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行政院農業委員會農糧署99年度科技計畫研究報告

計畫名稱： 桑葉萃取物開發為保健食品之研究 (第1年/全程1年)

(英文名稱) Study and application of Morus alba L. leaves extract to attenuate fatty liver formation by decreasing hepatic lipid accumulation

計畫編號： 99農科-3.1.3-糧-Z1(3)

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一、執行成果中文摘要：

當脂肪過度堆積在肝時會增加肝臟疾病之風險，與肥胖、脂血代謝異常、高血壓、胰島素抗性及糖尿病等有極高之關聯性，但因病狀容易遭到忽略而進展成更嚴重的脂肪性肝炎，所以降低肝臟脂肪堆積之化學藥品或是天然萃取物開發，已經成為當前治療脂肪肝性疾病一項重要的課題。來自乾燥桑葉 (*Morus spp.*) 中的桑葉多酚萃取物 (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MLPE是否具備抑制肝臟脂肪堆積之功效仍有待確認，故本論文以細胞模式和動物模式探討MLPE 減少肝臟脂肪堆積之作用與機制，並分別以油酸和高油脂飲食 (high fat diet, HFD) 誘導兩種模式中肝臟脂肪堆積。結果發現MLPE可降低血液和肝臟脂質 (三酸甘油酯、膽固醇、脂肪酸) 堆積的現象，並使高油脂飲食組動物體重減輕。同時還發現MLPE可以減少脂質生成酵素，例如：FAS (fatty acid synthase)、ACC (Acetyl-CoA carboxylase)、HMGCR (HMG-CoA reductase) 及其相關轉錄因子 (SREBP1, SREBP2, PPAR γ) 之蛋白表現。此外，MLPE主要是透過AMPK (AMP-activating kinase) 之傳訊路徑調控上述這些蛋白質，達到降低肝臟脂肪堆積之效果。此研究證實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，對於抑制脂肪肝確有不錯之開發潛力，然而，今後仍需進行更深入研究證實MLPE促進脂質代謝之作用。





二、執行成果英文摘要：

Fat accumulation in the liver increases the risk of developing progressive liver injuries, and may have implication about obesity, dyslipidemia, hypertension, insulin resistance and diabetes. It is a very important to develop specific pharmacological or nutritional agents to reduce lipid content and prevent the development of more severe forms of liver disease. Mulberry leaf polyphenol extracts (MLPEs) were extracted from dry mulberry leaves (*Morus spp.*), which have been known to modulate serum fasting glucose, lipid and antiatherosclerosis. However, the effect of MLPEs on regulating hepatic lipid metabolism is unclear. This study was evaluate the effects and mechanisms of MLPEs in reducing hepatic lipid accumulation when administered in fat overload conditions both in cell culture and in an animal experimental model. We found that MLPEs could regulate serum and hepatic lipid contents (e.g. triglyceride, cholesterol, and free fatty acid) and about range of weight gain was also reduced in HFD (high-fat diet) mice. Furthermore, numerous lipogenic enzymes, such as FAS (fatty acid synthetase), ACC (acetyl-CoA carboxylase), HMGCR (HMG-CoA reductase) and associated-lipogenic transcriptional factors (SREBP1, SREBP2, and PPAR γ) were suppressed by MLPEs. Our results show that MLPEs are able to reduce the hepatic lipid accumulation through the AMPK (AMP-activating protein kinase) signaling pathway. And it may have potential therapeutic implications for human dyslipidemias. However, more evidences are still required to demonstrate the effects of MLPEs on lipid metabolism.





三、計畫目的：

1. 評估桑葉萃取物減緩高脂肪食物所誘發動物脂肪肝。
2. 分析動物脂肪肝指標，如血脂肪、肝臟脂肪及肝臟功能酵素表現。
3. 建立油酸誘導肝細胞脂肪堆積之模式。
4. 完成桑葉萃取物抑制肝細胞脂肪堆積之效果。
5. 探討桑葉萃取物在肝細胞排除或抑制脂肪堆積之機轉。





四、重要工作項目及實施方法：

一、重要工作項目

1. 建立油酸誘導肝細胞脂肪堆積之模式
2. 完成桑葉萃取物抑制肝細胞脂肪堆積之效果
3. 評估桑葉萃取物減緩高脂飼糧所誘發動物之脂肪肝。
4. 分析動物脂肪肝指標，如血脂肪、肝臟脂肪及肝臟功能酵素表現
5. 探討桑葉萃取物在肝細胞排除或抑制脂肪堆積之機轉

二、實施方法

I. 高脂飼糧誘導小鼠產生脂肪肝之模式

動物模式以C57BL/6 雄性小黑鼠為實驗動物，購自國家研究院實驗動物中心，小鼠購入時約為六週齡，飼養於中山醫學大學動物中心。隨機將動物分成四組，控制組(normal control)、誘導組(HFD induced control)、試驗組I (MLE 0.5%)與試驗組II (MLE 1.5%)，以每組10隻為一組的方式飼養。誘導組餵食之飼料包含20% lard oil與0.5% cholesterol。飼料之給予，並紀錄每日小鼠之攝取情形。實驗期達8週，待實驗期間結束，犧牲取其血液做血清生化，包含肝功能、肝脂肪含量分析、血脂含量分析(包含TG、cholesterol)。

II. 肝細胞脂肪堆積

HepG2肝細胞處理不同濃度的MLE 後，將培養液移除，再以oil-Red O染劑覆蓋，靜置15-20分鐘後移除染劑，以PBS清洗三次。於光學顯微鏡 下，觀察細胞內深紅色的脂肪油滴，即為脂肪堆積。

III. 肝細胞脂肪堆積之定量

將HepG2細胞加入不同濃度的MLE 經過24小時後，移除培養基並以PBS清洗二次，再以nile red染劑覆蓋，在用trypsin將細胞打下，離心後將上清液倒掉，以PBS將細胞懸浮，以流式細胞儀進行分析。

IV. 肝細胞脂肪合成及分解之相關蛋白表現分析

將蛋白以西方點墨法分析，以5% blocking buffer作用，再以TBS-t漂洗三次。換上一級抗體(HMG-CoA reductase, FAS, SREBP-1與PPAR)，置於4 °C下，作用12~16小時，再以TBS-t漂洗三次。接著，換上二級抗體，於室溫下溫和作用1小時，再以TBS-t漂洗三次。最後加入ECL顯色，並以冷光儀偵測。

VI. 統計分析

每組皆三重複以上，採用Student's t-test統計分析。





五、結果與討論：

實驗結果

一、桑葉萃取物成分分析

以乾燥桑葉葉片進行萃取，得到水萃物產率為27%。多酚廣泛存在許多植物當中，具備良好降血脂、抗氧化功效，因此利用單環類多酚化合物 (Gallic acid, GA) 和多環類多酚化合物 (Rutin) 作為測定總多酚含量之標準品。結果顯示(Table 1)，MLE中之單環類多酚化合物含量為21.16%和多環類多酚化合物含量為26.6%。萃取MLE時發現桑葉具有特殊的清香甜味，可能與其富含多醣類有關，因此分析多醣含量發現MLE具有約25%的多醣成份存在。另外蛋白質佔2.37%；油脂佔8.4%。

二、油酸、桑葉萃取物及桑葉多酚萃取物對HepG2 細胞株之毒性測試

以不同濃度之OA、MLE及MLPE處理HepG2細胞株，24小時後分析細胞存活情形。Fig 1A, B結果顯示濃度在0.7 mM OA作用下，細胞的存活率不受影響，但超過0.9 mM 以上就開始對細胞產生傷害，尤其在1.0 mM OA作用下，細胞存活率只有20%左右。Fig 1C, D由MTT結果得知MLE和MLPE藥物半致死劑量 (IC_{50}) 分別是8.29 mg/ml和0.417 mg/ml，本實驗設計重點在觀察胞內脂質堆積前提是不傷害細胞，因此後續實驗將以1, 2, 3 mg/ml MLE和0.1, 0.2, 0.3 mg/ml MLPE為細胞實驗的處理劑量。

三、不同濃度油酸對HepG2 細胞脂質堆積之影響

由上述結果得知，0.1~0.7 mM OA細胞生長狀態良好，細胞存活率也都保持100%，因此選擇以0.2, 0.5, 0.7 mM OA培養HepG2細胞觀察脂肪堆積的情形，Fig 2A乃利用油紅染色試驗 (圖左) 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)；以及尼羅紅螢光染色試驗 (圖右) 之結果，脂肪在雷射光激發下散發紅色螢光。從兩種染色結果均發現細胞質內有脂肪存在，而且隨著濃度增加而明顯變多，接著，將OA處理後的細胞先以尼羅紅染色，再應用流式細胞儀可偵測螢光強度變化原理，螢光強度越強代表脂肪越多，將細胞內脂質含量量化並進行統計分析。Fig 2B 發現以0.2, 0.5, 0.7 mM 之OA濃度處理細胞後，分別有2~3倍不等的胞內脂肪增加 (** $p < 0.001$)。同時也發現，本實驗用來配製OA之溶劑 (Ethanol) 也具有些微誘導脂質堆積之情形，但不具顯著意義。此結果證實Fig 2A染色試驗肉眼觀察到的現象，最後本實驗選擇0.5 mM OA進行後續實驗。

四、桑葉萃取物及桑葉多酚萃取物減少HepG2 細胞內脂質堆積

由上述結果確定本實驗將使用的藥物處理濃度之後，接下來實驗加入MLE、MLPE以及搭配Statin (降膽固醇臨床用藥) 比較三種藥物減少細胞內脂質堆積之效果。Fig 3A, B為油紅染色及尼羅紅染色試驗之結果，細胞中紅色油滴清晰可見。OA誘導後的HepG2細胞脂肪含量較對照組增加2.3倍 ($^{\#}p < 0.05$)，分別加入1, 2, 3 mg/ml MLE和0.1, 0.2, 0.3 mg/ml MLPE進行染色和量化分析。Fig 3加入1, 2 mg/ml MLE作用下對細胞脂肪含量幾乎沒有變化，3 mg/ml MLE作用與OA誘導組比較，脂肪





含量由2.3倍減低至1.5倍，(* $p < 0.05$)，Statin與OA誘導組比較也有減少1.5倍的表現(* $p < 0.05$)。Fig 4A, B從兩種染色結果皆可見到，MLPE抑制胞內脂肪堆積的能力隨著濃度越高效果越明顯，Fig 4C 與OA誘導組對照之下0.1, 0.2, 0.3 mg/ml MLPE胞內脂肪含量分別是1.3倍，1.0倍，0.8倍 (* $p < 0.05$ ，** $p < 0.001$)，Statin也有減少的表現。由以上三種藥物的實驗結果證明皆有抑制胞內脂質堆積之效果，但以MPLE的效果最好，MLE和Statin次之。

五、桑葉萃取物與桑葉多酚萃取物對HepG2細胞脂質代謝蛋白之表達

由於Fig 3到Fig 4的結果證實MLE和MLPE對OA誘導脂質堆積有顯著的抑制作用，因此擬進一步研究其詳細的作用機轉。在脂質代謝機制中，幾個跟脂質生成有關的酵素扮演很重要的角色，例如：FAS、ACC、A-FABP代表脂肪酸的合成與運輸，HMGCR和LDLR與膽固醇生成及低密度膽固醇酯的代謝有關。藉由觀察這些關鍵酵素之表現，有助於了解MLE和MLPE降低肝細胞內脂質堆積的分子機轉。

1. FAS之表達

FAS在體內主要參與脂肪酸新生 (fatty acid synthesis)，所以當食物中的醣類或脂肪過多時，會刺激FAS大量表現。由Fig 5結果可見，經OA誘導之細胞其FAS表現為1.17倍。與對照組相較下，加入1, 2, 3 mg/ml MLE處理後蛋白表現量分別是1.02, 0.95, 0.97倍。而Fig 6結果，OA誘導之細胞FAS表現為1.24倍。加入0.1, 0.2, 0.3 mg/ml MLPE處理後表現量分別是1.20, 1.05, 0.60倍。顯示MLE和MLPE均能減少HepG2細胞以OA誘導後FAS的表現，且有dose-dependent關係。

2. HMGCR之表達

Fig 5中，OA誘導之細胞HMGCR蛋白表現為1.75倍。與對照組相較下，加入1, 2, 3 mg/ml MLE處理後蛋白表現量分別是0.97, 1.07, 0.98倍。Fig 6中，OA誘導之細胞HMGCR蛋白表現為1.20倍。與對照組相較下，加入0.1, 0.2, 0.3 mg/ml MLPE處理後表現量分別是0.92, 0.50, 0.39倍。顯示MLE和MLPE均能減少HepG2細胞以OA誘導後HMGCR的表現。

3. LDLR之表達

Fig 5 結果顯示，OA誘導之細胞LDLR表現為0.91倍。與對照組相比下，加入1, 2, 3 mg/ml MLE處理後，蛋白表現量分別是0.78, 0.64, 0.55倍。Fig 6結果顯示，OA誘導之細胞LDLR表現為1.07倍。與對照組相較下，加入0.1, 0.2, 0.3 mg/ml MLPE處理後，蛋白表現量分別是0.80, 0.74, 0.47倍。顯示MLE和MLPE均能減少HepG2細胞以OA誘導後LDLR的表現，且有dose-dependent關係。

