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植物化學物質對前發炎與抗發炎訊息傳遞途徑於HAEC與HASMC模式之影響與機制

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中文摘要

本研究主要探討植物化學物質 chlorophyll a、chlorophyll b、chlorophyllin、quercetin cyanidin 對前發炎與抗發炎訊息傳遞途徑相關因子於 HAEC 模式之影響。前發炎因子包括：IL-8、CD40；核轉錄因子包括：NF-κB、AP-1 與 STAT3；活化轉錄因子受體：PPARα 與 PPARγ。以 10μM chlorophyll a、chlorophyll b、chlorophyllin、quercetin、cyanidin and aspirin 處理 HAEC (human aortic endothelial cell)18 小時後，並以 2ng/mL TNF-α 誘導 6 小時，以 ELISA 分析 IL-8、以 Cell Flow Cytometry 偵測 CD40，使用 EMSA (Electrophoretic Mobility Shift Assays)偵測前發炎與抗發炎基因的活化區與 NF-κB、AP-1、STAT3 結合的情形，使用 Western Blotting 分析蛋白質表現。結果顯示 chlorophyll a、 chlorophyll b and chlorophyllin 顯著降低 IL-8（$P<0.05$）與 CD40 表現。同時 quercetin、chlorophyll b 顯著降低 TNF-α 所引起 NF-kB 於細胞質與細胞核內的活化。EMSA 印證 chlorophyllin、chlorophyll b、quercetin 與 cyanidin 顯著降低 NF-kB 於細胞核內的活化。同時從 Western Blotting 與 EMSA 顯示 quercetin 、chlorophyll a、chlorophyll b、chlorophyllin 可抑制 NF-κB p65、AP-1 與 STAT3 的活性。另外 PPARα 和 PPARγ 亦受到 chlorophyllin、chlorophyll b、quercetin 與 cyanidin 的影響。是以植物化學物質對於阻斷發炎相關因子具有相當程度影響。

關鍵詞：植物化學物質、HAEC、NF-κB、STAT3、PPARα/γ

Abstract

This research discussed the pytochemical such as chlorophyllin, chlorophyll a, chlorophyll b, quercetin, cyanidin effects on pro-inflammatory and anti-inflammatory signaling pathways in human aortic endothelial cells (HAEC) model and its underlying mechanisms.

The pro-inflammatory factors include interleukin 8(IL-8), CD40; the nuclear transcription factors include nuclear factor-kappa B (NF-κB), activating protein-1(AP-1) and signal transducer and activator of transcription 3 (STAT3); the activated transcription factors of receptor include proxisome poliferator-activated receptor α (PPARα) and proxisome poliferator-activated receptor γ (PPARγ).

After pretreated of HAEC with 10μM chlorophyll a, chlorophyll b, chlorophyllin, quercetin, cyanidin and aspirin for 18hours, we used 2ng/mL tumor necrosis factor-alpha (TNF-α) to induce HAEC for 6 hours. By enzyme-linked immunoassay (ELISA) to analyze IL-8; by cell flow cytometry to detect surface marker CD40; using electrophoretic mobility shift assays (EMSA) to determinate the active site of pro-inflammatory and anti-inflammatory gene that bind NF-κB, AP-1, STAT3; by western blotting to measure the protein expression in the system.

The result of this research showed that chlorophyll a, chlorophyll b and chlorophyllin significantly attenuated expressions of IL-8 ($P<0.05$) and CD40. Simultaneously, quercetin, chlorophyll b significantly decreased the TNF-α induced expression of NF-κB p65 in nuclear compartment, especially confirmed by the EMSA result showed that chlorophyllin, chlorophyll b, quercetin and cyanidin significantly decreased expression of NF-κB in nuclear compartment. Meanwhiles, results of western blotting and EMSA showed that quercetin, chlorophyll a, chlorophyll b, chlorophyllin also attenuated NF-κB, AP-1 and STAT3 activity. Moreover, chlorophyllin, chlorophyll b, quercetin and cyanidin could influence PPARα and PPARγ. Therefore phytochemicals blocked inflammatory factors with large degree of effects.

Key word: phytochemicals, HAEC, NF-κB, STAT3, PPARα/γ

Intruduction

Inflammation is thought to promote atherogenesis (Palinski, 2003). Atherosclerosis is a chronic inflammatory process with increased oxidative stress in which the adhesion of monocytes to the vascular endothelium and their subsequent migration into the vessel wall are the pivotal early events in atherogenesis (Libby, 1995; Ross, 1993). Inflammatory cytokines such as TNF-α could activate NF- κ B (Baeuerle and Baltimore, 1996; DiDonato *et al.*, 1997) and AP-1 (Kyriakis, 1999; Zhu *et al*., 1998; Martin *et al*., 1997), the 2 major redox-sensitive eukaryotic transcription factors that regulate genes relevant to the expression of adhesion molecules (Muller *et al.*, 1997; Manna *et al*., 1998). Because the activation of NF- κ B or AP-1 could be inhibited to various degrees by different antioxidants, it is strongly suggested that endogenous reactive oxygen species (ROS) may play an important role in these redox-sensitive transcription pathways in atherogenesis (Muller *et al.*, 1997; Manna *et al*., 1998; Palinski, 2003).

In the present study, the effects of chla, chlb, cyanidin and quercetin on TNF-α-induced NF- κ B activation were analyzed along with the NF- κ B downstream target genes of IL-8 and CD40. Our results show that pytochemicals treatment of HAEC inhibits the expression of these genes via suppression of the AP-1, NF- κ B and STAT3 signaling pathways and support the notion of the potential for developing pytochemicals as an anti-inflammatory for therapeutic use particularly in cytokine-induced vascular disorders.

