism of linoleic acid peroxidation (Vaya and Aviram, 2003).

Types of free radical

There are many kinds of free radicals. Reactive Oxygen Species (ROS), which deviate on the basis of oxygen, are involved more in the biological system. ROS includes superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH・),

peroxide (ROO・) and singlet oxygen ($^1O_2$), etc (Parham, 2001). ROS oxidize lipid substances, leading to the formation of oxidized LDL (Steinberg, 1997).

Natural Processes as Sources of Free Radicals

Formation of free radicals cannot be avoided. Even if we do not smoke, free radicals are still generated in our body. In mitochondrial respiration, superoxide and hydrogen peroxide are produced in the electron transport chain (Cimino, 1989; Leng *et al*., 1994). Oxidative degradation of the acyl chain of xenobiotics, dicarboxilic and monounsaturated acid by peroxisomes produces hydrogen peroxide (Olinescu and Smith, 2002). In phagocytosis, superoxide and hydrogen peroxide are released outside the cell and kill the pathogen by lipid peroxidation and thus deteriorate its membrane (Olinescu and Smith, 2002). In the peroxidation of PUFA, peroxi-radical and other radicals are produced (Vaya and Aviram, 2003).

Antioxidant

In order to fight against the free radicals, there is an antioxidation system in our body. Antioxidant is defined as “any substance when presents at low concentration

compared to those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate" (Halliwell and Gutteridge, 1995). Antioxidant can scavenge free radicals or prevent their formation. It reacts with free radicals and forms non harmful products (Olinescu and Smith, 2002). Therefore, it is used to fight against oxidative stress. Antioxidant can be divided into non-enzymatic antioxidant and enzymatic antioxidant.

- *Non-enzymatic antioxidants*

Antioxidants can donate or accept an electron so as to make the whole system stable and quench the free radical polymerization. Through other process the antioxidative substance can be regenerated. For instance, vitamin E (α-tocopherol) accepts one electron from a free radical, like singlet oxygen and superoxide, and produces a vitamin E free radical. However, this vitamin E free radical is stable, harmless and readily reacts with other antioxidants such as ascorbic acid and glutathione to regenerate vitamin E (Olinescu and Smith, 2002). While vitamin C (ascorbic acid) scavenges free radicals by producing dehydroascorbate when the ascorbate/dehydroascorbate ratio is high, until the unstable dehydro-ascorbate undergoes hydrolysis, it can be reduced back to ascorbic acid. Dehydroascorbate

will do no harm (Cathcart, 1985).

- *Enzymatic antioxidants*

Some enzymes act as antioxidants and react with free radicals. For example, Superoxide Dismutase (SOD) E.C.1.15.1.1 transforms superoxide to hydrogen peroxide. Catalase (CAT) E.C.1.11.1.6 transforms hydrogen peroxide to water and oxygen. Glutathione Peroxidase (GPx) E.C.1.11.1.9 transforms hydrogen peroxide and glutathione to water and oxidized glutathione (Fig. 11).

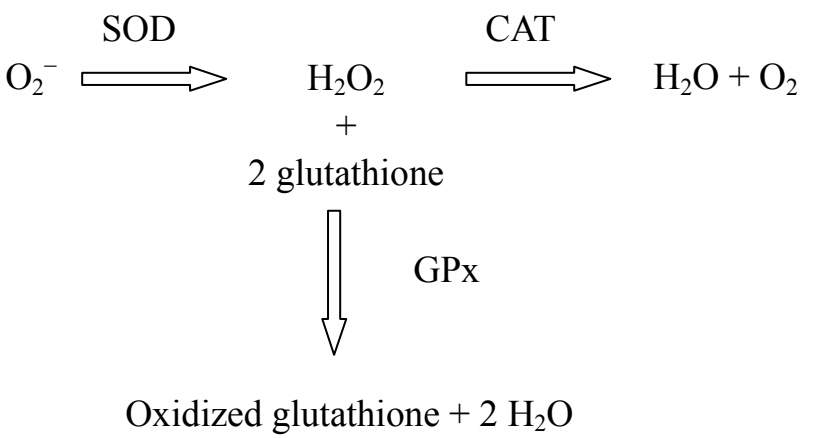


Fig. 11: Reactions of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) with free radicals.

The two risk factors of atherosclerosis are related to each other. After the excess cholesterol is packed into the LDL, oxidation of LDL occurs. There are two possible ways to prevent atherosclerosis, by lowering the cholesterol level or preventing the LDL from being oxidized. Shan Zha, a herb which acts on stomach and liver (Zhu, 1998), may be involved in the cholesterol metabolism. Apart from this, there are plenty of antioxidants, like quercetin, vitexin, hyperin, hyperoside, cyanin, quercitrin and catechin, found in Shan Zha (Zhu, 1998), which scavenge free radicals. Therefore, Shan Zha may help prevent atherosclerosis in both ways.

1.1.6: Shan Zha

1.1.6.1: Classification

Fig. 12: Photo of Shan Zha.

Crataegus is a fruit-bearing plant commonly found in China, Europe and North America. Shan Zha (Fig. 12) is the fruit of this plant. The genus *Crataegus* is classified in the tribe Crataegeae, which belongs to the subfamily Maloideae of the Rosaceae family (Sticher and Meier, 1998; Fineschi *et al*., 2005). More than 150 species of it have been found (Sticher and Meier, 1998). Since there are more than 150 species of *Crataegus*, the subspecies and hybridization among them are numerous. As a result, there are various morphologies of Shan Zha. Generally, most *Crataegus* species are medium-sized shrubs or small-sized trees with hard wood and thorny branches. They bear white flowers in May and produce red fruits in late summer (Sticher and Meier, 1998).

The name *Crataegus* originates from the Greek "krataios", meaning "strong", which refers to the hardness of this plant or "kratos", meaning "always having been there" (Upton, 1999). *Crataegus pinnatifida*, *Crataegus cuneata*, *Crataegus monogyna* and *Crataegus laevigata* are the most common species used for medicinal and research purposes. *Crataegus pinnatifida* and *Crataegus cuneata* are the two major species commonly found in China, while *Crataegus monogyna* and *Crataegus laevigata* are the main species found in Europe. The local names of *Crataegus* in different countries are listed in Table 1 (Upton, 1999; Zhu, 1998).

Table 1: Common names of *Crataegus* in different countries

Country	Common Names of Crataegus
China	Shan Zha
France	Aubépine, épine balanche, épine de mai
Germany	Weissdorn, hagedorn, mehlbeerbaum
Holland	Meidorn, haagdorn
Italy	Bianco spino
United Kingdom	Harthorne, hawthorn, hedgethorn, maythorn, whitethorn
United States	Hawthorn

1.1.6.2: Components

The kinds and quantity of the chemical components in Shan Zha may vary due to differences in the species investigated (Sticher and Meier, 1998), the part of the Shan Zha involved (Sticher and Meier, 1998; Upton, 1999; Quettier-Deleu *et al*., 2003), the harvesting time (Upton, 1999) and the environmental conditions where the Shan Zha is grown (Kirakosyan *et al*., 2003; Kirakosyan *et al*., 2004). Flavonoids, procyanidins, catechins, triterpene, as well as various ubiquitous compounds such as organic acids, essential oil, sugars, vitamins, and minerals are generally found in Shan Zha (Sticher and Meier, 1998; Rigelsky and Sweet, 2002; Zhang *et al*., 2002a). Among these components, antioxidants from Shan Zha are of major interests.

1.1.6.3: Antioxidants

Among the components of Shan Zha, antioxidants like flavonoids and procyanidins are of major interests. Common flavonoids found in Shan Zha are hyperoside, quercetin, quercitrin, rutin, vitexin, vitexin-2”-O-rhamnoside and acetylvitexin-2”-O-rhamnoside (Rigelsky and Sweet, 2002; Bahorun *et al*., 2002; Fong and Bauman, 2002). Flavonol-O-glycosides in the form of hyperoside, spiraeroside and rutin (Sticher and Meier, 1998; Fong and Bauman, 2002) and flavone-C-glycosides in the form of vitexin, vitexin-2”-O-rhamnoside, acetylvitexin-2”-O-rhamnoside, orientin and isoorientin (Sticher and Meier, 1998; Rigelsky and Sweet, 2002) are also found. Procyanidins which exist in Shan Zha are primarily composed of (–)-epicatechin unit, while (+)-catechin is reported in a lesser extent (Upton, 1999). The procyanidins can be divided into different forms according to the linkage of the interflavonoid bonds C4-C8 or C4-C6 and the level of polymerization (Sticher and Meier, 1998; Upton, 1999).

1.1.6.4: Pharmacological effects

Traditional use

Shan Zha has a long history as herbal medicine. The use of Shan Zha fruit to improve digestion, increase appetite and treat dyspnea can be traced back to the Song Dynasty (Year 960–1279) in China (Liu and Cui, 1986).

Modern use

Nowadays, apart from the fruit, the leaves and flowers of Shan Zha are also used as a supplement for treating a variety of diseases such as heart failure (Schwinger *et al*., 2000; Taubert, 2002), arrhythmia (Makdessi *et al*., 1999), hypertension (Voneiff *et al*., 1994; Mills and Bone, 2000) and hypercholesterolemia (Chen *et al*. 1995). An anti-inflammation property was also reported (Shahat *et al*., 1996). Most of the medical functions are related to CVDs and atherosclerosis, such as the hypocholesterolemic / hypolipidemic effect, the antioxidative effect and the effect of endothelial-dependent vasorelaxation.

- *Hypocholesterolemic / hypolipidemic effect*

Hypercholesterolemia is classified as one of the risk factors for causing atherosclerosis, especially when the LDL-cholesterol level is high (McGill, 1981). Therefore, preventing hypercholesterolemia is a way to reduce the risk of CVDs like atherosclerosis. Shan Zha is shown to have a hypocholesterolemic effect. The evidence and mechanisms involved are discussed below.

Chen and co-workers (1995) reported that consumption of a drink containing Shan Zha had a hypocholesterolemic effect on rats. Thirty-seven male Sprague Dawley (SD) rats weighing between 80–90 g were divided into three groups. In Group 1, rats drank a low-sugar, low-sodium, Shan Zha-containing product with vitamins C, B1, and B2, as well as iron and zinc added. Every 100 ml of the fluid contained 560 μg of Shan Zha flavones. In Group 2, rats drank 8 % sugar water and in Group 3, rats drank tap water. The amount of fluid drunk and diet ingested were recorded. After 2.5 months, the body weight, body fat, serum cholesterol and LDL-cholesterol level of the Group 1 rats were found to be significantly lower than that of the Group 2 and Group 3 rats. The serum triglyceride level of the Group 1 rats was significantly lower than that of the Group 2 rats, while the High-Density

Lipoprotein (HDL)-cholesterol level of the Group 1 rats was significantly higher than that of the Group 2 rats. Although a hypocholesterolemic effect was observed, it may not be an accurate comparison, as the food intake by rats in Group 3 was significantly higher than that of Groups 1 and 2, while the amount of liquid consumed by Group 2 was significantly higher than that of Groups 1 and 3. Furthermore, the drink contained other constituents, which may also cause this hypocholesterolemic effect.

Apart from the animal test, this Shan Zha-containing drink was also applied to humans to determine the effects of Shan Zha on human blood lipid levels (Chen *et al*., 1995). Thirty hyperlipidemia patients aged 43–59 participated in the trial. During the one month experimental period, the patients received 250 ml of the Shan Zha drink twice daily (1.4 mg of Shan Zha flavones per dose). Their usual daily activities and diet were maintained, with medications discontinued. Venous blood samples with fasting were collected at the beginning and end of the treatment period. Significant reductions were found in total cholesterol, serum triglycerides, LDL-cholesterol, Apolipoprotein B (Apo-B), and lipid peroxidate malonic dialdehyde in the Shan Zha treatment group. No significant change was observed in Apo-A or HDL-cholesterol. Although a positive effect was obtained in this

experiment, further studies are required to determine the role of Shan Zha in the management of hyperlipidemia.

A study performed by Zhang and co-workers (2002b) also demonstrated the hypolipidemic effect of Shan Zha (*Crataegus pinnatifida*) and showed that the mechanism involved was due to the decreased absorption of cholesterol by the down-regulation of intestinal ACAT, which converts cholesterol to cholesteryl esters and reduces cholesterol absorption. Twenty-four New Zealand white rabbits were divided into three groups, each group having one of the following: a normal diet with no cholesterol added (Group 1), a high cholesterol diet 1 g/100g (Group2) and a high cholesterol diet supplemented with 2 g/100g Shan Zha powder (Group 3). After 12 weeks, serum total cholesterol and triacylglycerols in Group 3 were significantly lowered by 23.4 and 22.2 % respectively compared with the Group 2 rabbits. Significantly, less cholesterol accumulation in aorta and a greater excretion of neutral and acidic sterols were observed in Group3 than in Group 2. Neutral and acidic sterols are formed by the hydrolyzation of esterified cholesterol. Their increased excretion causes a decrease in the cholesterol level. Supplementation of Shan Zha affected neither the activities of HMG-CoA-R, which is required for cholesterol synthesis, nor CH, which converts cholesterol to bile acids. However, it

significantly suppressed the activity of the intestinal ACAT. The results suggest that Shan Zha reduces serum cholesterol through a mechanism that involves the inhibition of cholesterol absorption mediated by the down-regulation of the intestinal ACAT activity.

Apart from rabbit, the hypocholesterolemic effect of Shan Zha was also observed in other animal models (Zhang *et al*., 2002a). Fifteen male Syrian golden hamsters were divided into two groups for a 4-week experiment. The diet of the control group was a semisynthetic one containing 0.1 % cholesterol while the diet of the tested group was the same but supplemented with 0.5 % Shan Zha aqueous ethanolic extract. The serum total cholesterol and triacylglycerols levels were lowered significantly by 10 and 13 % respectively in the Shan Zha group as compared with the control. Supplementation of Shan Zha aqueous ethanolic extract caused a greater excretion of both neutral and acidic sterols. The mechanisms of how Shan Zha reduced serum cholesterol were suggested by further enzymatic assays involving a greater excretion of bile acids mediated by the up-regulation of the hepatic CH activity, and the inhibition of cholesterol absorption mediated by the down-regulation of the intestinal ACAT activity.

More proofs of the hypocholesterolemic effect of Shan Zha were demonstrated by Rajendran and co-workers (1996). They proposed that mechanisms involved were related to the inhibition of cholesterol absorption and the enhancement of bile acid excretion. Furthermore, the enhancement of the LDL receptor activity was also involved. In their study, eighteen male Wistar rats weighing 120–150g were divided into three groups for a 6-week experiment. Group I was fed normal rat chow, Group II was fed an atherogenic diet and Group III was fed a tincture of Shan Zha simultaneously with an atherogenic diet. Plasma total cholesterol, LDL-cholesterol, Very-Low-Density-Lipoprotein (VLDL)-cholesterol and atherogenic index (LDL+VLDL cholesterol / HDL cholesterol) were significantly higher in Group II than in Group I and Group III. The liver total cholesterol level of Group III was significantly lower than Group II, while the liver bile acids and fecal bile acids of Group III were significantly higher than Group II. Liver cholesterol biosynthesis was reduced more significantly in Group III than in Group II. This indicated that Shan Zha prevented cholesterol accumulation in liver by enhancing the bile acid contents in the liver and feces while depressing hepatic cholesterol biosynthesis. An increase of the LDL binding sites could be observed in the liver plasma membrane in Group III rats, as the maximum binding capacity of the Scatchard plot of the specific binding data increased significantly. This means that Shan Zha increases the

influx of plasma cholesterol into the liver by increasing the number of hepatic LDL-receptors.

Li and co-workers (2004) further proposed that the hypocholesterolemic effect of Shan Zha was due to the components hyperoside and ursolic acid. Sixty-one mice were divided into six groups: normal group, egg-yolk induced hyperlipidemia group, hyperoside 0.05 g/kg group, hyperoside 0.15 g/kg group, ursolic acid 4 g/kg group and ursolic acid 8 g/kg group. All the hyperoside and ursolic acid groups were fed egg-yolk to induce hyperlipidemia. Results indicated that the serum total cholesterol level of the groups with hyperoside and ursolic acid were significantly lower than the egg-yolk induced hyperlipidemia group.

Generally, there are four ways to regulate the cholesterol metabolism in liver: a decrease in cholesterol synthesis, i.e. the inhibition of HMG-CoA-R; an activation of the LDL receptors; an inhibition of cholesterol absorption, i.e. the inhibition of ACAT and a conversion of cholesterol to bile acids, i.e. the activation of CH. Shan Zha is reported to have these properties to prevent the hypercholesterolemic effect. Although consuming Shan Zha did not alter the activity of HMG-CoA-R (Zhang *et al*., 2002a; Zhang *et al*., 2002b), a depression of hepatic cholesterol synthesis was

observed (Rajendran *et al*., 1996). The amount of LDL receptor was found to have increased after consuming Shan Zha (Rajendran *et al*., 1996). An up regulation of CH (Zhang *et al*., 2002a) and a down regulation of ACAT were observed (Zhang *et al*., 2002a; Zhang *et al*., 2002b), indicating that more cholesterol was converted to bile acids and less cholesterol was absorbed. Hyperoside and ursolic acid may be the causative agents.

- *Antioxidative effect*

Endothelial dysfunction caused by the oxidation of LDL is the initiation step for atherosclerosis (Woolf, 1999). An antioxidant is defined as any substance when presents at low concentration compared to those of an oxidisable substrate, significantly delays or prevents the oxidation of that substrate. As a result, antioxidants can minimize the oxidation of LDL and thus prevent atherosclerosis. Shan Zha was shown to be antioxidative.

Successive extraction was done on Shan Zha with ether, ethyl acetate, butanol and water. The ethyl acetate fraction was the only one to inhibit Cu^{2+}-mediated LDL oxidation. The components of this fraction included: ursolic acid, hyperoside,

isoquercitrin, epicatechin, chlorogenic acid, quercetin, rutin and protocatechuic acid. Except ursolic acid, all of these isolated phenolic compounds protected human LDL from Cu^{2+}-mediated LDL oxidation (Zhang, 2001).

