行政院國家科學委員會專題研究計畫 成果報告

扛香藤、續隨子與台灣欒木萃取物抑制 LDL 氧化及保護心血管疾病之功能與機制探討

研究成果報告(精簡版)

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行政院國家科學委員會補助專題研究計畫 ■成果報告
□期中進度報告

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中英文摘要

腦血管與心臟疾病在近幾年來則是分居台灣地區十大死因之第二及第三名，僅次於惡性腫瘤之後。動脈粥狀硬化(atherogenesis)引發的病理機制非常複雜，其形成的成因主要成因有: 氧化型低密度脂蛋白(oxLDL)經由產生自由基而刺激細胞內訊息傳遞分子造成(1.)血管內皮功能失調;(2.)皮滑肌細胞之增生與位移;(3.)並造成內皮細胞表面表現異常的黏著分子，例如: ICAM-1、VCAM-1，增加與單核球間之黏著作用;(4.)發炎反應; (5) 以致最終導致內皮細胞與 macrophage 的傷害甚至凋亡。由此可知心血管疾病的預防與治療，是一個急待解決與重視的課題。由實驗結果證實，扛香藤、續隨子與台灣欒木萃取物能十分有效地抑制 LDL 氧化，利用 relative electrophoretic mobility (REM) shift assays、electrophoresis of ApoB fragmentation、Diene conjugation assay、自由基清除率、證實扛香藤、續隨子與台灣欒木萃取物可有效抑制硫酸銅所誘導 LDL 的氧化。在氧化型低密度脂蛋白對人類血管內皮細胞所造成的傷害實驗中，台灣欒木萃取物與續隨子萃取物同時也可抑制氧化型低密度脂蛋白誘發人類臍靜脈內皮細胞的損傷，主要是透過抑制氧化型低密度脂蛋白所破壞粒腺體膜電位 (mitochondrial membrane potential) 及抑制細胞凋亡過程中所釋放出的 cytochrome C 和下游基因 caspase 3 之活化，此外，台灣欒木萃取物與續隨子萃取物同時也可降低氧化型低密度脂蛋白誘發人類臍靜脈內皮細胞的細胞內導致 ROS 的產生。綜合以上結果，台灣欒木萃取物與續隨子萃取物具有保護 LDL 氧化及防止 oxLDL 傷害內皮細胞的效果，黃連素或許可應用於臨床上保護氧化型低密度脂蛋白所造成內皮細胞的功能失調及傷害。

關鍵詞：動脈硬化; 氧化型低密度脂蛋白; 內皮細胞; 細胞凋亡; 抗氧化

Cerebrovascular and heart diseases are the second and third causes of death in Taiwan. Oxidized low-density lipoprotein (oxLDL) is a known major risk factor for accelerating atherogenesis. It involves several critical steps in atherosclerosis including the expression of adhesion molecules on endothelial cells, the production of various pro-inflammatory cytokines and growth factors, the proliferationand migration of vascular cells and the retardation of endothelial regeneration. The early stages of the atherosclerotic process are initiated by accumulation of oxLDL and activation of endothelial cells with subsequent expression of

adhesion molecules and increased binding of monocytes to the vascular endothelium. In this study, we evaluated the anti-oxidative activity of the extracts *of Mallotus repandus* (MRE), *Euphorbia lathyris L.* (ELE) *and Koelreuteria formosanas* (KFE) and how the extracts *of Mallotus repandus* (MRE), *Euphorbia lathyris L.* (ELE) *and Koelreuteria formosanas* (KFE) rescues human umbilical vein endothelial cells (HUVECs) from oxidized LDL (oxLDL)-mediated dysfunction. The anti-oxidative activity of *Euphorbia lathyris L.* (ELE) *and Koelreuteria formosanas* (KFE) was defined by the relative electrophoretic mobility of oxLDL, fragmentation of Apo B, and Diene conjugation assay of the Cu^{2+}-mediated oxidation of LDL. *Euphorbia lathyris L.* (ELE) *and Koelreuteria formosanas* (KFE) also inhibited the generation of ROS, and the subsequent mitochondrial membrane potential collapse, chromosome condensation, cytochrome c release, and caspase-3 activation induced by oxLDL in HUVECs. Our results suggest that berberine may protect LDL oxidation and prevent oxLDL-induced cellular dysfunction. In conclusion, we have demonstrated the prevention and its mechanism of *Euphorbia lathyris L.* (ELE) *and Koelreuteria formosanas* (KFE) on LDL oxidation and oxLDL-induced endothelial cell dysfunction.

Keyword： atherogenesis; oxLDL; endothelial cell; apoptosis; anti-oxidative activity

報告內容

一、前言及研究目的

心臟疾病是已開發國家最重要的死亡原因之一，國人在飲食西化，以及工作壓力急劇加大後，心臟疾病的發生率也隨之增加。根據衛生署近五年內調查統計，台灣的總死亡率近四十年來呈明顯下降，然而動脈硬化性疾病的死亡率都明顯增加，腦血管及心臟病近三十年來一直名列十大死因前茅，更是老人最主要的病因與死因；並且由全國健康訪問調查結果指出，十二歲以上的國人近一百四十五萬人罹患心臟病，其中六十五歲以上的老人大約每五位就有一人罹患心臟病。以民國八十九年為例，國民因心臟病死亡的人數高達 10,552 人，即平均每 49 分 48 秒就有一人因為心臟疾病而死亡，也就是一天之內至少有 29 人死於心臟病。調查同時發現，罹患心臟病的個案中，約百分之十八到百分之四十六的比例亦是高血壓、糖尿病、高血脂等三高疾病的高危險群。此外，腦血管與心臟疾病在近幾年來則是分居台灣地區十大死因之第二及第三名，僅次於惡性腫瘤之後，因此心血管疾病的預防與治療，是一個急待解決與重視的課題。

動脈粥狀硬化(atherogenesis)引發的病理機制非常複雜，其形成的成因主要成因有三: 1. 低密度脂蛋白氧化損傷; 2. 血管內皮功能破壞及損傷; 3.慢性發炎反應。動脈硬化疾病的危險因子，主要是高膽固醇血症、高血壓、糖尿病、年紀大、抽煙及早發性動脈硬化疾病家族史。具有這些危險因子的人，大多被證實會傷害內皮細胞或使其功能失常，因而容易發生動脈硬化。動脈硬化塊的組成，主要是以血脂肪（lipid core）為中心，主成份是膽固醇，包在其周圍的是平滑肌細胞和各種結締組織。對動脈硬化塊，一般可分為兩種，一種稱為穩定的動脈硬化塊，一種是所謂的不穩定動脈硬化塊（含膽固醇成分多）。不穩定動脈硬化塊，易發生破裂而造成急性栓塞。現在許多降血脂肪藥物（主要是statins類），皆稱為有穩動脈硬化塊及改善內皮細胞功能的作用。所以接受降膽固醇藥物治療，可以在短期內即可明顯減少血管急性栓塞，進而降低發生心肌梗塞和腦中鋒的機率[1-19]。腦血管與心臟疾病在近幾年來則是分居台灣地區十大死因之第二及第三名，僅次於惡性腫瘤之後，因此心血管疾病的預防與治療，也是一個急待解決的課題。動脈硬化(atherogenesis)是一種特殊型態的慢性發炎反應，特徵為脂質沉積、慢性發炎及慢性傷口癒合的過程，最後造成血

管引起血栓(thrombosis)，造成局部組織缺血、缺氧而壞死，可能會引起中風(stroke)及心肌梗塞(myocardial infarction)而致死[20,21]。動脈硬化的致病機轉的相關研究已證實：「內皮細胞功能喪失(endothelial dysfunction)」與「低密度脂蛋白氧化修飾(LDL oxidative modification)」是其中的兩大關鍵。而氧化型低密度脂蛋白(oxidized low density lipoprotein, oxLDL)的堆積不僅會誘發免疫反應更會使得內皮細胞功能受損。而功能受損的內皮細胞使得大分子容易通過，並在血管壁上堆積，進而導致許多趨化物質釋出[22]，終使病灶更加惡化[23-25]。

扛香藤(*Mallotus repandus* (Willd.) Muell-Arg.)，別名，桶交藤(台灣)、石岩楓、山龍眼、黃豆樹、大力王、倒掛金鉤、萬子藤、舒力起、犁頭柴、倒金鉤、青倒鉤、木賊楓藤，為大戟科野桐屬。灌木或喬木，有時藤本狀，長可達 10 公尺以上，全體密被黃色星狀柔毛。根、莖，全年均可採，洗淨，切片，曬乾。夏、秋季採葉，鮮用或曬乾。味苦、辛，性溫。具有除濕，利水，治風濕骨痛，水腫，頑癬，條蟲，白口瘡，清熱，解毒，止癢，皮膚潰瘍，過敏性皮炎，慢性喉炎，癰疽疔瘡，狂犬咬傷，肝病,肝硬化,腹水。可知扛香藤自古以來已為一多用途之作物，亦為民間常用的藥用保健植物。由扛香藤的莖可萃取出三種 D:A-friedo-oleanane lactones: 3-oxo-D:A-friedo-oleanan-27,16alpha-lactone 、3alpha-benzoyloxy-D:A-friedo-oleanan-27, 16alpha-lactone 和 3beta-hydroxy-D:A-friedo-oleanan-27,16alpha-lactone [26]; 而由扛香藤的莖和根可萃取出三種 triterpenoids: 分別是 3alpha-hydroxy-13alpha-ursan-28,12beta-olide 3-benzoate, 3alpha-hydroxy-28beta-methoxy-13alpha-ursan-28, 12beta-epoxide 3-benzoate, and 3alpha-hydroxy-13alpha-ursan-28-oic acid [27]。扛香藤水提取物，丙酮提取物及丁醇提取物均有抗潰瘍作用，其中以丁醇提取物最好。近年來的相關研究顯示, 扛香藤除了具有食用、藥用的優點之外，仍具有多項的保健功效，近來有許多文獻指出，扛香藤莖的萃取物具有清除自由基 superoxide radical (O2.-) 和 hydroxyl radical (OH.)的能力[28]。此外，有研究證實扛香藤莖的萃取物具有良好抗發炎的功效，且由動物實驗證明將老鼠餵食扛香藤莖的萃取物，可保護由四氯化碳所引起肝臟損傷，降低血清中的 GOT 與 GPT 的數值[29]。由初步結果顯示，扛香藤的粗萃取物具有很強的抗 LDL 氧化能力，並且可保護由氧化型 LDL 所誘導的內皮細胞凋亡(專利申請中)，且由細胞實驗結果得知，扛香藤的粗萃取物對於正

常的肺細胞(MRC5)與肝細胞(Chang liver)是沒有細胞毒性。

台灣欒木(*Koelreuteria formosanas*)，為台灣特有之欒木，又名：金苦楝、苦楝公、苦楝舅，屬落葉喬木，枝條上有許多一點一點細細的皮孔，二回羽狀複葉，卵形，互生，葉緣的粗鋸齒非常明顯，種子為黑色球形。原產地在台灣，九至十月會開出金黃色花穗，花朵凋謝後會結出紅褐色、像燈籠狀的蒴果。花果能誘鳥，常做為景觀樹和行道樹，有很好的綠化美化效果。目前並無 SCI 期刊文獻記載其功能與相關研究。且由我們的初步結果顯示，台灣欒木的粗萃取物具有很強的抗氧化能力(專利申請中)，且由細胞實驗結果得知，台灣欒木的粗萃取物對於正常的肺細胞(MRC5)與肝細胞(Chang liver)是沒有細胞毒性。

續隨子(*Euphorbia lathyris L.*)，又名千金子、千兩金、菩薩豆、拒冬、聯步。本品為大戟科植物續隨子 *Euphorbia lathyris* L.的乾燥成熟果子。可栽培或野生。主要產於河北、河南、浙江等地。夏秋二季果實成熟時採收，將雜質除去後，乾燥可製成藥材。其中藥材以粒飽滿，種仁色白，油性足者為佳。飲片分生用和製霜，味辛，性溫，種子含脂肪油：油中含多種脂肪酸；daphnetin、esculetin、euphorbetin、isoeuphorbetin、epoxylathyrol，製成之霜稱千金子霜。於本草綱目記載，續隨子辛溫，治癥瘕痰飲，冷氣脹滿，蠱毒鬼疰。利大小腸，下惡滯物，塗疥癬瘡，續隨子亦可用於治療小便不通、水腫與蛇咬傷。有文獻指出在老鼠的活體實驗中，續隨子中的 ingenol-3-hexadecanoate 具有抑制 Sarcoma 180 之抗癌活性[30]。並且將續隨子種子萃取物通過 silica gel column 可純化出具有 tyrosinase inhibitory activity 的 esculetin [31]。由細胞實驗結果初步得知，續隨子之萃取物對於正常的肺細胞(MRC5)與肝細胞(Chang liver)並無細胞毒性，且具有很好之抗氧化能力。。因此，本計劃擬利用扛香藤、續隨子與台灣欒木之萃取物，利用體外培養人類臍帶靜脈內皮細胞(human umbilical vein endothelial cells; HUVECs)以及人類血管內皮細胞株(EA-hy926)與巨噬細胞株(RAW264.7、THP-1、U937)，以氧化型低密度脂蛋白(oxLDL)誘導血管內皮細胞與巨噬細胞之細胞凋亡及巨噬細胞所誘導之發炎反應，用來模擬人體內粥狀動脈硬化的形成，探討扛香藤、續隨子與台灣欒木這些天然萃取物作用在血管內皮細胞、皮滑肌細胞與巨噬細胞之保護作用及其功能，並釐清其訊息傳遞路徑與作用機轉。

二、研究方法

1. 扛香藤、續隨子與台灣欒木成分之萃取[1]

扛香藤、續隨子與台灣欒木經洗滌，陰乾後，各稱取 100-200 公克，切碎放入圓底燒瓶中，分別以水或有機溶劑如 50%酒精，於 70℃水浴中迴流十二小時，萃取液趁熱過濾並保存之。重複迴流步驟共二次，並合併二次經過濾之萃取液，進行減壓濃縮，待去除有機溶劑則冷凍乾燥而存放於-20℃冰櫃中以備分析用。

2. 低密度之蛋白的分離及氧化[2]

將購自於台中捐血中之正常人類血漿 2ml，加入 0.7ml 的 o.15M NaCl 及 0.3 mM EDTA (pH7.4)離心 90000 rpm，10℃，10 分鐘，取下層液 2 ml 後，重複上述步驟後再離心 3.5 小時，再取下層液 2 ml，加入 KBr 離心 3.5 小時，取上層液中淡黃色油層即為 LDL。LDL 先通過 PD-10 desalting column 後以去除 EDTA，加入 10μM $CuSO_4$ 於 37℃反應 16 小時，再通過一次 PD-10 desalting column 以去除銅離子，作蛋白定量後置於 4 備用。