Object of Studies

The objective of the studies is:

To investigate the effects of phytochemicals such as: chla, chlb, cyanidin and quercetin on TNF− α -induced expression of pro-inflammatory and anti-inflammatory signaling pathways in HAEC model. The response factors as follows:

1. Proinflammatory factors: IL-8 and CD40.
2. MAPK: P38, p ERK1/2.
3. Nuclear transcription factors: NF-κB, AP-1 and STAT3.
4. Aactivated transcription factors of receptor: proxisome poliferator-activated receptor α (PPARα) and proxisome poliferator-activated receptor γ (PPARγ).

It is expected to evaluate the effects of pytochemicals on antiatherogenesis by regulating signal trasduction pathways, by playing a negative modulator of inflammation. Therefore, the phytochemicals may propose the therapeutic strategies to combat atherosclerosis.

Literature Review.

Atherosclerosis is a chronic inflammatory process (Palinski, 2003) with increased oxidative stress in which the adhesion of monocytes to the vascular endothelium and their subsequent migration into the vessel wall are the pivotal early events in atherogenesis (Libby, 1995; Ross, 1993). The interaction between monocytes and vascular endothelial cells could be mediated by adhesion molecules including vascular cell adhesion molecule (VCAM- 1)

(Cybulsky and Gimbrone, 1991), intercellular adhesion molecule 1 (ICAM-1) (Poston *et al.*, 1992), and E-selectin (Richardson *et al*., 1994) on the surface of the vascular endothelium. Inflammatory cytokines such as TNF-α could activate NF- κ B (Baeuerle and Baltimore, 1996; DiDonato *et al.*, 1997) and AP-1 (Kyriakis, 1999; Zhu *et al*., 1998; Martin *et al*., 1997), the 2 major redox-sensitive eukaryotic transcription factors that regulate genes relevant to the expression of adhesion molecules (Muller *et al.*, 1997; Manna *et al*., 1998). Because the activation of NF- κ B or AP-1 could be inhibited to various degrees by different antioxidants, it is strongly suggested that ROS may play an important role in these redox-sensitive transcription pathways in atherogenesis (Muller *et al.*, 1997; Manna *et al*., 1998; Palinski, 2003).

Cells exposed to various stimuli trigger an increase of ROS that modify proteins via phosphorylation of signaling molecules involved in the ERK **(**extracellular signal-regulated kinase), c-Jun N-terminal kinase (JNK) and p38 mitogen-activiated protein kinase (p38) pathways (Saito *et al.*, 2002). Most prominent among the oxidation-sensitive pathway is the NF- κ B system, which regulates leukocyte adhesion molecules, such as ICAM-1, VCAM-1, platelet/endothelial cell adhesion molecule-1 (PECAM-1), P-selectin and E-selectin (Gerard and Bollins, 2001; Lusis, 2002) and chemokines, growth-promoting and antiapoptotic factors, but also some proinflammatory and prothrombotic factors (Collins and Cybulsky, 2001). The antioxidants apparently inhibit NF-κB activation in macrophage and release of ROS in endothelial cells (Erl *et al.*, 1997). By diminishing NF- κ B activation, the antioxidants vitamins would diminish the cellular response to oxLDL, reducing monocyte adhesion, foam-cell formation, and cytotoxicity to vascular cells (Collins and Cybulsky, 2001). The proinflammatory factors included: TNF-α, interferon- γ (IFN- γ), interleukin -1(IL-1), interleukin -8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), macrophage-colony stimulating factor (M-CSF), cyclooxygenase-2 (COX-2), nitric oxide synthase (NOS) and CD40 (Gerard and Bollins, 2001; Lusis, 2002). These proinflammatory factors can module CD40 that can enhance the adhesion molecules, such as ICAM-1, VCAM-1 and E-selectin expression (Hollenbaugh *et al.*, 1995; Karmann *et al.*, 1995).

Kim et al. (2006) reported that anthcyanidin inhibited ICAM-1 and VCAM-1 expression through inhibited the nuclear appearance of NF- κ B. Quercetin, the most abundant flavonoid in the human diet and is an excellent free radical scavenging antioxidant **(**Ross and Kasum, 2002) attenuated expression of ICAM-1 and E-selectin in HAEC (Lotito and Frei, 2006) while De Stefano *et al.*(2007) further demonstrated that quercetin decreased the activities of iNOS (inducible nitric oxide synthase), COX-2, NF- κ B, p65/p50 NF- κ B, IRF-γ (interferon regulatory factor-γ) and STAT-1α (signal transducer and activator of transcription-1α) in RAW 264.7 macrophages. Min et al. (2007) demonstrated that quercetin attenuated PMACI-induced activation of NF-κB and p38 but not JNK or ERK. Recently Lee et al. (2008) reported that quercetin attenuated PMA-induced NF- κ B, AP-1, p-ERK, p-MEK activities and suggested that quercetin inhibited mitogen-activated protein kinase / ERK kinase (MEK) 1 activity through

formed a hydrogen bond with the backbone amide group of Ser^{212}, which inactive the activation loop of MEK1. Garciau-Mediavilla et al. (2007) further shown inhibitory effects by quercetin and kaempferol on NF-κB activation and protein concentration of the phosphorylated form of the inhibitory protein of nuclear factor-$\kappa B\alpha$ (I$\kappa B\alpha$) and of IκB kinase α (IKK α).