Shanthi and co-workers (1996) also suggested that the alcoholic extract of Shan Zha inhibited *in vivo* lipid peroxidation. Furthermore, the Shan Zha extract could maintain the antioxidant level and antioxidant enzyme activities in the body. Eighteen male Wistar rats weighing 120–150g were divided into three groups for a 6-week experiment. Group 1 was given a normal diet, Group 2 was given an atherogenic diet and Group 3 was given an atherogenic diet together with the alcoholic extract of Shan Zha. Results indicated that rats consuming the Shan Zha extract had significantly lower lipid peroxidation levels in serum, liver, aorta and heart compared with the rats in the atherogenic diet. The level of antioxidants like glutathione and α-tocopherol in the body was also higher in the Group 3 rats than in the Group 2 rats. The antioxidant activities of enzymes like SOD, CAT, GPx and glutathione-S-transferase in the liver, aorta and heart decreased in the Group 2 rats, but those activities were maintained in the Group 3 rats. The Shan Zha extract was able to inhibit Cu^{2+}-mediated LDL oxidation in human serum *in vitro*. The author believed the antiatherogenic effect of Shan Zha related to its antioxidant property.

Shan Zha is found to contain many phenolic antioxidants; among them are hyperoside, isoquercitrin, epicatechin, chlorogenic acid, quercetin, rutin and protocatechuic acid. They were shown to be LDL oxidation inhibitors (Zhang, 2001). Furthermore, Shan Zha helps the body to maintain a high level of antioxidants and antioxidant enzymes, which are commonly used to defend against oxidative stress (Shanthi, 1996).

Shan Zha has a therapeutic effect even when oxidized LDL is added to the human endothelial cells. Chang and Chen (2001) found that the addition of increasing concentrations of Shan Zha extract increasingly reduced the harmful effects of oxidized LDL on the human endothelial cells and inhibited the adhesion of monocytes.

- *Endothelial-dependent vasorelaxation*

A lack of vasoelasticity is one of the symptoms of atherosclerosis. Endothelial dysfunction reduces the elasticity of blood vessels, as EDRF like NO are produced and released in the endothelial cells (Mitani and Kimura, 2003; Behrendt and Ganz, 2002). The detailed mechanism of this endothelium-dependent vasorelaxation is

demonstrated in Fig. 6 (Kim *et al*., 2000; Moncada *et al*., 1986). Briefly, upon stimulation by various agonists, e.g. acetylcholine, histamine, substance P and isoproterenol, the concentration of intracellular Ca^{2+} increases, which causes the opening of the Ca^{2+}-dependent K^+ channel and an increase of K^+ efflux. The increase of K^+ efflux causes hyperpolarization of the endothelial cells, providing a driving force for transmembrane Ca^{2+} influx into the cells, which in turn triggers the NO production from L-arginine with the presence of NOS. The released NO stimulates soluble guanylate cyclase in the smooth muscle cells, leading to an increase in cGMP and finally leading to vasodilation. The restoration of vasorelaxation is a good proof of atherosclerosis prevention.

Kim and co-workers (2000) provided evidence that Shan Zha relaxed vascular tone. The active ingredients and the mechanism involved were also reported. Isolated thoracic aorta rings with or without endothelium from male SD rats weighing 270–330 g were precontracted with 1 μM phenylephrine in the presence of 1 μM indomethcin, so as to prevent the production of vasoactive prostanoids, and the cumulative relaxation response of the Shan Zha extract (1–100 μg/ml) were analyzed. The Shan Zha extract significantly relaxed the rat aortic rings with endothelium in a concentration-dependent manner, but those without endothelium

were not affected.

Since flavonoids and procyanidins are believed to be the major active components of Shan Zha (Rigelsky and Sweet, 2002), their effects on the endothelium-dependent vasorelaxation were also investigated. Kim and co-workers (2000) performed the above experiment by using procyanidins (10 – 100 μg/ml), which was isolated from the Shan Zha extracts (45.7 % procyanidins and less than 0.1ng flavonoids) and three flavonoids of Shan Zha, namely hyperoside (10 μg/ml), rutin (10 μg/ml) and vitexin (10 μg/ml). None of the extracted flavonoids showed the effect of endothelium-dependent relaxation. However, procyanidins significantly relaxed the phenylephrine (1 μM) contracted rat aortic rings with endothelium in a concentration-dependent manner. While the isolated procyandins was only nine times more concentrated than the Shan Zha extract, they induced eighteen times greater endothelium-dependent relaxation potency. The author therefore deduced that the endothelium-dependent relaxation by the Shan Zha extract was caused by the presence of procyanidins (Kim *et al*., 2000). The endothelium-dependent vasorelaxation effect of procyanidins was also confirmed by Aldini and co-workers (2003) using human internal mammary aortic rings.

Addition of Nω-nitro-L-arginine (10 μM), the nitric oxide synthase inhibitor, and methylene blue (1 μM), the soluble guanylate cyclase inhibitor, significantly reduced the relaxation effect of procyanidins. This indicated that the effect of procyanidins was nitric oxide synthase-dependent and guanylate cyclase-dependent. Preincubation of antagonists for muscarinic receptor, histamine receptor, substance P receptor and β-adrenoceptor before the addition of phenylephrine (1 μM) did not affect the relaxation effect of procyanidins, indicating that procyanidins did not interact with these receptors. Preincubation of tetraethylammonium, a non-selective K^+ blocker, before the addition of phenylephrine (1 μM) significantly inhibited the relaxation effect of the rat aortic rings treated with procyanidins, but preincubation of glibenclamide, an ATP-sensitive K^+ channel blocker, did not produce such effect. This study indicated that procyanidins might be the compound causing the endothelium-dependent relaxation effect of Shan Zha. The production of NO in the endothelial cells is increased, and the activation of tetraethylammonium-sensitive K^+ channels might be involved in the mechanism (Kim *et al*., 2000). Chen and co-workers (1998) also concluded that Shan Zha contained active components which caused the vasorelaxation in the isolated mesenteric rat arteries. NO, but not other EDRF, was the causative agent for this action.

1.1.7: Objectives

Atherosclerosis affects the vascular elasticity of blood vessels (Blankenhorn and Kramsch, 1989), in which the relaxation and contraction abilities are affected. In previous studies, researches mainly focused on the relaxation ability, but no information was found on the contraction responses. Reduction of the relaxation ability of blood vessels can be caused by the oxidation of LDL as a result of endothelial dysfunction (Betteridge *et al*., 1999). Since cholesterol is the major component of LDL, the reduction of cholesterol is believed to be antiatherogenic. Apart from this, oxidation of LDL can be prevented by the enhancement of the antioxidative enzyme activities (Halliwell and Gutteridge, 1995). As a result, Shan Zha, with its hypocholesterolemic effect (Zhang *et al*., 2002a; Zhang *et al*., 2002b) and the ability to enhance the antioxidative enzyme activities (Shanthi, 1996), is believed to be antiatherogenic. It is valuable to find out whether these two mechanisms are the only mechanisms involved in the antiatherogenic effect or other mechanisms are also involved.

Therefore, there are several questions have to be answered in this study. They are as follows:

1. Does Shan Zha have any antiatherogenic effect?

2. If the answer for the above question is yes, is this antiatherogenic effect caused by the hypocholesterolemic effect or the enhancement of antioxidative enzyme activities of Shan Zha, or both?

3. Are there any other mechanisms involved in the antiatherogenic effect?

In order to answer the above questions, the following objectives were set.

1) To confirm the beneficial effect of Shan Zha on atherosclerosis in terms of both vasorelaxation and vasoconstriction.

2) To find out the possible mechanisms for the antiatherogenic effect of Shan Zha, 3 parts of studies will be involved, they are:

- To find out the relations between the antiatherogenic effect and the hypocholesterolemic effect of Shan Zha.

- To find out the relations between the antiatherogenic effect and the enhancement of the antioxidative enzyme activities of Shan Zha.

- To find out other possible mechanisms for the antiatherogenic effect of Shan Zha.

After further proof of the antiatherogenic effect of Shan Zha and the understanding of the detailed mechanisms involved, people would be more confident to use Shan Zha as a food supplement in their daily lives. In addition, more potential ingredients from Shan Zha could be identified, isolated and purified for the medication development for atherosclerosis.

2: METHODOLOGY

2.1: Materials

2.1.1: Plant, animals and blood sample

The herb Shan Zha, harvested in Shan Dong, was brought from Kwok Shing Hong Co., Ltd., Hong Kong SAR, China. The voucher specimen was deposited in Dr. Peter Yu Hoi-fu's laboratory, Department of Applied Biology and Chemical Technology. SD rats were supplied from the Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, HKSAR, China. Whole blood from pig was collected at Tsuen Wan Central Butchery, Hong Kong, China.

2.1.2: Chemicals

2,2′-Azobis-(2-Amidinopropane) Dihydrochloride was bought from Cayman Chemical Company. Heparin, indomethacin, neostigmine, phenylephrine, acetylcholine, 1,1-Diphenyl-2-picrylhydrazyl Free Radical, standard compounds of

chlorogenic acid and quercetin, and other chemicals were purchased from Sigma (St. Louis, MO, USA). The HPLC grade acetonitrile were purchased from International Laboratory, USA. Extraction solvents like petroleum ether, ethyl acetate and ethanol, and all the other solvents were in analytical grade and purchased from BDH Laboratory Supplies (Poole, Dorset, England).

2.2: Preliminary determination of antiatherogenic effect of Shan Zha

In order to determine whether Shan Zha has the antiatherogenic effect, rats were used and the vascular elasticity was measured after consuming Shan Zha with an atherogenic diet. The vascular elasticity was measured as a parameter for atherosclerosis because loss of vascular elasticity is one of the symptoms of atherosclerosis. The weight of livers was measured and the appearance of livers was observed both by naked eyes and under microscope, in order to preliminarily examine the relation between the antiatherogenic effect and the hypocholesterolemic effect of Shan Zha.

2.2.1: Animals for preliminary determination of antiatherogenic effect of Shan Zha

8-month old male SD rats (~550 g) were housed at room temperature, in constant humidity and a 12 hr light-dark cycle. Free access of tap water was allowed. The experiment was conducted for twenty-eight days. Twenty-one rats were randomly divided into three groups with Sample Number (n)=7, which were named as Normal group (N), with a normal diet; Hypercholesterolemia group (H), with 1 % cholic acid, 2 % cholesterol and 5.5 % oil in a normal diet and Shan Zha powder group (SZ+H), with the same diet as the Hypercholesterolemia group, but with an addition of 2 % Shan Zha powder in the diet. The protocol was reviewed and approved by the Committee of Animal Ethics, The Hong Kong Polytechnic University.

2.2.1.1: Antiatherogenic effect of consuming Shan Zha for 28 days

The 28-day treated rats were killed by cervical dislocation. The thoracic aortas were rapidly dissected. The fat and the surrounding connective tissues were cleared. Aortic rings (about 4 mm) were prepared and mounted between two stainless steel

hooks, in 5 ml water-jacketed organ baths (37 °C) containing Krebs-Ringer bicarbonate solution of the following composition (mM): NaCl, 118.0; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $NaHCO_3$, 25.0; KH_2PO_4, 1.2 and glucose, 11.0. The bath solution was constantly gassed with carbogen (95 % oxygen and 5 % carbon dioxide). An optimal load of 15 ± 1 mN was applied to the rings. Changes in force were recorded by isometric force-displacement transducers which were connected to a PowerLab data acquisition system, where the data sampling rate was set at 40 per minute.

Subsequently, the tissues were allowed to equilibrate for 60 min under their resting tension. During the equilibration period, the tissues were washed with drug-free Krebs-Ringer bicarbonate solution for every 20 min and the resting tension was readjusted, whenever necessary, before commencing the experiments. After equilibration, the isolated aortic rings were sensitized with 40 mM KCl, until two consecutive contractile responses were reproducible.

Relaxation response

To investigate the relaxation effects of acetylcholine on the isolated aorta, after sensitization the aorta were pre-contracted with 1 μM phenylephrine, a concentration that produces approximate 85 % of maximum contraction (EC_{85}) in the tissues, in the presence of 1 μM indomethacin and 1 μM neostigmine. After a steady-state contraction was established, cumulative concentrations (10 nM – 100 μM) of acetylcholine were added to the organ bath.

Contraction response

To investigate the effect of contraction, the aorta was washed with a $CaCl_2$ free Krebs-Ringer bicarbonate solution after sensitization, and 40 μM KCl was added, after a steady-state was established and cumulative concentrations (1 μM – 1 mM) of $CaCl_2$ were added to the organ bath.

2.2.1.2: Observations of livers

Fatty liver can be a risk factor for atherosclerosis (Kurihara *et al*., 2001; Olga *et*

al., 2002). The formation of fatty liver is due to excessive fat, which is more than 5 % lipid weight of the liver, deposits in the hepatocytes (Kurihara *et al*., 1994). By observing the size, colour, lipid deposited and the weight of the liver, the effect of hyperlipidemia can be observed. By comparing the above phenomenon with the vascular elasticity, the relation between hyperlipidemia and atherosclerosis can be examined.

A B

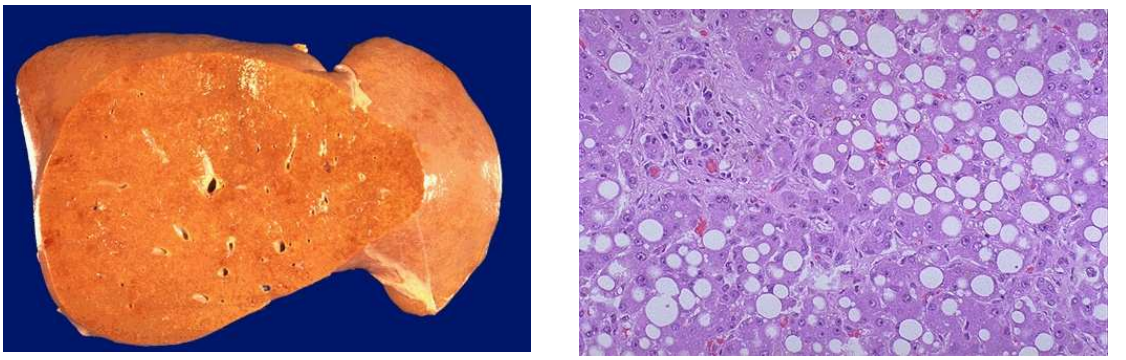


Fig. 13: Appearance of fatty liver. Observed by naked eye (A). Observed under microscope 400× (B) (Wu, 2004).

About 10 g of the rat livers were removed, rinsed with saline, and kept in 10 % formalin before sectioning. Waxed specimens were prepared and stained by hematoxylin and eosin using the method of Ratcliffe (1982).

2.3: Measurement of antioxidative power of Shan Zha

In the above experiment, Shan Zha showed a tendency to reduce the atherogenic effect caused by the hypercholesterolemic diet in terms of the relaxation ability. However, this trend is not significant. A larger dosage or using an extract which involved more active ingredients may help improve the effect. Furthermore, in order to determine the relation between the antiatherogenic effect and the antioxidative effect of Shan Zha, it was necessary to find out most antioxidative extract of Shan Zha. Since the preparation of many Chinese medicine involved heating, the antioxidative power of the Shan Zha extract under heat treatment was also examined.

2.3.1: Extraction of Shan Zha

In this experiment, Shan Zha was divided into the heated and unheated groups. The heated group was heated in a 99 ^{o}C water bath for 1 hr. In each group, Shan Zha was further extracted with four different solvents of different polarities in a ratio of 1:10 w/v. The solvents were petroleum ether, ethyl acetate, 80 % ethanol and water, with n=3. The Shan Zha-solvent mixtures were shaken in 300 rpm at 37 ^{o}C for 2 hr.

After filtering, the residuals were re-extracted for two more times. The water extract was dried in a freeze drier, while the others were dried in a vacuum oven below 50 ^{o}C. The dried extracts were re-dissolved in Dimethyl Sulfoxide (DMSO) to 1 μg/ml for the chemical tests mentioned in part 2.3.2.1. DMSO was set as blank for the tests.

2.3.2: Measurement of antioxidative power of Shan Zha

The antioxidative power of Shan Zha was measured chemically and *in vitro*. The chemical properties of antioxidants were determined by the Ferric Reducing / Antioxidant Power (FRAP) Assay, which measured the red-ox reaction, the 1,1-Diphenyl-2-picrylhydrazyl Free Radical (DPPH) Assay, which measured the free radicals scavenges capacity and the Folin-Ciocalteu Assay, which measured the amount of the phenolics content. The chemical tests measured the antioxidants level in a chemical way. However, it cannot represent the power of an antioxidant in a biological system. In order to measure the antioxidant power of the extract in a biological system, erythrocyte was used.

2.3.2.1: Chemical tests

FRAP Assay

In the FRAP assay, the antioxidant in the sample reduces Fe^{3+}-tripyridyltriazine (TPTZ) complex, to a blue colored ferrous (Fe^{2+}), which increases absorbance (Abs) at 593nm. The change in absorbance is proportional to the combined (total) FPAP value of the antioxidant in the sample (Benzie and Strain, 1996).

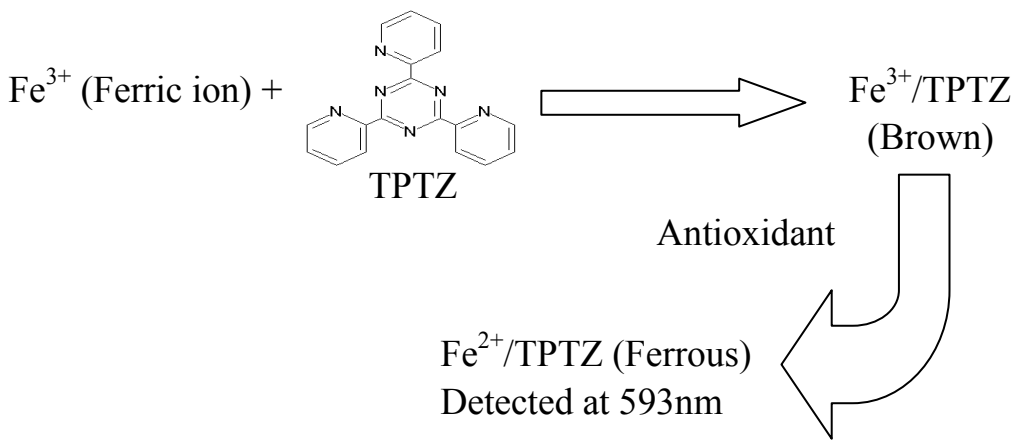


Fig. 14: Reaction of FRAP Assay.

