

科技部補助專題研究計畫成果報告
期末報告

桑葉成份抑制非酒精性脂肪肝及肥胖促進肝癌及抗藥性之研究
(第3年)

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計畫參與人員：博士後研究：楊孟元

中華民國 105 年 10 月 13 日

中文摘要：來自乾燥桑葉 (*Morus spp.*) 中的桑葉水萃物(MLE)與多酚萃取物 (MLPE) 被證實具有降血糖、降血脂、對抗粥狀動脈硬化等心血管疾病危險因子。第一年我們發現MLE可以減少脂質生成酵素，例如：FASN、ACC、HMGCR及其相關轉錄因子 (SREBP1, PPAR γ) 之蛋白表現。此外，MLPE也可降低脂肪細胞分化，並透過誘導細胞凋亡的方式，減少脂肪細胞的脂肪堆積，進而達到降低體脂肪之效果。第二年我們探討MLE 抑制肥胖促肝癌作用，將HepG2 細胞培養於含有15% 3T3-L1 脂肪細胞CM 共同培養在無血清之DMEM 培養基內顯示 MLPE 可抑制CM 誘導HepG2 細胞增生之作用，MLPE 抑制CM 所誘導之肝癌細胞增生是透過TNF- α 下游調控之NF κ B 路徑，而非p38 MAPK/AP-1 路徑。當細胞處理MLE 其pStat3/Stat3 及 PI3K/Akt/mTOR 也減少。顯示MLPE 為MLE 的主要功能成份，其作用是透過抑制TNF- α 及IL-6 途徑，進而抑制CM 誘導HepG2 細胞的增生作用。進一步的動物試驗，顯示MLE 減少HFD 促進DEN 致肝癌的作用，其作用包含MLE 降低TNF- α 及IL-6，增加抗氧化酵素的活性。第三年研究探討桑葉多酚萃取物(MLPE)是否能夠改善經化學藥物治療後所帶來的抗藥性問題。首先利用內質網壓力(ER stress)會增加肝癌細胞的藥物抵抗作用(drug resistance)之模式，再以化療藥物doxorubicin (DOX)處理人類肝癌細胞株HepG2，發現ER stress確實會減緩DOX的細胞毒殺作用，再處理MLPE之後則能夠降低ER stress相關蛋白COX2與p38的表現，MLPE增加肝癌細胞對於DOX的敏感度，促進肝癌細胞走向細胞凋亡，並進一步探討釐清其中的相關分子機轉。由以上研究確認桑葉成份具有抑制非酒精性脂肪肝、降低肥胖促進肝癌及改善抗藥性之作用，是一可提供為保健及醫藥的素材。

中文關鍵詞：桑葉萃取物；桑葉多酚；非酒精性脂肪肝病；抑制肝脂肪；促進脂肪細胞分化；肥胖；肝癌；發炎；抗氧化；抗藥性；內質網壓力；阿黴素

英文摘要：Dietary fat is one of the most important environmental factors associated with the incidence of obesity. In the first year study, the antiobesity effects of Mulberry leaf extract (MLE) and Mulberry leaf polyphenol extracts (MLPE) were investigated. The results showed that intake of MLE and MLPE can be beneficial for the suppression of HFD-induced dyslipidemia, hepatosteatosis, and obesity. Moreover, the MLE and MLPE induced 3T3-L1 adipocytes apoptosis and inhibited on the differentiation of preadipocytes. In the second year, we assayed the effect of MLE on inhibiting hepatocarcinogenesis promoted by obesity. HepG2 cells were cultured in no serum DMED contained 15% conditioned medium (CM) obtained from 3T3 -L1 cell cultured medium. The results indicated that MLPE inhibited proliferation of HepG2 cells induced by CM via TNF- α regulating NF κ B but not p38 MAPK/ AP1 signaling. The decreased expression of pStat3/Stat3 and TNF- α and IL-6 in MLE treated cells was also investigated. In conclusion,

MLPE, the functional ingredient of MLE, could inhibit the proliferation of HepG2 induced by CM from 3T3 cells through suppressing TNF- α and IL-6 activation. Further animal study showed that MLE reduced TNF- α and IL-6 expression, increased antioxidant enzymes activation, thereby lessened DEN-induced hepatocarcinogenesis promoted by high fat diet (HFD). In the third year, We first established an ER stress microenvironment inducing resistance to chemotherapeutic in HCC. Based on this mimicked ER stress microenvironment and then exposed to doxorubicin in HepG2 cells. The discovery provide important new insights that ER stress may improve resistance to doxorubicin-mediated HepG2 cell cytotoxicity by co-treatment with MLPE. It significantly decreased the levels of COX-2 and p38 associated with ER stress. MLPE can increase the sensitivity of doxorubicin-induced apoptosis in human hepatocellular carcinoma cells. It is needed to explore the molecular mechanisms. These findings will help us to further evaluate the potential for the development of new hepatocellular carcinoma therapy.

英文關鍵詞：Mulberry Leaf Polyphenolic Extract; Non-Alcoholic Fatty Liver; Obesity; Lipogenesis; Adipocyte Apoptosis; Differentiation; HCC; Inflammation; Antioxidation; drug resistance; ER stress; doxorubicin

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(期中進度報告/期末報告)

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計畫主持人：王朝鐘

計畫參與人員：張純華、楊孟元

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中 華 民 國 105 年 10 月 10 日

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三年總摘要

來自乾燥桑葉 (*Morus spp.*) 中的桑葉水萃物(MLE)與多酚萃取物 (MLPE) 被證實具有降血糖、降血脂、對抗粥狀動脈硬化等心血管疾病危險因子。**第一年**我們發現 MLE 可以減少脂質生成酵素，例如：FASN、ACC、HMGCR 及其相關轉錄因子 (SREBP1, PPAR γ) 之蛋白表現。此外，MLPE 也可降低脂肪細胞分化，並透過誘導細胞凋亡的方式，減少脂肪細胞的脂肪堆積，進而達到降低體脂肪之效果。**第二年**我們探討 MLE 抑制肥胖促肝癌作用，將 HepG2 細胞培養於含有 15% 3T3-L1 脂肪細胞 CM 共同培養在無血清之 DMEM 培養基內顯示 MLPE 可抑制 CM 誘導 HepG2 細胞增生之作用，MLPE 抑制 CM 所誘導之肝癌細胞增生是透過 TNF- α 下游調控之 NF κ B 路徑，而非 p38 MAPK/AP-1 路徑。當細胞處理 MLE 其 pStat3/Stat3 及 PI3K/Akt/mTOR 也減少。顯示 MLPE 為 MLE 的主要功能成份，其作用是透過抑制 TNF- α 及 IL-6 途徑，進而抑制 CM 誘導 HepG2 細胞的增生作用。進一步的動物試驗，顯示 MLE 減少 HFD 促進 DEN 致肝癌的作用，其作用包含 MLE 降低 TNF- α 及 IL-6，增加抗氧化酵素的活性。**第三年**研究探討桑葉多酚萃取物(MLPE)是否能夠改善經化學藥物治療後所帶來的抗藥性問題。首先利用內質網壓力(ER stress)會增加肝癌細胞的藥物抵抗作用 (drug resistance)之模式，再以化療藥物 doxorubicin (DOX)處理人類肝癌細胞株 HepG2，發現 ER stress 確實會減緩 DOX 的細胞毒殺作用，再處理 MLPE 之後則能夠降低 ER stress 相關蛋白 COX2 與 p38 的表現，MLPE 增加肝癌細胞對於 DOX 的敏感度，促進肝癌細胞走向細胞凋亡，並進一步探討釐清其中的相關分子機轉。由以上研究確認桑葉成份具有抑制非酒精性脂肪肝、降低肥胖促進肝癌及改善抗藥性之作用，是一可提供為保健及醫藥的素材。

Abstract

Dietary fat is one of the most important environmental factors associated with the incidence of obesity. **In the first year study**, the antiobesity effects of Mulberry leaf extract (MLE) and Mulberry leaf polyphenol extracts (MLPE) were investigated. The results showed that intake of MLE and MLPE can be beneficial for the suppression of HFD-induced dyslipidemia, hepatosteatosis, and obesity. Moreover, the MLE and MLPE induced 3T3-L1 adipocytes apoptosis and inhibited on the differentiation of preadipocytes. **In the second year**, we assayed the effect of MLE on inhibiting hepatocarcinogenesis promoted by obesity. HepG2 cells were cultured in no serum DMEM contained 15% conditioned medium (CM) obtained from 3T3 -L1 cell cultured medium. The results indicated that MLPE inhibited proliferation of HepG2 cells induced by CM via TNF- α regulating NF κ B but not p38 MAPK/ AP1 signaling. The decreased expression of pStat3/Stat3 and TNF- α and IL-6 in MLE treated cells was also investigated. In conclusion, MLPE, the functional ingredient of MLE, could inhibit the proliferation of HepG2 induced by CM from 3T3 cells through suppressing TNF- α and IL-6 activation. Further animal study showed that MLE reduced TNF- α and IL-6 expression, increased antioxidant enzymes activation, thereby lessened DEN-induced hepatocarcinogenesis promoted by high fat diet (HFD). **In the third year**, We first established an ER stress microenvironment inducing resistance to chemotherapeutic in HCC. Based on this mimicked ER stress microenvironment and then exposed to doxorubicin in HepG2 cells. The discovery provide important new insights that ER stress may improve resistance to doxorubicin-mediated HepG2 cell cytotoxicity by co-treatment with MLPE. It significantly decreased the levels of COX-2 and p38 associated with ER stress. MLPE can increase the sensitivity of doxorubicin-induced apoptosis in human hepatocellular carcinoma cells. It is needed to explore the molecular mechanisms. These findings will help us to further evaluate the potential for the development of new hepatocellular carcinoma therapy.

第一年度 研究報告

桑葉成份抑制非酒精性脂肪肝及肥胖促進肝癌及抗藥性之研究(第1 年)

中文摘要及關鍵字

關鍵字：桑葉多酚，非酒精性脂肪肝病，抑制肝脂肪，促進脂肪細胞分化，肥胖。

中文摘要

當脂肪過度堆積時會增加相關疾病之風險，與肥胖、脂血代謝異常、高血壓、胰島素抗性及糖尿病等有高度的關聯性。來自乾燥桑葉 (*Morus spp.*)中的桑葉水萃物(MLE)與多酚萃取物 (MLPE) 被證實具有降血糖、降血脂、對抗粥狀動脈硬化等心血管疾病危險因子，然而MLE 與MLPE 是否具備降低脂肪堆積之功效仍有待確認，故本研究以細胞模式和動物模式探討MLE與MLPE減少體脂肪堆積之作用與機制，並分別以3T3-L1 adipocyte和高油脂飲食(high fat diet, HFD) 誘導動物的兩種模式中探討MLE 與MLPE 抑制脂肪堆積之功效。結果發現MLE可降低血液和肝臟脂質，並使高油脂飲食組動物體重減輕，體脂肪減少。同時還發現MLE可以減少脂質生成酵素，例如：FAS、ACC、HMGCR及其相關轉錄因子(SREBP1, PPAR γ) 之蛋白表現。此外，MLPE也可降低脂肪細胞分化，並透過誘導細胞凋亡的方式，減少脂肪細胞內脂肪的堆積作用，進而達到降低體脂肪之效果。此研究證實MLE與MLPE可以有效降低血液和肝臟中脂質含量，且無肝腎毒性之不良副作用，對於改善肥胖及抑制體脂肪確實具有作用可開發為保健食品之潛力。

Abstract

Keywords: Mulberry Leaf Polyphenolic Extract; Non-Alcoholic Fatty Liver; Obesity; Lipogenesis; Adipocyte Apoptosis; Differentiation

Dietary fat is one of the most important environmental factors associated with the incidence of obesity. In this study, the antiobesity effects of Mulberry leaf extract (MLE) and Mulberry leaf polyphenol extracts (MLPE) were investigated. C57Bl/6 mice were divided into normal and obese groups, and obese rats were fed a high-fat diet (HFD) containing 20% lard oil and 2% cholesterol. Then, MLE was given as a supplement to obese mice at doses of 0.5% and 1.5%, respectively, for a period of 6 weeks. The results showed that body, liver organ, and adipose tissue weights of peritoneal and epididymal fat pads in the HFD + 0.5% MLE and HFD + 1.5% MLE groups were significantly decreased as compared to those in the HFD group. Serum lipid profiles were significantly decreased in the HFD + 0.5% MLE and HFD + 1.5% MLE groups as compared to those in the HFD group. Hepatic triacylglycerol and cholesterol levels were significantly decreased in the HFD + 0.5% MLE and HFD + 1.5% MLE groups as compared to those in the HFD group. MLE markedly lowered fatty acid synthase, HMG-CoA reductase and SREBPs, the enzymes of lipid synthesis. Moreover, the MLE and MLPE induced 3T3-L1 adipocytes apoptosis and inhibited on the differentiation of. These results demonstrate that intake of MLE and MLPE can be beneficial for the suppression of HFD-induced dyslipidemia, hepatosteatosis, and obesity.

(三)報告內容：前言、研究目的、文獻探討、研究方法、結果與討論

(1) 前言、研究目的、文獻探討

1. 非酒精性脂肪肝 (non-alcoholic fatty liver disease, NAFLD)

1-1. 肥胖與非酒精性脂肪肝

在臨床上所謂的「脂肪肝」是指肝臟內所屯積的脂肪(主要是三酸甘油酯)的重量超過全肝臟重量的百分之五，或是超過百分之十以上的肝細胞呈現脂肪空泡變性的現象而謂之；其中非酒精性脂肪肝病(non-alcoholic fatty liver disease, NAFLD)的病因，包括某些藥物及毒物、先天性代謝疾病、及後天代謝性疾病，其中最常見病因的就是肥胖症、糖尿病及高血脂症[1]。NAFLD 是目前肝臟醫學界對非酒精性脂肪肝病最廣為接受的一種命名，它所涵蓋的肝臟疾病很廣泛，從單純脂肪肝，脂肪肝炎，脂肪纖維化，到脂肪肝硬化都包含。事實上，NAFLD 病程中的脂肪變性及其所衍生成NASH 的真正致病機轉目前尚不很明確，由近年來研究所得的假說，得知NAFLD 的成因是多樣性的，其中最為經典的假說則以 Day 與James[2]提出胰島素抗性(insulin resistance, IR)以及脂質過氧化作用對肝細胞進行攻擊，導致肝臟發生瀰漫性脂肪浸潤、炎症反應、壞死、凋亡、再生損害以及肝星狀細胞活化等一系列病理連鎖性免疫攻擊反應。換言之，是由於IR 而導致脂肪過度貯積，並在胰島素增加以及脂肪酸本身調控不良之下，使得肝臟細胞易於遭受進一步的氧化作用，而造成肝細胞膜本身脂質發生過氧化作用，並誘發炎症細胞激素以及氧自由基的釋放，更再次導致肝臟進一步發生壞死性炎症以及纖維化的病變。

1-2. NAFLD 致病機轉

一旦肝臟受到損傷後，均可導致脂質(尤其是三酸甘油酯)在肝細胞內異常的代謝及屯積[3]。理論上，各種致肝傷害因素可以通過下述一種或多種機制來導致脂肪肝(包括NAFLD 中的脂肪變性)的衍生：一、由於個體攝取過多的高脂肪類飲食並導致高脂血症，相對會使血清FFA 異常增加；二、肝細胞合成FFA 或由碳水化合物轉化成三酸甘油酯增加；三、FFA 在肝細胞粒線體內氧化及利用率降低，而轉變為三酸甘油酯；四、由於VLDL 本身合成的減少，而導致三酸甘油酯轉運發生障礙，結果脂肪組織以及肝細胞間的脂肪代謝動態失衡，以及肝細胞中的三酸甘油酯合成及排泄之間的平衡發生障礙，因而導致三酸甘油酯在肝細胞內過度屯積，最終則衍生脂肪變性[4]。此外，某些荷爾蒙如雌性荷爾蒙、類固醇、生長激素或昇糖素，亦可通過能量代謝變化，促使碳水化合物轉化為脂肪，並增加FFA 產生、抑制脂肪酸氧化、減少膜磷脂組成，也因而造成肝臟本身對其他致病因素的敏感度增加，更進一步誘發甚至加劇肝細胞脂肪變性的衍生及其嚴重度。

2. 調控脂質代謝相關蛋白

脂肪酸生成酶(fatty acid synthase ; FAS)在催化長鏈脂肪酸新生過程中，參與在malonyl-CoA 轉變為palmitate 之步驟，FAS 的表現都受到飲食及激素的調控[5]。ACC(Acetyl-CoA carboxylase) 主要功能是將acetyl-CoA 催化malonyl-CoA 的合成，是脂肪酸合成的速率決定步驟，其活性受到 AMPK 調控[6]。HMG-CoA reductase 位於內質網上的膜蛋白，在膽固醇合成過程中扮演著速率決定的角色，哺乳類動物的HMG-CoA reductase 之基因表現會受到mevalonate 所衍生之固醇或非固醇類isoprenoids 的代謝產物所影響或藉由其他複雜機制來負回饋調節膽固醇之恆定[7]。甘油酯合成之起始及速率限制步驟是glycerol-3-phosphate 與 long-chain fatty acyl-CoA 經醯化作用形成1-acyl-glycerol-3-phosphate (LPA)。反

應過程是由兩種 glycerol-3-phosphate acyltransferase (GPAT) isoenzymes 所催化。mtGPAT 被認為參與在三酸甘油酯之合成，動物實驗證實在脂肪細胞及肝細胞之TG 的合成，mtGPAT 為一關鍵蛋白[8]。固醇調節結合蛋白(sterol regulatory element binding-proteins; SREBPs)是與膽固醇脂肪酸合成有關的轉錄因子，為肝細胞中調節醣類代謝與脂質代謝的一種蛋白。在哺乳類動物中可被分為三種型，分別為SREBP-1a，SREBP-1c 與SREBP-2。SREBP-1a 是所有SREBP-responsive gene 的活化者(activator)，包括膽固醇合成、脂肪酸合成及三酸甘油酯合成之調節；SREBP-1c 則調控脂肪酸及三酸甘油酯的生成，如：FAS 與GPAT及葡萄糖之代謝；SREBP-2 為調控與膽固醇的生成代謝相關蛋白的表達如HMG-CoA reductase、LDLR[9]。過氧化體增殖活化受體 PPARs 為一群核內荷爾蒙接受體，其功能為一轉錄因子，作用在目標基因的啟動子的專一性序列(PPAR response element)上，調控基因的表現。PPARs 在體內作為脂質sensors，在全身扮演代謝作用之調控者，幾乎控制各方面的脂肪酸代謝 [10] [11]。carnitine palmitoyltransferase I (CPTI)為粒線體內的脂肪酸 β -氧化作用之反應速率限制酵素，存在骨骼肌肌肉和肝臟細胞中，功能為脂肪酸的載體，能將脂肪酸送入粒線體內進行氧化，CPTI表達增加時，將會使更多的脂肪酸被運送進入可使其被氧化的粒線體內[12]。AMP-activated protein kinase (AMPK)在體內作為一個能量平衡的偵測器，當細胞內AMP/ATP 比例升高時，例如：缺氧、局部缺血、飢餓、運動、葡萄糖缺乏等情況下，AMP 會與AMPK 結合，AMPK 上游激酶LKB1 將AMPK threonine residue (Thr-172)的位置磷酸化，而活化了 α 次單位的活性，提升細胞內的能量，同時關閉消耗ATP 的代謝路徑，比如抑制脂肪和膽固醇的合成。因此AMPK 是細胞內非常重要的能量調控因子，協調體內代謝和能量需要 [13]。AMPK 可以調控許多不同功能，而在脂質合成代謝方面，AMPK 可經由

磷酸化多種酵素如：FAS，ACC，HMGCoR 及GPAT而使得脂肪酸、膽固醇及三酸甘油酯合成受到抑制[14]。

3. Obesity and cancer

一些大型的流行病學研究指出，超重和肥胖(BMI > 25)會導致罹患癌症的風險增加[15]。有幾種機制被提出來解釋肥胖會增加罹患癌症的風險，包括糖尿病 II 型和胰島素抗性在肥胖者的患病率，導致提高胰島素和胰島素生長因子 1(IGF-1)的血液循環濃度，以及由脂肪組織釋出的性類固醇激素和細胞激素的產生增加。此外，肝臟脂肪堆積，脂肪肝和肝癌發展之間的關聯已久負盛名[16]肥胖引起的脂肪肝，以及更嚴重的併發症酒精性脂肪性肝炎(NASH)，或非酒精性脂肪性肝病(NAFLD)，非酒精性脂肪肝，其可協同造成更大的肝癌風險的增加，導致肝實質細胞的內質網(ER)壓力上升和積累活性氧物種(ROS)，造成慢性肝損傷。據認為，慢性肝損傷，是原本靜止的肝細胞，經代償性作用使肝細胞分化增生，是肝癌發展的主要致病機制之一[17]。在高脂肪食物下使用diethylnitrosamine作為誘導肝癌[18]的作用也有顯著的促進IL-6 及TNF α 表現作用。

4. Obesity promoted hepatocellular carcinoma

肥胖，對某些惡性腫瘤，包括乳腺癌，子宮內膜癌，結腸癌，腎細胞癌，食管腺癌，胰腺癌，肝癌具潛在性影響[19]。在台灣相關連的研究，代謝性疾病(肥胖和糖尿病)和病毒性肝炎之間的協同作用，HBV 或HCV 攜帶者的肝癌風險與肥胖和糖尿病增加超過100 倍[20]。肥胖與代謝性症候群相關，包括胰島素抗性和第2 型糖尿病及一連串的非癌性肝臟疾病，如NAFLD 和NASH，肝纖維化，和肝硬化等。另一方面，肥胖是脂肪肝的主要危險因素，但早期階段是可以通過運動和飲食控制來減肥治療。但是，如果沒有適當的管理，NAFLD 可能發展為慢性肝臟炎症，稱為脂肪性肝炎(NASH)，這是一個嚴重的脂肪肝發炎狀況，

可以進一步發展為肝纖維化和肝硬化，造成了嚴重的併發症，包括肝衰竭和肝癌。值得注意的是，肥胖和NAFLD 可誘導肝細胞的增殖和降低在小鼠模型中的肝細胞的凋亡，在纖維化的情況下，導致肝增生，炎症，和肝硬化[21]。

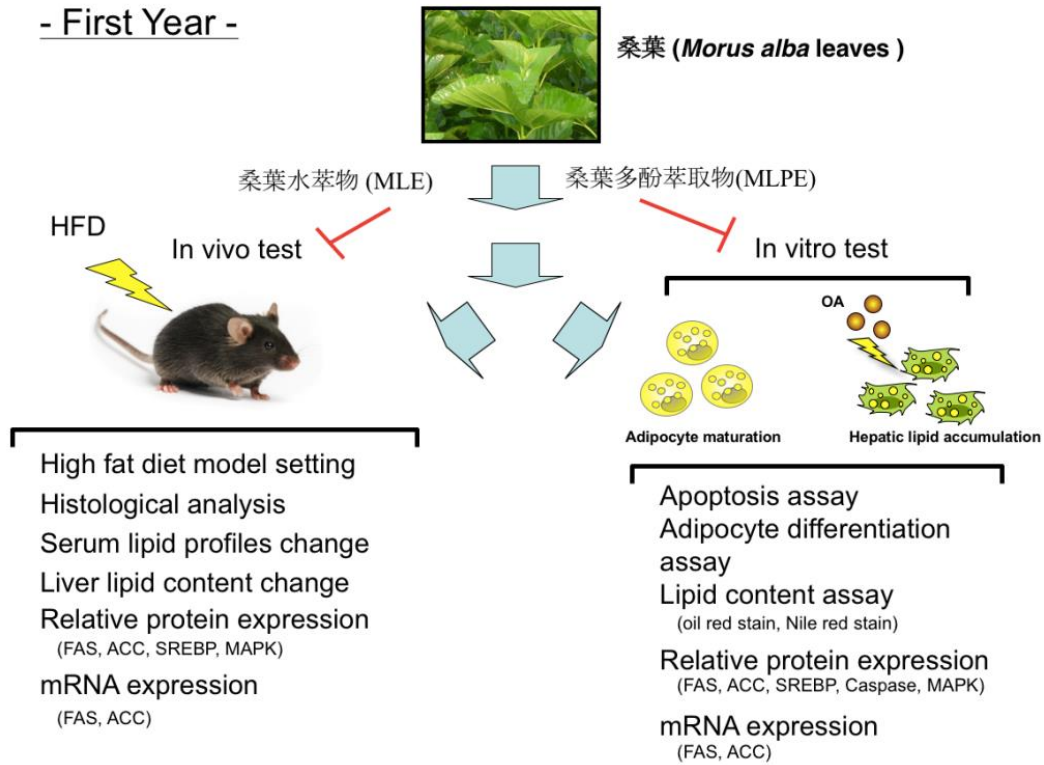
5. 桑葉醫學用途

已有許多研究證實桑葉的醫學功能，包括具有(a)抗菌及抗氧化能力[22,23]，也具有抗發炎之能力[24]，可以提升抗氧化酵素glutathione reductase、glutathione peroxidase、glutathione-S-transferase、superoxide dismutase 和catalase 含量，有助於改善糖尿病患者的紅血球容易發生脂質過氧化現象[25]；(b)抑制動脈硬化症：桑葉中的植物甾醇能抑制腸道內膽固醇的吸收，軟化疏通微細血管，抑制血栓形成。桑葉的類黃酮物質quercetin 3- (6-malonylglucoside)會抑制LDL 的氧化修飾作用，因而抑制動脈粥樣硬化形成的作用[26]；(c) 抗糖尿病：在飲食中添加桑葉萃取物可以減少飯後血糖濃度過高，主要是因為桑葉萃取物的成份中具有含氮的糖基，可抑制 α -glucosidase 活性，進而達到調節血糖的功效[27,28]；(d)抗發炎：桑葉萃取物也被證實會抑制發炎因子調控者COX-2 和iNOS，也能抑制過敏反應[24]；(e)神經保護：桑葉萃取物已被應用於Alzheimer's disease 的治療，因為桑葉萃取物會抑制amyloid β -peptide(1-42) fibril 的形成和減弱amyloid β -peptide(1-42) 所誘發的神經毒性作用[29]；(f) 抑制皮膚色素：從桑樹(Morus nigra)的莖部分離出一種酪氨酸酶(ty 抑制劑成分TMBC 會抑制B16 melanoma 細胞合成黑色素[30]。桑葉能有效抑制DOPA oxidase 和antityrosinase 的活性[31]。目前我們已發表四篇有關於桑葉及主成份調節血脂及抑制血管病變及機轉之研究[32-35]，(附件一)包括J. Agric. Food Chem., 57, 9147-9153. 2009；58, 2536-2542. 2010；59, 1985-1995. 2011；J. Food Sci., 76, 65-72. 2011。及發現桑葉成份具調節AMPK 促進肝癌細胞凋亡作用[36] (J. Agric. Food Chem., 60, 6891- 6898, 2012, 附件二)，MLE 及MLPE

也抑制脂肪細胞分化(附件三)從以上的背景本計劃擬進行開發研究桑葉抑制肥胖，非酒精性脂肪肝，及肥胖促進肝癌之研究。第一年探討MLE 及MLPE 抑制肝臟脂肪堆積，脂肪細胞分化及肥胖。

(2) 研究架構

第一年：MLE 及MLPE 抑制肝臟脂肪堆積，脂肪細胞分化及肥胖



(3) 研究方法

3-1. 桑葉萃取物之製備

100 g 乾燥桑葉加入3 L 逆滲透水熬煮，水滾後計時40 分鐘，放涼，以濾紙過濾得到的桑葉汁液經由減壓濃縮，最後進行真空冷凍乾燥機，所得之粉末即為桑葉萃取物。在各種細胞實驗之前，均以此冷凍乾燥粉末配製成MLE 溶液後，再經過0.22 μm filter 過濾除菌進行後續實驗。

3-2. 桑葉多酚之製備

取100 g 乾燥桑葉加入300 ml 甲醇於50°C 水浴萃取3 小時後，過濾，收集濾液，反覆萃取3~5 次，減壓濃縮後，再以500 ml 二次水回溶萃取物，加入200 ml 正己烷，利用分液漏斗混合均勻兩種溶液，靜置隔夜，收集水層溶液。再加入180 ml 乙酸乙酯萃取溶於水層中的多酚成分，靜置隔夜，收集上層溶液，重覆萃取3~5 次，利用減壓濃縮乾燥後，最後溶於250 ml 二次水，經過真空冷凍乾燥後所得之粉末即為桑葉多酚。在各種細胞實驗之前，以此乾燥粉末溶於50 % 酒精溶液後，再經過0.22 μm filter 過濾除菌成為桑葉多酚萃取液(MLPE)進行後續實驗。

3-3. 桑葉總多酚含量測定

以gallic acid(GA)和rutin 溶液作為測定酚含量之標準品，使其最後體積為1ml，各加入0.5 ml 之2N Folin-Ciocalteu phenol reagent，以及3 ml Na_2CO_3 (200 g/l) 混合均勻，室溫下靜置數分鐘，再加入5 或10 ml 去離子水混合均勻，以1250 \times g 離心5 分鐘，分別在波長765 nm 及510 nm 下測定吸光值，繪製標準迴歸曲線。桑葉多酚萃取液樣品事先稀釋，取0.1 ml 樣品加入0.9 ml 甲醇反應後測定吸光，依迴歸方程式計算酚含量。

3-4. 細胞培養

HepG2 培養於MEM (HyClone®) 培養液中(pH 7.3)，培養液中含10 % fetal bovine serum(FBS)、1 % glutamine (200 mM)、1 % non-essential amino acids、2.2 g/l sodium bicarbonate 與1 % sodium pyruvate(100 mM/ml)。解凍後繼代培養在37 °C，5 % CO₂ 之培養箱。每次實驗前依各實驗需求，計算不同之細胞密度，將HepG2 細胞培養於 culture dish，待細胞型態展開後，加入0.5 mM 油酸(oleic acid, OA) 作用16 或24 小時誘導細胞脂質堆積，油酸事先需與BSA 混合均勻。接著，加入不同濃度的桑葉萃取物(MLE)、桑葉多酚 (MLPE) ，在37 °C 恆溫培養箱中反應24 小時。之後移除培養液，並以PBS 潤洗細胞1~3 次後，進行以下分析試驗。

3-5. 油紅染色 (Oil red staining)

Oil red 是一種對脂質具有親和性之染劑，利用染色後呈現的紅顏色反應細胞內中性脂肪的含量。將欲染色的細胞以PBS 沖洗2~3 次，加入4 % paraformaldehyde 固定1 小時，加入Oil Red 染劑(3 mg/ml)室溫下染色15 分鐘，之後，將多餘染劑後洗去，在每盤細胞內滴入少許PBS，以倒立式顯微鏡拍照。

3-6. 尼羅紅染色和定量試驗 (Nile red staining and quatitation)

螢光染劑可針對脂質染色，並且可以在雷射光激發下，發出紅色螢光，螢光強度表示脂質濃度，藉此反應細胞內中性脂肪的含量。將欲染色的細胞以PBS 沖洗2~3 次，加入4 % paraformaldehyde 固定1 小時，以PBS稀釋Nile red 染劑(1 µg/ml)室溫下，避光，染色5 分鐘，之後，將多餘染劑後洗去，在每盤細胞內滴入少許PBS，立刻以雷射光488 nm 激發觀察。定量試驗則以流式細胞儀偵測細胞螢光強度。

3-7. 脂質代謝轉錄因子分析 (Reverase transcriptase RT-PCR)

首先配製hexane : isopropanol (3 : 2, v/v) 作為油脂之溶劑，加入處理好的細胞沉澱物中，室溫下震盪1 小時，離心取上清液至乾淨試管，待其自然風乾，測試前以少許isopropanol 回溶。每盤細胞取total RNA(8 µg/µl)為模板，加入混合液 (5X RT-buffer, dNTP, oligo dT, revers transcriptase)，進行反轉錄。反轉錄條件為42°C 1 小時，99°C 10分鐘，4°C 30 分鐘。cDNA 可以長期儲存於-20°C。接著，取2.5 µl cDNA 與目標基因的primer，加入混合液 (10X PCR-buffer, dNTP, Taq DNA polymerase, DEPC-H2O) 進行PCR 反應。primer 序列如下：

Gene	Forward primer	Reverse primer
FAS	TACAT CGACT GCATC AGGCA	GATAC TTTCC CGTCG CATA C
ACC α 1	TGAAG GCTGT GGTGA TGGAT	CCGTA GTGGT TGAGG TTGGA
HMGCR	AGGTT CCAAT GGCAA CAACA GAAG	ATGCT CCTTG AACAC CTAGC ATCT
LDLR	CAATG TCTCA CCAAG CTCTG	TCTGT CTCGA GGGGT AGCTG
GAPDH	CGGA GTCA ACGG ATTT GGTC GTAT	AGCC TTCT CCAT GGTT GGTG AAGA

3-8. MLE 抑制脂肪肝及肥胖動物試驗

實驗動物為鼠齡6 週之C57BL/6 品系公鼠，購自國家實驗動物中心，個別飼養在不鏽鋼絲網籠，給予Purina Lab Chow 飼料餵食，蒸餾水飲用不限制，每週記錄一次飼料攝取量及體重變化。飼育期共6 週。將C57BL/6 分成4 組，每組20 隻，不同組別老鼠飼料成分如下：

Group	Control (C)	High fat diet (HFD)	0.5 % (MLE)	1.5 % (MLE)
Protein (%)	23	23	23	23
Lard (%)	0	20	20	20
Cholesterol (%)	0	2	2	2
MLE (%)	0	0	0.5	1.5

飼育動物6週後，已先將動物空腹12小時為原則，動物抽取血液樣品以及肝臟組織，採樣後進行下列項目分析：

3-9. 血清生化測定分析

血脂質(TG、TC、LDL-C 及HDL-C)、血糖(glucose)、肝功能(GOT、GPT)、腎功能(BUN、CRE)、電解質(Na、K)之測定其測定方法，利用酵素作用與比色測定原理，在定量的血清中加入呈色劑，以分光光度計於適當波長下測定吸光值，使用血液生化自動分析儀器分析。血清游離脂肪酸之測定依照free fatty acid Quantification Kit(Biovision)說明書指示操作。取出待測樣品，以酵素作用與比色測定原理，加入測定試劑反應1小時後，在波長570 nm 下測定吸光值。根據試劑說明書提供之計算公式求得待測物濃度。

3-10. 肝臟總膽固醇和三酸甘油酯含量分析

秤取0.1 g 肝臟組織均質之後，加入2 ml chloroform/methanol (2/1, v/v)，震盪1小時後，離心，收集有機層，在室溫下使其自然揮發至全乾。測定前加入少許異丙醇回溶。取出待測樣品，以酵素作用與比色測定原理，加入膽固醇測定試劑或三酸甘油酯測定試劑，以分光光度計在波長500 nm 下測定吸光值，根據試劑說明書提供之計算公式，換算成三酸甘油酯與總膽固醇之含量。

3-11. MLE 和MLPE 抑制前脂肪細胞(3T3-L1)分化及促進脂肪細胞凋亡

將前脂肪細胞preadipocytes 3T3-L1 培養在DMEM 培養基中包含10 % FBS，4 mM L- glutamine，1 % penicillin-streptomycin，待細胞長滿後48 h，加入MDI 誘導培養液(day 0)(DMEM 包括10 % FBS、0.5 mM 3-isobutyl-1-methylxanthine，1 uM dexamethasone 及1 ug/ml insulin) 48 h，之後更換培養基(day 2)(DMEM 包括1 ug/ml insulin)繼續培養48 h 後(day 4)，每兩天更換培養基(DMEM 包含10 % FBS)至第14 天完全分成脂肪細胞。在細胞分化過程的第8 天，細胞加入不同濃度的

MLE 和MLPE，並觀察抑制脂肪細胞的分化。包括oil red stain(方法1-5)；
triglyceride assay(方法1-10)；western blot analysis 分析蛋白的表現，包括G3PDH、
PPAR γ 、C/EBP α 、CD36、SREBP-1c、FASN、BCL2、Bax、caspase、p21、p27
等，並以Flow cytometry (FACScan)分析細胞週期及細胞凋亡。

(4) 結果與討論

C57BL/6 小鼠飼養6週期間，各組動物之毛色隨飼育期有逐漸灰暗且脫毛現象，對高油脂飼料攝取量較正常飼料差，飲水狀況正常，但鼠隻活動力旺盛，反應敏捷。表一顯示各組動物在飼養期間體重變化的情形，各組體重隨週齡逐漸增加，但以雙週體重變化百分比來看，發現在0.5%和1.5% MLE組之老鼠體重增加的幅度均較HFD組低，以第6週為例，HFD組體重變化率為31.19%，而0.5%和1.5% MLE組分別為20.78%、19.46%，顯示MLE有抑制高脂肪誘導之小鼠體重增加($p < 0.05$)。各組老鼠犧牲後取出肝臟、腎臟周邊脂肪組織，以生理食鹽水清洗後，瀝除水分秤重並統計重量變化。由外觀明顯可見HFD誘導組與餵食0.5% MLE組的肝臟顏色偏粉(正常為暗紅色)，但餵食1.5% MLE之後肝臟顏色有稍微偏紅。將各組動物肝臟重量與各自體重相除，比較對肝臟的重量變化，結果發現各組動物肝臟重量並無差異，將各組動物肝臟檢體進行均質，萃取油脂進行含量分析。從HFD誘導組的變化可證明高油、高膽固醇飲食的確會增加肝臟三酸甘油酯、膽固醇濃度。三酸甘油酯含量分析結果(Fig 2)與總膽固醇含量有降低之趨勢，在1.5% MLE組有意義減少的作用($p < 0.05$)，由體內試驗結果証實MLE確有排除肝臟油脂堆積之效。新鮮肝臟取下最大葉送交病理科進行切片及染色分析，觀察肝細胞內脂質堆積情形，此法為脂肪肝診斷之黃金標準。Figure 3顯示，HFD誘導組明顯可見白色空泡出現在肝細胞中，堆積的部位以中央靜脈(central vein)周圍最為嚴重，而0.5% MLE組之結果與HFD組相近，1.5% MLE組白色空泡不論是大小、數量都相對減少許多，顯示桑葉可能有助於減少肝臟脂肪堆積。由脂肪組織重量變化評估桑葉對體脂肪是否具調節的作用。Fig. 4顯示脂肪組織大小，在經過高脂肪食物誘導的組別中，單九週邊脂肪組織堆積情形明顯上升

(1.91 ± 0.48 %), 然而在給予0.5% MLE 餵食組和1.5% MLE 餵食組雖然有觀察到脂肪組織減少的趨勢, 1.5% MLE 有意義降低 (Fig.4)。體內所有堆積的脂肪將全部都停留在血液中, 因此血脂檢查為重要的評估指標 (Table 2)。在各組動物血中, 以0.5 % MLE 及1.5 % MLE 之總膽固醇含量 (101.11 ± 9.75 , $p < 0.05$; 81.37 ± 8.11 , $p < 0.05$); 三酸甘油酯 (42.11 ± 8.84 , $p < 0.05$; 32.75 ± 5.75 , $p < 0.05$); 游離脂肪酸 (6.52 ± 0.17 , $p < 0.05$; 5.57 ± 0.92 , $p < 0.05$), 均有意義降低。此結果顯示, 桑葉萃取物可降低血中三酸甘油酯、膽固醇與游離脂肪酸的堆積 (Table 2)。

肥胖所引發相關併發病譬如糖尿病、高脂血症、胰島素抗性等, 皆影響血糖之穩定。良好的血糖控制得以事先預防或延緩其發生機率。以HFD 餵食動物血糖濃度明顯上升 (155.22 ± 29.23 , $p < 0.05$), 以0.5% MLE (91.00 ± 6.12 , $p < 0.05$) 或1.5% MLE 餵食 (70.62 ± 8.12 , $p < 0.05$) 與血糖值都有明顯減少, 顯示桑葉具有降血糖的功能 (Table 2)。由Table 2 比較發現, 血中LDL 濃度變化, 在分別以0.5%及1.5% MLE 餵食之後, 其表現皆有達到統計意義之降低 (68.66 ± 7.76 , $p < 0.05$; 43.25 ± 4.50 , $p < 0.001$)。另外比較血漿HDL 濃度, 在HFD 的組別則中有增加的情形 (68.88 ± 20.28 , $p < 0.05$), 而在兩種MLE 試驗組中呈降低的趨勢 (33.11 ± 3.06 , $p < 0.05$; 33.75 ± 4.30 , $p < 0.05$), 可能是由於高油脂飲食增加總膽固醇含量的結果。但由於HFD 組之HDL 含量出現非預期之偏高, 間接影響LDL/HDL ratio 的換算, 因此無法就本實驗結果評估桑葉萃取物是否能降低心血管疾病之風險。