Materials and Methods

Cell Cultures

Human aortic endothelial cells (HAEC, Clonetics) were grown in Medium 200 (Cascade Biologics) supplemented with low serum growth supplement (Cascade Biologics) in an atmosphere of 95% air and 5% CO2 at 37°C in plastic flasks. The final concentrations of the components in Medium 200 contained 2% FBS (Gibco-BRL), 1 μg/mL hydrocortisone, 10 ng/mL human epidermal growth factor, 3 ng/mL human fibroblast growth factor, 10μg/mL heparin, and 1% antibiotic-antimycotic mixture (GibcoBRL) (Vielma, 2004). The human monocytic cell line U937 (American Type Culture Collection) was grown in suspension culture in RPMI-1640 (GibcoBRL) containing 10% FBS and 1% antibiotic-antimycotic mixture in an atmosphere of 95% air and 5% CO2 at 37°C. After incubation with pytochemicals and aspirin, or TNF-α, cell viability was always greater than 90% by using trypan blue exclusion method or MTT assay.

Cell Enzyme–Linked Immunosorbent Assay

To examine whether pytochemicals and aspirin could modify the expression of IL-8, cell ELISA was conducted. The expression of IL-8 on HAEC surface was quantified as previously described(Kaneko, *et al.*, 1996). Briefly, at 95% confluence in 96-well microplates, antioxidants were added to HAEC 18 hours before activation or during the 6h TNF-α activation period. The monolayers were washed and then incubated with goat anti-human IL-8 monoclonal antibodies (R&D Systems) at a final concentration of 0.5 μg/mL in HBSS containing 1% skim milk to detect the surface expression of these adhesion molecules. After incubation of cells at room temperature for 30 minutes, the plates were washed 4 times with HBSS containing 0.05% Tween-20 and then treated with 0.1 mL/well of peroxidaseconjugated rabbit anti-goat IgG (1:2000 dilution in HBSS containing 1% skim milk). After 1-hour incubation at room temperature, the plates were washed 5 times with HBSS containing 0.05% Tween-20 and incubated at room temperature in 100μL of 3% o-phenylenediamine and 0.03% H_2O_2 in a mixture of 50 mmol/L citrate buffer and 100 mmol/L phosphate buffer, pH 7.4. After incubation for 15 minutes in a dark place, 50μL/well of 2 mol/L H_2SO_4 was added, and spectrophotometric readings were made at 490 nm using a microplate reader. Because the cells were not permeabilized, this ELISA detected cell surface–expressed protein.

Western Blot Analysis

Western blot analysis was conducted to determine whether the changes in expression of nuclear transcriptional factors and MAPKs by pytochemicals and aspirin depend on the changes in amounts of protein synthesis. The total, cytosolic and nuclear-cell lysates were subjected to

SDS-polyacrylamide (12%) gel electrophoresis, followed by electroblotting onto PVDF membrane. Membranes were probed with a mouse or rabbit monoclonal antibody directed to NF-κB p65, PI3K, P38, ERK1/2 (BD Transduction Laboratories, Upstate, Chemicon, Upstate, respectively), Incubate blot in secondary antibody of Goat anti-mouse IRDYE800CW STREPTAVIDIN or Goat anti-rabbit IRDYE680CW STREPTAVIDIN for **60** minutes at room temperature with gentle shaking. Protect from light during incubation and processing. Wash membrane 4 times for 5 minutes each at room temperature in PBS wirh 0.1% Tween-20 with gentle shaking. Rinse membrane with PBS to remove residual Tween-20. The membrane is ready to scan. Using AlphaEaseFC to analysis the spot density and using the internal control as 100 % to calculate the relative sample's density.

Electrophoretic Mobility Shift Assay for NF-κB, AP-1, STAT3

For NF-κB IRDye™ 700 (NF-κB oligo-IRDye™ 700, AP-1 oligo-IRDye™ 700, STAT3 oligo -IRDye™ 700) infrared dye labeled oligonucleotides the binding reaction flow order of added 1μL 10X binding buffer (100mM TRIS, 500mM NaCl, 10mM DTT, pH 7.5), 5μL H_2O, 2μL 25mM DTT/2.5%Tween-20, 1μL oligonucleotide-IRDye 700 (NF-κB oligo-IRDye™ 700, AP-1 oligo-IRDye™ 700, STAT3 oligo -IRDye™ 700), 1μL poly(dI•dC) and 1μL nuclear extract, then incubated at room temperature for 20 min in dark. After the incubation period, 1X Orange Loading Dye (LI-COR) is added to the binding reaction, then load on a gel (4% polyacrylamide) for electrophoresis at 90V for 40 min. Prepare 4% native polyacrylamide gel (40% polyacryamide stock, polyacrylamide-BIS ratio 29:1) containing 50mM Tris, pH 7.5, 0.38M glycine, 2mM EDTA, 10%APS, TEMED and H_2O. Scan the gel inside the glass plates using 1.5 mm focus offset (assuming 1mm thick gel and glass plates are 1 mm thick), and start with Scan Intensity setting of 8 for 700 channels using Odyssey Infrared Imaging System (LI-COR Biosciences). The 22-mer synthetic double-stranded olinucleotides used as NF-κB (5' AGT TGA GGG GAC TTT CCC AGG C 3', 3' CGC TTG ATG ACT CAG CCG GAA 3'**),** AP-1 (5'CGC TTG ATG ACT CAG CCG GAA3'. 3' GCG AAC TAC TGA GTC GGC CTT 5'), STAT3 **(**5'GAT CCT TCT GGG AAT TCC TAG ATC 3', 3' CTA GGA AGA CCC TTA AGG ATC TAG 5') probs in the gel shift assay.