300 µl freshly prepared working FRAP reagent, 10 µl sample or standard solution and 30 µl distilled water were mixed. Absorbance at 593 nm was measured by a Cobas Fara centrifugal analyzer. The change in absorbance during the monitoring period was calculated and changed into Fe^{2+} value by comparing with the absorbance of the Fe^{2+} standard solution (Benzie and Strain, 1996).

DPPH Assay

In the DPPH• assay, the antioxidants reduce the free radical 2,2-diphenyl-1-picrylhydrazyl, which has an absorption maximum at 515 nm. The free radical solution is prepared by dissolving 2.4 mg DPPH• in 100 ml methanol. For the photometric assay, 1.95 ml DPPH• solution and 50 µl sample solution were mixed. At first, the extinction of the cuvette with 1.95 ml DPPH• was measured as blank, the antioxidant solution was then added and mixed. The reaction was measured after 2, 3, 4, 5 and 10 min and then in intervals of 5 min afterwards until the change in absorbance is < 0.003 / min (Schlesier *et al*., 2002). The percentage of Free Radical Scavenging Capacity (SR %) of the antioxidant is:

SR % = (1 – Abs sample / Abs control) × 100 %

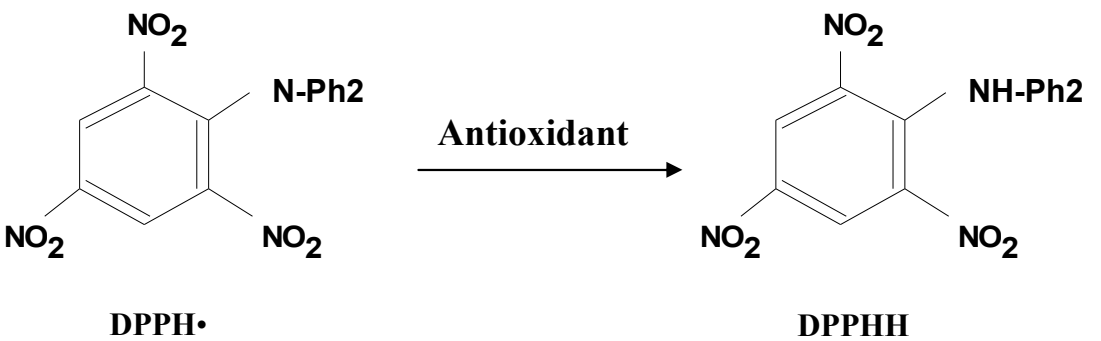


Fig. 15: Reaction of DPPH• Assay.

Folin-Ciocalteu Assay

In the Folin-Ciocalteu Assay, the phenolics content of the samples were oxidized by phosphomolybdic and phosphotungstic acid in Folin-Ciocalteu reagent, the yellow color of Folin-Ciocalteu reagent turns blue, which can be detected at 750nm. (Singleton *et al*., 1999)

100 μl sample were mixed with 500 μl Folin-Ciocalteu reagent and 400 μl 0.7 M Na_2CO_3. Absorbance at 750 nm was measured after 2 hr of reaction time (Schlesier *et al*., 2002). Gallic acid was used as the standard and the total phenolics content was expressed as gallic acid equivalent in 1 g of the sample.

2.3.2.2: Protective effect of Shan Zha extracts against hemolysis caused by AAPH

The chemical tests measured the antioxidants level in a chemical way. However, it cannot represent the power of antioxidant in a biological system. In order to measure the antioxidant power of the extract in a biological system, erythrocyte is used.

The water-soluble azo compound 2,2´-Azobis-(2-amidinopropane) dihydrochloride (AAPH) is a free radical generator. The free radical AAPH attacks PUFA of the erythrocyte membrane and causes hemolysis, while the protective effect of the antioxidant can be detected by the degree of hemolysis.

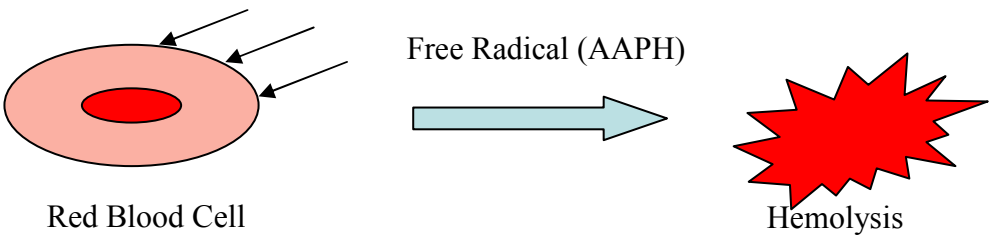


Fig. 16: Reaction of AAPH Assay.

Whole blood from pig with 100 units/ml heparin was centrifuged at 625 g for 10 min. The pellet, erythrocyte, was washed 3 times with Phosphate Buffered Saline (PBS), which consisted of (mM) NaCl, 137; KCl, 2.7; Na_2HPO_4, 8.1; KH_2PO_4, 1.5. 20 % erythrocyte was made. 600 µl erythrocyte suspension was mixed with 600 µl sample extracts, which were prepared by dissolving 10 mg/ml in DMSO, and then further diluted to 0.1 mg/ml by PBS. The mixture was incubated at 37 °C for 10 min and rolled at 22 rpm. After that 300 µl 400 mM AAPH in PBS was added and incubated at 37 °C and rolled at 22 rpm. After 2 hr, 100 µl mixture was added to 1.25 ml ice cold PBS and water. The mixtures were centrifuged at 385 g for 10 min.

The Abs of the supernatant was measured at 540 nm by a microplate reader BioRad 550.

Hemolysis % = (Abs PBS / Abs water) × 100 %

Controls:

With AAPH only: Erythrocyte suspension + PBS (instead of sample) + AAPH

Without AAPH: Erythrocyte suspension + PBS (instead of sample and AAPH)

2.4: Examination of antiatherogenic effect of consuming 80 % ethanol Shan Zha extract

Since all the results from the antioxidative power tests indicated that 80 % ethanol Shan Zha extract has the highest antioxidative power, this extract was applied to examine the antiatherogenic effect. The mechanisms involved were also determined, which included the involvement of endothelial cell, the involvement of Apo-B, the activities of the antioxidative enzymes and blood pressure.

2.4.1: Animals for examination of antiatherogenic effect of consuming 80 % ethanol Shan Zha extract

10-month old male SD rats (~ 600 g) were housed at room temperature, in constant humidity and a 12 hr light-dark cycle. Free access to tap water was allowed. The experiment was conducted for twenty-eight days. Thirty-two rats were randomly divided into four groups, n=8, and named as Normal group (N), with a normal diet; Hypercholesterolemia group (H), with 1 % cholic acid, 2 % cholesterol and 5.5 % oil in a normal diet; Shan Zha 80% ethanol extract 30 mg/kg group (SA), with the same diet as the Hypercholesterolemia group and force fed with 30 mg/kg 80 % ethanol extract and Shan Zha 80% ethanol extract 100 mg/kg group (SB), with the same diet as the Hypercholesterolemia group and force fed with 100 mg/kg 80 % ethanol extract. The Shan Zha extracts were fed once per day. The protocol was reviewed and approved by the Committee of Animal Ethics, The Hong Kong Polytechnic University.

Blood from rats which had fasted for 12 hr was collected from the eyes on day 1 and day 28 of the experiment respectively. The 100 unit/ml heparin-containing blood was centrifuged at 1500g for 15 min. The supernatants, plasma, were stored at

-20 ^{o}C before the determination of the plasma cholesterol contents, Apo-B contents and the activities of the antioxidative enzymes.

2.4.1.1: Relaxation response of aorta with and without endothelium

With endothelium

The same method was used as shown in part 2.2.1.1. “Relaxation response”.

Without endothelium

The 28-day treated rats were killed by cervical dislocation. The thoracic aortas were rapidly dissected. The fat and the surrounding connective tissues were cleared. Aortic rings (about 4 mm) were prepared, the endothelial cell was removed by gentle rubbing of the lumen and they were mounted between two stainless steel hooks, in 5 ml water-jacketed organ baths (37 °C) containing Krebs-Ringer bicarbonate solution of the following composition (mM): NaCl, 118.0; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $NaHCO_3$, 25.0; KH_2PO_4, 1.2 and glucose, 11.0. The bath

solution was constantly gassed with carbogen (95 % oxygen and 5 % carbon dioxide). An optimal load of 15 ± 1 mN was applied to the rings. Changes in force were recorded by isometric force-displacement transducers which were connected to a PowerLab data acquisition system, where the data sampling rate was set at 40 per minute. Subsequently, the tissues were allowed to equilibrate for 60 min under their resting tension. During the equilibration period, the tissues were washed with the drug-free Krebs-Ringer bicarbonate solution for every 20 min and the resting tension was readjusted, whenever necessary, before commencing the experiments. After equilibration, isolated aortic rings were sensitized with 40 mM KCl, until two consecutive contractile responses were reproducible. After sensitization, 1 μM noradrenalin and 10 μM acetylcholine were added to the organ bath. If no relaxation occurred, the bath was washed with the Krebs-Ringer bicarbonate solution for about 30 min and the aorta were pre-contracted with 1 μM phenylephrine, a concentration that produces approximate 85 % of maximum contraction (EC_{85}) in the tissues, in the presence of 1 μM indomethacin and 1 μM neostigmine. After a steady-state contraction was established, cumulative concentrations (10 nM – 30 μM) of acetylcholine were added to the organ bath.

2.4.1.2: Determination of lipid content in livers

The appearance of livers was observed, their weight was measured and their lipid content was examined. Part of the rat livers were removed, rinsed with saline, weighed and kept at -80 ^{o}C before homogenization. 1 g of liver tissues was homogenized with 2:1 chloroform-methanol mixture (v/v) to a final dilution of 1:20 w/v using an Ultra-turrax T-25 homogenizer. After filtration, 10 ml of filtrate was added to 2 ml water and the mixture was centrifuged at 900 g for 20 min. The lower phase was dried and its weight measured (Folch *et al*., 1956). The liver lipid content was expressed as the weight of the lipid per g of the liver.

2.4.1.3: Measurement of hypocholesterolemic effect

The plasma Total Cholesterol content (TC), the HDL-cholesterol content, the LDL-cholesterol content, and the mRNA expression level of the LDL receptor were measured in order to estimate the hypocholesterolemic effect.

Plasma cholesterol contents: TC, HDL-cholesterol and LDL-cholesterol

LDL is a risk factor of atherosclerosis, because it can be oxidized and the Ox-LDL attracts the engulfment of macrophage and form foam cell (Betteridge *et al*., 1999). LDL transports cholesterol to cells, so it is regarded as "bad cholesterol". HDL transports cholesterol away from cells to liver for metabolism. Apart from that it also reduces oxidative modification of LDL, and inhibits the cytokine-induced expression of cellular adhesion molecules on endothelial cells (Brewer and Fojo, 2003), so it is regarded as "good cholesterol". By analyzing the total plasma cholesterol concentration and its amount in HDL and LDL, the distribution of cholesterol can be determined and the risk of getting atherosclerosis can be estimated.

Plasma concentration of TC, HDL-cholesterol and LDL-cholesterol were measured by the ALCYON 300i system following the instruction of kits from Zhongsheng Beikong Bio-technology and Science Inc.

mRNA expression level of LDL receptor

Since cholesterol is the major component of LDL, the metabolism of cholesterol is related to the amount of LDL, which can be oxidized and form atherosclerosis. In this study the mRNA expression level for the gene of the LDL receptor (LDLR), which receives LDL to the liver, was determined.

Three isolated livers from each group of rats were homogenized and the total RNA was isolated using the Trizol reagent (Life Technologies Inc., Rockville, MD, USA) for the determination of the gene expression levels in the various groups. cDNA was obtained in 20 µl of Reverse Transcriptase (RT)-reaction mixture using the Super Script First-Strand Synthesis System for RT-Polymerase Chain Reaction (PCR) (Life Technologies Inc.) with oligo $d(T)_{12–18}$. Real time RT-PCR was performed with Cepheid SmartCycler with the SYBR green real time PCR detection kit, iQ SYBR Green Supermix (BioRad), in 25 µl of the total reaction mixture containing 0.5 µl of the RT-reaction mixture and 200–300 nM of each primer (forward and reverse). The primer sets used are shown in Table 2. The expression levels of the cDNA of interest were related to an internal standard: housekeeping gene Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH), to correct the differences in quantity and quality between

different RNA samples. The amplification protocol consisted of predenaturation for 180 s at 95 °C, and then 40 PCR cycles consisting of denaturation for 10 s at 95 °C, annealing for 30 s at 60–62°C and extension for 30s at 72 °C. The threshold cycle for each amplification curve was determined and plotted against the standard curve to calculate the amount of product. Values from each group were normalized to the product produced by GAPDH. Each real-time RT-PCR reaction was performed in duplicate for each liver sample. A standard curve was generated each time for assessing the level of cDNA.

Table 2: The primer sets used for the real-time PCR

Gene name	Forward primer	Reverse primer	Product size (bp)
LDLR	5'CTG TTC CCA CCT CTG TTT AC 3'	5' AGT GAG ATA CGG CGA ATA GA3'	146
GAPDH	5'TGC ACC ACC AAC TGC TTA G3'	5'AGT GGA TGC AGG GAT GAT GT3'	180

2.4.1.4: Apo-B contents

Apo-B is essential for the binding of LDL particles to the LDL receptor, allowing cells to internalize LDL and thus absorb cholesterol. Only one Apo-B molecule is present in each lipoprotein particle (Walldius and Jungner, 2004) and therefore the total Apo-B value indicates the total number of potentially atherogenic

lipoproteins.

Plasma Apo-B contents were measured by the ALCYON 300i system following the instruction of kits from Zhongsheng Beikong Bio-technology and Science Inc.

2.4.1.5: Antioxidative enzymes

ROS is generated from all aerobic cells (Cimino, 1989; Leng *et al*., 1994; Olinescu and Smith, 2002; Vaya and Aviram, 2003). LDL can be oxidized by ROS (Steinberg *et al*., 1989), while ROS in tissues can be cleared by antioxidant enzymes, including SOD, which transform superoxide to hydrogen peroxide; CAT, which transforms hydrogen peroxide to water and oxygen and GPx, which transforms hydrogen peroxide and glutathione to water and oxidized glutathione.

SOD, CAT and GPx activities were measured by using commercial kits (Nanjing Jiancheng Bioengineering Institute, China). SOD activity is expressed as U/ml plasma and 1 unit of SOD is defined as the amount of enzyme required to produce 50 % inhibition of SOD in 1 ml kit-reagent. CAT activity is expressed as U/ml plasma and 1 unit of CAT is defined as the amount of enzyme required to

degrade H_2O_2 in the reagent at the absorbance of 0.5–0.55. GPx activity is expressed as U/L plasma and 1 unit of GPx is defined as the enzyme activity required to decrease 1 μmol/L glutathione per min without the non-enzymatic action.

2.4.1.6: Measurement of blood pressure

Hypertension is one of the risk factors of atherosclerosis. At day 1 and day 28 of the experiment, the blood pressure of the rats was measured non-invasively. The rats were preheated and maintained at around 28 ^{o}C during the measurement. The tail of the rat was connected to the pressure cuff and pulse transducer MLT125R, the systolic pressure were measured and recorded by the NIBP Controller and PowerLab data acquisition system, where the data sampling rate was set as 40 per minute.

2.5: Relaxation response to 80 % ethanol Shan Zha extract on isolated aorta with endothelium

In order to examine whether ingredients from this extract cause direct relaxation on aorta, the 80 % ethanol Shan Zha extract was directly applied to the isolated aorta without consumption.

4-month old male SD rats (~400 g) were killed by cervical dislocation. The thoracic aorta was rapidly dissected. The fat and the surrounding connective tissues were cleared. Aortic rings (about 4 mm) were prepared and mounted between two stainless steel hooks, in 50 ml water-jacketed organ baths (37 °C) containing the Krebs-Ringer bicarbonate solution of the following composition (mM): NaCl, 118.0; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $NaHCO_3$, 25.0; KH_2PO_4, 1.2 and glucose, 11.0. The bath solution was constantly gassed with carbogen (95 % oxygen and 5 % carbon dioxide). An optimal load of 15 ± 1 mN was applied to the rings. Changes in force were recorded by isometric force-displacement transducers which were connected to a PowerLab data acquisition system, where the data sampling rate was set at 40 per minute.

Subsequently, the tissues were allowed to equilibrate for 60 min under their resting tension. During the equilibration period, the tissues were washed with the drug-free Krebs-Ringer bicarbonate solution for every 20 min and the resting tension was readjusted, whenever necessary, before commencing the experiments. After equilibration, the isolated aortic rings were sensitized with 40 mM KCl, until two consecutive contractile responses were reproducible.

To investigate the relaxant effects of the 80 % ethanol Shan Zha extract, on isolated aorta, after sensitization the aorta were pre-contracted with 1 μM phenylephrine, a concentration that produces approximate 85 % of maximum contraction (EC_{85}) in the tissues, in the presence of 1 μM indomethacin and 1 μM neostigmine. After a steady-state contraction was established, cumulative concentrations (0.1 μg/ml – 10 μg/ml) 80 % Shan Zha extract and (0.1 μM – 10 μM) of acetylcholine, which were dissolved in 50 % DMSO, were added to the organ bath. The 50 % DMSO solvent acted as control, the total DMSO content in the bath was around 0.5 %. The number of repetition was four.

2.6: Chlorogenic acid and quercetin, the ingredients of Shan Zha

Chlorogenic acid and quercetin are two of the ingredients of Shan Zha (Zhang *et al*., 2001). Their protective effects against hemolysis caused by the free radical were compared with that of ascorbic acid, for estimating the antioxidative strength. In order to examine whether these two compounds cause direct relaxation on aorta, chlorogenic acid and quercetin were directly applied to the isolated aorta without consumption. Since these compounds were the ingredients of Shan Zha, a High

Performance Liquid Chromatography (HPLC) analysis method was developed for the quantitative detection of them.

2.6.1: Protective effect of chlorogenic acid and quercetin against hemolysis caused by AAPH

Chlorogenic acid and quercetin were added as sample as mentioned in part 2.3.2.2. The protective effects of them were compared with that of ascorbic acid.

