# 行政院國家科學委員會專題研究計畫成果報告

中文題目：抑制 HMG-CoA Reductase 單寧成份之研究

英文題目：The In Vitro Inhibitory Effect of Flavonoid Astilbin on 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase on Vero Cells

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主持人：陳保羅

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## 中文摘要

動脈硬化相關疾病在許多國家仍是最重要致死原因。高脂血症是導致動脈硬化的重要因子。降血脂藥如 HMG-CoA reductase 抑制劑，可降低冠狀動脈疾病死亡率。Flavonoids 是富含於蔬果，紅酒及綠茶中天然抗氧化物質。以前的研究顯示一些抗氧化劑有降血脂的效果而 flavonoids 服用會降低冠狀動脈疾病死亡率。本研究的目的即探討從中草藥分離出的 Flavonoids 成份是否有抑制 HMG-CoA reductase 的作用。

培養中的非洲綠猴腎細胞(Vero Cell Lines)是過去研究 HMG-CoA reductase 抑制劑的材料，在加入 Pravastatin 之後，細胞生長會被抑制。但在加入 1 mM mevalonate 之後，這種抑制作用會減少。從中草藥分離出來的 flavonoid compounds (flavonols, flavones, flavanones)加入含有或沒有 mevalonate 的 Vero cells 中，觀察細胞生長情形。

在約 40 多種 flavonoid 物質中，只有黃鹼醇(flavonol group)中的 astilbin 有類似 Pravastatin 抑制 Vero cells 生長的作用。

本研究發現在某些中草藥萃取出的 Flavonoid 成份有類似 HMG-CoA reductase inhibitor 的作用，可提供未來中草藥降血脂效用研究上的參考。



關鍵詞 動脈粥狀硬化、中草藥、高血脂。

## ABSTRACT

Epidemiological studies have shown that hypercholesterolemia is a major risk factor for coronary heart disease. In clinical trials of lipid lowering therapy, 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase inhibitor has been shown to decrease cardiac events and mortality. Flavonoids are polyphenolic natural antioxidants existing in vegetables, fruits and beverages such as tea and wine. Previous studies have shown that some antioxidants had hypocholesterolemic effect, and flavonoid intake was associated with the decrease of mortality from coronary artery disease. The aim of this study was to evaluate the inhibitory effect of flavonoids on HMG-CoA reductase.The methods for analysis of specific inhibitors of mevalonate biosynthesis have been well-established, using Vero cells, a cell line obtained from kidneys of African green monkeys. Flavonoids isolated from different traditional Chinese herbs were dissolved in DMSO and incubated with Vero cells with or without the addition of 1 mM mevalonate or 5 mM sodium acetate in order to observe cell growth for 24 h. Concentrations of 1mM mevalonate or 5 mM sodium acetate were added into culture medium in order to observe the effect on cell growth. Different concentrations of pravastatin to inhibit cell growth were used as a positive control. About 40 flavonoid compounds were used for study, only one compound, astilbin (belonging to the flavonol group), showed significant inhibition of Vero cell growth. This study shows that one flavonoid compound, isolated from traditional medicinal herbs, may be an effective HMG-CoA reductase inhibitor which might be developed into a new hypocholesterolemic agent.

***Key Words***: atherosclerosis, Chinese herbs, hperlipidemia

## INTRODUCTION

Flavonoids are a large group of polyphenolic compounds possessing antioxidant activity that occur naturally in a variety of foods from vegetable origin such as apples, onions and beverages such as tea and red wine.[1,2] The most important groups of flavonoids are anthrocyanins, flavonols, flavones and flavonones. Flavonols are scavengers of superoxide anions,[3] singlet oxygen,[4] and lipid peroxy radicals,[5] and they can sequester metal ions through liganding.[6] Oxidized low-density lipoproteins OX-(LDL) are cytotoxic and atherogenic, and are believed to be an important step in the formation of atherosclerotic plaques.[7] A previous report showed that flavonols and flavones had antiplatelet effect and reduced thrombogenic tendencies, and the mechanism was probably due to inhibition of cyclo-oxygenase.[8] Flavonoids have also been studied in relation to their improvement of vascular fragility.[9]

Multiple epidemiological studies have shown that increased vegetable and fruit consumption has inverse relationship to the occurrence of stroke and CHD in different populations.[10-12] The explanation of this phenomenon is probably due to an increase of flavonoid intake. For example, the Zutphen Study has shown that flavonoid intake was significantly inversely associated with mortality from CHD and showed an inverse relation with incidence of myocardial infarction.[10] The relative risk of coronary heart disease mortality in the highest versus the lowest tertile of flavonoid intake was 0.42.[10] Besides beneficial antioxidant effect of flavonoid, we presume that the positive results of the above memtioned epidemiological studies were probably due to hypocholesterolemic effect. Previous studies have demonstrated that some antioxidants, such as beta-carotene, vitamin C and vitamin E have hypocholesterolemic effect, which is related to the inhibition of HMG-CoA reductase.[13-15] This study was undertaken to evaluate whether flavonoids have HMG-CoA reductase inhibitory effect.