CD40 Flow Cytometry Assay

Flow Cytometry HAEC were analyzed for surface expression of CD40 (Pharmingen) using a FACScan (Becton Dickinson) as described (Ferran, 1993).

Results and Discussion

Cell Enzyme–Linked Immunosorbent Assay

Cell-ELISA showed that pretreated of HAEC with 10 μM quercetin , cyanidin, chlorophyll a, chlorophyll b, chlorophyllin and aspirin for 18h significantly decreased IL-8 by the treatments.

Western Blot Analysis and Electrophoretic Mobility Shift Assay for Nuclear Transcription Factors

Chlorophyll a and chlorophyll b decreased the expression of NF-κB p65 in nuclear compartments. Meanwhile, quercetin and cyanidin significantly decreased the expression of NF-κB p65 in nuclear compartments, especially confirmed by the EMSA result, of witch may further influence on the expression of adhesion molecules and there by may attenuate the atherosclerosis and inflammatory responses. PI3K and p38MAPK expresion were also attenuated by the treatments while ERK1/2 expression increased by chl a and chl b treated.

CD40 Flow Cytometry Assay

CD40 also significantly reduced by the treatments.

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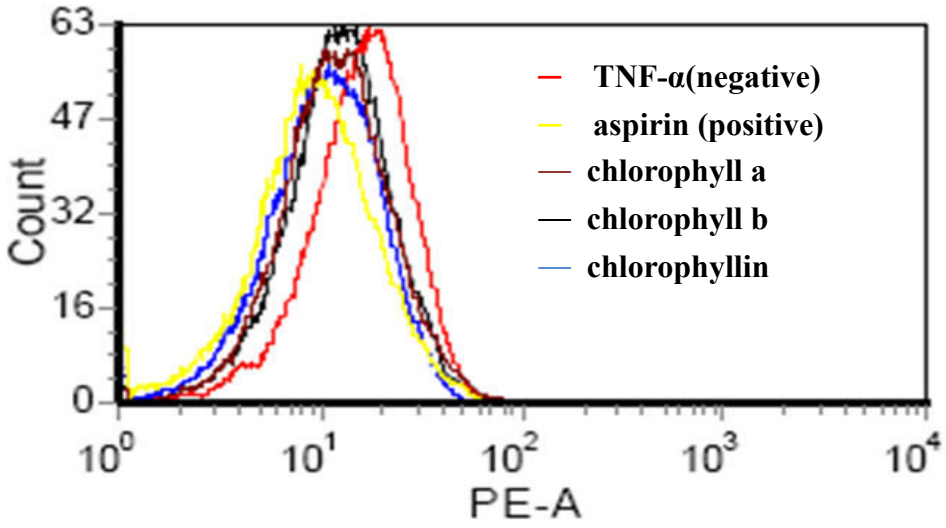
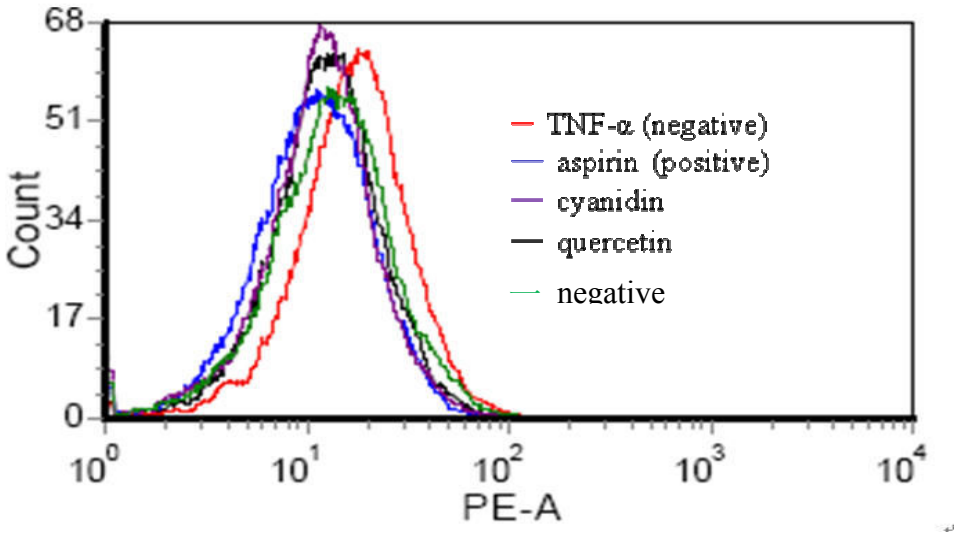
Appendix

(A)



(B)



(C)