3. LDL electrophoretic motility assay [2]

利用 lipoprotein 氧化後其帶電荷會由正電性轉為負電性的特性，將氧化後的 oxLDL 90 μl 加入 0.1% sudden black 染劑 10 μl，在 30℃混合作用 20 分鐘後，與 10 μl 50%之甘油混和後，loading 到 1% agarose 電泳膠片上，通電 100 伏特，30 min 後，依電泳結果以 band 移動的距離（electrophoretic mobility；EM）判定 LDL 氧化程度，以未氧化之 LDL 之 EM 為 1，以相對程度作為定量。

4. ApoB 蛋白片段化 (fragmentation) 的測試[2]

在 LDL 氧化反應結束之後，樣本須以 denaturing bugger (3% SDS, 10% glycerol 及 5% 2-mercaptoethanol) 在 95 ℃ 加熱 5 分鐘。接著配製 3-15% gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE)，取樣本 40 μl 注入膠的孔洞 中，然後以 48 V 進行電泳 150 分鐘。在電泳結束之後以 Coomassie Brilliant blue R250 染色 2 小時後再進行乾膠

5. Diene conjugation assay

脂質過氧化之重要特徵為其氧化產物共軛雙烯(diene)之生成量，會隨氧化時間增長而增加。利用共軛雙烯可在 230～235nm 吸光值下被偵測之原理，將 sample 以銅離子於 37℃誘導 LDL 氧化 360 min（約 6 小時），反應開始起每 10 分鐘測量一次吸光值，以共軛雙烯

生成量（吸光值）為縱座標，氧化時間為橫座標作圖。

6. TBARS assay [3]

LDL 氧化會生成過氧化物 malondialdehyde（MDA），一分子的 MDA 與二分子的 thiobarbituric acid（TBA）會生成具螢光性質的聚合物 TBARS（thiobarbituric acid reactive substance），此物質於激發波長為 532 nm，發射波長為 600 nm 時可被螢光光讀盤儀（Labsystem, CA）計偵測到。以 1,1,3,3-tetramethoxypropane（TMP）為標準液做標準曲線而可定量 LDL 氧化程度，單位為 nM MDA/mg protein。

7. 細胞培養[4]

人類血管內皮細胞 human umbilical vein endothelial cell (HUVEC) 以含 15% FBS、150 g/ml endothelial cell growth supplement、5 units/ml heparin sodium、100 units/ml penicillin-streptomycin，及 50 μg/ml gentamycin 的 M199 培養液初代培養，持續培養三週，期間進行所需實驗。人類血管內皮細胞株(EA-hy926) 培養在含有 20mM D-glucose、10% FBS、HAT media supplement 的完整 DMEM 培養液(包含 2mM glutamin 及 100 units/ml penicillin-streptomycin)。人類巨噬細胞株(THP-1, U937) 培養在含有 10% FBS、2mM glutamin 及 100 units/ml penicillin-streptomycin 的 RPMI 培養液。巨噬細胞株(RAW264.7) 則培養在含有 10% FBS、2mM glutamin 及 100 units/ml penicillin-streptomycin 的 DMEM 培養液。

8. 分離人類血管內皮細胞 (HUVECs) [2]

利用 PBS (內含 1% penicillin 及 streptomycin) 清洗臍帶後，將 1% trypsin 灌滿臍帶內壁，以針筒固定兩端臍帶開口，作用約 6 分鐘，來回沖洗臍帶內壁，收集此溶液以含 10% FBS 的 M199 培養液終止 trypsin 的活性，之後再以 M199 培養液 (不含 FBS) 沖洗臍帶內壁約 20 次，兩部分混合後離心 1200 rpm 5 分鐘，去掉上清，以含 10% FBS 的 M199 培養液打散細胞，將細胞培養在 37℃，5%CO_2 overnight，換新鮮的 M199 培養液 (含 15% FBS、150 μg/ml endothelial cell growth supplement、5 units/ml heparin sodium、100 units/ml penicillin-streptomycin，及 50 μg/ml gentamycin) 持續培養。

9. MTT (Microculture tetrazolium) 分析[5]

本實驗室用來測試細胞是否有活性以及是否存活的方法，將細胞以 3~5×10^4 細胞數分

至 24 孔盤中，37 ℃培養 16 小時後，以不同濃度扛香藤、續隨子與台灣欒木處理 1 小時後再加入 200 μg/ml oxLDL 處理 24 小時後，去除加藥的細胞培養液，再加入 1 ml 以細胞培養液 10 倍稀釋的 MTT reagent (final concentration 0.5 mg/ml)，待此作用 4 小時之後再以異丙醇將結晶溶出，於 O.D. 565 nm 下測定溶液吸光，由吸光強度可得知存活的細胞數多寡。

10. 內皮細胞成管作用 (Capillary-like tube formation assay) [6]

將 0.04 ml/well 的 Matrigel (10 mg/ml) (BD Bioscience Pharmingen) 鋪敷於 96 well 的培養皿上於 37℃ 凝固 1 小時,之後在每個 well 加入 0.15 ml 含有 20,000 個 HUVEC cell 的 M199 培養液，分別加入不同濃度的中草藥處理 1 小時後，再加入 200 μg/ml oxLDL 處理 24 小時後，，在 37℃ 培養 6 小時，將培養液移除，細胞經由甲醇固定，在顯微鏡下觀察管狀結構的形成。

11. Oil Red O 染色 [7]

將以不同濃度扛香藤、續隨子與台灣欒木處理 1 小時後再加入 200 μg/ml oxLDL 處理 24 小時後的 macrophage 細胞以 PBS 清洗兩次，以 citrate-acetone fixative solution 固定細胞 1 分鐘，再以 ddH_2O 清洗，加入 1% oil red O (溶於 60% Isopropanol)染色 15 分鐘，以 60% Isopropanol 清洗 5 分鐘,再以 ddH_2O 清洗,加入 0.05% Sodium carbonate solution 加強效果，再以蘇木紫 Hematoxylin 作對比染色，以 ddH_2O 清洗數次。

12. 低密度之蛋白的分離及氧化 [8]

將購自於台中捐血中之正常人類血漿 2ml,加入 0.7ml 的 o.15M NaCl 及 0.3 mM EDTA (pH7.4)離心 90000 rpm，10℃，10 分鐘，取下層液 2 ml 後，重複上述步驟後再離心 3.5 小時，再取下層液 2 ml，加入 KBr 離心 3.5 小時，取上層液中淡黃色油層即為 LDL。LDL 先過 PD-10 desalting column 後以去除 EDTA，加入 10 μM $CuSO_4$ 於 37℃反應 16 小時，再過一次 PD-10 desalting column 以去除銅離子。

13. DAPI stain [10]

將人類血管內皮細胞或巨噬細胞以不同濃度扛香藤、續隨子與台灣欒木萃取物處理 1 小時後，加或不加 oxLDL 培養 24 小時，以 PBS 清洗兩次，然後以 4% para-formaldehyde 固定作用 30 min 後，以 PBS 清洗，加入染劑(DAPI)染 30 min，以 PBS 清洗，於螢光顯微鏡下觀察(UV 461nm)。

14. ROS 含量測定 [2]

將人類血管內皮細胞或巨噬細胞種於96-well，以不同濃度扛香藤、續隨子與台灣欒木萃取物處理1小時後，加或不加oxLDL培養24小時，以PBS清洗後，將DCF-AM (2',7-dihydrodichlorofluorescein acetoxymethy ester) 螢光劑處理細胞一小時，以450-490nm波長下激發，在515-550nm波長下會發射出螢光，在螢光顯微鏡下觀察，或使用螢光讀盤儀(Labsystem, CA)來測量細胞內之螢光強度。

15. Western blotting 分析 [12]

用 western blotting 的方法測定 caspase、PARP、cytochrome C、Bcl-2 的蛋白量首先製備 12.5% SDS-PAGE 電泳膠片, 置於電泳槽中, 並加入電泳緩衝液, 取 16μl sample (蛋白總量 20μg), 加入 4μl loading buffer, 將 sample denature (95℃，10 min)之後再 loading 到電泳片中, 以 140V 進行電泳分離。大約 3 小時之後, 將膠拆下後進行蛋白轉漬, 將膠體置入冰冷之 transfer buffer, 將預先浸濕的 NC paper 蓋在膠體上面後裝入 transfer holder, 於 4℃下, 以 100V 進行轉漬 1 小時之後，取出 NC paper 加入 blocking buffer, 在室溫下搖動一個小時。然後加入一級抗體於 TBS buffer, 在 4℃下反應 overnight, 之後以 washing buffer (TBS+0.05% Tween 20)清洗三次, 每一次 10 分鐘。接著再加入二級抗體於 TBS buffer, 於室溫作用二個小時後以 washing buffer 清洗三次, 每一次 10 分鐘。最後以 ECL 冷光系統紀錄。

16. cell-cell adhesion 分析

在 24 wells dishes 培養人類血管內皮細胞至 100% confluency，加入另一事先以 [^{3}H]-thymidine 培養之巨噬細胞及不同濃度扛香藤、續隨子與台灣欒木萃取物處理，於細胞培養箱中培養 3 小時後, 以 PBS 洗去尚未 adhesion 之[^{3}H]-thymidine cancer cell，經 trypsinized 之後以 liguid scitillation counter 計算 radioactivity。

17. 統計分析

所有數據以電腦統計軟體SigmaStat (Jandel Scientific Software, USA)進行one-way analysis of variance(one-way ANOVA)分析。

三、結果與討論

氧化型低密度脂蛋白(oxLDL)在血管內的堆積會使得內皮細胞功能受損甚至死亡。我們取純化之LDL (100 μg/ml)，加入10μM $CuSO_4$使其氧化，同時處理不同濃度(高濃度: 0, 25, 50, 75, 100 μg/mL或是低濃度:5, 10, 15, 20 μg/mL)的扛香藤、續隨子與台灣欒木萃取物或維他命E類似物Trolox (10, 50 μM)，在37℃下共同反應4小時(ApoB fragmentation assay)、Diene conjugation assay、16小時 LDL electrophoretic motility，以Trolox作為positive control，首先分析台灣欒木萃取物(KFEE)保護LDL氧化的效果。結果顯示，10 μg/mL的台灣欒木萃取物(KFEE)對於ApoB斷裂化 **(Fig. 1)**、LDL泳動率 **(Fig. 2)**及共軛雙烯(diene)之生成量**(Fig. 3)**、自由基的清除率(DPPH radical scavenging assay) **(Fig. 4)**皆有很好的保護效果，而25 μg/mL的台灣欒木萃取物其保護效果更是超過50%。証實台灣欒木萃取物具有保護LDL不受氧化的效果。以不同濃度(0, 25, 50, 75, 100 μg/ml)的台灣欒木萃取物(KFEE)處理靜脈血管內皮細胞(HUVEC細胞)，同時再加入200 μg/ml的氧化型低密度脂蛋白(oxLDL)處理，共同培養24小時。利用MTT assay偵測細胞的存活率，發現台灣欒木萃取物(KFEE)可以保護由oxLDL所造成的內皮細胞死亡 **(Fig. 5A)**。再以Trypan blue exclusion assay，計數HUVEC細胞死細胞與活細胞的數目，證實台灣欒木萃取物(KFEE)的確可以保護oxLDL處理下內皮細胞的存活細胞以及降低死亡細胞數 **(Fig. 5B)**。而內皮細胞的型態及其細胞的完整性會影響內皮細胞控制分子通透的功能，且這個功能的缺失與動脈粥狀硬化的形成有關。因此，我們同時也以相位差顯微鏡觀察細胞型態，證實台灣欒木萃取物(KFEE)不僅有效提高oxLDL處理下內皮細胞的存活，且存活細胞的細胞型態與control組相比並無明顯不同。進一步以DAPI染色及Flow cytometry分析oxLDL的處理是否造成HUVEC細胞的細胞凋亡。證實oxLDL的處理會造成chromosome condensation，而台灣欒木萃取物(KFEE)可以明顯降低這個現象 **(Fig. 6)**。也證實oxLDL的處理會造成subG1 phase (hypodiploid cell)的大量增加，而台灣欒木萃取物(KFEE)可以有效的減少sub G1 phase，甚至完全回復 **(Fig. 7)**。針對oxLDL造成HUVEC細胞的細胞凋亡及台灣欒木萃取物(KFEE)的保護效果，探討其中的分子機制。首先，以DCFH-DA染色再以Flow cytometry進行分析細胞內ROS的產生，發現oxLDL會造成HUVEC細胞內的ROS增加，而台灣欒木萃取物(KFEE)則會抑制ROS的增加 **(Fig. 8)**。另外，以JC-1染色再以螢光顯微鏡觀察HUVEC細胞的粒線體膜電位，發現oxLDL會造成HUVEC細胞的