2.6.2: Relaxation responses of chlorogenic acid and quercetin on isolated aorta with endothelium

This part of study aimed to examine whether the ingredients of Shan Zha had any direct effect of vasorelaxation. 4-month old male SD rats (~400 g) were killed by cervical dislocation. The thoracic aortas were rapidly dissected. The fat and the surrounding connective tissues were cleared. Aortic rings (about 4 mm) were prepared and mounted between two stainless steel hooks, in 50 ml water-jacketed organ baths (37 °C) containing Krebs-Ringer bicarbonate solution of the following composition (mM): NaCl, 118.0; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $NaHCO_3$, 25.0;

KH_2PO_4, 1.2 and glucose, 11.0. The bath solution was constantly gassed with carbogen. An optimal load of 15 ± 1 mN was applied to the rings. Changes in force were recorded by isometric force-displacement transducers which were connected to a PowerLab data acquisition system, where the data sampling rate was set at 40 per minute.

Subsequently, the tissues were allowed to equilibrate for 60 min under their resting tension. During the equilibration period, the tissues were washed with the drug-free Krebs-Ringer bicarbonate solution for every 20 min and the resting tension was readjusted, whenever necessary, before commencing the experiments. After equilibration, the isolated aortic rings were sensitized with 40 mM KCl, until two consecutive contractile responses were reproducible.

To investigate the relaxant effects of chlorogenic acid and quercetin on the isolated aorta, after sensitization the aorta were pre-contracted with 1 μM phenylephrine, a concentration that produced approximately 85 % of maximum contraction (EC_{85}) in the tissues, in the presence of 1 μM indomethacin and 1 μM neostigmine. After a steady-state contraction was established, cumulative concentrations (0.1 μM – 10 μM) of acetylcholine, chlorogenic acid and quercetin,

which were dissolved in 50 % DMSO, were added to the organ bath. The 50 % DMSO solvent acted as control, the total DMSO content in the bath was around 0.5 %. The number of repetition was four.

2.6.3: HPLC analysis of Chlorogenic acid and quercetin in Shan Zha extracts

Since chlorogenic acid and quercetin are two of the ingredients found in Shan Zha, an HPLC analysis method was developed for quantifying the extracted chlorogenic and quercetin.

2.6.3.1: Sample extraction

The sample extraction method was the same as part 2.3.1.

2.6.3.2: HPLC system

To identify and quantify the ingredients of Shan Zha, a HPLC method modified from Baranowski and co-workers (2004) was applied using Agilent 1100 series, which

involved a degasser, a quaternary pump, an autosampler and a diode array-multiple wavelength detector. The standards or sample extracts were injected into the HPLC column (SUPERLCOSIL TM LC-18, 25 cm × 4.6 mm, 5 μm) and eluted at a flow rate of 1 ml/min at 25 ^{o}C, with a gradient mobile phase composed of 2 solvents: 0.05 % trichloromethane in water (solvent A) and 0.05 % trichloromethane in acetonitrile (solvent B) 0 min: A:B=88:12; 25 min: A:B=79:21; 30 min: A:B=75:25; 35 min: A:B=25:35; 60 min: A:B=0:100. Absorption spectra were registered in the range of 190–400nm. Registration of chromatograms was carried out at 360 nm.

0.025–0.250 μg chlorogenic acid and 0.01–0.50 μg quercetin were injected into the HPLC system, and the peak areas obtained were used for calibration. The retention time and the absorption spectra were used for identification. Analysis of inter- and intra-day for the standards were measured for the reliability, which was represented by the Relative Standard Deviation (RSD) and accuracy calculated, where RSD (%) = (standard deviation / mean) × 100 %; accuracy (%) = [(1 – (mean weight measured – weight spiked) / weight spiked)] × 100 %. 0.1 μg sample extracts were injected. The amounts of chlorogenic acid and quercetin extracted in the extracts were measured from the calibration curves.

2.7: Statistical Analysis

All data are presented as means ± Standard Errors of Means (SEM). Statistical analysis was performed by Analysis of Variance (ANOVA), Bonferroni post test: comparison of selected pairs of columns. A probability (p) value of <0.05 was considered to be statistically significant. All statistical analysis tests were performed by using Graph Pad Prism 4.02 for Windows (GraphPad Software, San Diego California).

3: RESULTS

3.1: Preliminary determination of antiatherogenic effect of Shan Zha

3.1.1: Effects on vascular elasticity of consuming Shan Zha for 28 days

A typical relaxation response observed to the cumulative acetylcholine concentrations of aorta with endothelium isolated from the Normal rats is shown in Fig. 17. Relaxation responses to the cumulative acetylcholine (Ach) concentrations and contraction responses to the cumulative calcium chloride ($CaCl_2$) concentrations of aorta isolated from the N group, H group and SZ+H group are shown in Fig. 18. Rats from the N group had the highest percentage relaxation ability, followed by rats from the SZ+H group. Rats from the H group had the lowest percentage relaxation ability. At acetylcholine concentration at 0.1 mM, a significant difference was observed in the percentage relaxation ability of rats from the N group compared with that of the H group. No significant difference can be observed in the contraction responses among the groups.

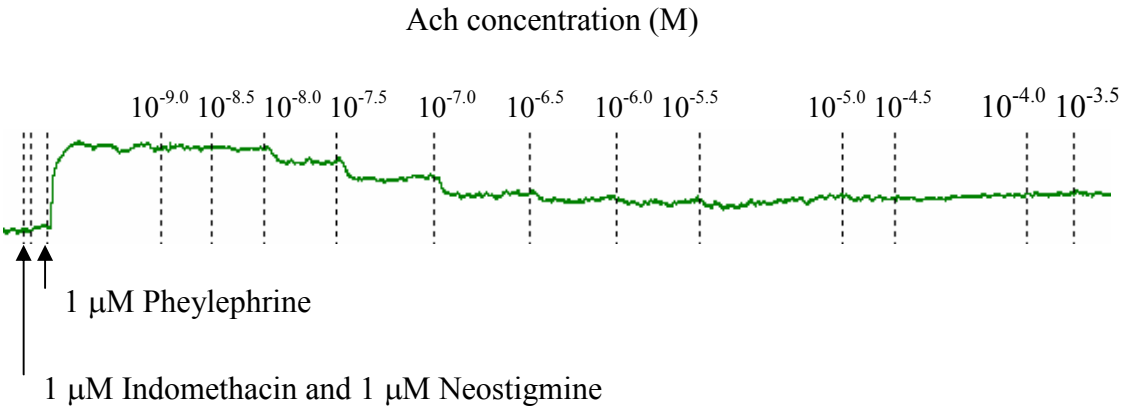


Fig. 17: Typical relaxation response observed to cumulative acetylcholine (Ach) concentrations of aorta with endothelium isolated from the Normal group.

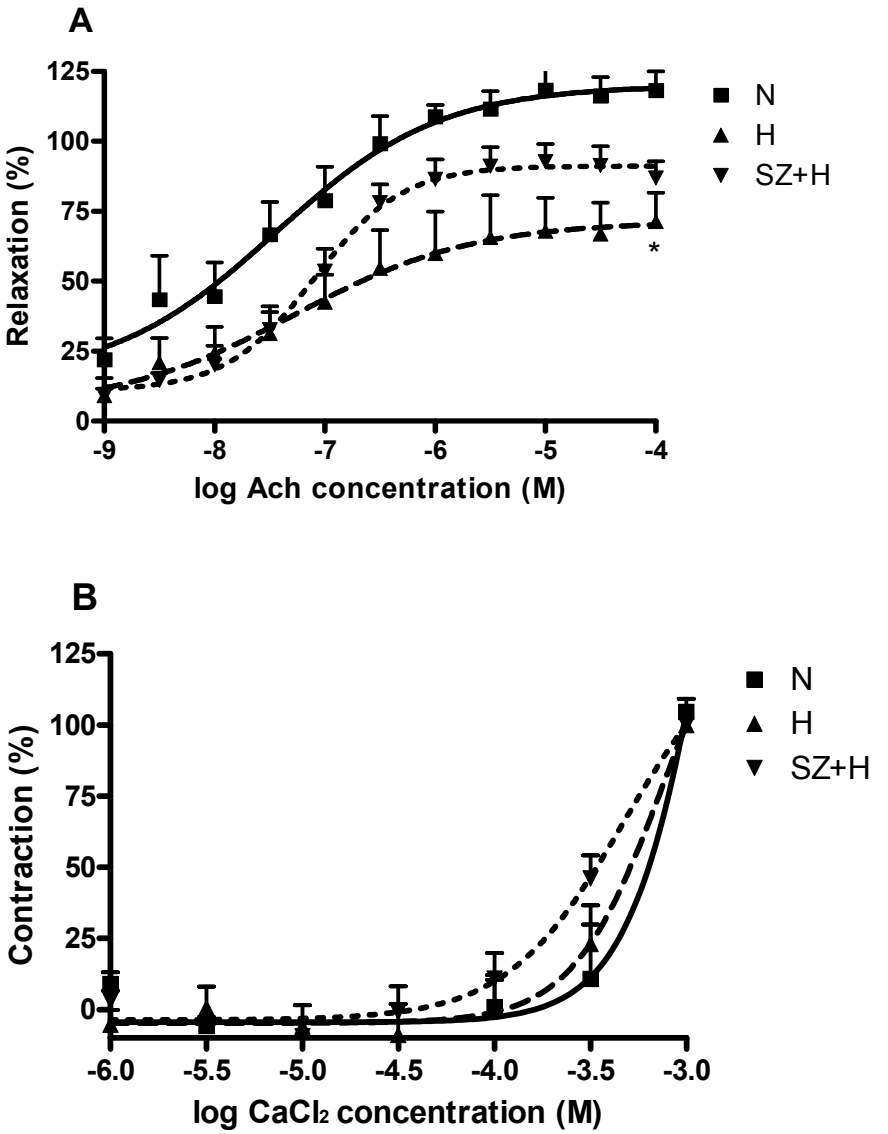


Fig. 18: Relaxation responses to cumulative acetylcholine (Ach) concentrations (A) and contraction responses to cumulative calcium chloride ($CaCl_2$) concentrations (B) of aorta isolated from the Normal (N), Hypercholesterolemia (H) and Shan Zha powder (SZ+H) groups.

Values are expressed as mean ± S.E.M., n=5–6. * Differ significantly from the Normal group at $p < 0.05$.

3.1.2: Observations of livers

Changes in body weight and the weight of liver of the N, H and SZ+H groups of rats are shown in Fig. 19. The body weight of the N group increased 21.57 ± 13.4 g while the weight of the H and SZ+H groups decreased 40.57 ± 13.4 g and 49.00 ± 11.59 g respectively. A significant difference could be observed between the N and H group at $p<0.01$, while the liver weight of the N group was significantly less than the H group at $p < 0.001$. Enlarged and yellowish livers were observed in the H group and the SZ+H group, while the size and colour of livers from the N group remained normal and dark red (Fig. 20). When the livers were viewed under microscope, lipid depositions in and enlargement of the liver cells could be observed in the rats of the H group and the SZ+H group but not in the rats of the N group (Fig. 20).

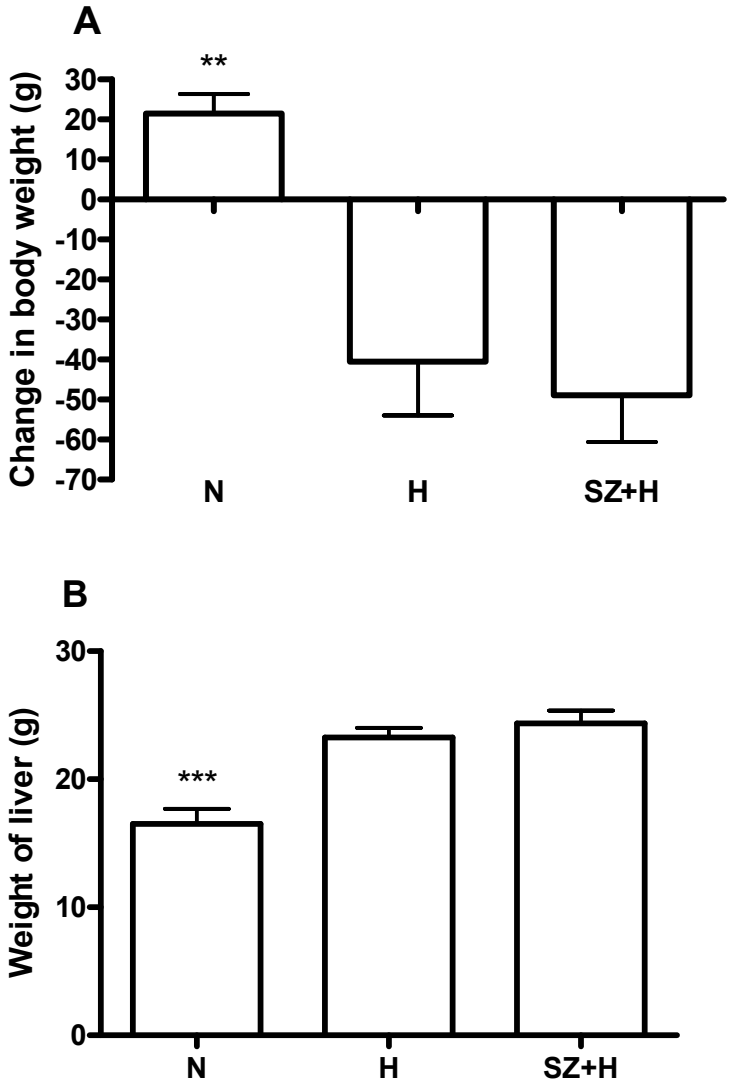


Fig. 19: Changes in body weight (A) and liver weight (B) of the Normal (N), Hypercholesterolemia (H) and Shan Zha powder (SZ+H) groups.

Values are expressed as mean ± S.E.M., n=7. ** and *** Differ significantly from the Hypercholesterolemia group at $p < 0.01$ and 0.001.

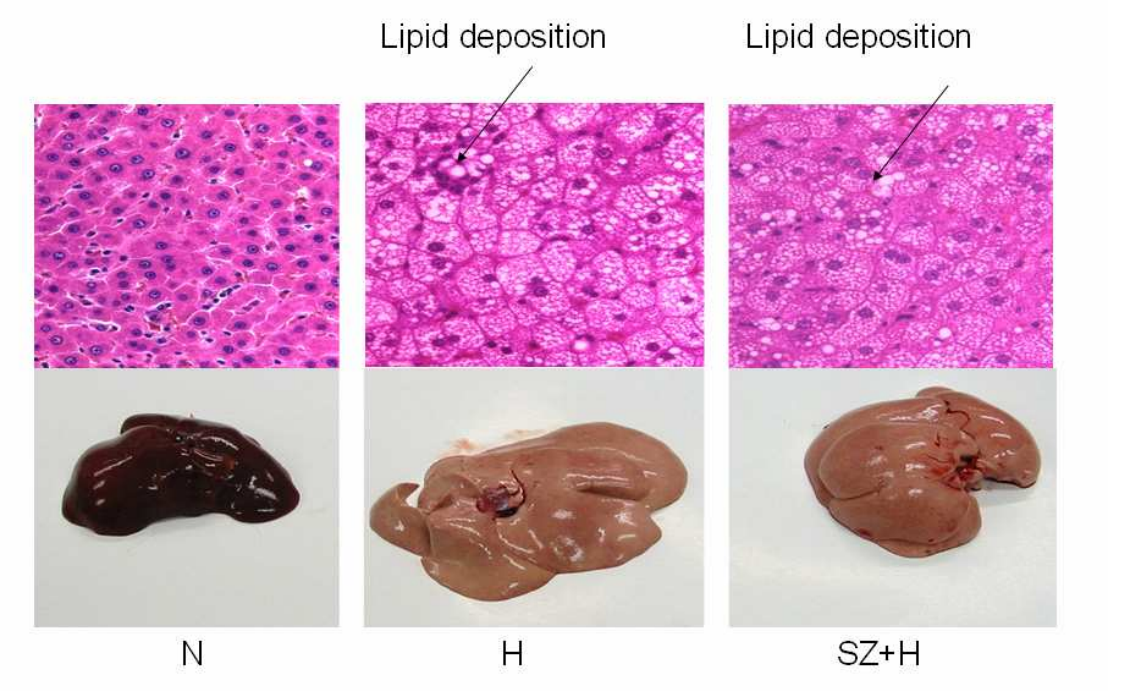


Fig. 20: Appearance of livers from the Normal (N), Hypercholesterolemia (H) and Shan Zha powder (SZ+H) groups. Observed by naked eye (bottom). Observed by microscope (400x) (top).

3.2: Measurement of antioxidative power of Shan Zha

3.2.1: Chemical tests

The FRAP values, SR % and phenolic contents of the Shan Zha extracts were shown in Tables 3, 4 and 5 respectively. More antioxidants were extracted with 80 % ethanol and water with or without the heat treatment of the sample. However when comparing the antioxidant content with or without the heat treatment, significant

differences were observed in the 80 % ethanol and water extracts only. Correlations between the chemical tests: FRAP Assay vs DPPH Assay; FRAP Assay vs Folin-Ciocalteu Assay; and DPPH Assay vs Folin-Ciocalteu Assay are shown in Fig. 21. Positive correlations between each test were observed.

Table 3: FRAP values of the Shan Zha extracts.

FRAP value (μmol/kg)	Heated	Unheated	
Petroleum Ether	18.90 ± 0.98$^{\wedge\wedge\wedge}$	15.44 ± 2.02$^{\#\#\#}$	
Ethyl Acetate	103.72 ± 16.46$^{\wedge\wedge\wedge}$	115.06 ± 16.30$^{\#\#\#}$	
80 % Ethanol	272.89 ± 8.92	438.88 ± 14.79	***
Water	246.27 ± 3.73	383.57 ± 6.30$^{\#\#\#}$	***

Values are expressed as mean ± S.E.M., n=3. $^{\wedge\wedge\wedge}$ Differ significantly with the heated 80 % ethanol at $p<0.001$; $^{\#\#\#}$ Differ significantly with the unheated 80 % ethanol at $p<0.001$; *** Differ significantly between the heated and unheated group at $p<0.001$.

Table 4: Free radical scavenging power (SR %) of the Shan Zha extracts.