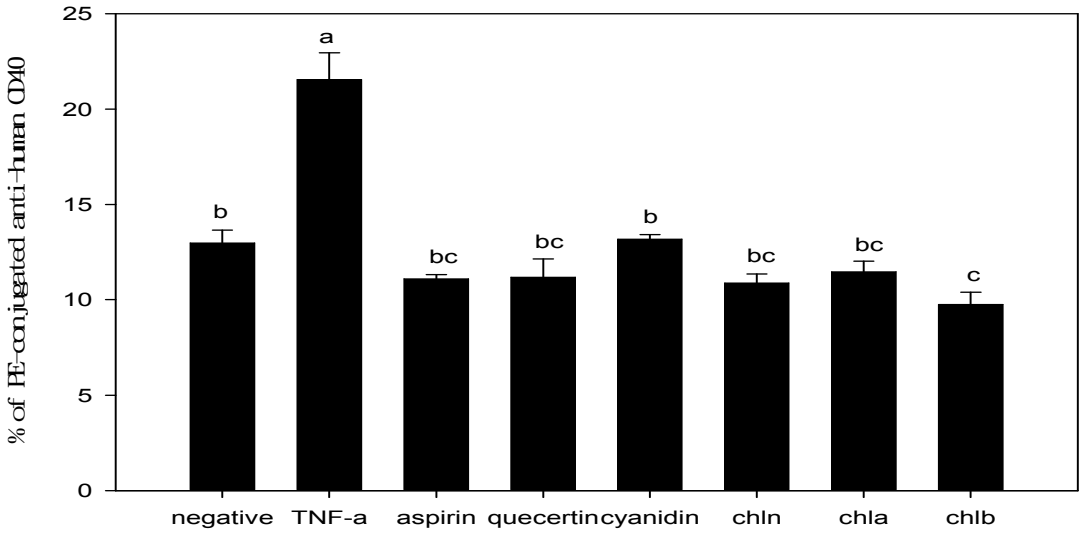
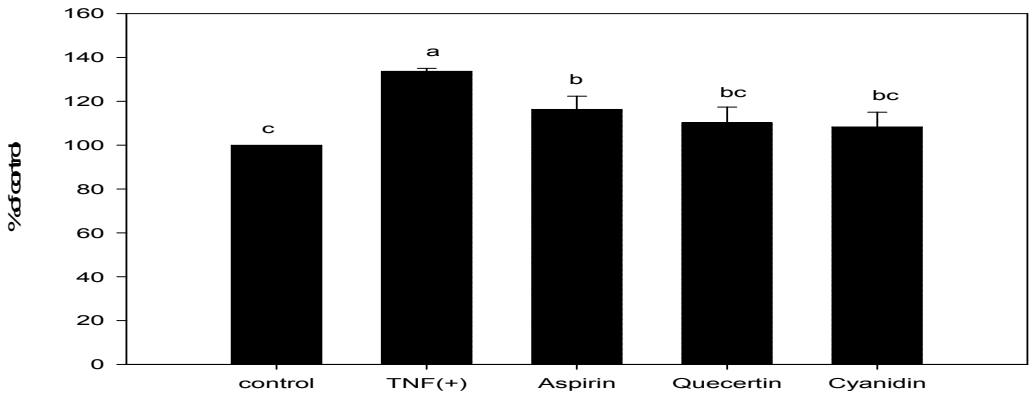


Fig 1. (A)(B)(C) 以 10μM 之 chlorophyll a、chlorophyll b、chlorophyllin、quercetin、cyanidin and aspirin 處理細胞 18 小時後，以 2ng/mL TNF-α 誘導細胞 6 小時後，藉由 PE mouse anti-human CD40 染細胞表面抗原所得到 CD40 的結果。

Fig1(A) Chlorophyll related compounds block upregulation of CD40 expression was quantified by flow cytometry. Green line represent untreated cells, red line represent TNF-α treated cells, yellow line represent aspirin treated cells, maroon line represent chl a treated cells and black line represent chl b treated cells.

Fig1(B) The compounds as described below block upregulation of CD40 expression was quantified by flow cytometry. Green line represent untreated cells, red line represent TNF-α treated cells, blue line represent aspirin treated cells, black line represent qucercetin treated cells and purple line represent cyanidin treated cells.

(A)



(B)

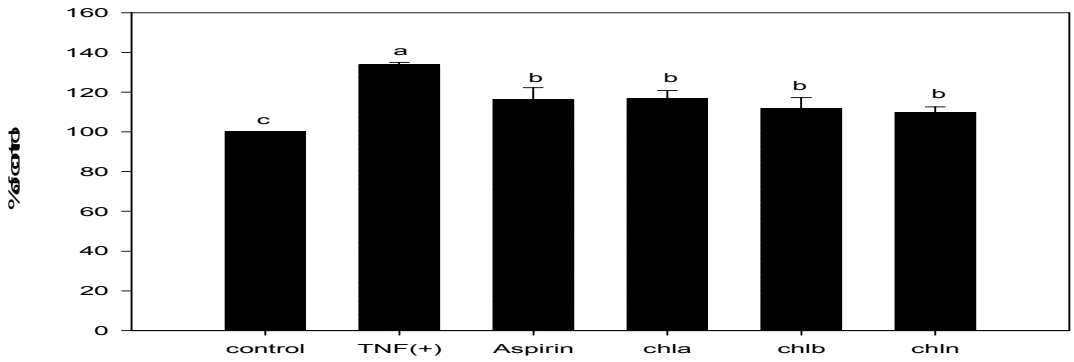
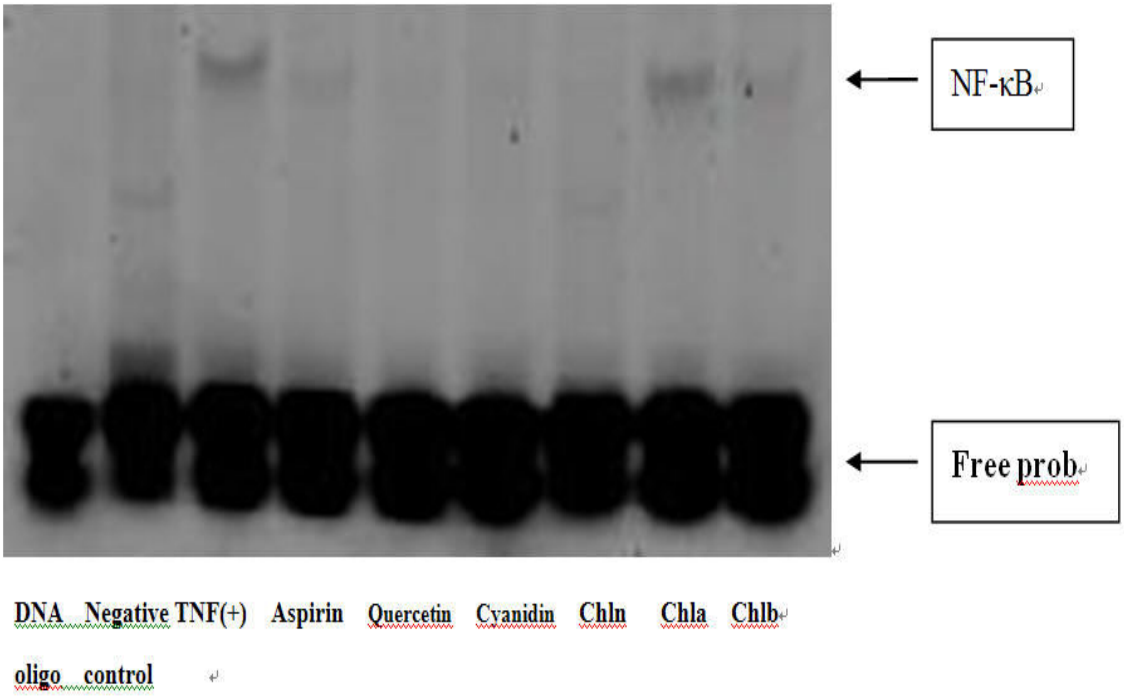