由於細胞實驗結果證明MLE 及MLPE 分別都具有抑制脂質相關蛋白之作用, 所以同樣觀察這些蛋白在動物實驗的表現情形。由Fig .4 結果得知, HFD 組別明顯提升脂質生成酵素蛋白(FAS, HMGCR, LDLR, A-FABP) 表現, 顯示高油飲

食的確會促進肝臟脂肪合成蛋白之表現。以0.5% MLE 及1.5% MLE 餵食 C57BL/6 小鼠6 週之後，這些蛋白在體內分別都呈現減少的趨勢。此實驗之結果證明桑葉具有抑制脂肪生成蛋白表現之能力，推測可能與MLE 降低三酸甘油酯和總膽固醇之作用機轉有關。Figure 5 顯示ACC 蛋白在HFD 組中表現最顯著 (2.02 倍)，而0.5% MLE (1.08 倍)及1.5% MLE 組 (0.92 倍) 表現量降低，證明桑葉抑制ACC 之活性。觀察AMPK 活性變化。AMPK 在HFD 組的表現量降低 (0.89 倍)，而在兩種桑葉餵食組則無太大差異 (0.96 與1.01 倍)；但p-AMPK 蛋白的表現量在1.5% MLE 餵食組有明顯增加，經計算p-AMPK/AMPK ratio 後發現，AMPK 活性表現的確在1.5% MLE 餵食組明顯上升，約為HFD 誘導組之2 倍，(Fig. 5, $p < 0.05$)。此結果推測，以桑葉萃取物餵食高油脂飲食之C57BL/6 小鼠可以透過活化AMPK 平衡體內能量代謝，調節脂質生成蛋白的表現。

桑葉調節脂肪代謝的分子機制可能是透過抑制轉錄因SREBP1、SREBP2、PPAR γ 蛋白質層次表現，以高油飼料餵食C57BL/6 小鼠之動物模式，結果也發現SREBP1 降低約0.84 倍(0.5% MLE) 和0.81 倍 (1.5% MLE)；SREBP2 降低約1.19 倍 (0.5% MLE) 和1.07 倍 (1.5% MLE)；PPAR γ 降低約1.00 倍(0.5% MLE) 和0.91 倍 (1.5% MLE)。證明桑葉具有抑制脂生性轉錄因子在體內的蛋白表現，且有dose-dependent 趨勢，此現象與細胞試驗相符合 (Fig.5)。

觀察3T3-L1 脂肪細胞對於桑葉萃取物之敏感度為何，以決定後續細胞試驗所須之萃取物劑量。由Fig. 6 中之結果可以得知，MLE 與MLPE 對於要造成3T3-L1 前脂肪細胞並無致死毒性，所需之劑量相當大(IC₅₀ > 4 mg/mL)，因此後續實驗選用1 ~ 2 mg/mL 之MLE 而MLPE 則選用0.25~ 0.5 mg/mL 之劑量。

由Fig. 7 的結果中可以得知，MLE 與MLPE 皆具有抑制脂肪細胞分化之作用。在低劑量的MLE 即可抑制32%的細胞分化為成熟的脂肪細胞，而較高劑量

的MLE 的效果更為顯著，具有83%的抑制效果。相對於MLE，MLPE 也同樣具有抑制脂肪細胞分化之作用，在以較低劑量的MLPE 處理後，可降低42% 的脂肪細胞分化，而在高劑量的MLPE 共同處理之下，可明顯降低74% 的脂肪細胞分化。

有文獻證實抑制脂肪細胞分化之方式可能是透過促進脂肪細胞死亡，進而降低脂肪細胞分化之數目。故我們接著以流式細胞儀分析，在經由MLE 與MLPE 處理後的3T-L1 細胞株，是否會造成細胞之凋亡，結果發現，在MLE 刺激之下，確實會誘導3T-L1 脂肪細胞走向細胞凋亡的路徑(apoptosis)，且有dose-dependant 的趨勢(Fig. 8)。相似的結果，也出現在MLPE處理過後的細胞株，MLPE 在以更低的劑量作用下，即可達到細胞凋亡的作用，此點在3T-L1 脂肪細胞未分化之前不同(Fig. 6)。顯示MLE 與MLPE 所誘導的細胞凋亡作用可能僅針對已分化之脂肪細胞，但其中的詳細機制尚待進一步的實驗證明。

我們接著以西方點墨法分析脂肪細胞分化指標之相關蛋白表現，結果發現，MLE 與MLPE 確實可以抑制脂肪細胞的分化，且是透過降低相關轉錄因子的表現量，進而影響脂肪細胞之分化作用，而我們也同時分析了數種在脂肪細胞分化過程當中，皆扮演相當重要角色的轉錄因子，發現無論是SREBP1，或是PPAR γ ，以及ALK-7 皆會受到MLE 與MLPE 的處理過後，而導致蛋白表現量有所降低(Fig.9)，說明MLE 與MLPE 確實可以抑制脂肪細胞的分化作用，進而降低體脂肪的堆積。

(5) 結論

隨著人口年齡的老化，平均壽命之延長，所遭遇到的疾病及健康問題也隨之增加，因此現代人不僅是要活得長，更要活得健康，有尊嚴，也相對顯示預防醫學的重要，飲食所造成的肥胖實乃代謝性相關疾病形成之主因。依目前健康食品之開發趨近成熟，而現今在市面上所流通之保健食品亦不計其數，而目前市場上僅有少數產品通過相關規範，並不足以滿足日趨增加的市場需求，據研究指出桑葉含豐富抗氧物質及多酚成份，所以具代表性及說服力的功效為抗氧化與降血脂兩大方面，本實驗室目前已針對桑葉萃取物之功能性做相關深入探討，發現桑葉萃取物具有降血脂、抑制心血管疾病之功效，相關數據已發表在國際期刊當中。而本研究證實桑葉萃取物確實可以有效降低血液和肝臟中脂質含量及抑制脂肪細胞分化及促進凋亡，進一步減少體重及非酒精性脂肪肝(Fig.10)，且無肝腎毒性之不良副作用，而桑葉本身目前用途不多，經濟效益不大，因此材料成本不大，栽植方便，若能開發為健康食品一定具有相當之市場潛力，及經濟效益。

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Mulberry 1-Deoxynojirimycin Pleiotropically Inhibits Glucose-Stimulated Vascular Smooth Muscle Cell Migration by Activation of AMPK/RhoB and Down-regulation of FAK

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ABSTRACT: Mulberry 1-deoxynojirimycin (DNJ), an inhibitor of α -glucosidase, has been reported to help prevent diabetes mellitus and suppress lipid accumulation. The aim of this study was to determine whether mulberry DNJ has pleiotropic effects on the development of atherosclerosis. The mechanisms by which mulberry DNJ might inhibit migration of A7r5 vascular smooth muscle cells (VSMCs) under hyperglycemic conditions mimicking diabetes were investigated. The antimigratory effects of DNJ on VSMCs were assessed by Western blot analysis of migration-related proteins and by electric cell-substrate impedance sensing (ECIS) and visualization of F-actin cytoskeleton. Two pathways of DNJ-mediated inhibition of VSMC migration were identified. The first involved AMPK activation to inhibit fatty acid synthase (FASN) and Akt activity and then RhoB activation to inhibit nuclear factor- κ B (NF- κ B) and matrix metalloproteinase-2 (MMP) activity. The second involved inhibition of focal adhesion kinase (FAK), Ras, and RhoA activity leading to inhibition of F-actin activity.

KEYWORDS: DNJ, VSMC, migration, atherosclerosis, AMP activated protein kinase

INTRODUCTION

Compared to the general population, diabetic patients are 2–4 times more likely to develop atherosclerosis. Many risk factors, such as hyperglycemia, hypertension, hyperlipidemia, and smoking, may cause endothelial dysfunction, which is the first step in the atherosclerotic process. Subsequently, chemotactic cytokines and adhesion molecules may attract monocytes and cause their migration into the intimal layer of the vascular wall, where they become macrophages. Thereafter, macrophages take up oxidized low-density lipoprotein (LDL) and then transition into foam cells, a key component of atherosclerotic plaque. Monocyte-derived cytokines and growth factors will further stimulate vascular smooth muscle cell (VSMC) proliferation and migration. Monocytes may also secrete matrix metalloproteinases (MMPs) to induce plaque instability and then rupture. In atherosclerosis, the VSMC contributes to vascular wall inflammation, lipo-protein retention, and fibrous cap formation (which stabilizes plaque). Upon vascular injury, VSMCs transform into a more synthetic phenotype from their normally quiescent and contractile condition, then proliferate and migrate into the intimal layer of the artery wall to induce intimal hyperplasia, which is the mechanism of restenosis after percutaneous coronary intervention (PCI).^{1–4} Understanding the mechanisms underlying VSMC proliferation and migration is important for evaluating the involvement of these processes in the pathophysiology of atherosclerosis and restenosis after PCI.

Mulberry leaf and dietary mulberry leaf have been reported to have hypoglycemic, hypolipidemic, and antioxidative effects and to prevent coronary artery disease.^{5–11} Mulberry 1-deoxynojirimycin (DNJ) has been reported to be the main component of mulberry leaf that prevents diabetes mellitus (DM) by inhibiting α -glucosidase in the small intestine and in postprandial hyperglycemia.^{5,6,12} Intake of DNJ also suppresses lipid accumulation.^{12,13} Our previous studies have shown that mulberry leaf extract can effectively inhibit proliferation and migration of VSMCs and thereby atherosclerosis by up-regulating p53, inhibiting cyclin-dependent kinase, and blocking small GTPase, Akt/NF- κ B signals.^{13,14} Our aim in this study was to examine whether the pleiotropic effect of mulberry DNJ on the development of atherosclerosis goes beyond antidiabetes and antihyperlipidemia effects. Thus, we investigated mechanisms underlying mulberry DNJ inhibition of VSMC migration under hyperglycemic conditions mimicking diabetes.

MATERIALS AND METHODS

Cell Culture. The cell line A7r5, a smooth muscle cell line derived from rat thoracic aorta, was obtained from the American Type Culture Collection (ATCC no. CRL-1444; Manassas, VA, USA). A7r5 cells

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Mulberry Leaf Extract Inhibits the Development of Atherosclerosis in Cholesterol-Fed Rabbits and in Cultured Aortic Vascular Smooth Muscle Cells

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ABSTRACT: This study used high-cholesterol-fed New Zealand white rabbits and aortic vascular smooth muscle cells (VSMCs) to investigate the impact of mulberry leaf extract (MLE) on the development of atherosclerosis. The results show that the major components of MLE are polyphenols, flavonoids, carbohydrates, proteins, and lipids, and the major constituents of mulberry leaf polyphenol extract (MLPE) are polyphenols and flavonoids. In addition to improvement of liver function, the atheroma burden and levels of serum cholesterol, triglycerides, and low-density lipoprotein (LDL) are also significantly reduced after MLE treatment. MLE and MLPE improved endothelial function, inhibited proliferation and migration of aortic VSMCs, and reduced atheromas in the vascular wall. In conclusion, this study demonstrates that, in addition to exerting hypolipidemic effects, MLE and MLPE can effectively inhibit proliferation and migration of aortic VSMCs, improve vascular endothelial function, and reduce atheroma burden, thereby preventing atherosclerosis.

KEYWORDS: atherosclerosis, proliferation, migration, mulberry leaf extract

■ INTRODUCTION

The endothelium is a complex endocrine and paracrine organ that plays an important role in the maintenance of vascular homeostasis. Damage to the endothelium initiates a number of processes that promote atherosclerosis, including increased endothelial permeability, platelet aggregation, leukocyte adhesion, and cytokine production. Oxidative stress, caused by smoking, diabetes mellitus, hypertension, hypercholesterolemia, and aging, can lead to endothelial dysfunction. Structural regression of atherosclerosis is preceded by improvements in endothelial function.^{1,2}

Atherosclerosis, the major cause of coronary artery disease, is a low-density lipoprotein (LDL)-related chronic inflammatory process that involves endothelial cells, monocytes, vascular smooth muscle cells (VSMCs), and many soluble mediators. Stimulation of the vascular wall by oxidative stress leads to permeation of LDL into the subendothelial space, where it becomes oxidized. Monocytes recruited by the activation of the endothelium differentiate into macrophages. The oxidized LDL particles are then taken up by macrophages, which evolve into foam cells. Cytokines and growth factors secreted by macrophages also induce the proliferation and migration of VSMCs into the subendothelial space. These processes participate in the formation of atherosclerotic plaque. Plaques that contain inflammatory cells and have a fibrous cap covering

the lipid-rich core are at high risk for rupture, which can lead to acute coronary syndrome.^{3,4}

The polyphenol-rich mulberry leaf has been reported to have hypoglycemic,^{5,6} hypolipidemic,⁷ and antioxidant properties,^{8–11} and its administration may thus reduce the risk of cardiovascular disease. Our previous studies have shown that mulberry leaf extract (MLE) is rich in polyphenols and can effectively inhibit VSMC proliferation and migration. This inhibition occurs through up-regulation of p53, inhibition of cyclin-dependent kinase, and suppression of small GTPase and Akt/NF- κ B signaling,^{12,13} thereby inhibiting the development of atherosclerosis. In the present study, we use an animal model (cholesterol-fed New Zealand white rabbits) and aortic VSMCs to investigate the effect of polyphenol-rich MLE on the development of atherosclerosis.

■ MATERIALS AND METHODS

Materials. Mulberry water extracts were prepared from the fruit of *Morus alba* L. (mulberry), which was obtained from Dada township in Taichung, Taiwan. Rat aortic smooth muscle cell line A7r5 was obtained from Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu,

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Mulberry leaf polyphenol extracts reduced hepatic lipid accumulation involving regulation of adenosine monophosphate activated protein kinase and lipogenic enzymes

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ABSTRACT

Fat accumulation in the liver increases the risk of developing progressive liver injury. It can induce all the symptoms of metabolic syndrome, which is associated with many additional health problems, including increased risk of obesity, hypertension, insulin resistance, and non-alcoholic fatty liver disease (NAFLD). Therefore, prevention and treatment of fat accumulation in the liver are relevant to health promotion. Mulberry leaf polyphenol extracts (MLPE) have been known to modulate serum fasting glucose, lipid and antiatherosclerosis. However, the effect of MLPE on regulating hepatic lipid metabolism is unclear. This study evaluated the effects and mechanisms of MLPE in reducing hepatic lipid accumulation in cell culture. We found MLPE could regulate hepatic lipid accumulation. Further, numerous lipogenic enzymes, such as FAS (fatty acid synthetase), ACC (acetyl-CoA carboxylase), HMGCR (HMG-CoA reductase) and associated lipogenic transcriptional factors (SREBP1 and SREBP2) were suppressed by MLPE. Our results show MLPE is able to reduce hepatic lipid accumulation through activation of the AMPK (AMP-activating protein kinase) signaling pathway. It may have potential therapeutic implications for human NAFLD.

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1. Introduction

Unburnt energy is conserved in the form of fat in adipose tissue, leading to obesity and obesity-associated fatty liver disease (FLD) (Evans, Barish, & Wang, 2004). Obesity is closely

associated with diseases such as non-alcoholic fatty liver disease (NAFLD), hypertension, hyperlipidemia, arteriosclerosis and cancer (Kopelman, 2000; Tilg & Moschen, 2006; Willen & Hotamisligil, 2005). Obesity seems to be an important risk factor for non-alcoholic fatty liver disease (Ruderman, Chis-

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(7) 研究結果

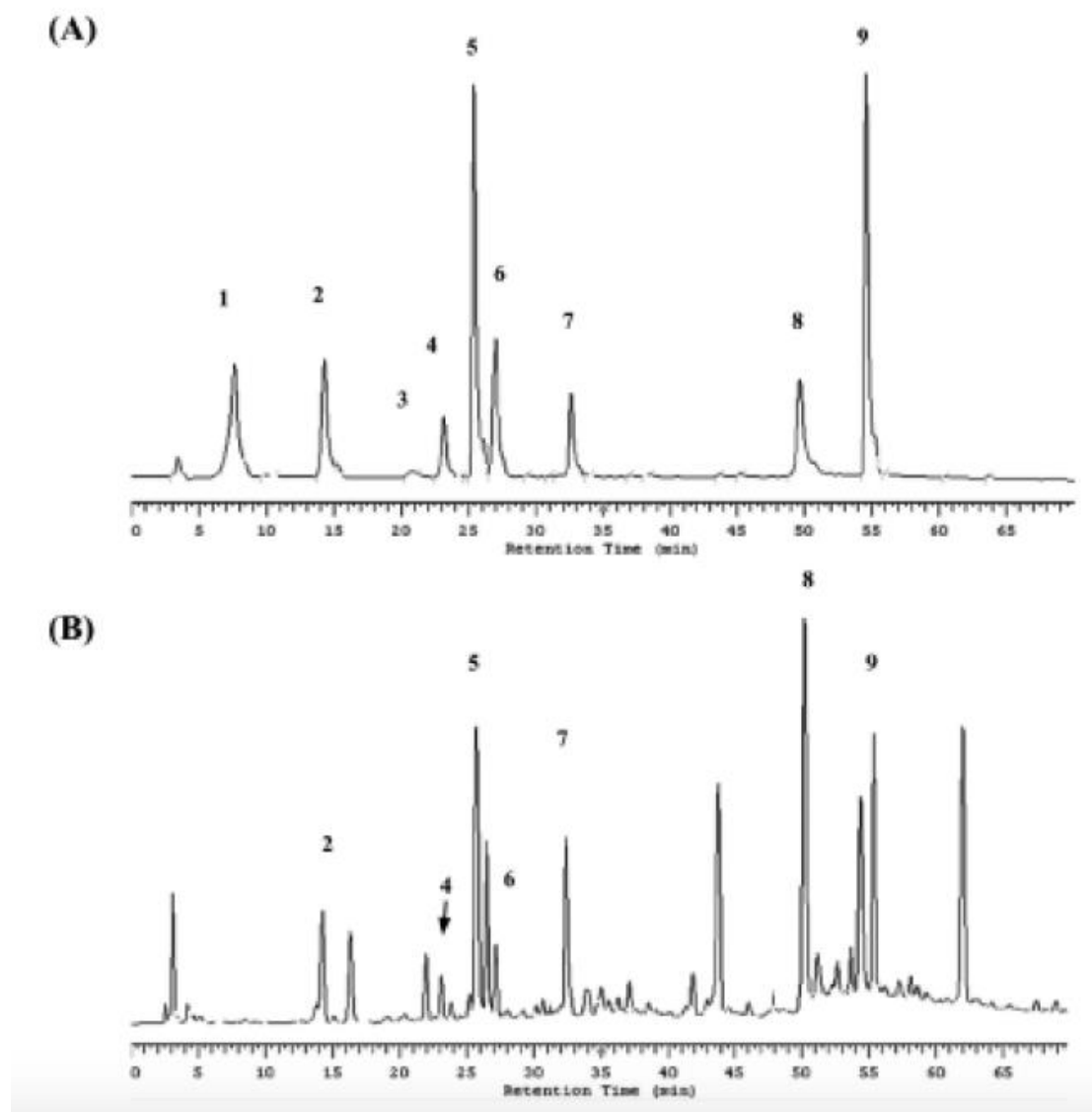


Figure 1. The HPLC chromatogram of MLPE. (A) HPLC chromatogram of nine kinds of standard polyphenols. Peaks: 1, gallic acid; 2, protocatechuic acid; 3, catechin; 4, gallic acid catechin; 5, gallic acid gallate; 6, caffeic acid; 7, rutin; 8, quercetin; 9, naringenin. (B) HPLC chromatogram of free polyphenols from MLPE.

Table 1. Body weight change of C57BL/6 mice fed basal laboratory diet or high fat diet during the 6 weeks feeding period.

Group	Weeks			
	0	2	4	6
C	21.55±1.54	22.63±1.69	24.56±1.39	25.57±1.38
		(5.05%)	(14.09%)	(18.79%)
HFD	21.80±1.26	22.73±1.93	25.96±1.5	28.60±1.7 ^b
		(8.87%)	(19.08%)	(31.19%)
MLE 0.5%	21.65±0.47	23.02±0.69	24.83±1.09	26.15±1.01 ^c
		(6.32%)	(14.68%)	(20.78%)
MLE 1.5%	22.50±0.66	23.79±0.71	25.33±0.69	26.87±0.71 ^c
		(5.75%)	(12.58%)	(19.46%)

()=((week X – week 0) / week 0)×100 %

b, *p* < 0.05 compared with the ND group.

c, *p* < 0.05 compared with the HFD group.

Table 2. Biochemical characteristics of C57BL/6 mice after 6 weeks on experimental diets.

Variable	C	HFD		
		—	MLE	
			0.5%	1.5%
GOT (U/L)	16.22±3.07	279.28±116.61 ^b	28.55±12.94 ^c	24.12±6.38 ^d
GPT (U/L)	10.33±1.12	60.66±12.45	14.33±7.45 ^c	13.50±4.00 ^d
BUN (mg/dL)	30.98±7.39	29.70±2.04	29.94±3.41	21.63±5.00
CRE (mg/dL)	0.24±0.04	0.54±0.12	0.33±0.08	0.30±0.12
GLU (mg/dL)	70.00±8.5	155.22±29.23 ^b	91.00±6.12 ^c	70.62±8.12 ^d
Na (mEq/L)	143.00±3.35	145.22±3.31	147.22±2.91	144.75±3.73
K (mEq/L)	4.92±0.24	4.90±0.21	4.73±0.25	4.88±0.17
TCHO (mg/dL)	71.66±7.43	157.33±38.58 ^b	101.11±9.75 ^c	81.37±8.11 ^d
TG (mg/dL)	29.00±5.61	125.77±34.95 ^b	42.11±8.84 ^c	32.75±5.75 ^d
HDL (mg/dL)	16.66±2.35	33.88±2.28 ^b	33.11±3.06 ^c	33.75±4.30 ^d
LDL (mg/dL)	45.88±4.04	79.33±12.7 ^b	68.66±7.76 ^c	43.25±4.50 ^d
LDL/HDL-C	2.80±0.47	2.34±0.27 ^b	2.07±0.19 ^c	1.30±0.22 ^d
Ketone body (mmol/L)	0.51±0.37	0.77±0.27	0.77±0.29	0.68±0.18
Free fatty acid (nmol)	6.42±0.51	6.89±0.44 ^b	6.52±0.17 ^c	5.57±0.92 ^d

HFD group were maintained on a high-fat diet containing 20% lard oil and 2% cholesterol for 6 weeks (n=10). C, normal group; HFD, high fat diet without MLE group; 0.5% MLE, C57BL/6 mice fed high fat diet with 0.5% MLE powder; 1.5% MLE, C57BL/6 mice fed high fat diet with 1.5% MLE powder. All data are expressed as the mean±SD. C, control; HFD, high fat diet; GOT, Glutamic-oxaloacetic transaminase; GPT, Glutamic-pyruvic transaminase; BUN, blood urea nitrogen; CRE, serum creatinine; GLU, glucose; Na, sodium; K, potassium; TCHO, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

b, p<0.05 for HFD group versus Control

c, p<0.05 for 0.5% MLE-treated group versus HFD

d, p<0.001 for 1.5% MLE-treated group versus HFD

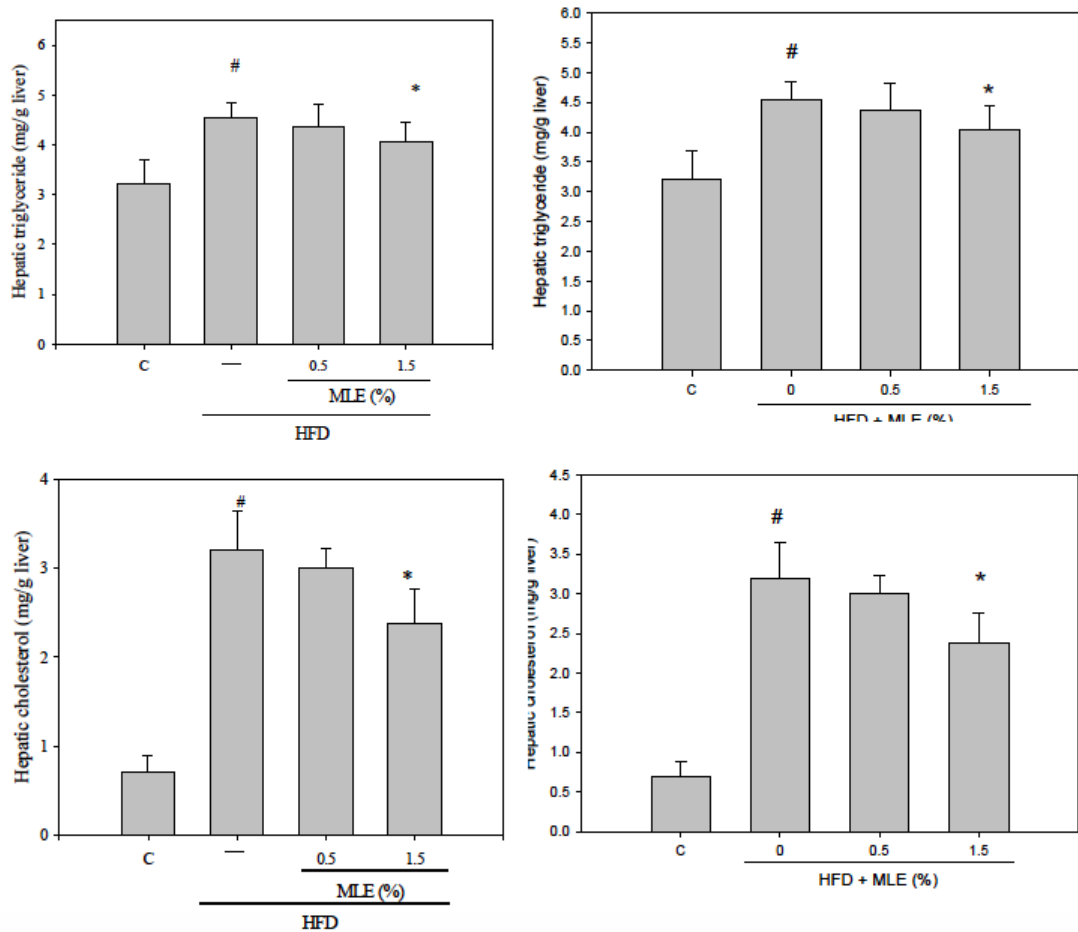


Figure 2. MLE reduced hepatic triglyceride and cholesterol content in HFD-fed C57BL/6 mice. Relative amounts of liver triglyceride (A) and cholesterol (B) contents in C57BL/6 mice (n=10). Lipids were extracted from liver tissue obtained from four group mice and analyzed by spectrometry. C, control; HFD, high fat diet; MLE-0.5%, C57BL/6 mice fed high fat diet with 0.5% MLE powder; MLE-1.5%, C57BL/6 mice fed high fat diet with 1.5% MLE powder. #, $p < 0.05$ compared with the control group. *, $p < 0.05$ compared with the HFD group.

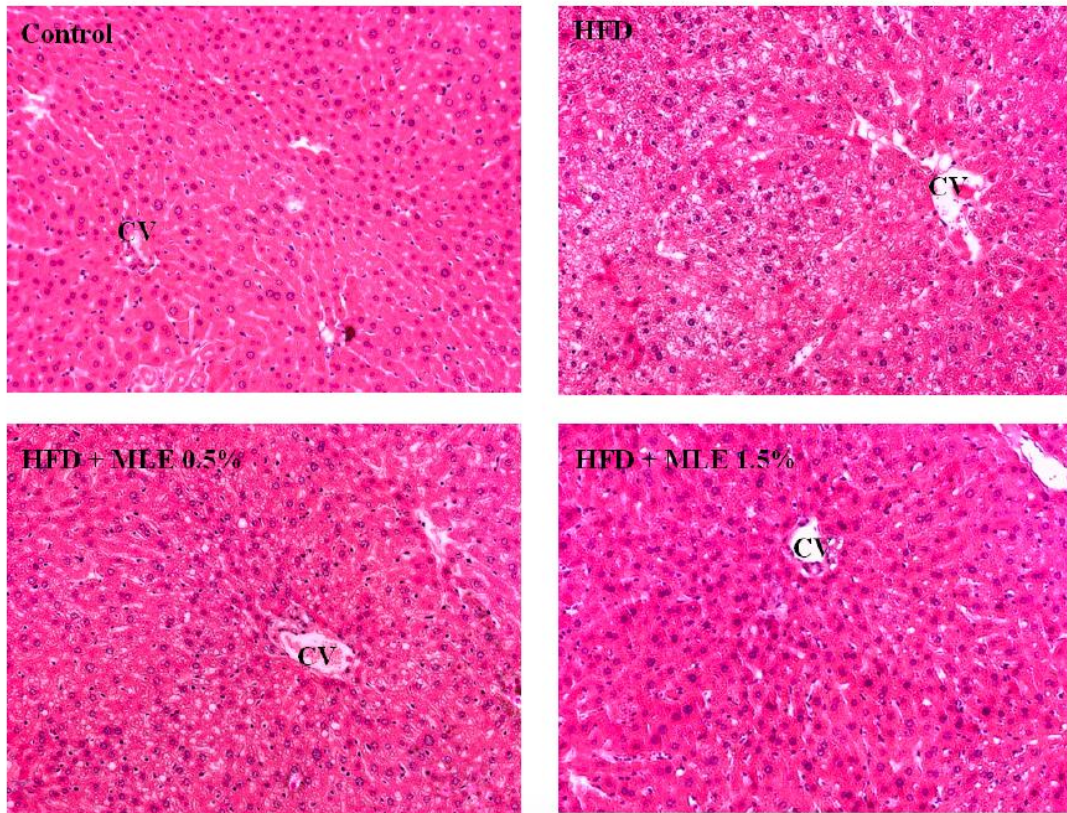


Figure 3. Histologic features of lipid accumulation in livers of C57BL/6 mice. HFD group were maintained on a high-fat diet containing 20% lard oil and 2% cholesterol for 6 weeks (n=10). C, normal group; HFD, high fat diet without MLE powder; MLE-0.5%, C57BL/6 mice fed high fat diet with 0.5% MLE powder; MLE-1.5%, C57BL/6 mice fed high fat diet with 1.5% MLE powder. Paraffin-embedded sections of liver from C57BL/6 mice were stained with hematoxylin and eosin. Representative photomicrographs are shown. (100×). CV, central vein

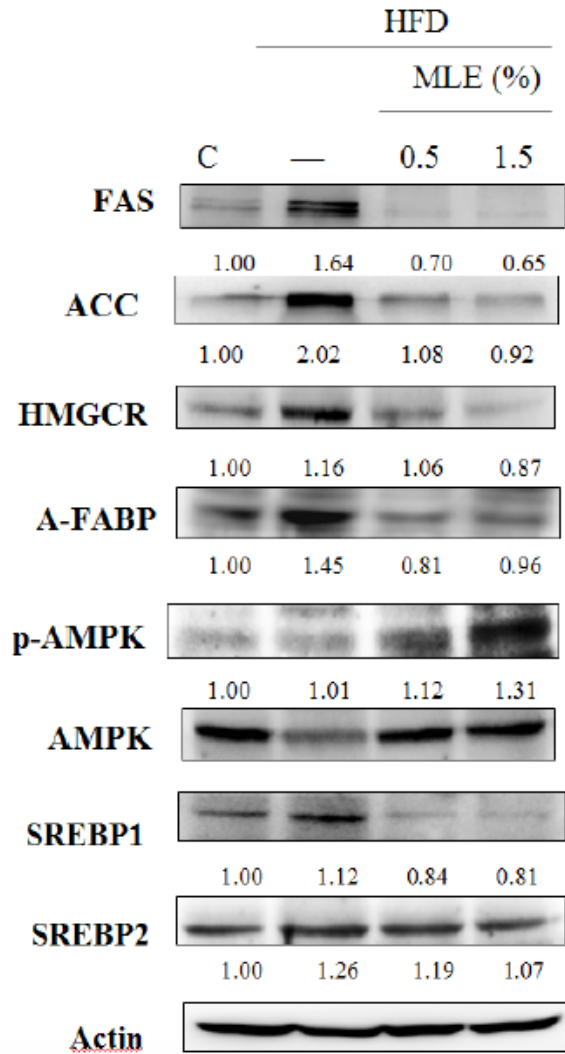


Figure 4. MLE suppressed lipogenic protein expression of liver in HFD-fed C57BL/6 mice. HFD group were maintained on a high-fat diet containing 20% lard oil and 2% cholesterol for 6 weeks (n=10). Protein expressions were detected by Western blot analysis. C, normal group; HFD, high fat diet without MLE powder; MLE-0.5%, C57BL/6 mice fed high fat diet with 0.5% MLE powder; MLE-1.5%, C57BL/6 mice fed high fat diet with 1.5% MLE powder. The data were shown as means \pm SD from three replicates per treatment.

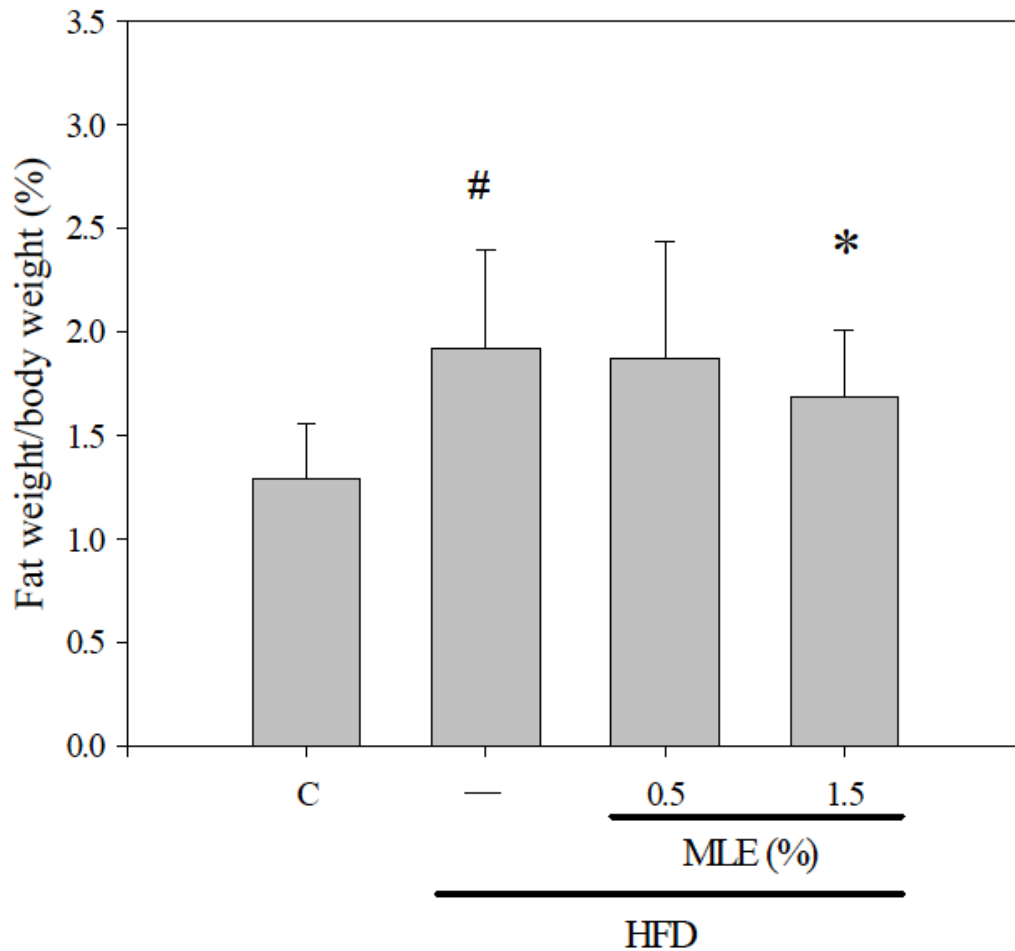


Figure 5. MLE attenuated total fat tissue in HFD-fed C57BL/6 mice. HFD group were maintained on a high-fat diet containing 20% lard oil and 2% cholesterol for 6 weeks (n=10). Total fat tissue content epididymal fat, perirenal fat, inguinal fat and subcutaneous fat tissue. C, normal group; HFD, high fat diet without MLE powder; MLE-0.5%, C57BL/6 mice fed high fat diet with 0.5% MLE powder; MLE-1.5%, C57BL/6 mice fed high fat diet with 1.5% MLE powder. #, $p < 0.05$ compared with the control group. *, $p < 0.05$ compared with the HFD group.

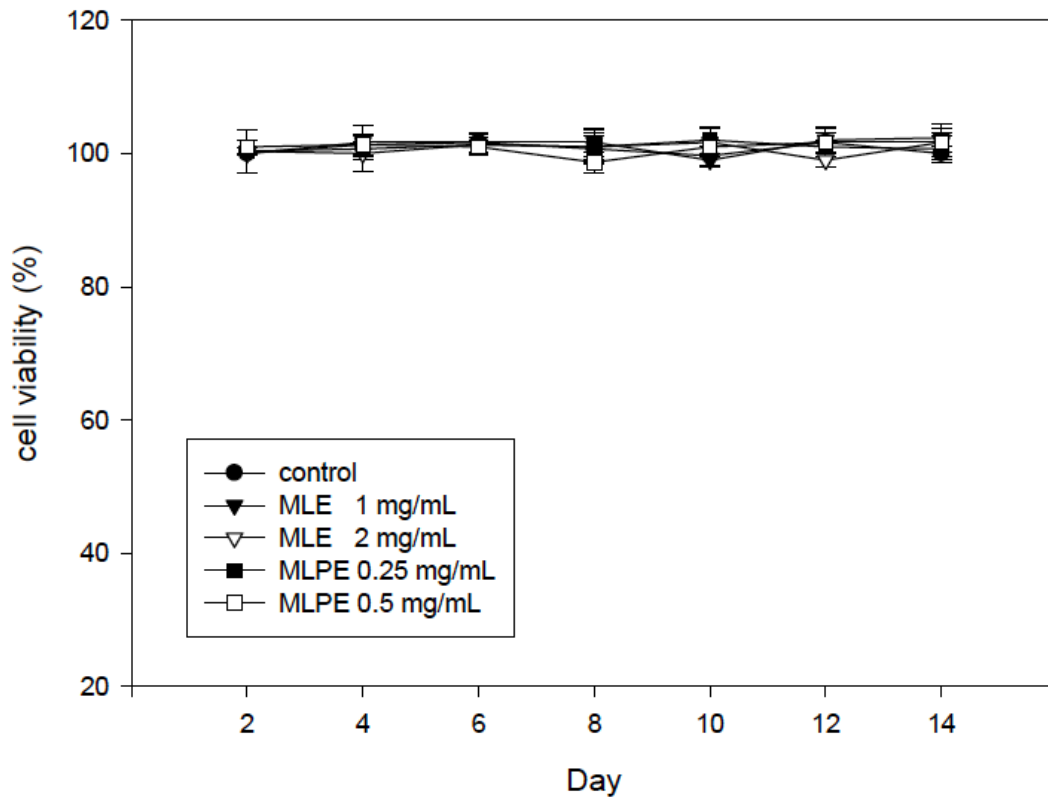


Figure 6. The cytotoxicity effect of MLE or MLPE on 3T3-L1 preadipocyte cells. 3T3-L1 preadipocyte cells were incubated with various concentrations of MLE (1 or 2 mg/mL) or MLPE (0.25 or 0.5 mg/mL) at 37°C for indicated time. Cell viability was measured by MTT assay. The data were shown as means \pm SD from three replicates per treatment.