粒線體膜電位降低，而台灣欒木萃取物(KFEE)則會保護粒線體膜電位的降低 **(Fig. 9)**。進一步以Western Blot分析，証實oxLDL會促使caspase 3的活化以及PARP的切割，同時，也發現Bcl-2減少而BAX增加的現象，而台灣欒木萃取物(KFEE)對於osLDL所造成的apoptosis相關蛋白的變化都有保護的效果 **(Fig. 10)**。綜合以上結果，台灣欒木萃取物(KFEE)不僅可以有效保護LDL不受氧化，且對於oxLDL所造成的內皮細胞的apoptosis也有相當好的保護效果，而其中機制，可能是透過降低oxLDL所造成細胞內ROS的增加，進而保護粒線體不受傷害且防止細胞的apoptosis。在扛香藤萃取物(MRE)的抗氧化能力部分，結果顯示，10 μg/mL的扛香藤萃取物(MRE)對於LDL泳動率 **(Fig. 11)**、ApoB斷裂化 **(Fig. 12)**及自由機清除率 **(Fig. 13)**皆有很好的保護效果，証實扛香藤萃取物具有保護LDL不受氧化的效果。利用oil-red O stain 觀察foam cell的形成，扛香藤萃取物(MRE)可以有效抑制RAW264.7細胞吞食氧化型LDL的能力**(Fig. 14)** 。最後分析續隨子木萃取物(ELE)保護LDL氧化的效果。結果顯示，利用lipoprotein氧化後其帶電荷會由正電性轉為負電性的特性，隨子木萃取物(ELE)可有效抑制LDL的氧化**(Fig. 16)**，此外對於ApoB斷裂化 **(Fig. 15)**及共軛雙烯(diene)之生成量**(Fig. 17)**、自由基的清除率 **(Fig. 18)**皆有顯著的保護效果。証實台灣欒木萃取物具有保護LDL不受氧化的效果。以不同濃度(0, 25, 50, 75, 100 μg/ml)的續隨子木萃取物(ELE)處理靜脈血管內皮細胞(HUVEC細胞)，同時再加入200 μg/ml的氧化型低密度脂蛋白(oxLDL)處理，共同培養24小時。利用MTT assay偵測細胞的存活率，發現續隨子木萃取物(ELE)可以保護由oxLDL所造成的內皮細胞死亡 **(Fig. 19)**。再以Trypan blue exclusion assay，計數HUVEC細胞死細胞與活細胞的數目，證實續隨子木萃取物(ELE)的確可以保護oxLDL處理下內皮細胞的存活細胞以及降低死亡細胞數 **(Fig. 20)**。而內皮細胞的型態及其細胞的完整性會影響內皮細胞控制分子通透的功能，且這個功能的缺失與動脈粥狀硬化的形成有關。進一步以DAPI染色及Flow cytometry分析oxLDL的處理是否造成HUVEC細胞的細胞凋亡。同時將細胞處理oxLDL會造成subG1 phase (hypodiploid cell)的大量增加，而續隨子木萃取物(ELE)可以有效的減少sub G1 phase，甚至完全回復 **(Fig. 20)**。利用DAPI染色證實oxLDL的處理會造成chromosome condensation，而續隨子木萃取物(ELE)可以明顯降低這個現象 **(Fig. 23)**。針對oxLDL造成HUVEC細胞的細胞凋亡及續隨子木萃取物(ELE)的保護效果，探討其中的分子機制。首先，以DCFH-DA染色再以Flow cytometry進行分析細胞內ROS的產生，發現

oxLDL會造成HUVEC細胞內的ROS增加，而續隨子木萃取物(ELE)則會抑制ROS的增加 **(Fig. 21)**。另外，以JC-1染色再以螢光顯微鏡觀察HUVEC細胞的粒線體膜電位，發現oxLDL會造成HUVEC細胞的粒線體膜電位降低，而續隨子木萃取物(ELE)則會保護粒線體膜電位的降低 **(Fig. 22)**。

四、計畫成果及自評

關於探討台灣欒木萃取物(KFEE)、續隨子木萃取物(ELE)與扛香藤萃取物(MRE)保護 LDL 的氧化及保護內皮細胞不受 oxLDL 傷害的效果及機制，已正在整理。本計劃有系統的分析扛香藤、續隨子與台灣欒木之萃取物對於降低 LDL 氧化的效果，以及對血管內皮細胞的保護效果，期望能在心抗血管疾病的應用上有所貢獻。未來希望透過動物活體實驗我們將證實扛香藤、續隨子與台灣欒木之萃取物具有預防心血管疾病的功效及應用性。

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附表及附圖

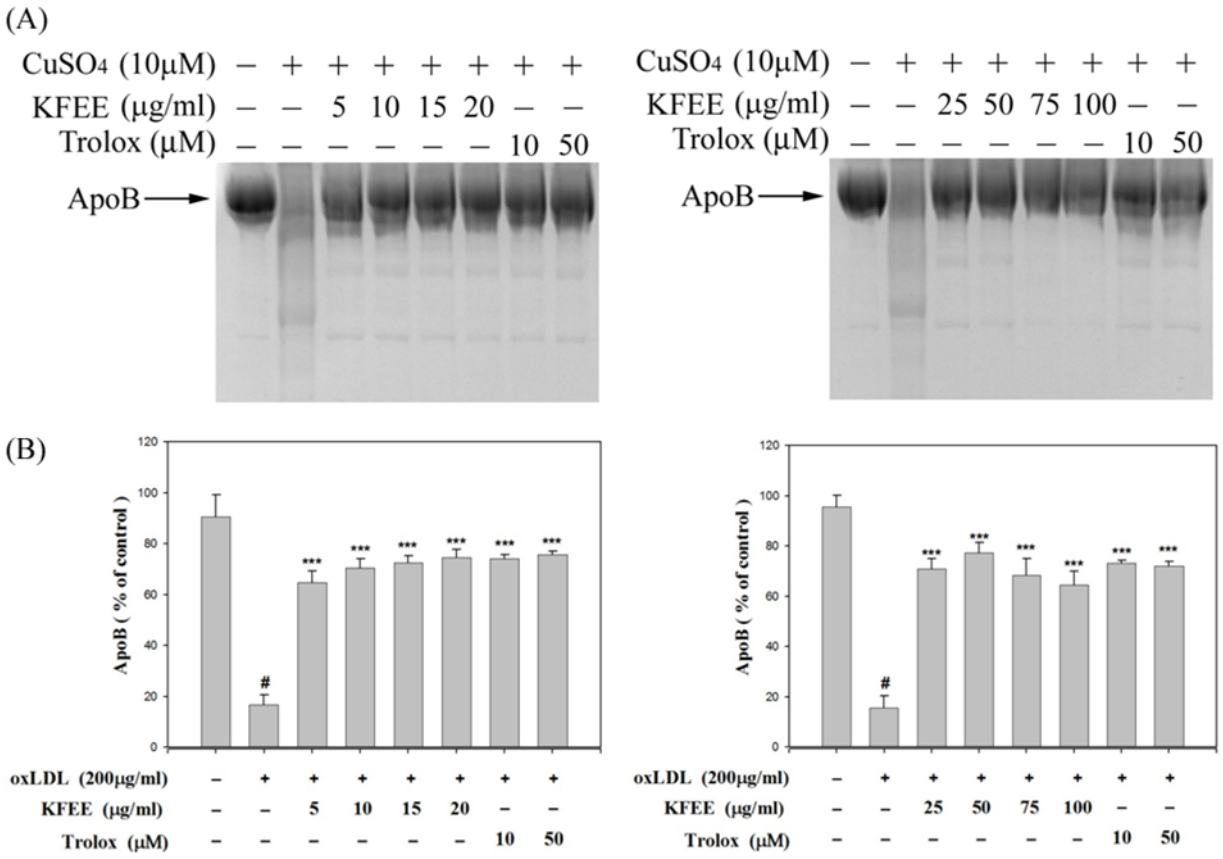

Figure 1. Effect of KFEE on the Cu^{2+}-mediated the ApoB fragmentation in LDL. (A) LDL (200 μg/mL) was incubated with 10 μM $CuSO_4$ at 37 ˚C in the absence or presence of KFEE or Trolox for 4 h and applied to 7.5% SDS-PAGE as described in the Materials and Methods. (B) Quantification of the ApoB fragmentation assay using densitometry is presented as means ± SD of three independent experiments. The signal intensity of the native LDL was assigned as 100% arbitrarily. #, P < 0.001 compared with control. *, P < 0.05; **, P < 0.01; and ***, P < 0.001 compared with the oxLDL-treated group.

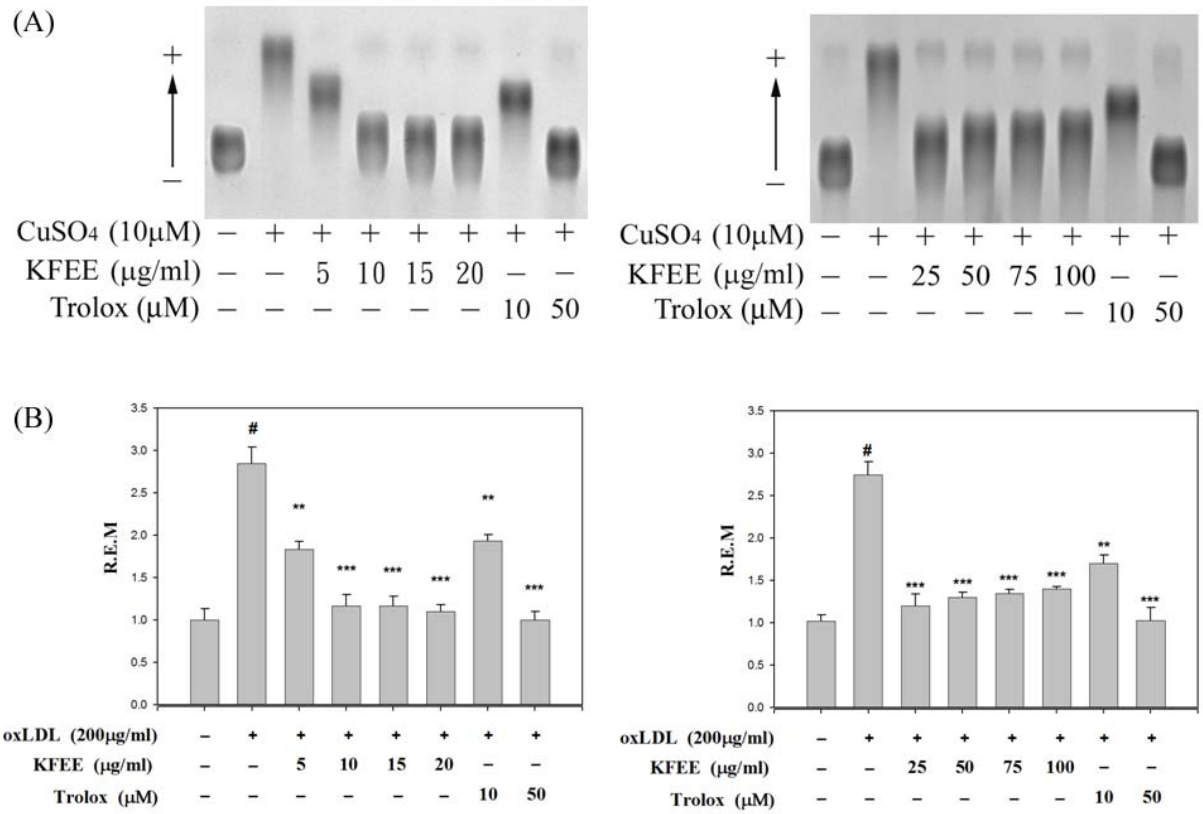

Figure 2. Effect of KFEE on the Cu^{2+}-mediated shift of electrophoretic mobility in LDL. (A) LDL was incubated with 10 μM $CuSO_4$ for 16 h at 37 ° C in the presence or absence of KFEE or Trolox, as positive control, and applied to 0.6% agarose gels as described in the Materials and Methods. (B) The results from the agarose gel electrophoresis were quantified and expressed in the form of relative electrophoretic mobility (REM). The distance traveled in the agarose gel by the native LDL was assigned the arbitrary unit 1

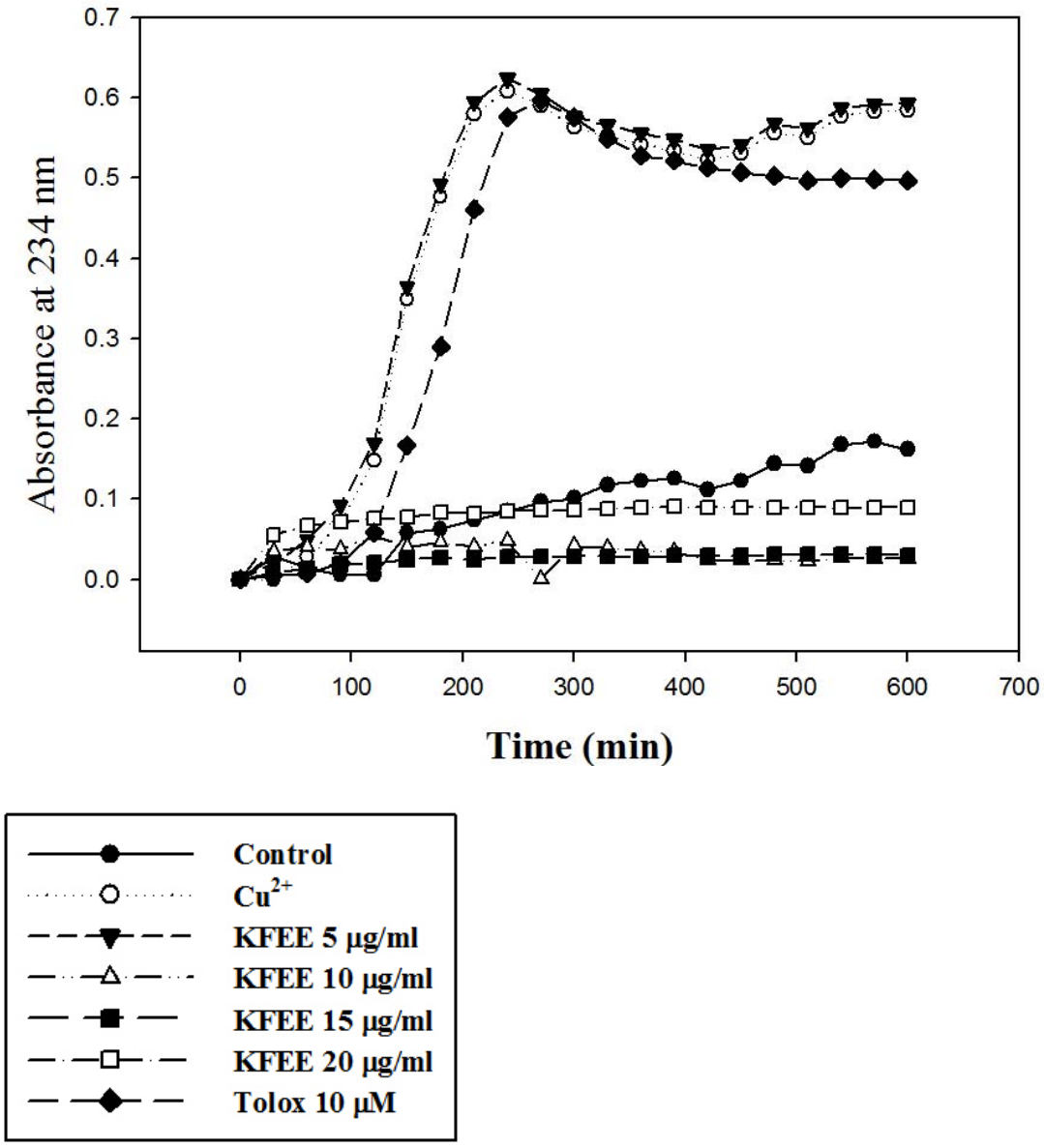

Figure 3. Effect of KFEE on copper-mediated LDL oxidation. LDL preincubated with increasing concentration of KFEE (5, 10, 15, 20 µg/mL). Oxidation was induced with the addition of $CuSO_4$ (10 µM). The formation of conjugated dienes.