SR %	Heated	Unheated	
Petroleum Ether	3.10 ± 0.21$^{\wedge\wedge\wedge}$	3.05 ± 0.26$^{\#\#\#}$	
Ethyl Acetate	6.88 ± 0.74$^{\wedge\wedge\wedge}$	6.67 ± 0.50$^{\#\#\#}$	
80 % Ethanol	11.77 ± 0.26	15.93 ± 0.19	***
Water	8.67 ± 0.32$^{\wedge\wedge\wedge}$	11.95 ± 0.36$^{\#\#\#}$	***

Values are expressed as mean ± S.E.M., n=3. $^{\wedge\wedge\wedge}$ Differ significantly with the heated 80 % ethanol at $p<0.001$; $^{\#\#\#}$ Differ significantly with the unheated 80 % ethanol at $p<0.001$; *** Differ significantly between the heated and unheated group at $p<0.001$.

Table 5: Phenolic contents of the Shan Zha extracts in terms of gallic acid equivalent (mg/g).

Gallic acid equivalent (mg/g)	Heated	Unheated	
Petroleum Ether	-0.36 ± 0.09$^{\wedge\wedge\wedge}$	-2.41 ± 0.15$^{\#\#\#}$	
Ethyl Acetate	10.60 ± 1.09$^{\wedge\wedge\wedge}$	9.61 ± 1.95$^{\#\#\#}$	
80 % Ethanol	31.16 ± 1.65	44.16 ± 0.83	***
Water	24.43 ± 0.77$^{\wedge\wedge\wedge}$	34.06 ± 0.61$^{\#\#\#}$	***

Values are expressed as mean ± S.E.M., n=3. ^^^ Differ significantly with the heated 80 % ethanol at $p<0.001$; $^{\#\#\#}$ Differ significantly with the unheated 80 % ethanol at $p<0.001$; *** Differ significantly between the heated and unheated group at $p<0.001$.

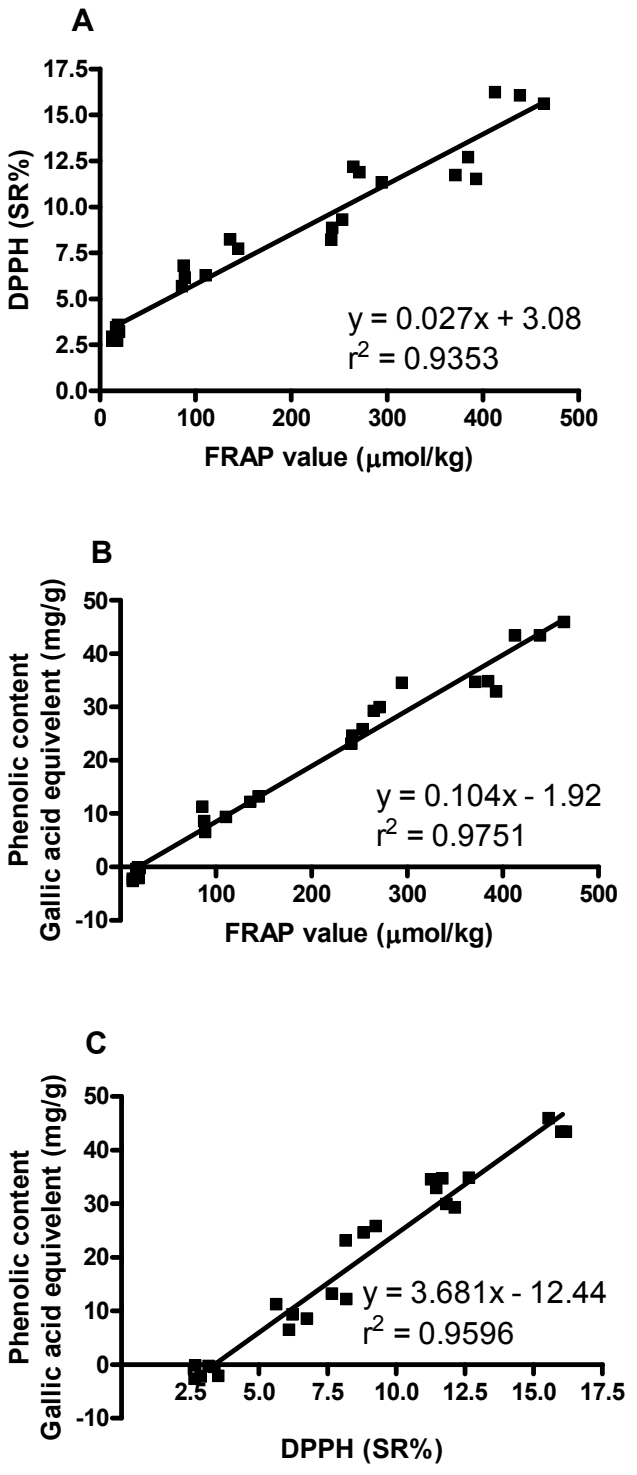


Fig. 21: Correlations between the chemical tests. FRAP Assay vs DPPH Assay (A), FRAP Assay vs Folin-Ciocalteu Assay (B) and DPPH Assay vs Folin-Ciocalteu Assay (C).

3.2.2: Protective effect of Shan Zha extracts against hemolysis caused by AAPH

Table 6: Hemolysis percentage of different Shan Zha extracts.

Hemolysis Percentage (%)	Heated	Unheated
Petroleum Ether	63.73 ± 9.17	53.75 ± 7.64
Ethyl Acetate	62.68 ± 10.05	56.59 ± 7.31
80 % Ethanol	59.46 ± 12.32	33.18 ± 5.64*
Water	61.92 ± 8.99	46.85 ± 8.04
With AAPH only	63.77 ± 5.79	
Without AAPH	2.87 ± 1.62***	

Values are expressed as mean ± S.E.M., n=6. * and *** Differ significantly from the "With AAPH Only" group at $p<0.05$ and <0.001 respectively.

The hemolysis percentages of erythrocyte from pig caused by the AAPH in different groups are shown in Table 6. A significant difference, $p<0.001$, could be observed in AAPH group vs Without AAPH group. In the unheated groups, 80 % Ethanol extract had the lowest hemolysis effect among the extract groups and it was significantly lower than the control group "With AAPH only". No significant difference could be observed among the heated groups. None of the comparisons between the heated and unheated groups of the same extract showed significant difference.

3.3: Examination of antiatherogenic effect of consuming 80 % ethanol extract

3.3.1: Relaxation response with / without endothelium

The result of relaxation responses to cumulative Ach concentrations on aorta with and without endothelium isolated from the N, H, SA and SB rats are shown in Fig. 22. Having endothelium, rats from the N group and the SB group, which consumed the hypercholesterolemic diet with 100 mg/kg 80 % ethanol extract, had a higher percentage relaxation ability than the rats from the H group and SA group, which consumed the hypercholesterolemic diet with 30 mg/kg 80 % ethanol extract. At Ach concentration ranged 0.1 – 0.3 mM, significant differences were observed in the percentage relaxation ability of rats from the N group compared with the H group and the SB group compared with the H group. Without endothelium, no significant difference could be observed among the groups.

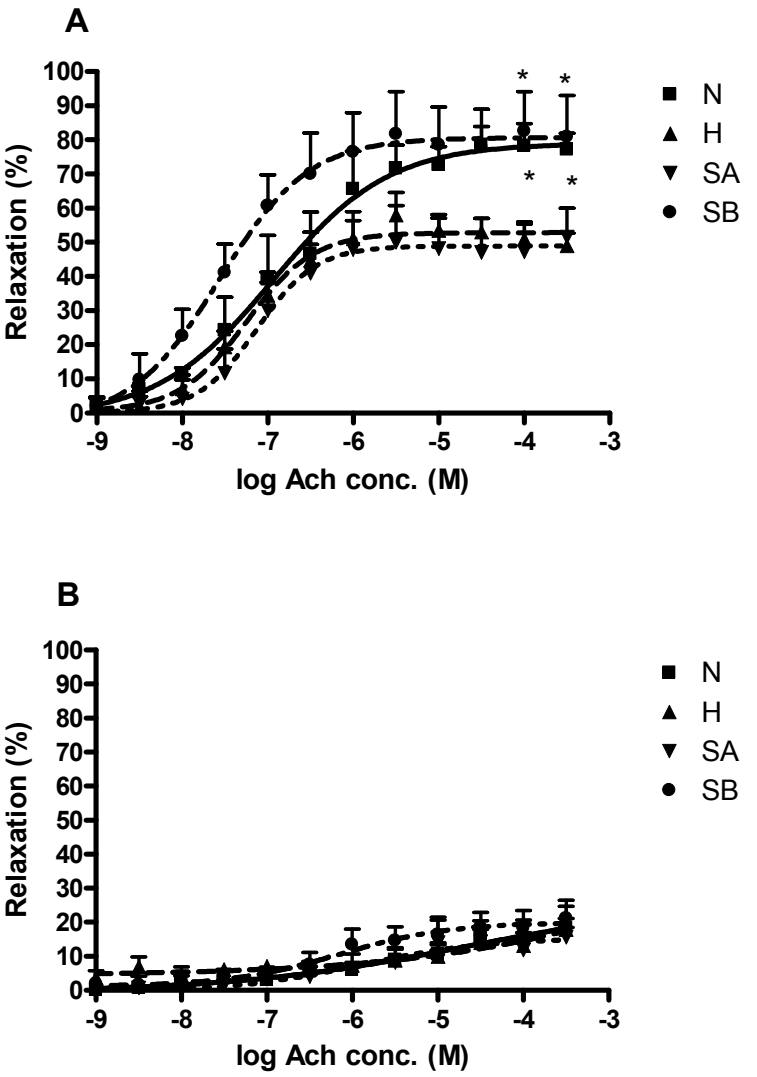


Fig. 22: Relaxation responses to cumulative acetylcholine concentrations of aorta with endothelium (A) and without endothelium (B) isolated from the Normal (N), Hypercholesterolemia (H), Shan Zha 80 % ethanol extract 30 mg/kg (SA) and Shan Zha 80% ethanol extract 100 mg/kg (SB) groups.

Values are expressed as mean ± S.E.M., n=4–5. * Differ significantly from the H group at $p<0.05$.

3.3.2: Determination of lipid content in livers

The appearance of livers of the N, H, SA and SB groups are shown in Fig. 23. Enlarged and yellowish livers were observed in the H, SA and SB groups, while the size and colour of livers from the N group rats remained normal and dark red. The liver weight from the N, H, SA and SB groups are shown in Fig. 24A. The liver weight of the N group was significantly lower than that of the H group (18.71 ± 1.05 g and 27.32 ± 0.80 g respectively). The liver weight of the SA and SB groups do not show any significant difference when compared with the H group. The lipid content of rats from the N, H, SA and SB groups are shown in Fig. 24B. The lipid content of the N group was the lowest 0.041 ± 0.01 g/g, while that of the H, SA and SB groups were high, they were 0.168 ± 0.01, 0.178 ± 0.02 and 0.174 ± 0.02 g/g respectively. A significant difference of $p<0.001$ could be observed when the lipid content of the N group was compared with that of the H group.

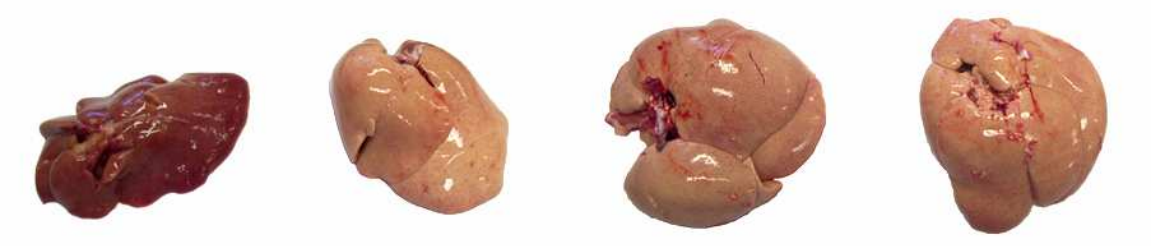


Fig. 23: Appearance of livers from the Normal (N), Hypercholesterolemia (H), Shan Zha 80% ethanol extract 30 mg/kg (SA) and Shan Zha 80% ethanol extract 100 mg/kg (SB) groups.

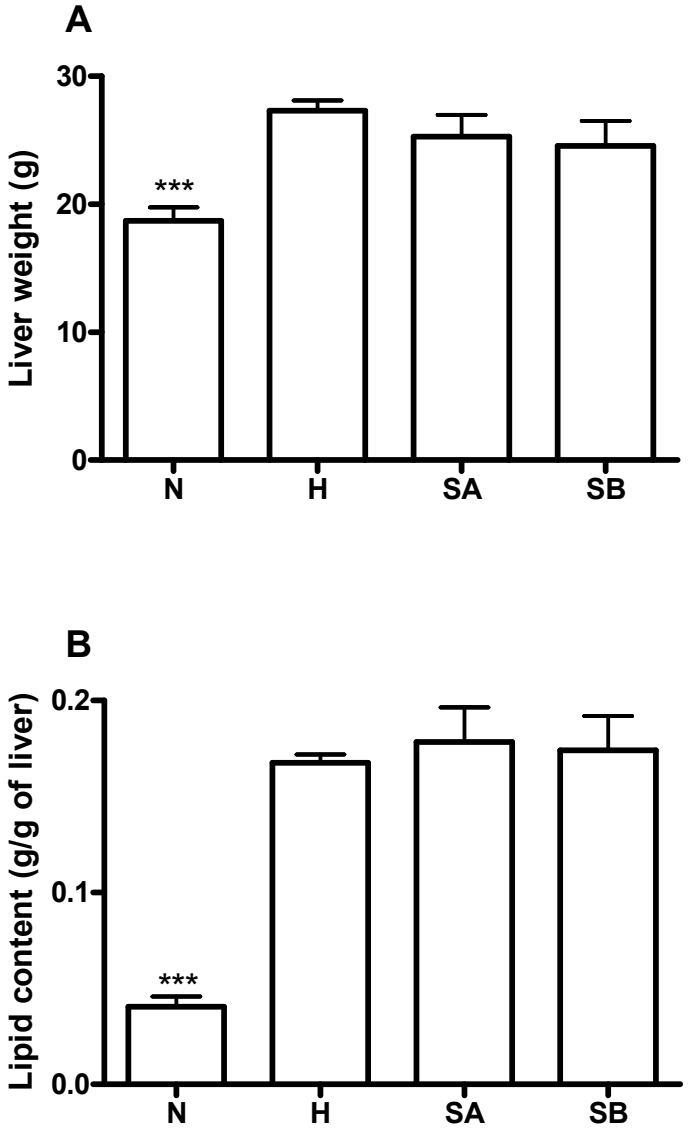


Fig. 24: Liver weight (A) and lipid content (B) of the rats from the Normal (N), Hypercholesterolemia (H), Shan Zha 80% ethanol extract 30 mg/kg (SA) and Shan Zha 80% ethanol extract 100 mg/kg (SB) groups.

Values are expressed as mean ± S.E.M., n=8. *** Differ significantly from the H group at $p<0.001$.

3.3.3: Hypocholesterolemic effect

Plasma cholesterol content: TC, LDL-cholesterol and HDL-cholesterol

Changes in the concentrations of total cholesterol, LDL-cholesterol and HDL-cholesterol of the rat plasma from the N, H, SA and SB groups are shown in Fig. 25. There were nearly no changes in the plasma total cholesterol and LDL-cholesterol content of the N group, while those changes in the H, SA, SB groups were large (Figs. 23 and 24). Significant differences could be observed in the change in the plasma total cholesterol and LDL-cholesterol content when the H group was compared with the N group. The HDL-cholesterol content of the N group increased 0.68 ± 0.16 mM after a 28-day experiment, while that of the H, SA and SB groups decreased 0.61 ± 0.54, 0.37 ± 0.39 and 0.62 ± 0.52 mM respectively. However, no significant difference could be observed among these groups.

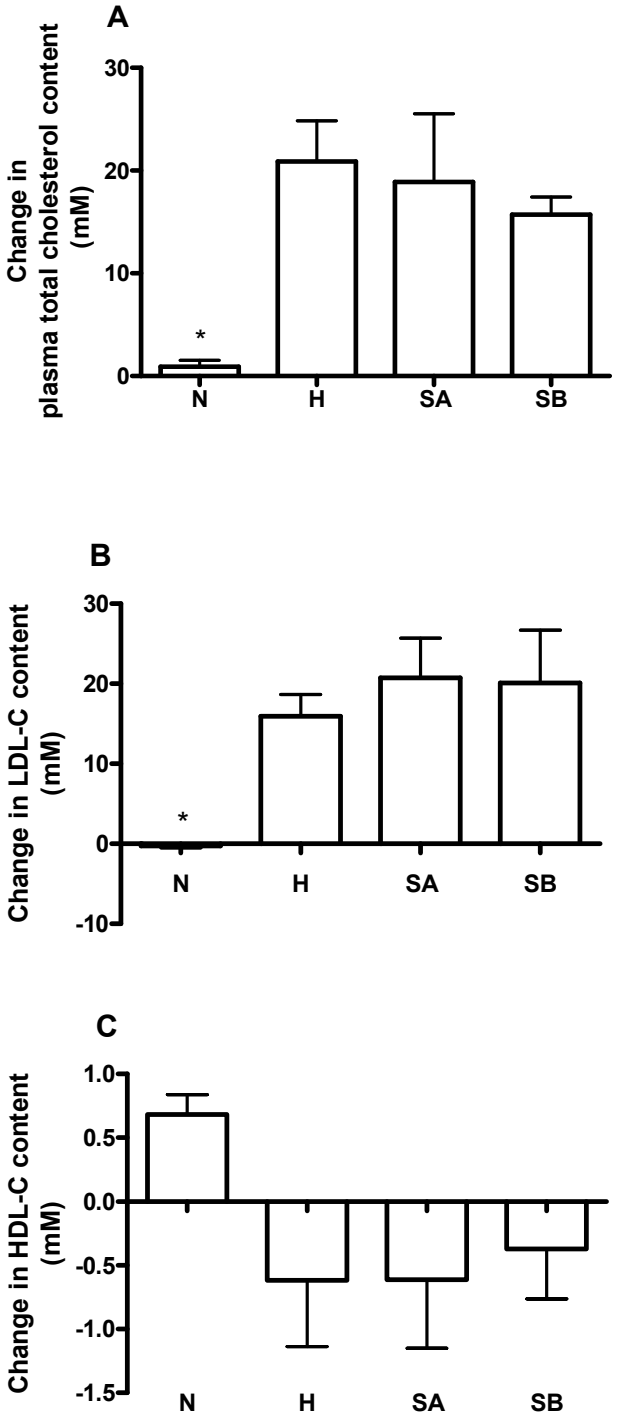


Fig. 25: Changes in the plasma total cholesterol content (A), LDL-cholesterol content (B) and HDL-cholesterol content (C) of the rats from the Normal (N), Hypercholesterolemia (H), Shan Zha 80% ethanol extract 30 mg/kg (SA) and Shan Zha 80% ethanol extract 100 mg/kg (SB) groups.