4. A-FABP之表達

Fig 5經OA誘導之細胞A-FABP蛋白表現為1.24倍。與對照組相較下，加入1, 2, 3





mg/ml MLE處理後蛋白表現量分別是1.03, 0.89, 0.89倍。Fig 6經OA誘導之細胞A-FABP蛋白表現為1.50倍。與對照組相較下, 加入0.1, 0.2, 0.3 mg/ml MLPE處理後表現量分別是1.61, 1.51, 1.30倍。顯示MLE和MLPE均能減少HepG2細胞以OA誘導後A-FABP的表現, 且有dose-dependent關係。從Fig 5和Fig 6結果證明, 經OA誘導後的細胞其FAS、HMGCR、A-FABP蛋白表現量均上升, 而無論是MLE或MLPE皆可抑制這些蛋白之表現, 推測桑葉可能具有抑制脂肪酸、膽固醇合成之作用。

5. ACC與AMPK

ACC的功能和FAS同樣屬於合成脂肪酸之酵素, ACC負責催化acetyl-CoA轉變成malnoyl-CoA; FAS則將malnoyl-CoA合成長鏈脂肪酸。AMPK可以藉由磷酸化作用(phosphorylation) 抑制ACC活性以減少脂肪酸合成, 然而AMPK本身也需要藉由上游蛋白質激酶(AMPK kinase) 磷酸化後始有活性。除此之外, AMPK在醣類、脂肪代謝機轉中是個很重要的中樞調節者。

Fig 7A以OA 誘導後, 細胞內ACC蛋白表現量分別為1.81倍, 顯示細胞進行脂肪酸合成作用十分旺盛; 與對照組相較下, MLE蛋白表現量分別為1.70, 1.68, 1.59倍。p-ACC表現方面, 與對照組相較下, MLE處理後蛋白表現量明顯的增加為1.63, 1.68, 2.51倍。在AMPK蛋白表現方面, 在所有細胞組別中AMPK蛋白量皆沒有變化; 而p-AMPK蛋白表現方面, 與對照組相較下, MLE處理後蛋白表現量明顯的增加為1.18, 1.35, 1.80倍。將蛋白表現以p-ACC/ACC ratio計算3次Mean±SD統計, 觀察ACC活性表現情形。由Fig 7B得知, p-ACC/ACC ratio變化量隨著MLE處理濃度而增加, 在3 mg/ml 時表現量最高, 而在OA誘導組表現量最低, 代表MLE除了降低ACC蛋白表現量外, 也會抑制ACC活性 (**p<0.001)。為了進一步證實調控ACC活性是來自上游蛋白AMPK所調控的, 接下來進一步觀察AMPK和p-AMPK之表現。將p-AMPK/AMPK蛋白變化量計算3次Mean±SD結果顯示於Fig 7C。如圖所示, p-AMPK/AMPK ratio在3 mg/ml MLE和處理後有上升的趨勢 (*p<0.05), 代表MLE會活化AMPK。證明此點之後, 同樣比較MLPE對ACC與AMPK蛋白之影響。由Fig 8A得知, MLPE在ACC蛋白表現方面分別是0.1 mg/ml (2.07倍), 0.2 mg/ml (1.31倍), 0.3 mg/ml (1.07倍), 與OA誘導組 (2.30倍) 相較下有減少的趨勢; p-ACC的表現方面分別是0.1 mg/ml (1.40倍), 0.2 mg/ml (2.10倍), 0.3 mg/ml (2.01倍), 與OA誘導組 (1.24倍) 相較下有增加的趨勢; AMPK蛋白表現分別是0.1 mg/ml (0.98倍), 0.2 mg/ml (1.46倍), 0.3 mg/ml (1.28倍), 與OA誘導組 (0.83倍) 相較下有增加的趨勢; 在p-AMPK表現方面分別是0.1 mg/ml (1.43 倍), 0.2 mg/ml (1.66倍), 0.3 mg/ml (2.07倍), 與OA誘導組 (1.10倍) 相較下有增加的趨勢。於是將蛋白變化計算p-ACC/ACC ratio和p-AMPK/AMPK ratio顯示於Fig 8B, C。由Fig 8B得知, OA誘導組細胞會減少p-ACC/ACC ratio (#p<0.05), 而0.3 mg/ml MLPE會增加p-ACC/ACC ratio約2.0倍左右 (**p<0.001), 顯示MLPE會抑制ACC活性表現。由Fig 8C得知, 0.3 mg/ml MLPE會增加p-AMPK/AMPK ratio約2.3倍左右 (*p<0.05), 顯示MLPE會活化AMPK活性表現。由此實驗結果證明MLE和MLPE都可透過活化AMPK路徑的方式抑制ACC活性, 進而降低細





胞進行脂質合成作用。

6. SREBP1、SREBP2、PPAR γ 之蛋白表現

SREBP1、SREBP2分別負責進行脂肪酸與膽固醇的合成調控，例如轉錄FAS、ACC、HMGCR、LDLR等蛋白。PPAR γ ，在肝臟負責轉換游離脂肪酸成為三酸甘油酯形式儲存，在脂肪細胞促進其分化。當體內脂肪酸濃度升高時會活化，轉錄FABP、LDLR等蛋白。由Fig 9A, B結果得知，與OA誘導組相較下，MLE和MLPE兩者可以降低SREBP1、SREBP2、PPAR γ 之蛋白表現，且有dose-dependent的關係。由此數據變化更加驗證了，桑葉可能透過這些轉錄因子的抑制作用，調控脂肪酸、膽固醇的合成。

六、MLE、MLPE和Statin調控脂質蛋白之表現

從前面的實驗證明MLE和MLPE經由抑制FAS、HMGCR、LDLR等蛋白之表現，而減少脂肪酸、膽固醇合成作用，也確認其上游為AMPK所調控。接下來實驗加入臨床降膽固醇藥—Statin，與MLE、MLPE比較對上述這些蛋白調控之效果。將細胞以OA誘導，分別加入Statin (5 μ M)、MLE (3 mg/ml) 和MLPE (0.3 mg/ml)，利用西方墨點法分析蛋白表現 (Fig 10)。在FAS蛋白表現方面，OA誘導組的變化是1.07倍，Statin、MLE和MLPE各組表現量分別是1.10、0.85、0.80倍，顯示MLPE的抑制效果最好，而Statin沒有變化；HMGCR蛋白表現方面，OA誘導組的變化是1.14倍，Statin、MLE和MLPE各組表現量分別是1.06、0.88、0.90倍，顯示MLE和MLPE的效果差不多，甚至比Statin好；LDLR蛋白表現方面，OA誘導組的變化是0.96倍，Statin、MLE和MLPE各組表現量分別是1.05、0.95、0.94倍，對提升LDLR的效果三種藥都沒有作用。此實驗結果證明MLE和MLPE都具有抑制脂肪酸、膽固醇合成蛋白表現的能力，而Statin藥理機轉乃HMGCR抑制劑，故只能調控HMGCR之蛋白表現量，但由本實驗結果尚可知以5 μ M之Statin處理HepG2細胞及能達到降低膽固醇合成之效果，但因劑量遠低於人體治療濃度故效果不如MLE和MLPE。

七、桑葉萃取物、桑葉多酚萃取物及Statin對細胞脂質代謝基因之表達

利用reverse transcription-PCR分析脂質代謝基因在MLE、MLPE和Statin作用下之影響，欲分析之基因包括：FAS、ACC、HMGCR與LDLR (Fig 11)。與對照組相比，FAS、ACC皆未有顯著變化；唯獨HMGCR在MLPE作用下似有減少之趨勢 (約0.84-0.89倍)。另外，從圖中也發現LDLR在OA誘導組表現量降低。由此實驗結果推測，MLE、MLPE和Statin作用無法反應在基因層次上。

動物實驗：

一、生理狀況與體重變化

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。Fig 12表示各組動物在飼養期間體重變化的情形，結果顯示，各組體重隨週齡逐漸增加





，但彼此之間無明顯差異，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，但不具顯著意義。以第6週為例，HFD組體重變化率為22.01%，而0.5%和1.5% MLE組分別為19.46%、20.78%。

二、 肝臟變化

各組老鼠犧牲後取出肝臟，以生理食鹽水清洗後，瀝除水分秤重並統計重量變化。

Fig 13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。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。結果顯示，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（Fig 14），顯示桑葉可能有助於減少肝臟脂肪堆積。

三、 肝臟三酸甘油酯和總膽固醇含量分析

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 15A）與總膽固醇含量分析結果雷同（Fig 15B, * $p < 0.05$ ），僅1.5% MLE組有減少的趨勢，由體內試驗結果証實MLE確有排除肝臟油脂堆積之效。

四、 血清生化檢查

血清中所含的脂肪，主要是指膽固醇、三酸甘油酯（又稱中性脂肪）、磷脂質以及游離脂肪酸，膽固醇又分為總膽固醇、低密度膽固醇酯和高密度膽固醇酯，最後都到肝臟進行分解代謝，一旦肝臟對脂肪代謝發生異常，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）。

1. 總膽固醇（TCHO）、三酸甘油酯（TG）與游離脂肪酸（FFA）

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。

2. 血漿低密度脂蛋白膽固醇（LDL）、高密度脂蛋白膽固醇（HDL）與LDL/HDL ratio
血中60~70%的膽固醇是由低密度脂蛋白膽固醇攜帶，主要是將膽固醇由肝臟帶到周邊組織，一旦血液中低度脂蛋白膽固醇過多，容易在血管壁形成斑塊導致血管硬化。而高密度脂蛋白膽固醇負責將末梢組織之膽固醇帶回肝臟形成膽鹽（bile salt）





後排除，膽鹽隨膽汁排泄於消化道，可幫助分解食物中脂肪，故有「好的膽固醇」之稱。臨床上認為低度脂蛋白膽固醇超過160 mg/dl，或高密度脂蛋白膽固醇小於35 mg/ml就有罹患冠狀動脈硬化和心臟疾病之危險，因此，低度脂蛋白膽固醇通常被視為引起此類疾病的危險因子(44)。

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 mg/dl, $^*p < 0.05$)，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。

五、 肝臟脂質代謝相關蛋白之表達

1. FAS、HMGR、LDLR、A-FABP之蛋白表現

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 16結果得知，HFD組別明顯提升脂質生成酵素蛋白

(FAS, HMGR, 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。

2. ACC與AMPK蛋白表現分析

Fig 17結果，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，與細胞實驗結果符合。p-ACC在HFD組中表現增加 (1.29倍)，而0.5% MLE及1.5% MLE餵食組卻呈降低趨勢 (0.80倍和0.91倍)，但仍舊可觀察到1.5% MLE餵食組有較0.5% MLE略升。因此直接觀察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 17B, $^*p < 0.05$)。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。

3. 轉錄因子SREBP1、SREBP2、PPAR γ 之表現

細胞實驗結果顯示桑葉調節脂肪代謝的分子機制可能是透過抑制轉錄因子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 18)。

結果討論

本研究室先前對桑葉的研究著重於脂質過氧化以及粥狀動脈硬化的探討，雖然都與脂質的調控有關，但心血管細胞和肝細胞畢竟不同，設計實驗架構與觀察重點蛋白上有很大的落差。從近幾年國內外臨床調查發現脂肪肝在全球人口大約有20%的佔有率，其中並不包括酒精性脂肪肝和肝炎病毒引起的人口。因為肥胖人口的增加，使得脂肪肝罹患率逐年攀升，在十大死因中至少有五項的致病原因就包含脂肪肝，估計脂肪肝將是未來越來越值得重視的公共問題。

為了研究脂肪肝之分子機制，本實驗建立了以油酸 (Oleic acid, OA) 為誘導劑的細胞模式。油酸為C18：1單元不飽和脂肪酸，是血液中含量最多的脂肪酸(48)，也是所有最容易與甘油酯化成三酸甘油酯 (triglyceride) 堆積的脂肪酸(53)。以其誘導肝細胞脂肪堆積之結果非常明顯，但是在油酸稀釋配製上有幾點需要注意，否則將影響誘導的成效：(一) 油酸本身即是油脂，加入細胞培養液之前必須與固定比例的白蛋白 (bovine serum albumin, BSA) 混合均勻，提升油脂溶解度。(二) 油酸事先以絕對酒精配成原液，實驗開始前再依照使用比例稀釋之，因此酒精濃度對實驗設計的影響也必需在考量範圍之內。一般而言，0.1%以下的劑量是可以被接受的範圍。接下來，就本實驗結果與操作過程作討論。

評估肝細胞內脂肪堆積程度

肝細胞本身即擁有合成脂肪之能力，細胞膜雙層磷脂結構亦包含脂肪，如何評估細胞質堆積的是額外攝入的脂肪呢？使用兩種親脂性染劑—油紅染劑 (oil red O) 以及尼羅紅染劑 (nile red) 試驗雙重檢驗脂肪堆積的程度。Fig 2A兩種染色結果有一致增加的趨勢，然而油紅染劑使用上必須新鮮配製，否則常會產生沉澱顆粒，在顯微鏡底下觀察十分顯著，因而影響實驗結果之判斷，所以本論文另外以尼羅紅染色評估細胞內脂質堆積程度。Fig 3和Fig 4針對桑葉萃取物 (MLE) 和桑葉多酚萃取物 (MLPE) 抑制胞內脂質堆積效果所做的試驗，雖然可以見到細胞質內的小油滴散佈，但無法以肉眼區分出兩種桑葉萃取物之間的差異，也無法察覺不同濃度之間的差異。經過文獻搜尋找出一個可行的解決方案：細胞先以尼羅紅螢光染色後，再應用流式細胞儀偵測螢光強度變化之功能，螢光強度越強代表脂肪越多，將胞內脂質量化並進行統計分析，此法可以扣除對照組基本的螢光量，就初步評估脂肪堆積程度上，的確有很大的幫助。