## METHODS

Flavonoid compounds (flavonols, flavones, flavanones) were isolated in the laboratory of Dr. Kuo of China Medical College, Tai-Chung, Taiwan. HMG-CoA, DL-mevalonate (lactone form), penicillin, streptomycin, methylrosaniline chloride and trypsin were purchased from Sigma (Sigma Chemical Co., MO, USA). Pravastatin was a generous gift from Sankyo Company (Tokyo, Japan). Eagle's minimum essential medium (MEM) and calf serum were obtained from Gibco Co. Munich, Germany. Microplates (96 wells) were obtained from Corning Co. California, USA. Other materials were commercially available. All flavonoid compounds were dissolved in 100% DMSO. Cell culture medium had 0.01% DMSO (cells $3\times10^4$). After incubation for 24 h, cell growth and morphology was examined by light microscope (Olympus, Japan). Concentrations of 1mM mevalonate or 5 mM sodium acetate were added into culture medium in order to observe the effect on cell growth. Different concentrations of pravastatin to inhibit cell growth were used as a positive control.

**Vero cells**

Vero cells, an established cell line from kidney cells of African green monkey, were grown in a humidified incubator (5% $CO_2$) at 37 ℃ in a 1-1 glass flask containing 200 ml of Eagle's minimum essential medium (MEM) supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), 0.075% (w/v) $NaHCO_3$, 0.03% (w/v) glutamine, and calf serum at 5% (v/v) (5% CS-MEM). Cells were subcultured according to standand trysinization procedures. Vero cells ($3\times10^4$ cells) in 100 μl of the above mixture, but now with calf serum at 2% (v/v), were inoculated in each well of microplates and incubated at 37 ℃ for 1 h. Then, various amounts of flavonoid compounds (flavonols, flavones, flavonones) 100 μg/ml with and without 1 mM mevalonate or 5 mM sodium acetate were added to the wells. Vero cells were grown in a humidified incubator (5% $CO_2$) at 37 ℃ for 24 h.

**Measurement of cell growth**

Cell growth was measured by the method of Armstrong.[16] Cells grown on each well of 96-well microplates were washed twice with 100 μl of calcium- and magnesium-free phosphate-buffered saline (PBS) and stained with 50 μl of staining solution (methylrosaniline 0.5%, NaCl 0.85%, formamide 5% and ethanol 50% ) for 20 minutes. Then the staining solution was removed and the cells were washed with water. The absorbance at 540 nm was measured by microplate photometer (Titertek Co., Osaka, Japan).

## RESULTS

**Concentrations of cells**

Vero cells were seeded in each well of a 96-well microplate at $1.0 \sim 6.0 \times10^4$ cells in 100 μl of 2% CS-MEM. Confluent growth was observed after a 24-hour incubation when the cells were seeded at $3.0 \sim 4.0 \times10^4$ cells/well. Thus, a concentration of $3.0\times10^4$ cells/well was adopted in subsequent experiments.

**Effect of various chemicals on growth of Vero cells**

Fig. 1 shows the effect of pravastatin on growth of Vero cells. Pravastatin at the dosage of 100 μM inhibited cell growth completely. However, when 1 mM mevalonate was added to the culture medium, both morphological changes and growth inhibition were overcome and the cells grew normally. Addition of 5 mM sodium acetate had no effect. Morphological changes and growth inhibition of various flavonoid compounds on Vero cells were observed at a uniform dose of 100 μg/ml. These data indicated that this method was highly sensitive to mevalonate biosynthesis inhibitors.

The results indicated that most of the flavonoid compounds could not inhibit mevalonate

biosynthesis in cultured cells (Tables 1-3). Although some flavonoid compounds had the inhibitory effect on growth of Vero cells, the effect was not overcome after adding mevalonate into the culture medium. Only the astilbin (Table 1) showed potent inhibitory effect on growth of Vero cells, just like the effect of pravastation.