Values are expressed as mean ± S.E.M., n=4–8. * Differ significantly from the H group at $p<0.05$.

mRNA expression level of LDL receptor

The LDLR expression levels of all the groups were similar and no significant difference was observed among the groups (Fig. 26).

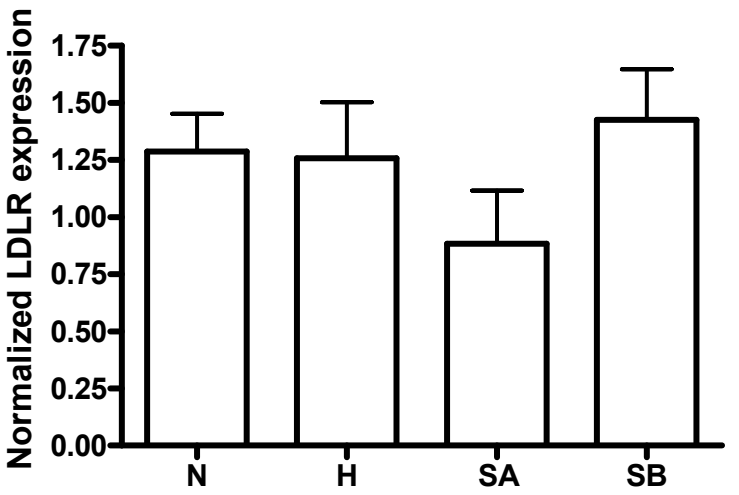


Fig. 26: Gene expression level of LDLR of the rats from the Normal (N), Hypercholesterolemia (H), Shan Zha 80% ethanol extract 30 mg/kg (SA) and Shan Zha 80% ethanol extract 100 mg/kg (SB) groups.

Values are expressed as mean ± S.E.M., n=3.

3.3.4: Apo-B content

Changes in the plasma Apo-B content of the rats from the N, H, SA and SB groups are shown in Fig. 27. The Apo-B content of the N group did not increase after the 28-day experiment, while that of the H, SA and SB groups increased 1.30 ± 0.28, 1.24 ± 0.56 and 1.48 ± 0.57 g/L respectively. However, no significant difference could be observed among these groups.

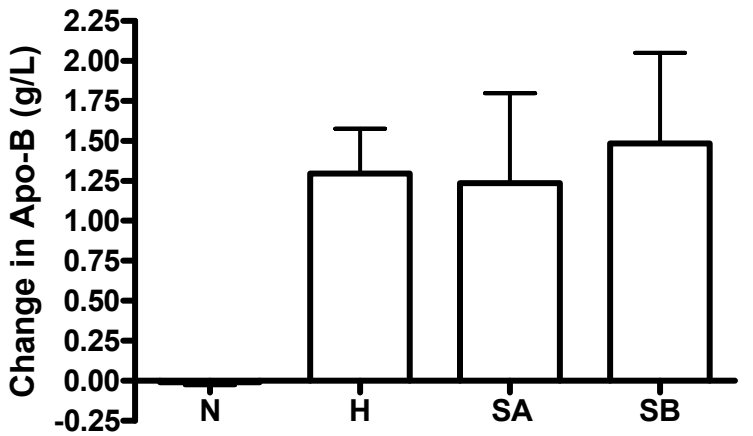
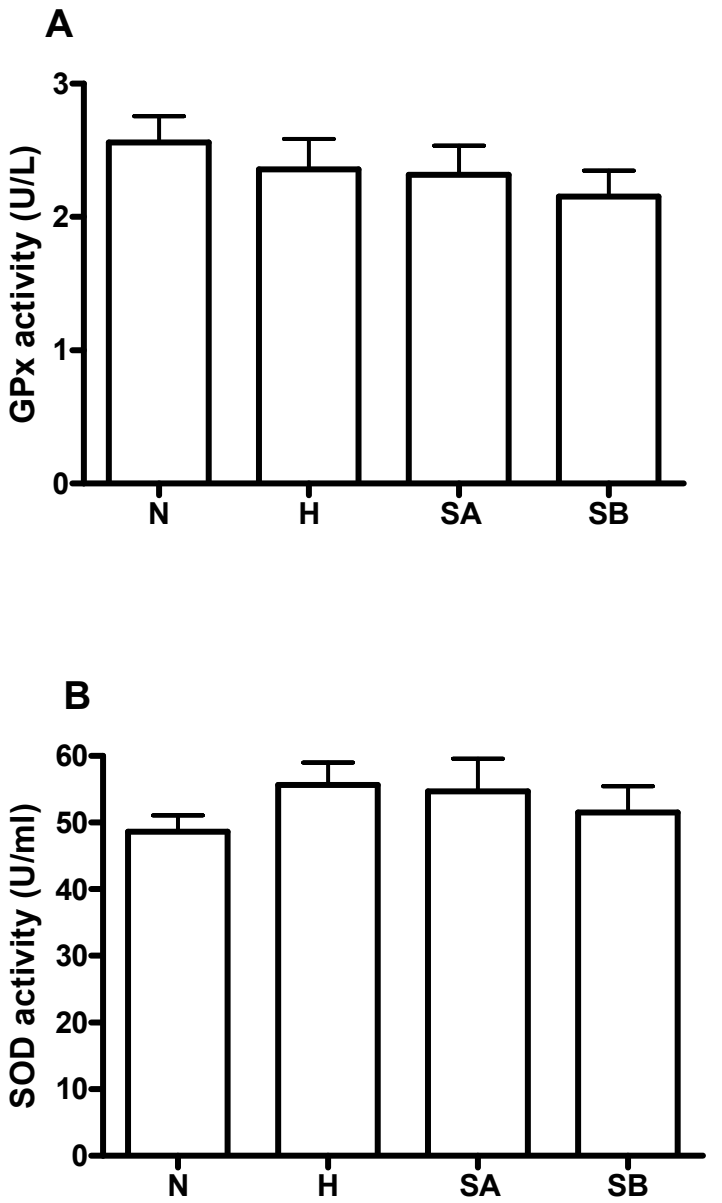


Fig. 27: Changes in plasma Apo-B content of the rats from the Normal (N), Hypercholesterolemia (H), Shan Zha 80% ethanol extract 30 mg/kg (SA) and Shan Zha 80% ethanol extract 100 mg/kg (SB) groups.

Values are expressed as mean ± S.E.M., n=6–7.

3.3.5: Antioxidative enzymes

The plasma GPx, SOD and CAT activities from rats of the N, H, SA and SB groups are shown in Fig. 28. There were no significant differences in the GPx, SOD and CAT activities among the groups.



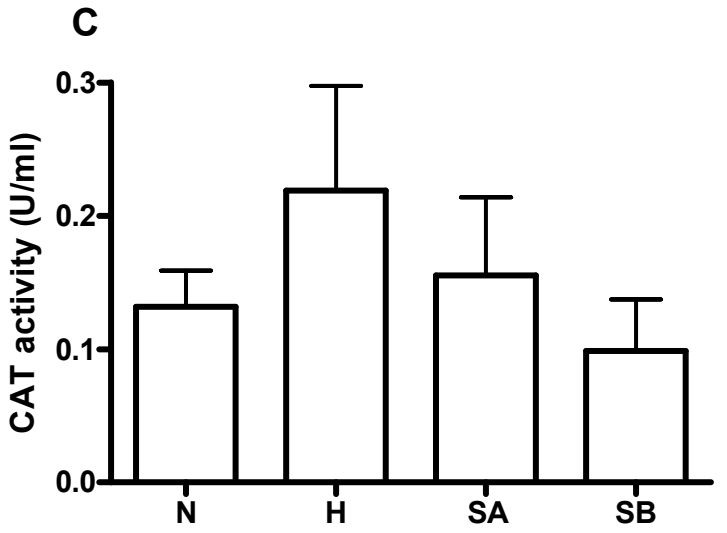


Fig. 28: Glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) activities of plasma from the Normal (N), Hypercholesterolemia (H), Shan Zha 80% ethanol extract 30 mg/kg (SA) and Shan Zha 80% ethanol extract 100 mg/kg (SB) groups.

Values are expressed as mean ± S.E.M., n=8.

3.3.7: Blood pressure

Changes in blood pressure from the N, H, SA and SB groups are shown in Fig. 29. The blood pressure of the N and SB groups decreased, while that of the H and SA groups increased. A significant difference could be observed between the H and SB groups at $p<0.05$.

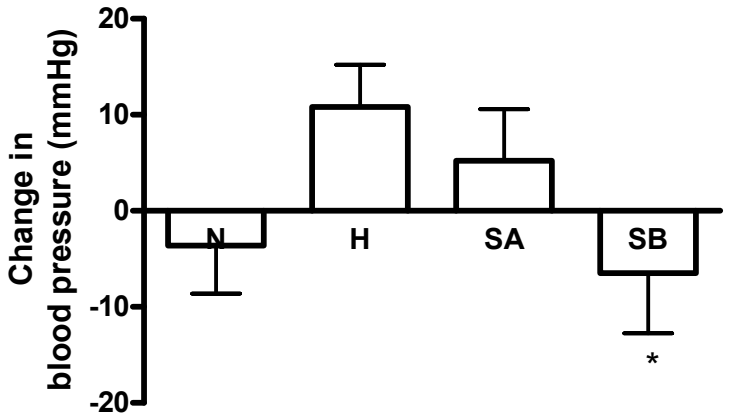


Fig. 29: Changes in blood pressure from the Normal (N), Hypercholesterolemia (H), Shan Zha 80% ethanol extract 30 mg/kg (SA) and Shan Zha 80% ethanol extract 100 mg/kg (SB) groups.

Values are expressed as mean ± S.E.M., n=6–7. * Differ significantly from the H group at $p<0.05$.

3.4: Relaxation response of 80 % ethanol Shan Zha extract on isolated aorta

Ach caused a significant relaxation response with 50 % DMSO in nearly all the concentrations (Fig. 30). However, SZ 80 % Ethanol extract did not cause any significant relaxation in the (0.1 – 10 μM or μg/ml) concentration (Fig. 30).

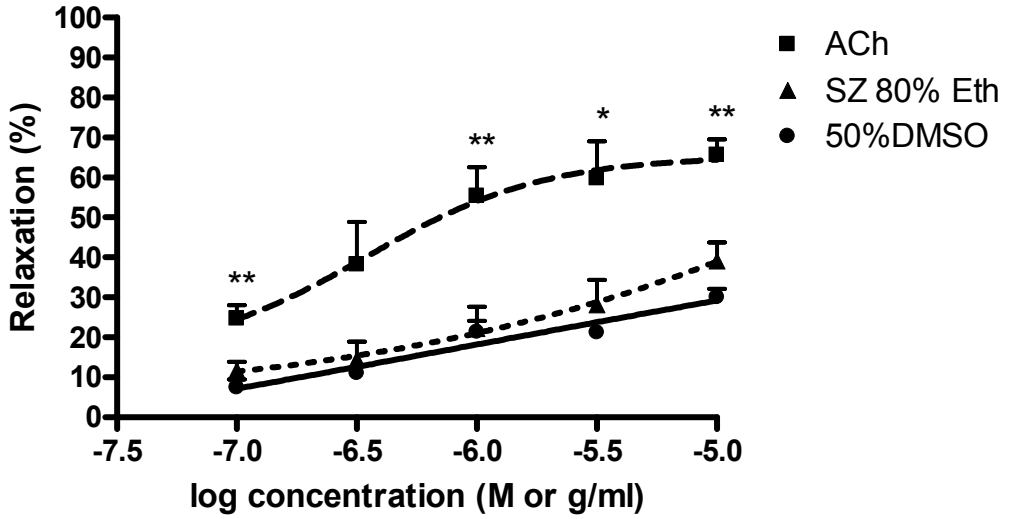


Fig. 30: Relaxation responses to cumulative concentrations (0.1 – 10 μM) of acetylcholine (ACh), (0.1–10 μg/ml) Shan Zha 80 % ethanol extract (SZ 80 % Eth) and 50 % DMSO of aorta with endothelium isolated from rats.

Values are expressed as mean ± S.E.M., n=3. * and *** Differ significantly with 50 % DMSO at $p<0.05$ and 0.001 respectively.

3.5: Chlorogenic acid and quercetin, the ingredients of Shan Zha

3.5.1: Protective effect of chlorogenic acid and quercetin on isolated aorta caused by AAPH

The hemolysis percentages of the different groups are shown in Table 7. AAPH caused a significant increase in the hemolysis percentages from 0.51 ± 0.62 % to 69.72 ± 6.98 % $p<0.001$. Ascorbic acid, chlorogenic acid and quercetin significantly protected blood from hemolysis ($p<0.001$). They protect the erythrocyte at different levels. Chlorogenic acid and quercetin had a greater protection on erythrocyte than ascorbic acid at $p<0.05$ and 0.01 respectively.

Table 7: Hemolysis percentage of different pure compound groups.

	Hemolysis percentage (%)
Ascorbic Acid	$26.81 \pm 8.30^{***}$
Chlorogenic Acid	$6.20 \pm 2.55^{***, \#}$
Quercetin	$1.45 \pm 0.52^{***, \#\#}$
With AAPH Only	$69.72 \pm 6.98^{\#\#\#}$
Without AAPH	$0.51 \pm 0.62^{***, \#\#\#}$

Values are expressed as mean ± S.E.M., n=6. *** Differ significantly with the "With AAPH Only" group at $p<0.001$, $^{\#, \#\#, \#\#\#}$ Differ significantly with the Ascorbic Acid group at $p<0.05$, 0.01 and 0.001 respectively.

3.5.2: Relaxation response of chlorogenic acid and quercetin on isolated aorta with endothelium

The relaxation responses to cumulative concentrations (0.1 – 10 μM) of ACh, chlorogenic acid, quercetin, and 50 % DMSO of aorta with endothelium isolated from rats are shown in Fig. 31. Ach caused a significant difference with 50 % DMSO in all the concentrations. Chlorogenic acid caused a significant relaxation at the concentration of 10 μM. Quercetin did not cause any significant relaxation in the (0.1 – 10 μM) concentrations.

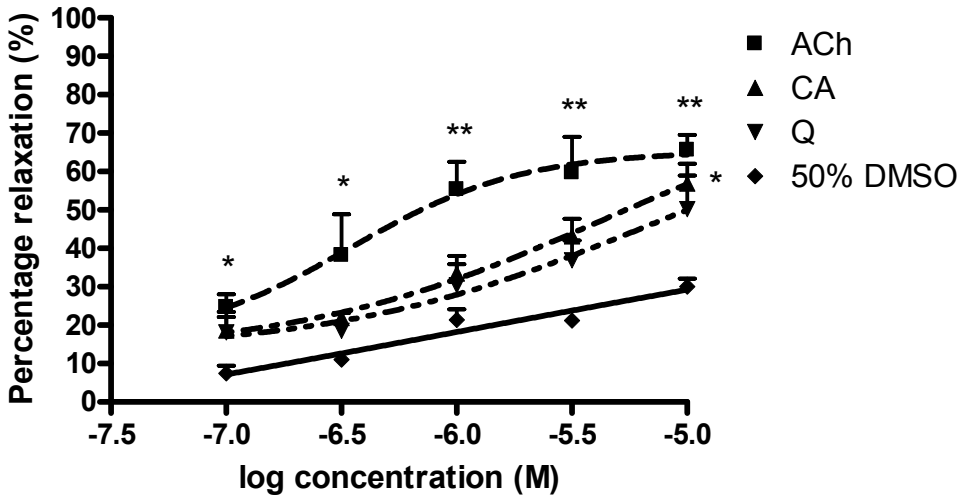


Fig. 31: Relaxation responses to cumulative concentrations (0.1–10 μM) of acetylcholine (ACh), chlorogenic acid (CA), quercetin (Q) and 50 % DMSO of aorta with endothelium isolated from rats. Values are expressed as mean ± S.E.M., n=3. * and ** Differ significantly with the 50 % DMSO at p<0.05 and 0.01 respectively.

3.5.3: HPLC analysis of chlorogenic acid and quercetin in Shan Zha extracts

The absorption spectrum from 190nm to 400nm and the calibration curve of chlorogenic acid and quercetin are shown in Figs. 32 and 33 respectively. The accuracy of chlorogenic acid and quercetin for intra- and interday are around 90 % (Table 8). The HPLC chromatograms of standards and Shan Zha extracts are shown in Fig. 34. The chlorogenic acid contents extracted by different solvents are shown in Table 9. More chlorogenic acid could be extracted without the heat treatment. In the ethyl acetate extract and 80 % ethanol extract, the amount of chlorogenic acid extracted were significantly higher in the unheated treatment at $p<0.01$ and 0.001 respectively. Heated or unheated, chlorogenic acid could be extracted in ethyl acetate, 80 % ethanol and water in descending performance. The quercetin contents extracted by different solvents are shown in Table 10. Under the heat treatment more quercetin could be extracted in the ethyl acetate, than in the 80 % ethanol ($p<0.001$) and 80 % ethanol is preferable to water ($p<0.001$). Apart from the ethyl acetate extracts, the amount of quercetin extracted under the heated and unheated treatment did not show any significant difference.