AMPK在肝臟的調控角色

AMPK可以因應細胞狀態決定行脂肪酸氧化作用或合成作用，在脂質代謝路徑中扮演





很重要的角色，而其下游蛋白主要是ACC (acetyl-CoA carboxylase)，FAS (fatty acid synthase) 和HMGCR (HMG-CoA reductase)。本實驗證實桑葉具有抑制脂肪合成蛋白表現的能力，經由Fig 7, 8, 18 *in vitro*與*in vivo*結果雙雙顯示這些蛋白上游調控因子為AMPK；另有研究報導以紅茶多酚 (theaflavins) (49)與葡萄多酚 (resveratrol) (54) 為對象，探討降低肝細胞HepG2脂肪堆積之作用機轉。結果發現，這兩種多酚類化合物皆是經由活化AMPK進而調控下游脂質生成蛋白之表現，並且降低細胞內三酸甘油酯含量，與本實驗結果不謀而合。

ACC (acetyl-CoA carboxylase) 的作用是催化malonyl-CoA合成，malonyl-CoA是脂肪酸合成過程中作為C₂ donor，透過FAS (fatty acid synthase) 連結成長鏈的脂肪酸結構；但malonyl-CoA同時也是促進脂肪酸氧化作用的抑制劑。ACC在體內有兩種不同的形式—ACC1與ACC2，受AMPK磷酸化位置在ACC1主要是Ser80和Ser81；在ACC2主要是Ser219和Ser220，分別調控兩種不同的脂質代謝機制。

ACC1 (acetyl-CoA carboxylase 1) 的活性表現受AMPK磷酸化作用而被抑制，導致脂肪酸合成的原料—malonyl-CoA合成量減少，降低脂肪合成量。因此，ACC1大多出現在促進脂肪生成的組織較多 (lipogenic tissue)，如肝臟、脂肪組織和乳腺。肝臟缺乏ACC1表現的動物 (liver ACC1 knockout mice, LACC1KO)，會表現2倍以上的FAS轉錄活性，以及提升PPAR γ 表現量，但肝細胞中三酸甘油酯含量仍然大量減少 (55)。在本實驗Fig 7, 8結果中可以清楚見到，細胞以OA誘導後對於ACC1表現增加，而加入桑葉萃取物 (MLE) 和桑葉多酚 (MLPE) 處理之後ACC1的表現有遞減的趨勢，p-ACC1則漸增。顯示桑葉可以抑制ACC1活性表現而影響脂肪酸合成作用。

ACC2產生的malonyl-CoA屬於carnitine palmitoyl transferase1 (CPT-1) 內生性抑制劑，而ACC2的活性受AMPK抑制後導致malonyl-CoA減少，因此CPT-1傳遞脂肪酸的功能得以進行，使存在細胞質的游離脂肪酸加速進入粒線體進行氧化作用。所以ACC2主要表現在心臟和肌肉組織的粒線體上，供給能量，少量存在於肝臟。Fig 8結果顯示，細胞加入桑葉多酚萃取物 (MLPE) 之後的對於ACC2表現減少，顯示其能有效促進細胞行脂肪酸氧化作用。但在Fig 7以桑葉萃取物 (MLE) 處理之結果卻相反，推測可能是多酚含有的成分與粗萃取物不同所致。

SREBP 在肝臟的調節角色

承如先前介紹所言，SREBP (sterol regulatory element-binding protein)在體內調節脂肪酸、膽固醇合成相關基因表現，而目前發現的三種SREBP在調節轉錄活性上各有不同的功能。例如：SREBP-1a，SREBP-1c同樣來自SREBP1基因所產生，但SREBP-1c與脂肪酸合成有關；SREBP-1a和SREBP-2則主要調控膽固醇合成(39)。而在本實驗中發現，SREBP1, 2蛋白表現量不管在*in vitro*或*in vivo*的加藥試驗組中都是減少的，因此推測桑葉可能透過轉錄因子抑制FAS、ACC、HMGCR (41-43) 和LDLR (56) 之轉錄活性而減少脂肪酸、膽固醇的合成。有趣的是，LDLR乃身體代謝LDL-C的主要路徑，同時對降低心血管風險因子有極大幫助，但在Table 2結果卻發現血液LDL-C含量並沒有因為LDLR表現降低而減少，這是否顯示LDL可能透過其它特殊途





徑而被代謝，仍需要進行更多的實驗來證明。Metformin，一種治療糖尿病患胰島素抗性的藥物。在胰島素抗性 ob/ob 小鼠試驗中發現，其可藉由抑制SREBP1之表現，改善小鼠脂肪堆積的情況 (57)。同時，Metformin也會刺激AMPK活化，使下游脂生性酵素 (lipogenic enzymes) 失去活性後，抑制脂肪合成作用。將此藥物注射兔子體內誘導AMPK表現，發現也具有降低SREBP-1c mRNA表現的作用，但其相關機制尚未釐清 (58)。

本實驗細胞模式所使用的油酸 (OA) 是以酒精稀釋配製而成的，因此在部分討論蛋白質表現的實驗中 (例如在Fig 5的FAS增加1.17倍)，發現酒精對照組 (Solvent control) 也有些許的影響。此一結果正好呼應脂肪肝成因中包含長期飲酒過量所導致的酒精性脂肪肝 (AFLD)，本實驗雖然僅探討單純肥胖引起之脂肪肝 (NAFLD)，但亦有研究證明酒精會促進SREBP1 (sterol regulatory element-binding protein1)和peroxisome proliferators activate receptor γ (PPAR γ) coactivator α (PGC-1 α)轉錄因子調節脂質生成 (lipogenesis) 的作用。而且酒精可能藉由活化sirtuin 1-AMPK路徑，降低PGC-1 α 或提高SREBP1轉錄活性增加肝臟合成脂肪(59)。

非酒精性脂肪肝的治療

非酒精性脂肪肝在病程發展上十分緩慢，病因眾多，且約80%的患者都不見有絲毫症狀，因此一般皆建議改變生活習慣，例如減少西方飲食、減重及運動為主，藥物控制為輔，才能真正有效控制脂肪肝發生率。針對治療脂肪肝之臨床試驗藥物有：Thiazolidinedions (TZD，一種PPAR γ agonist) 可增加胰島素敏感度，改善血清GOT和肝細胞脂肪堆積 (steatosis) 組織學積分評估，但使用期間病患有體重增加，以及肝損害之副作用(60)，因此長期使用效果尚待評估。至於曾被報導過的抗氧化藥物 (vitamins C, E) 和肝臟庇護劑Ursodeoxycholic acid (or metformin) (61) 雖然投入臨床試驗研究，並取得部份的效果，但尚未有令人信服的大規模隨機臨床試驗資料。第二代的TZD藥物Rosiglitazone、Pioglitazone去除了第一代藥物肝毒性的影響，使用上較安全，但仍有些用藥禁忌存在(60, 62)。Statin的作用機轉為抑制膽固醇合成 (cholesterol synthetase)，增加肝臟LDL接受體，使肝臟提升對non-HDL lipoprotein的再吸收，減少血中LDL、VLDL、IDL(45)。而LDLR存在於肝細胞表面，亦可協助清除血漿過多LDL、VLDL脂蛋白含量，但在本實驗結果Fig5, 6, 17卻見到LDLR無論處理MLE或MLPE皆呈現減少之趨勢，然而血漿LDL並無升高之情形，此現象可能與LDL進入細胞的方式有關(63)。其中一類Statin藥物—simvastatin，用於有心臟病、中風、糖尿病或周邊血管阻塞病史的患者，可降低心臟血管疾病25~30%的發生率 ($p < 0.00001$) (64)。這些藥物同時也適用於治療代謝症候群中最為重要的典型症狀—胰島素抗性，因此，早期篩選代謝性症候群，早期提供治療更可以有效預防脂肪肝的發生。





六、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化物質及多酚成份，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，發現荷葉萃取物具有降血脂、抑制心血管疾病之功效(65-67)，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，且無肝腎毒性之不良副作用，對於減緩脂肪肝的發生確有不錯之開發潛力，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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實驗圖表與說明

Table 1 Composition of MLE (mulberry leaf extract)

MLE	%
Polyphenol	
Gallic acid	10.20 ± 0.05
Quercetin	6.74 ± 0.09
Carbohydrate	25.43 ± 3.56
Protein	2.43 ± 0.26
Lipid	5.44 ± 1.83
Fiber	42.64 ± 12.87

Gallic acid and quercetin are the standards of polyphenol content assay. The MLE was prepared as described in Materials and Methods.





Table 2 Clinical 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	28.55±12.94	24.12±6.38
GPT (U/L)	10.33±1.12	60.66±12.45	14.33±7.45	13.50±4.00
TCHO (mg/dL)	71.66±7.43	157.33±38.58*	101.11±9.75 [#]	81.37±8.11 [§]
TG (mg/dL)	29.00±5.61	125.77±34.95*	42.11±8.84 [#]	32.75±5.75 [§]
HDL (mg/dL)	16.66±2.35	68.88±20.28*	33.11±3.06 [#]	33.75±4.30 [§]
LDL (mg/dL)	45.88±4.04	79.33±12.7*	68.66±7.76 [#]	43.25±4.50 [§]
LDL/HDL-C	2.80±0.47	1.21±0.27*	2.07±0.19 [#]	1.30±0.22 [§]
Free fatty acid (nmol)	6.42±0.51	6.89±0.44*	6.52±0.17 [#]	5.57±0.92 [§]

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 \pm SD. C, control; HFD, high fat diet; GOT, Glutamic-oxaloacetic transaminase; GPT, Glutamic-pyruvic transaminase; TCHO, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

* $p < 0.05$ for HFD group versus Control

[#] $p < 0.05$ for 0.5%MLE-treated group versus HFD

[§] $p < 0.001$ for 1.5%MLE-treated group versus HFD



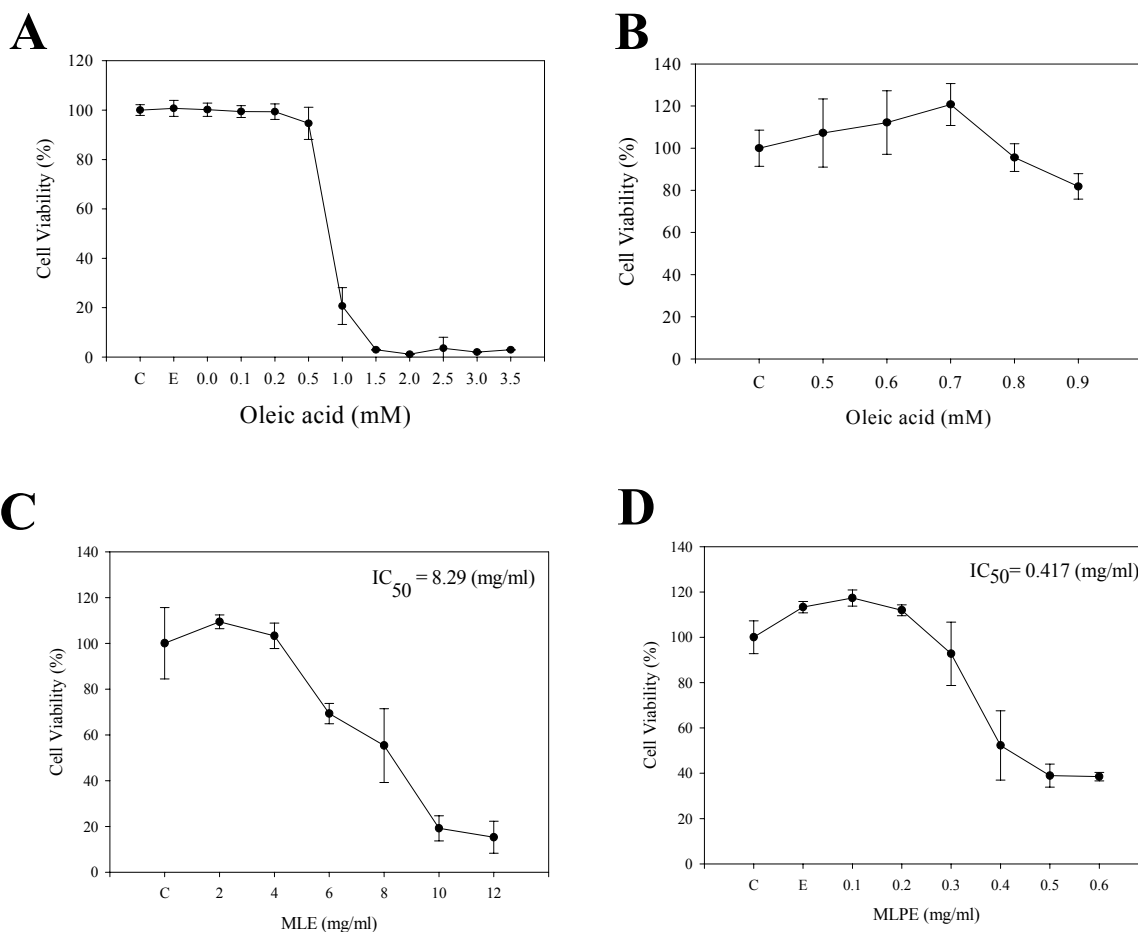
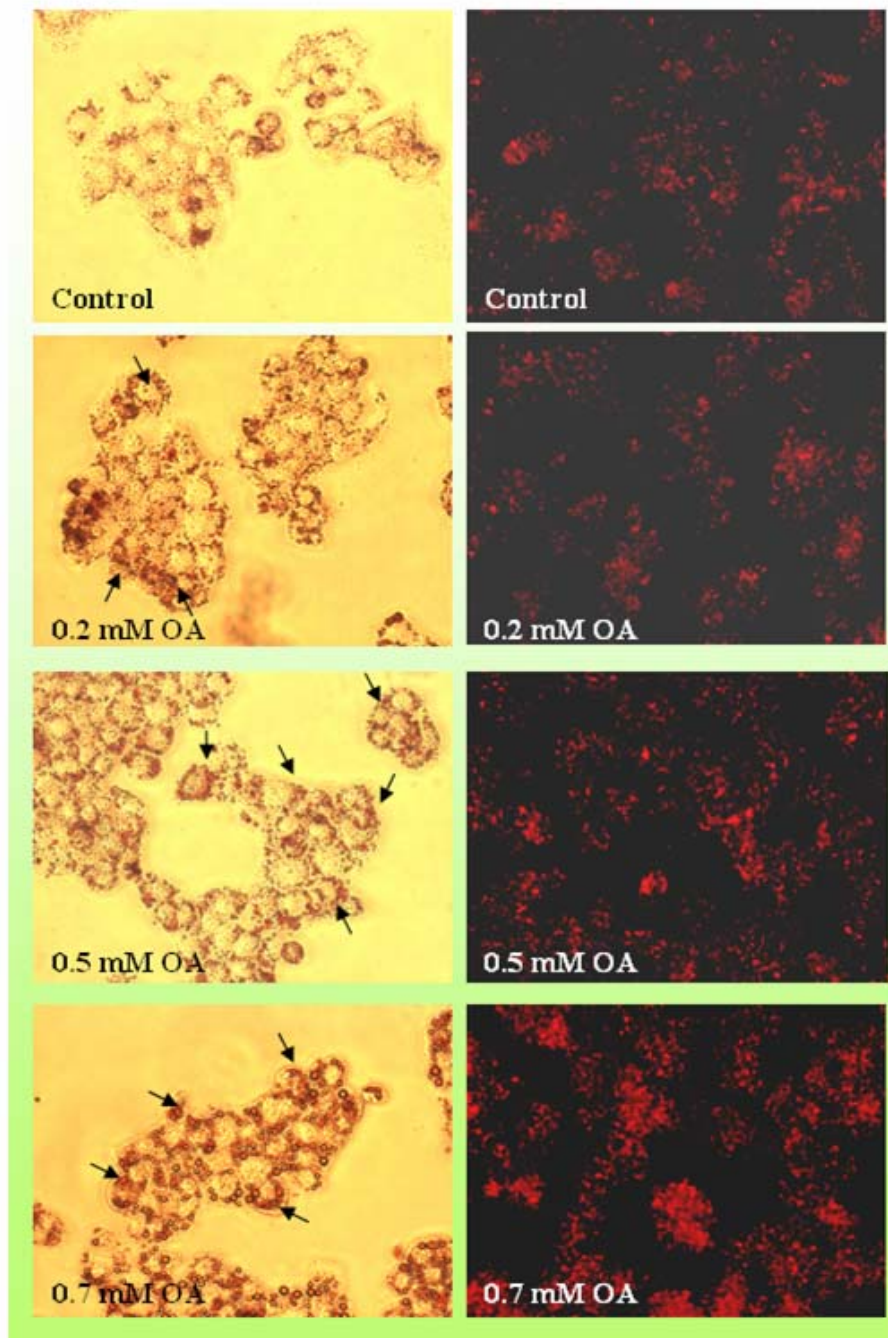