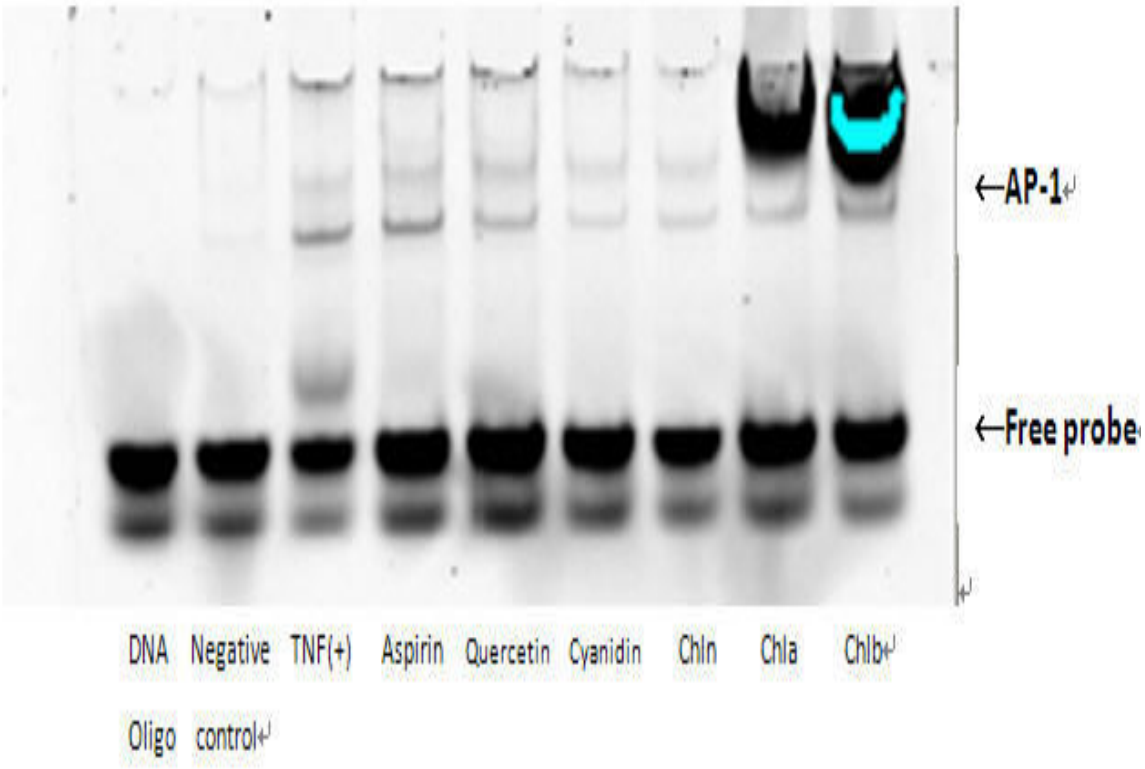
Fig 2. (A)(B) 以 10μM 之 chlorophyll a、chlorophyll b、chlorophyllin、quercetin、cyanidin and aspirin 處理細胞 18 小時後，以 2ng/mL TNF-α 誘導細胞 6 小時，收集細胞分泌至培養基中的 IL-8 以 ELISA 作分析所得知結果。$^{a\sim c}P < 0.05$

Fig 2. (A)(B) HAEC pretreated with the indicated samples for 18h then with TNF-α 2ng mL^{-1} for 6h, and expression of IL-8 was measured by cell-ELISA. Data are expressed as the mean±S.D. of three experiments. Results were statistically significant with different superscripts $^{(a\sim c)}$ at $P < 0.05$.