## DISCUSSION

The Zutphen Elderly Study suggested that lower mortality from CHD and lower incidence of myocardial infarction was due to increased flavonoid intake.[10] The predominant flavonoid in foods is quercetin, and higher intake of quercetin revealed inverse relationship with CHD mortality. The present study also included the compound quercetin, but the results showed that only astilbin possessed significant HMG-CoA reductase inhibition.

Quercetin also inhibits the cytotoxicity of oxidised-LDL in vitro.[17] It is possible that quercetin and other flavonoids reduce the rate of formation of oxidised-LDL and thus inhibit the growth of atherosclerotic plaques. In addition, flavonoids inhibit cyclo-oxygenases, which may reduce thrombosis.[8]

Hypercholesterolemia contributes substantially to the development and clinical expression of coronary and other forms of atherosclerosis.[18-20] Considerable evidence suggested that cholesterol lowering stabilizes plaques and reduces cardiovascular events, including all-cause mortality.[21-23] Major clinical trials have demonstrated significant reductions in cardiovascular events in patients with a wide range of cholesterol levels.[24] These findings suggest that aggressive lipid management is beneficial to patients and that manufacturing new lipid-lowering drugs is still a worthwhile pursuit in pharmaceutical science.

It is expected that this study's methodology would be advantageous over the conventional in vitro enzyme assay for a primary screening system for the following reasons: 1) The intracellular environment where an enzyme exists is maintained. It would be important to keep the enzyme environments as they are in living cells for screening enzyme inhibitors; 2) Primary assays can be done without radioactive substrates; 3) A pro-drug type of inhibitor which is activated after being incorporated into living cells might be discovered. On the other hand, some disadvantages also exist. To culture cells is rather troublesome and the running cost is high for routine screening work. It will take more time to evaluate the inhibitory potency by this method. So, unstable inhibitors might be overlooked.

Mevalonate is a key intermediate in cholesterol biosynthetic pathway. It is produced from acetyl–CoA by three enzymes, namely, acetoacetyl-CoA thiolase, HMG-CoA synthase and HMG-CoA reductase. These enzymes are expected to provide promising target sites for the pharmaceutical intervention of hypocholesterolemic agents. LDL particles contain endogenous antioxidants which are consumed during LDL oxidation,[25,26] and addition of exogenous antioxidants can protract the lag period or even prevent LDL oxidation, as shown with α-tocopherol, butylhydroxytoluene, urate, ascorbate, flavonoids, and probucol.[27,28] Flavonoids (derivatives of phenylchromone ring) are a large group of compounds naturally occurring in higher and lower plants.[29] Flavonoids have been shown to be able to affect various biological functions: capillary permeability, cellular secretory processes involved in the inflammatory response and inhibition of enzymes, receptors and carriers.[29] Some flavonoids are strong oxygen free radical scavengers and good metal chelators effective in preventing lipid peroxidation.[30]

Although flavonoid compounds have been shown to have antioxidant effect or probably vascular protection from atherosclerosis, some of these compounds may also inhibit the growth of Vero cells, like pravastatin. In conclusion, the flavonoid compound astilbin may have potent HMG Co-A reductase inhibitory effect, and whether it has cholesterol-lowering

effect needs further study.

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**Table 1. Effects of flavonoids (flavonols) on the growth of Vero cells with or without mevalonate**

| Compound | Conc. (µg/ml) | Absorbance at 540 nm | |
|---|---|---|---|
| | | None | Mevalonate (1mM) |
| Control | 1% DMSO | 0.837 | 0.872 |
| Galangin | 100 | N | N |
| Fisetin | 100 | N | N |
| Quercetin | 100 | 0.323 | 0.249 |
| Quercitrin | 100 | 0.104 | 0.185 |
| Rutin | 100 | N | N |
| Quercetin-3-0- | 100 | N | N |
| Myricetin | 100 | 0.576 | 0.510 |
| Myricitrin | 100 | 0.392 | 0.339 |
| Kaempferol | 100 | N | N |
| Rhamnustrioside | 100 | N | N |
| Astilbin | 100 | 0.385 | 0.816 |

N =non-detectable.

**Table 2. Effects of flavonoids (flavanones) on the growth of Vero cells with or without mevalonate**

| Compound | Conc. (µg/ml) | Absorbance at 540 nm | |
|---|---|---|---|
| | | None | Mevalonate (1mM) |
| Control | 1% DMSO | 0.187 | 0.802 |
| Naringenin | 100 | N | N |
| Naringin | 100 | N | N |
| Hespretin | 100 | 0.613 | 0.645 |
| Hesperidin | 100 | N | N |
| (+) Taxifolin | 100 | N | N |
| (+) Catechin | 100 | N | N |
| (-) Epicatechin | 100 | N | N |

N=non-detectable.