Figure 1 Cell viability of HepG2 cells were treatment with oleic acid, MLE and MLPE. (A-B) HepG2 cells were treated with Oleic acid under different concentration (0.1-3.5 mM) for 16 hours and analyzed by MTT assay. The data were mean \pm SD from 4 samples for each group. (C-D) Cultured HepG2 cells were treated with various concentrations of MLE and MLPE for 24 hours and analyzed by MTT assay. The data were mean \pm SD from 4 samples for each group. C, control; E, ethanol.





A





B

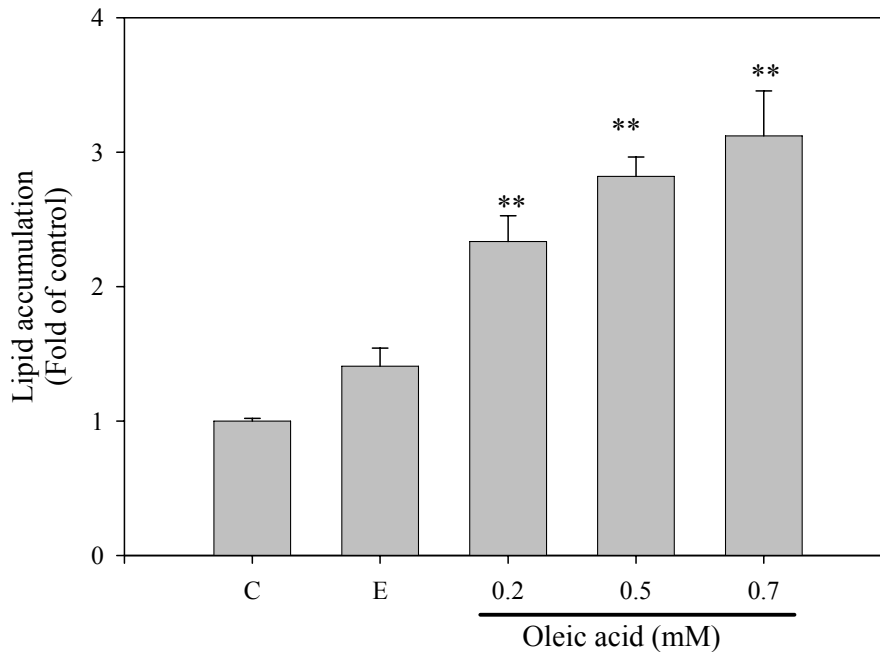
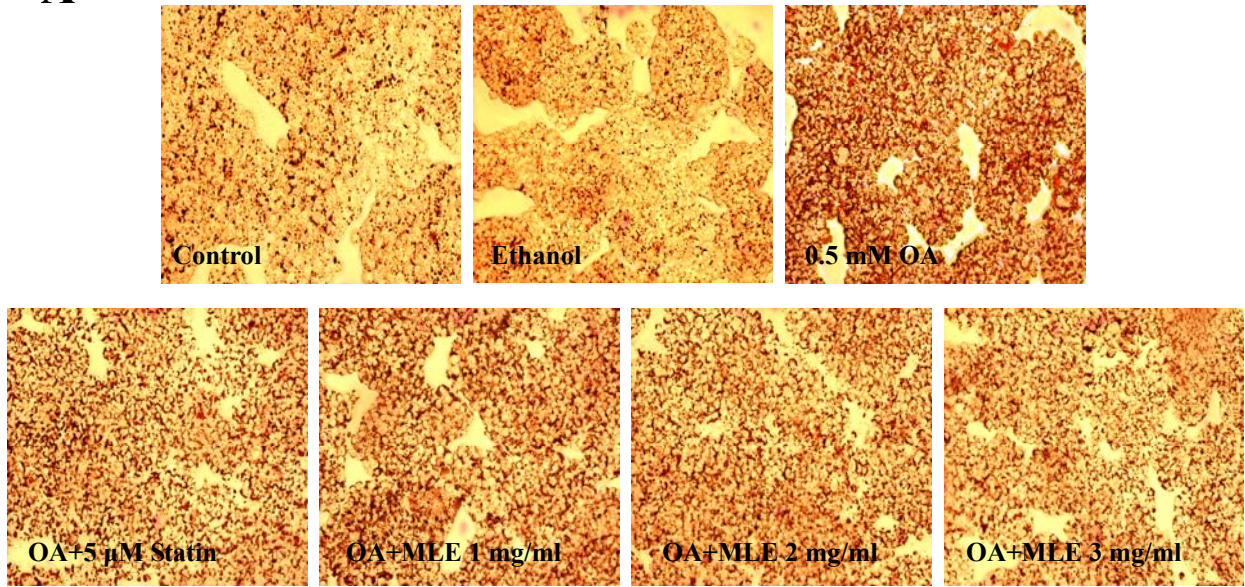


Figure 2 Oleic acid (OA) induced lipid accumulation in HepG2 cells. (A) Cultured cells were induced lipid accumulation with oleic acid (OA). To measure cellular neutral lipid accumulation by using Oil red O staining (left) and Nile red staining. (right) The white arrow indicated lipid droplet. HepG2 cells were incubated at 2.4×10^6 cells/well. A representative fluorescent photomicrographs are shown ($\times 200$). (B) Quantification of intracellular fat content with flow cytometric analysis. The data were mean \pm SD from 3 samples for each group. ** $p < 0.001$ in relation to control group. C, control; E, ethanol