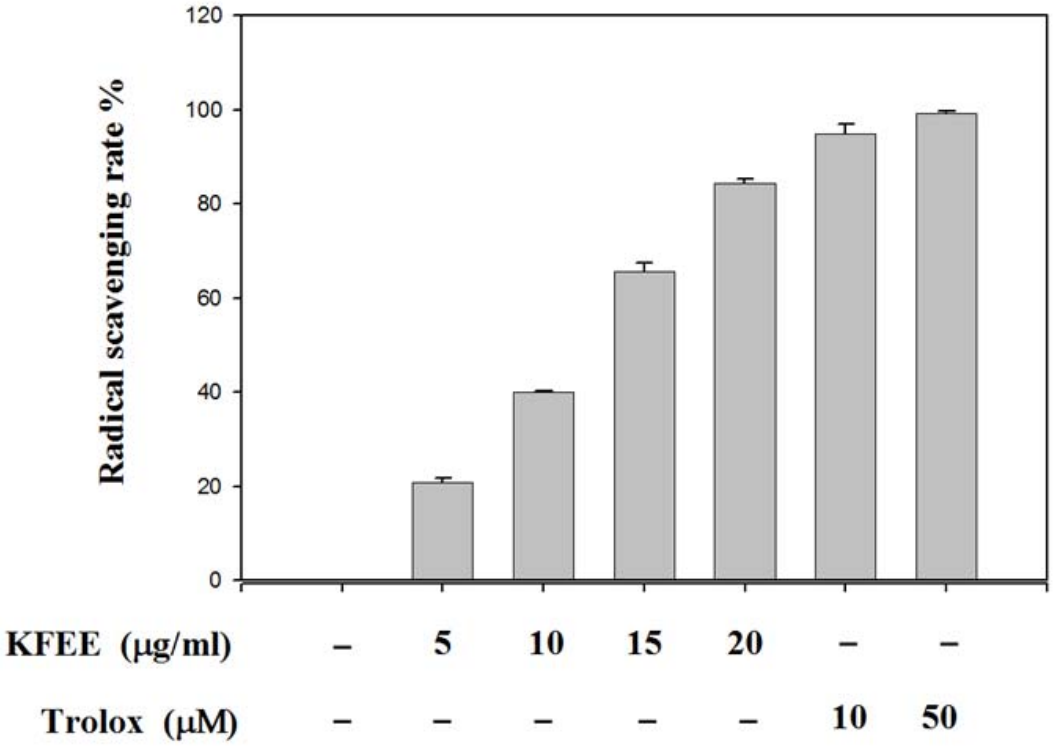

Figure 4. Effect of KFEE DPPH scavenging capability. The radical-scavenging activities of KFEE and Trolox were evaluated by using the DPPH radical scavenging assay. The absorbance of the sample without adding KFEE or Trolox was assigned as 100%, and its radical scavenging rate was assigned as 0% consequently. The quantitative data were presented as means (SD of three independent experiments. *, P < 0.05; **, P < 0.01; and ***, P < 0.001 compared with the oxidative LDL-treated group.

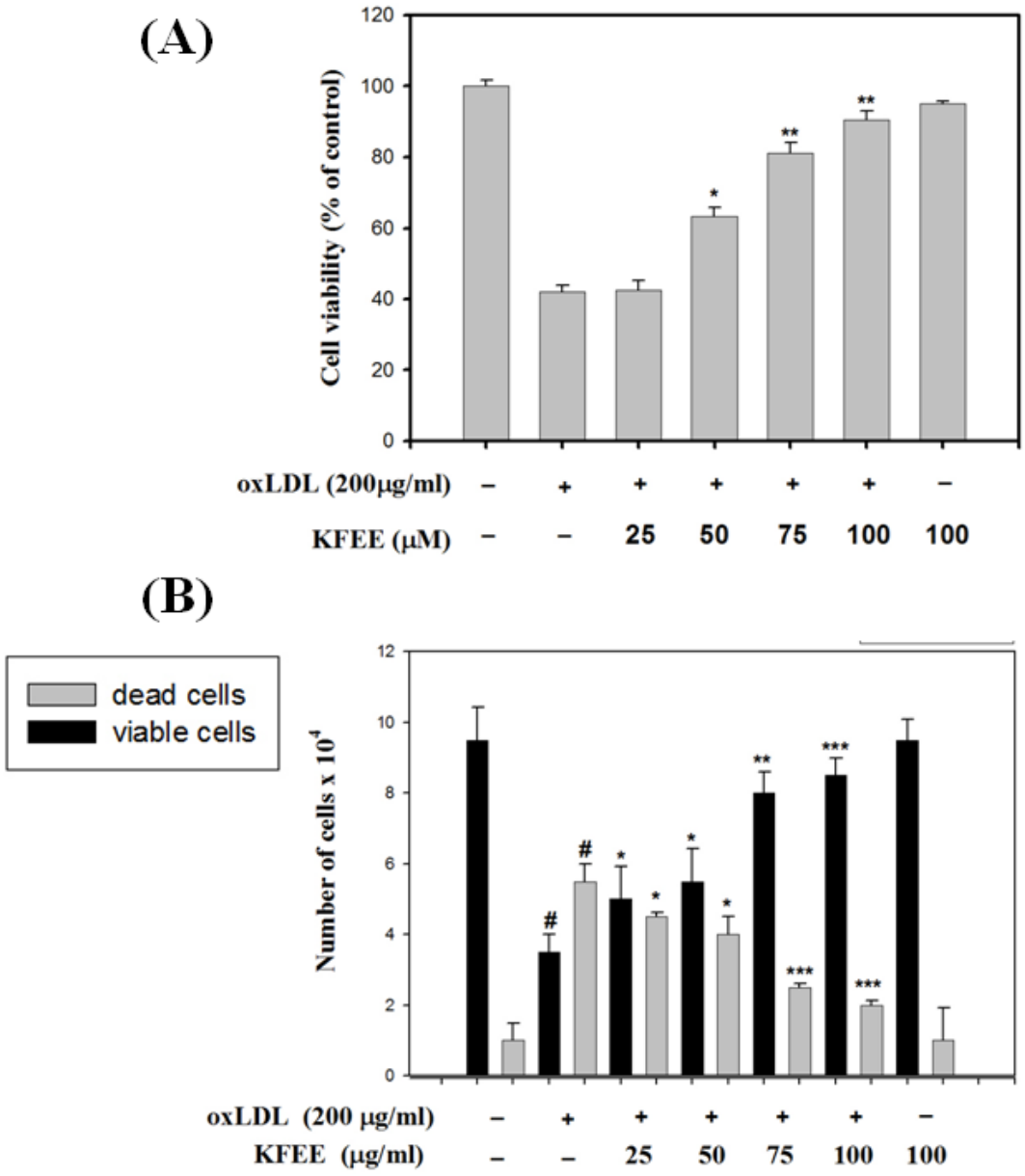

Figure 5. Effect of KFEE on oxLDL-induced endothelial cell death. HUVECs were incubated with oxLDL (200 μg/mL) in the absence and presence of KFEE (25, 50, 75, and 100 μg/mL) for 16 h. (A) The viability of treated HUVEC cells was detected using the MTT assay as described in the Materials and Methods. (B) Viable cells and dead cells were counted using the Trypan blue exclusion assay. The quantitative data were presented as means (SD of three independent experiments. #, $P < 0.001$ compared with control. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ compared with the oxLDL-treated group.

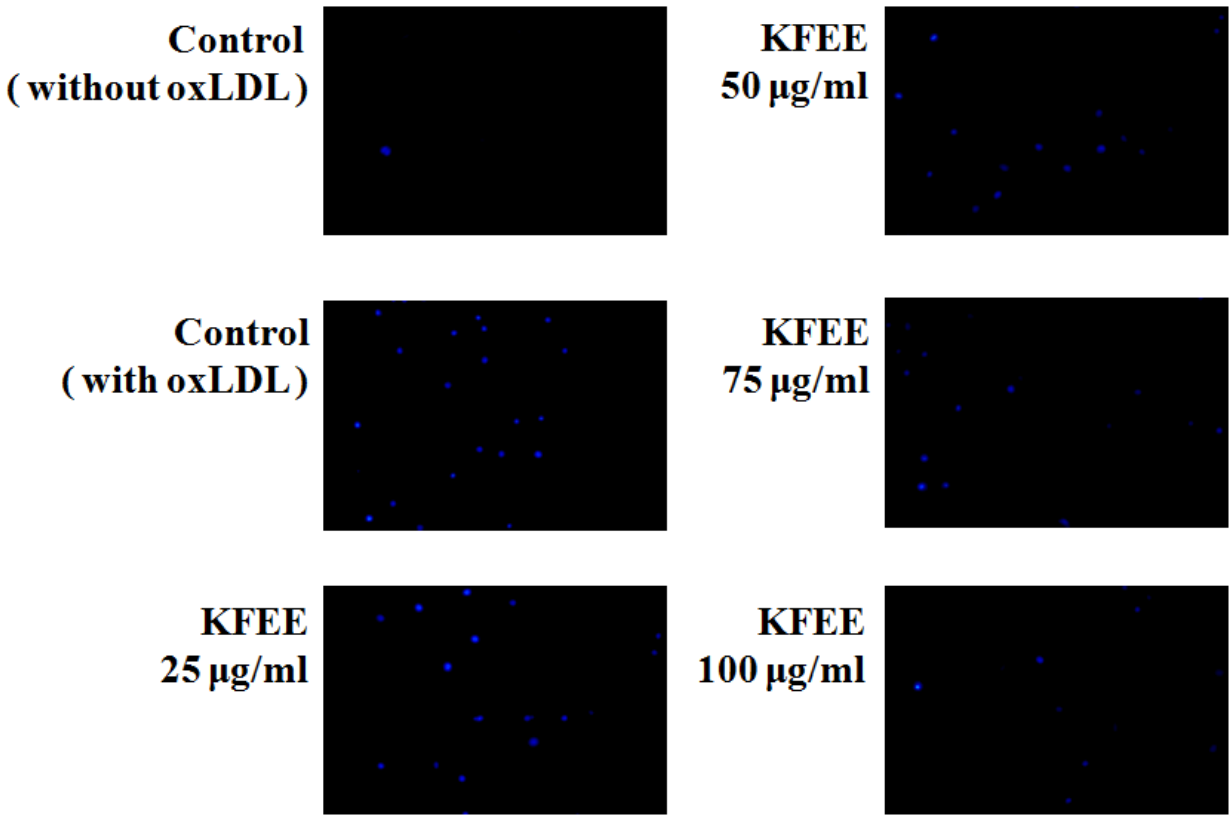

Figure 6. Protective effect of KFEE on oxLDL-induced endothelial cell apoptosis. HUVECs were incubated with oxLDL in the presence and absence of KFEE or Trolox for 16 h. The nuclear morphology of the treated cells was observed by fluorescence microscopy using DAPI stain (at a magnification of 200×). Arrows showed areas of intense fluorescence staining with condensed nuclei.

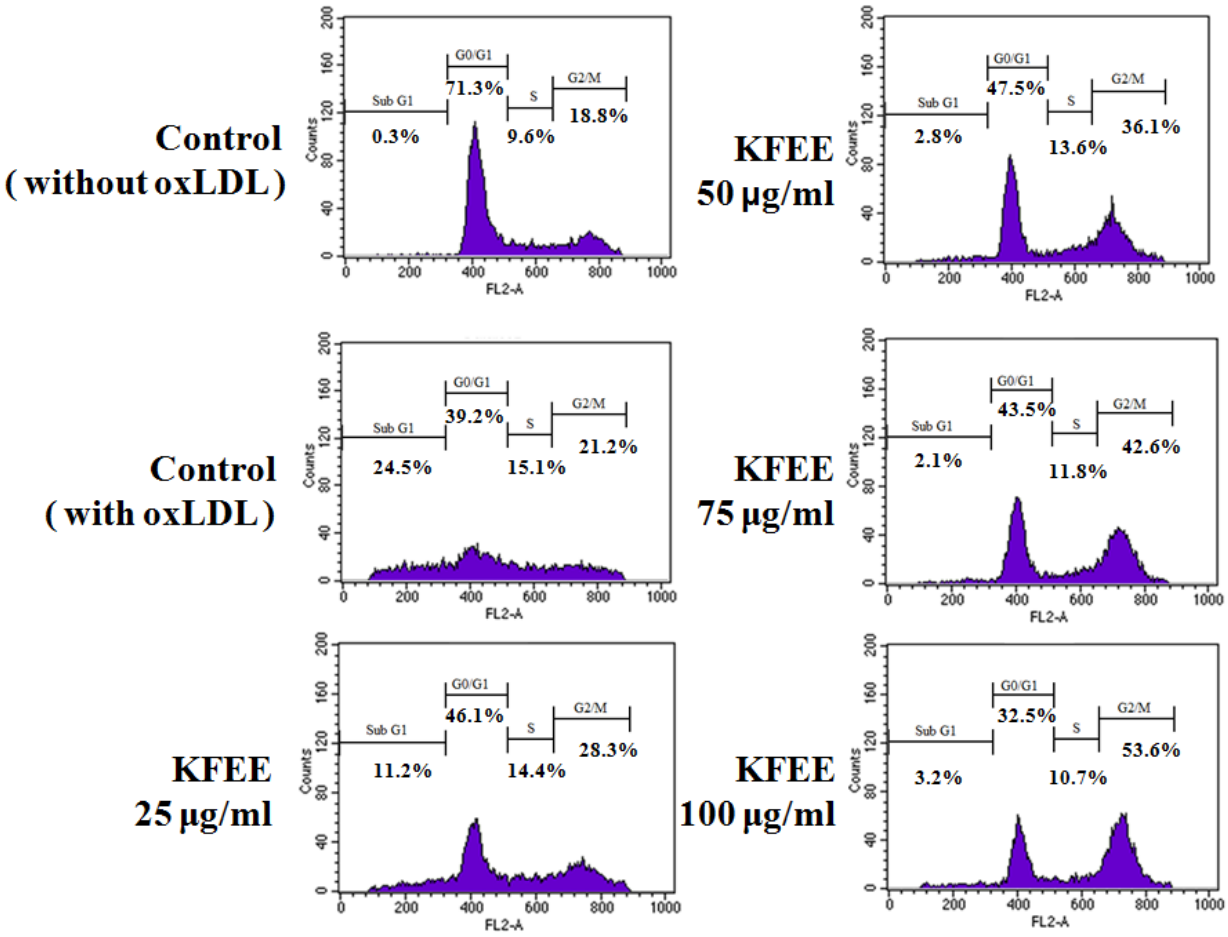

Figure 7. Protective effect of KFEE on oxLDL-induced endothelial cell apoptosis. HUVECs were incubated with oxLDL in the presence and absence of KFEE or Trolox for 16 h. The hypodiploid cell population (sub G1 phase) of the treated HUVEC cells was analyzed by flow cytometry using PI stain, and at last, 10000 events of total cells were analyzed for each experimental treatment.