**Table 3. Effects of flavonoids (flavones) on the growth of Vero cells with or without mevalonate**

| Compound | Conc. (µg/ml) | Absorbance at 540 nm | |
|---|---|---|---|
| | | None | Mevalonate (1Mm) |
| Control | 1% DMSO | 0.655 | 0.619 |
| 3-Hydroxyflavone | 100 | N | N |
| 7-Hydroxyflavone | 100 | N | N |
| Baicalcin | 100 | N | N |
| Chrysin | 100 | 0.455 | 0.413 |
| Apigenin | 100 | N | N |
| Apigenin-7-0 | 100 | N | N |
| Luteolin-7-0-glucoside | 100 | 0.371 | 0.395 |
| Linarin | 100 | N | N |
| Pectolinarin | 100 | N | N |
| Cirsimarin | 100 | 0.465 | 0.524 |

N =non-detectable

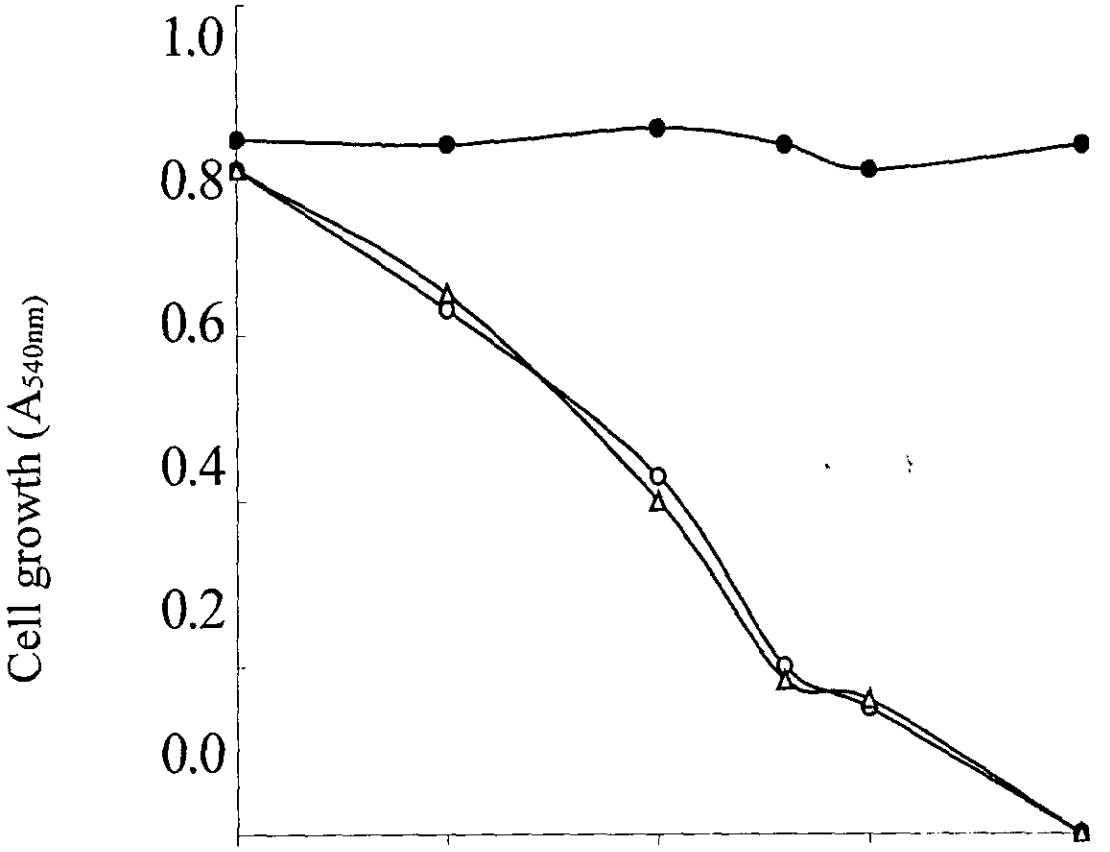


**Fig. 1.** Effects of pravastatin (o) alone or in combination with 1mM mevalonate ( •) or 5 mM sodium acetate ( Δ) on the growth of Vero cells