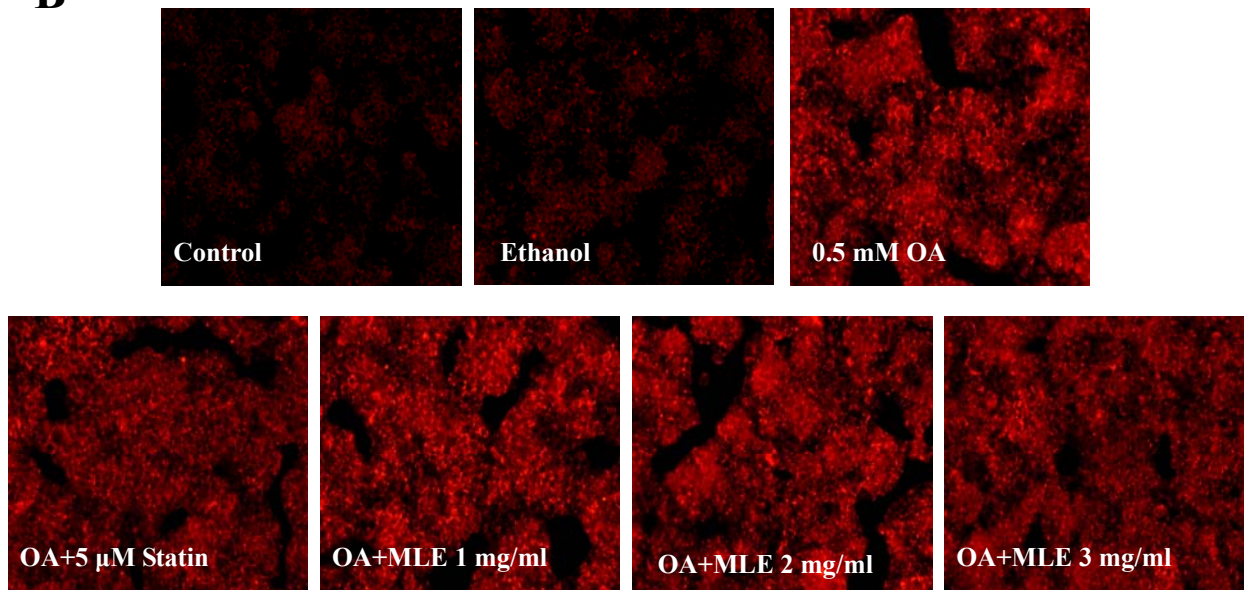




A



B





C

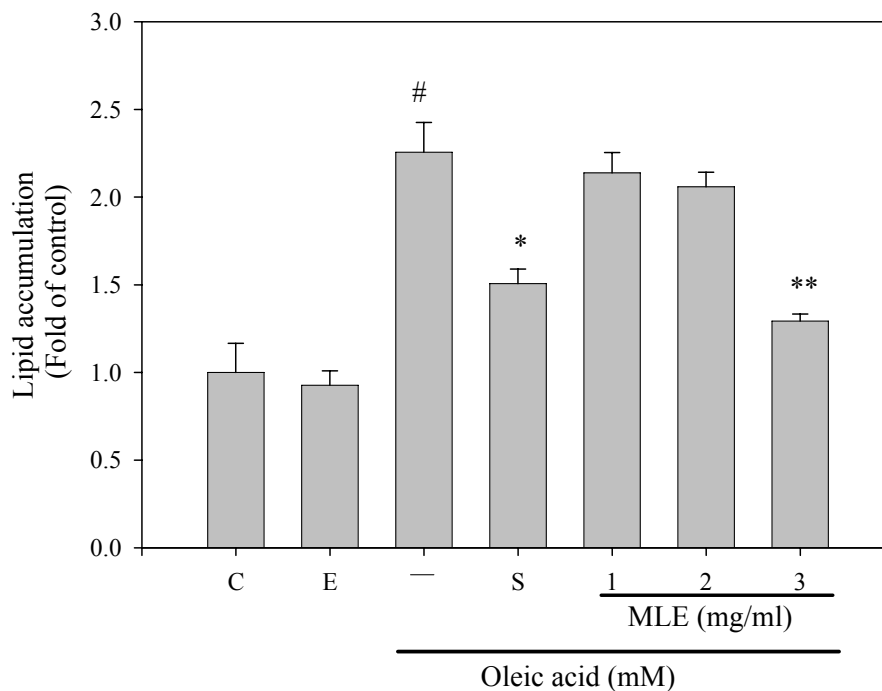


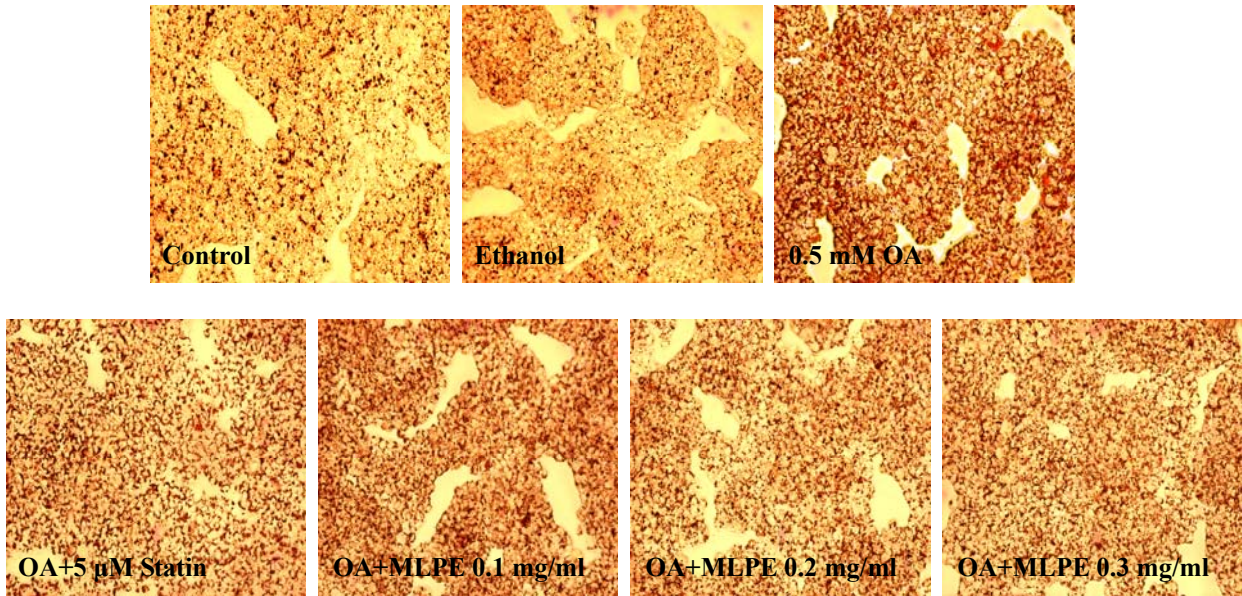
Figure 3 Inhibition of OA-induced lipid accumulation by MLE in HepG2 cells.

(A-B) Cultured cells were induce lipid accumulation with oleic acid (OA) for 16 hours and treated with MLE (1-3 mg/ml) or statin (5 μ M) for 24 hours. To measure cellular neutral lipid accumulation by using Oil red O statining and Nile red staining. HepG2 cells were incubated at 2.4×10^6 cells/well. A representative fluorescent photomicrographs are shown ($\times 100$). (C) Quantification of intracellular fat content with flow cytometric analysis. The data were mean \pm SD from 3 samples for each group. # $p < 0.05$ in relation to control group. * $p < 0.05$ in relation to OA-induced group. ** $p < 0.001$ in relation to oleic acid-induced group. C, control; E, ethanol; S, statin

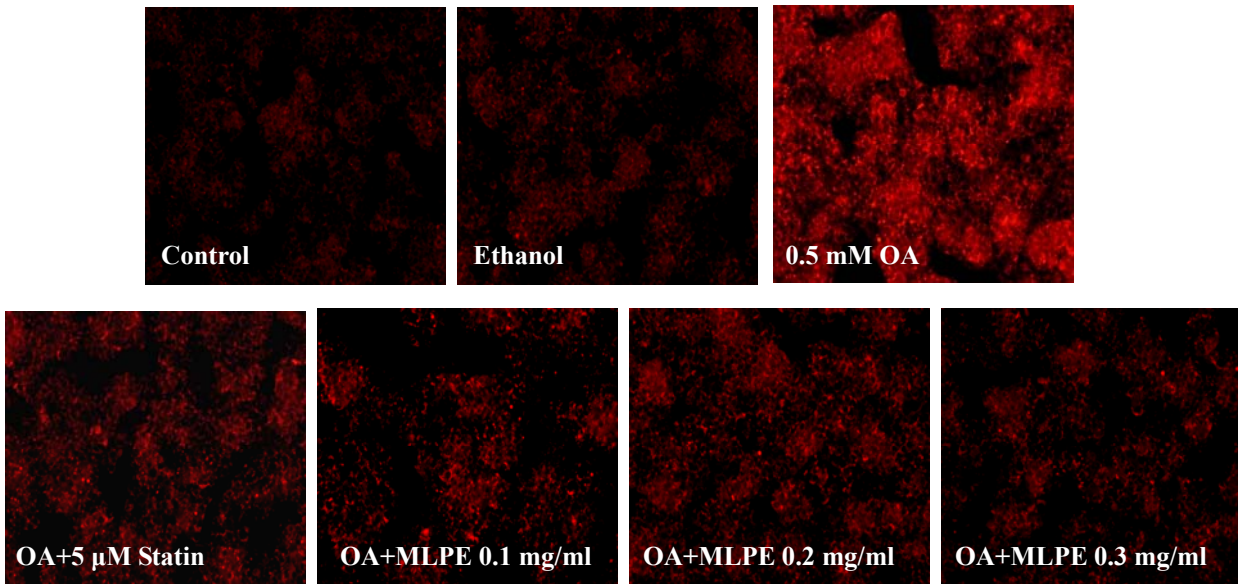




A



B





C

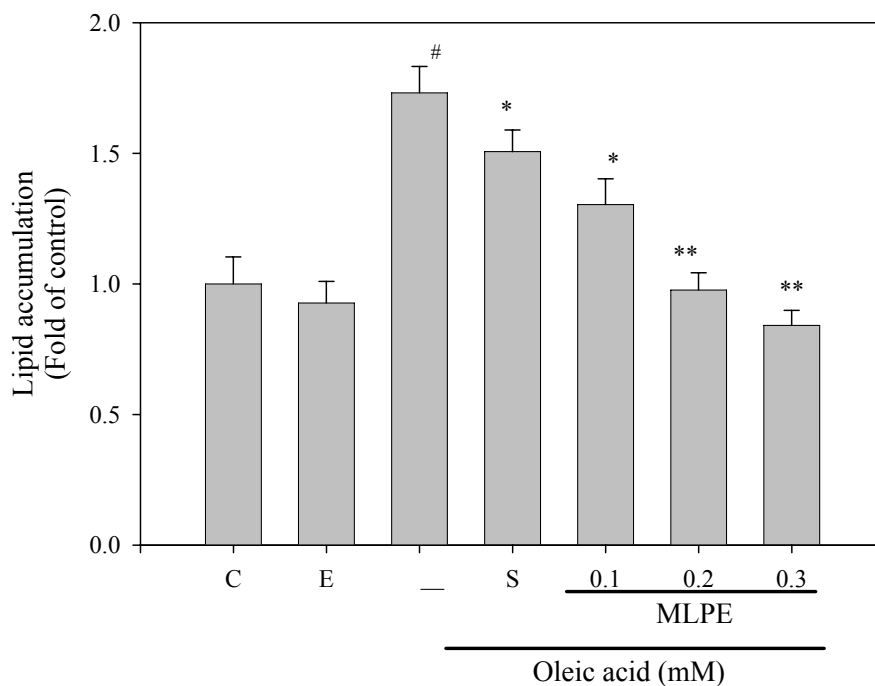


Figure 4 Inhibition of OA-induced lipid accumulation by MLPE in HepG2 cells.

(A-B) Cultured cells were induce lipid accumulation with oleic acid (OA) for 16 hours and treated with MLPE (0.1-0.3 mg/ml) or statin (5 μ M) for 24 hours. To measure cellular neutral lipid accumulation by using Oil red O statining and Nile red staining. HepG2 cells were incubated at 2.4×10^6 cells/well. A representative fluorescent photomicrographs are shown ($\times 100$). (C) Quantification of intracellular fat content with flow cytometric analysis. The data were mean \pm SD from 3 samples for each group. # $p < 0.05$ in relation to control. * $p < 0.05$ in relation to OA-induced group. ** $p < 0.001$ in relation to oleic acid-induced group. C, control; E, ethanol; S, statin



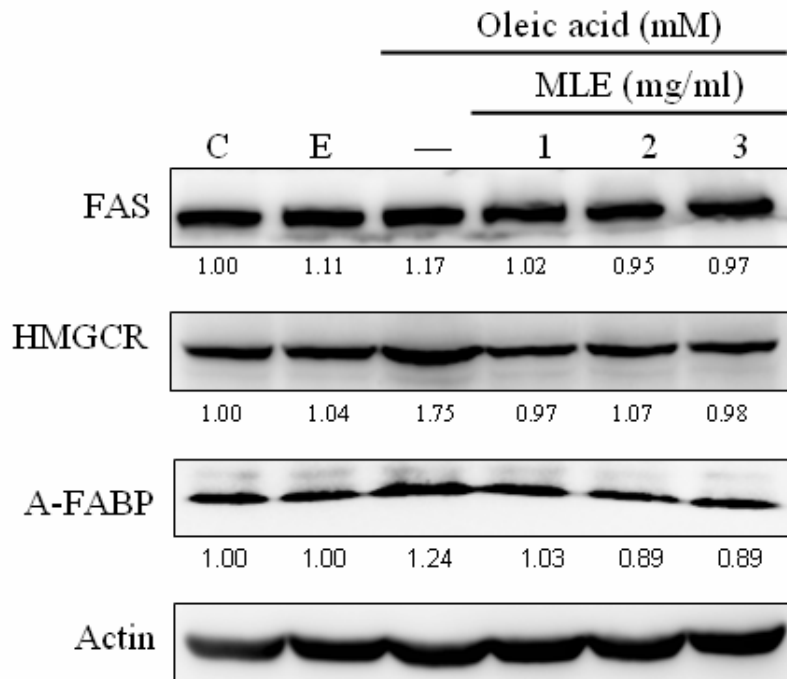


Figure 5 MLE can influence the expression of several enzymes involved in lipid metabolism. Cultured cells were induced lipid accumulation with oleic acid (OA) for 24 hours and treated with MLE (1, 2, 3 mg/ml). Protein expressions were detected by Western blot analysis. BSA was used as a control. C, control; E, ethanol



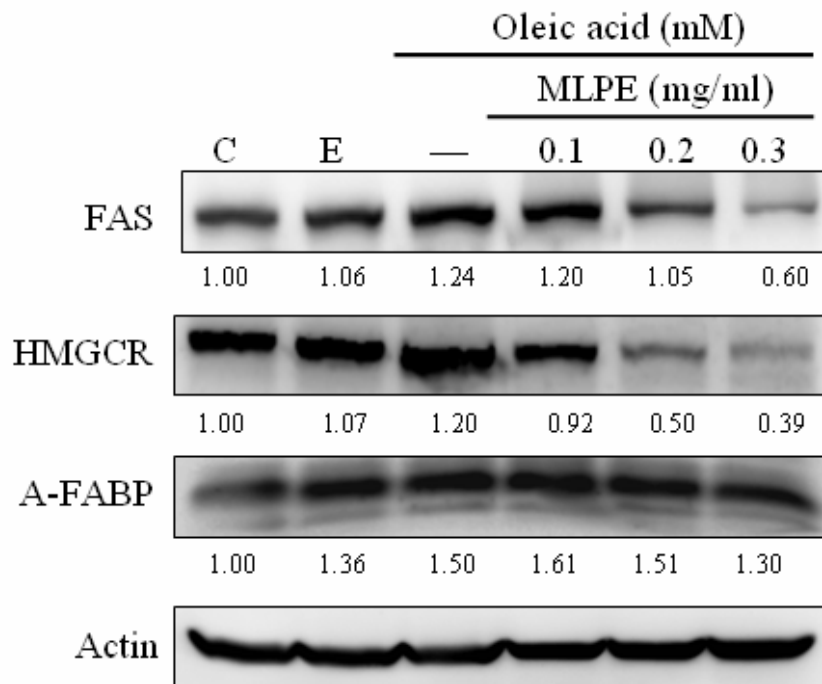


Figure 6 MLPE can influence the expression of several enzymes involved in lipid metabolism. Cultured cells were induced lipid accumulation with oleic acid (OA) for 24 hours and treated with MLPE (0.1, 0.2, 0.3 mg/ml). Protein expressions were detected by Western blot analysis. BSA was used as a control. C, control; E, ethanol