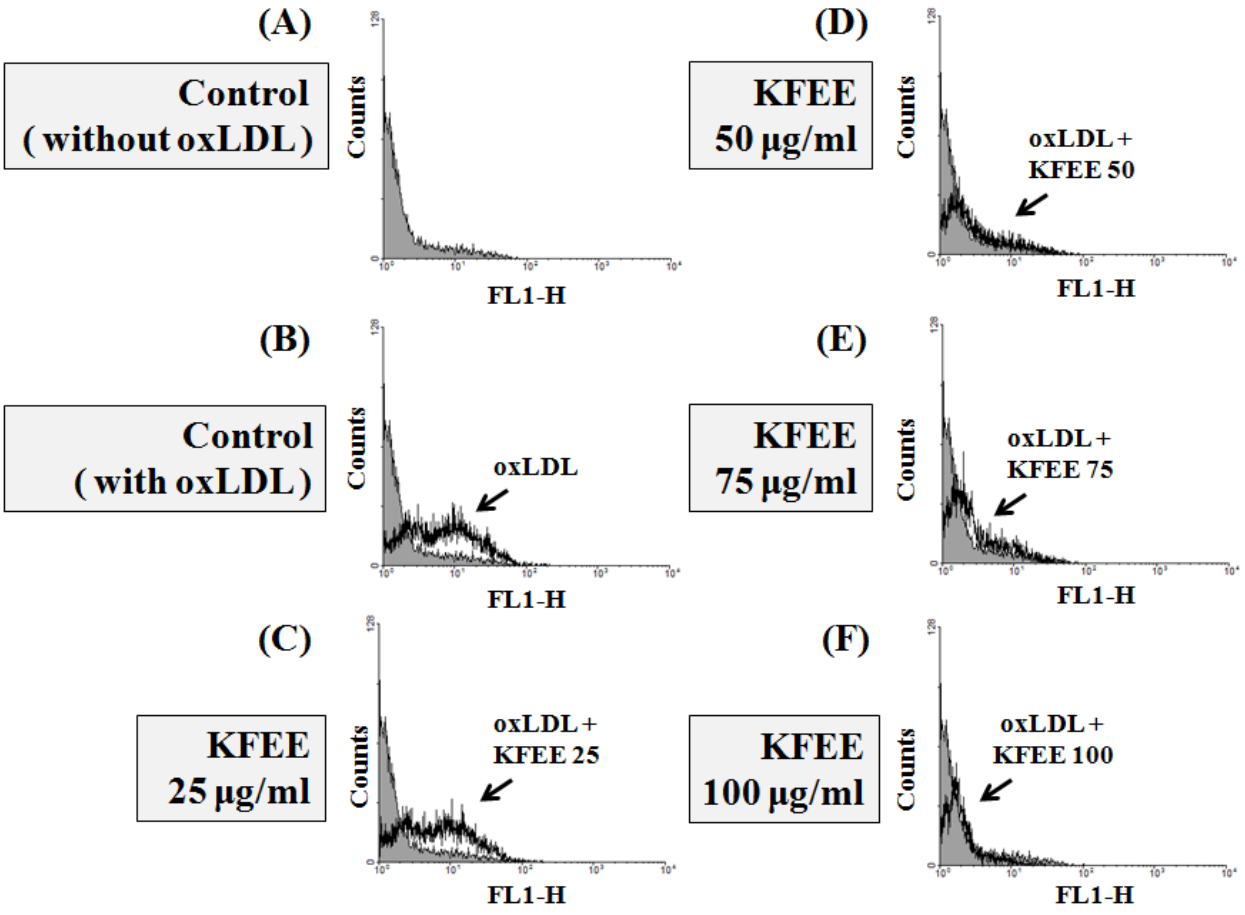

Figure 8. Effects of KFEE on oxLDL-induced ROS production in HUVECs. The ROS levels of (A) the HUVEC cells without treatment, control (without oxLDL)(gray trace); (B) the HUVEC cells with treatment of oxLDL(200 µg/mL), control (with oxLDL)(dark trace); (C) the HUVEC cells with treatment of oxLDL and KFEE(25 µg/mL), KFEE 25 µg/mL (dark trace); (D) the HUVEC cells with treatment of oxLDL and KFEE(50 µg/mL), KFEE 50 µg/mL (dark trace); (E) the HUVEC cells with treatment of oxLDL and KFEE(75 µg/mL), KFEE 75 µg/mL (dark trace); and (F) the HUVEC cells with treatment of oxLDL and KFEE(100 µg/mL), KFEE 100 µg/mL (dark trace), were measured by flow cytometry using DCFH staining.

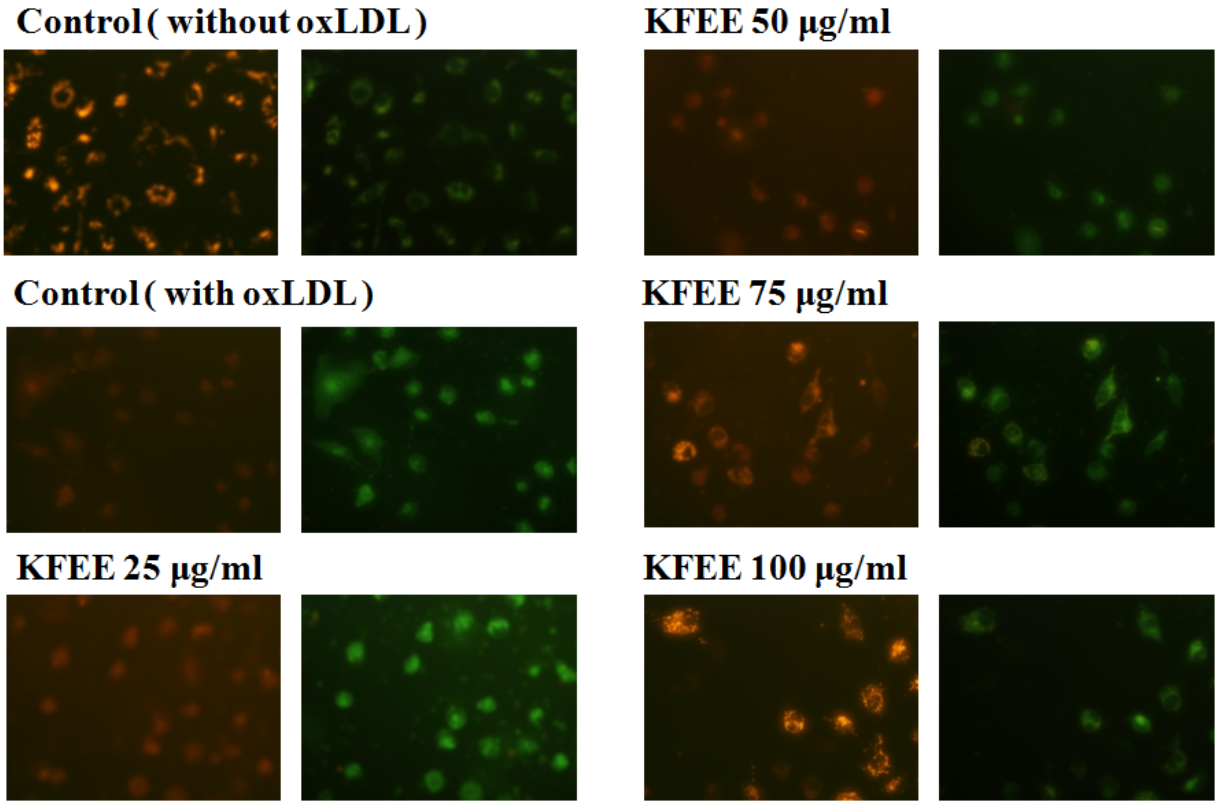

Figure 9. Effects of KFEE on oxLDL-induced changes in the mitochondrial membrane potential in endothelial cells. HUVECs were incubated with oxLDL (200 μg/mL) in the absence and presence of KFEE (25, 50, 75, and 100 μg/mL) for 16 h. The changes of the mitochondrial membrane potential (ΔΨm) were assessed by using fluorescent lipophilic cationic JC-1 dye. JC-1 is selectively accumulated within intact mitochondria to form multimer J-aggregates emitting fluorescence light at 590 nm (red) at a higher membrane potential, left, and monomeric JC-1 emits light at 527 nm (green) at a low membrane potential, right.

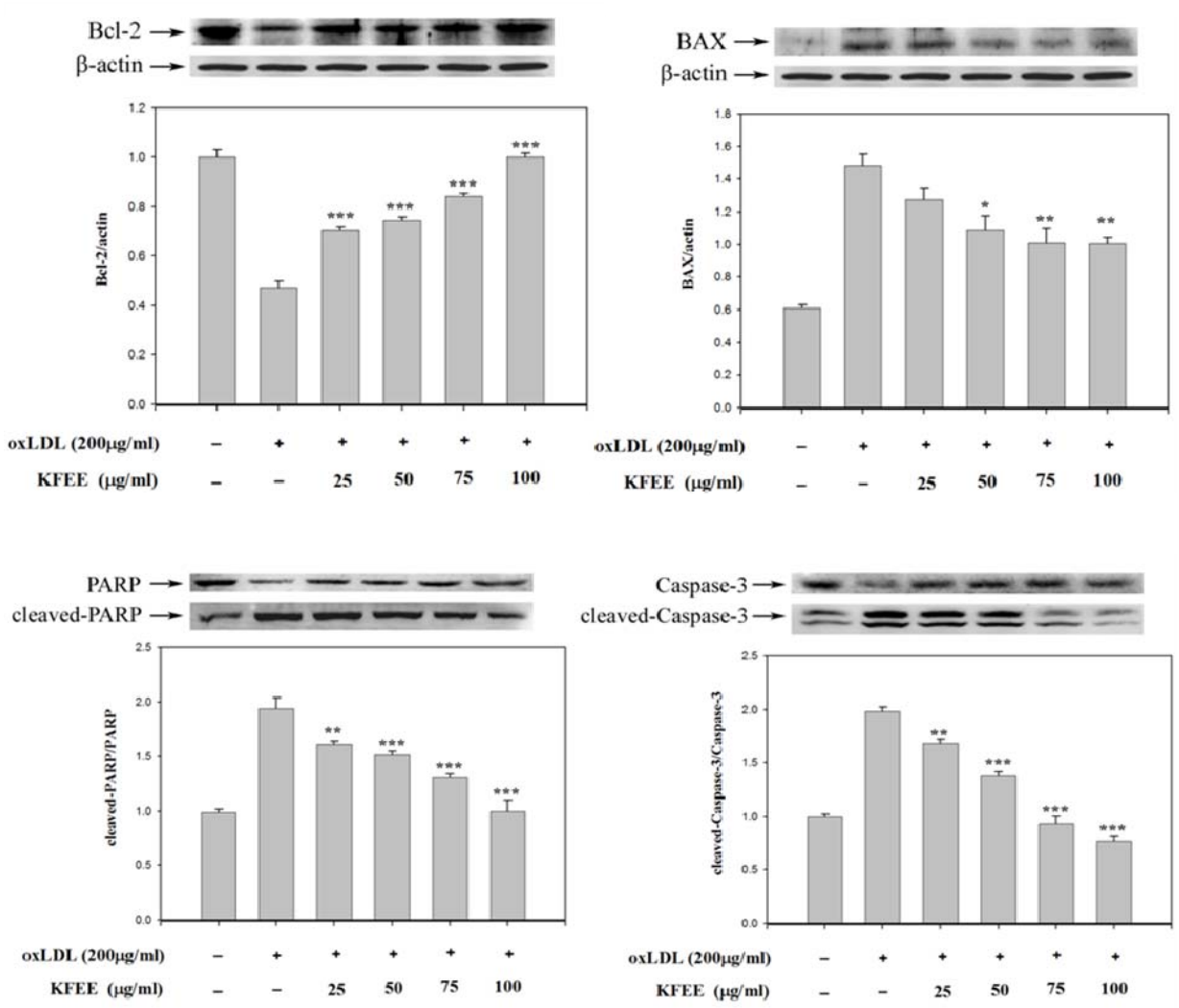

Figure 10. Effects of KFEE on oxLDL-induced caspase-3 and PARP activation. In the Western blot assay, cell lysates were subjected to SDSPAGE, with β-Actin used as an internal control. Signals of proteins were visualized with an ECL detection system. The results were representative of three independent experiments.

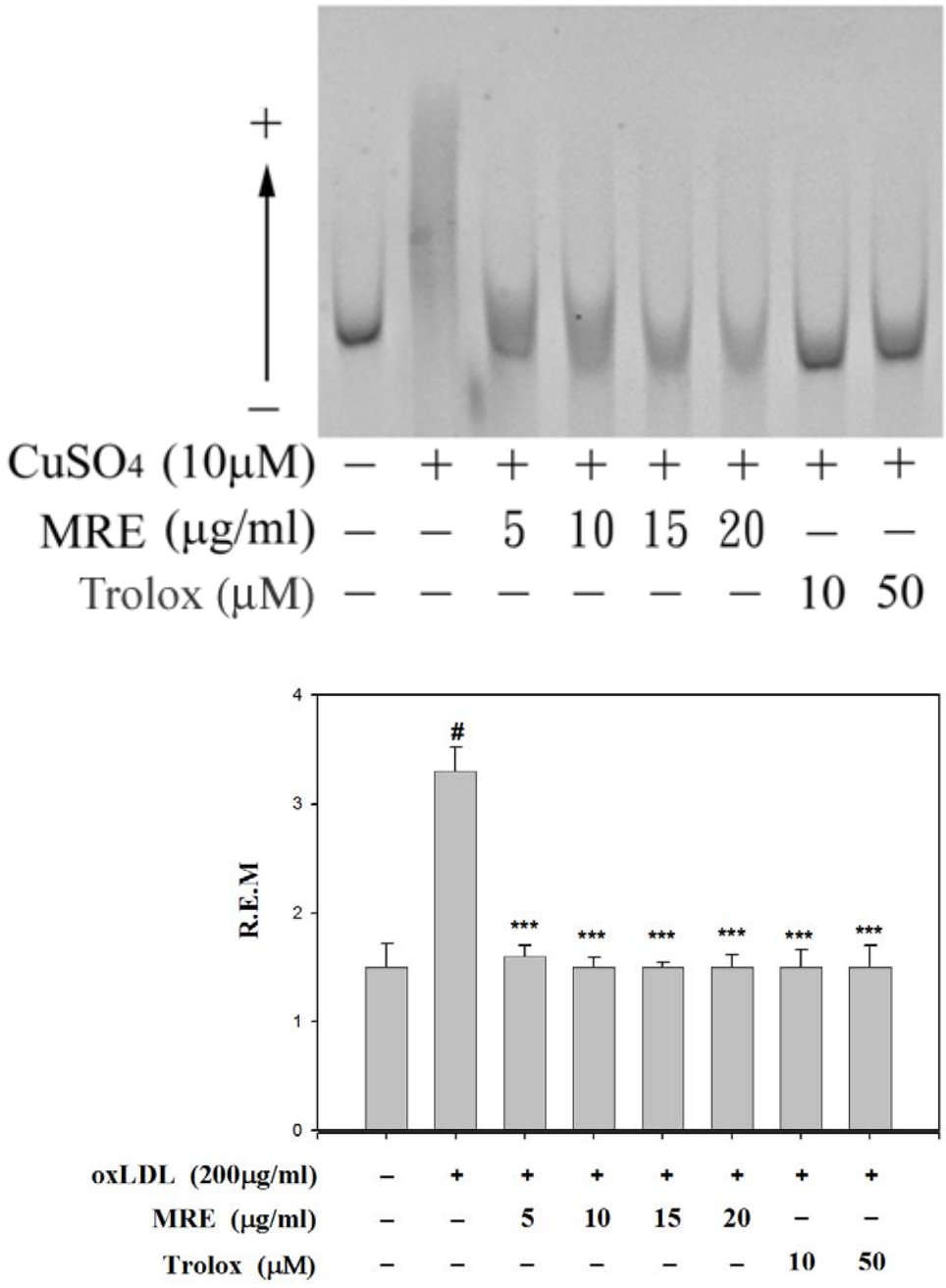

Figure 11. Effect of MRE on the Cu^{2+}-mediated the ApoB fragmentation in LDL. (A) LDL (200 μg/mL) was incubated with 10 μM $CuSO_4$ at 37 ˚C in the absence or presence of MRE or Trolox for 4 h and applied to 7.5% SDS-PAGE as described in the Materials and Methods. (B) Quantification of the ApoB fragmentation assay using densitometry is presented as means ± SD of three independent experiments. The signal intensity of the native LDL was assigned as 100% arbitrarily. #, P < 0.001 compared with control. *, P < 0.05; **, P < 0.01; and ***, P < 0.001 compared with the oxLDL-treated group.