Pre-adipocyte

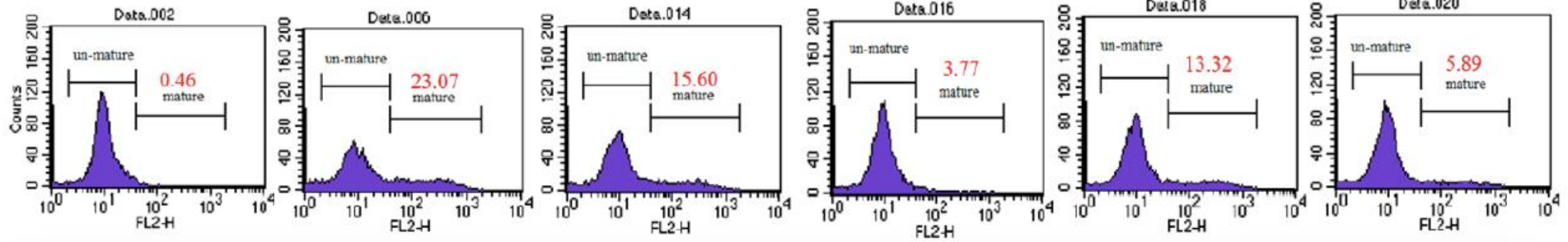
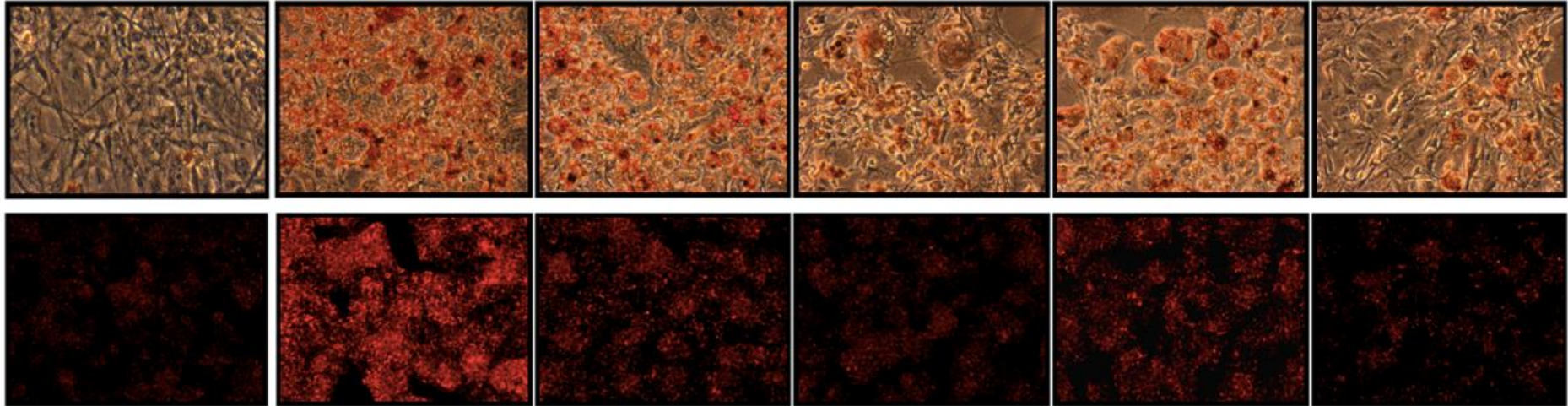
Induced

MLE
1 mg/mL

MLE
2 mg/mL

MLPE
0.25 mg/mL

MLPE
0.5 mg/mL



(B)

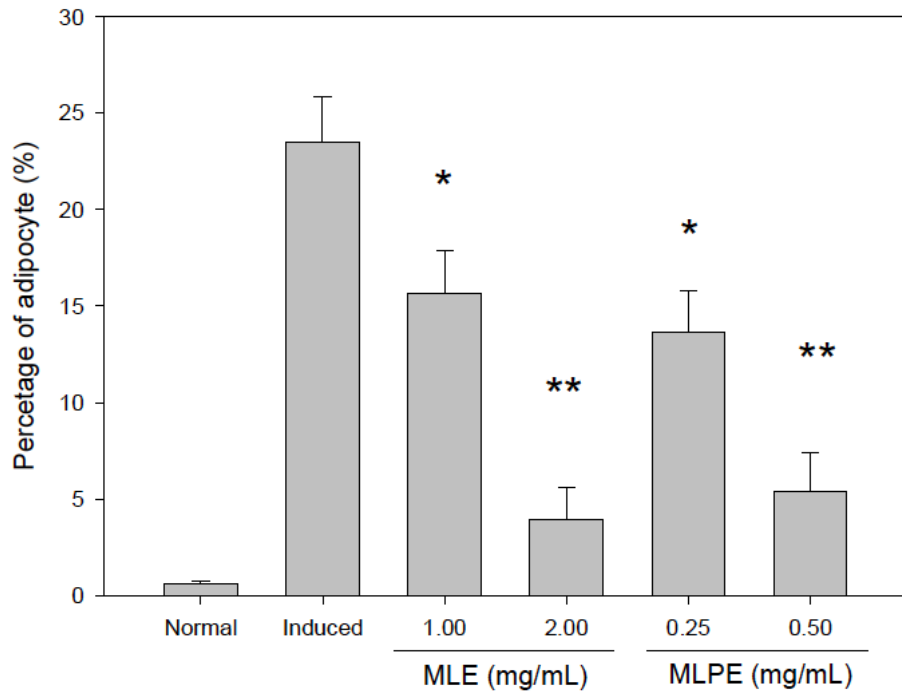
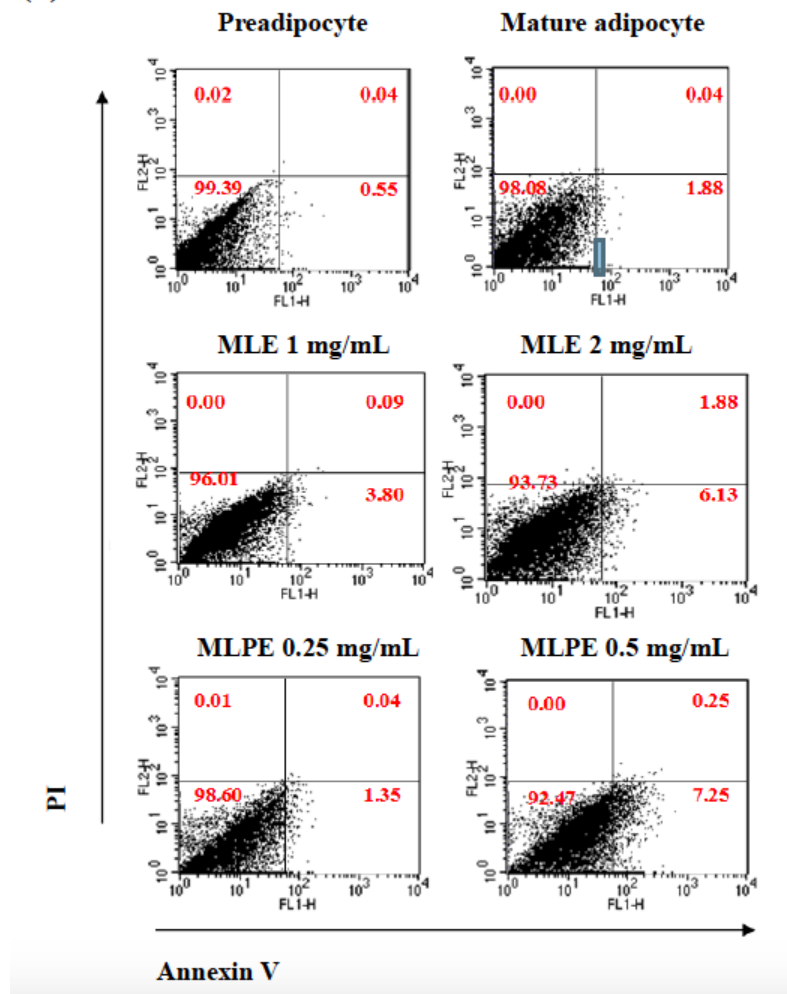


Figure 7. MLE and MLPE inhibited the fat accumulation in mature adipocyte cell. Postconfluent 3T3-L1 mature adipocytes were treated with MLE or MLPE. 3T3-L1 cell were stained with O oil red (top, A) Nile red (medial, A) and flow cytometry (bottom, A). The fluorescence was quantified by flow cytometry (B). The data were shown from three replicates per treatment.

(A)



(B)

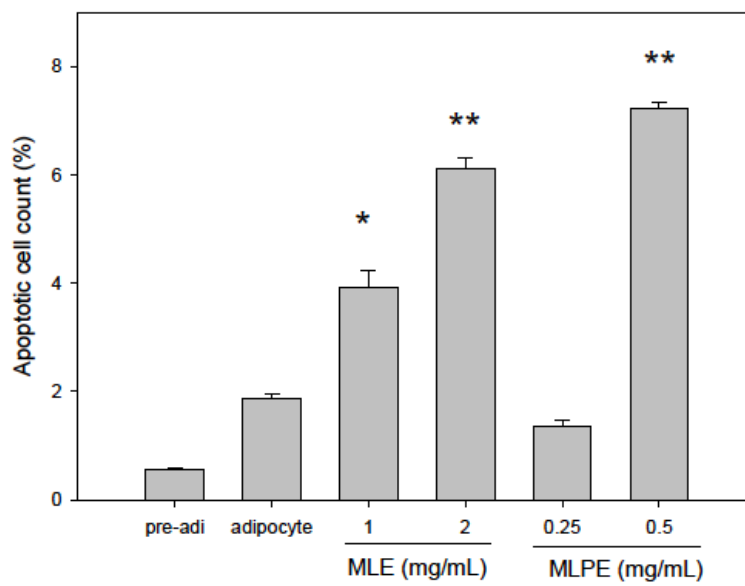


Figure 8. MLE and MLPE induced apoptosis in mature adipocyte. Postconfluent 3T3-L1 mature adipocytes were treated with various concentration MLE or MLPE. 3T3-L1 cell were stained with Annexin V/PI then analysed by flow cytometry (A). The lower-right phase is the early apoptotic cells. The fluorescence was quantified by flow cytometry (B). PI: propidium iodide. The data were shown from three replicates per treatment.

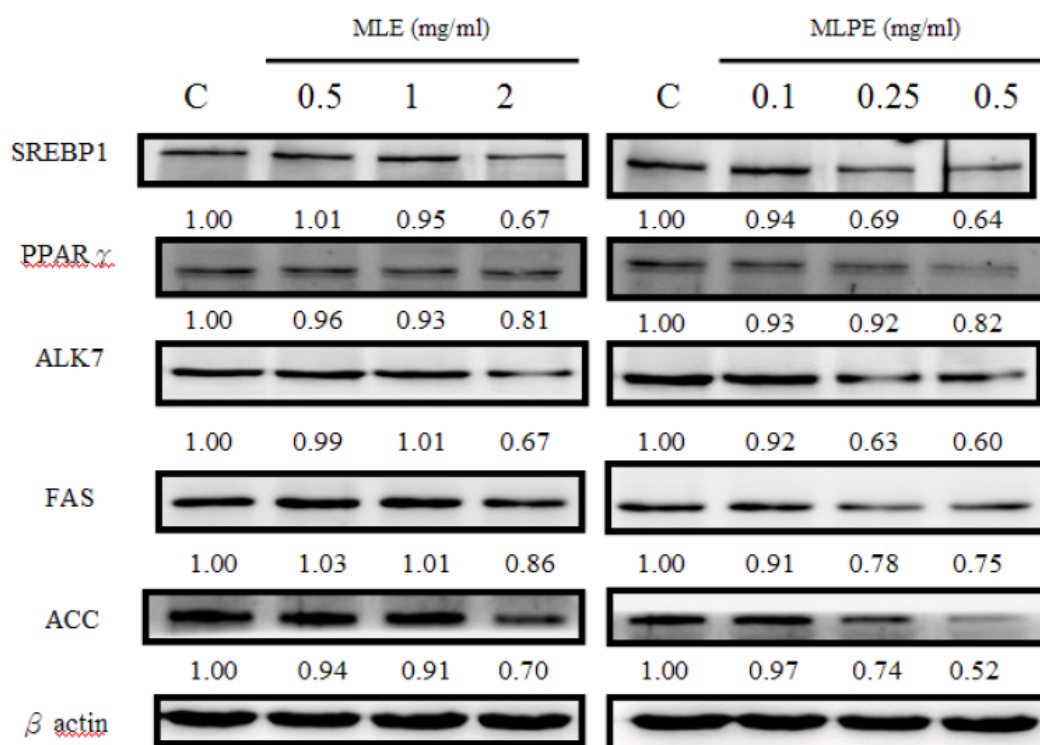


Figure 9. MLE and MLPE inhibited mature adipocyte differentiation. MLE and MLPE reduced the expression of adipocyte differentional markers SREBP1, PPAR γ and ALK7. Postconfluent 3T3-L1 mature adipocytes were treated with MLE or MLPE. The proteins isolated from cell were analyzed via Western blot with anti-SREBP1, PPAR γ and ALK7 antibody. The detailed method is described under "Experimental Procedures." The indicated arrow on the right side represents indicated protein. All data are expressed as a fold relative to control untreated cells. The data were shown as means \pm SD from three replicates per treatment.

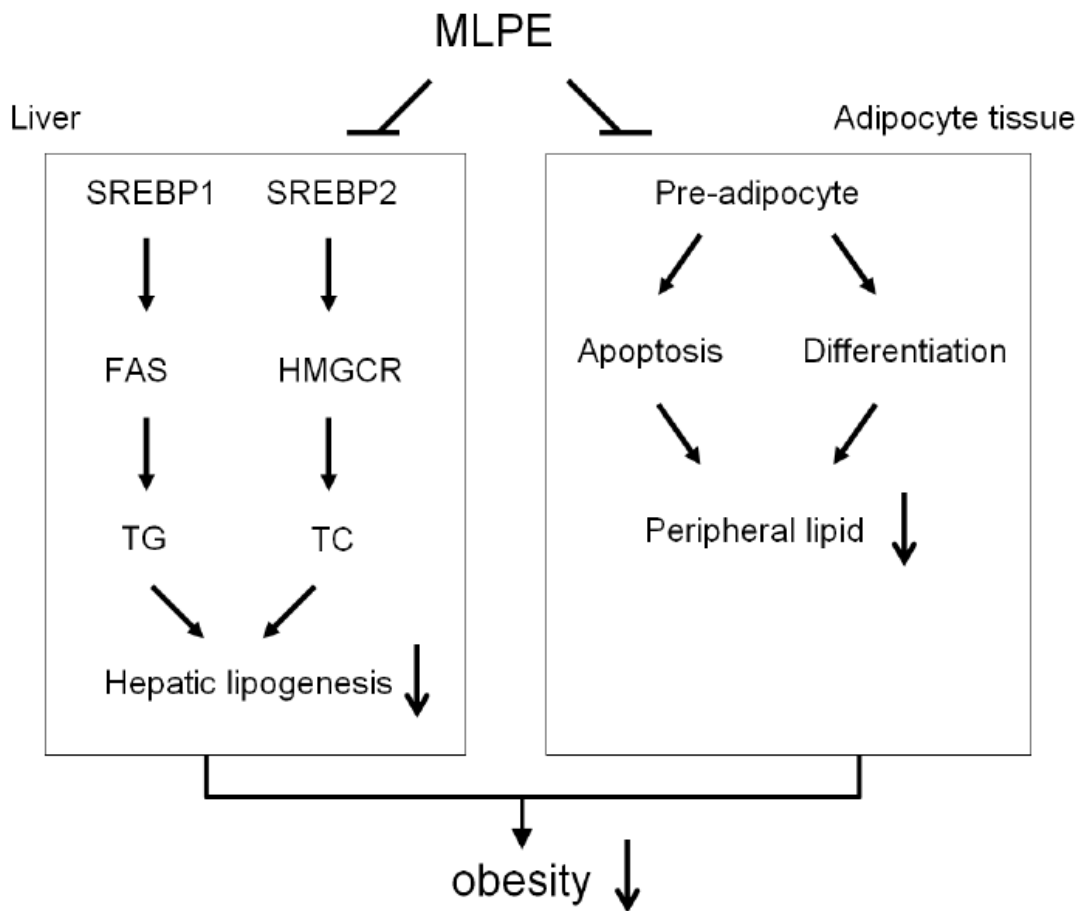


Figure 10. Schematic model of the MLPE ameliorate obesity. MLPE were shown to be capable of inhibiting hepatic lipogenesis through the mechanism, attenuation of SREBP1/FASN/triglyceride pathway and SREBP2/HMG CoA reductase /total cholesterol pathway. MLPE also reduced peripheral lipid accumulation by inducing mature adipocyte apoptosis and suppressing adipocyte differentiation. These results suggested that MLPE can be beneficial for the suppression of high-fat-diet-induced dyslipidemia, hepatosteatosis and obesity.

第二年度 研究報告

桑葉成份抑制非酒精性脂肪肝及肥胖促進肝癌及抗藥性之研究(第2年)

(二)中、英文摘要及關鍵詞

關鍵字：桑葉多酚；肥胖；肝癌；發炎；抗氧化。

中文摘要

來自乾燥桑葉 (*Morus spp.*)中的桑葉水萃物(MLE)與多酚萃取物 (MLPE) 被證實具有降血糖、降血脂、對抗粥狀動脈硬化等心血管疾病危險因子，本研究第一年證實MLE 與MLPE 減少體脂肪堆積之作用與機制(已發表於Journal of Functional Foods,5,620-632,2013)，並分別以3T3-L1 adipocyte 分化模式證明MLPE 也可降低脂肪細胞分化，並透過誘導細胞凋亡的方式，減少脂肪細胞內脂肪的堆積作用，降低體脂肪之效果(Mulberry leaf extract inhibits adipocyte differentiation through regulation of adipogenesis in 3T3-L1 cells and high-fat diet-induced obesity. Food Chem.投稿中)。第二年我們探討MLE 抑制肥胖促肝癌作用，將HepG2 細胞培養於含有15% 3T3-L1 脂肪細胞CM 共同培養在無血清之DMEM 培養基內顯示MLPE 可抑制CM 誘導HepG2 細胞增生之作用，MLPE 抑制CM 所誘導之肝癌細胞增生是透過TNF- α 下游調控之NF κ B 路徑，而非p38 MAPK/ AP1 路徑。當細胞處理MLE 其pStat3/Stat3 及PI3K/Akt/mTOR 也減少。顯示MLPE 為MLE 的功能成份，其作用是透過抑制了TNF- α 及IL-6 的活化途徑，使得CM 誘導之HepG2 細胞的增生被抑制。進一步的動物試驗，顯示MLE 減少HFD 促進DEN 致肝癌的作用，其作用包含了MLE 抑制TNF- α 及IL-6，增加

antioxidant enzymes 的活性，透過這些作用調節targets 減少肝癌化作用，目前正在蛋白質體研究中。

Abstract

Keywords: Mulberry Leaf Polyphenolic Extract; Obesity; Hepatocarcinoma; Inflammation; Antioxidation

Dietary fat is one of the most important environmental factors associated with the incidence of obesity. In the first year study, the antiobesity effects of Mulberry leaf extract (MLE) and Mulberry leaf polyphenol extracts (MLPE) were investigated. The results showed that intake of MLE and MLPE can be beneficial for the suppression of HFD-induced dyslipidemia, hepatosteatosis, and obesity. Moreover, the MLE and MLPE induced 3T3-L1 adipocytes apoptosis and inhibited on the differentiation of preadipocytes. In the second year, we assayed the effect of MLE on inhibiting hepatocarcinogenesis promoted by obesity. HepG2 cells were cultured in no serum DMEM contained 15% conditioned medium (CM) obtained from 3T3-L1 cell cultured medium. The results indicated that MLPE inhibited proliferation of HepG2 cells induced by CM via TNF- α regulating NF κ B but not p38 MAPK/ AP1 signaling. The decreased expression of pStat3/Stat3 and TNF- α 及 IL-6 in MLE treated cells was also investigated. In conclusion, MLPE, the functional ingredient of MLE, could inhibit the proliferation of HepG2 induced by CM from 3T3 cells through suppressing TNF- α and IL-6 activation. Further animal study showed that MLE reduced TNF- α and IL-6 expression, increased antioxidant enzymes activation, thereby lessened DEN-induced hepatocarcinogenesis promoted by high fat diet (HFD). Next, we will clarify the related targets using proteomic research.

(三)報告內容：前言、研究目的、文獻探討、研究方法、結果與討論

(1) 前言、研究目的、文獻探討

1. 非酒精性脂肪肝 (non-alcoholic fatty liver disease, NAFLD)

1-1. 肥胖與非酒精性脂肪肝

在臨床上所謂的「脂肪肝」是指肝臟內所屯積的脂肪(主要是三酸甘油酯)的重量超過全肝臟重量的百分之五，或是超過百分之十以上的肝細胞呈現脂肪空泡變性的現象而謂之。非酒精性脂肪肝病(non-alcoholic fatty liver disease, NAFLD)的病因也有許多，包括某些藥物及毒物、先天性代謝疾病、及後天代謝性疾病，其中最常見病因的就是肥胖症、糖尿病及高血脂症[1]。NAFLD 是目前肝臟醫學界對非酒精性脂肪肝病最廣為接受的一種命名，而NASH(non-alcoholic steatohepatitis)的也屬於NAFLD 的一種狀況。事實上，NAFLD 病程中的脂肪變性及其所衍生成NASH 的真正致病機轉目前尚不很明確，最為經典的假說則以Day 與James[2]提出胰島素抗性(insulin resistance, IR)以及脂質過氧化作用對肝細胞進行攻擊，導致肝臟發生瀰漫性脂肪浸潤、炎症反應、壞死、凋亡、再生損害以及肝星狀細胞活化等一系列病理連鎖性免疫攻擊反應，導致肝臟進一步發生壞死性炎症以及纖維化的病變。

1-2. NAFLD 致病機轉

肝臟是脂肪代謝的重要器官，一旦肝臟受到損傷後，均可導致脂質(尤其是三酸甘油酯)在肝細胞內異常的代謝及屯積[3]。理論上，由於個體攝取過多的高脂肪類飲食並導致高脂血症、肝細胞合成FFA 或由碳水化合物轉化成三酸甘油酯增加、FFA 在肝細胞粒線體內氧化及利用率降低，而轉變為三酸甘油酯、由於VLDL 本身合成的減少，而導致三酸甘油酯轉運發生障礙，結果脂肪組織以及

肝細胞間的脂肪代謝動態失衡，以及肝細胞中的三酸甘油酯合成及排泄之間的平衡發生障礙，因而導致三酸甘油酯在肝細胞內過度屯積，最終則衍生脂肪變性[4]。

2. Obesity promoted hepatocellular carcinoma

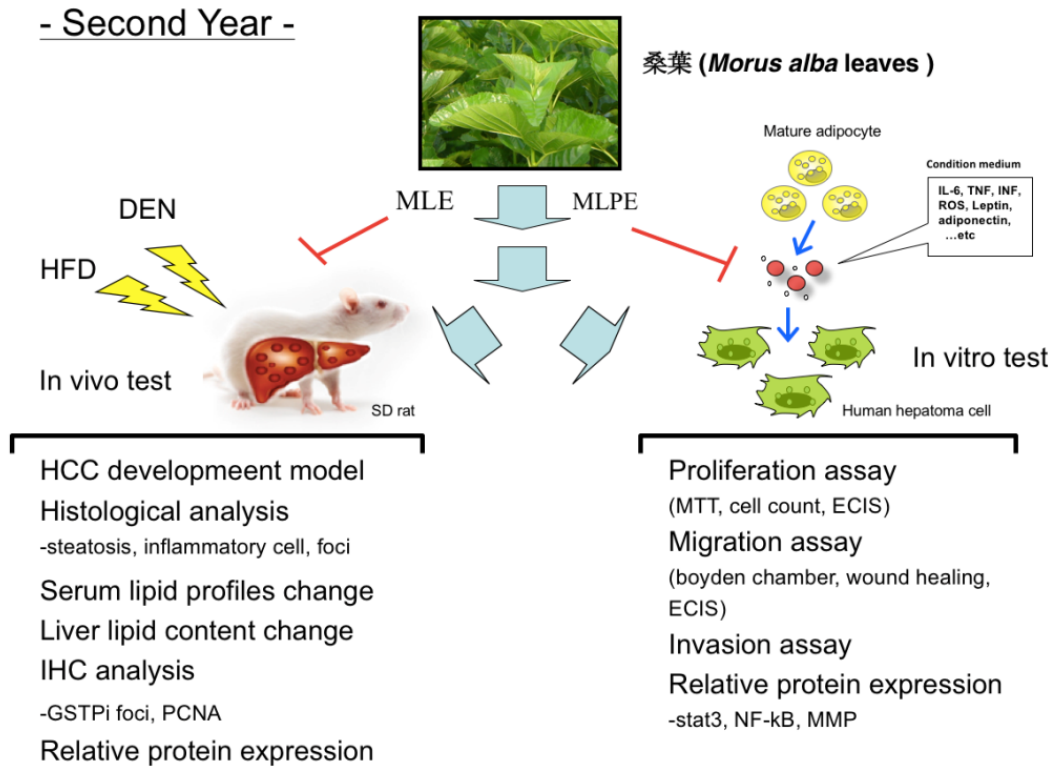
肥胖已被證明是一個獨立的危險因素，對某些惡性腫瘤，包括乳腺癌，子宮內膜癌，結腸癌，腎細胞癌，食管腺癌，胰腺癌，肝癌具潛在性影響，肥胖也代表一個獨立的HCC 危險因素[5]。研究指出，代謝性疾病(肥胖和糖尿病)和病毒性肝炎之間的協同作用，HBV 或HCV 攜帶者的肝癌風險與肥胖和糖尿病增加超過100 倍[6]。肥胖與代謝性症候群相關，包括胰島素抗性和第2 型糖尿病及一連串的非癌性肝臟疾病，如NAFLD 和NASH，肝纖維化，和肝硬化等。值得注意的是，肥胖和NAFLD 可誘導肝細胞的增殖和降低在小鼠模型中的肝細胞的凋亡，在纖維化的情況下，導致肝增生，炎症，和肝硬化[7]。

3. 桑葉醫學用途

已有許多研究證實桑葉的醫學功能，包括具有(a)抗菌及抗氧化能力[8, 9]，也具有抗發炎之能力[10, 11]；(b)抑制動脈硬化症[12]；(c) 抗糖尿病[13, 14]；(d) 抗發炎[10]；(e)神經保護[15]；(f) 抑制皮膚色素[16]。桑葉能有效抑制DOPA oxidase 和antityrosinase 的活性[17]。目前我們已發表四篇有關於桑葉及主成份調節血脂及抑制血管病變及機轉之研究[18-21]。

(2) 研究架構

第二年：MLE 和MLPE 抑制脂肪細胞分化培養液(CM)之促進肝癌作用



(3) 研究方法

3-1. 誘導3T3-L1 分化及Conditioned medium(CM)製備

誘導3T3-L1 至第14 天完全分化成脂肪細胞。繼續培養細胞48 h 收集脂肪細胞培養液，離心後取上清液稱CM。將肝癌細胞HepG2 cell 培養於FBS-free media: CM= 1:1 的比例混合培養HepG2 cell 48 h 做以下的實驗。

3-2. CyQuant for cell proliferation assay

將HepG2 cell 處理medium 組別、CM 組別、CM+MLE 組別及CM+MLPE 組別處理200 μ l CyQuant GR kit 分析細胞增生情形。

3-3. Wounding assay 及ECIS assay

藉由Yellow tip 在培養皿中劃出一道所謂的“傷口”來持續觀察細胞移動的能力。採用加入正常medium 組別、CM 組別、CM+MLE 組別及CM+MLPE 組別進行測試，每隔一段固定時間（分別是0、12、24、48、72 小時）將細胞以倒立顯微鏡在400X 下觀察傷口癒合的情形；並拍照計數爬進傷口的細胞數量。ECIS assay 此方法利用是利用電學參數的變化偵測細胞的黏附和伸展、成長曲線、活動力與屏障功能等。

3-4. Invasion assay

將transwell insert 覆蓋一層matrix gel，經過24 或48 小時的反應時間後，利用倒立顯微鏡在400X 下觀察細胞移動情形並且計數有多少的細胞能夠移動到下層。

3-5. Quantitative real time PCR (qRT-PCR)

利用Q-PCR 觀察不同組別之TNF- α 與IL-6 之mRNA 表現量。

3-6. Western blot

以SDS-PAGE 分析蛋白的表現。包括AMPK, ACC, FASN, MMPs, TIMP1, p-p38, p-Stat3, NF- κ B, MAPK 等，並進一步進行蛋白質體分析。

3-7. IL-6 and TNF- α antibody depletion

誘導3T3-L1 分化而來的CM 培養在2 μ g/ml anti-mIL-6-antibody 或是IgG1 (R&D Systems), 取上清液的CM 與HepG2 cells一起培養並做invasion、migration、western blot 等實驗。藉以釐清IL-6 及TNF- α 對細胞增生、invasion、migration 機制。

3-8. MLE 抑制肥胖促進DEN 致肝癌作用

3-8-1. Animal and diets

實驗動物隨機分成下列組別(N=8): Group 1:正常對照組，給予正常飲食Purina Lab Chow 及正常飲水; Group 2: HFD 組，Purina Lab Chow (78%), lard oil (20%), cholesterol (2%); Group 3:實驗組，HFD+0.5% MLE; Group 4: 實驗組,HFD+1% MLE。餵食12 週後犧牲動物，動物犧牲前空腹約14~16 小時，斷頭採集血液，並取肝臟組織作HE stain、 蛋白分析及免疫組織染色，進行組織病理學觀察，包含steatosis, inflammatory cells(per mm²), ballooning degeneration of hepatocytes。

3-8-2. Immunohistochemical staining (PCNA and P-GST foci)

將石蠟包埋的組織切片以fresh xylene 浸泡5 min 2 次，進行脫蠟。接著再以100% EtOH 浸泡5 min, 95% EtOH 浸泡5 min 1 次, 85% EtOH 浸泡5 min 1 次，70% EtOH 浸泡5 分鐘1 次，ddH₂O 浸泡5 min 1 次，目的為rehydration。接著在做些前處理，首先先用sodium citrate buffer (0.1 M citrate buffer, 0.1 M sodium buffer in ddH₂O)煮沸15 min，放置室溫自然冷卻，再用1% H₂O₂ 浸泡5 min 後以PBS 清洗3 次，用3 % FCS (fetal calf serum) blocking 20 min，將玻片傾斜，吸去多餘的FCS。以primary antibody (PCNA及P-GST antibody)在室溫作用1 h 後，以

PBS 清洗5 min 2 次，在加入secondary antibody (biotin labeled) 1 : 500 於室溫下作用1 h 後以PBS 清洗5 min 2 次，加入ABC solution (kit)作用45 min 後以PBS 清洗5 min 2 次，再用DAB 於室溫下避光、呈色5 min 後以Tris 清洗乾淨，再用hematoxylin 染色，來回震盪20 下，以H₂O 洗去多餘的染劑，ddH₂O 浸泡5 min 1 次，70% EtOH 浸泡2 min 1 次，85% EtOH 浸泡2 min 1 次，90% EtOH 浸泡2 min 1 次，100% EtOH 浸泡2 min 1 次，xylene 浸泡2 min 3 次，最後以aqueous mounting medium 進行封片，於顯微鏡底下觀察。

3-8-3. Quantification of cell apoptosis

主要利用Terminal dUTP Nick End Labeling (TUNEL) assay 評估肝臟組織中凋亡的細胞。將石蠟包埋的肝臟組織切片進行脫蠟後，用100 mM sodium citrate buffer 煮沸20 min，隨後置於3% H₂O₂ 浸泡15 min 冷卻後，將肝臟切片加入TMR-dUTP 和TdT (Roche Diagnostic) 在37°C下避光1 h，於螢光顯微鏡下觀察細胞的變化。

3-8-4. Hepatic lipid peroxidation

主要是測量肝臟組織中的脂質過氧化物malondialdehyde(MDA)。使用不同濃度1,1,3,3- tetramethoxypropane (TEP)作為標準液並畫出標準曲線，進行肝臟脂質過氧化的定量程度。取稀釋10 倍的肝臟組織液200 µl TCA buffer (trichloroacetic acid)混合後，於4°C下離心 (1000 rpm、30 min)後，取上清液200 µl 再加入200 µl TBA buffer (thiobarbituric acid)混合後，於95°C下加熱並避光反應40 分鐘後，以螢光光度計在532 nm/600 nm 下測定。

3-8-5.肝臟中抗氧化酵素之測定

麩胱苷肽過氧化酶活性分析(glutathion peroxidase activity)以H₂O₂當受質，當H₂O₂ 與GSH (glutathion) 作用時，GPx 會將GSH 催化成氧化型的麩胱苷肽

(oxidized glutathione, GSSG)，而GSSG 利用GR (glutathion reductase) 與NADPH 作用就可還原成GSH，並於波長340 nm 下遞減的速度，就可以間接求得GPx 的活性。超氧化物歧化酶活性分析 (superoxide dismutase activity) 利用pyrogallol 在鹼性環境下會進行自氧化作用，而當發生自氧化作用時pyrogallol 會反應形成 purpurgallin，且在反應的初期會產生大量的超氧自由基，但當有存在SOD 時則會將超氧自由基歧化，進而抑制pyrogallol 自氧化速率，所以可經由此換算SOD 活性。過氧化氫酶活性分析 (catalase activity)

catalase 具有分解 H_2O_2 的能力，形成 H_2O 與 O_2 。而 H_2O_2 在波長240 nm 下具有吸光值，所以當存在catalase 則 H_2O_2 的吸光值會逐漸減少，所以可以經由吸光值之變化得知過氧化氫酶的活性。

3-8-6.肝臟中解毒酵素測定

將保存在 $-80^{\circ}C$ 的老鼠肝臟取出，加入適量之homogenization buffer，以組織研磨機進行均質，將均質後組織液於 $4^{\circ}C$ 以39000 rpm 離心30 分鐘，吸取上清液即含有肝臟解毒酵素之萃取液，取1.5 mL GSH substrate buffer 及1.5 mL CDNB substrate buffer 置入石英管中，再加入20 μ L 之萃取液，迅速混合均勻，在340 nm 下，反應3 分鐘，並測定OD 值變化，以公式換算活性之變化量。

3-8-7.血中發炎因子之測定

本實驗採用定量三明治原理的酵素免疫分析(quantitative sandwich enzyme immunoassay, RayBio[®] Rat TNF- α ELISA Kit and RayBio[®] Rat IL-6 ELISA Kit)測量血液中TNF- α or IL-6 的濃度。此分析中96 孔分析盤中已結合大鼠 anti-TNF- α and IL-6 之單株抗體，血液中的TNF- α or IL-6 會與分析盤中的單株抗體結合。取100 μ L 的血液檢體或標準品在低溫 $4^{\circ}C$ 下反應12~16 小時後，未結合的物質會在清洗的過程中洗去，另外再加入100 μ L 能與TNF- α or IL-6 結

合的單株抗體(biotinylated antibody)，室溫反應1 小時後，未結合的抗體會在清洗過程中洗去，再加入100 μL 帶有酵素會與biotin 形成高親和性抗體酵素結合體的streptavidin solution，室溫反應45 分鐘後，未結合的抗體酵素結合體會在清洗過程中洗去，接著加入100 μL 酵素受體(TMB One-Step Substrate Reagent)呈色，避光反應30 分鐘後，最後以50 μL 終止液(stop solution)終止酵素呈色反應，於波長450 nm下測其吸光值變化。當血液中的TNF- α and IL-6 越多時，則抗體酵素結合體經血液TNF- α or IL-6 與分析盤中單株抗體之結合數目會增加，此時酵素標定反應物呈色會越深，即呈色與血液中TNF- α or IL-6 含量呈正比。

3-8-8. 血中肥胖因子之測定

本實驗採用定量三明治原理的酵素免疫分析(quantitative sandwich enzyme immunoassay, RayBio[®] Rat Leptin ELISA Kit and AssayPro Rat Adiponectin ELISA Kit))測量血液中Leptin and Adiponectin 的濃度。此分析中96 孔分析盤中已結合大鼠anti-Leptin or antiadiponectin 之單株抗體，血液中的Leptin or adiponectin 會與分析盤中的單株抗體結合。取100 μL 的血液檢體或標準品在低溫4 $^{\circ}\text{C}$ 下反應12~16 小時後，未結合的物質會在清洗的過程中洗去，另外再加入100 μL 能與Leptin or adiponectin 結合的單株抗體(biotinylated antibody)，室溫反應1 小時後，未結合的抗體會在清洗過程中洗去，再加入100 μL 帶有酵素會與biotin 形成高親和性抗體酵素結合體的streptavidin solution，室溫反應45 分鐘後，未結合的抗體酵素結合體會在清洗過程中洗去，接著加入100 μL 酵素受體(TMB One-Step Substrate Reagent)呈色，避光反應30 分鐘後，最後以50 μL 終止液(stop solution)終止酵素呈色反應，於波長450 nm 下測其吸光值變化。當血液中的Leptin or adiponectin 越多時，則抗體酵素結合體經由血液Leptin or adiponectin 與分析盤

中anti-Leptin or antiadiponectin 單株抗體之結合數目會增加，此時酵素標定反應物呈色會越深，即呈色與血液中Leptin or adiponectin 含量呈正比。

3-9. Proteomic analysis

誘導3T3-L1 分化而來的CM 培養於肝癌細胞HepG2 cell 48 h，收蛋白。

A: 第一維電泳: 150 µg 的去蛋白血清使之在水和溶解於含7 M urea, 2 M thiourea, 2 % CHAPS, 0.002% bromophenol blue, 2% IPG buffer (pH 3-10 linear)和65 mM DTE 之rehydration 緩衝液。先將樣品放入holder 中，再輕輕放入strip gel，確定strip gel 下無任何氣泡，最後覆蓋上少量cover oil，以防止strip 乾掉。接著使用IPGphor 等電膠系統，先以30 V 將strip rehydration 12 h，使strip 膨潤；100 V，0.5 h；4000 V，0.5 h；8000 V/小時。待IEF 結束將strip 以二次水清洗以去除cover oil，進行SDS 電泳分析。

B: 第二維電泳 (SDS-PAGE): 將預先製備好的SDS 膠片取出，將strip 置於含50 mM Tris-HCl pH 8.8, 6 M urea, 2 % DTT 和2.5 % iodoacetamide 之 bromophenol blue 之平衡液15 分鐘，進行電泳分析。在膠體上層放入SDS running buffer，再放入strip，檢查是否有氣泡，移除running buffer 以0.5 % agar 進行封膠並使strip 固定。等電膠電泳結束在12 % acrylamide gradient gels 於 24 mA/gel 條件下電泳 5 h，接著將膠體進行銀染，以ImageScanner 攝影。

C: 二維電泳分離之蛋白質點進行交內水解及MALDI-TOF 質譜儀分析: 將目標蛋白質點從polyacrylamide gel 切出，SDS-PAGE 中之蛋白質以trypsin裂解後，以100 µl含50% acetonitril和25 mM ammonium bicarbonate pH 8.0 之緩衝液沖洗2 次每次作用15 分鐘，以100 µl 100%之acetonitrile 沖洗一次，再乾燥。乾燥的膠體片加入10 µl 的25 mM ammonium bicarbonate 含0.1 µg trypsin (Promega, Madison, WI, USA)，再以siiconized blue stick 弄碎並培養於37°C至少16 h。胜肽

持續以50 μ l 的50% acetonitrile 和5% trifluoroacetic acid 萃取出來後再次乾燥。
胜肽接著以10 μ l 的0.1% trifluoroacetic acid 再懸浮並以手動方式取2 μ l 樣品至
於sample target 上。0.2 μ l 胜肽混和物加入0.2 μ l matrix，內含20 mg/ml
a-cyano-4-hydroxycinnamic acid 和 standard peptide BSA fragment 和
adrenocorticotropic hormone fragment 之內標準。這些檢體再以Ultraflex TM 質譜
儀(Bruker Daltonics, Bremen, Germany)作分析。測量知單點質量以Swissport
database 之ExPASy 所提供如Mascot 搜尋程式來做測量分析。

(4) 結果與討論

3T3-L1 前脂肪細胞分化為脂肪細胞

3T3-L1 前脂肪細胞是由老鼠的初代培養胚胎細胞中，分離出具有細胞持續分裂能力的 3T3 纖維母細胞，是目前最常用來研究脂肪細胞發展和脂肪代謝 (adipogenesis) 的細胞株之一。3T3-L1 前脂肪細胞在未分化前其形態和特性與纖維母細胞相似，經過添加四種分化劑 0.45 μM rosiglitazone (RSG)、1 $\mu\text{g/ml}$ insulin、0.25 μM dexamethasone (DEX) 及 0.5 mM isobutylmethylxanthine (IBMX) 經過 14 天的分化之後，細胞內會產生三酸甘油酯的油滴狀堆積，細胞形態則會由紡錘狀之纖維母細胞轉變成球狀脂肪細胞，在顯微鏡底下可明顯看出細胞內油滴的堆積 (Fig. 1A-1B)，Figure 1C 係利用油紅染色試驗確認脂肪細胞之結果，由圖中可見細胞質內油滴堆積之現象(箭頭所指紅色部分為油滴)。