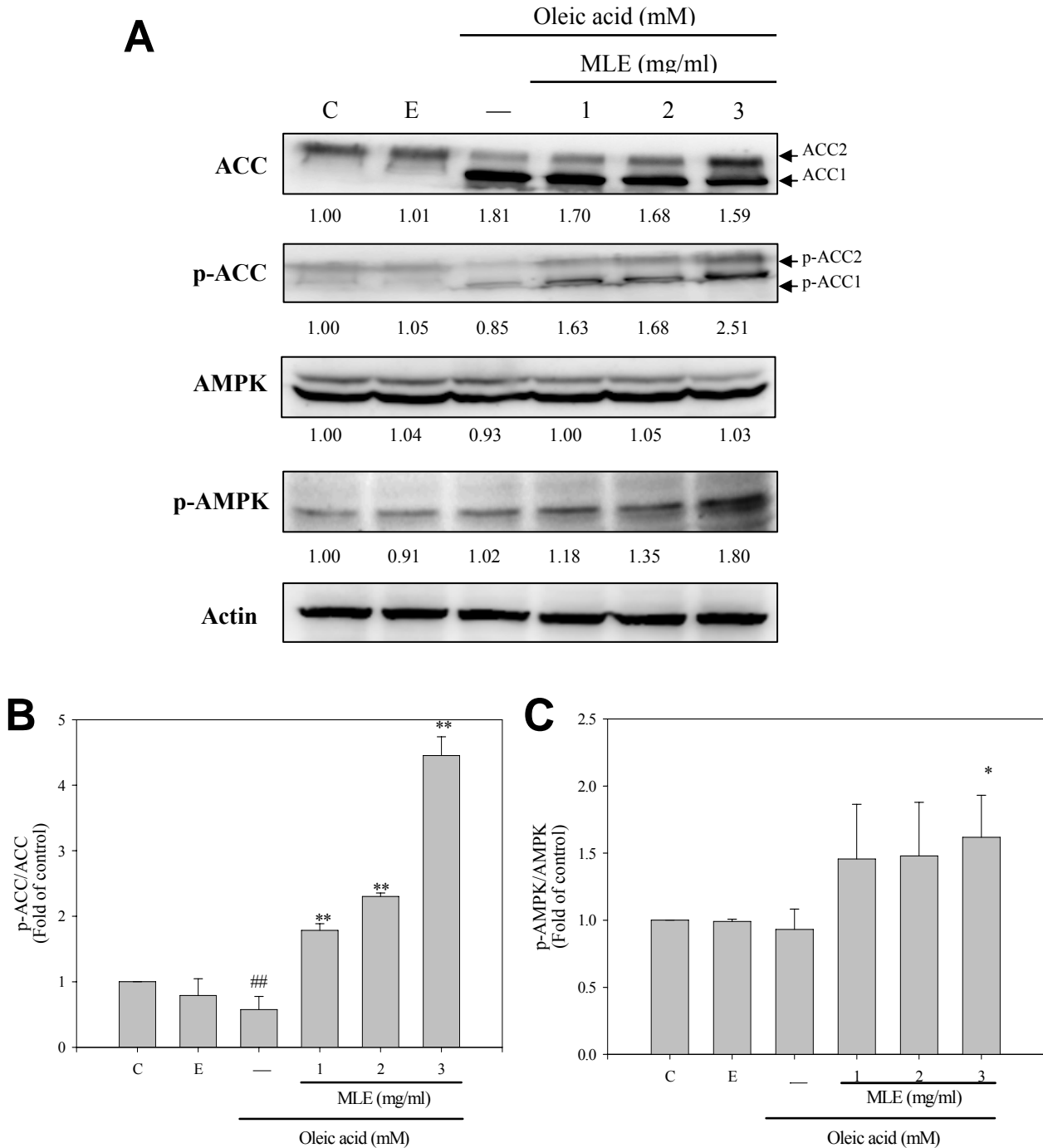
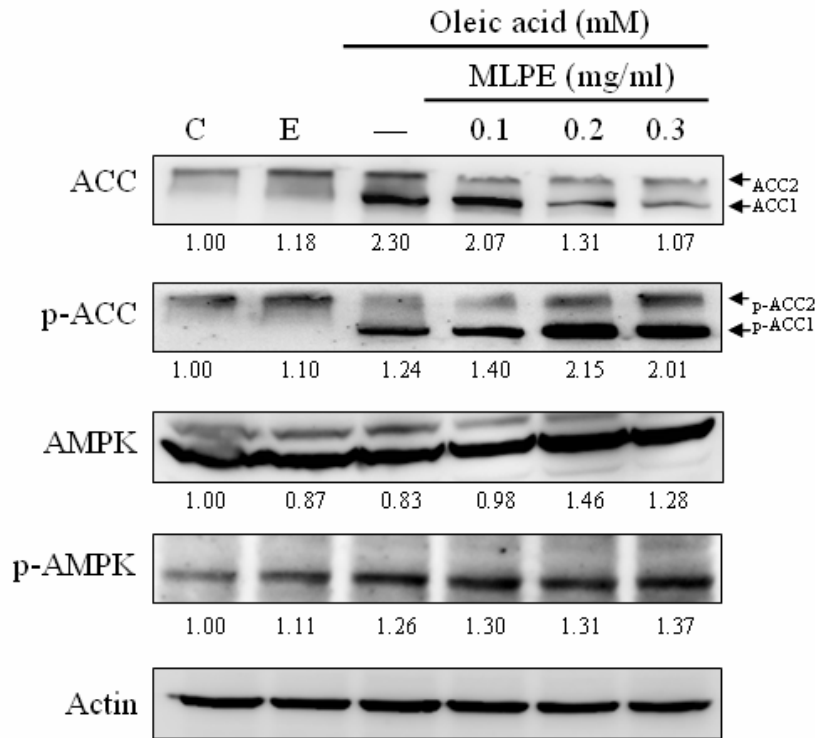
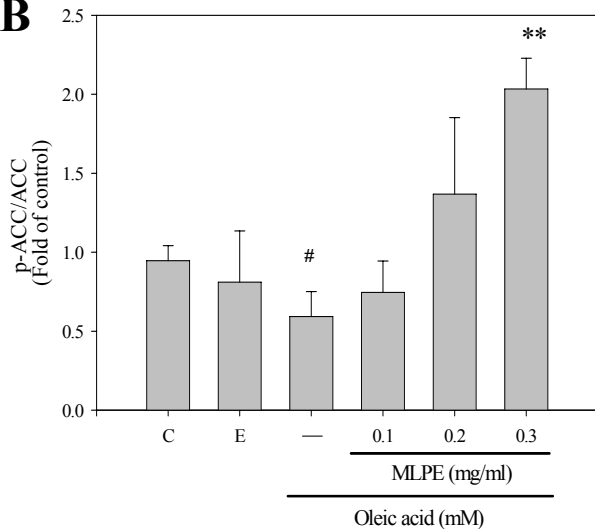
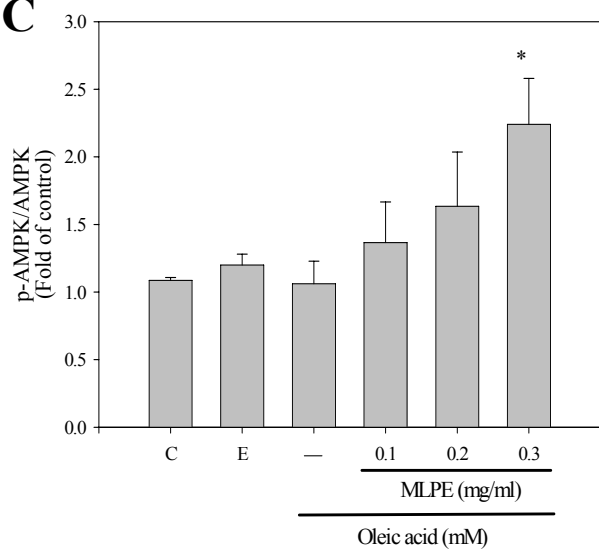


Figure 7 MLE stimulated AMPK activity in OA-induced HepG2 cells. (A) Cultured cells were induced lipid accumulation with oleic acid (OA) for 24 hours and treated with MLE (1, 2, 3 mg/ml). BSA was used as a control. AMPK phosphorylation and its substrate ACC phosphorylation were detected by Western blot analysis. (B-C) Quantification of p-ACC/ACC and p-AMPK/AMPK were depicted means \pm SD from 3 samples for each group. $^{\#\#}$ $p < 0.001$ in relation to control. * $p < 0.05$ in relation to OA-induced group. ** $p < 0.001$ in relation to OA-induced group. C, control; E, ethanol.



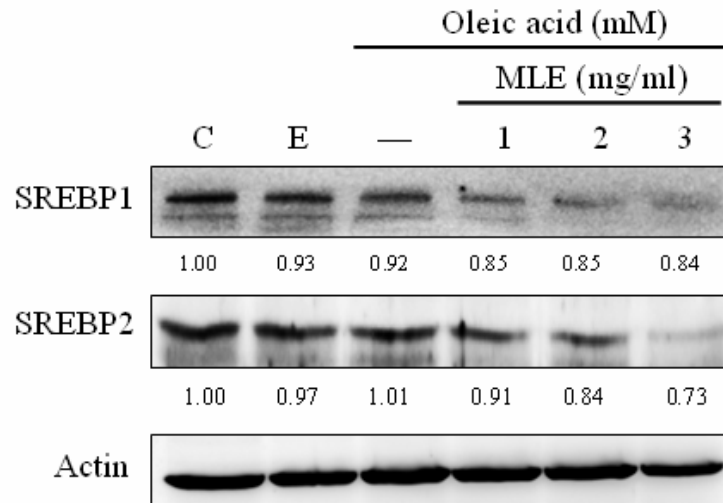
**A****B****C****Figure 8 MLPE stimulated AMPK activity in OA-induced HepG2 cells.** (A)

Cultured cells were induce lipid accumulation with oleic acid (OA) for 24 hours and treated with MLPE (0.1, 0.2, 0.3 mg/m). BSA was used as a control. AMPK phosphorylation and its substrate ACC phosphorylation were detected by Western blot analysis. (B-C) Quantification of p-ACC/ACC and p-AMPK/AMPK were depicted means \pm SD from 3 samples for each group[#] p < 0.05 in relation to control. * p < 0.05 in relation to OA-induced group. ** p < 0.001 in relation to OA-induced group. C, control; E, ethanol.





A



B

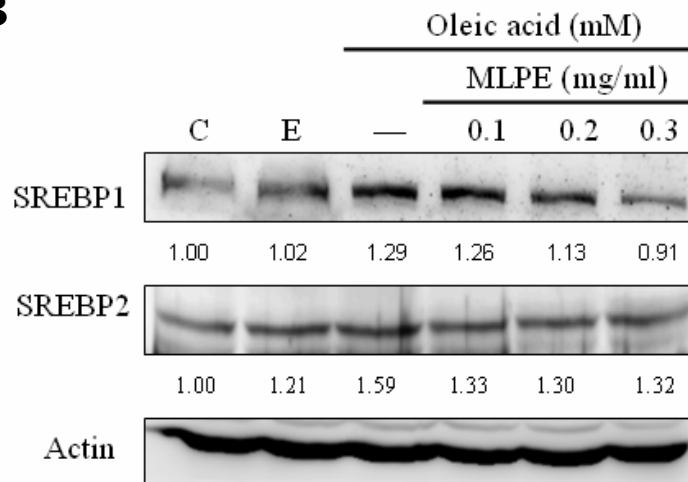


Figure 9 MLE and MLPE can influence the expression of several transcriptional factors involved in lipid metabolism. (A) Cultured cells were induced lipid accumulation with oleic acid (OA) for 24 hours and treated with MLE (1, 2, 3 mg/ml) or (B) MLPE (0.1, 0.2, 0.3 mg/ml). Protein expressions were detected by Western blot analysis. BSA was used as a control. C, control; E, ethanol



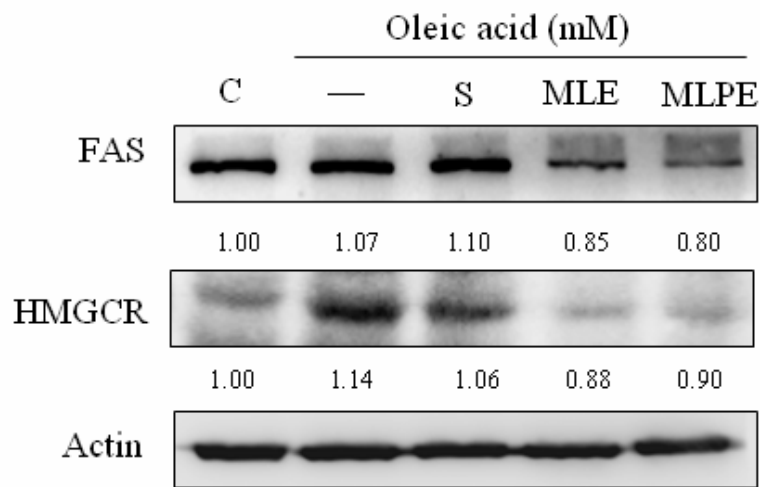


Figure 10 Comparative MLE, MLPE and Statin for lipogenic enzymes expression. Cultured cells were induced lipid accumulation with oleic acid (OA) for 24 hours and treated with MLE (3 mg/ml), MLPE (0.3 mg/ml) and statin (5 μ M). Protein expressions were detected by Western blot analysis. BSA was used as a control. C, control; E, ethanol; S, statin