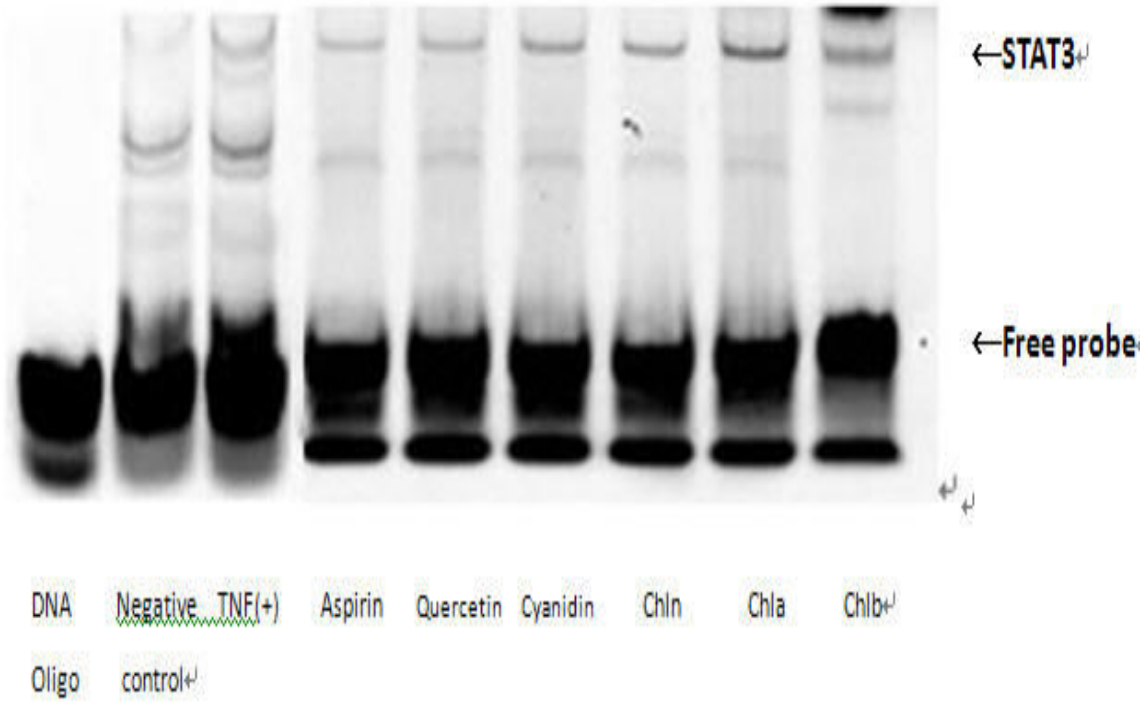
(A)


NF-κB
Free prob
DNA
Negative control
TNF(+)
Aspirin
Quercetin
Cyanidin
Chln
Chla
Chlb

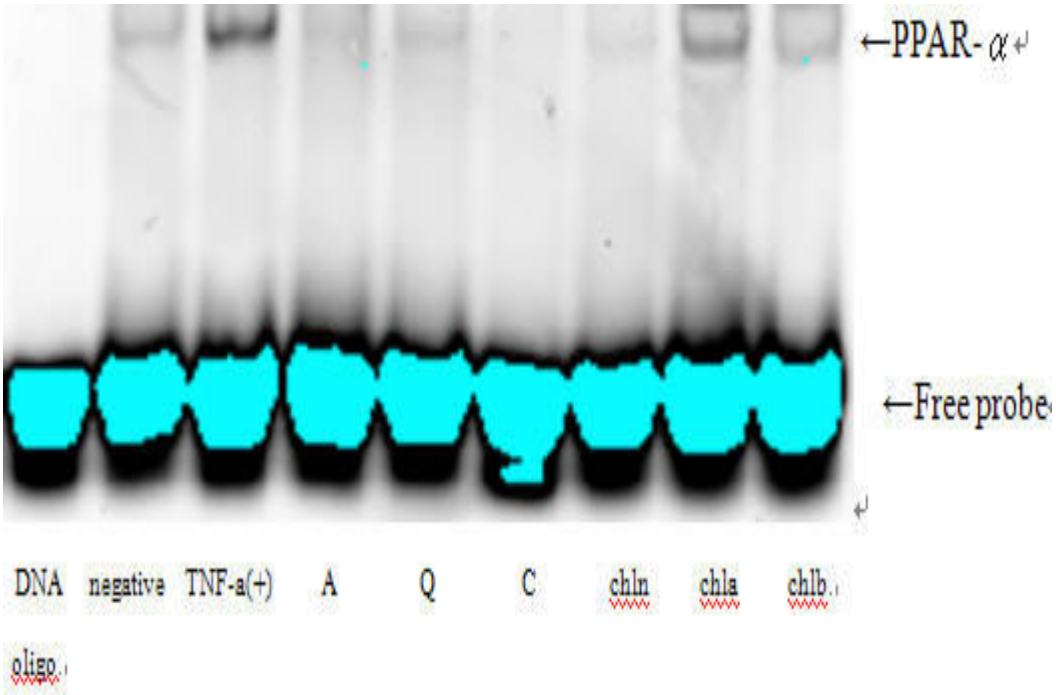
(B)


←AP-1
←Free probe
DNA
Negative Oligo control
TNF(+)
Aspirin
Quercetin
Cyanidin
Chln
Chla
Chlb

(C)


←STAT3
←Free probe
DNA Oligo
Negative control
TNF(+)
Aspirin
Quercetin
Cyanidin
Chln
Chla
Chlb

(D)


←PPAR-α
←Free probe
DNA
negative
TNF-a(+)
A
Q
C
chln
chla
chlb
oligo

(E)