3T3-L1 脂肪細胞誘導肝癌細胞增生

將 Hep-G2 細胞 (5×10^4 cell/well) 培養於含 10% FBS 及不含血清 (serum free) 之 24 well 培養皿中，給予 0、5、10、15、20、25 % 由脂肪細胞分泌之 CM，處理 24 小時。由 Figure.2A 結果發現在不含血清的條件之下給予 CM，Hep-G2 細胞有明顯的增生現象 ($p < 0.05$)；而含 10% FBS 的組別，給予同樣條件之 CM，Hep-G2 細胞生長卻不受培養液之影響。由於血清成分十分複雜，除了包含蛋白質、代謝物、無機鹽外，另含許多生長因子、激素、細胞附著因子等，當這些成分存在的情況下，會影響 CM 內細胞激素作用的效果，也因此無法明顯誘導肝癌細胞增生之現象。同樣的將 Hep-G2 細胞 (5×10^4 cell/well) 培養於不含血清之 24 well 培養皿中，給予 0、5、10、15、20、25% 由脂肪細胞所分泌之 CM，處理 24 及 48 小時後，結果顯示 Hep-G2 細胞皆有明顯增生的表現，並且以添加 15% 脂肪前驅細

胞所分化之CM 的培養皿中的Hep-G2 細胞增生最為顯著，與控制組相比較大約為1.26-1.30 倍 ($p < 0.05$)(Fig. 2B)。

桑葉萃取物抑制脂肪細胞誘導肝癌細胞增生

將HepG2 細胞培養於不同濃度的桑葉萃取液(0.5 mg/ml - 4.0 mg/ml MLE)且加入含有15% 3T3-L1 脂肪細胞CM 共同培養在無血清之DMEM 培養基內，分別處理8、16、24 及48 小時後，以WST-1 分析，結果顯示48 小時後，CM 的組別與控制組相比較細胞增生的現象比其他時間點顯著，約為控制組1.5 倍，因此後續皆以48 小時的時間點為實驗條件。接著將同樣條件的HepG2 細胞加入不同濃度的桑葉萃取液(1.0 mg/ml - 4.0 mg/ml MLE)培養8、16、24 及48 小時後發現，HepG2 細胞增生現象被抑制了，且隨著MLE 劑量的增加，抑制的現象也越顯著，這顯示桑葉萃取液(MLE)可抑制脂肪細胞誘導HepG2 細胞增生之作用。

MLE抑制脂肪細胞培養液誘導之HepG2 細胞的增生是經由TNF- α 及IL-6 的活化途徑

已知脂肪細胞分泌之培養液包括了一些發炎性細胞激素，如IL-1、IL-6、TNF- α 以及MCP-1 等等。而TNF- α 及IL-6 活化路徑是造成肝癌細胞增生的主要途徑，為了解桑葉萃取物(MLE)對於抑制脂肪細胞培養液所誘導之肝癌細胞增生之作用機轉，我們針對TNF- α 及IL-6 這兩條主要訊息路徑以西方墨點法進行分析。TNF- α 活化調控下游p38 MAPK/ AP1(c-jun/c-fos) 路徑及NF κ B 路徑，由Figure. 4A 的結果得知即使在最高濃度4 mg/ml MLE 的作用下，p38 與c-jun 蛋白表現並沒有受到MLE 之影響。然而當細胞處理1、2 及4 mg/ml MLE，NF κ B顯著的受到不同濃度MLE 的抑制，其蛋白表現減少約9%、18%及47%。磷酸化I κ B 其蛋白表現增加約為控制組的1.22 倍、1.36 倍及2.24 倍。由上述結果得知MLE 抑

制脂肪細胞培養液所誘導之肝癌細胞增生是透過TNF- α 下游調控之NF κ B 路徑，而非p38 MAPK/ AP1 路徑。

IL-6 下游Stat3 是主要影響細胞增生的路徑，同樣的我們將脂肪細胞培養液與HepG2 細胞培養48 小時之後，pStat3/Stat3 的比值與控制組比較約為1.2 倍 (P<0.05)。同時將脂肪細胞培養液與不同濃度之MLE 同時處理48 小時之後，西方墨點法的結果發現，當細胞處理1、2 及4 mg/ml MLE 其pStat3/Stat3 約為1.23 倍、1.12 倍與1.03 倍(P< 0.05) (Fig. 4B)。除以上兩條路徑，文獻也指出HGF, IGF, IL-6 TNF- α 等生長因子亦會透過活化PI3K/Akt 路徑影響肝癌細胞之增生。我們針對PI3K/Akt 下游蛋白進行西方墨點法分析，結果發現CM 會透過PI3K/Akt/mTOR 路徑促進HepG2 細胞的增生，當細胞處理1、2 及4 mg/ml MLE 其pAkt/Akt 磷酸化比值約為115%、100% 與92% (P< 0.05)；pmTOR/mTOR 比值約為100%、88% 與90% (P< 0.05)。PI3K/Akt 下游另一路徑GSK3 β 蛋白表現則不受到任何影響(Fig. 4C)。我們接著利用anti-hIL-6 與anti-TNF α 來確認脂肪細胞分泌之培養液是否透過此兩條訊息路徑，導致HepG2 細胞的增生。以西方墨點法偵測STAT3 蛋白的表現，由Figure 5A 的結果發現HepG2 細胞處理CM 後 pStat3/Stat3 的比值為控制組的1.21 倍，處理anti-hIL-6 後 pStat3/Stat3 的比值與CM 組比較之下減少17%，與控制組的表現量相近。Figure 5B 的結果顯示 HepG2 細胞處理CM 後NF- κ B 的表現為控制組的1.22 倍，處理anti-TNF α 後 NF- κ B 的表現降為與控制組相近。而當細胞處理CM 後， pI κ B/I κ B 的比值為控制組的1.22 倍，處理anti-TNF- α 後pI κ B/I κ B 的比值則有表現回復的情況。此外，我們亦將HepG2 細胞處理4.0 mg/ml MLE 且加入含有15% 3T3-L1 脂肪細胞 CM 共同培養在無血清之DMEM 培養基內，再加入anti-hIL-6(10 μ g/ml)或 anti-TNF- α (1 ng/ml) 於0、24、48 及72 小時之後以WST 增生實驗進行分析。

結果發現HepG2 細胞與CM 共同培養72 小時之後，細胞增生為控制組的1.63 倍；處理4.0 mg/ml MLE 與CM 的組別相較之下，細胞增生減少32%；若同時處理MLE 與anti-hIL-6 或anti-TNF- α 則細胞增生分別減少36%與35%，而單純處理4.0 mg/ml MLE 與同時處理MLE 和anti-hIL-6 或anti-TNF- α 的組別相較之下在統計學上並無明顯差異。這顯示MLE可抑制脂肪細胞誘導HepG2 細胞增生之作用是透過IL-6 與TNF- α 兩條訊息路徑(Fig. 5C)。

總結這些實驗結果得知桑葉萃取物(MLE)對TNF- α 及IL-6 活化途徑皆具有抑制之作用，並且對TNF- α 、IL-6 之共同活化途徑PI3K/Akt/mTOR 也具有相同的抑制作用，這顯示桑葉萃取物(MLE)是透過抑制了TNF- α 及IL-6 的活化途徑，而使得脂肪細胞培養液誘導之HepG2 細胞的增生也被抑制了(Fig. 6)。

MLE 抑制肥胖促進肝癌發生之作用

大鼠飼養期間，各組動物之毛色不變且無脫毛現象，此外各組大鼠反應敏捷，活動狀況正常。實驗期間以包含40% 牛油 (beef tallow) 的HFD 飼料餵食誘導大鼠體重增加產生肥胖現象，並測量體重確定高於正常飼料對照組作為誘導成功之依據。誘導肝癌發生之模式是利用雄性SD 大鼠，以飲用水中含有0.01% DEN 之模式，長期慢性誘導大鼠自發性產生肝癌之模式，實驗為期共12 週大鼠，每週測量體重一次並紀錄之。結果顯示，在飼養期間統計體重變化率中HFD 明顯地增加大鼠體重(Fig. 7)，顯示此模式確實可以誘導大鼠產生肥胖現象，在伴隨著DEN 的誘導產生HCC 的模式下，藉以評估肥胖是否能夠有效增加肝癌產生機率。由結果中可以觀察到，在HFD 同時伴隨著DEN 誘導的模式下(HD 組)，與單獨餵食DEN 的組別相比，大鼠的體重增加率也有明顯地上升($p < 0.05$, Fig. 7A)，顯示即使在同時餵食DEN 的情況下，HFD 也可以增加大鼠的體重。在MLE 餵食之大鼠體重與高脂肪餵食組別比較，在餵食後第6 週時，即開始有效地減緩控制

大鼠體重的增加，但是與HD 組相比較卻是無統計學上的差異(Fig. 7A)。而在餵食MLE 後長達12 週時，與HD 組相比較，MLE 也明顯地降低大鼠體重($p < 0.05$, Fig. 7A)，顯示即使在DEN 的誘導下MLE 仍然具有調節體重之功效。由大鼠之肝臟外觀可以發現，在經過DEN 的刺激之後，肝臟明顯出現眾多的大小不同之不正常結節，明顯有肝腫瘤之現象(Fig. 7B)，而這情形在HD 組中更為明顯，意味HFD 確實有增加HCC 的產生(Fig. 7B)。而同時餵食MLE 的組別，肝臟的外觀則有明顯改善現象，肝臟外觀結節與腫瘤數目皆有顯著地減少(Fig. 7B)。老鼠犧牲後取肝臟並秤其重量紀錄之，將每組中肝臟重量以統計軟體分析，結果發現DEN 處理組別的肝臟與體重比率(liver weight/bw)明顯高於正常組別，顯示肝臟確實在因為DEN 的刺激下而造成相當程度的破壞，進而有不正常肥大的情形，約有2 倍的增加(Fig. 7C)。而在同時誘導肥胖與HCC 的組別中(HD 組)，其肝臟與體重比率更是高於DEN 單獨誘導組，肝臟肥大的現象更為顯著，說明在有誘導肥胖因素條件下，肝臟的機能受到嚴重破壞($p < 0.05$, Fig. 7C)。在同時給予MLE 之組別中，與HD 組相比，MLE 有明顯降低肝臟的比重，顯示其肝腫大現象有回復的趨勢，也說明MLE 具有保護肝臟之能力($p < 0.05$, Fig. 7C)。接著以H&E stain 觀察肝臟組織的病理變化，從正常組大鼠肝臟組織之肝細胞排列規則整齊，而肝癌細胞會有核仁出現多型現象且大小不一致與核濃染的情形，從結果來看DEN 處理之組別都有癌化細胞發生，且有明顯地空泡化之現象(Fig. 8A)，而在同時誘導肥胖與HCC 的組別中(HD 組)中，HCC 出現的情形與數目更為顯著(Fig. 8A)，說明肥胖確實可以增加HCC 的產生。而從各組染色結果來看，隨著MLE 的劑量增加，癌化細胞數量與區域明顯有減少的情形(Fig. 8A)，而觀察大鼠各組肝細胞癌發生比率，隨著MLE 劑量的增加，肝癌發生比率也有減緩之情形(Fig. 8B)，顯示肝臟確實有逐漸好轉，MLE 具有保護肝臟之能力。

MLE 對血液中肝功能指標的影響

AST 主要存在於肝臟、心肌、肌肉，紅血球之內也有，ALT 則主要存在於肝細胞內。當這些細胞因為壞死而破壞之後，細胞內的AST，ALT 就會釋出到血液中，所以肝炎、心肌梗塞、肌肉發炎或溶血時，AST 與ALT 值都可能上升。而 γ -GT 是一種分解蛋白質的酵素，主要存在於肝和腎組織。有少部份存在於脾臟、攝護腺和心臟。 γ -GT 常用於檢驗肝膽道系統方面的疾病，也是偵測膽汁鬱積性肝疾病的敏感指標。而當肝臟功能受損，其代謝脂肪的能力將會降低，也會導致血液裡的總膽固醇(total cholesterol)與三酸甘油酯(total triglyceride)的數值上升。由結果顯示DEN 確實有造成肝臟之受損，使得相關之肝發炎指標AST、ALT 與 γ -GT 的數值上升(Fig. 9)，而在同時誘導肥胖與HCC 的組別中(HD 組)中，相關肝功能指標上升情形更為顯著($p < 0.05$, Fig. 9)，說明肥胖確實可以導致肝臟的惡化，加速HCC 的產生。

由結果得知因為DEN 破壞肝臟功能，導致肝臟機能受損，無法正常代謝肝臟中的脂質，使得脂質堆積在肝臟當中，肝臟中total cholesterol 與total triglyceride 增加(Fig. 4C)，進一步阻礙正常肝臟細胞，影響正常脂肪代謝路徑，使得血液中的total cholesterol 與total triglyceride 也隨之增加(Fig. 10)。而在同時誘導肥胖與HCC 的組別中(HD 組)中，此情形更為嚴重，說明肥胖確實可以導致肝臟的惡化，導致幫助HCC 的產生($p < 0.05$, Fig. 10)。與HD 組相比，餵食隨著MLE 餵食劑量增加，有效改善低血液中AST、ALT 與 γ -GT 數值變化($p < 0.05$, Fig. 10)。同時MLE 能夠降低血中total cholesterol與total triglyceride 數值($p < 0.05$, Fig. 10)，減緩肝臟脂質堆積現象，無論是肝臟中total cholesterol 與total triglyceride 數值皆有下降($p < 0.05$, Fig. 10)，根據上述結果，再一次說明MLE 具有保護肝臟，避免肝損傷之能力。

MLE 保護肝臟之作用機制

瘦素Leptin 以及脂聯素Adiponectin 與脂肪的調節有密切關係，兩者都是由脂肪所分泌，其中 Leptin 在生理功能上與控制食慾有很大的關係，當空腹或限制能量攝取的時候，血漿中的 Leptin 濃度將下降，當進食後，Leptin 濃度將增加以刺激大腦下視丘來達到抑制食慾的效果。而Leptin 的分泌也會受到胰島素調節所控制(22)。Adiponectin 在能量代謝上的主要功能為降低血糖，減少肝臟葡萄糖輸出，降低血中脂肪酸濃度，增加脂肪酸在肌肉中之氧化反應，進而提升胰島素的敏感度(23)。

而從實驗結果 Figure 11 可以觀察到HFD 組別其 Leptin 的表現會增加而 Adiponectin 分泌會減少，而大鼠經過DEN 誘導不會影響obesity factor 的變化。但是在HD 組中，大鼠因為有經過HFD 的誘導，所以導致體內obesity factor 數值改變，Leptin 的表現增加，而 Adiponectin 分泌則下降($p < 0.05$, Fig. 11)。而給予 MLE 後與HD 組相比較，無論是Leptin 或是Adiponectin 的數值皆有改善的現象($p < 0.05$, Fig. 11)，。所以證實，MLE 對於obesity factor 確實會有影響，能增加血漿中 Adiponectin 的分泌，進一步達到降低血中游離脂肪酸之作用。

MLE 對發炎相關因子的影響

大鼠利用高脂飲食誘導後，並在犧牲時採集血液，並測試其促發炎相關因子 TNF- α 以及 IL-6。TNF- α 是從單核球細胞及巨噬細胞中所產生出來，可以誘導單核白血球及巨噬細胞分化、活化巨噬細胞、刺激補體及凝血系統。在脂多醣(lipopolysaccharide, LPS)誘導的發炎反應中，TNF- α 是一個最早期被誘發的調節因子，其次誘發IL-1、IL-6 及IL-8 的產生，進而誘導感染區域血管表達黏附分子，促使內皮細胞產生血小板活化因子，以催化血液凝結及局部血管阻塞，並且可以防止病原菌擴散，當其產生量不足或過度時反而會傷害宿主，因此產生適量

的 TNF- α , IL-6 調控是相當重要(24)。從結果 Figure 11 可以觀察到，經由高脂飲食餵食的大鼠其血清中的促發炎因子 TNF- α 的表現量明顯在誘導組 HFD 上升(Fig. 11)，在餵食DEN 的組別中，其肝臟也因為受到DEN破壞導致肝臟出現發炎現象，使得TNF- α 與IL-6 的數值也有所上升，而HD 組也一樣，在有HFD 與DEN 雙重誘導下，大鼠血中的TNF- α 與IL-6 數值也明顯地上升，但是與DEN 組相比無統計學上的差異(Fig. 11)。而在給予 MLE 後，與HD 組相比較，無論是TNF- α 或是IL-6 的數值皆有下降的趨勢($p < 0.05$, Fig. 11)，顯示MLE 確實可以降低促發炎因子的表現，減低肝臟發炎的情形發生。

MLE 對抗氧化酵素表現之影響

過去文獻中肝臟受損時，會增加組織內氧化壓力，而氧化壓力又會增加DNA 被攻擊的機會，惡性循環，進而導致HCC 的發生(25, 26)。因此我們想要探討MLE 是否可以透過降低氧化壓力而阻止肝癌的發生。

從肝臟組織內脂質過氧化程度(TBARs assay)分析結果顯示，在DEN 誘導下會造成肝臟大量脂質過氧化(Fig. 12)，而在同時誘導肥胖與HCC 的組別中(HD 組)中，此情形更為嚴重，說明肥胖確實可以導致肝臟的惡化($p < 0.05$, Fig. 12C)。與HD 組相比，餵食MLE 的組別皆有明顯改善肝臟脂質過度氧化之情形($p < 0.05$, Fig. 12C)，說明MLE 具有保護肝臟，避免肝損傷之能力。而從測量GSH, catalase, GPx 與SOD 抗氧化酵素活性發現，DEN 組別之抗氧化酵素活性較低，而HD 組也一樣具有較低的抗氧化酵素活性(Fig. 12A)，但是隨著MLE 的餵食劑量增加，抗氧化酵素的活型明顯有改善情形，顯示MLE可回復抗氧化酵素活性，提供肝臟的保護。

MLE 促進肝臟解毒酵素GST 表現

而肝臟是人體重要的解毒器官，而GST 為重要的解毒酵素，其活化可降低致癌物對 .胞的傷害。從肝臟組織內解毒酵素活性分析結果顯示，GST α 、GST μ 與total GST 在DEN 誘導組別之酵素活性較低(Fig. 12B)，而HD 組也一樣具有較低的解毒酵素活性($p < 0.05$, Fig. 12B)。與HD 組相比，隨著餵食MLE 的劑量上升，MLE 有增加解毒酵素活性的趨勢($p < 0.05$, Fig. 12B)，顯示MLE 確實可增加肝臟的解毒功能，而達到阻止肝癌發生的效果。

(5) 結論

隨著人口年齡的老化，平均壽命之延長，所遭遇到的疾病及健康問題也隨之增加，因此現代人不僅是要活得長，更要活得健康，有尊嚴，也相對顯示預防醫學的重要，飲食所造成的肥胖實乃代謝性相關疾病形成之主因。依目前健康食品之開發趨近成熟，而現今在市面上所流通之保健食品亦不計其數，而目前市場上僅有少數產品通過相關規範，並不足以滿足日趨增加的市場需求，據研究指出桑葉含豐富抗氧物質及多酚成份，所以具代表性及說服力的功效為抗氧化與降血脂兩大方面，本實驗室目前已針對桑葉萃取物之功能性做相關深入探討，發現桑葉萃取物具有降血脂、抑制心血管疾病之功效，相關數據已發表在國際期刊當中。而本研究第一年證實桑葉萃取物確實可以有效降低血液和肝臟中脂質含量及抑制脂肪細胞分化及促進凋亡，進一步減少體重及非酒精性脂肪肝。第二年證實桑葉成份抑制肥胖因子促進肝癌細胞增生發炎及oxidant stress，進一步減少高脂肪促進DEN 致肝癌作用，而肥胖促進肝癌target 目前正以蛋白質體探討進行中。

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(7) 研究結果

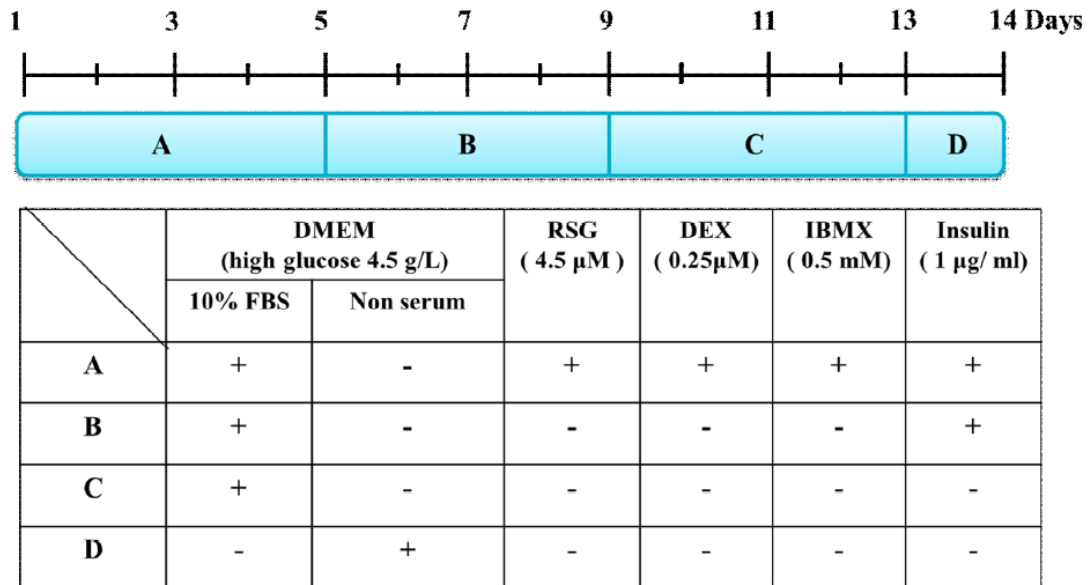


Table 1. Induce of adipogenic differentiation from preadipocytes (3T3-L1). Adipose differentiation of 3T3-L1 preadipocytes was induced as described in Materials and Methods.

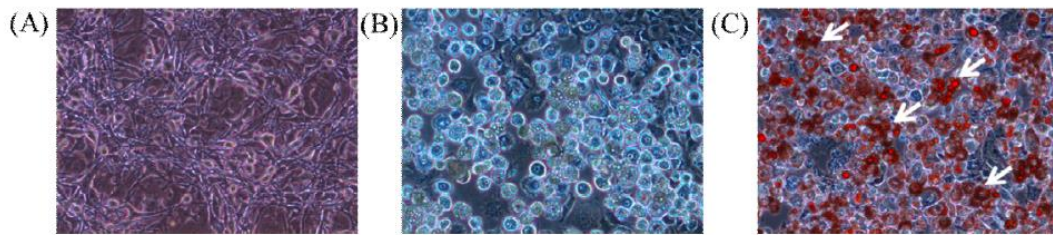
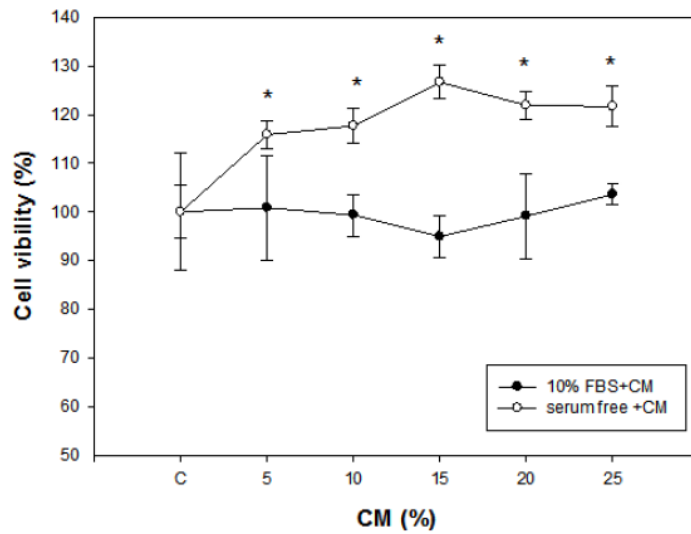


Figure 1. Adipose differentiation of 3T3-L1 preadipocytes. (A) Mouse preadipocytes, 3T3-L1 cell line showed a fibroblast-like shape. (B) The preadipocytes were induced into adipogenic differentiation by differentiation-inducing media. (C) Adipose differentiation of 3T3-L1 preadipocytes evaluated by Oil-red-O staining of lipid droplets. The adipogenic-differentiated cell showed positive staining for Oil-red-O. Red-colored expression indicated lipid droplet of differentiated adipocyte.

(A)



(B)

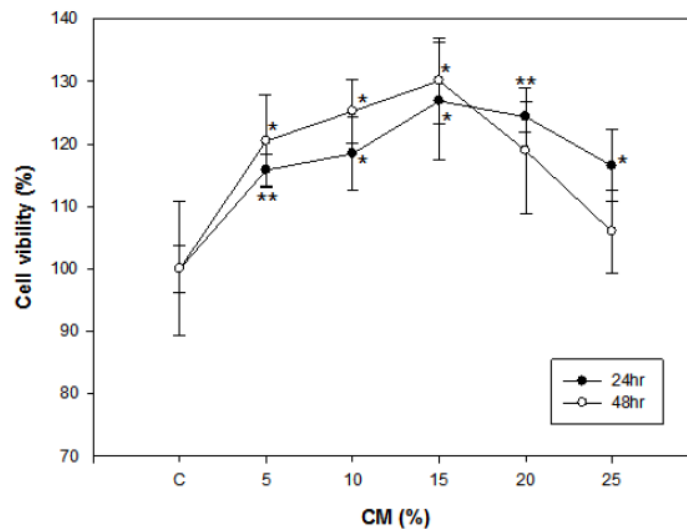


Figure 2. Cell proliferation of HepG2 cells induced with a dipocyte-conditioned medium (CM). (A) 5×10^4 HepG2 cells were treated with different percentage CM (5 - 25%) in with or without 10% FBS medium for 24 h. Cell viability was analyzed by the MTT assay. (B) HepG2 cells were treated with different percentage CM (5 - 25%) in serum free culture medium for 24 h and 48 hr. Cell viability was analyzed by the MTT assay. The result represents an average of four independent experiments \pm SD. *, $P < 0.05$ and **, $P < 0.005$ compared with the control. The four independent experiments were significantly different according to a one-way ANOVA.

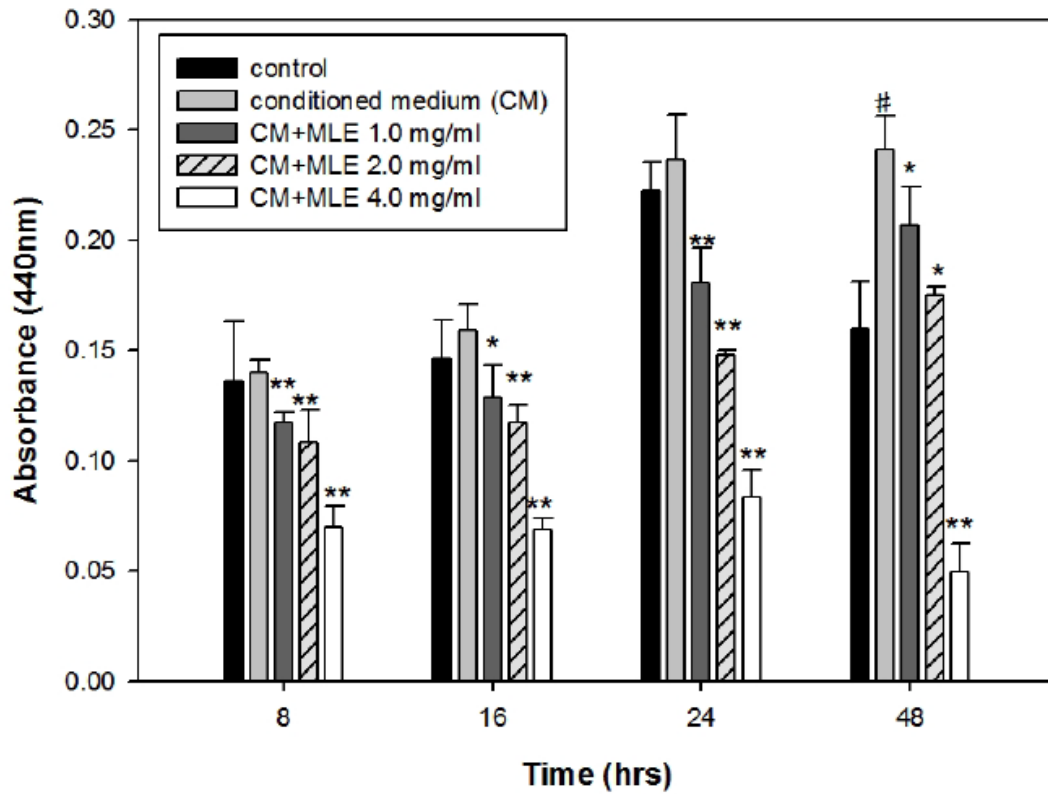
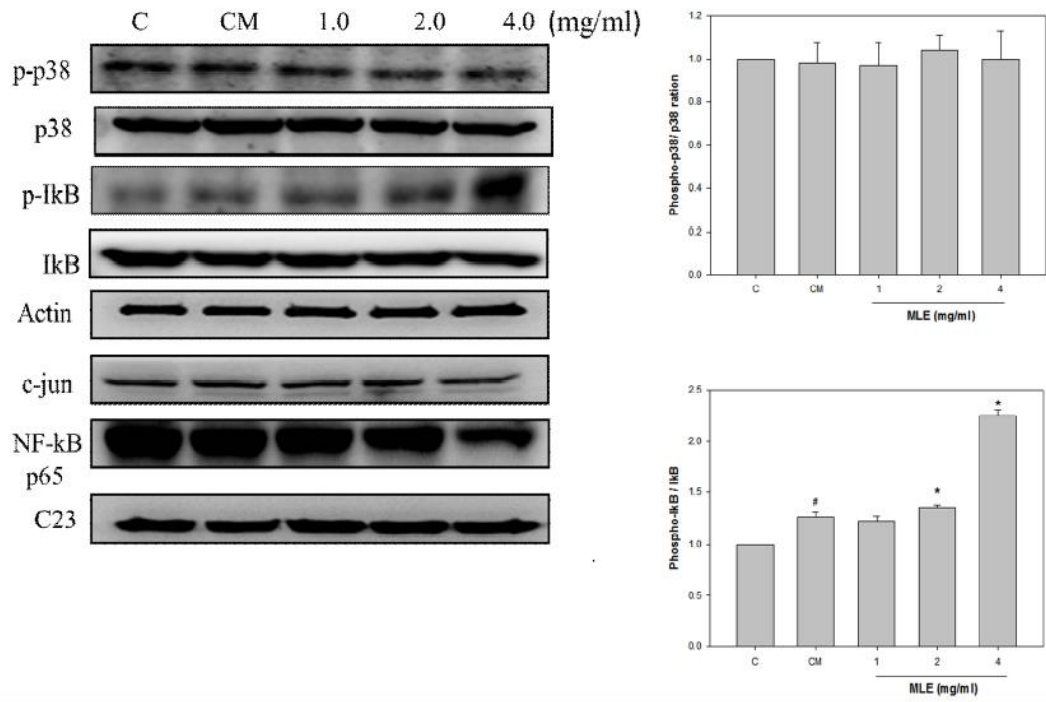
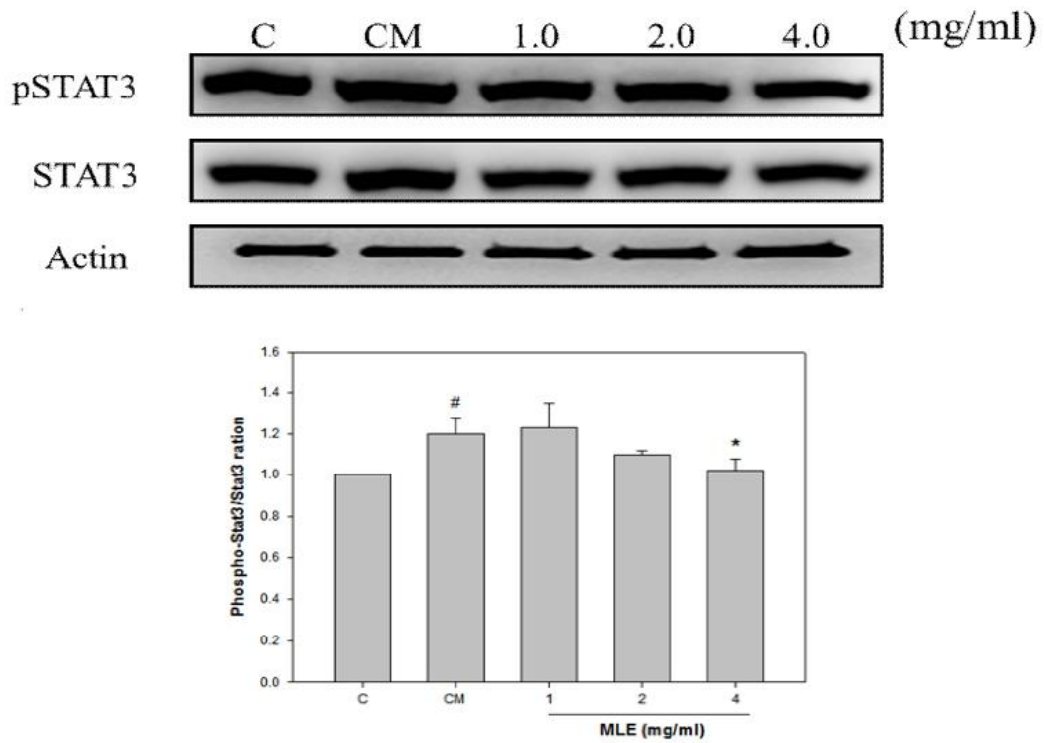


Figure 3. Inhibition of adipocyte-conditioned medium (CM) induced cell proliferation by MLE in HepG2 cells. 5×10^4 HepG2 cells were induced with adipocyte-conditioned medium and treated with MLE under different concentrations (1-4 mg/ml) for 8, 16, 24, and 48 hours. Cell proliferation was evaluated by the WST assay. The result represents an average of three independent experiments \pm SD. #, $P < 0.05$ compared with the control. *, $P < 0.05$ and **, $P < 0.005$ versus CM group. The three independent experiments were significantly different according to a one-way ANOVA.

(A)



(B)



(C)

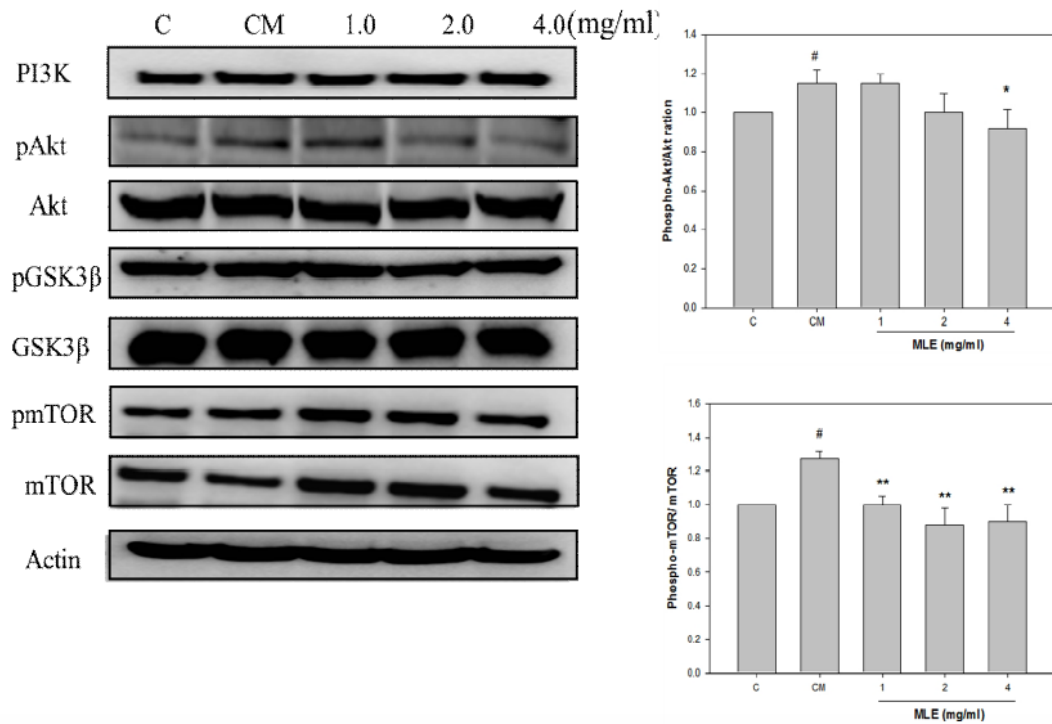


Figure 4. Effects of adipocyte-conditioned medium (CM) induced cell proliferation by MLE on the protein levels of TNF- α /IL-6 signaling related proteins in HepG2 cells. HepG2 cells were induced with adipocyte-conditioned medium and treated with MLE under different concentration (1- 4 mg/ml) for 48 hours. (A) Quantification of phosph-p38/p38 and phosph-I κ B/I κ B. (B) Quantification of phosph-Stat3/Stat3. (C) Quantification of phosph-Akt/Akt, phosph-mTOR/mTOR and phosph-GSK3 β /GSK3 β . The graph represents mean values of three independent experiments, and error bars represent the means \pm SD of these experiments. # $P < 0.05$ compared with the control. *, $P < 0.05$ and **, $P < 0.005$ versus CM group. The three independent experiments were significantly different according to a one-way ANOVA. C, control; CM, adipocyte-conditioned medium.

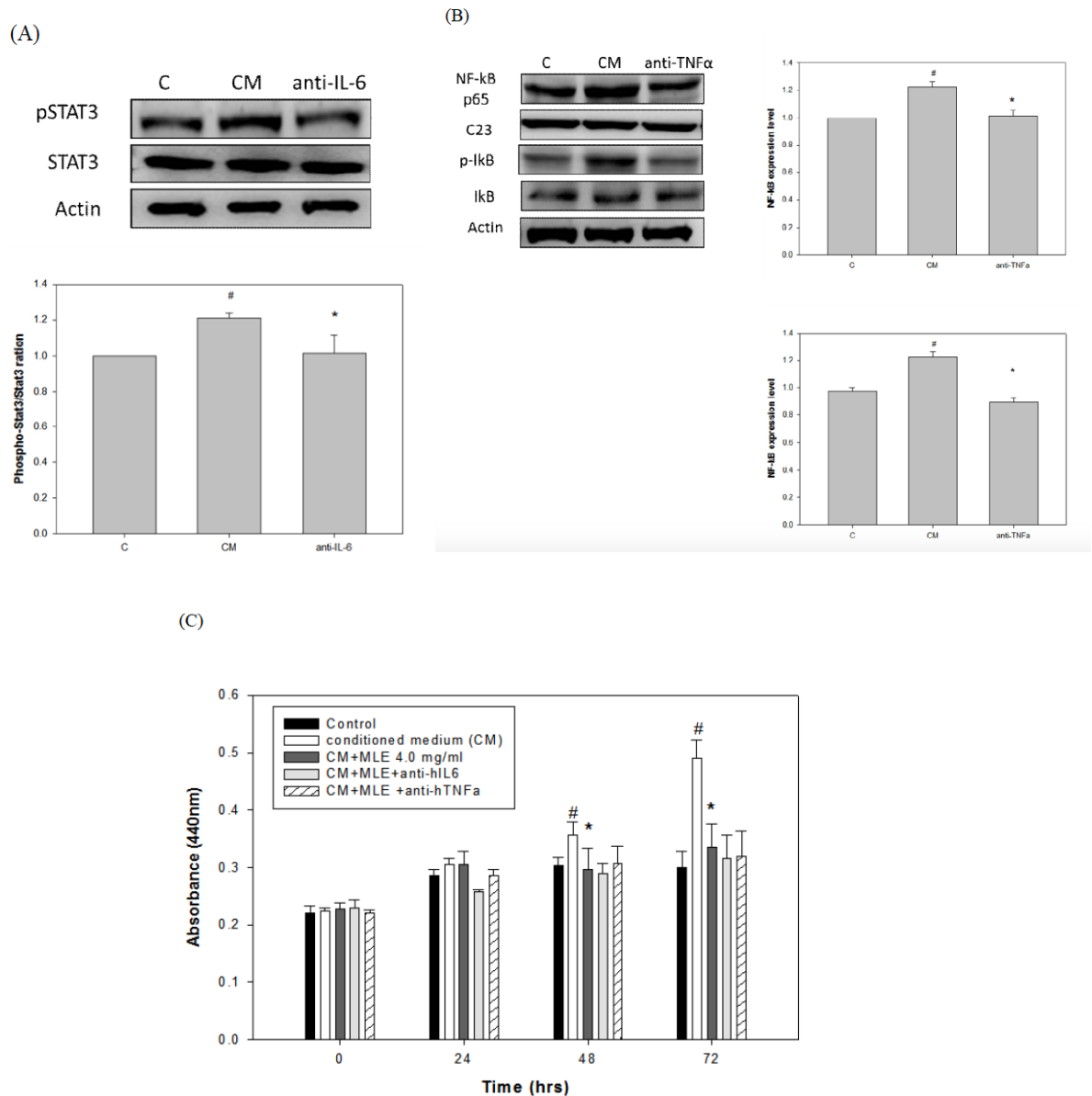


Figure 5. Inhibition of adipocyte-conditioned medium (CM) induced cell proliferation by anti-hIL-6 and anti-hTNF- α in HepG2 cells. (A) and (B) HepG2 cells were induced with adipocyte-conditioned medium and treated with anti-hIL-6 or (10 μ l/ml) anti-hTNF- α (1 ng/ml) for 48 hours. (C) HepG2 cells were treated with or without adipocyte-conditioned medium and the HepG2 cells were presence MLE 4 mg/ml with anti-hIL-6 or anti-hTNF- α for 0, 24, 48, and 72 hours. Proliferation was measured by the WST assay. All the experiments were performed thrice in triplicates. #, $P < 0.05$ versus control group and *, $P < 0.05$ versus CM group The three independent experiments were significantly different according to a one-way ANOVA. C, control; CM, adipocyte-conditioned medium.