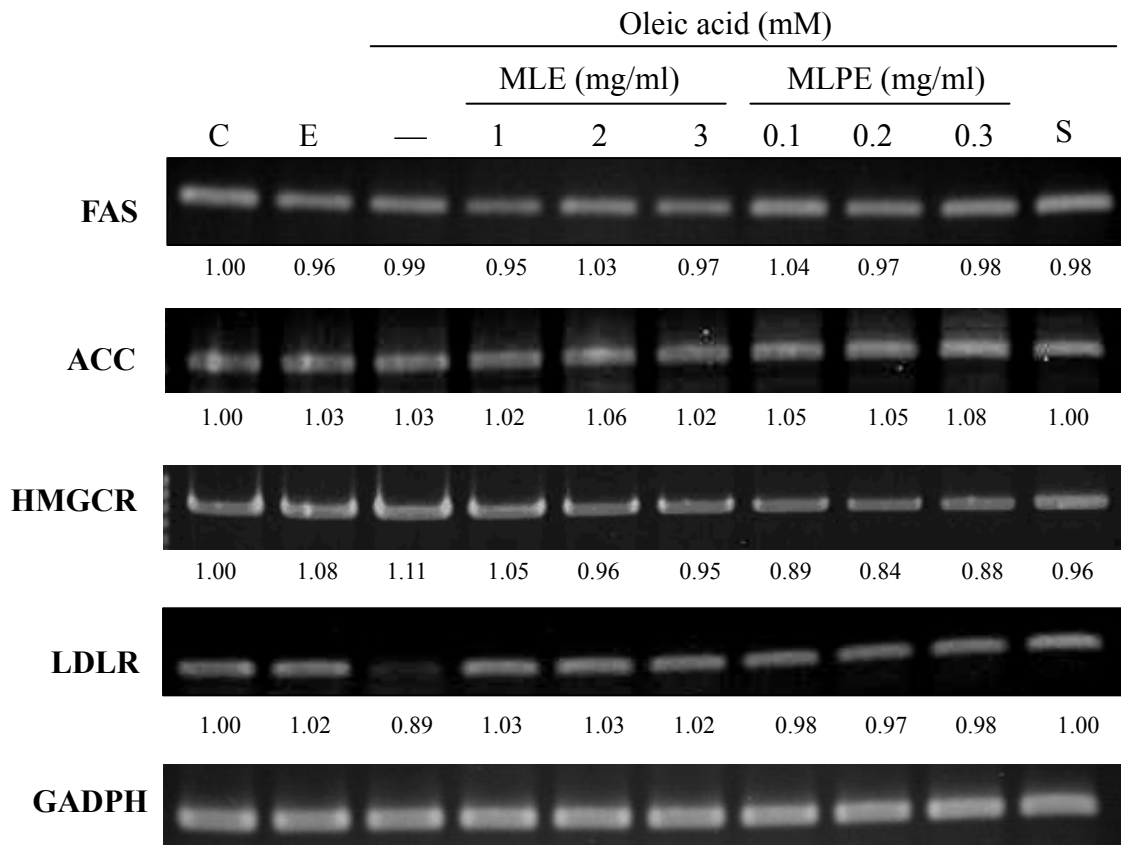


Figure 11 Effect of MLE, MLPE and Statin on mRNA level in lipid metabolism.

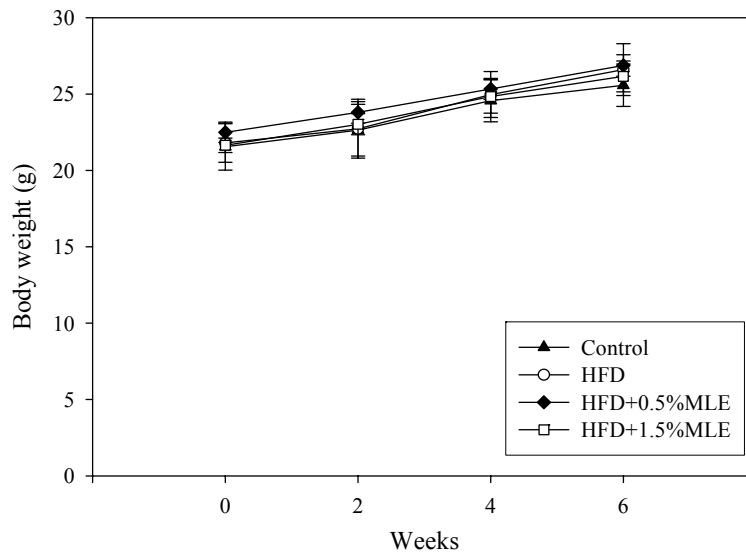
Cultured cells were induce lipid accumulation with oleic acid (OA) and treated with MLE (1, 2, 3 mg/ml), MLPE (0.1, 0.2, 0.3 mg/ml) or statin (5 μ M) for 24 hours.

Cellular mRNA expression levels were measured by RT-PCR. GADPH as a internal control. C, control; E, ethanol; S, statin





A



B

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	24.96±1.5	26.60±1.7
		(8.87%)	(14.52%)	(22.01%)
MLE 0.5%	22.50±0.66	23.79±0.71	25.33±0.69	26.87±0.71
		(5.75%)	(12.58%)	(19.46%)
MLE 1.5%	21.65±0.47	23.02±0.69	24.83±1.09	26.15±1.01
		(6.32%)	(14.68%)	(20.78%)

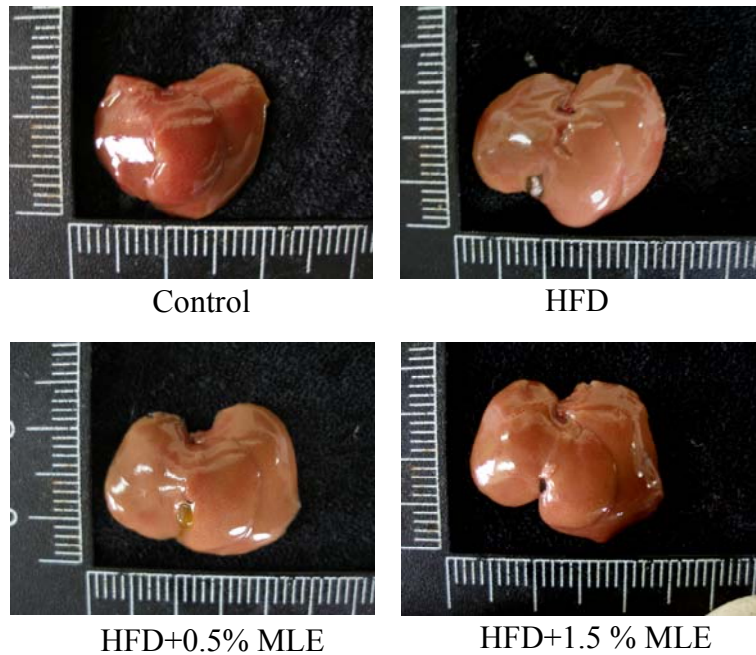
() = ((week X - week 0) / week 0) × 100 %

Figure 12 Body weight change of C57BL/6 mice fed basal laboratory diet or high fat diet during the 6 weeks feeding period. (A) Body weight curve of four group C57BL/6 mice during 6 weeks. (B) All values are expressed as the mean±S.D and body weight change (%) every two weeks. HFD group were maintained on a high-fat diet containing 20% lard oil and 2% cholesterol for 6 weeks (n=20). 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.





A



B

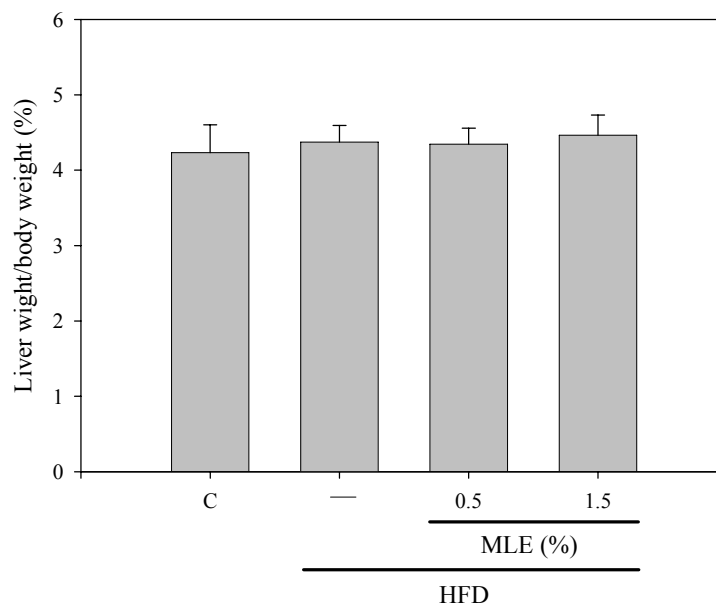


Figure 13 The ratio of liver weight/body weight in C57BL/6 mice. HFD group were maintained on a high-fat diet containing 20% lard oil and 2% cholesterol for 6 weeks (n=20). C, normal group; HFD, high fat diet without MLE powder; MLE-0.5%, C57BL/6 mice fed high fat diet with 0.5% MLE powder; MLE-1.5%, C57BL/6 mice fed high fat diet with 1.5% MLE powder.



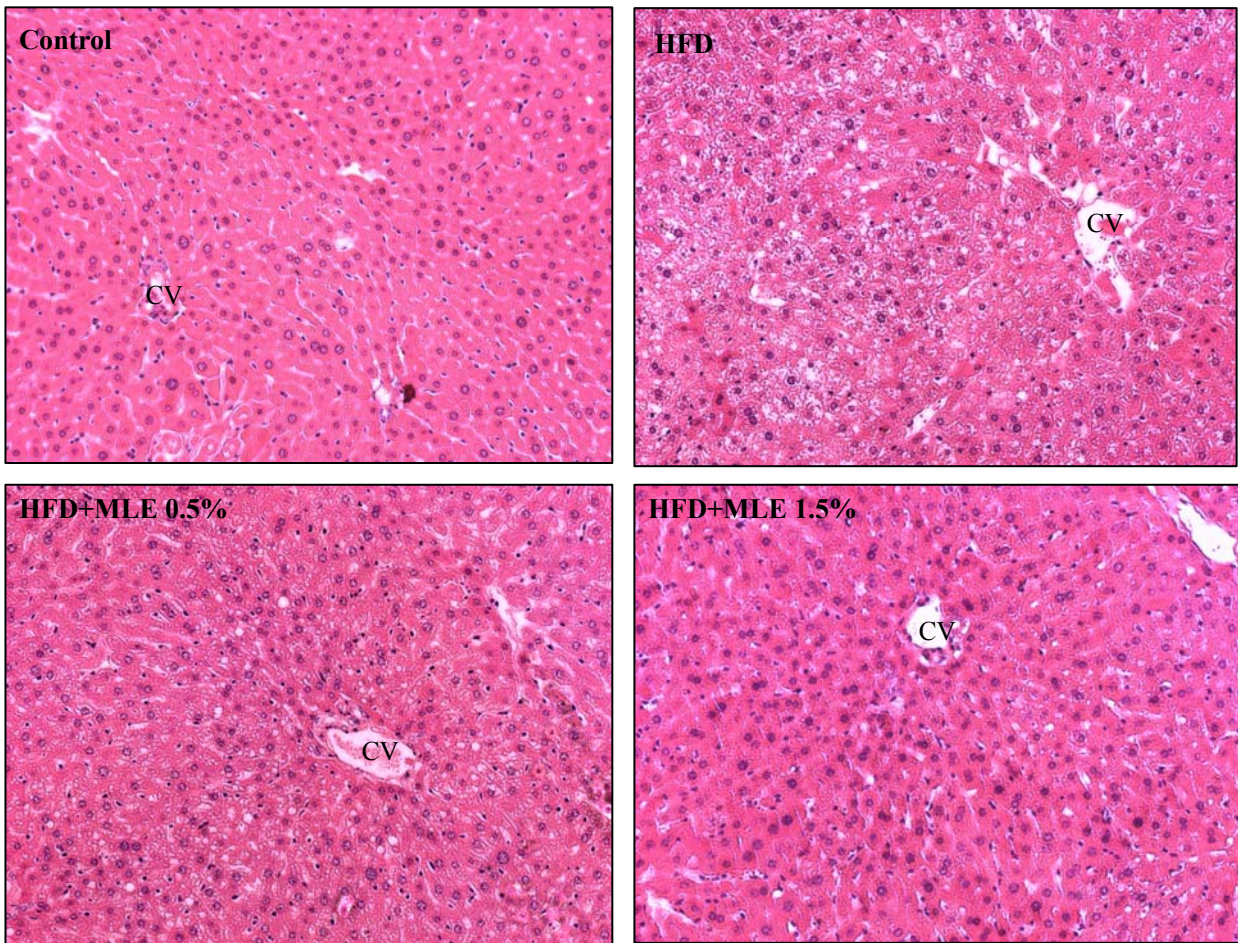


Figure 14 Histologic features of lipid accumulation in livers of C57BL/6 mice.

HFD group were maintained on a high-fat diet containing 20% lard oil and 2% cholesterol for 6 weeks (n=20). 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. ($\times 100X$). CV, central vein.