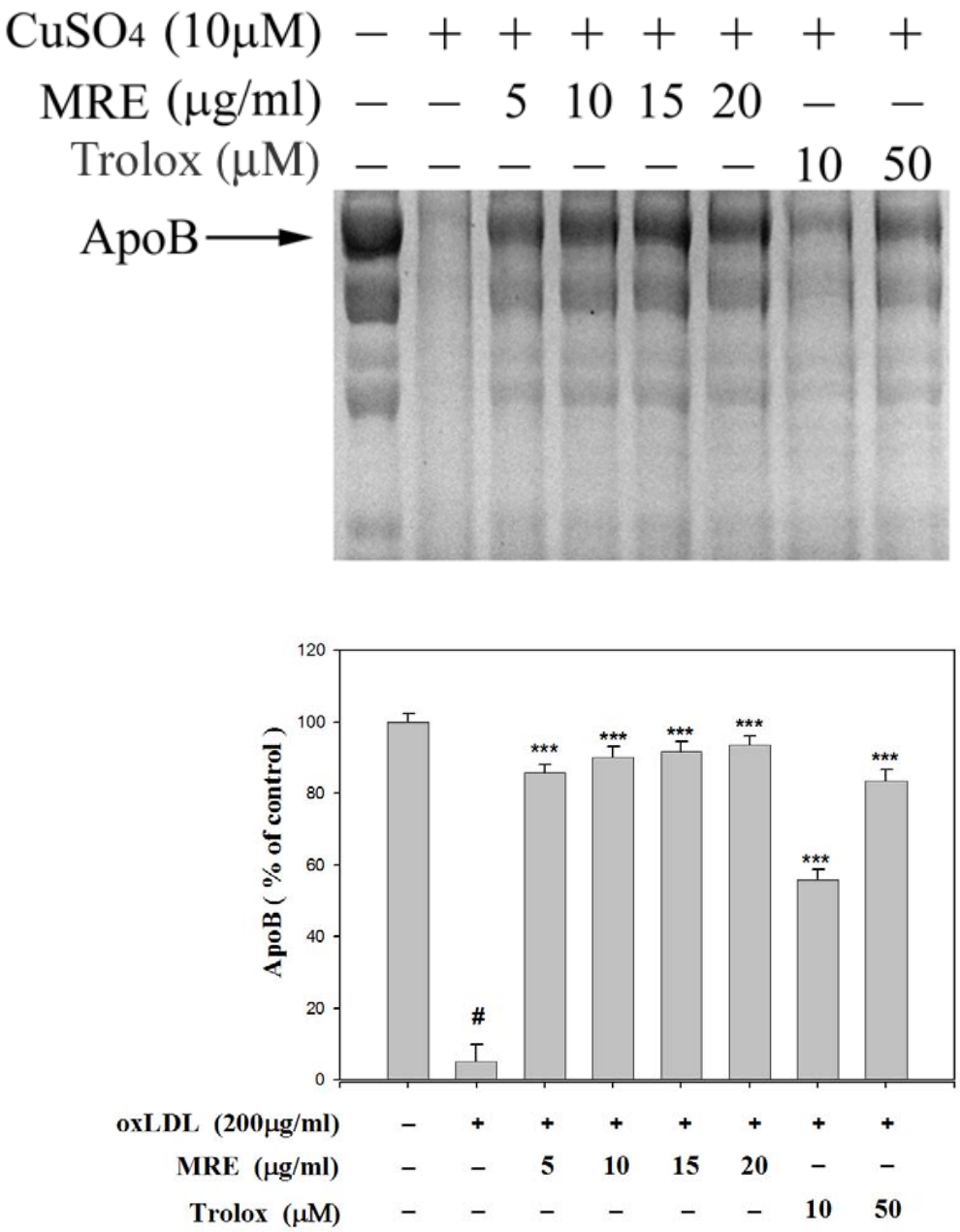

Figure 12. Effect of MRE on the Cu^{2+}-mediated shift of electrophoretic mobility in LDL. (A) LDL was incubated with 10 μM $CuSO_4$ for 16 h at 37 ° C in the presence or absence of MRE or Trolox, as positive control, and applied to 0.6% agarose gels as described in the Materials and Methods. (B) The results from the agarose gel electrophoresis were quantified and expressed in the form of relative electrophoretic mobility (REM). The distance traveled in the agarose gel by the native LDL was assigned the arbitrary unit 1

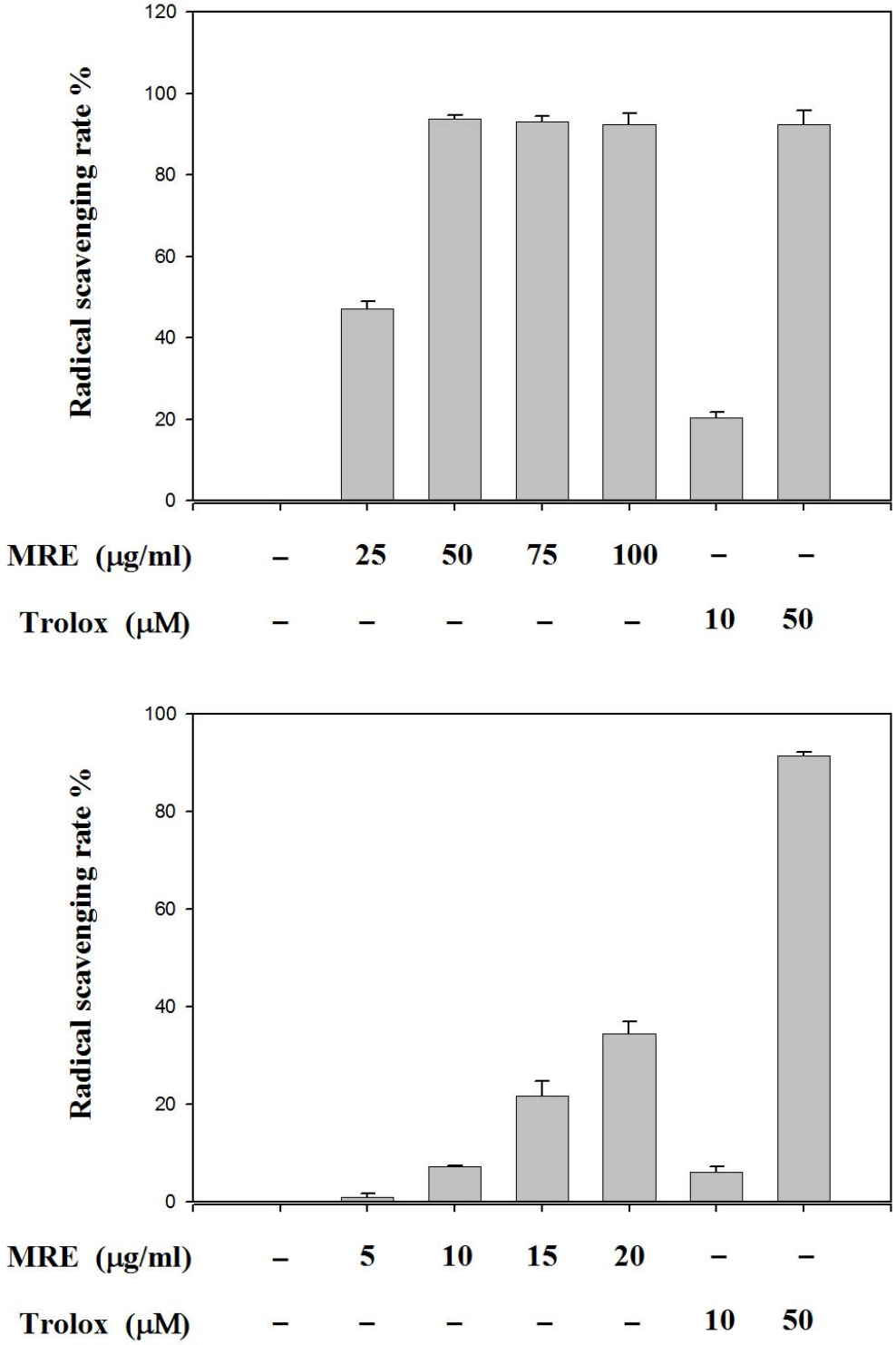

Figure 13. Effect of MRE DPPH scavenging capability. The radical-scavenging activities of MRE and Trolox were evaluated by using the DPPH radical scavenging assay. The absorbance of the sample without adding KFEE or Trolox was assigned as 100%, and its radical scavenging rate was assigned as 0% consequently. The quantitative data were presented as means (SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ compared with the oxidative LDL-treated group.

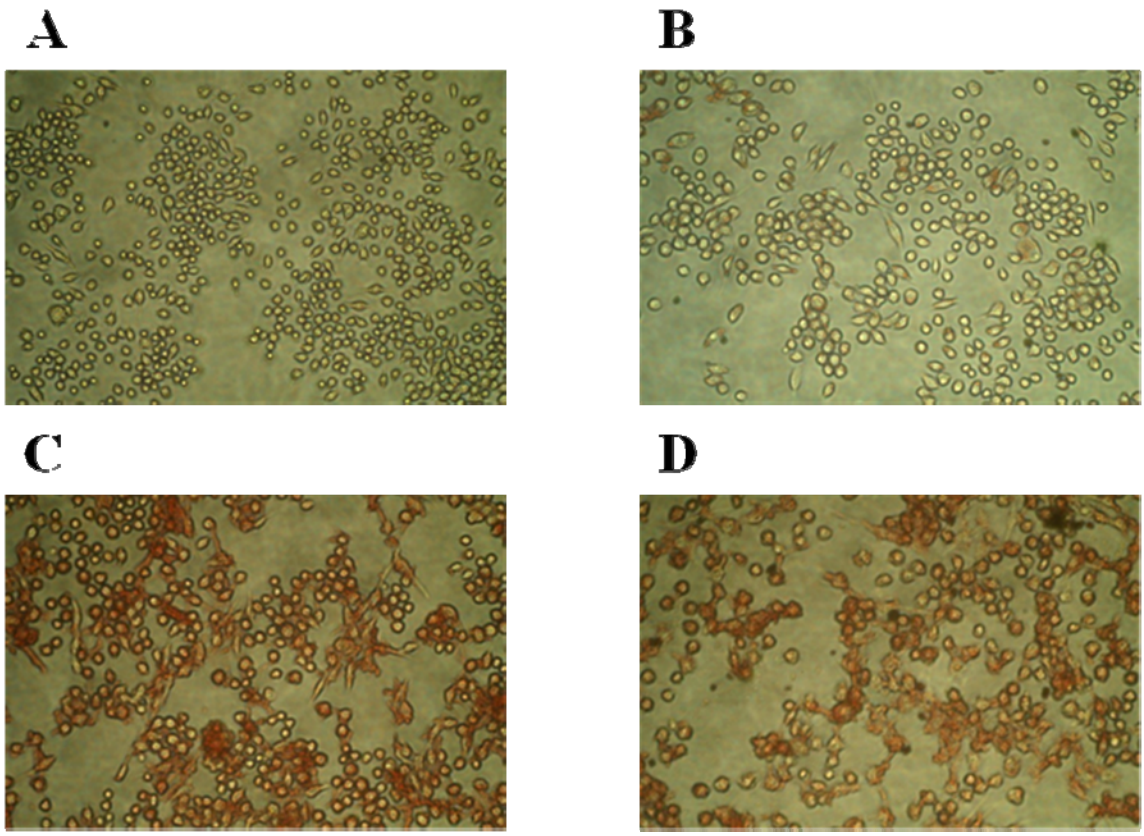

Figure 14. Effect of MRE on lipid accumulation in RAW 264.7 cells. Cells were incubated with copper-mediated LDL (100 μg/mL) or native LDL (nLDL) in the absence or presence of MRE (75 μg/mL) for 24 h. After incubation, cells were washed with PBS and stained with Oil Red O (ORO). Panel A, macrophages (400× magnification); Panel B, macrophages incubated with nLDL; Panel C, macrophages incubated with ox- LDL; Panel D, macrophages incubated with ox-LDL and treated with MRE.