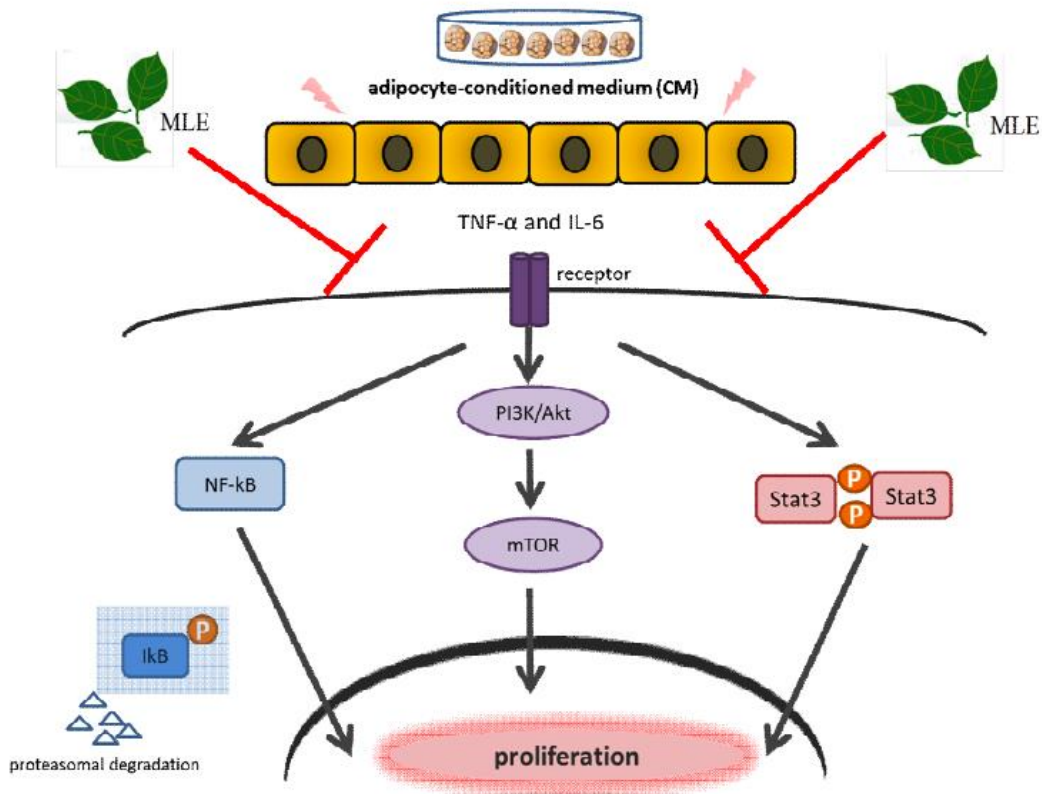


Figure 6. Proposed scheme for the role of mulberry leaf extracts in adipocyte-conditioned medium (CM) induced hepatocyte proliferation. This graph shown MLE suppression of PI3K/Akt/mTOR and Stat3 signaling. MLE also increase IκB degradation and inhibited NF-κB activation. Therefore, MLE inhibition of adipocyte-conditioned medium (CM) induced cell proliferation via the TNF-α /IL-6 signaling in HepG2 cells.

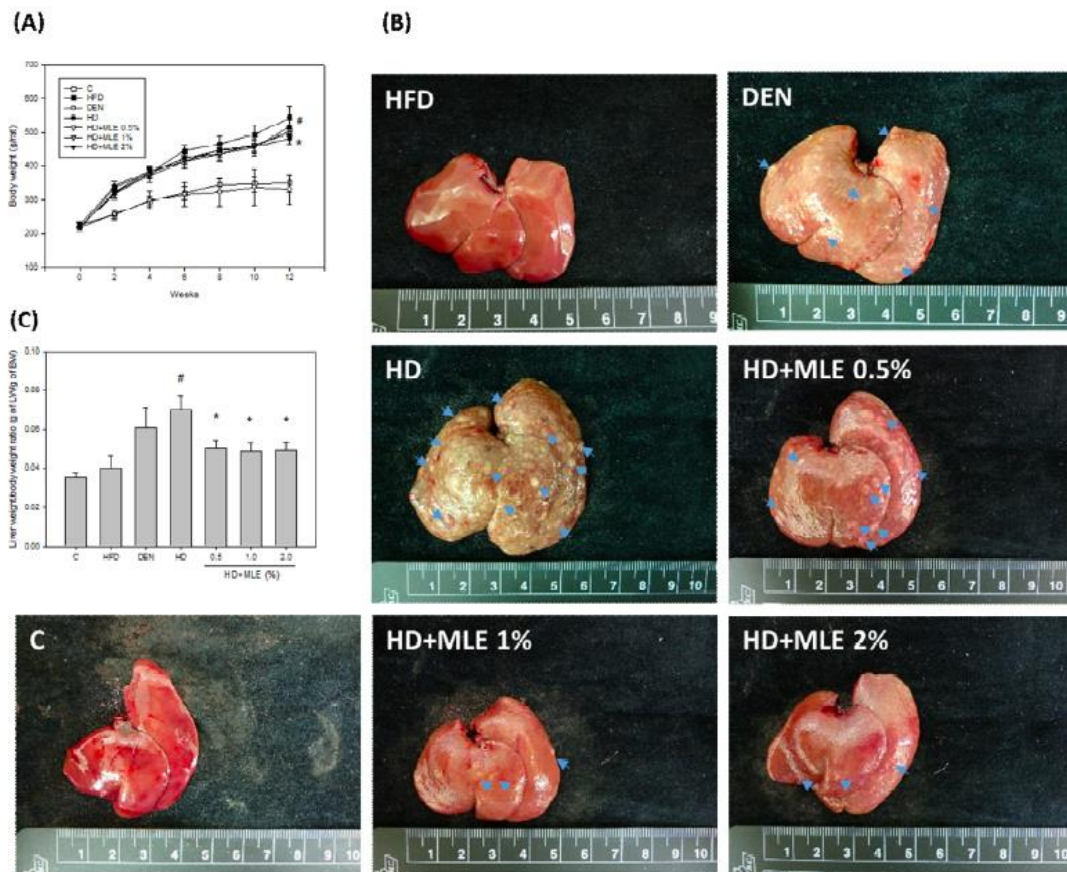


Figure 7. The effect of MLE supplement on body weight, liver appearance and liver weight in the DEN with HFD-fed rats. (A) The body weight change, (B) liver appearance and (C) liver weight in DEN-induced rats. SD rats fed on normal diet without DEN water (control). HFD, SD rats fed on high fat diet without DEN water. DEN, normal diet with DEN water. HD, HFD with DEN water. Data are shown as the means \pm SD. #, $p < 0.05$ as compared with the DEN group; *, $p < 0.05$ as compared with the HFD+DEN group.

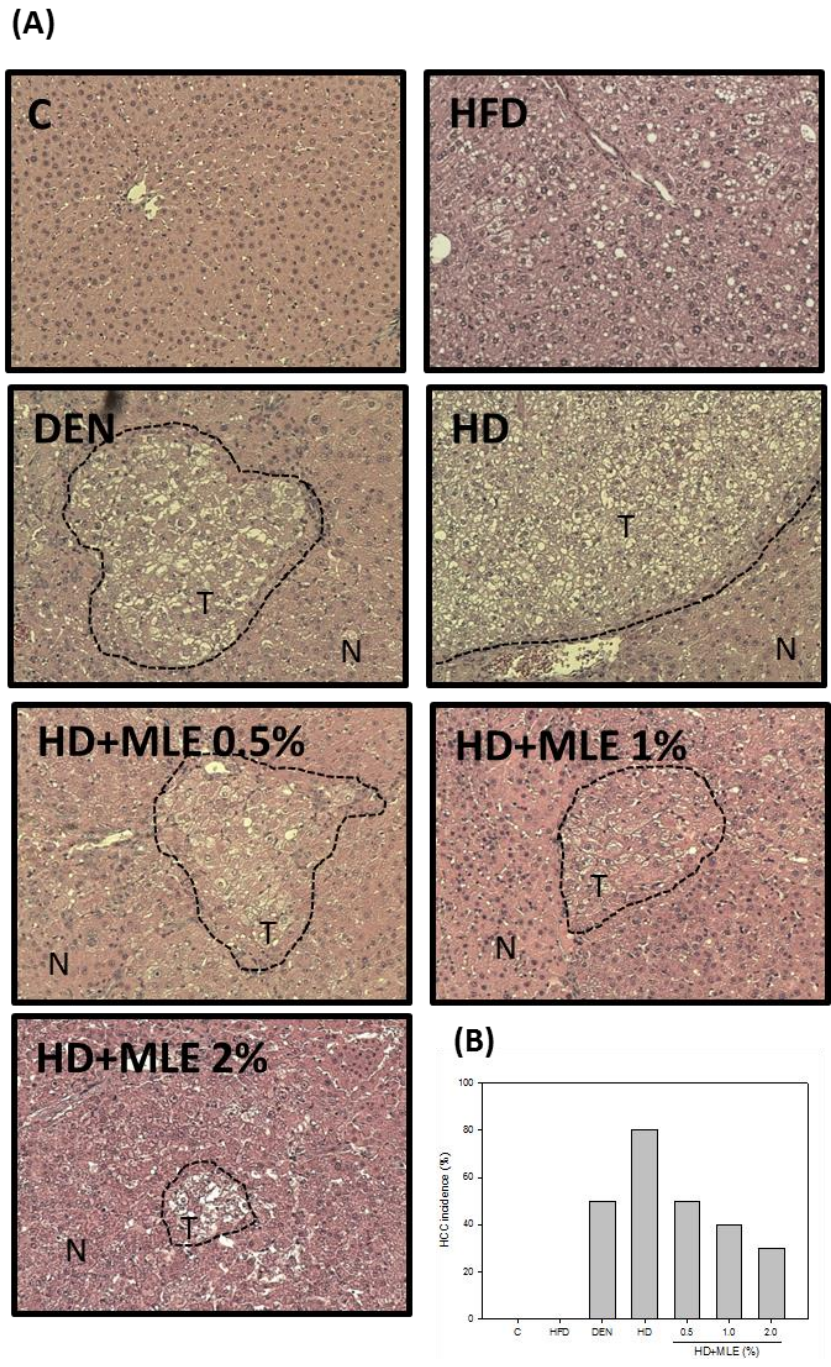


Figure 8. MLE supplement improved the formation and the incidence of HCC in DEN with HFD-fed rats. (A) The sections were stained with H&E and examined by microscope at 100x. N, normal hepatocyte; T, tumor. (B) The incidence of Hepatocellular carcinoma in DEN-induced Rat. Livers from SD rats fed on normal diet without DEN water (control). HFD, SD rats fed on high fat diet without DEN water. DEN, normal diet with DEN water. HD, HFD with DEN water.

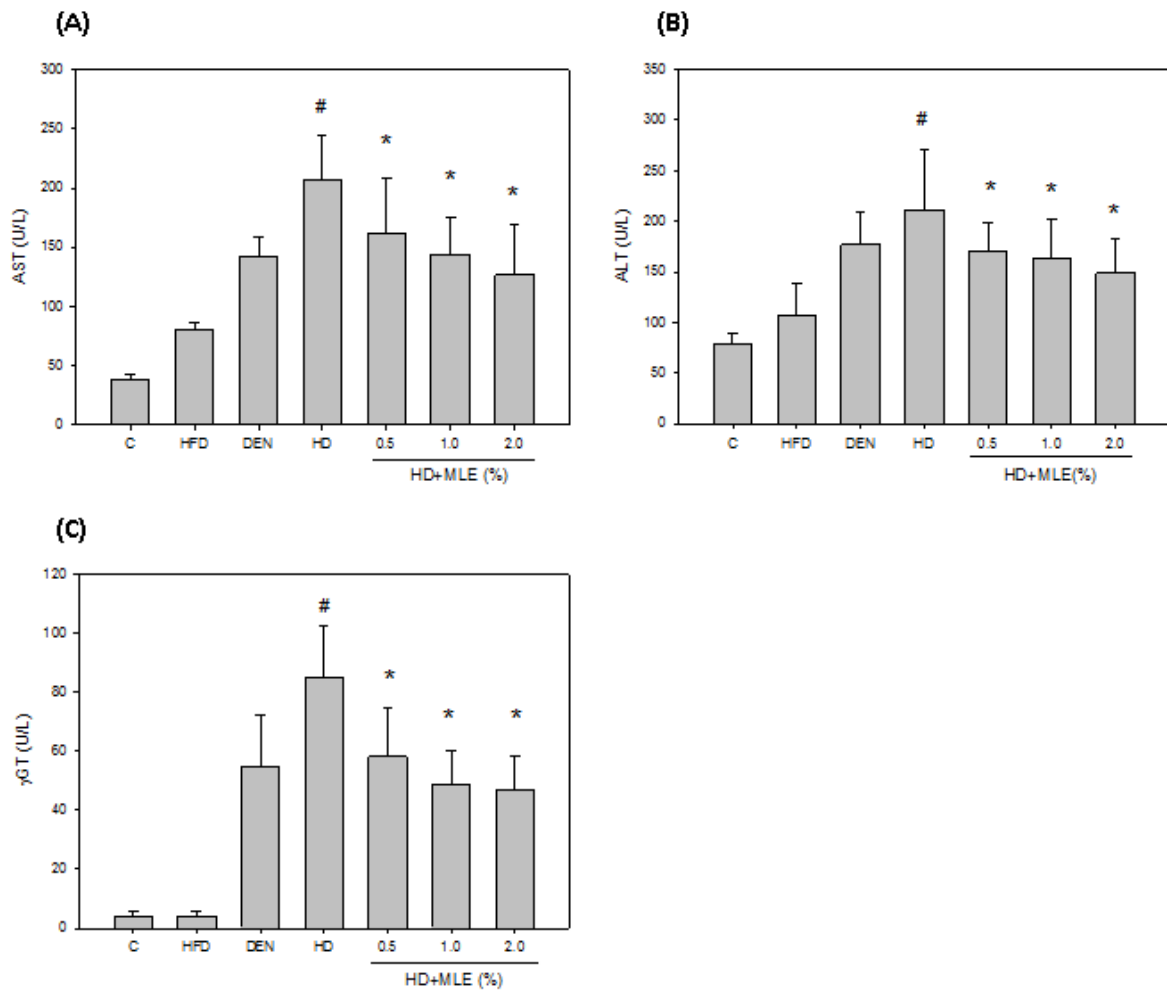


Figure 9. MLE supplement ameliorated the biomarkers expression of hepatic injury in DEN with HFD-fed rats. The biomarkers being quantitated were (A) AST, (B) ALT and (C) γ GT level in plasma from DEN-induced rats. SD rats fed on normal diet without DEN water (control). HFD, SD rats fed on high fat diet without DEN water. DEN, normal diet with DEN water. HD, HFD with DEN water. Data are shown as the means \pm SD. #, $p < 0.05$ as compared with the DEN group; *, $p < 0.05$ as compared with the HFD+DEN group.

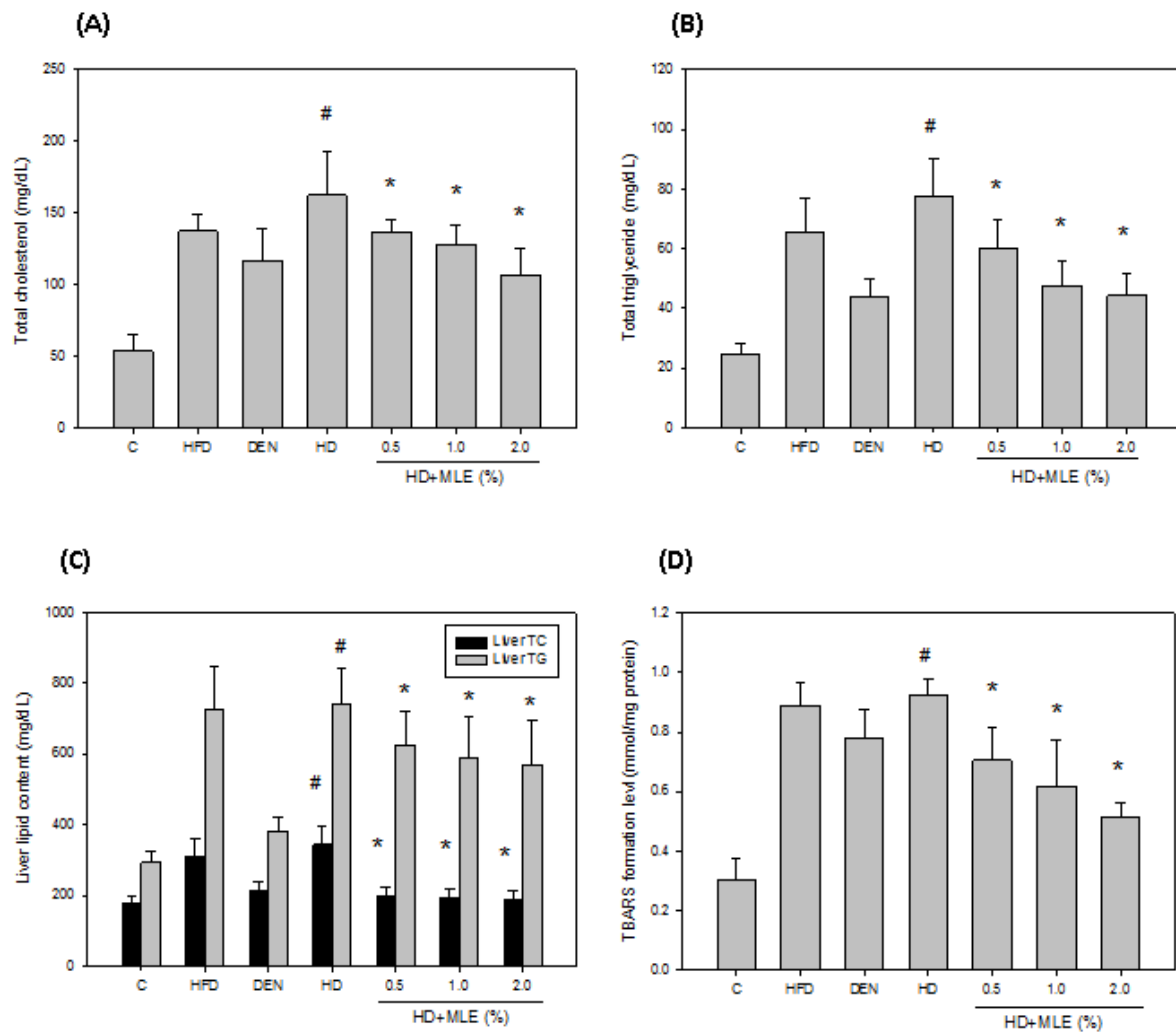


Figure 10. MLE supplement ameliorated the serum lipid and hepatic lipid in DEN with HFD-fed rats. The biomarkers being quantitated were (A) total cholesterol, (B) total triglyceride level in plasma from DEN-induced rats. (C) Total cholesterol and total triglyceride level in liver from DEN-induced rats. (D) The levels of TBARS formation in liver was quantitated by spectrophotometric analysis. SD rats fed on normal diet without DEN water (control). HFD, SD rats fed on high fat diet without DEN water. DEN, normal diet with DEN water. HD, HFD with DEN water. Data are shown as the means \pm SD. #, $p < 0.05$ as compared with the DEN group; *, $p < 0.05$ as compared with the HFD+DEN group.

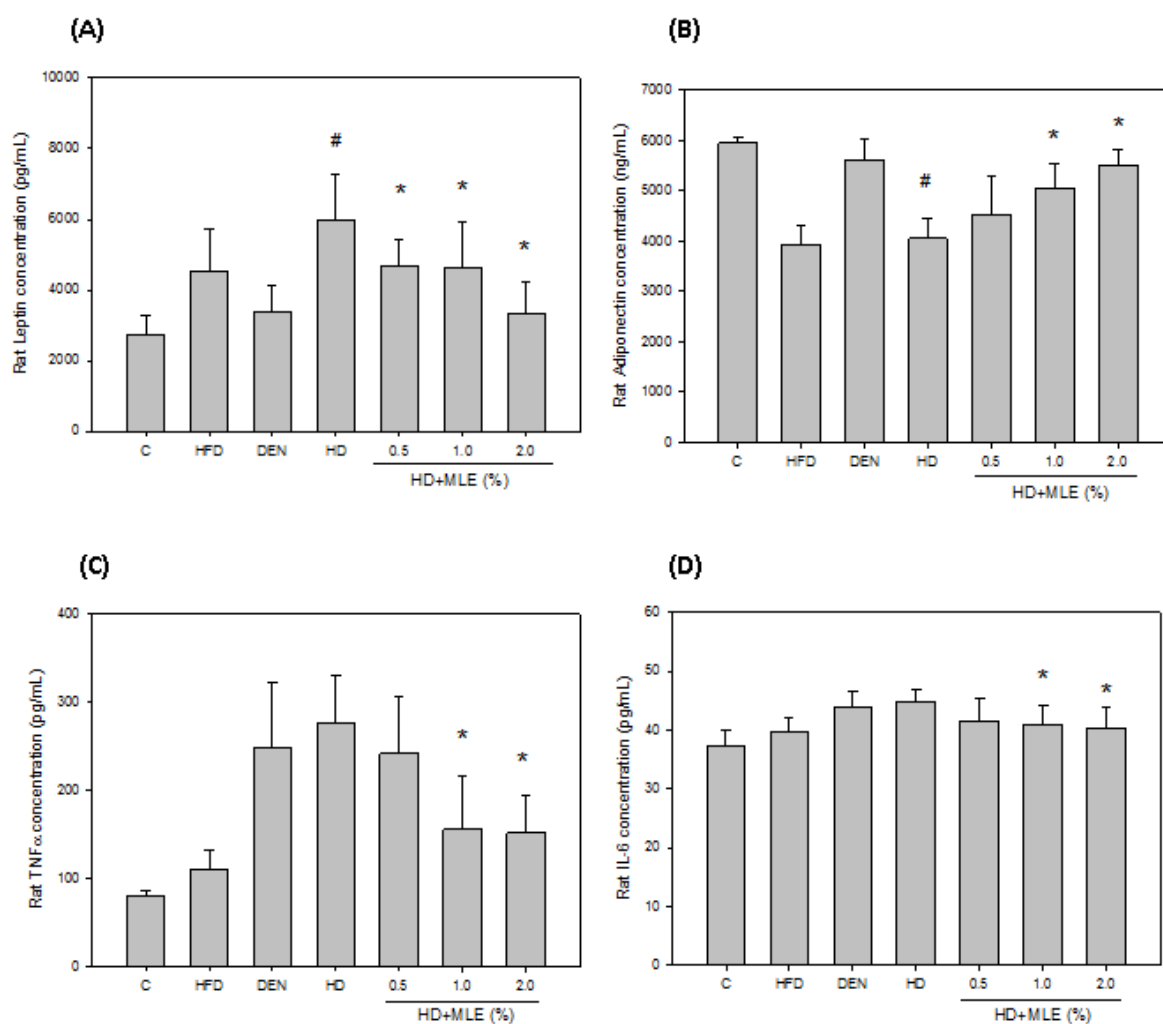


Figure 11. MLE supplement reduced the obesity factors in DEN with HFD-fed rats. The obesity factors being quantitated were (A) leptin, (B) adiponectin, (C) TNF α and (D) IL-6 level in plasma from DEN-induced rats. SD rats fed on normal diet without DEN water (control). HFD, SD rats fed on high fat diet without DEN water. DEN, normal diet with DEN water. HD, HFD with DEN water. Data are shown as the means \pm SD. #, $p < 0.05$ as compared with the DEN group. *, $p < 0.05$ as compared with the HD+DEN group.

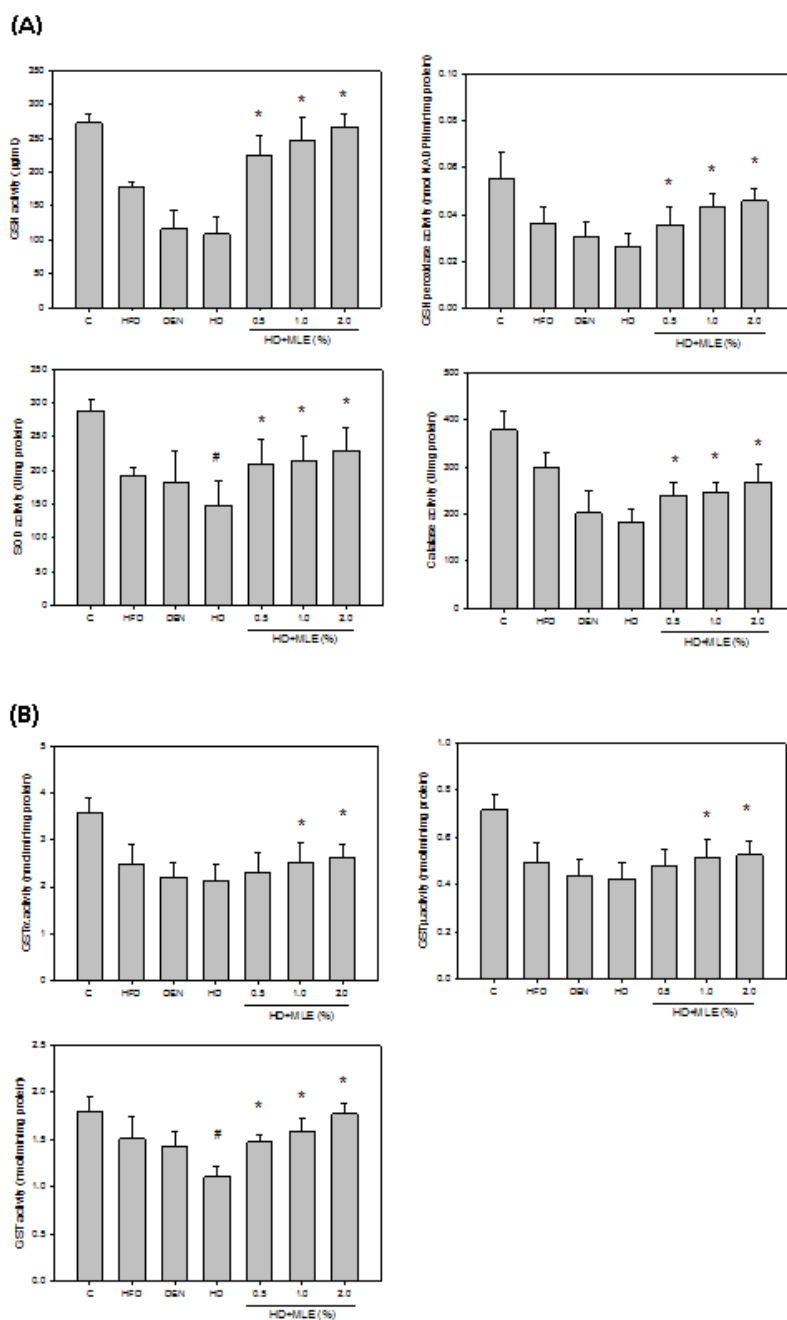


Figure 12. MLE supplement enhanced the activation of antioxidant enzyme and GSTs in liver in DEN with HFD-fed rats. (A) The levels of GSH, GPx, SOD and catalase activity of liver from DEN-induced rat. (B) The levels of GST- α , GST- μ , and total GST enzymatic activity of liver from DEN-induced rat. Enzyme activity was quantitated by spectrophotometer analysis. The crude extracts of liver obtained from DEN-induced rats. SD rats fed on normal diet without DEN water (control). HFD, SD rats fed on high fat diet without DEN water. DEN, normal diet with DEN water. HD, HFD with DEN water. Data are shown as the means \pm SD. #, $p < 0.05$ as compared with the DEN group; *, $p < 0.05$ as compared with the HFD+DEN group.

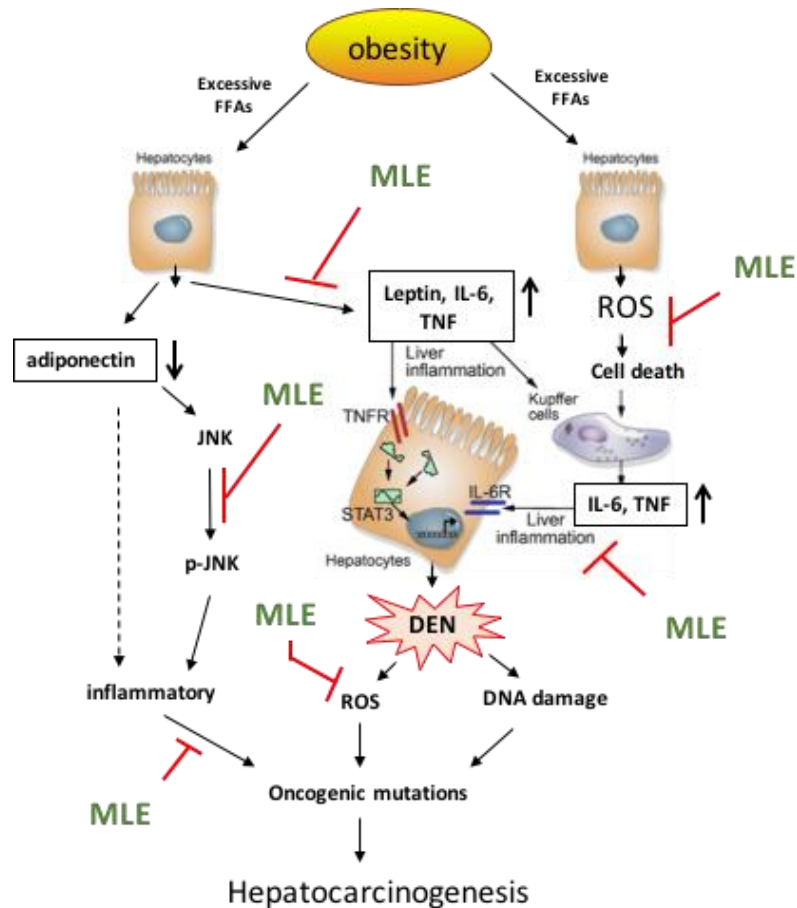


Figure 13. Proposed scheme for the role of mulberry leaf extracts inhibits obesity promoting in DEN-induced hepatocellular carcinoma in rat. This graph shown MLE suppressed the release level of leptin, IL-6 and TNF α . MLE also decreased ROS and liver inflammation in DEN-induced rats. Our results demonstrate that obesity enhanced DEN-induced HCC development and MLE could inhibit it. These findings will help to further evaluate the potential for the development of new hepatocellular carcinoma therapy.

第三年度 研究報告

桑葉成份抑制非酒精性脂肪肝及肥胖促進肝癌及抗藥性之研究(第3 年)

(二)中、英文摘要及關鍵詞

中文摘要

關鍵字：桑葉萃取物；肝癌；抗藥性；內質網壓力；阿黴素。

肝癌長年位居於國人癌症死亡原因的前兩名。肝癌在早期不易察覺，多數病人確診時已經進入中、晚期。對於無法手術治療或轉移性肝癌，大都採用化學治療，但治療的效果有限。對於化療藥物所產生的抗藥性，則是目前在藥物治療肝癌中最主要的問題，因此如何改善抗藥性問題，或開發相關輔助治療方式是目前肝癌研究的主要方向。近年來的許多研究指出，天然萃取物能夠輔助於癌症的治療。桑葉為桑科落葉高木，其主要化學成分含有 DNJ (1-deoxynojirimycin)，類黃酮 (Flavonoids)，多酚成分(polyphenolic acid)及 GABA (γ -aminobutyric acid)等，而桑葉中富含多種類黃酮、多酚物質能有效抑制癌症的侵潤、增生及轉移。本研究擬探討桑葉多酚萃取物(MLPE)是否能夠改善經化學藥物治療後所帶來的抗藥性問題。首先利用內質網壓力(ER stress)會增加肝癌細胞的藥物抵抗作用(drug resistance)之模式，再以化療藥物 doxorubicin (DOX)處理人類肝癌細胞株 HepG2，發現 ER stress 確實會減緩 DOX 的細胞毒殺作用，再處理 MLPE 之後則能夠降低 ER stress 相關蛋白 COX2 與 p38 的表現，MLPE 能夠增加肝癌細胞對於 DOX 的敏感度，促進肝癌細胞走向細胞凋亡，並進一步探討釐清其中的相關分子訊息機轉。這些研究結果將有助於我們進一步評估開發輔助肝癌治療的潛力。

Abstract

Keywords: MLPE; HCC; drug resistance; ER stress; doxorubicin;

Hepatocellular carcinoma (HCC) ranks second among leading cause of cancer-related deaths for many years in Taiwan. It is difficult to observe the HCC patients at the early stage, which usually been diagnosed at middle or late-stage. For those who cannot receive the operations, the HCC patients are mainly taken chemotherapy to remedy their disease. However, it is limited the effects of the treatment. Therefore, the development of improving the drug resistance and novel strategies which aimed at improving the chemotherapeutic effects of hepatocellular carcinoma is significantly important. Recently, there are many studies have demonstrated that the natural extracts could be a successful cancer treatment. Mulberry leaf, the leaf of *Morus alba*, the main chemical component containing DNJ (1-deoxynojirimycin), Flavonoids, polyphenolic acid and GABA (γ -aminobutyric acid) etc. Mulberry leaf have many kinds of Flavonoids and polyphenolic acid that can inhibit invasion, proliferation and metastasis of tumor cells. This study is trying to investigate whether MLPE can reverse the chemotherapy induce drug resistance in HCC. We first established an ER stress microenvironment inducing resistance to chemotherapeutic in HCC. Based on this mimicked ER stress microenvironment and then exposed to doxorubicin in HepG2 cells. The discovery provide important new insights that ER stress may improve resistance to doxorubicin-mediated HepG2 cell cytotoxicity by co-treatment with MLPE. It significantly decreased the levels of COX-2 and p38 associated with ER stress. MLPE can increase the sensitivity of doxorubicin-induced apoptosis in human hepatocellular carcinoma cells. It is needed to explore the molecular mechanisms. These findings will help us to further evaluate the potential for the development of new hepatocellular carcinoma therapy.