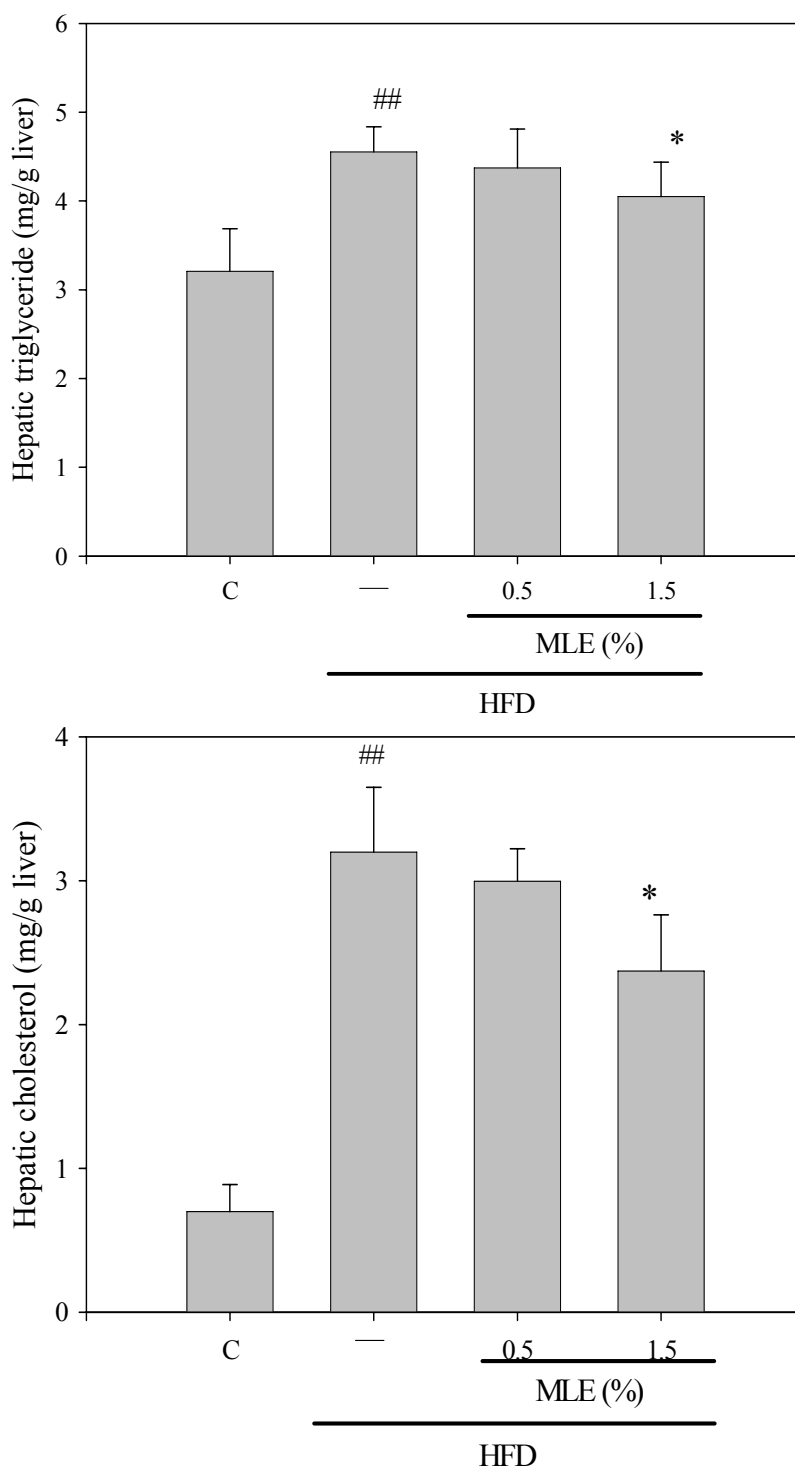


Figure 15 Quantitative determination revealed that hepatic triglyceride and cholesterol content in C57BL/6 mice. Relative amounts of liver triglyceride (A) and cholesterol (B) contents in C57BL/6 mice (n=20). Lipids were extracted from liver tissue obtained from four group mice and analyzed by spectrometry. ^{##}p < 0.001 in relation to control group. ^{*}p < 0.05 in relation to HFD group. 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.



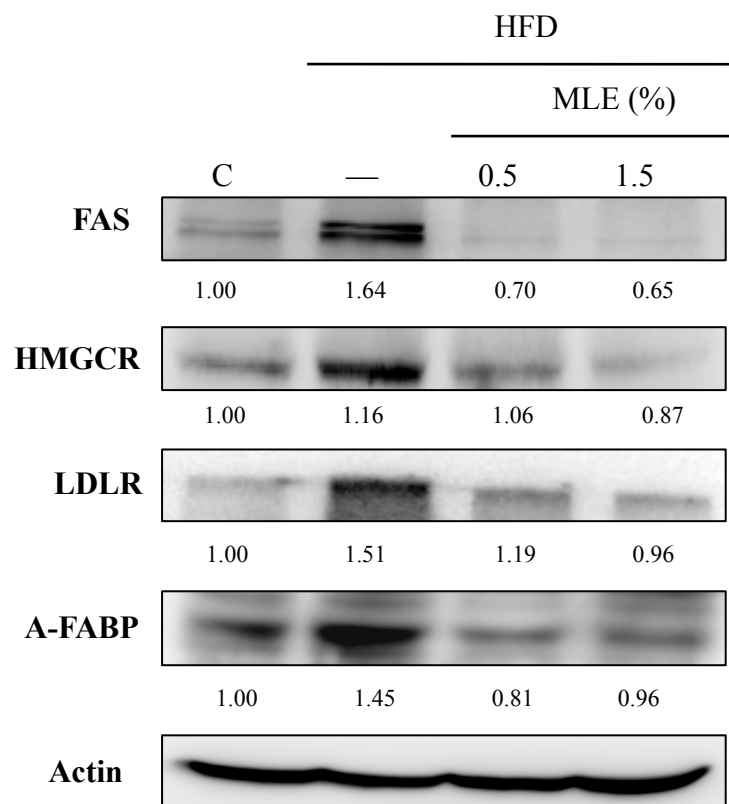


Figure 16 Hepatic expressions of several lipogenic enzymes in C57BL/6 mice.

HFD group were maintained on a high-fat diet containing 20% lard oil and 2% cholesterol for 6 weeks (n=20). 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.



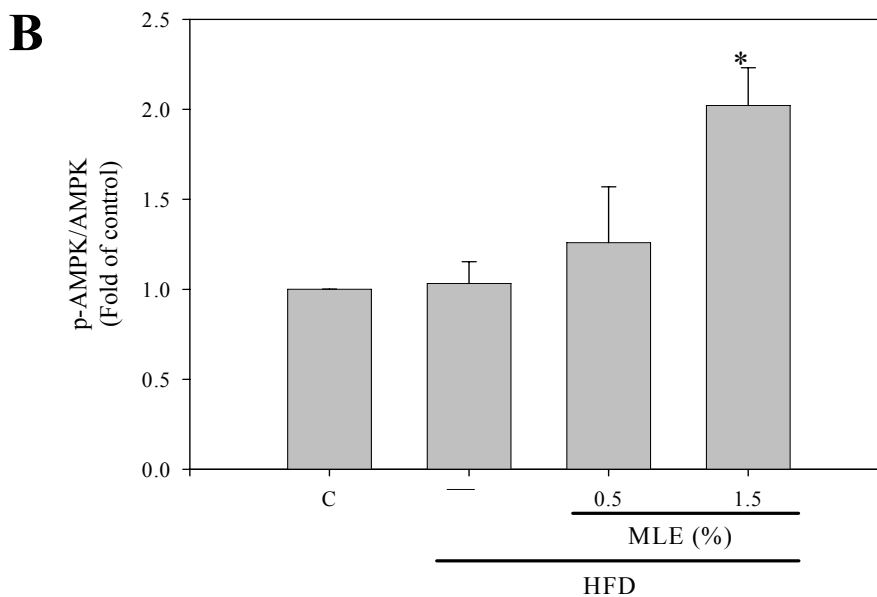
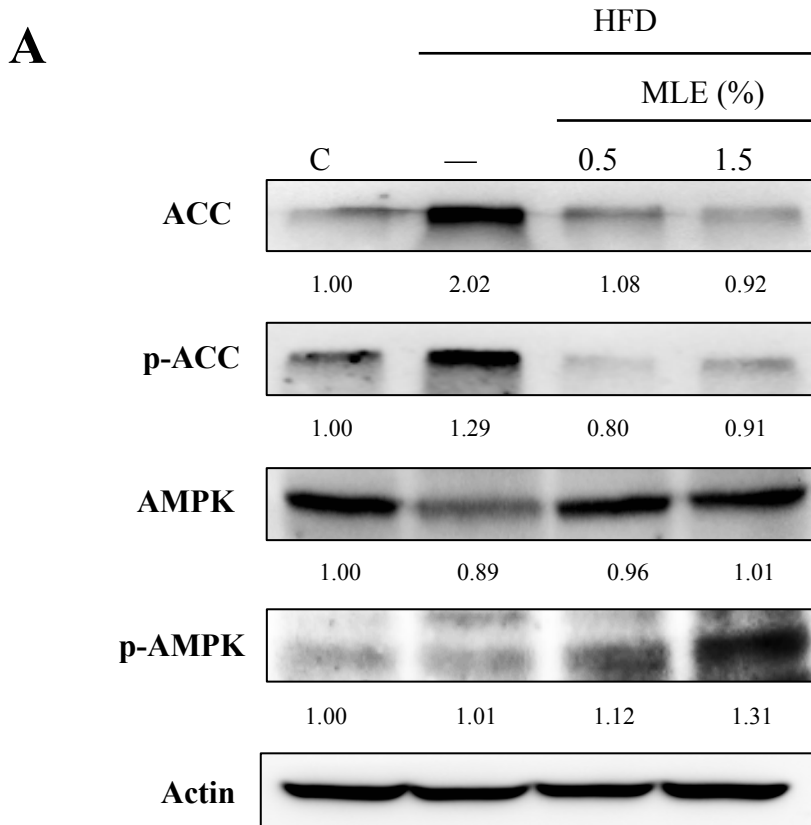


Figure 17 MLE treated increase AMP-activated protein kinase (AMPK) and acetyl-coA carboxylase (ACC) phosphorylation in C57BL/6 mice. (A) AMPK phosphorylation and its substrate ACC phosphorylation were detected by Western blot analysis. (B) All values are expressed as the mean \pm S.D. * $p < 0.05$ in relation to high fat diet group. C, control; HFD, high fat diet group



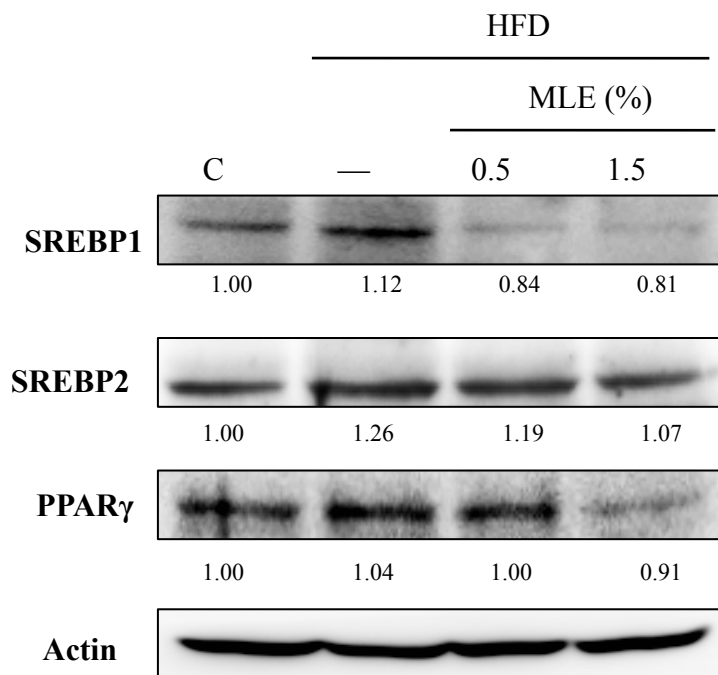


Figure 18 MLE suppressed expression of several lipogenic transcriptional factors in C57BL/6 mice. HFD group were maintained on a high-fat diet containing 20% lard oil and 2% cholesterol for 6 weeks (n=20). Protein expressions were detected by Western blot analysis. C, normal group; HFD, high fat diet 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.





Mulberry leaf extracts and polyphenols

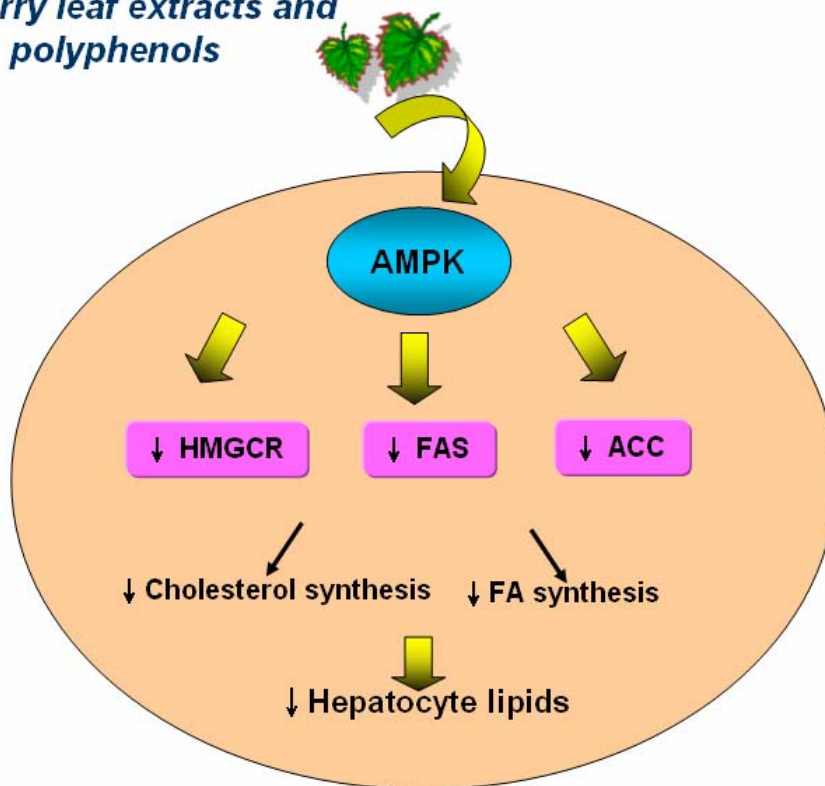


Figure 19 Proposed scheme for the role of mulberry leaf extracts and polyphenols in hepatocyte lipid metabolism.