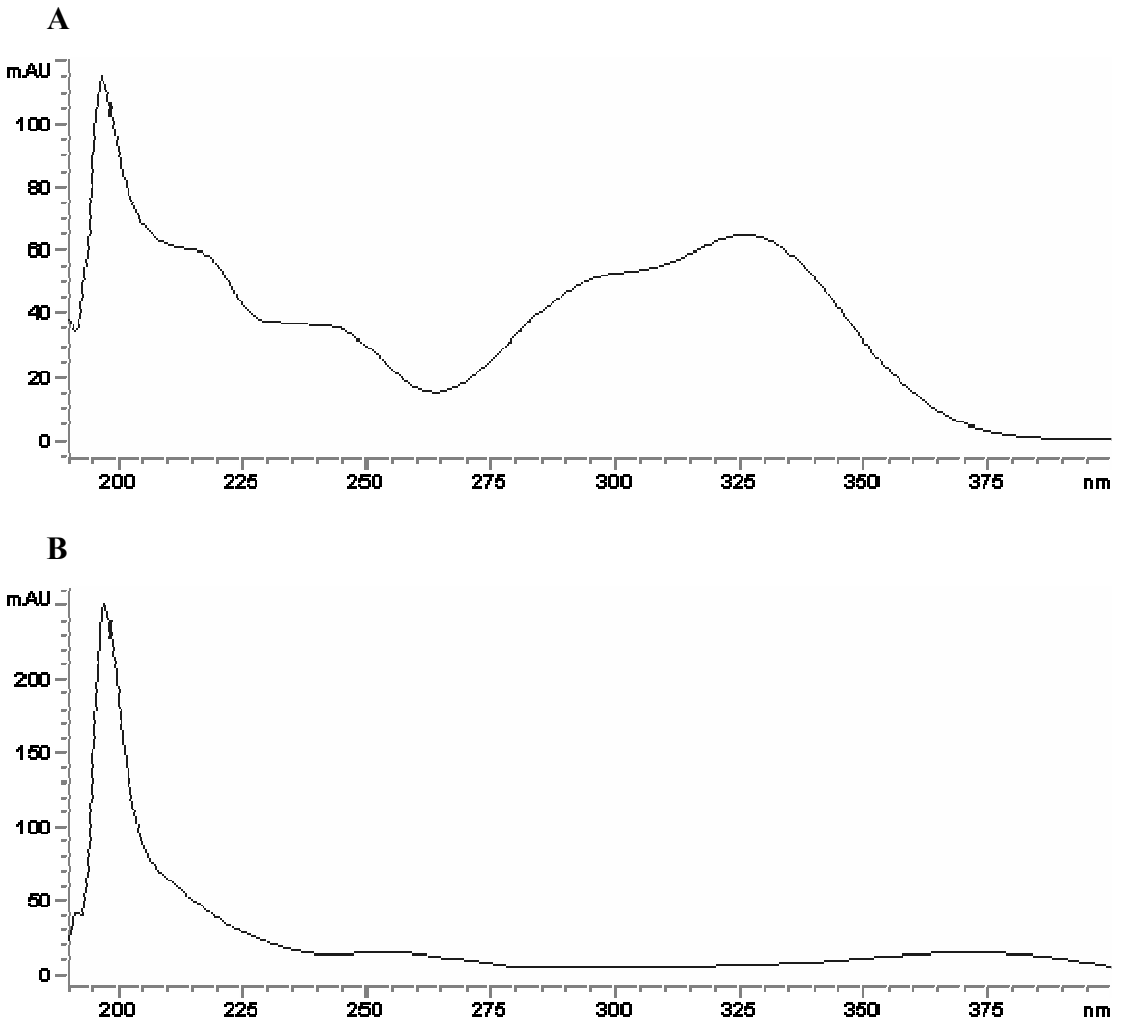


Fig. 32: Absorption spectrums of chlorogenic acid (A) and quercetin (B) from 190nm to 400nm.

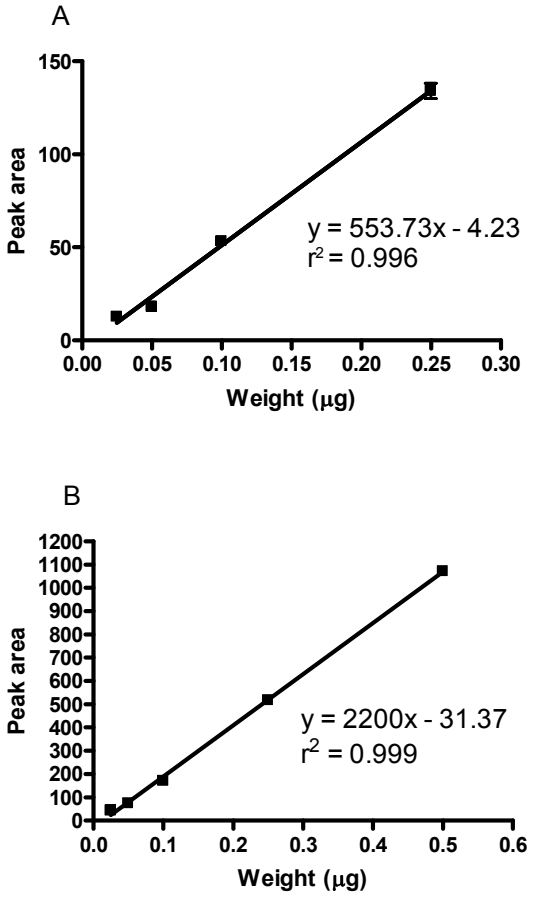


Fig. 33: Calibration curves for chlorogenic acid (A) and quercetin (B).

Values are expressed as mean ± S.E.M., n=3.

Table 8: Intra- and interday variability for the HPLC detection of chlorogenic acid and quercetin.

		Weight injected (µg)	Weight detected (µg)	RSD[a] (%)	Accuracy[b] (%)
Interday	Chlorogenic acid	0.1	0.098 ± 0.008	8.42	101.52
	Quercetin	0.1	0.113 ± 0.003	2.28	87.30
Intraday	Chlorogenic acid	0.1	0.092 ± 0.012	12.54	108.25
	Quercetin	0.1	0.110 ± 0.002	1.49	89.98

Values are expressed as mean ± S.D. n=3.

[a]RSD (%)(Relative standard deviation) = (Standard deviation / mean) x 100 %.

[b]Accuracy (%) = [(1-(mean weight measured – weight spiked) / weight spiked)] × 100 %

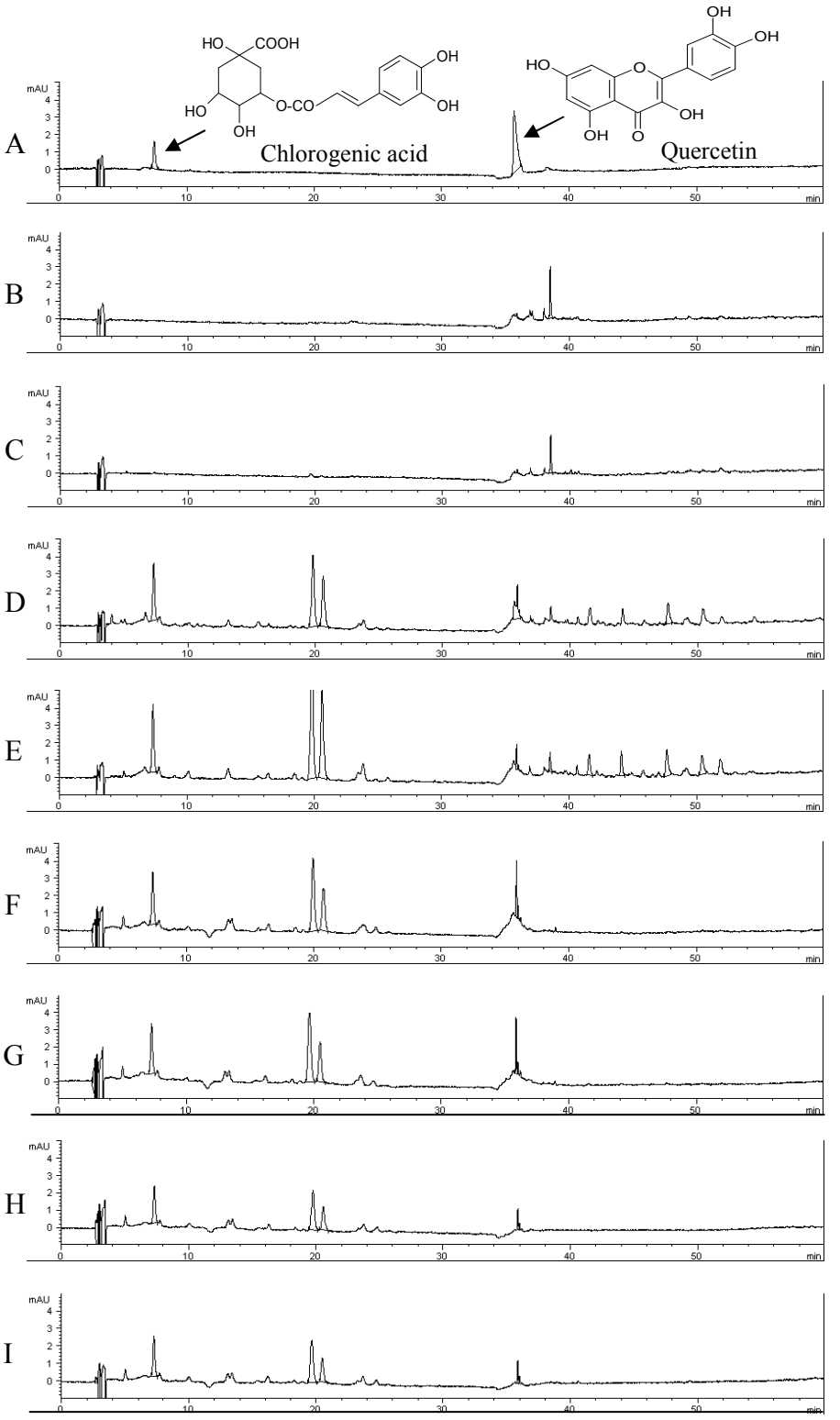


Fig. 34: HPLC chromatograms of the standards chlorogenic acid and quercetin (A), heated petroleum extract (B), unheated petroleum extract (C), heated ethyl acetate extract (D), unheated ethyl acetate

extract (E), heated 80 % ethanol extract (F), unheated 80 % ethanol extract (G), heated water extract (H) and unheated water extract (I).

Table 9: Chlorogenic acid contents (μg/g Shan Zha) of the Shan Zha extracts.

Chlorogenic acid contents (μg/g Shan Zha)	Heated	Unheated	
Petroleum Ether	NA	NA	
Ethyl Acetate	$0.75 \pm 0.02^{\wedge\wedge\wedge}$	$0.85 \pm 0.01^{\#\#\#}$	***
80 % Ethanol	0.62 ± 0.02	0.69 ± 0.02	**
Water	$0.52 \pm 0.01^{\wedge\wedge\wedge}$	$0.53 \pm 0.01^{\#\#\#}$	

Values are expressed as mean ± S.E.M., n=3. NA: immeasurable; ^^^ Differ significantly with the heated 80 % ethanol at $p<0.001$; ### Differ significantly with the unheated 80 % ethanol at $p<0.001$; ** and *** Differ significantly between the heated and unheated group at $p<0.01$ and 0.001 respectively.

Table 10: Quercetin contents (μg/g Shan Zha) of the Shan Zha extracts.

Quercetin contents (μg/g Shan Zha)	Heated	Unheated	
Petroleum Ether	NA	NA	
Ethyl Acetate	$0.25 \pm 0.01^{\wedge\wedge\wedge}$	$0.17 \pm 0.00^{\#\#}$	***
80 % Ethanol	0.20 ± 0.00	0.21 ± 0.00	
Water	$0.17 \pm 0.00^{\wedge\wedge}$	$0.17 \pm 0.00^{\#\#\#}$	

Values are expressed as mean ± S.E.M., n=3. NA: immeasurable; ^^ and ^^^ Differ significantly with the heated 80 % ethanol at $p<0.01$ and 0.001 respectively; ## and ### Differ significantly with the unheated 80 % ethanol at $p<0.01$ and 0.001 respectively; *** Differ significantly between the heated and unheated group at $p< 0.001$.

4: DISCUSSIONS

The present study not only provided evidence that Shan Zha had a beneficial effect on atherosclerosis by improving the vascular elasticity through the vasorelaxation of aorta, but also suggested the possible mechanisms involved in the protective effect.

4.1: Examination of antiatherogenic effect of Shan Zha

This experiment aimed to examine the antiatherogenic effect of Shan Zha. Rats are believed to be an atherosclerosis-resistant species, because they do not have plasma cholesteryl ester transfer protein, and HDL is the major carrier of plasma cholesterol (Moghadasian, 2002). As a result, rats are generally hypo-responsive to dietary cholesterol (Moghadasian, 2002). Nevertheless, rats can still be a model for atherosclerosis experiments, as hypercholesterolemia, hyperlipidemia and atherogenesis can be induced in rats by high-cholesterol / high-fat diets containing cholic acid, which increases cholesterol absorption and decreases cholesterol excretion by a concomitant suppression of CH (Moghadasian, 2002). The diet used

in this study: 2 % cholesterol, 1 % cholic acid and 5.5 % oil is commonly used to induce aortic atherosclerotic lesion in rats and mice (Nakamura *et al*., 1989; Nishina *et al*., 1990).

Hypercholesterolemia was successfully induced in the rats, although the weight of rats from the H and SZ+H groups dropped (Fig. 19A). The liver weight of the H and SZ+H groups were higher (Fig. 19B) and the liver color of them became yellowish after the experiment (Fig. 20). It is believed that the weight of the rats should have been increased in all the groups, especially those fed with the hypercholesterolemic diet (Sato et al., 2002). However, the result from this experiment (Fig. 19A) showed that the liver weight of the rats from the H and SZ+H groups dropped. This might be due to the rats disliking the hypercholesterolemic diet. As a result, they consumed less and their weight dropped. Since the hypercholesterolemic diet was taken, even though the amount was small, the hypercholesterolemic effect still occurred.

The loss of elasticity is one of the symptoms of atherosclerosis, rats from the N group had the highest percentage of relaxation ability, followed by rats from the SZ+H group, while rats from the H group had the lowest percentage of relaxation

ability (Fig. 18) and the N and H groups had a significant difference at $p<0.05$. However, no significant difference could be observed in the contraction response. This indicates that consuming Shan Zha showed a tendency to reduce the atherogenic effect caused by the hypercholesterolemic diet in terms of relaxation ability. This effect might not be due to the hypolipidemic effect of Shan Zha because the liver weights of the H and SZ+H groups were significantly higher than that of the N group (Fig. 19). The heavy liver weight might be due to the lipid deposition in liver (Fig. 20). This indicated that the hypolipidemic effect of Shan Zha cannot be observed in this study. Our results were not in agreement with Zhang and co-workers' (2002b), in which New Zealand white rabbits fed with 2 % Shan Zha and 1 % cholesterol diet showed a hypolipidemic effect.

The reasons that our results do not agree with Zhang and co-workers' (2002b) is due to the species difference of the animal model. Different animal model may cause different results. Apart from this, in order to induce hypercholesterolemia in the rats, cholic acid was added to the diet, which affects the metabolism of cholesterol in the rat model.

In the above experiment, Shan Zha showed a tendency to reduce the atherogenic effect caused by the hypercholesterolemic diet in terms of the relaxation ability. However, this trend was not significant. A larger dosage or using an extract which involves more active ingredients may help improve the effect. Furthermore, in order to determine the relation between the antiatherogenic effect and the antioxidative effect of Shan Zha, finding out the most antioxidative extract of Shan Zha was required. Since the preparation of many Chinese medicines involved heating, the antioxidative power of Shan Zha extract under heat treatment was also examined.

4.2: Chemical tests for measurement of antioxidative power of Shan Zha

The oxidation of LDL is a crucial stage for the initiation of atherosclerosis (Betteridge *et al*., 1999). Antioxidant and antioxidative enzymes delay or prevent the oxidation of an oxidisible substrate like LDL. The current chemical studies aimed to find out the most antioxidative extract and the effect of heating on the antioxidant, because heating is a common preparation procedure for Chinese medicine.

In this study, the antioxidative power was measured by three chemical tests, the FRAP Assay, the DPPH Assay and the Folin-Ciocalteu Assay, which measured the red-ox reaction, free radicals scavenges capacity, and the amount of the phenolics content respectively. All the chemical tests showed that most antioxidant dissolved in 80 % ethanol and these antioxidants could be affected by heating (Tables 3–5). A positive co-relation could be found between each test (Fig. 21). The antioxidant power (from FRAP Assay) and the free radical scavenging activity (from DPPH• Assay) dropped together with the phenolics content (from Folin-Ciocalteu Assay), which implies that the antioxidants from the Shan Zha extracts are mainly phenols.

Different researchers have different opinions of whether the heating of Shan Zha affects the antioxidant contents. It is suggested that heat treatment of apple juice helps prevent the polyphenols from oxidation, because heat inactivates the enzyme polyphenol oxidase, so oxidized products are hardly formed. As a result, a larger part of the polyphenols originally present in apples can be preserved (Addie *et al*., 2004). Furthermore, heat treatment of ginseng at a temperature and under pressure higher than those applied to the conventional preparation of red ginseng, gives rise to an increased production of saponins, such as Rg_3, Rg_5, Rg_6, Rh_2, Rh_3, Rh_4, and Rs_3, which are absent or present in trace amounts in conventional white or red

ginseng (Keum *et al*., 2000). These results suggested that the antioxidant contents is richer after heating. However, it is also suggested that the heat treatment of onion causes the loss of polyphenols (Ioku *et al*., 2001). Boiling causes the phenolic compounds of olive oil transformed to simple phenols in the water phase and it also causes a rapid degradation of α-tocopherol and the glyceridic fraction of the oil (Manuel *et al*., 2002). These suggest that after heat treatment, the phenolics contents will be lost. Since the result from the Folin-Ciocalteu assay showed that the phenolic content of the unheated 80 % ethanol and water extract was significantly higher than those with the heat treatment, as a result, antioxidant contents in Shan Zha could be affected by heat treatment.

4.3: Protective effect of Shan Zha extracts against hemolysis caused by AAPH

The chemical tests measured the antioxidant levels in a chemical way. However, they could not represent the antioxidative power in a biological system. In order to measure the antioxidant power of the extract in a biological system, erythrocyte was used. Erythrocyte was chosen because the erythrocyte membrane is rich in PUFA, which is very susceptible to free radical mediated peroxidation (Ma *et al*., 2000) and

causes hemolysis, while antioxidant can prevent hemolysis. The erythrocyte is an ideal model for this study, because it neither has nucleus, mitochondria nor other subcellular structures, so less substance is going to affect the system. (Olinescu and Smith, 2002).