(三)報告內容：前言、研究目的、文獻探討、研究方法、結果與討論

(1) 前言、研究目的、文獻探討

3-1. 肝癌

肝細胞癌(Hepatocellular carcinoma, HCC)，簡稱肝癌。在醫學界被認為是最具挑戰性的惡性疾病，世界衛生組織(WHO)的最新統計，肝癌是全球第六大常見的癌症，也是癌症死亡的第三位原因，每年死亡人數超過60 萬人，其中八成出現在亞洲與非洲國家。根據中華民國衛生福利部國民健康署的2011年資料顯示，惡性腫瘤患者死亡中排名第二。台灣每年約有8 千個新增肝癌病例，每年約有7 千人死於肝癌[5]。誘導肝癌發生可能的主要因素有：1. B型肝炎病毒：在亞洲和非洲流行地區，B型肝炎感染的傳播主要是經由母親垂直傳染給新生兒，高達90%的感染者會演變成慢性感染，B型肝炎會頻繁地嵌入到宿主的DNA中。台灣是B型肝炎的盛行區，B型肝炎帶原者約占15~20%。B型肝炎感染者易產生肝癌的風險包括：男性或年齡超過50歲[6]、已經感染很長一段時間、有肝癌家族史、有接觸過黃麴毒素、喝酒、抽菸、合併感染C型肝炎或合併感染D型肝炎病毒(hepatitis delta virus)、高B型肝炎病毒量及B型肝炎基因型C (genotype C)等[7-8]。2.C型肝炎病毒：根據流行病學相關研究指出，C型肝炎帶原者罹患肝癌的機率是非C型肝炎帶原者肝癌的機率15至20倍[9]。C型肝炎感染者易造成肝癌的風險包括：感染時的年齡大、男性、與人類免疫缺陷病毒(HIV)或B型肝炎合併感染、患有糖尿病或肥胖的人有關[10-11]。3.酗酒(定義為每天攝入60克以上酒精)：長期大量飲酒者罹患肝癌比未酗酒者高出1.5至2.0倍風險[12]。4.肝硬化：肝硬化可能是肝癌的先兆，也可能是造成肝癌之原因。最近有研究指出門脈壓力肝硬化程度和肝癌發生率有線性關係[13-14]。5.肝毒性物質：能使肝臟受損之毒性物質，其

中最具代表性是由黴菌所產生的黃麴毒素B1。6.肥胖/糖尿病/非酒精性脂肪化肝
炎(NASH)：會造成潛在性的肝硬化，造成肝癌的產生。7.其他因素：遺傳性血鐵
沉積(hemochromatosis)、 α 1-抗胰島蛋白酶缺乏症、自體免疫性肝炎、紫質症和威
爾森式症等亦有相關報告與肝癌有關。目前對於初期小型肝癌最佳治療方式為外
科治療，但肝癌早期症狀不明顯，如未持續追蹤檢查，常不易及早發現，以至於
肝癌確診通常已經無法接受外科手術治療[15]。而對於無法接受外科手術的病人
化學治療仍有其可行性，但化學藥物所引發的副作用及肝癌細胞的抗藥性，使得
治療效果不如預期。

3-2. 肝癌臨床藥物治療

臨床上肝癌的治療方法包括手術及非手術治療(肝動脈栓塞、酒精注射、電射
頻熱治療及化學治療等)。而過去對於肝癌最能有效根治性治療就是外科切除，
小型肝癌的手術治癒率相當高，但問題是許多病患常合併有肝硬化，影響到手術
的可行性，因此肝癌患者在診斷時，大約不到三分之一的人能有機會考慮外科手
術，有希望藉由手術治療的病人約只占全部肝癌患者的 15-20%。針對無法接受
手術治療的病患，或者可選擇局部腫瘤動脈血管栓塞或放射線照射等，局部非根
除性治療方式，但是對於有肝癌遠端轉移或重大血管侵犯之病人，全身化學治療
則是往往唯一可以選擇的治療方式。

臨床上常見的肝癌用藥包含：1.順鉑(Cisplatin)：經由靜脈給藥，會破壞 DNA
造成細胞死亡，是一種對細胞增生的抑制劑無特異性選擇的廣效性化療藥物[16]。
2.Doxorubicin：為 Topo II 的抑制劑，作用在結合 DNA，抑制核酸合成，廣泛使
用於各種癌症的化學治療，結構屬於環黴素(anthracycline)類抗生素[17]。3.
Paclitaxel：防止細胞分裂和使細胞週期停在 G2/M 期。4.Mitomycin C：為
alkylating 藥劑，具抑制 DNA 合成、核分裂的能力[18]。5.5-fluorouracil (5-FU)

會抑制 thymidylate 合成酶，抑制 DNA 合成，使細胞週期停留在 S 期[19]。在臨床應用上化學治療並沒有明顯提高生存率，不管是單一或是合併療法，效果都不佳[20]。典型的肝癌都保有活躍的代謝系統，可能造成化學治療藥品的內在抗藥性[21-22]，以及肝癌病人的多重藥品抗藥性蛋白質家族攜帶基因，通常有強化的表現[23]，還有其他因素也可能對治療有不利影響，例如大部分的化療藥品原本具有肝毒性，可能因此惡化肝臟功能，而肝門靜脈高壓(portal hypertension)會導致脾臟隔離效應(splenic sequestration)所帶來的白血球與血小板缺乏，也可能使藥品無法發揮。6.Sorafenib：分子標靶治療，一種口服小分子激酶抑制劑(tyrosine kinase inhibitors)，具有抗細胞增生和抗血管增生，且不受限各種原因引起的肝癌，也不會對肝功能有很大的傷害，但是療效低僅只有 2-3%[24]。

3-3. 肝癌抗藥性相關之機制

肝細胞本身帶有許多抗藥基因，而肝細胞癌化後巧妙地利用細胞內的多種機制變化，降低化學藥物的作用，減緩藥物致死性傷害，增加肝癌細胞的存活率[3]。目前已知的幾種可能參與在肝癌細胞的抗藥性之相關機制有：

3-3-1. 細胞凋亡 (Apoptosis)

細胞凋亡是指細胞進行有計畫性的死亡(programmed cell death, PCD)。細胞凋亡早期細胞型態變得不規則、細胞微絨毛消失、細胞膜皺縮、細胞染色體濃縮凝聚、DNA 裂解、phosphatidyl-serine (PS)會由細胞膜內轉移到細胞膜外等；細胞凋亡晚期，細胞膜仍保持完整性，最後則會形成凋亡小體(apoptotic body)並由吞噬細胞或上皮細胞所清除[25-27]。由於細胞膜仍維持其完整性，分解後有毒物質不會釋放出來，因此不會引起發炎反應。細胞凋亡的途徑主要可以分為內生性路徑(intrinsic pathway)與外生性路徑(extrinsic pathway)[28-29]：1.內生性的細胞凋亡：與內質網或粒線體有關[30]。當鈣離子平衡被改變或內質網累積過多錯誤或未折

疊完全的蛋白質可能會造成內質網壓力，細胞會進行自我修復一旦無法修復就會進而誘導細胞凋亡[31]；或是改變粒線體膜的通透性(mitochondrial membrane permeabilization, MMP)，藉由 Bcl-2 家族蛋白、cardiolipin 和 mitochondrial permeability transition pore (PTP) 形成通道孔洞[32]。MMP 造成粒線體膜電位 ($\Delta\Psi_m$)下降，而釋放細胞凋亡因子，例如 cytochrome c、apoptosis inducing factor (AIF)、endonuclease G (Endo G)、Second mitochondria-derived activator of caspase (SMAC)/DIABLO 等進入細胞質中[33]。其中 cytochrome c 由粒線體被釋放到細胞質後，會與 apoptotic protease activating factor-1 (Apaf-1)、pro-caspase-9、dATP 結合形成 apoptosome，進而使蛋白水解 pro-caspase-9 形成活化態 caspase-9[34-35]。caspase-9 再活化下游的 caspase-3，-6 與-7 接著誘發細胞凋亡[36-37]。另外，在哺乳類的細胞中，可以經由破壞粒線體膜電位，使 AIF 和 Endo G 進入細胞核內，誘發細胞凋亡導致 DNA 斷裂、細胞染色體濃縮凝聚，此路徑與 caspase 無關[38]。

2. 外生性的細胞凋亡：又稱為死亡受體路徑，細胞表面的死亡受體包括 CD95、tumor necrosis factor receptor 1(TNF-R1)、TRAIL-R1 (DR4)、TRAIL-R2 (DR5)等[39]。當細胞膜上的死亡受體與其配體結合後，造成受體的聚合，進而吸引下游蛋白 Fas-associated Death Domain (FADD)形成 death induced signaling complex (DISC)，並活化下游的 procaspase-8，使其形成活化型的 caspase-8[40-41]。caspase-8 活化後，會引發兩種訊息傳遞的路徑，第一型路徑為 Caspase-8 活化下游 procaspase-3 形成 caspase-3[42]。當 caspase-3 進入細胞核內，會作用於 inhibitor of caspase-activated DNase I (ICAD)與 caspase-activated DNase I (CAD)，導致 ICAD 的裂解釋放出 CAD 並且活化 DNase I，進而將 DNA 分解[43]；第二型路徑中 caspase-8 會使 Bid 裂解形成 truncated Bid (tBid)，並與粒線體的 Bax 和 Bak 結合，促使 cytochrome c 被釋放到細胞質中，誘發細胞凋亡[44]。細胞凋亡機制

失衡與癌症發生有關，因此現今許多抗癌藥物的研發，藉由活化癌細胞凋的凋亡路徑，誘導癌細胞死亡為主要的目標 [45-47]。

3-3-2. 內質網壓力 (ER stress)

內質網是細胞內修飾、折疊分泌性蛋白質和膜蛋白的一種重要胞器，數種細胞壓力，例如營養缺乏、缺氧、蛋白質 N 端醮化作用受抑制，造成內質網恆定性改變，而在內質網中累積過多錯誤或未折疊完全的蛋白質稱為內質網壓力 [48-49]。在內質網的膜上，有三種主要偵測內質網壓力的蛋白，double stranded RNA (PKR)-like endoplasmic reticulum kinase (PERK)、inositol-requiring enzyme 1 (IRE1)、activating transcription factor 6 (ATF6)。在正常運作的內質網中，這三種蛋白質於內質網腔內與 GRP78 結合，此時為不活化狀態[50]。當內質網腔內的錯誤蛋白或未折疊完全蛋白開始累積，會引發內質網相關降解反應 (endoplasmic reticulum-associated -associated degradation, ERAD)與未折疊蛋白質反應(Unfolded protein response, UPR)以避免細胞受到傷害[51-52]。ERAD 能將錯誤或是未折疊完全的蛋白質運送至細胞質中，利用 26S 蛋白質解體(proteasome)和溶小體(Lysosome)將其分解再利用，而 UPR 可以藉由將 PERK 磷酸化 eukaryotic initiation factor 2 α (eIF2 α)磷酸化，藉此減少蛋白質的合成；IRE1 的活化促使 X-box binding protein(XBP1) mRNA 剪切，產生具有活性的 spliced XBP1(XBP1s) mRNA，片段化的 XBP1 會結合 ER stress response element 及 UPR element 並活化 chaperones 基因的轉錄，將訊息傳遞至細胞質中[53]；ATF6 在高基氏體裡被活化，在進入細胞核中與內質網壓力反應單元(endoplasmic reticulum stress response element, ERSE)結合，增加蛋白質的合成，例如 PDIA4、endoplasmic oxidoreductin-1 like protein(ERO1L)、unspliced XBP1 (XBP1u)[53]。這些蛋白的活化會增加蛋白質折疊、加速錯誤折疊蛋白分解或是抑制蛋白質合成來達到減緩內質網壓力[54-55]。

然而若蛋白質持續或過度累積且此情況來不及緩解，會造成訊息傳導由促進生存 (pro-survival)轉為促進細胞凋亡(pro-apoptosis)[56-58]。內質網壓力導致的細胞凋亡主要是經由 CCAAT/enhancer-binding protein homologous protein (CHOP)、B-cell lymphoma 2 (Bcl-2) 家族與 Caspase-4，而 CHOP 又調節著 Bim 基因的表現，且 p38 MAPK、c-jun N-terminal kinase (JNK)也都可調控 CHOP 的活性[59-61]。大部分的腫瘤中常因血管新生快速來不及供應不正常組織大量增生的養分，容易有養份缺乏、缺氧與改變 pH 值，並活化 UPR[62]。因此內質網壓力對腫瘤是十分常見的，且其中 GRP78 的表現量增加與癌症惡化程度有關[63-64]。GRP78 的表現雖可避免正常組織及器官內質網造成的傷害，然而卻會造成癌細胞增生並產生抗藥性[65-66]。

3-4. 肝癌抗藥性相關蛋白

3-4-1. P-糖蛋白(P-glycoprotein)

抗藥性為肝癌在化學治療上重要問題之一，當腫瘤細胞產生抗藥性時，抗癌藥物只能發揮少許的功效。腫瘤的抗藥性通常不只發生在單一細胞毒素藥物 (cytotoxic drug)的使用，也會對其他的藥物產生多重的抗藥性(multidrug resistance, MDR)[67]。多重抗藥性與調控 MDR 的 ABC 傳遞系統家族有關(ATP binding cassette superfamily)有關 [68]，其主要原因是由於細胞膜上 P-糖蛋白 (P-glycoprotein, P-gp)的過度表現，其分子量為 170kDa [69]，為 ABC 傳遞系統家族中的膜上運輸蛋白，基因型為 *mdr1*[70]，P-gp 利用 ATP 能量主動運輸可將許多不同種類的化療藥物排出腫瘤外，而使藥物在細胞內的濃度降低，無法達到毒殺腫瘤細胞的作用[71]，另外，此蛋白也是參與解毒功能的最重要蛋白之一[72]。根據臨床研究指出，腫瘤有高度表現 P-gp 的病人比沒有高度表現的病人對化療的預後較差[73]，近年來研究肝癌的抗藥性其他蛋白有 Annexin A3 (ANXA3)，在

腫瘤的增生、轉移和抗藥性有相當重要的作用[74]。

3-4-2. Caspase

Cysteiny l aspartate-specific proteinases (Caspase) 家族蛋白，在細胞凋亡中佔有重要的角色[75]。在正常情況下，會以不活化型 pro-caspase 存在於細胞內，須接受細胞凋亡相關訊息後，切除細胞內蛋白質的 aspartate 之後的鍵結，產生自我分解或藉由其他的 protease 而被活化引發 Caspase cascade，使細胞凋亡的機制被啟動[76-77]。Caspase 依其功能可分為兩種類型：1. initiator caspase: 位於調控細胞凋亡途徑上游的位置，在活化狀態時必須被切割，才具有活性，藉由活化下游的 effector caspase 將細胞凋亡訊息傳遞下去，例如 caspase-2、-8、-9、-10 及 12[78]。2. effector caspase: 在細胞凋亡的過程中，位於下游的調控位置，須由 initiator caspase 或 proteases 作用後才可被活化，effector caspase 活化後會轉移到細胞核中造成染色體的斷裂，例如 Caspase-3、-6 及 -7[79-80]。Caspase-3 為細胞凋亡最重要的蛋白，參與了內生性與外生性的細胞凋亡路徑[81]。Procaspase-3 被活化後形成 caspase-3，會進入到細胞核內將 poly ADP-ribose polymerase (PARP)、mouse double minute-2 (MDM2)、actin 等裂解，導致細胞失去正常功能而促使細胞進行細胞凋亡[82-83]。在許多的研究中發現在人類許多癌症中 procaspase-3 會被過度表達，其中包括了肺癌、乳癌、肝癌等[84-86]。Inhibitors of Apoptosis (IAP) 家族蛋白，為內生性的抑制細胞凋亡[87]。常見的 IAP 家族蛋白有 XIAP、Survivin，能抑制 caspase 的活性，因此能阻止細胞凋亡的發生[88-89]。

3-4-3. B-cell lymphoma 2 (Bcl-2) 家族蛋白

Bcl-2 家族蛋白可依據其功能與 Bcl-2 homology (BH) 序列區域分成三類，主要藉由調控粒線體的穩定，進而調控細胞凋亡[90]。第一類為抑制細胞凋亡，具有 BH1、BH2、BH3 和 BH4 序列區及疏水性 C 端和 transmembrane domain，例

如 Bcl-2、Bcl-xL 及 Mcl-1 等。其中 Bcl-2/Bcl-xL 會抑制 voltage-dependent anion channel (VDAC) 的活性並阻止 cytochrome c 的釋放[91]。此類蛋白能利用各個 BH 序列區形成類似手套的結構，和促進細胞凋亡的 Bcl-2 蛋白家族的 BH3 序列區結合，而抑制細胞凋亡[92]。第二類為促進細胞凋亡，擁有 BH1、BH2 和 BH3 序列區及 TM 與疏水性 C 端，例如 Bak 和 Bax。這類的蛋白會形成聚合體，而在粒線體的外膜上形成孔道造成 cytochrome c、AIF、Smac/DIABLO 等促凋亡的蛋白從粒線體釋放到細胞質中，進而活化下游 caspase，促進細胞凋亡[93-94]。Bax 為內生性的細胞凋亡重要調控蛋白[95]。第三類其僅具有 BH3 序列區，例如 Bad、Bim 和 Bid 等。此類蛋白可連接外生性與內生性的途徑，影響細胞凋亡訊息的傳遞[96]。Bcl-2 家族蛋白在內質網壓力所誘導的細胞凋亡調控路徑為重要的調控蛋白[97]。其中 Bid 結合在粒線體的外膜上，會抑制 Bcl-2 的表現，而促進細胞的凋亡[98]。Bcl-2 家族蛋白的促進細胞凋亡與抑制細胞凋亡的蛋白比值會影響細胞的存活，然而許多研究指出多種癌症，因為促進細胞凋亡與抑制細胞凋亡的蛋白表達比例失調，導致細胞的異常增生，也會造成抗藥性的產生 [99-101]。

3-4-4. Mitogen-activated protein kinases (MAPKs)

MAPKs 是屬於 serine/threonine 蛋白激酶家族之一，在細胞的訊息傳遞中扮演非常重要的角色，例如增生、分化、發育及細胞凋亡等。當細胞受到外來刺激時，會活化細胞內 MAPKs 的訊息傳遞[102]。在哺乳類細胞中，MAP 激酶分主要為三大類，包括 ERK、JNK/SAPK(C-Jun N-terminal Kinase/ Stress-Activated Protein Kinase)與 p38 激酶[103]。然而當細胞接收到外界不同的訊息或刺激時，會活化不同的 MAPKs 調控路徑[104]。其中當細胞受到傷害及環境的壓力時，會活化 JNK 和 p38 激酶，進而誘發細胞凋亡；而 ERK 的活化通常與細胞的增生及細胞週期調控較為相關[105]。目前有許多的研究發現 P38 激酶會促進 Bax 進入

粒線體，而誘發細胞凋亡[106]；JNK 磷酸化會抑制抗凋亡蛋白 Bcl-2 的活化[107]；藉由誘導 Bcl-2 的表達，使 ERK 抑制細胞進行細胞凋亡[108]，然而也有文獻發現 ERK 調控細胞的增生與否，主要取決於細胞的類型與 ERK 活化的程度[109]。

3-4-5. Phosphoinositide 3-kinase (PI3K)/AKT

PI3K/AKT 訊息傳遞路徑主要調控細胞週期、生長、代謝及腫瘤新生[110]。PI3K 是屬於 lipid kinase family，含有兩個次單元 p85 及 p110。其中 p110 是具催化次單元，能將 phosphatidylinositol-4,5-bisphosphate (PIP₂) 磷酸化成 phosphatidylinositol-3,4,5-trisphosphate (PIP₃)。PIP 是一個二級傳遞訊號，可將訊息傳遞至下游的路徑，使 AKT 被活化，進入到細胞質[111]。AKT 又稱為 Protein kinase B (PKB)，為 serine-threonine kinase，與各種癌細胞的增生、存活相關[112]。當 AKT 磷酸化後，會調控下游的細胞凋亡因子，例如 Bad、Caspase-9，進而抑制細胞凋亡來增加細胞的存活[113]。因此，現今許多的研究將抑制 AKT 的活化為靶向，應用在癌症的治療中[114]。先前的研究也發現 PI3K/AKT 調控路徑的活化與癌症的化學治療，所導致的抗藥性有關[115-116]。

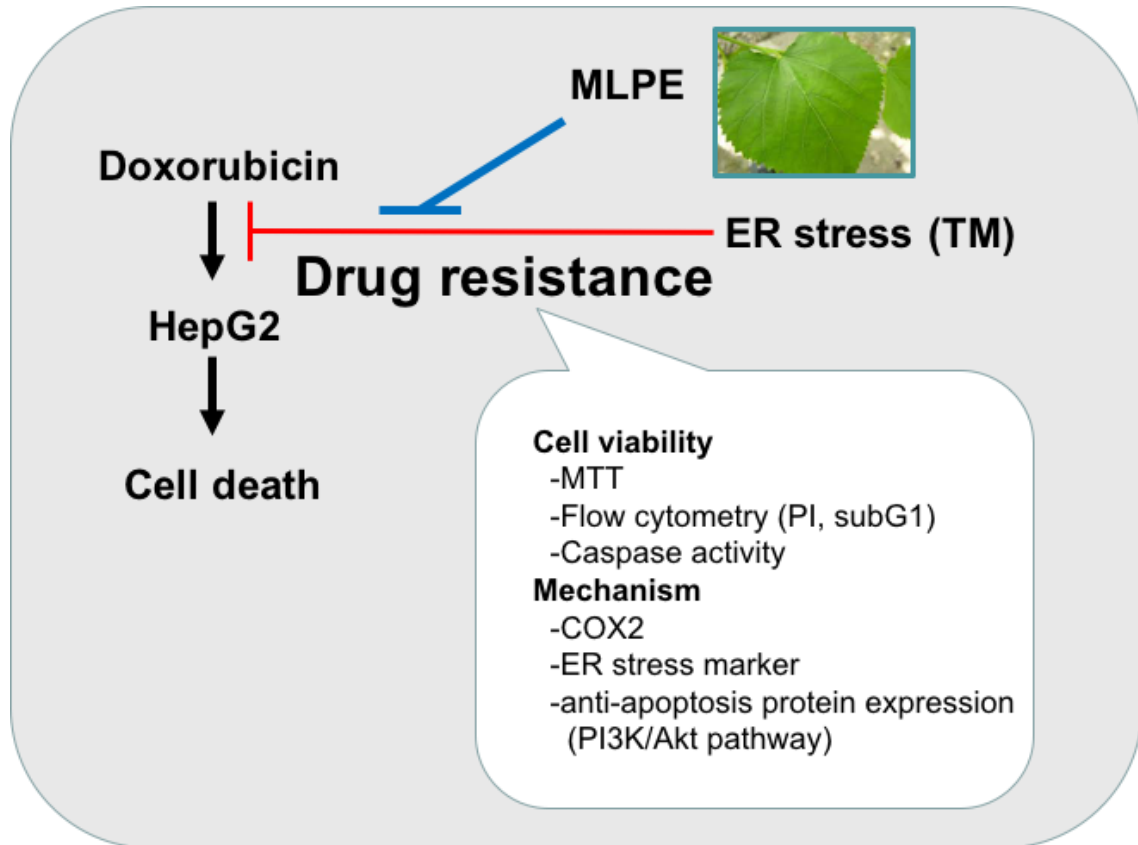
3-5. 桑葉之背景介紹

桑葉(*Morus alba* L.)屬於桑科落葉高木，為多年生之灌木。世界各國對於桑樹的栽培，超過 1000 餘種，然而大部分均屬於山桑(*Morus bombycis*)、唐山桑(*Morus alba*)及魯桑(*Morus multicaulis*)三種。約有 10 種桑種分布於北半球的溫暖地區，其中以中國及日本分布最廣[117]在台灣桑樹分布於海拔 1500 公尺以下的山野，分布廣泛。桑葉在中國傳統用藥已有很長一段歷史，主要功用為降血糖、消炎、利尿及鎮咳等。桑葉主要的生物活性成分，如 DNJ (1-deoxynojirimycin)、類黃酮化合物(flavonoids)、GABA (γ -aminobutyric acid)等[118-119]。目前已有許多研究證實桑葉的醫學功能，包括具有 1.抑制皮膚素：桑葉中的類黃酮，能有

效的抑制多巴胺酶(DOPA oxidase)和酪胺酸酶(antityrosinas)，減少黑色素生成[120]。2.降血糖：桑葉萃取物中的含氮醣基，可抑制 α -glucosidase 活性，而達到調節血糖之功效[121-122]。3.降血脂:桑葉萃取物可改善血漿中甘油三酯(TG)和增加血漿中高密度脂蛋白(HDL)和血漿低密度脂蛋白(LDL)的含量，並增加動脈內黏附細胞的表達，而達到降血脂和保護血管的功效[123-124]、4.抑制動脈硬化症：槲皮酮 quercetin 會抑制 LDL 的氧化修飾作用，因而抑制動脈粥樣硬化形成的作用[125]。5.抗氧化能力:類黃酮化合物，如槲皮酮(quercetin)、芸香苷(rutin)、異槲皮素(isoquercitrin)具有抗氧化能力，能有效清除超氧因離子[126-127]。此外，在近年來的癌症研究中，類黃酮化合物能有效的預防各種癌症[128-129]並有許多相關的研究指出多酚成分能誘導肝癌細胞凋亡[130]。

(2) 研究架構

第三年：MLE 及MLPE降低肝癌化療藥物的抗藥性之作用



(3) 研究方法

桑葉萃取物之製備 (preparation of MLE)

100 g 乾燥桑葉加入 3 L 逆滲透水熬煮，水滾後開始計時 40 分鐘，放涼，以濾紙過濾去除葉渣，得到的桑葉汁液經由減壓濃縮機去除大部分水分，最後置於真空冷凍乾燥機進行製粉階段，所得之粉末即為桑葉萃取物。在各種細胞實驗之前，均以此冷凍乾燥粉末配製成 MLE 溶液後，再經過 0.22 μm filter 過濾除菌進行後續實驗。

桑葉多酚之製備 (preparation of MLPE)

取 100 g 乾燥 MLE 加入 300 mL 甲醇於 50°C 水浴萃取 3 小時後，過濾，收集濾液，反覆萃取 3~5 次，以減壓濃縮法乾燥之，再以 500 mL 二次水回溶萃取物，加入 200 mL 正己烷，利用分液漏斗混合均勻兩種溶液，靜置隔夜，收集水層溶液。再加入 180 mL 乙酸乙酯萃取溶於水層中的多酚成分，靜置隔夜，收集上層溶液，重覆萃取 3~5 次，利用減壓濃縮乾燥後，最後溶於 250 mL 二次水，經過真空冷凍乾燥後所得之粉末即為桑葉多酚。在各種細胞實驗之前，以此乾燥粉末溶於 50% 酒精溶液後，再經過 0.22 μm filter 過濾除菌成為桑葉多酚萃取液 (MLPE) 進行後續實驗。

細胞株培養 (cell culture)

細胞株來自人類肝臟腫瘤分化良好之肝癌細胞(HepG2，ATCC HB-8065)。HepG2 培養於 RPMI-1640 培養液中(pH 7.3)，培養液中含 10% FBS、200 mM glutamine，1% penicillin- streptomycin。細胞培養在 5% CO₂，37°C 之培養箱中。

細胞毒性試驗 (MTT assay)

MTT (3-(4,5-dimethylthiazol-yl)-2,5-di-phenyltetrazolium bromide) 是一種黃色的染劑，會被活細胞所吸收並經由粒線體中的 succinate dehydrogenase 還原成

藍紫色的 formazan，可用來檢測細胞存活與生長變化。在 24 well 的細胞培養皿，種入 4×10^4 細胞，培養於 5% CO_2 ， 37°C 之培養箱中，待隔夜細胞展開，更新培養基，同時給予 doxorubicin (DOX, 0~10 $\mu\text{g}/\text{mL}$)、tunicamycin (TM, 0~6 μM) 與不同 MLPE 之濃度劑量(0~4 mg/mL)，每個劑量三重複。給藥處理 24 小時後，加入 MTT solution 於培養箱 4 小時，隨後將所有培養液去除，再加入 1mL isopropanol 避光 10 分鐘，將 isopropanol 吸至微量離心管中離心 1000 rpm 5 min，以波長 563 nm 測定吸光值，並觀察細胞存活率。

細胞凋亡分析 (apoptosis detection assays)

Propidium iodide (PI) 能與核酸(包含 DNA 與 RNA) 結合，在以雷射光 488 nm 波長的激發下可放出橘紅色波長的螢光。而流式細胞儀則利用壓力將待測細胞在鞘液的包圍之下呈單行排列，依次通過雷射照射區，由雷射光源激發出 488 nm 波長的光波打在細胞上，會依細胞的不同特性而產生不同的螢光強度與光散射，在雷射光束垂直的 90 度位置收集紅色螢光訊號 (617 nm)，可偵測細胞中染上 PI 的 DNA 含量。

將人類肝癌細胞株 HepG2 (2×10^6 cell/100 mm dish) 加入 TM 或 MLPE 後 8 小時，再加入 10 μM 的 DOX 處理 16 小時後，先以 PBS 清洗數次，再利用 0.5% trypsin-EDTA 與細胞作用，使細胞與培養皿脫離，離心去除上清液，沉澱物即細胞。隨即將細胞打散後加入 1 mL 事先保存於 -20°C 的 70% 乙醇固定細胞，最少 12~16 小時。分析前先將檢體離心去除乙醇，再以 PBS 清洗，離心後取沉澱物。將細胞輕彈打散，加入 1 mL 的 PI 染劑(20 $\mu\text{g}/\text{mL}$ PI, 20 $\mu\text{g}/\text{mL}$ RNase A, 0.1% triton-X 100)，於室溫下避光染色 15 分鐘，以流式細胞儀(flow cytometry)分析，藉以觀察 MLPE 是否具有影響 HepG2 細胞之細胞週期的能力。當細胞出現細胞凋亡的情形時，細胞核內的 DNA 會裂解成為間斷的 DNA 片段，在流式細胞儀

的細胞週期圖譜上會出現在 G0/G1 phase 之前，稱之為 subG1 phase，故我們藉由觀察經過加藥處理後的細胞之 subG1 phase 變化來評估細胞凋亡的情形。

西方墨點法 (Western blot analysis)

將 HepG2 cell (1×10^4 /well)，分盤於 10 cm dish 中，與不同濃度的 TM 與 MLPE 處理，再以 DOX 刺激的細胞，以 trypsin-EDTA 將細胞由培養皿打下，以 PBS 沖洗，加入 RIPA buffer (15 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris-base, pH 7.5)，於冰上震盪 30 分鐘，取上層液定量蛋白，將定量後之蛋白取 50 μ g，取定量後之肝組織均質液配置電泳檢體(sample)，加入等量的 Sample Buffer (2 mL 0.5 M Tris-HCl pH 6.8, 1.6 mL Glycerol, 3.2 mL 10% SDS, 0.8 mL 2- β -mercaptoethanol, 0.4 mL 0.5% bromophenol blue)，以 95°C 加熱 10 分鐘，迅速至入冰中冷卻，以離心機將檢體 spin down 後再 loading 至電泳槽膠片上每個 well 中，其上層膠以 80 伏特，下層以 100 伏特進行電泳，待電泳結束後接者進行蛋白質的轉漬至 Nitrocellular paper 上，完成轉漬後以 5% 脫脂牛奶於室溫下進行 blocking 1 小時，以 washing buffer (TBS with 0.5 % tween-20) 沖洗 3 次，將 NC paper 移至於 4°C 冰箱中與初級抗體反應 overnight，再以 washing buffer 沖洗 3 次後，以分別經二級抗體於室溫下反應 1 小時，再以 washing buffer 沖洗 3 次，最後加入 Western Blot Chemiluminescence Reagent Plus 反應 1 分鐘後，於冷光螢光數位影像分析儀(LAS-4000 plus system)顯影觀察並定量。

統計分析 (statistical analysis)

每個實驗至少進行三次，所有數據資料以 SigmaPlot 套裝軟體進行 student t-testm 計算，比較各組別間之差異，P 小於 0.05 者為具統計學上意義。

(4) 結果與討論

誘發內質網壓力可降低 DOX 所導致的細胞凋亡

為了探討內質網壓力(ER stress)的增加，是否會改變人類肝癌細胞株對於化學藥物的感受度。首先我們分別找出化學藥物 doxorubicin (DOX) 與桑葉多酚萃取物(MLPE)各自對於人類肝癌細胞株(HepG2)的半致死劑量(IC₅₀)，HepG2 細胞在處理 DOX 經過 24 小時之後，得到 IC₅₀ 大於 10 µg/mL (Fig. 1A)。而同樣的模式下，HepG2 細胞在經過 MLPE 處理 24 小時之後，可以得到 IC₅₀ 為 1.57 mg/mL (Fig. 1B)，但是在 MLPE 1 mg/mL 經過 24 小時處理之下細胞存活率，仍有 80% 以上(Fig. 1B)。根據文獻指出，細胞在經過 Tunicamycin (TM)刺激之後，會自發性產生 ER stress。接著我們利用 TM 刺激 HepG2 細胞 8 小時之後，再加入 DOX 處理細胞，觀察 DOX 對於 HepG2 細胞的毒殺作用是否有所改變。結果發現，HepG2 細胞在經過 TM 刺激之後，能夠明顯地降低 DOX 的細胞毒殺作用，提高肝癌細胞的存活率(Fig. 2)。而增加 TM 的劑量(從 1.5 µM 到 6 µM)也能夠增加肝癌細胞對於 DOX 藥物毒殺抵抗能力，使肝癌細胞的存活率有著明顯上升的趨勢(Fig. 2)。說明 TM 所誘導的 ER stress 導致肝癌細胞對於化學致癌藥物 DOX 產生一定程度的藥物抵抗作用(resistance)，進而避免肝癌細胞走向死亡。

另一方面，我們利用流式細胞儀分析 HepG2 的細胞週期變化，當細胞出現細胞凋亡(apoptosis)的情形時，細胞會出現大量斷裂的 DNA 片段，藉由螢光染劑標定之後，會出現在 G0/G1 phase 之前，稱之為 subG1 phase。結果發現，HepG2 細胞在經過 DOX 處理下，肝癌細胞會大量的 subG1 phase (20.64%, Fig. 3B)。但是 HepG2 細胞在 TM 提前刺激之下再經過 DOX 處理後，肝癌細胞的 subG1 數目則會明顯地降低 40% (12.76%, Fig. 3B)。說明肝癌細胞在提前藉由 TM 刺激產

生 ER stress 後，能夠有效地減緩 DOX 所具有的細胞毒殺作用，降低對於 DOX 的敏感性。接著我們觀察 caspase 3 的活化與否(cleaved caspase 3)，評估 DOX 對於肝癌細胞所造成的 apoptosis 程度是否會受到 ER stress 的影響。由結果可以得知，DOX 確實會造成 HepG2 細胞產生 cleaved caspase 3，讓肝癌細胞走向凋亡(Fig. 3C)，而 TM 的處理下則會顯著地降低 cleaved caspase 3 的活化(Fig. 3C)，說明著在肝癌細胞中誘發 ER stress 會讓癌細胞對於化學藥物 DOX 產生藥物抵抗作用(resistance)，避免肝癌細胞走向死亡。

MLPE 降低 ER stress 所對抗 DOX 的細胞毒殺作用

接著我們探討天然萃取出物 MLPE 是否能夠改善 ER stress 針對 DOX 所產生的 resistance。在相同的模式下，我們先以 TM 刺激 HepG2 細胞，同時加入 MLPE，經過 8 小時後，再加入 DOX 誘導肝癌細胞走向 apoptosis。結果發現，TM 的刺激會讓 HepG2 細胞對於 DOX 的毒殺作用產生藥物抵抗性，避免肝癌細胞走向死亡(Fig. 3A)，然而在加入 MLPE 同時處理的情況之下，MLPE 有效地降低 TM 所誘導產生的藥物抵抗作用，再次造成肝癌細胞的死亡現象(Fig. 3A)。即使是增加 MLPE 的劑量也可以觀察到相似的趨勢(Fig. 3A)。我們進一步利用流式細胞儀分析 MLPE 對於 ER stress 所產生的藥物抵抗作用，發現 MLPE 確實可以減緩 TM 所造成的藥物抵抗作用，讓肝癌細胞株再次因為 DOX 的誘導而走向死亡(22%, Fig. 3B)。緊接著我們在 caspase 3 的表現中，觀察到肝癌細胞在 MLPE 的處理下，又再次出現活化型 caspase 3 (cleaved form, Fig. 3C)。說明 MLPE 確實可以減緩 ER stress 所產生的藥物抵抗作用，而同時處理 MLPE 與 TM 則可以提高肝癌細胞對於化學藥物 DOX 的敏感度(Fig. 3)。

COX2 參與在 ER stress 幫助肝癌細胞抵抗 DOX 所誘導的細胞凋亡

接著我們分析 TM 所誘導的 ER stress 是透過調控什麼蛋白或是影響什麼機制，

來達到抵抗 DOX 的細胞毒殺作用。根據過去的文獻發現，細胞在 TM 刺激之下，COX2 與 GRP78 會有所改變。因此我們觀察在 TM 刺激 HepG2 細胞的 8 小時之內，COX2 與 GRP78 的蛋白質變化。結果發現，隨著 TM 處理時間的增加，GRP78 的蛋白表現量也有所上升，而 GRP78 又是 ER stress 過程中會大量表現的關鍵蛋白，可視為是 ER stress 的代表(a hallmark of ER stress, Fig. 4A)。另外，我們也發現到 HepG2 細胞在 TM 處理 4 小時下，就可以觀察到 COX2 的蛋白表現量增加的趨勢，隨著 TM 處理的時間延長，COX2 的蛋白表現量也跟著上升(Fig. 4A)。同時間，我們也發現肝癌細胞 HepG2 在經過 TM 刺激下，會增加細胞存活路徑(survival pathway)相關蛋白的表現量，我們觀察到 PI3K/Akt pathway 的蛋白磷酸化，街友隨著 TM 處理時間的增加而有上升的趨勢(Fig. 4C)。這些數據明白地指出，肝癌細胞在 TM 刺激之下會誘導細胞產生 ER stress，並活化細胞生存作用相關路徑蛋白的表現量，而 COX2 是參與在其中。

因此我們利用 COX2 蛋白的抑制劑(Celecoxib)驗證 COX2 在 ER stress 抵抗化學藥物 DOX 的細胞毒殺作用中所扮演的角色。首先，在細胞存活率試驗中發現，加入 COX2 抑制劑(Celecoxib)後，確實能夠減緩 DOX 所造成的細胞毒殺作用，即使是增加 DOX 的劑量(10 $\mu\text{g}/\text{mL}$)，Celecoxib 也能夠提高 HepG2 細胞的存活率(Fig. 5A)。而由 Figure 5B 的結果可以證明，Celecoxib 確實可以降低 HepG2 細胞內的 COX2 蛋白表現量，進而抑制 COX2 的作用。接著我們利用流式細胞儀分析肝癌細胞的細胞週期變化，發現 COX2 抑制劑降低 COX2 的蛋白表現後，也能夠影響 ER stress 對於 DOX 的藥物抵抗作用(subG1 5.46%)，增加 HepG2 細胞對於 DOX 的敏感度造成肝癌細胞的凋亡(subG1 11.56%, Fig. 5C)。同樣地，Celecoxib 確實可以讓因為 ER stress 而降低活化的 caspase 3，再一次因為 DOX 的刺激而活化(cleaved form, Fig. 5D)。上述的結果證明，抑制 COX2 的表現，可

以阻止 ER stress 所造成的藥物抵抗作用，讓肝癌細胞又因為 DOX 的細胞毒殺作用而走向死亡，也說明 COX2 在 ER stress 的藥物抵抗作用中扮演著相當重要的角色。

p38 參與在 ER stress 針對 DOX 所造成的藥物抵抗作用當中

另外，我們知道 MAPK 家族蛋白在細胞內負責調控許多細胞生理作用，包含細胞生存(survival)，細胞生長(proliferation)甚至是細胞凋亡作用(apoptosis)。在我們的模式下，我們發現到 p38 MAPK 蛋白有參與在 TM 所誘導的 ER stress 作用當中(Fig. 4B)。在 TM 刺激肝癌細胞 4 小時後，即可發現到 p38 的活化也隨著 TM 處理的時間增加而上升，而其下游的蛋白 c-Jun 也有同樣的趨勢(Fig. 4B)。說明 p38 也參與在 TM 所誘導的 ER stress 作用當中。因此我們利用 p38 的抑制劑 SB203580 來反證 p38 是否在 ER stress 產生的藥物抵抗作用中所扮演的角色。首先，在細胞存活率試驗中發現，加入 p38 抑制劑(SB203580)後，明顯降低 DOX 所造成的細胞毒殺作用(Fig. 6A)，同時，在增加 DOX 的劑量(由 0.63 $\mu\text{g}/\text{mL}$ 到 10 $\mu\text{g}/\text{mL}$)，SB203580 也能夠有效地提高 HepG2 細胞的存活率(Fig. 6A)。再由 Figure 6B 的結果中證明，SB203580 確實可以降低 HepG2 細胞內 p38 的磷酸化表現，抑制 p38 的活化。接著我們利用流式細胞儀分析肝癌細胞的細胞週期變化，發現 p38 抑制劑降低 p38 的活化後，也確實能夠影響 ER stress 對於化學藥物 DOX 的藥物抵抗作用(subG1 5.46%)，增加 HepG2 細胞對於 DOX 的敏感度造成肝癌細胞的凋亡(subG1 10.59%, Fig. 6C)。同樣地，SB203580 確實可以讓原本因為 ER stress 而降低活化的 caspase 3，再一次接受 DOX 刺激而轉為活化態(cleaved form, Fig. 6D)。上述的結果證明抑制 p38 的活化，可以阻止 ER stress 所造成的藥物抵抗作用，讓肝癌細胞又因為 DOX 的細胞毒殺作用而走向死亡，也說明 p38 在 ER stress 的藥物抵抗作用中扮演著相當重要的角色。

MLPE 透過抑制 COX2 或 p-p38 表現改善 ER stress 產生的藥物抵抗作用

上述的結果說明，降低 COX2 的蛋白表現量會影響肝癌細胞內 ER stress 作用，改善 ER stress 對於 DOX 產生的藥物抵抗反應。因此我們接著觀察，桑葉多酚萃取物 MLPE 是否能夠抑制 COX2 的表現，或是阻止 p38 的活化。由結果可以得知，HepG2 細胞在 TM 處理 8 個小時後，會造成細胞內的 ER stress 產生，讓 ER stress 的指標蛋白 GRP78 的表現量增加，然而 HepG2 細胞在同時處理 MLPE 之後，確實可以改善細胞內的 ER stress，降低 GRP78 的蛋白表現量(Fig. 7A)。同時我們可以觀察到，肝癌細胞在 TM 的處理下 COX2 的表現量也有所上升，但是其表現卻會被 MLPE 所抑制(Fig. 7A)。說明 MLPE 確實可以透過降低 COX2 的表現，調節 ER stress 的作用。

另外一方面，我們之前也證實 p38 的活化會影響 ER stress 的作用。因此我們也觀察到肝癌細胞在 MLPE 處理下，確實會抑制 TM 所誘導的 p38 pathway 活化。由結果中發現，MLPE 不僅是降低磷酸化的 p38，而其下游的 c-Jun 的磷酸化表現也有所下降，再一次證實 MLPE 的確可以抑制 p38 路徑的傳遞(Fig. 7B)。

同時，肝癌細胞在有 MLPE 處理的情況下，確實可以降低細胞生存作用 (survival) 之相關蛋白的表現。HepG2 細胞在 TM 的刺激之下，會造成 PI3K 與其下游 Akt 的活化，進一步增加細胞的存活率，而細胞在有 MLPE 存在的情況下，MLPE 確實抑制 PI3K/Akt 的磷酸化表現，證明 MLPE 阻止 PI3K/Akt 路徑的訊息傳遞(Fig. 7C)。

(5) 討論

肝癌為常見的癌症之一，然而其具有高度的抗藥性，使得在化學治療效果並不佳。化學治療藥物 Doxorubicin 會促進肝癌細胞走向細胞凋亡，但是又會因為癌細胞體內所帶有的內源性或抗藥性基因的作用，保護癌細胞逃脫死亡。有文獻指出，腫瘤快速的生長與血管供應不足會導致缺氧、營養缺乏及代謝改變[132]，而在許多研究也發現缺氧、營養缺乏等會造成腫瘤對化療藥物產生抗藥性增加[133-134]，但是這如何讓肝癌產生抗藥性仍然是未知。根據先前的研究指出缺氧、營養缺乏等為環境的壓力會使內質網產生內質網壓力[48-49]。內質網壓力會導致相關蛋白的活化，增加蛋白質折疊、加速錯誤折疊蛋白分解或是抑制蛋白質合成來達到減緩內質網壓力[54-55]。過去已有文獻說明，肝癌細胞會產生內質網壓力 (ER stress)，即使是面對其他的化療藥物 Cisplatin，也能夠保護癌細胞，避免肝癌細胞走向 Cisplatin 所誘導的細胞凋亡作用[135]。而 ER stress 產生的藥物抵抗作用，並不僅出現在 HepG2 當中，在其他不同的肝癌細胞株 SMMC-7721、Hep3B 中也發現會因為 GRP78 的表達量增加，形成 ER stress，來達到保護肝癌細胞，減緩化療藥物的毒殺作用，避免走向細胞死亡之途[136]。

Tunicamycin 的作用是抑制 glucosaminyl-1-phosphate transferase 而阻止蛋白質 N 端醣化反應，形成 N-glycosidic protein-carbohydrate，使蛋白質無法折疊而造成內質網壓力的產生。本篇利用 Tunicamycin (TM) 誘發肝癌細胞株產生 ER stress，同樣地，利用其他 ER stress 誘導物質 (dithiothreitol, DTT) 所引發的 ER stress，隨著 DTT 處理的時間的增加，與 DTT 劑量的上升，皆被證實可以降低肝癌細胞株因為化療藥物的刺激而走向細胞凋亡的數量[136]。再次說明 ER stress 確實可以增加肝癌細胞的藥物抵抗作用。