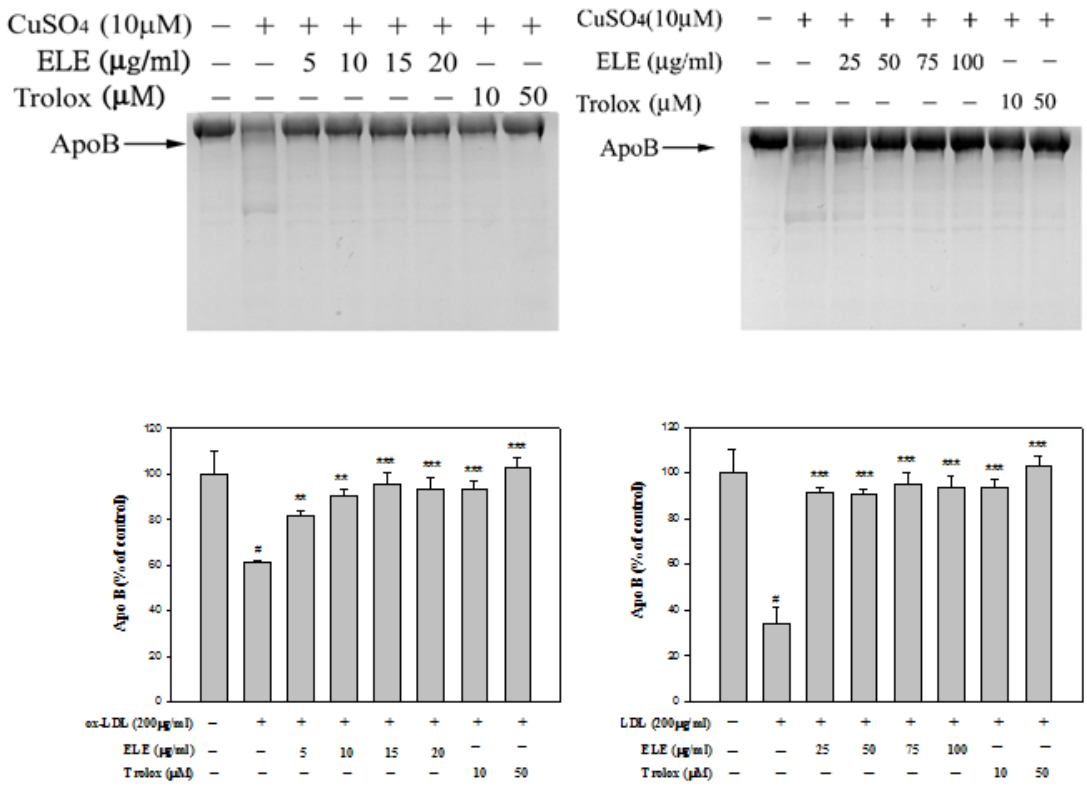

Figure 15. Effect of ELE on the Cu^{2+}-mediated the ApoB fragmentation in LDL. (A) LDL (200 μg/mL) was incubated with 10 μM $CuSO_4$ at 37 ˚C in the absence or presence of KFEE or Trolox for 4 h and applied to 7.5% SDS-PAGE as described in the Materials and Methods. (B) Quantification of the ApoB fragmentation assay using densitometry is presented as means ± SD of three independent experiments. The signal intensity of the native LDL was assigned as 100% arbitrarily. #, P < 0.001 compared with control. *, P < 0.05; **, P < 0.01; and ***, P < 0.001 compared with the oxLDL-treated group.

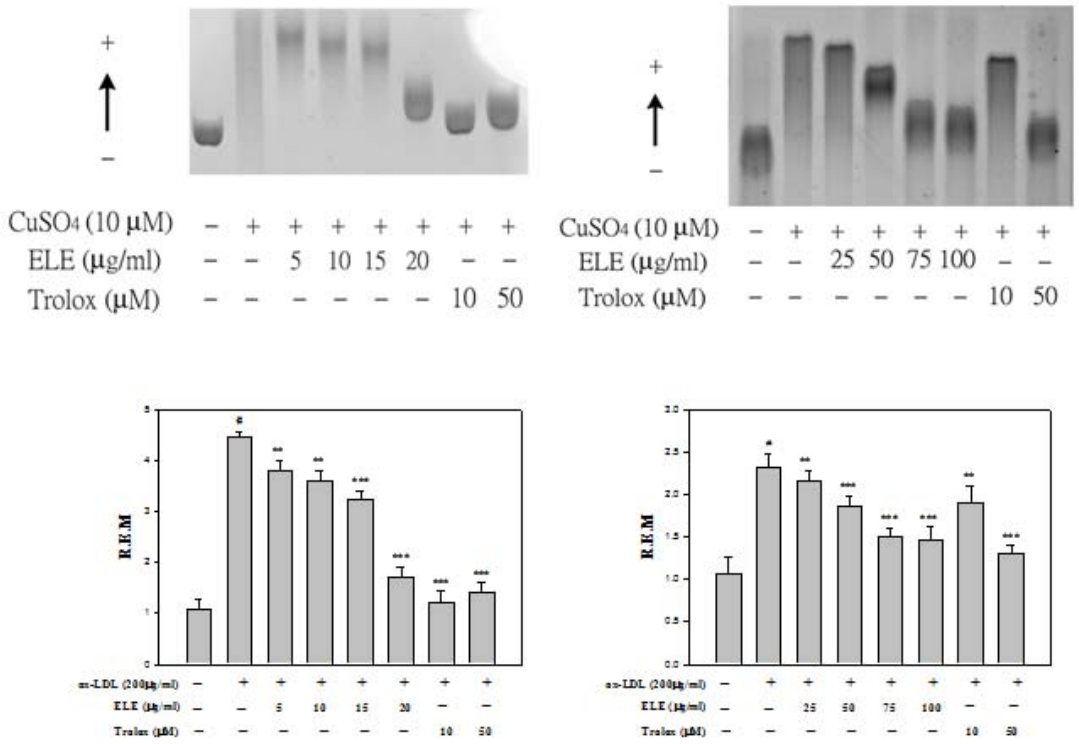

Figure 16. Effect of ELE on the Cu^{2+}-mediated shift of electrophoretic mobility in LDL. (A) LDL was incubated with 10 μM $CuSO_4$ for 16 h at 37 ° C in the presence or absence of ELE or Trolox, as positive control, and applied to 0.6% agarose gels as described in the Materials and Methods. (B) The results from the agarose gel electrophoresis were quantified and expressed in the form of relative electrophoretic mobility (REM). The distance traveled in the agarose gel by the native LDL was assigned the arbitrary unit 1

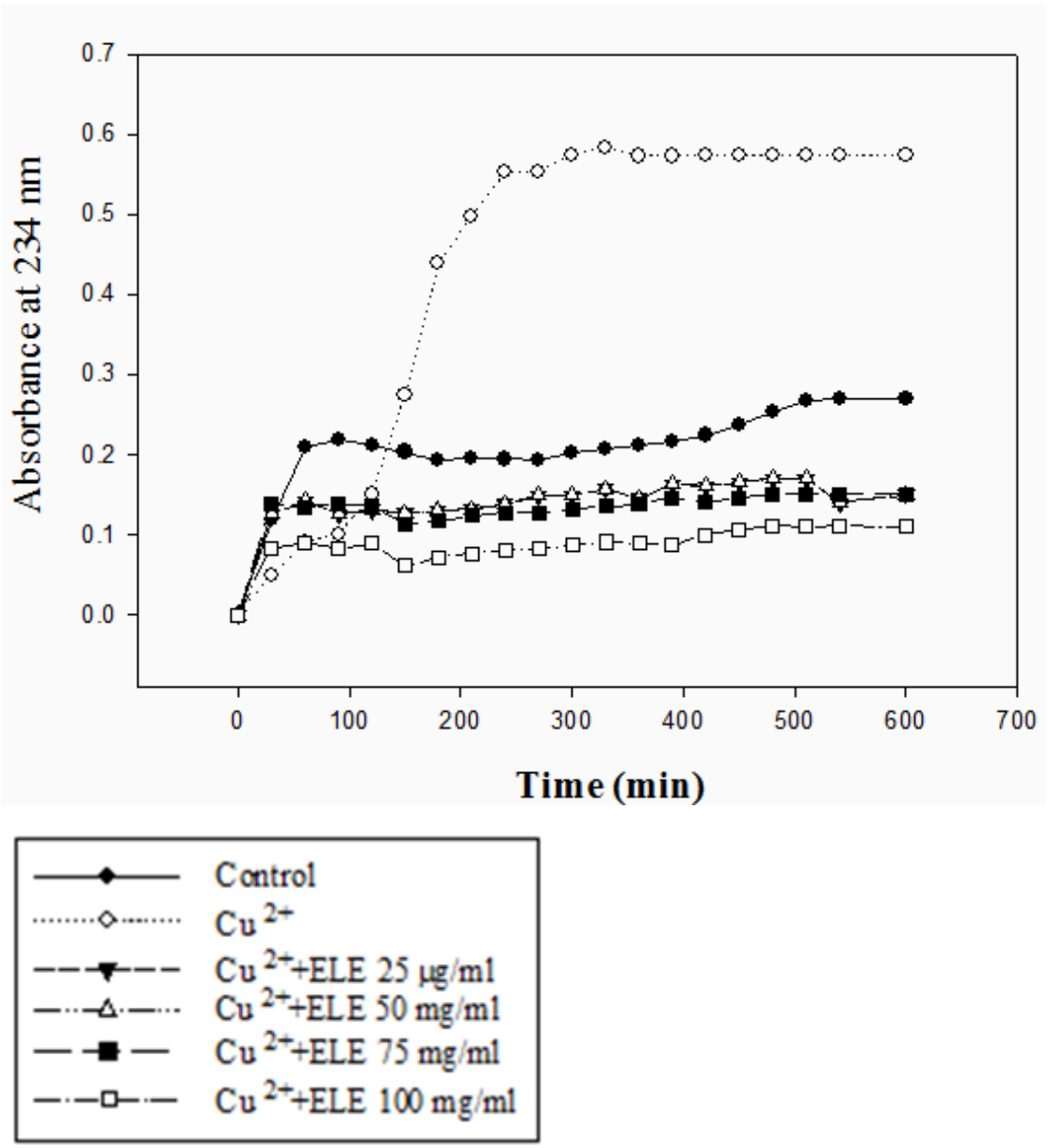

Figure 17. Effect of ELE on copper-mediated LDL oxidation. LDL preincubated with increasing concentration of ELE (25, 50, 75, 100 µg/mL). Oxidation was induced with the addition of $CuSO_4$ (10 µM). The formation of conjugated dienes.

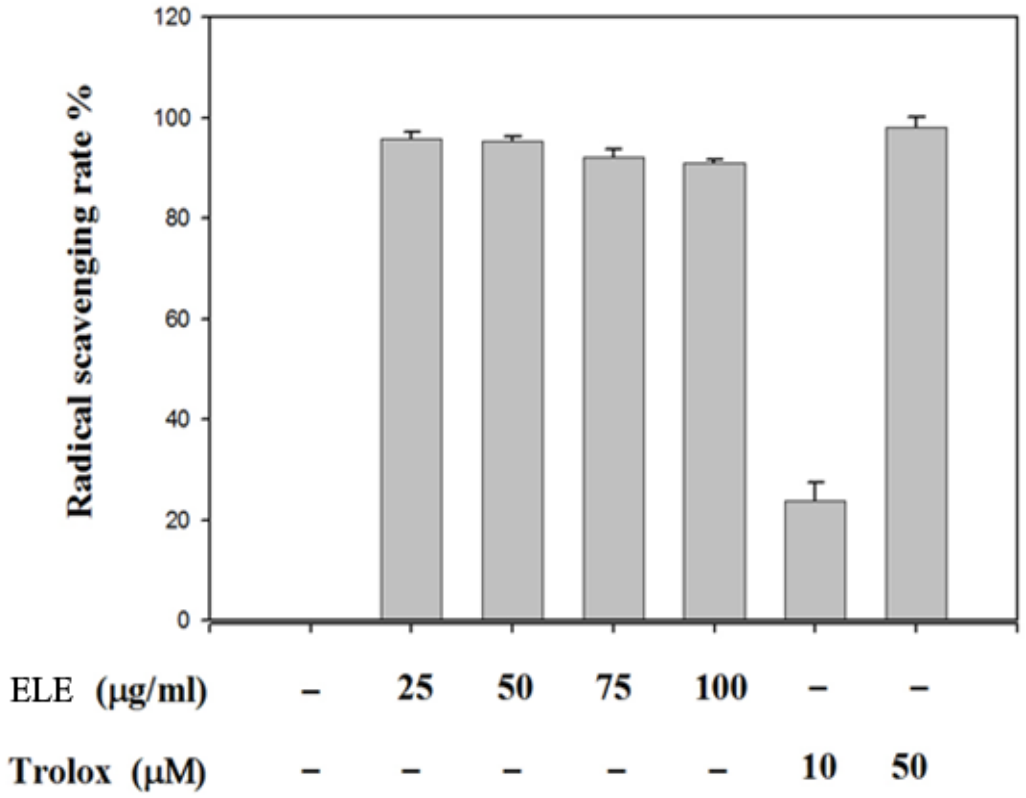

Figure 18. Effect of ELE DPPH scavenging capability. The radical-scavenging activities of ELE and Trolox were evaluated by using the DPPH radical scavenging assay. The absorbance of the sample without adding ELE or Trolox was assigned as 100%, and its radical scavenging rate was assigned as 0% consequently. The quantitative data were presented as means (SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ compared with the oxidative LDL-treated group.