It is indicated that the 80 % ethanol extract of Shan Zha was the best protective extract against hemolysis (Table 6). However, there was no significant difference between the heated and unheated groups. This means that the antioxidant which showed an antioxidative activity in the unheated extract in the FRAP Assay, did not show any protective effect in this *in vitro* test. The protective effect may be due to some specific ingredients of Shan Zha.

4.4: Examination of antiatherogenic effect of consuming 80 % Shan Zha extract

Since all the results from the antioxidative power tests indicated that 80 % ethanol Shan Zha extract had the highest antioxidative power, this extract was applied to the *in vivo* system to examine the antiatherogenic effect.

4.4.1: Relaxation Response of aorta with / without endothelium of rat which consumed 80 % Shan Zha extract.

A lack of vasoelasticity is one of the symptoms of atherosclerosis. Rats from the N and SB groups, which consumed a normal diet and 100 mg/kg 80 % ethanol Shan Zha extract with the hypercholesterolemic diet respectively, had the highest percentage of relaxation ability, while rats from the H group and the SA group, which consumed the hypercholesterolemic diet and 30 mg/kg 80 % ethanol Shan Zha extract with the hypercholesterolemic diet respectively, had the lowest percentage relaxation ability (Fig. 22). There was a significant difference between the N and SB groups and with H group at $p<0.05$ at Ach concentrations of 0.1 mM and 0.3 μM, while the SA group did not show any significant difference with the H group. This indicates that the consumption of Shan Zha helps reduce the atherogenic effect caused by the hypercholesterolemic diet in terms of the relaxation ability in a concentration-dependent manner.

Endothelial dysfunction reduces the elasticity of blood vessels, as EDRF like NO are produced and released in the endothelial cells (Behrendt and Ganz, 2002;

Mitani and Kimura, 2003). In order to examine the effect of NO, endothelium of the aortas were removed, and the vascular elasticity in terms of relaxation was performed once more. No significant difference could be observed among the groups (Fig. 22). The relaxation ability was significantly lower when the endothelial cell was removed in the N group and the group with Shan Zha consumption. However, there was no significant difference of those with or without endothelial cell of the H group. This indicates that the protective effect was related to the endothelium cell. The reduction of relaxation ability of the H group might be due to the damage of the endothelial-dependent vasodilator. The high cholesterol content in the hypercholesterolemic diet caused an increase in the LDL-cholesterol concentration. Lipid peroxidation occurred under the attack of free radicals. As a result, the endothelial cell was damaged and endothelial dysfunction occurred. The dysfunction of the endothelium decreased the release of EDRF like NO. As a result, the relaxation ability of the smooth muscle decreased. In order to find out whether the LDL content was increased for oxidation, the effect of Shan Zha on the blood cholesterol content and the antioxidative enzyme activities were measured.

4.4.2: Relation between atherosclerosis and hypolipidemic / hypocholesterolic effect of consuming 80 % ethanol Shan Zha

Many products of Shan Zha claim that Shan Zha has hypocholesterolemic and hypolipidemic effects, which prevent atherosclerosis. A limited study of the relation between them has been performed. Hypercholesterolemia is one of the risk factors of atherosclerosis, thus preventing hypercholesterolemia could prevent atherosclerosis. However, the beneficial effect of atherosclerosis may not be necessarily due to the hypocholesterolemic effect, it may involve some other mechanisms. This is the first study to indicate that the mechanism of the cardiovascular protective effect of Shan Zha is not due to the presence of the hypocholesterolemic effect. Fatty liver is one of the risk factors of atherosclerosis (Brea, *et al*., 2005). The formation of fatty liver is due to excessive fat deposits (>5 % lipid in liver) in the hepatocytes (Kurihara *et al*., 1994). Enlarged and yellowish livers were observed in the H, SA and SB groups, while the size and colour of livers from the N group rat remained normal and dark red (Fig. 24). The weight of liver from the N group was significantly less than that of the H group (18.71 ± 1.05 g and 27.32 ± 0.80 g respectively), while the liver weights of the SA

and SB groups did not show any significant difference with that of the H group. The lipid content of the N group was the lowest 0.041 ± 0.01 g/g, while that of the H, SA and SB groups were high, they were 0.168 ± 0.01, 0.178 ± 0.02 and 0.174 ± 0.02 g/g respectively. A significant difference $p<0.05$ could be observed when the lipid content of the N group was compared with that of the H group. A hypolipidemic effect of Shan Zha could not be observed in this study.

LDL is the major carrier of cholesterol in blood (Zhao and Shen, 2005). The oxidation of LDL initiates endothelial dysfunction and thus atherosclerosis (Steinberg *et al*., 1989). In our study, there was almost no change in the plasma total cholesterol and the LDL-cholesterol content of the N group, while a large increase of the plasma total cholesterol and LDL-cholesterol contents in the H, SA, SB groups were observed (Fig. 25). Significant differences could be observed in the change in the plasma total cholesterol and LDL-cholesterol content when the H group was compared with the N group. The HDL-cholesterol content of the N group increased 0.68 ± 0.16 mM after the 28-day experiment, while that of the H, SA and SB groups decreased, but no significant difference could be observed among these groups. Hypocholesterolemic effect of Shan Zha was not expressed in this experiment.

This result does not agree with the result done by Chen and co-workers (1995). They claimed that SD rats consuming 560 μg Shan Zha flavones containing drink had a significantly lower body weight and body fat than the rats consuming tap water and sugar drink instead. Although the results of these two experiments are not directly comparable, as the diet which caused the hypercholesterolemic effect and the amount of Shan Zha applied are not the same, both of them should have a hypolipidemic effect at different levels. Our results do not agree with the results from Rajendran and co-workers (1996), who showed that the liver total cholesterol level decreased significantly after consuming 6-week tincture of Shan Zha by Wistar rats, in which Shan Zha inhibited the absorption of cholesterol. Zhang *et al*. (2002a) reported that Shan Zha causes a hypocholesterolemic effect by an up-regulation of CH. Since cholic acid was used in this study, which caused a hypercholesterolemic effect by increasing the cholesterol absorption and decreasing the cholesterol excretion by a concomitant suppression of CH (Moghadasian, 2002). As a result, Shan Zha could not induce a hypocholesterolemic effect in rats taking cholic acid.

4.4.3: mRNA expression level of LDL receptor

Since cholesterol is the major component of LDL, the metabolism of cholesterol is related to the amount of LDL, which can be oxidized and form atherosclerosis. In this study, the mRNA expression level of LDL receptor (LDLR) was determined.

No significant difference can be observed in the LDLR expression from the N, H, SA and SB groups (Fig. 26). Therefore, it is believed that the hypercholesterolemic diet and Shan Zha extract do not affect the expression of LDLR.

4.4.4: Effect of Shan Zha on Apo-B content

Apo-B is essential for the binding of LDL particles to the LDL receptor, allowing cells to internalize LDL and thus absorb cholesterol. Only one Apo-B molecule is present in each lipoprotein particle (Walldius and Jungner, 2004) and therefore the total Apo-B value indicates the total number of potentially atherogenic lipoproteins. In our result, the Apo-B content of the N group did not increase after

the 28-day experiment, while that of the H, SA and SB groups increased 1.30 ± 0.28, 1.24 ± 0.56 and 1.48 ± 0.57 g/L respectively. However, no significant difference could be observed among these groups (Fig. 27). This result was in agreement with the change in the plasma LDL-cholesterol (Fig. 25). There was almost no change in the Apo-B and LDL-cholesterol in the N group, while the Apo-B content increased with LDL-cholesterol in the H, SA and SB groups.

4.4.5: Effect of Shan Zha on antioxidative enzyme activities

ROS is generated from all aerobic cells through mitochondrial respiration (Cimino, 1989; Leng *et al*., 1994), oxidative degradation of acyl chain of xenobiotics, dicarboxilic and monounsaturated acid by peroxisomes (Olinescu and Smith, 2002), phagocytosis (Olinescu and Smith, 2002) and the peroxidation of PUFA (Vaya and Aviram, 2003). LDL can be oxidized by ROS (Steinberg *et al*., 1989), while ROS in tissues can be cleared by antioxidant enzymes, including SOD, which transforms superoxide to hydrogen peroxide; CAT, which transforms hydrogen peroxide to water and oxygen and GPx, which transforms hydrogen peroxide and glutathione to water and oxidized glutathione. In this study, the GPx, SOD and CAT activities of the plasma from rats of the N, H, SA and SB groups did

not show any significant difference (Fig. 28). This indicates that the effect of Shan Zha on the antioxidative enzyme activities could not be confirmed. Although there was an absence of changes in the antioxidative enzyme activities in our experiment, the amount of oxidant reduced after the ingestion of the garlic extract (Durak *et al*., 2004), which is also a common traditional Chinese medicine rich in antioxidants, and thus might help prevent atherosclerosis.

Among the enzymes SOD, CAT and GPx, SOD is the most important for the prevention of atherosclerosis. Oxidized LDL increases the level of superoxide in endothelial cell culture (Galle *et al*., 1999), while the apoptosis or proliferation caused can be inhibited by SOD (Vergnani *et al*., 2000).

Since CAT and GPx share the same substrate H_2O_2, through unknown mechanism, under oxidative stress, the activity of GPx decreases, while that of CAT increases. It is suggested that the increase in CAT activity may be a compensatory mechanism against the lowered GPx activity (Durak *et al*., 2004).

4.4.6: Effect of Shan Zha on blood pressure

Risk factors of atherosclerosis such as hypercholesterolemia and hypertension are associated with an impaired vasodilatory response (Tuteja *et al*., 2004; Kawashima, 2004). Our result is in agreement with the above observations. The highest relaxation response was obtained from the SB group, which consumed 100 mg/kg 80 % ethanol Shan Zha extract with the hypercholesterolemic diet (Fig 22), while this group had the greatest decrease in blood pressure in the 28-day experiment (Fig. 29). Rats from the H and SA groups, which consumed the hypercholesterolemic diet and 30 mg/kg 80 % ethanol Shan Zha extract with the hypercholesterolemic diet respectively, had the lowest relaxation response (Fig. 22), while their increase in blood pressure were the highest (Fig. 29). High blood pressure causes macrophages and T-cells grow easily in the vessel, which can cause endothelial dysfunction, the initiation step for atherosclerosis (Betteridge *et al*., 1999), while the endothelial dysfunction caused abnormalities of endothelial cells, affecting the release of NO, the vasodilator, which is considered to be the main factors responsible for the enhancement of the total systemic vascular resistance, leading to an increase in the arterial blood pressure (Tuteja *et al*., 2004).

4.5: Relaxation response of 80 % ethanol Shan Zha extract on isolated aorta

The antiatherogenic effect of the consumption of Shan Zha is presented in part 3.1.1. In order to examine whether ingredients from this extract cause direct relaxation on aorta, the 80 % ethanol Shan Zha extract was directly applied to the isolated aorta without consumption. It is indicated that the 80 % ethanol Shan Zha extract did not cause any relaxation response in the isolated aorta (Fig. 30), which was not in agreement with the study performed by Kim and co-workers (2000). They claimed that applying tincture Shan Zha (a mixture of *Crataegus oxyacantha*, L. and *C. monogyna* Jacq.) extract 1–100 μM to the isolated aorta from SD rats, precontracted with 1 μM phenylephrine in the presence of 10 μM indomethacin, significantly caused vasorelaxation. There are some differences between our and their experiments. Apart from the different species of Shan Zha used, the solvent used for extraction were also not the same. In our experiment, 80 % ethanol was used, while they used methanol. They further purified the extract before applied to the aorta, and only the most effective fractions were shown, while we did not further extract it. So theoretically, although the concentration added was the same, the concentration in terms of effective ingredients was not the same. In addition, the

concentration that started to have a relaxation effect was 0.3 mM, while our addition of sample terminated at 0.01 mM, so our sample may not be concentrated enough. As a result, our data was not comparable to theirs.

4.6: Chlorogenic acid and quercetin content of Shan Zha extract

Chlorogenic acid and quercetin are two of the antioxidant ingredients of Shan Zha (Zhang *et al*., 2001). Both chlorogenic acid and quercetin protected the erythrocyte against hemolysis better than ascorbic acid, with quercetin more efficient than chlorogenic acid (Table 7). However, the unheated 80 % ethanol extract was the most protective extract, instead of the heated ethyl acetate extract (Table 6), which contained more quercetin. It is suggested that antioxidants in the 80 % ethanol unheated extract is more antioxidative than the ethyl acetate heated extract.

The ingredients of Shan Zha may have a direct effect of vasorelaxation. Chlorogenic acid caused a significant relaxation at concentration 10 μM, while quercetin did not cause any significant relaxation response. Chlorogenic acid did not

increase the eNOS promoter activity and the eNOS mRNA expression in human endothelial cell culture (Li *et al*., 2004), so it is believed that chlorogenic acid would not cause relaxation response. In this study, significant relaxation was observed *in vitro* test, but it may not represent the condition *in vivo*.

In order to develop a method for the detection of chlorogenic acid and quercetin, An HPLC system was developed. In this study, the quantity of chlorogenic acid and quercetin in each extract was analyzed. More chlorogenic acid could be extracted without the heat treatment. Whether heated or unheated, chlorogenic acid could be extracted in ethyl acetate, 80 % ethanol and water in descending performance (Table 9). For quercetin, the same results can be achieved when under heat treatment. Apart from the ethyl acetate extracts, the amount of quercetin extracted under heated and unheated treatments did not show any significant difference (Table 10). Generally, more chlorogenic acid and quercetin could be extracted in the ethyl acetate layer. It was not in agreement with the chemical test results for antioxidant, in which the unheated 80 % ethanol extraction was the most antioxidative. This indicated that apart from chlorogenic acid and quercetin, 80 % ethanol had a higher amount of other antioxidants, or some antioxidants in this layer were better than chlorogenic acid and quercetin.

5. CONCLUSION

CVDs are the major cause of morbidity and mortality in the developed world. Among the CVDs, atherosclerosis is the most serious because it can lead to other severe diseases such as stroke, heart attack and coronary heart disease. Shan Zha has long been a folk medicine in China, Europe and North America. Many medical functions of Shan Zha are related to CVDs. Since the mechanisms involved are not fully understood, this low-cost and effective use of Shan Zha is not widely accepted. If the detailed mechanisms involved are unveiled, people would become more confident to use Shan Zha as a food supplement in their daily lives. In addition, more potential ingredients from Shan Zha can be identified, isolated and purified for the medication development for atherosclerosis. This study aimed to study the mechanisms on the antiatherogenic effect of Shan Zha.

In the preliminary study of this project, the consumption of Shan Zha by rats was found to show a tendency to reduce the atherogenic effect caused by the hypercholesterolemic diet. Three groups of male Sprague-Dawley rats (n=7) were fed a normal diet, a hypercholesterolemic diet, and a hypercholesterolemic diet supplemented with 2 % Shan Zha powder respectively for four weeks. The

relaxation percentage of the rat isolated aortas from the normal group was significantly higher than that of the hypercholesterolemic group (p<0.05) at the acetylcholine concentration greater than 100 μM and the Shan Zha group showed a tendency to reduce the atherogenic effect of the hypercholesterolemic diet. However, there is no difference in the contraction response among the groups, indicating that vasoconstriction is independent for the antiatherogenic effect.

Antioxidant is believed to be antiatherogenic. Shan Zha was divided into groups with and without heat treatment for extraction in different solvents. Chemical tests for antioxidant showed that most antioxidant could be extracted in 80 % ethanol extract and the heat treatment might damage the phenolic contents. Thus, the 80 % ethanol extract without heat treatment was used for the mechanistic studies.

Consuming this 80% ethanol Shan Zha extract for twenty-eight days significantly prevented hypertension and the loss of vascular elasticity caused by the hypercholesterolemic diet on rats. These effects were in a concentration-dependent manner. The protective effect existed without the presence of any hypocholesterolemic effect. The plasma total cholesterol and low density lipoprotein of rat consuming Shan Zha was as high as those which consumed the

hypercholesterolemic diet only. The protective effect was independent of the increase of the antioxidative enzyme activities, as there were no significant differences in the superoxide dismutase, catalase and glutathione peroxidase activities among the groups. Furthermore, the relaxation ability was significantly lower when the endothelial cell was removed in the normal group and the group with Shan Zha consumption. However, there was no significant difference of those with or without endothelial cell of the hypercholesterolemia group. This indicated that the protective effect was related to the endothelium cell.

In this project, the antiatherogenic effect was measured basing on the enhancement of relaxation ability. However, ingredients of Shan Zha might directly cause vessel relaxation. In order to further examine whether the ingredients of Shan Zha caused vessel relaxation directly, the 80 % ethanol Shan Zha extract, chlorogenic acid and quercetin, two of the flavonoids found in Shan Zha were added instead of acetylcholine to the isolated aorta. Chlorogenic acid significantly caused vessel relaxation at 10 μM. A reliable HPLC analysis method was also developed for quantifying chlorogenic acid and quercetin.

5.1: Recommendations for future work

This study indicated that the cardiovascular protective effect of Shan Zha exists without the presence of the hypocholesterolemic effect of Shan Zha and is independent of the enhancement of the antioxidative enzyme activities. Since oxidative LDL causes endothelial dysfunction, an early phenomenon of atherosclerosis, antioxidants from Shan Zha may protect the endothelium and thus protect the blood vessel. Furthermore, chlorogenic acid, an ingredient of Shan Zha, may enhance the relaxation ability of aorta and prevent the blood vessel from hardening.

The antiatherogenic effect of consuming 80 % ethanol Shan Zha extract is shown, which is believed to be related to the endothelium and deduction of blood pressure. However, the detailed mechanisms involved are not fully understood, the involvement of eNOS and the release of NO should be further investigated.

In this study, chlorogenic acid, an ingredient of Shan Zha, caused relaxation response in the isolated aorta with endothelium. There are many potential ingredients found in Shan Zha. In this study, only 2 ingredients were screened for

their pharmacological effect. More ingredients of Shan Zha should be examined for the pharmacological effect for antiatherosclerosis.

An antiatherogenic effect was observed in rat, but this *in vivo* model may not represent the condition in human. The toxicity of Shan Zha is not well-addressed in literature. As a result, after the pre-clinical and toxicity studies, clinical studies should be performed.

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