有許多文獻說明，過度表現細胞體內的 ER stress，會活化下游蛋白 CHOP，導致癌細胞走向死亡的路途[137, 138]。但是本篇發現，TM 所誘導之 ER stress 卻會保護肝癌細胞，避免 DOX 的刺激而死亡。經比較過後發現，可能是因為 ER stress 的程度不同，導致相關蛋白質持續或過度累積，而且此情況得不到適時地緩解，造成癌細胞的傷害，經由訊息傳導，促使癌細胞由促進生存機制 (pro-survival) 轉為細胞凋亡 (pro-apoptosis)。而不論是由 Wang C 團隊或是 Dai R 實驗結果中皆證實，ER stress 確實會導致癌細胞走向細胞凋亡，但是比較其中的差異點就可以發現，同樣都是以 Tunicamycin (TM) 誘發癌細胞株產生 ER stress，但是兩間實驗室的 TM 處理時間或是劑量皆大於本篇所做，所以可以推測同樣是誘導 ER stress 的產生會導致與我們不同的結果，原因就在於所誘導出的 ER stress 的程度不同，再次呼應不同程度的 ER stress 可能會影響細胞走向不同的生理作用。

COX-2 為環氧化酶，在大部分的組織中並無表現，但研究發現 COX-2 會在腫瘤中會大量被表現。因此，COX-2 對於腫瘤新生有重要的影響性，包括轉移、血管新生及調控細胞的凋亡[139]。先前的文獻發現，內質網壓力會使人類的乳癌細胞株 MCF-7 大量的表達 COX-2[140]，而在人類肝癌細胞株 HepG2 也發現內質網壓力的產生會讓 COX-2 大量的表現[141]。根據這些研究指出內質網壓力與 COX-2 有重要的相關。在本篇研究中發現，TM 會促進肝癌細胞 HepG2 的 COX2 表現量增加，形成 ER stress，降低肝癌細胞走向細胞凋亡數目。而我們再利用 COX2 的抑制劑 celecoxib 的抑制效果下，證實降低 COX2 的表現，可以增加肝癌細胞對於 DOX 敏感度。再次說明 COX2 可能參與在 ER stress 調控肝癌細胞的藥物抵抗作用當中。

近年來的研究發現，CHOP 為內質網壓力使細胞走向細胞凋亡的重要轉錄蛋

白。內質網壓力誘導細胞凋亡路徑，會活化 PERK 調控下游蛋白 CHOP，讓 CHOP 過度表現，進而抑制促凋亡蛋白 Bcl-2 並活化凋亡蛋白 Bim，使細胞走向凋亡[142]，而目前的研究對於 doxorubicin 在內質網壓力下，桑葉多酚是否會經由 CHOP 調控路徑促使細胞凋亡，是我們未來值得繼續研究的方向。桑葉多酚應用在癌症的治療尚且還需要更多的基礎與臨床試驗。

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(7) 研究結果

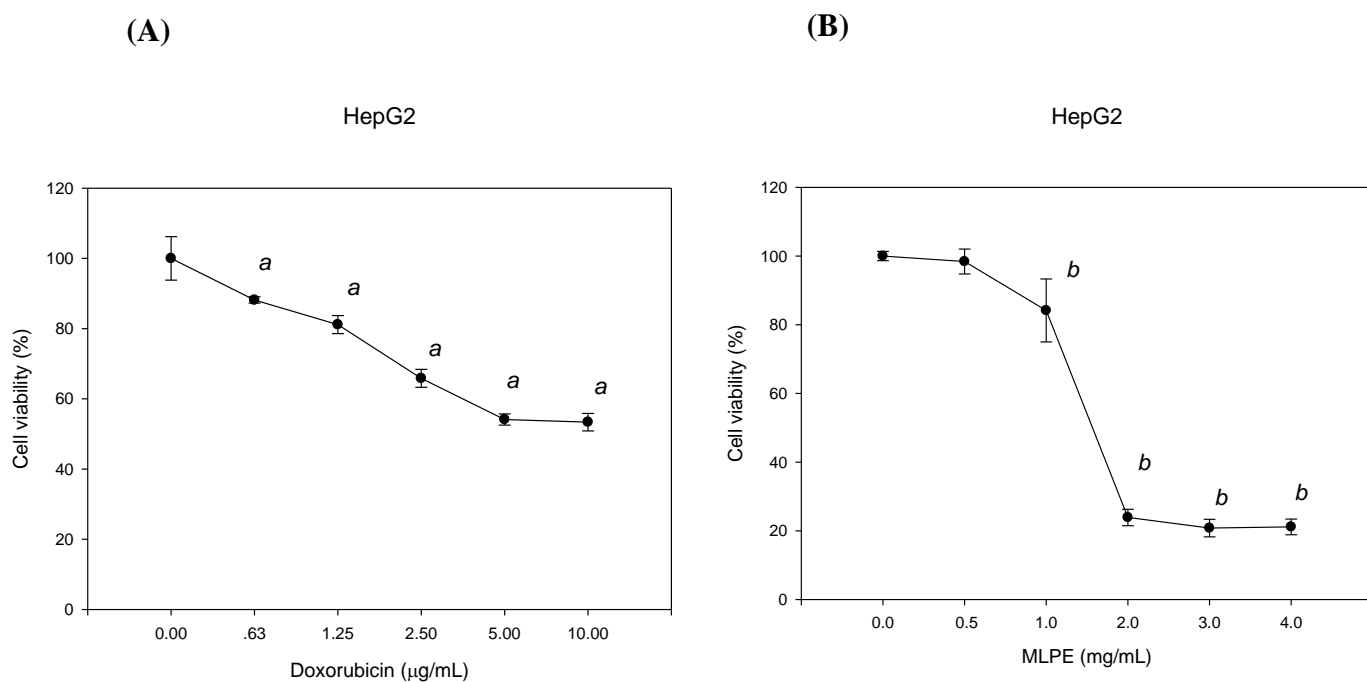


Figure 1. Effect of doxorubicin and MLPE on cell viability in HepG2 cells. HepG2 cells were pretreated with doxorubicin (0, 0.63, 1.25, 2.5, 5 and 10 $\mu\text{g/mL}$) or MLPE (0, 0.5, 1.0, 2.0, 3.0 and 4.0 mg/mL) for 24 hr. Cell viability of HepG2 cells was determined by the MTT assay. Data are expressed as the mean \pm SD of three independent experiments. a, $p < 0.05$, compared with HepG2 cells treated without doxorubicin. b, $p < 0.05$, compared with HepG2 cells treated without MLPE.

HepG2

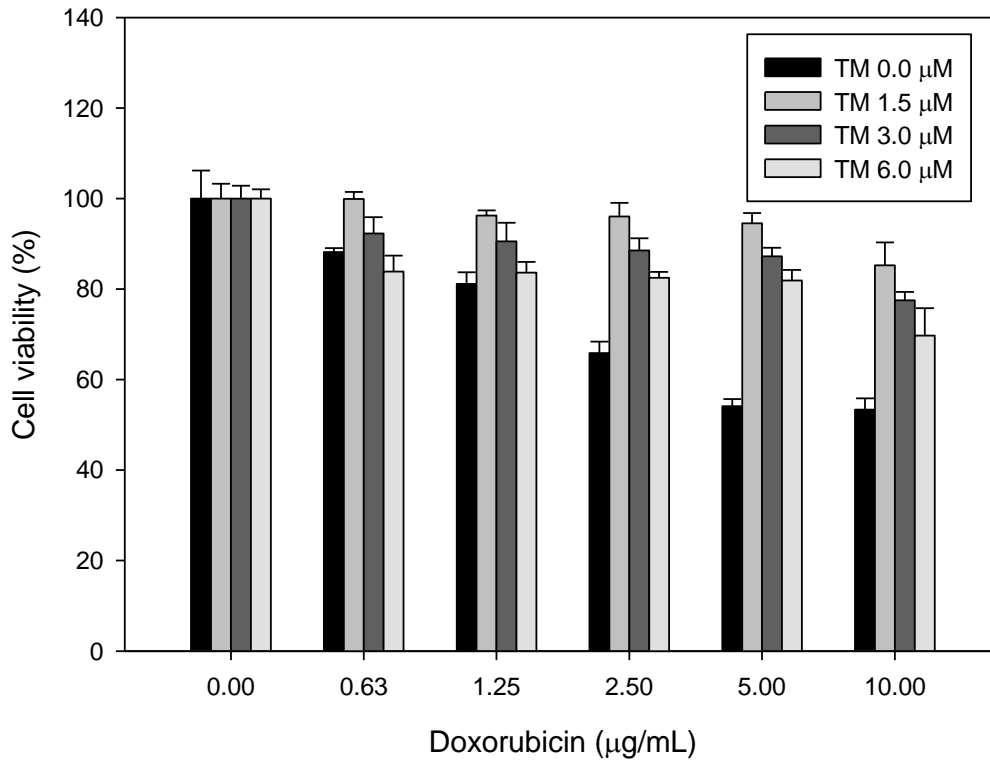
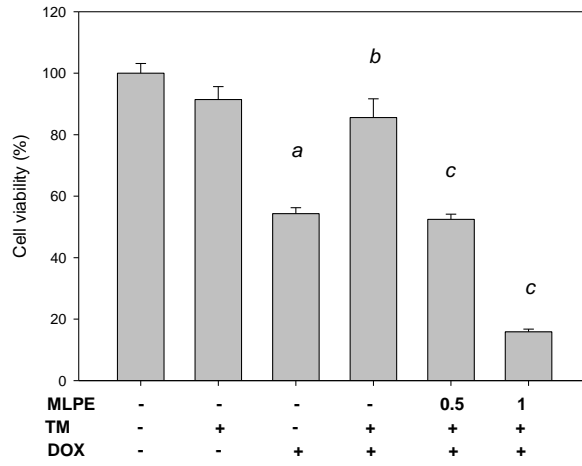


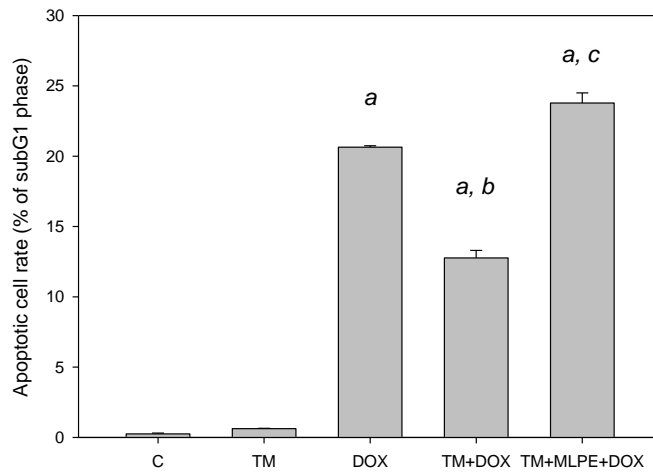
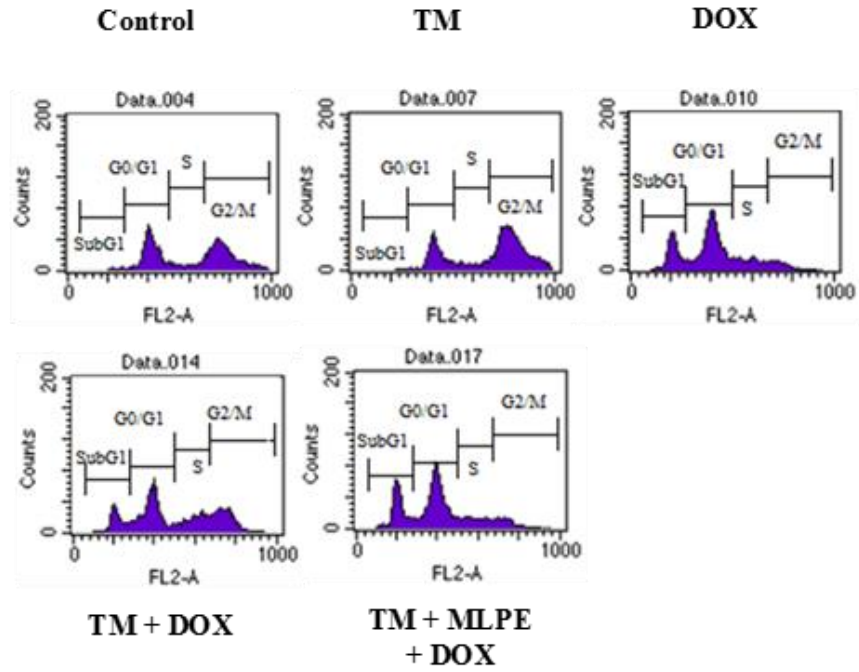
Figure 2. Tunicamycin reduced the cell toxicity induced by doxorubicin in HepG2 cells. HepG2 cells were treated with tunicamycin (0, 1.5, 3 and 6 µM) only for for 8 hr then exposed to different concentrations of doxorubicin (0, 0.63, 1.25, 2.5, 5 and 10 µg/mL) for 24 hr. Cell viability of HepG2 cells was determined by the MTT assay. Data are expressed as the mean \pm SD of three independent experiments.

(A)

HepG2



(B)



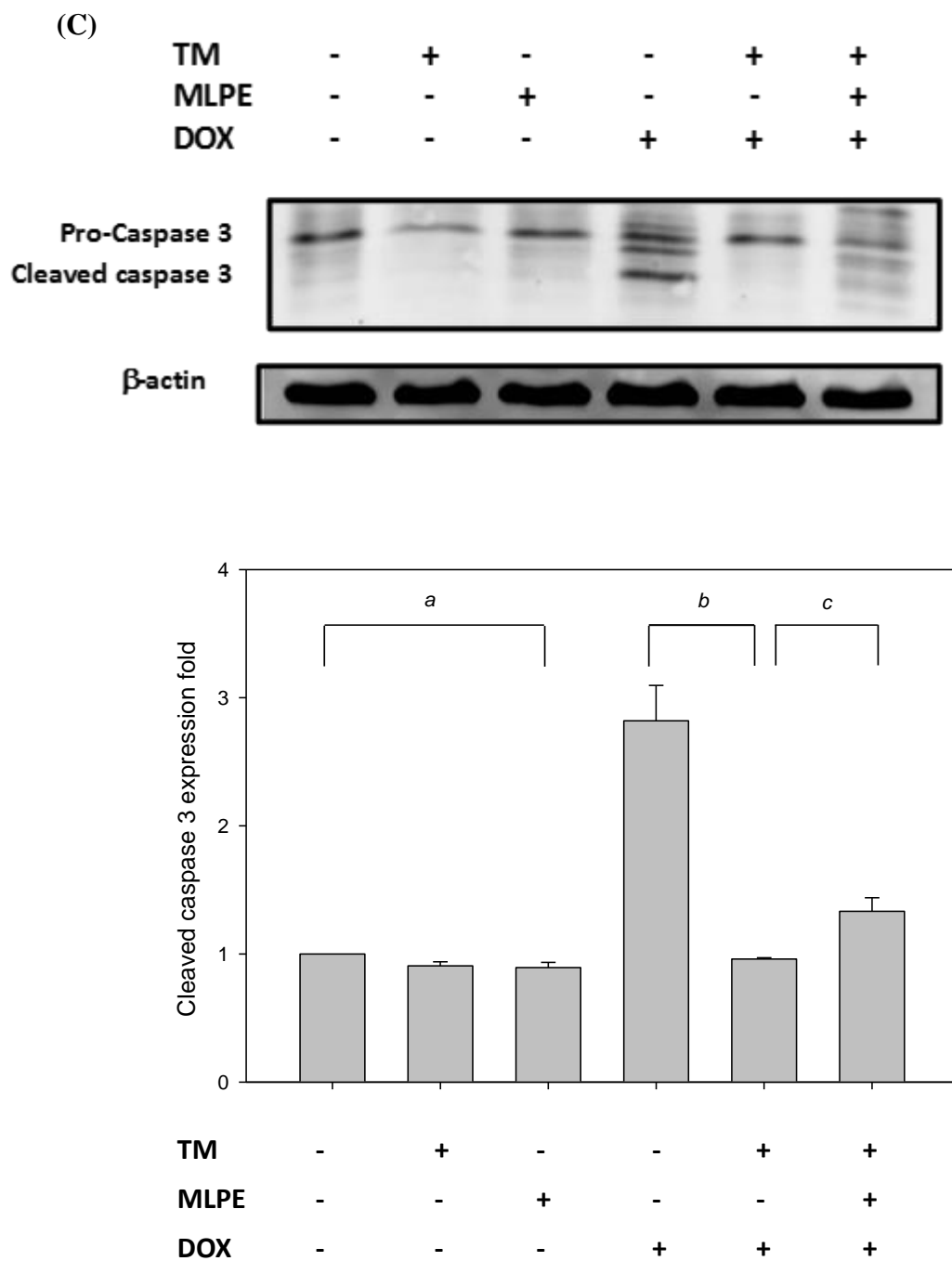
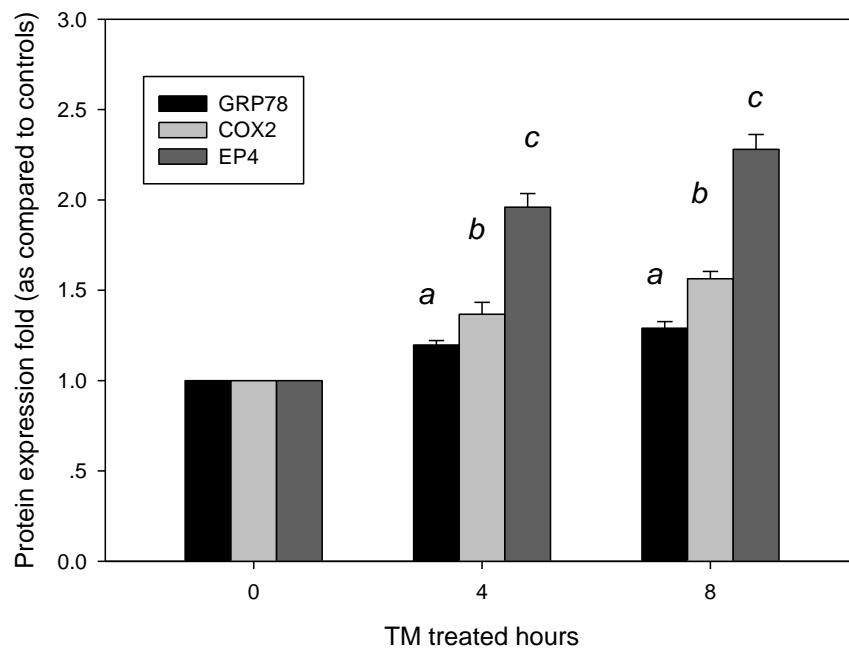
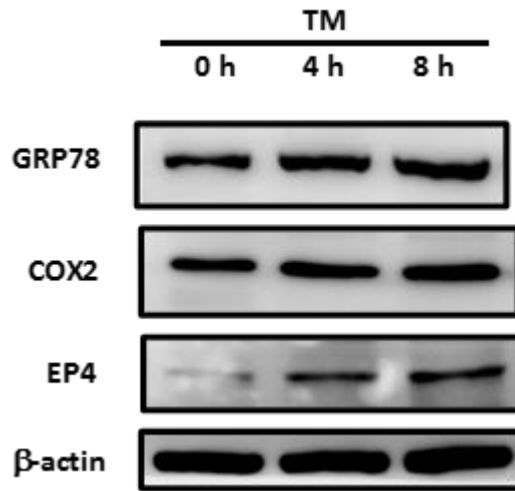


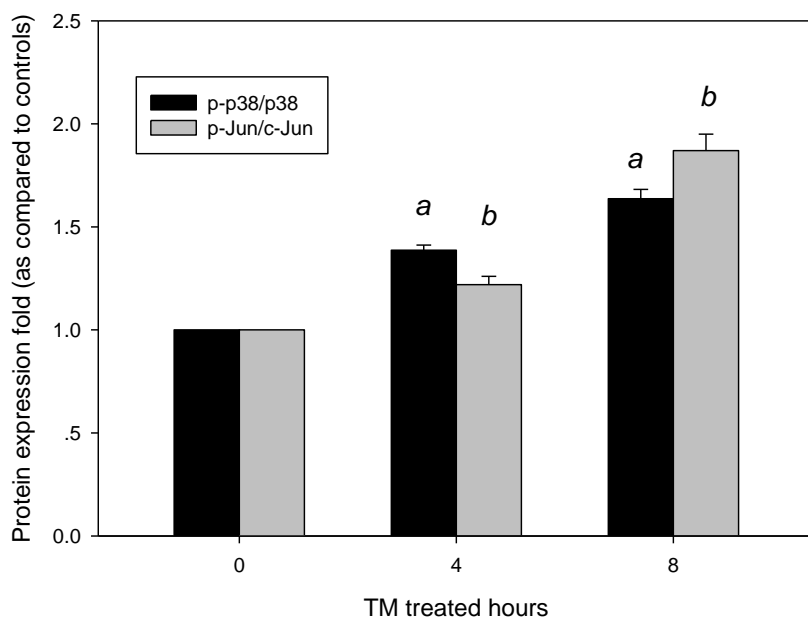
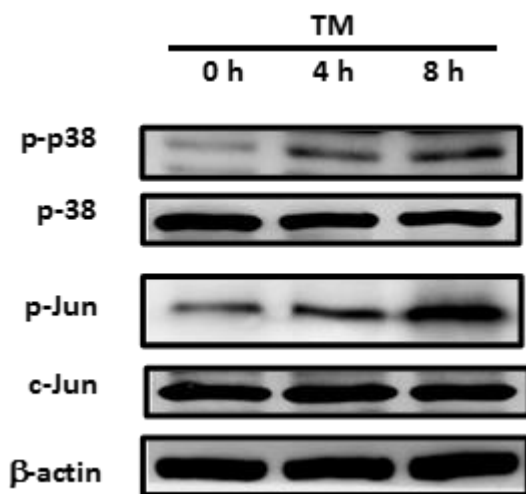
Figure 3. Effect of co-pretreatment with MLPE and tunicamycin on apoptosis induced by doxorubicin in HepG2 cells. HepG2 cells were treated with 1.5 μ M tunicamycin for 8 hr, either in the absence or the presence different concentrations of

MLPE (0.5 and 1 mg/mL) and then exposed to doxorubicin (10 μ g/mL) for 24 hr. (A) Cell viability of HepG2 cells was determined by the MTT assay. (B) Apoptosis was analyzed as the sub-G1 fraction by flow cytometry. Cell and percentage of apoptotic cells in subG1 phase was examined by flow cytometry. C: Untreated HepG2 cells as control; TM: HepG2 cells pretreated with tunicamycin; DOX: HepG2 cells treated with doxorubicin alone; TM+DOX: HepG2 cells pretreated with tunicamycin, and then exposed to doxorubicin; TM+MLPE+DOX: HepG2 cells co-pretreated with 1.5 μ M tunicamycin and 0.5 mg/mL MLPE and then exposed to 10 mg/mL doxorubicin. (C) Cleaved caspase-3 as an apoptotic marker was measured by western blot using specific anti-caspase-3 antibody. β -actin in the same HepG2 cells extract was used as an internal control. Data are presented as mean \pm SD for the three independent experiments. a $p < 0.05$, compared with HepG2 cells alone, b, $p < 0.05$, compared with HepG2 cells treated with doxorubicin for 24 hr, c, $p < 0.05$, compared with HepG2 cells pretreated with tunicamycin for 8 hr and then exposed to doxorubicin for 24 hr.

(A)



(B)



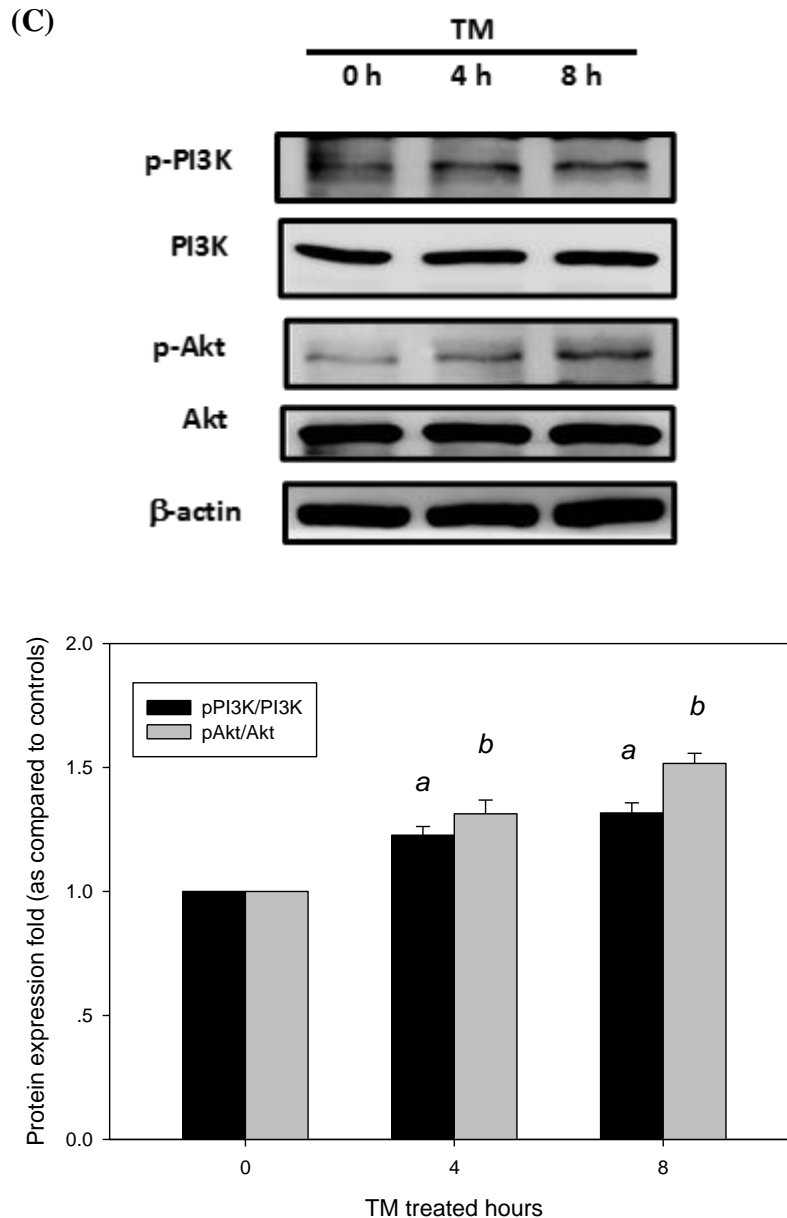


Figure 4. Tunicamycin treatment induced the ER stress related protein expression in doxorubicin-induced cells. HepG2 cells were treated with 1.5 μ M tunicamycin (TM) for 0 (control), 4 and 8 hr. Equal protein amounts of cell lysates were subjected to western blot assay using specific antibody as described in material and method. β -actin in the same HepG2 cells extract was used as an internal control. Optical density reading values of the specific protein versus the loading control protein β -actin are represented as fold of the control values. a, b, c, $p < 0.05$, compared with HepG2 cells alone.

(A)

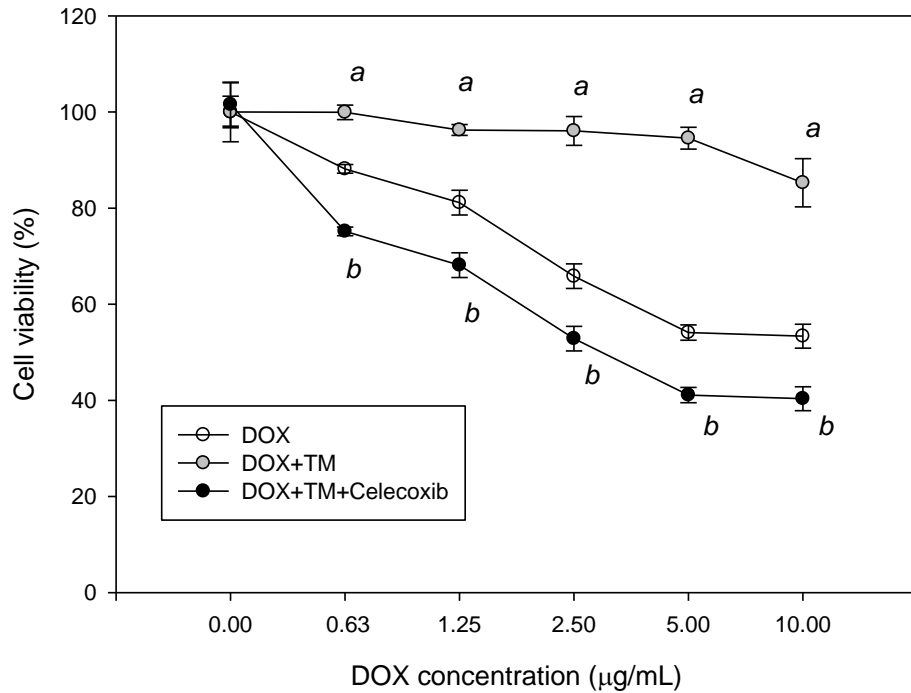


Figure 5A. Effect of co-pretreatment with tunicamycin and celecoxib on cell toxicity induced by doxorubicin in HepG2 cells. HepG2 cells were pretreated with 1.5 µM tunicamycin for 8 hr, either in the absence or the presence of celecoxib (50 µM) and then exposed to doxorubicin (10 µg/mL) for 24 hr. Cell viability of HepG2 cells was determined by the MTT assay. Data are expressed as the mean ± SD of three independent experiments. a, $p < 0.05$, compared with HepG2 treated with doxorubicin for 24 hr; b, $p < 0.05$, compared with HepG2 pretreatment with tunicamycin for 8 hr and then treated with doxorubicin for 24 hr.

(B)

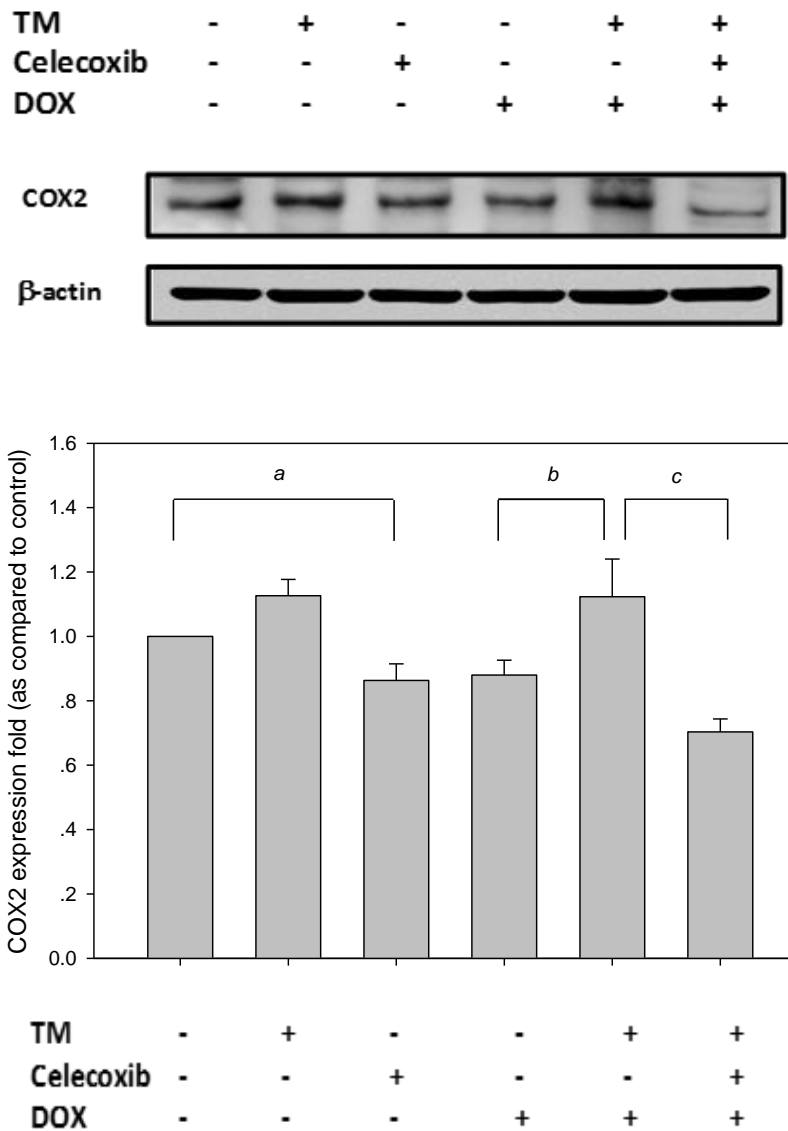
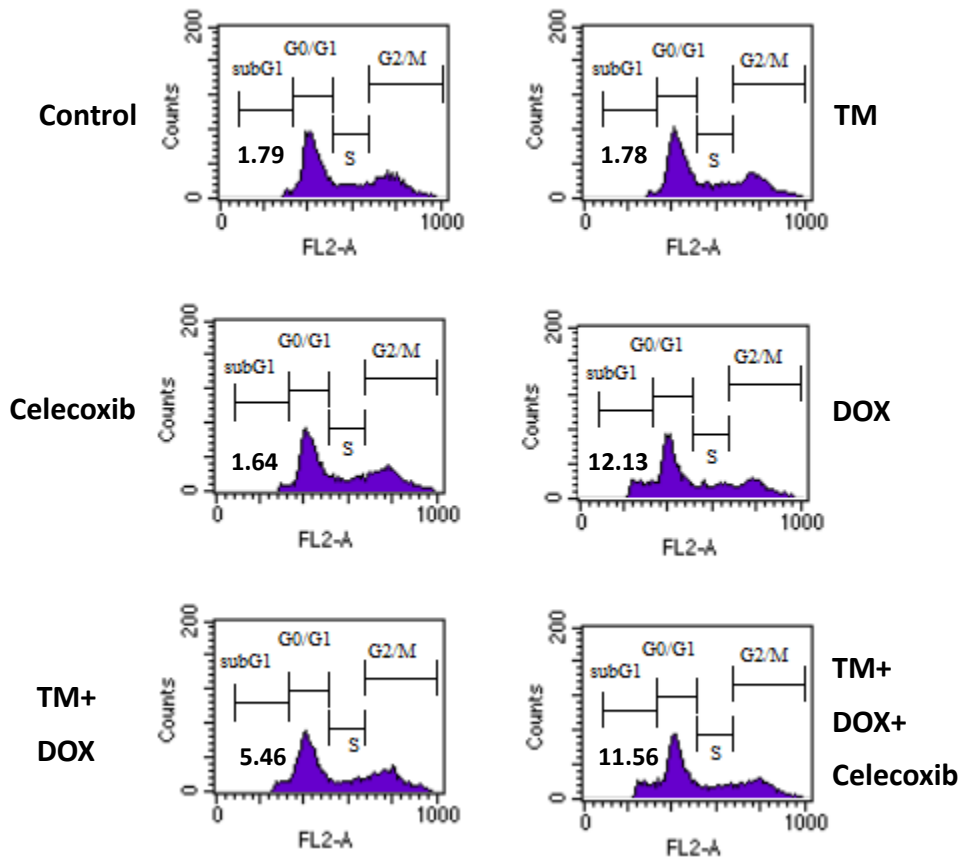


Figure 5B. Effect of co-pretreatment with tunicamycin and celecoxib on COX2 expression in HepG2 cells induced by doxorubicin. HepG2 cells were treated with 1.5 μ M tunicamycin for 8 hr, either in the absence or the presence of 50 μ M celecoxib and then exposed to doxorubicin (10 μ g/mL) for 24 hr. Cleaved caspase-3 as an apoptotic marker were measured by western blot using specific anti-caspase-3 antibody. β -actin in the same HepG2 cells extract was used as an internal control. Data are presented as mean \pm SD for the three independent experiments. a, $p < 0.05$, compared with untreated HepG2; b, $p < 0.05$, compared with HepG2 treated with doxorubicin for 24 hr; c, $p < 0.05$, compared with HepG2 pretreatment with tunicamycin for 8 hr and then treated with doxorubicin for 24 hr.

(C)



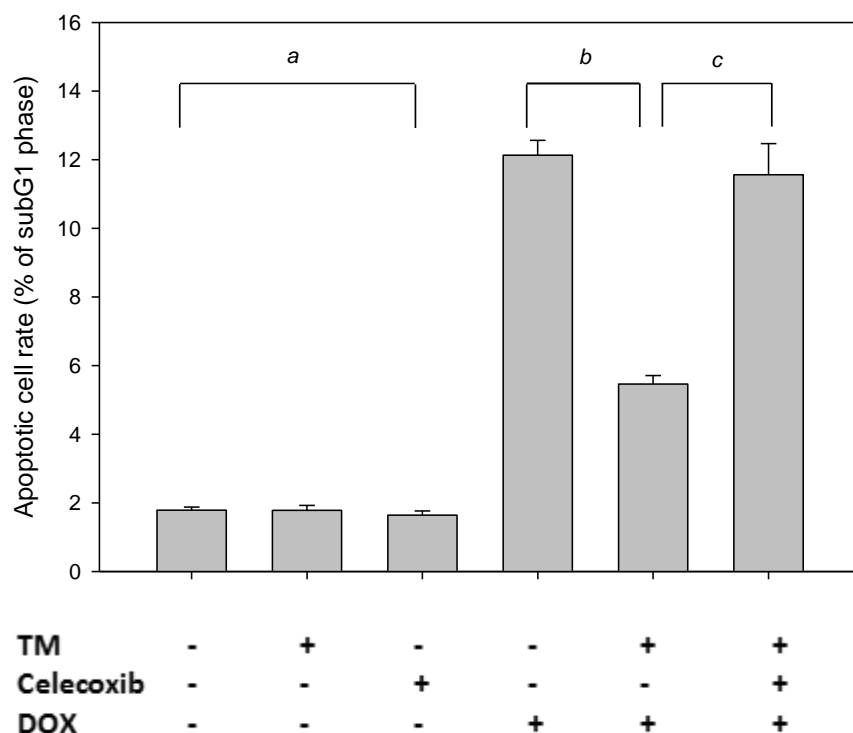


Figure 5C. Effect of co-pretreatment with tunicamycin and celecoxib on apoptosis induced by doxorubicin in HepG2 cells. (A) HepG2 cells were treated with 1.5 μ M tunicamycin and 50 μ M celecoxib for 8 hr, either in the absence or the presence of 1 mg/mL MLPE and then exposed to doxorubicin (10 μ g/mL) for 24 hr. Apoptosis was analyzed as the sub-G1 fraction by flow cytometry. (B) Cell and percentage of apoptotic cells in subG1 phase was examined by flow cytometry. C: Untreated HepG2 cells as control; TM: HepG2 cells pretreated with tunicamycin alone; Celecoxib: HepG2 cells pretreated with celecoxib alone; DOX: HepG2 cells treated with doxorubicin alone; TM+DOX: HepG2 cells pretreated with tunicamycin and then exposed to doxorubicin; TM+celecoxib+DOX: HepG2 cells co-pretreated with tunicamycin and celecoxib and then exposed to doxorubicin. Data are presented as mean \pm SD for the three independent experiments. a, $p < 0.05$, compared with HepG2 cells alone, b, $p < 0.05$, compared with HepG2 cells treated with doxorubicin, c, $p < 0.05$, compared with HepG2 cells pretreated with tunicamycin, and then exposed to doxorubicin.