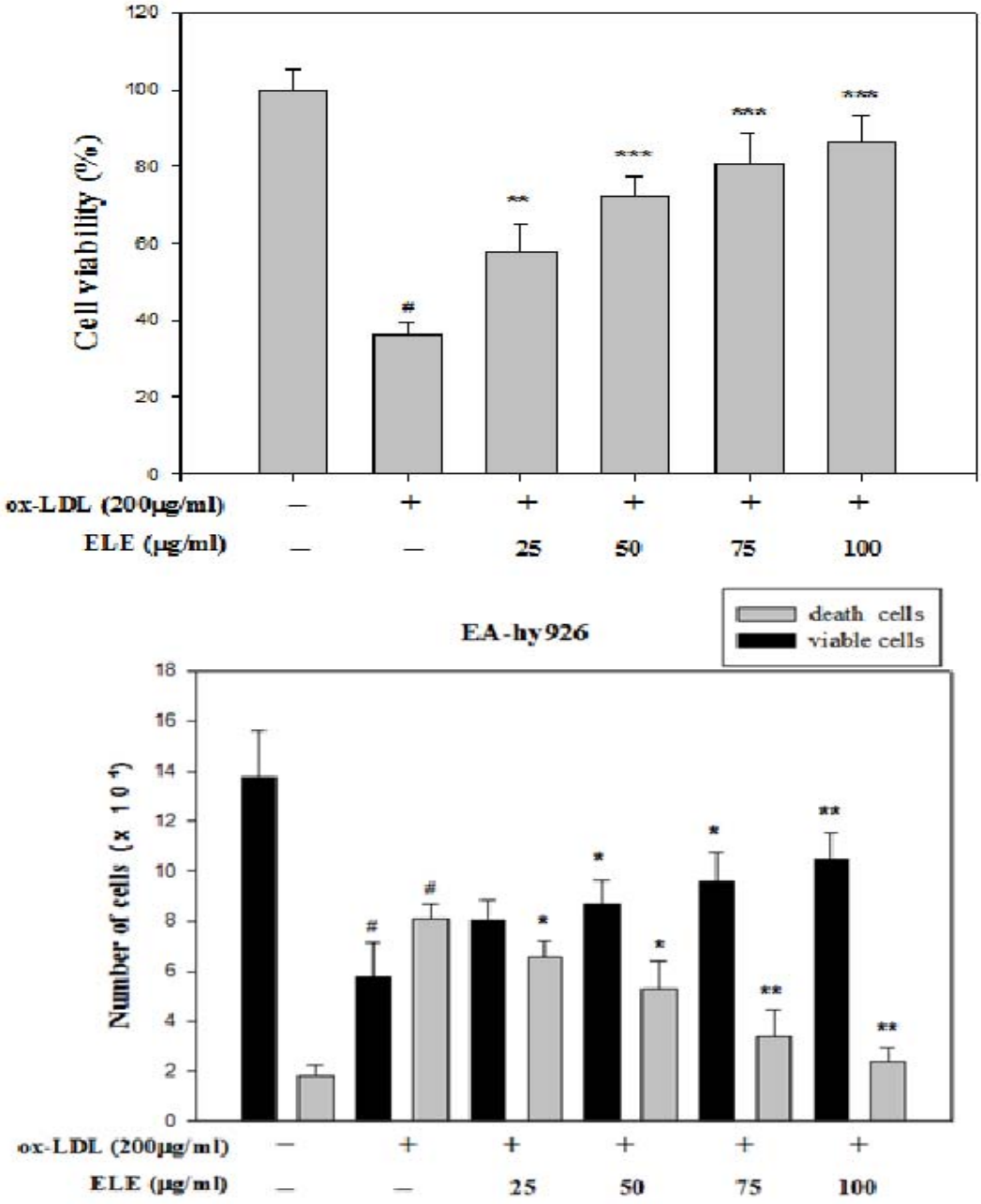

Figure 19. Effect of ELE on oxLDL-induced endothelial cell death. HUVECs were incubated with oxLDL (200 μg/mL) in the absence and presence of ELE (25, 50, 75, and 100 μg/mL) for 16 h. (A) The viability of treated HUVEC cells was detected using the MTT assay as described in the Materials and Methods. (B) Viable cells and dead cells were counted using the Trypan blue exclusion assay. The quantitative data were presented as means (SD of three independent experiments. #, $P < 0.001$ compared with control. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ compared with the oxLDL-treated group.

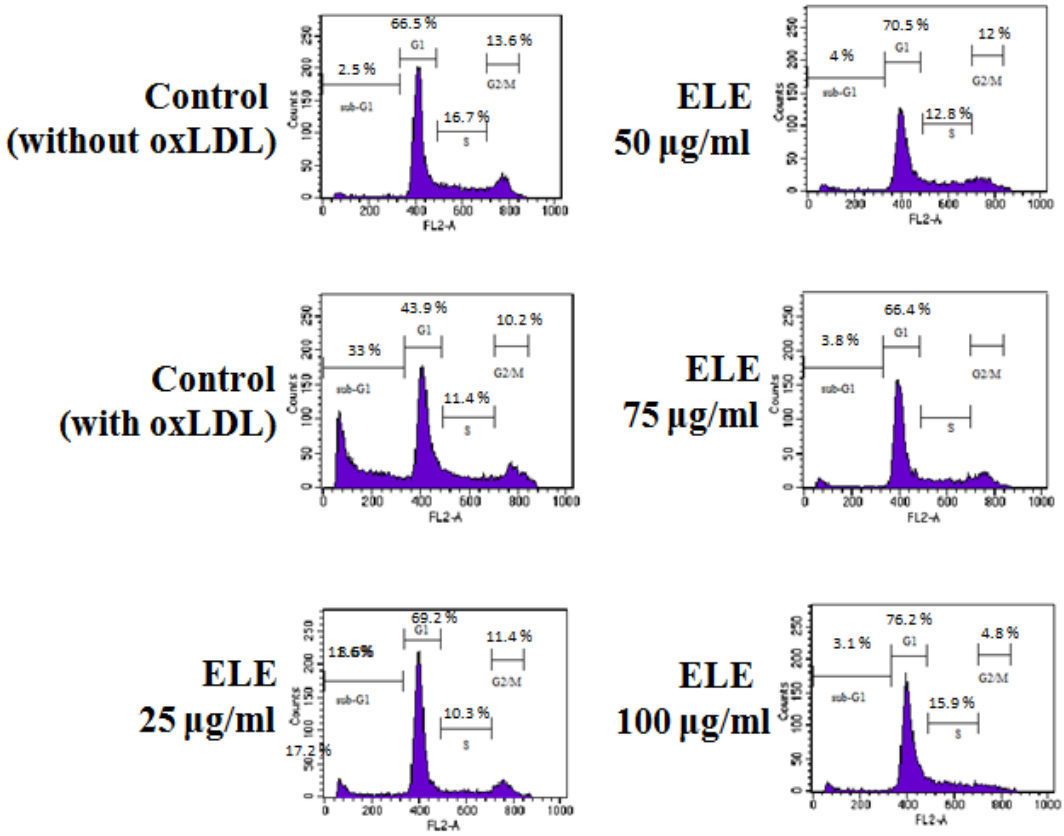

Figure 20. Protective effect of ELE on oxLDL-induced endothelial cell apoptosis. HUVECs were incubated with oxLDL in the presence and absence of ELE or Trolox for 16 h. The hypodiploid cell population (sub G1 phase) of the treated HUVEC cells was analyzed by flow cytometry using PI stain, and at last, 10000 events of total cells were analyzed for each experimental treatment.

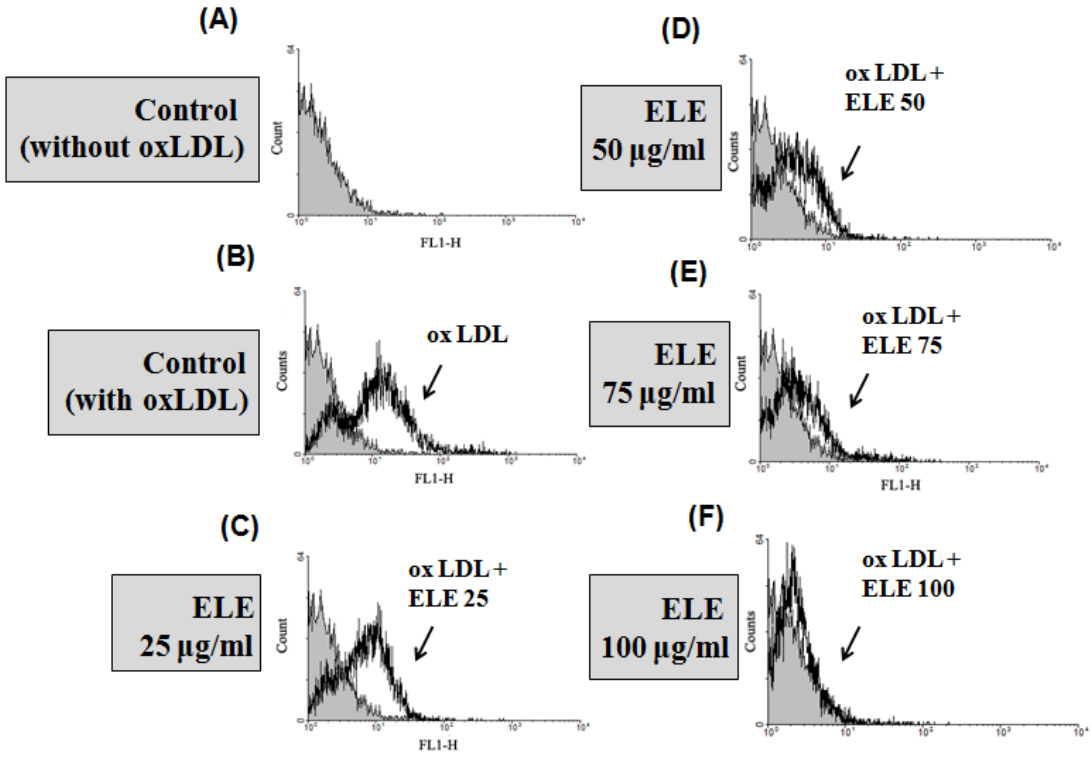

Figure 21. Effects of ELE on oxLDL-induced ROS production in HUVECs. The ROS levels of (A) the HUVEC cells without treatment, control (without oxLDL)(gray trace); (B) the HUVEC cells with treatment of oxLDL(200 µg/mL), control (with oxLDL)(dark trace); (C) the HUVEC cells with treatment of oxLDL and ELE(25 µg/mL), ELE 25 µg/mL (dark trace); (D) the HUVEC cells with treatment of oxLDL and ELE(50 µg/mL), ELE 50 µg/mL (dark trace); (E) the HUVEC cells with treatment of oxLDL and ELE(75 µg/mL), ELE 75 µg/mL (dark trace); and (F) the HUVEC cells with treatment of oxLDL and ELE(100 µg/mL), ELE 100 µg/mL (dark trace), were measured by flow cytometry using DCFH staining.

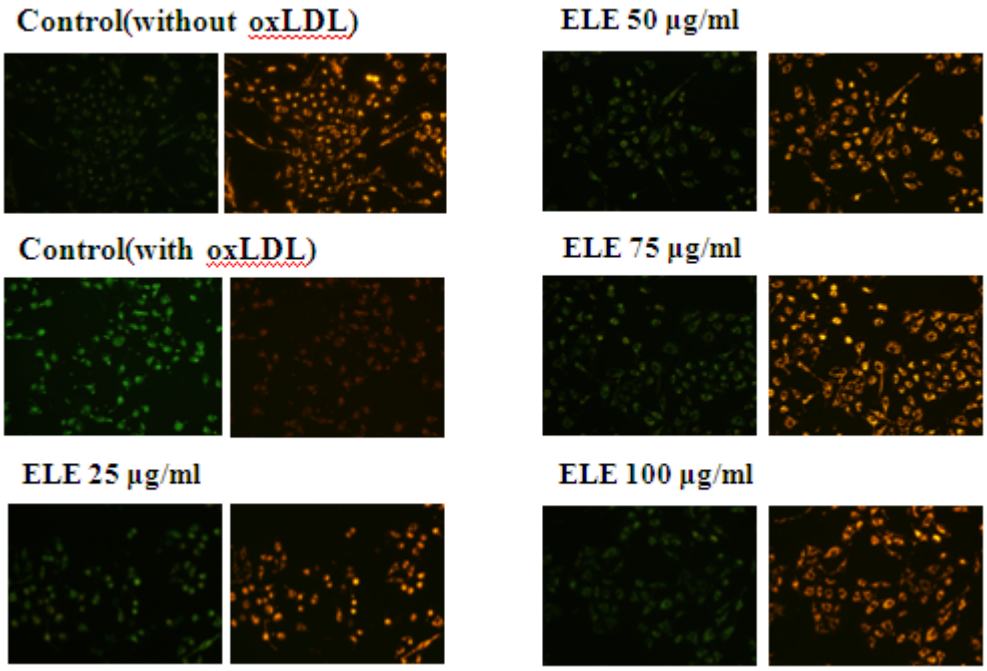

Figure 22. Effects of ELE on oxLDL-induced changes in the mitochondrial membrane potential in endothelial cells. HUVECs were incubated with oxLDL (200 µg/mL) in the absence and presence of ELE (25, 50, 75, and 100 µg/mL) for 16 h. The changes of the mitochondrial membrane potential (ΔΨm) were assessed by using fluorescent lipophilic cationic JC-1 dye. JC-1 is selectively accumulated within intact mitochondria to form multimer J-aggregates emitting fluorescence light at 590 nm (red) at a higher membrane potential, left, and monomeric JC-1 emits light at 527 nm (green) at a low membrane potential, right.

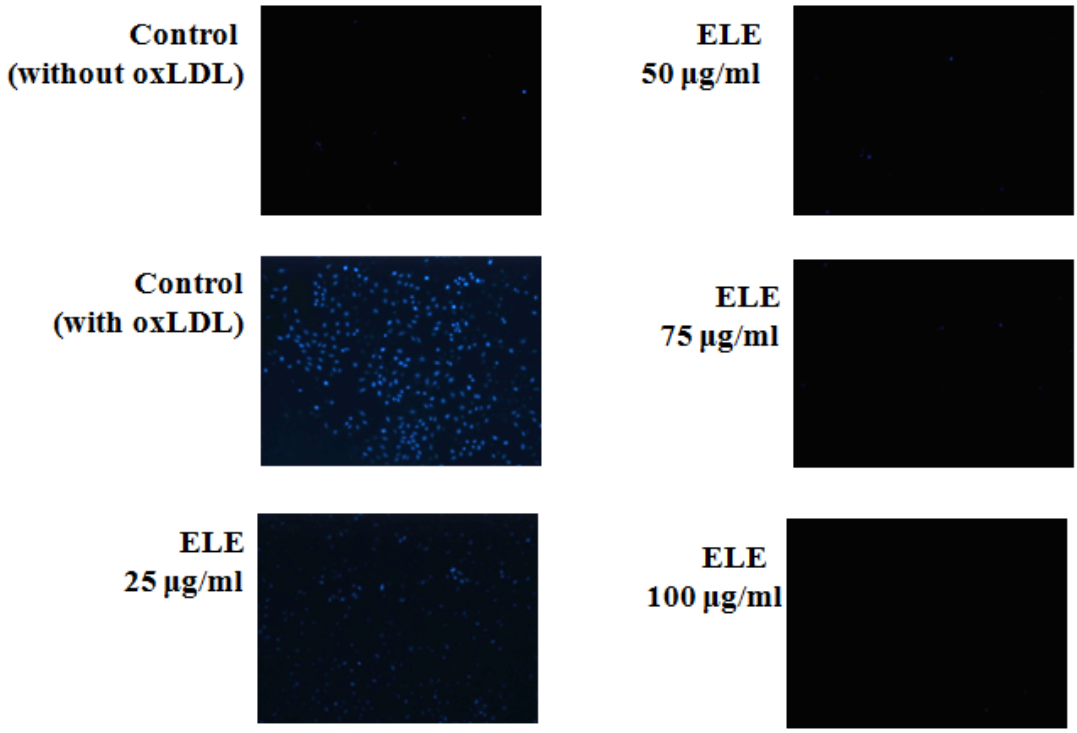

Figure 23. Protective effect of ELE on oxLDL-induced endothelial cell apoptosis. HUVECs were incubated with oxLDL in the presence and absence of ELE or Trolox for 16 h. The nuclear morphology of the treated cells was observed by fluorescence microscopy using DAPI stain (at a magnification of 200×). Arrows showed areas of intense fluorescence staining with condensed nuclei.