(D)

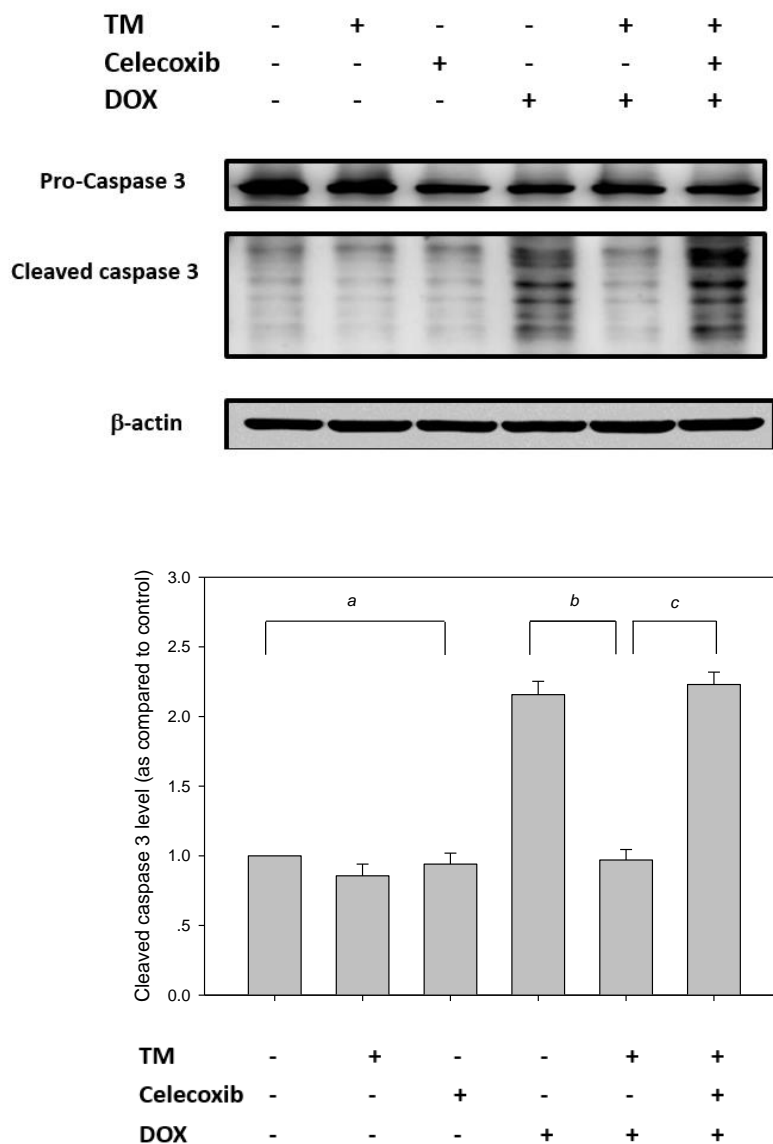


Figure 5D. Effect of co-pretreatment with tunicamycin and celecoxib on caspase 3 activated induced by doxorubicin in HepG2 cells. HepG2 cells were treated with 1.5 μ M tunicamycin for 8 hr, either in the absence or the presence of 50 μ M celecoxib and then exposed to doxorubicin (10 μ g/mL) for 24 hr. Cleaved caspase-3 as an apoptotic marker were measured by western blot using specific anti-caspase-3 antibody. β -actin in the same HepG2 cells extract was used as an internal control. Data are presented as mean \pm SD for the three independent experiments. a, $p < 0.05$, compared with untreated HepG2; b, $p < 0.05$, compared with HepG2 treated with doxorubicin for 24 hr; c, $p < 0.05$, compared with HepG2 pretreatment with tunicamycin for 8 hr and then treated with doxorubicin for 24 hr.

(A)

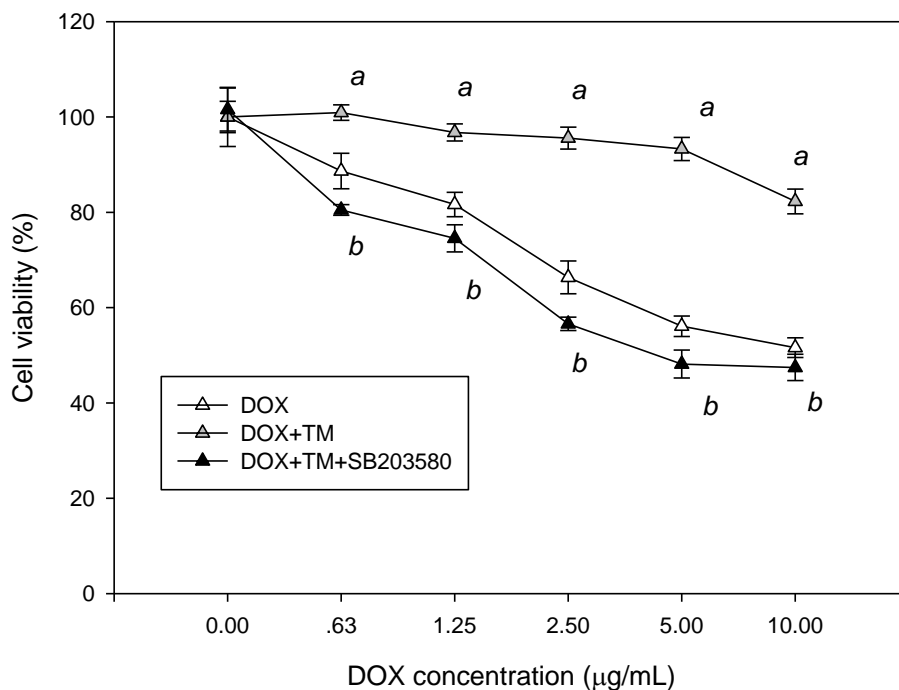
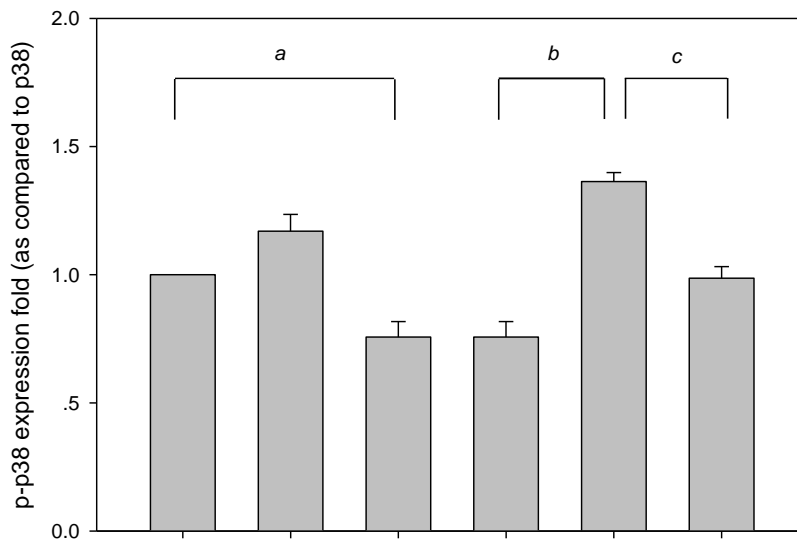
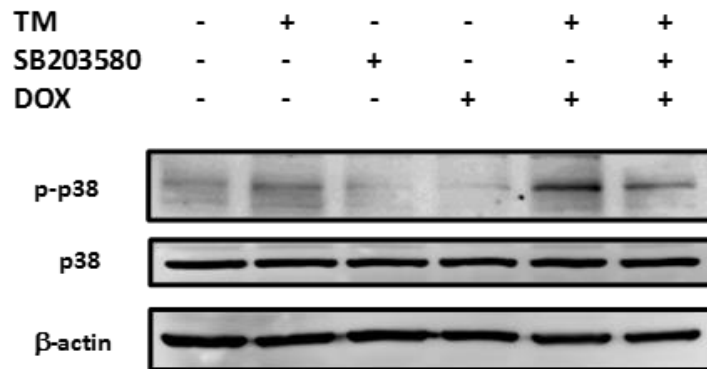


Figure 6A. Effect of co-pretreatment with tunicamycin and SB203580 on cell toxicity induced by doxorubicin in HepG2 cells. HepG2 cells were pretreated with 1.5 µM tunicamycin for 8 hr, either in the absence or the presence of SB203580 (20 µM) and then exposed to doxorubicin (10 µg/mL) for 24 hr. Cell viability of HepG2 cells was determined by the MTT assay. Data are expressed as the mean ± SD of three independent experiments. a, $p < 0.05$, compared with HepG2 treated with doxorubicin for 24 hr; b, $p < 0.05$, compared with HepG2 pretreatment with tunicamycin for 8 hr and then treated with doxorubicin for 24 hr.

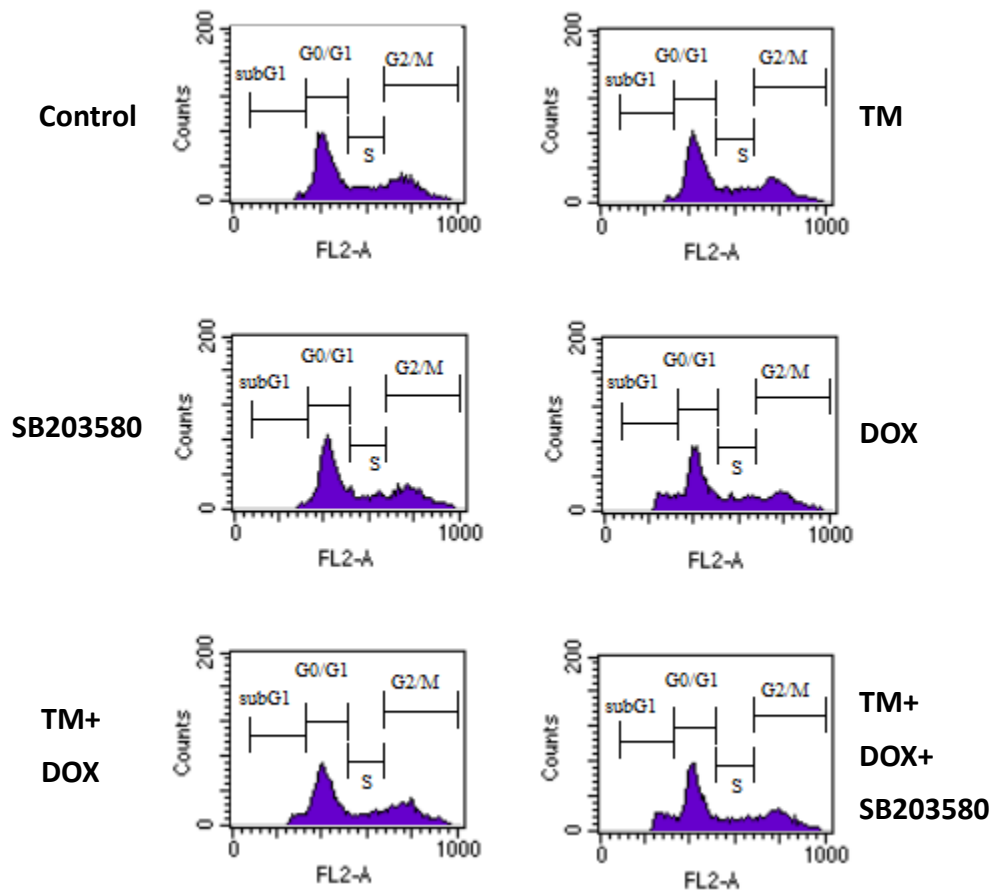
(B)



TM	-	+	-	-	+	+
SB203580	-	-	+	-	-	+
DOX	-	-	-	+	+	+

Figure 6B. Effect of co-pretreatment with tunicamycin and SB203580 on p-p38 expression in HepG2 cells induced by doxorubicin. HepG2 cells were treated with 1.5 μ M tunicamycin for 8 hr, either in the absence or the presence of 20 μ M SB203580 and then exposed to doxorubicin (10 μ g/mL) for 24 hr. Cleaved caspase-3 as an apoptotic marker were measured by western blot using specific anti-caspase-3 antibody. β -actin in the same HepG2 cells extract was used as an internal control. Data are presented as mean \pm SD for the three independent experiments. a, $p < 0.05$, compared with untreated HepG2; b, $p < 0.05$, compared with HepG2 treated with doxorubicin for 24 hr; c, $p < 0.05$, compared with HepG2 pretreatment with tunicamycin for 8 hr and then treated with doxorubicin for 24 hr.

(C)



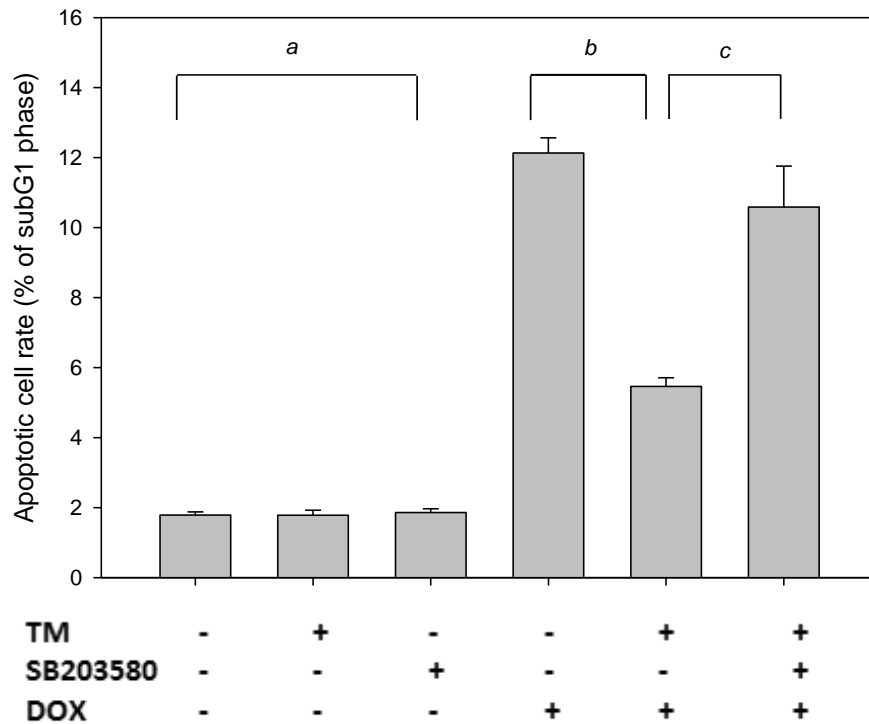


Figure 6C. Effect of co-pretreatment with tunicamycin and SB203580 on apoptosis induced by doxorubicin in HepG2 cells. (A) HepG2 cells were treated with 1.5 μ M tunicamycin and 20 μ M SB203580 for 8 hr, either in the absence or the presence of 1 mg/mL MLPE and then exposed to doxorubicin (10 μ g/mL) for 24 hr. Apoptosis was analyzed as the sub-G1 fraction by flow cytometry. (B) Cell and percentage of apoptotic cells in subG1 phase was examined by flow cytometry. C: Untreated HepG2 cells as control; TM: HepG2 cells pretreated with tunicamycin alone; SB203580: HepG2 cells pretreated with SB203580 alone; DOX: HepG2 cells treated with doxorubicin alone; TM+DOX: HepG2 cells pretreated with tunicamycin and then exposed to doxorubicin; TM+SB203580+DOX: HepG2 cells co-pretreated with tunicamycin and celecoxib and then exposed to doxorubicin. Data are presented as mean \pm SD for the three independent experiments. a, $p < 0.05$, compared with HepG2 cells alone, b, $p < 0.05$, compared with HepG2 cells treated with doxorubicin, c, $p < 0.05$, compared with HepG2 cells pretreated with tunicamycin, and then exposed to doxorubicin.

(D)

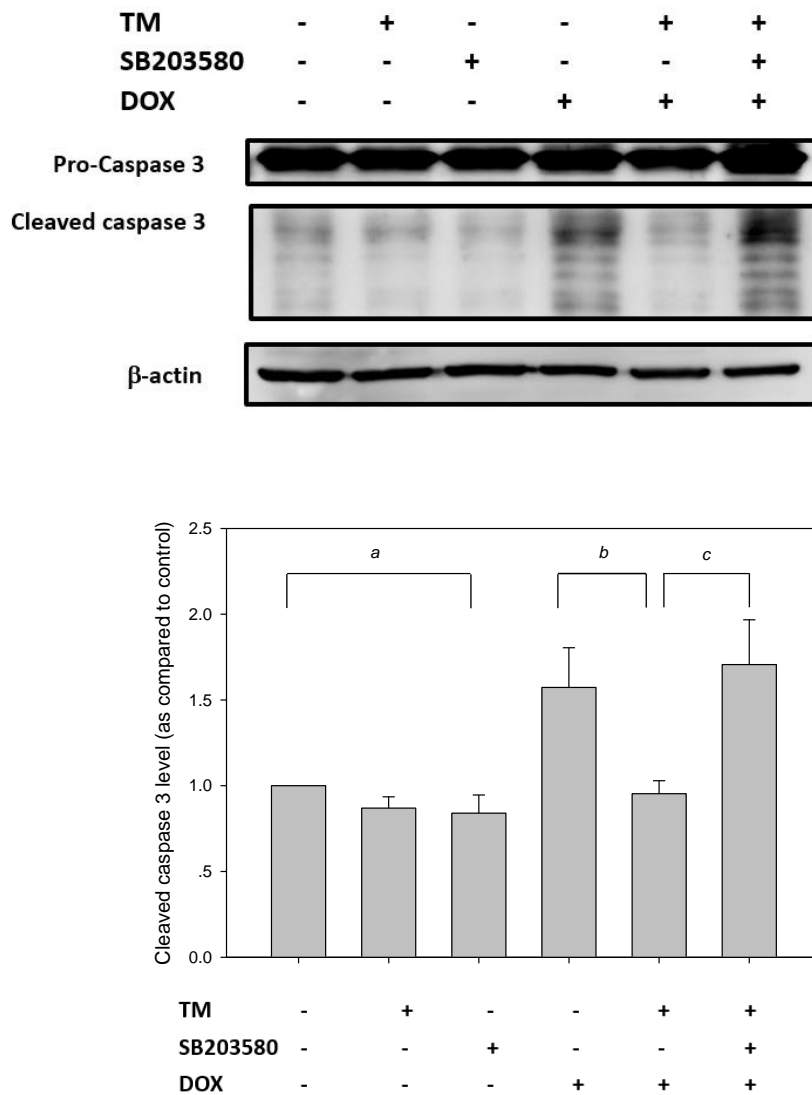


Figure 6D. Effect of co-pretreatment with tunicamycin and SB203580 on caspase 3 activated induced by doxorubicin in HepG2 cells. HepG2 cells were treated with 1.5 μ M tunicamycin for 8 hr, either in the absence or the presence of 20 μ M SB203580 and then exposed to doxorubicin (10 μ g/mL) for 24 hr. Cleaved caspase-3 as an apoptotic marker were measured by western blot using specific anti-caspase-3 antibody. β -actin in the same HepG2 cells extract was used as an internal control. Data are presented as mean \pm SD for the three independent experiments. a, $p < 0.05$, compared with untreated HepG2; b, $p < 0.05$, compared with HepG2 treated with doxorubicin for 24 hr; c, $p < 0.05$, compared with HepG2 pretreatment with tunicamycin for 8 hr and then treated with doxorubicin for 24 hr.

(A)

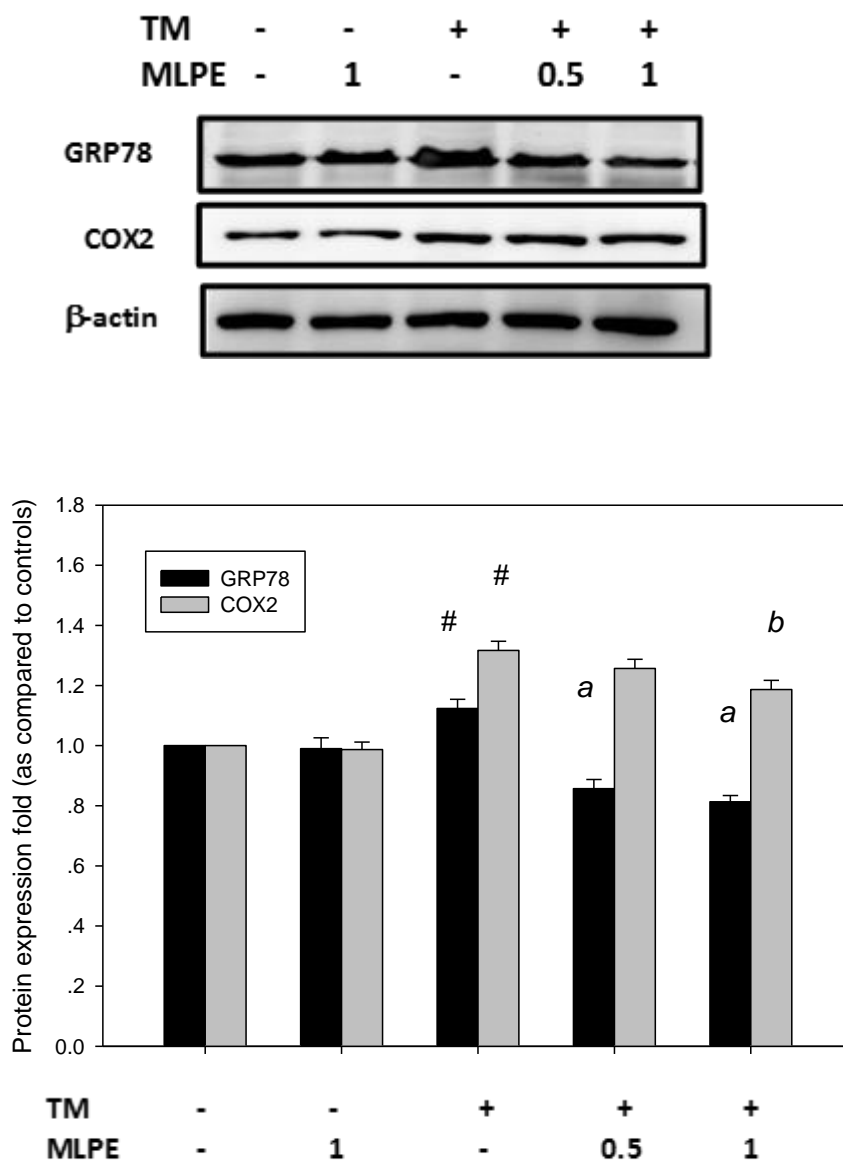


Figure 7A. Effect of co-treatment with MLPE and tunicamycin on the expression of COX-2 and GRP78 in HepG2 cells. Whole cell lysates from HepG2 cells with treatment with 1.5 μ M tunicamycin (TM) in either the absence (control) or the indicated concentration of MLPE (0.5 and 1 mg/mL) for 8 hr were subjected to western blotting analysis. β -actin in the same HepG2 cells extract was used as an internal used as an internal reference. Optical density reading values of the specific protein versus the loading control protein β -actin are represented as fold of the control values. #, $p < 0.05$, compared with untreated HepG2 cell. a, b, $p < 0.05$, compared with HepG2 pretreatment with tunicamycin for 8 hr.

(B)

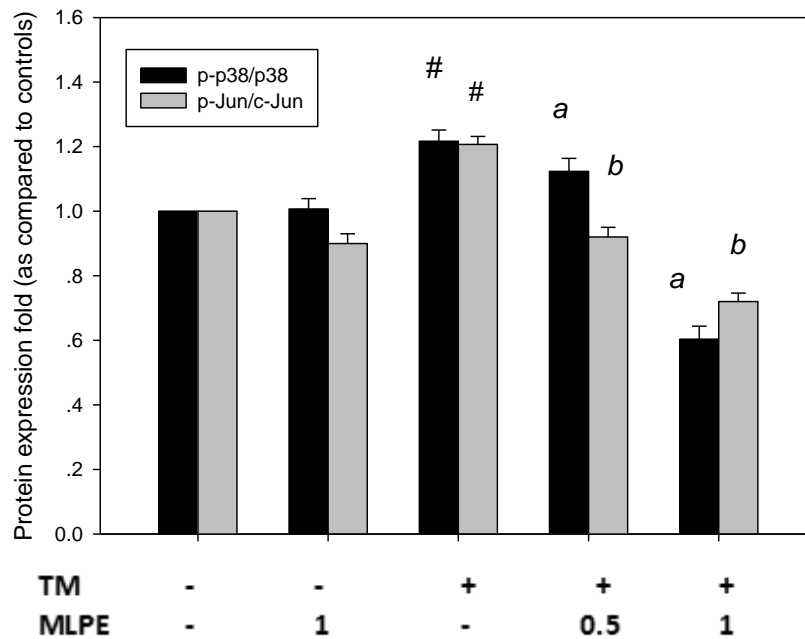
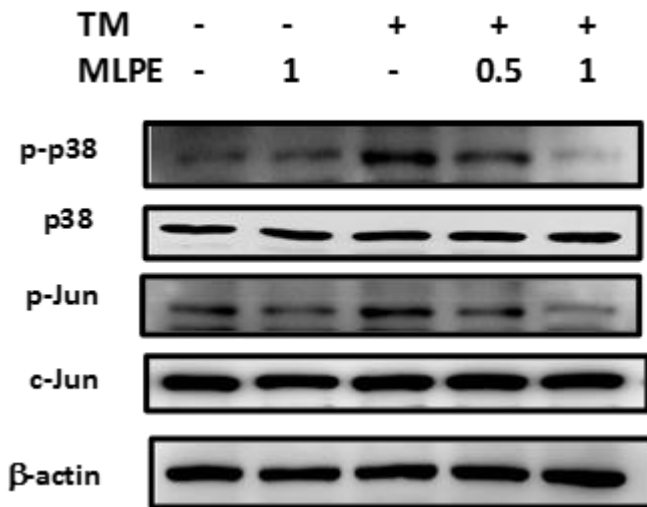


Figure 7B. Effect of co-treatment with MLPE and tunicamycin on the expression of p-p38 and p-Jun in HepG2 cells. Whole cell lysates from HepG2 cells with treatment with 1.5 μ M tunicamycin (TM) in either the absence (control) or the indicated concentration of MLPE (0.5 and 1 mg/mL) for 8 hr were subjected to western blotting analysis. β -actin in the same HepG2 cells extract was used as an internal used as an internal reference. Optical density reading values of the specific protein versus the loading control protein β -actin are represented as fold of the control values. #, $p < 0.05$, compared with untreated HepG2 cell. a, b, $p < 0.05$, compared with HepG2 pretreatment with tunicamycin for 8 hr.

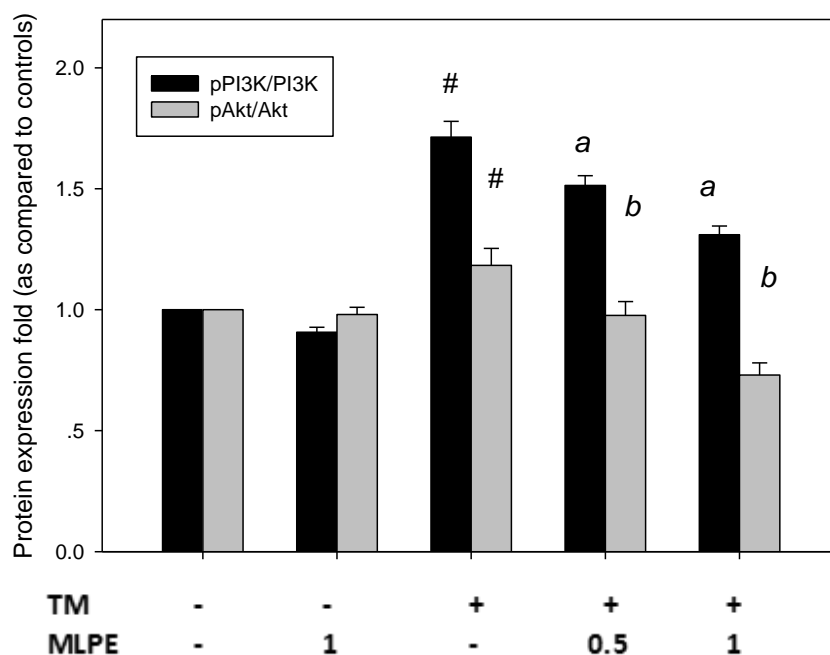
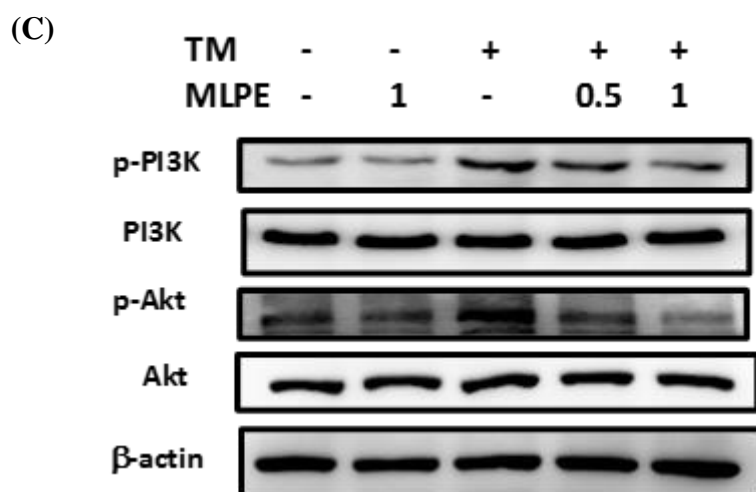


Figure 7C. Effect of co-treatment with MLPE and tunicamycin on the expression of p-PI3K/p-Akt in HepG2 cells. Whole cell lysates from HepG2 cells with treatment with 1.5 μ M tunicamycin (TM) in either the absence (control) or the indicated concentration of MLPE (0.5 and 1 mg/mL) for 8 hr were subjected to western blotting analysis. β -actin in the same HepG2 cells extract was used as an internal used as an internal reference. Optical density reading values of the specific protein versus the loading control protein β -actin are represented as fold of the control values. #, $p < 0.05$, compared with untreated HepG2 cell. a, b, $p < 0.05$, compared with HepG2 pretreatment with tunicamycin for 8 hr.

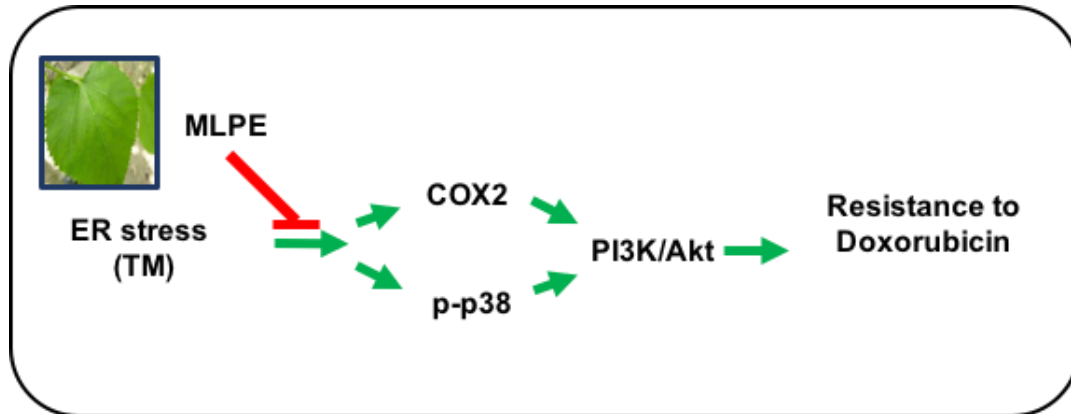


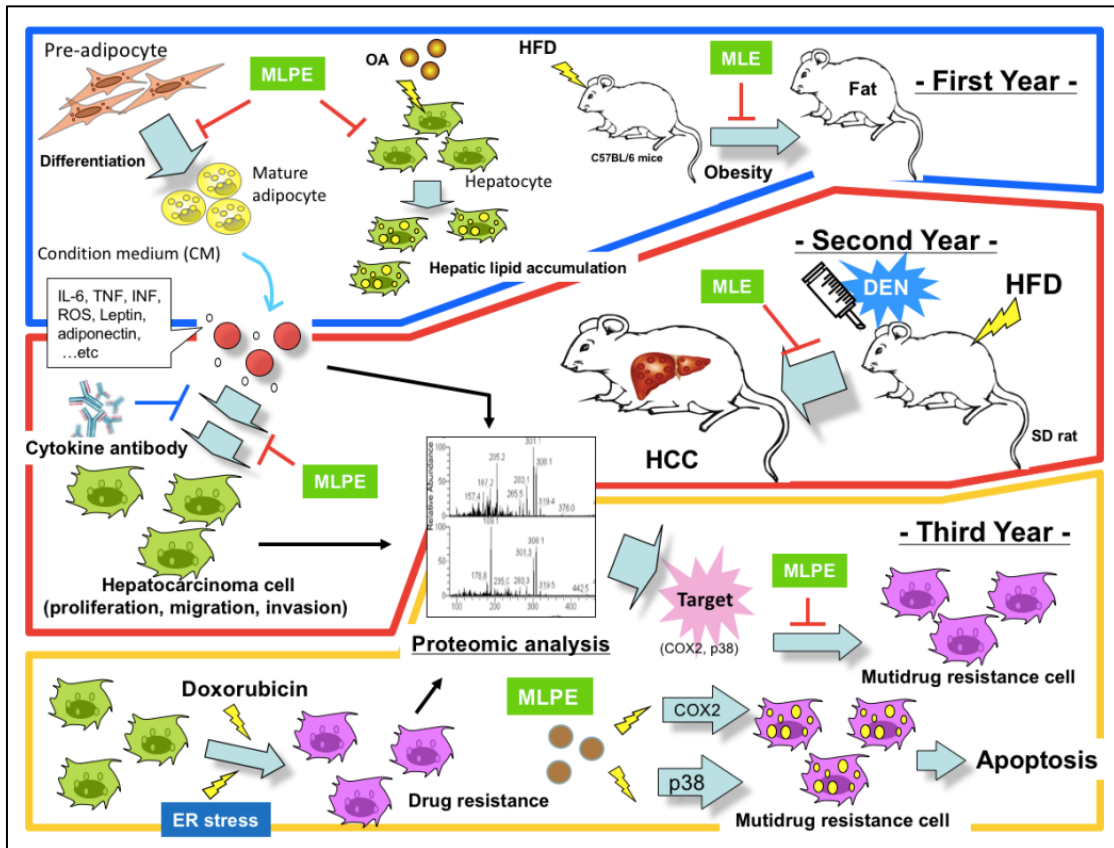
Figure 8. Our results demonstrate that MLPE reverses ER stress–induced resistance to doxorubicin in human hepatocellular carcinoma cells by targeting COX-2 mediated inactivation of PI3K/Akt pathway. These findings will help to further evaluate the potential for the development of new hepatocellular carcinoma therapy.

三年總結論

隨著人口年齡的老化，平均壽命之延長，所遭遇到的疾病及健康問題也隨之增加，因此現代人不僅是要活得長，更要活得健康，有尊嚴，也相對顯示預防醫學的重要，飲食所造成的肥胖實乃代謝性相關疾病形成之主因。依目前健康食品之開發趨近成熟，而現今在市面上所流通之保健食品亦不計其數，而目前市場上僅有少數產品通過相關規範，並不足以滿足日趨增加的市場需求，據研究指出桑葉含豐富抗氧物質及多酚成份，所以具代表性及說服力的功效為抗氧化與降血脂兩大方面，本實驗室目前已針對桑葉萃取物之功能性做相關深入探討，發現桑葉萃取物具有降血脂、抑制心血管疾病之功效，相關數據已發表在國際期刊當中。

而本研究**第一年**證實桑葉萃取物確實可以有效降低血液和肝臟中脂質含量及抑制脂肪細胞分化及促進凋亡，進一步降低體重增加及減緩非酒精性脂肪肝之生成，且無肝腎毒性之不良副作用。**第二年**證實桑葉成份抑制肥胖因子促進肝癌細胞增生發炎及氧化壓力，進一步減少肥胖所促進DEN 致肝癌發生之作用，其中也證實桑葉成份是透過降低氧化壓力與增加解毒酵素活性，進而達到保護肝臟之作用。而肝癌一直被視為復發率高、對化療呈抗藥性的癌症，故本研究於**第三年**針對肝癌的抗藥性方面作深入研究，結果發現，桑葉具有改善肝癌的抗藥性之作用。深入探討後發現桑葉萃取物是透過降低COX2與p38的路徑，進而能夠影響肝癌細胞對於化療藥物感受度，造成肝癌細胞的死亡。而桑葉本身目前用途不多，經濟效益不大，因此材料成本不大，栽植方便，由以上研究確認桑葉成份具有抑制非酒精性脂肪肝及肥胖促進肝癌及抗藥性之作用是一可提供為保健及醫藥的素材。

三年之總結



科技部補助計畫衍生研發成果推廣資料表

日期:2016/06/15

科技部補助計畫	計畫名稱: 桑葉成份抑制非酒精性脂肪肝及肥胖促進肝癌及抗藥性之研究
	計畫主持人: 王朝鐘
	計畫編號: 102-2313-B-040-003-MY3 學門領域: 食品及農化
無研發成果推廣資料	

102年度專題研究計畫成果彙整表

計畫主持人：王朝鐘			計畫編號：102-2313-B-040-003-MY3			
計畫名稱：桑葉成份抑制非酒精性脂肪肝及肥胖促進肝癌及抗藥性之研究						
成果項目			量化	單位	質化 (說明：各成果項目請附佐證資料或細項說明，如期刊名稱、年份、卷期、起訖頁數、證號...等)	
國內	學術性論文	期刊論文		3	篇	
		研討會論文		0		
		專書		0	本	
		專書論文		0	章	
		技術報告		0	篇	
		其他		0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件
				已獲得	0	
			新型/設計專利		0	
		商標權		0		
		營業秘密		0		
		積體電路電路布局權		0		
		著作權		0		
		品種權		0		
		其他		0		
	技術移轉	件數		0	件	
		收入		0	千元	
	國外	學術性論文	期刊論文		0	篇
			研討會論文		1	
			專書		0	本
專書論文			0	章		
技術報告			1	篇		
其他			0	篇		
智慧財產權及成果		專利權	發明專利	申請中	0	件
				已獲得	0	
			新型/設計專利		0	
		商標權		0		
		營業秘密		0		
		積體電路電路布局權		0		
		著作權		0		
		品種權		0		
其他		0				

	技術移轉	件數	0	件	
		收入	0	千元	
參與計畫人力	本國籍	大專生	1	人次	
		碩士生	1		
		博士生	1		
		博士後研究員	1		
		專任助理	0		
	非本國籍	大專生	0		
		碩士生	0		
		博士生	0		
		博士後研究員	0		
		專任助理	0		
其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)		目前我們已發表9篇有關於桑葉及主成份調節血脂、抑制血管病變、非酒精性脂肪肝、肝癌及肥胖之研究，包括J. Agric. Food Chem., 57, 9147-9153, 2009; 58, 2536-2542, 2010; 59, 1985-1995, 2011; 60, 6891- 6898, 2012; 61, 2780-2788, 2013; 61, 9867-9875, 2013; J. Food Sci., 76, 65-72, 2011 及發現桑葉成份具調節 AMPK作用，發表於 J. Fun. Food, 5, 1620-1632, 2013。及發現桑葉成份具調節及MLPE 也抑制脂肪細胞分化，發表於J. Fun. Food, 21, 249-262, 2016，從以上的成果擬與業界進行開發桑葉抑制肥胖，非酒精性脂肪肝，及肥胖促進肝癌之健康食品。			

科技部補助專題研究計畫成果自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現（簡要敘述成果是否具有政策應用參考價值及具影響公共利益之重大發現）或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

達成目標

未達成目標（請說明，以100字為限）

實驗失敗

因故實驗中斷

其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形（請於其他欄註明專利及技轉之證號、合約、申請及洽談等詳細資訊）

論文： 已發表 未發表之文稿 撰寫中 無

專利： 已獲得 申請中 無

技轉： 已技轉 洽談中 無

其他：（以200字為限）

目前我們已發表9篇有關於桑葉及主成份調節血脂、抑制血管病變、非酒精性脂肪肝、肝癌及肥胖之研究，包括J. Agric. Food Chem., 57, 9147-9153. 2009；58, 2536-2542.

2010；59, 1985-1995. 2011；60, 6891- 6898, 2012；61, 2780-2788, 2013；61, 9867-9875, 2013；

J. Food Sci., 76, 65-72. 2011；及發現桑葉成份具調節AMPK

J. Fun. Food, 5, 1620-1632。

及發現桑葉成份具調節及MLPE 也抑制脂肪細胞分化J. Fun. Food, 21, 249-262, 2016，從以上的成

果擬與業界進行開發桑葉抑制肥胖，非酒精性脂肪肝，及肥胖促進肝癌之健康食品。

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性，以500字為限）

目前我們已發表9篇有關於桑葉及主成份調節血脂、抑制血管病變、非酒精性脂肪肝、肝癌及肥胖之研究，包括J. Agric. Food Chem., 57, 9147-9153. 2009；58, 2536-2542.

2010；59, 1985-1995. 2011；60, 6891- 6898, 2012；61, 2780-2788, 2013；61, 9867-9875, 2013；

J. Food Sci., 76, 65-72. 2011；及發現桑葉成份具調節AMPK

J. Fun. Food, 5, 1620-1632。

及發現桑葉成份具調節及MLPE 也抑制脂肪細胞分化J. Fun. Food, 21, 249-262, 2016，從以上的成

果擬與業界進行開發桑葉抑制肥胖，非酒精性脂肪肝，及肥胖促進肝癌之健康食品。

4. 主要發現

本研究具有政策應用參考價值：否 是，建議提供機關

（勾選「是」者，請列舉建議可提供施政參考之業務主管機關）

本研究具影響公共利益之重大發現：否 是

說明：（以150字為限）