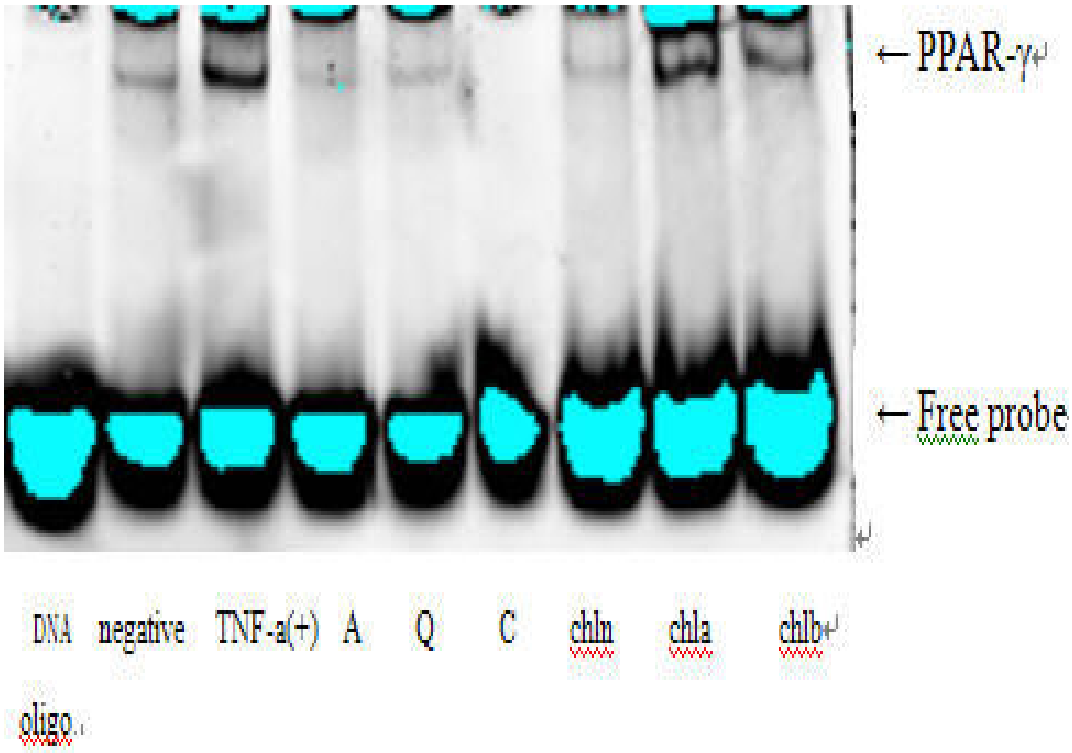


Fig 3. (A)(B)(C)(D)(E) 此為 EMSA 之結果；以 10μM 之 chlorophyll a、chlorophyll b、chlorophyllin、quercetin、cyanidin and aspirin 處理細胞 18 小時後，以 2ng/mL TNF-α 誘導細胞 6 小時後，萃取細胞核蛋白與標記 IR-Dye700 之 oligonucleotide 測量 NF-κB、AP-1、STAT3、PPARα、PPARγ 的結合作用。

Fig 3. NF-κB, AP-1, STAT-3, PPARα and PPARγ EMSA using IRDye 700 end-labeled oligonucleotides duplex.

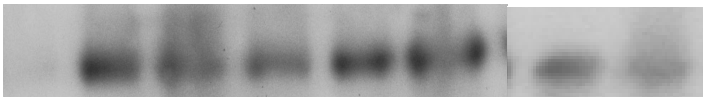
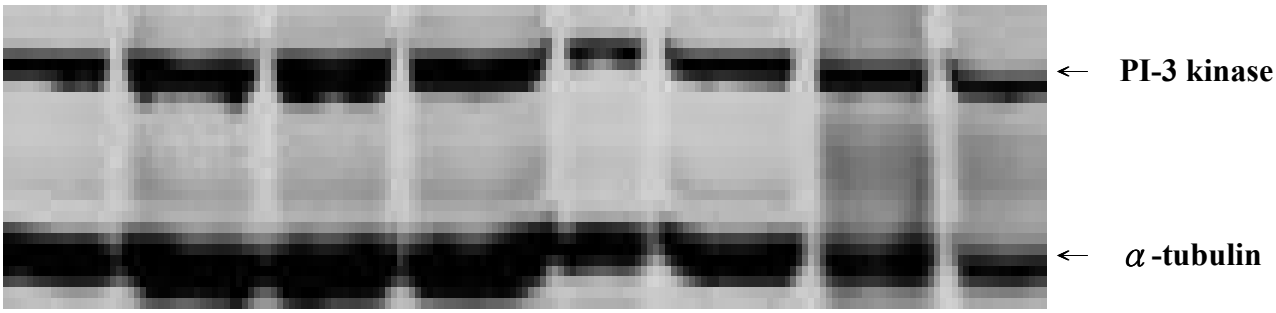


Fig 4. 此為西方點墨法(western blotting)測定 NF-κB 之結果。以 10μM 之 chlorophyll a、chlorophyll b、chlorophyllin、quercetin、cyanidin and aspirin 處理細胞 18 小時後，以 2ng/mL TNF-α 誘導細胞 6 小時後、萃取核蛋白與抗體反應測定 NF-κB 所產生之結果。

Fig 4. HAEC pretreated with chlorophyll related compounds and aspirin for 18h then with TNF-α 2ng mL-1 for 6h, and the expression of NF-κB p65 in nuclear compartment was shown by Western Blot.



Negative TNF-α Aspirin Quercetin Cyanidin Chln Chla Chlb

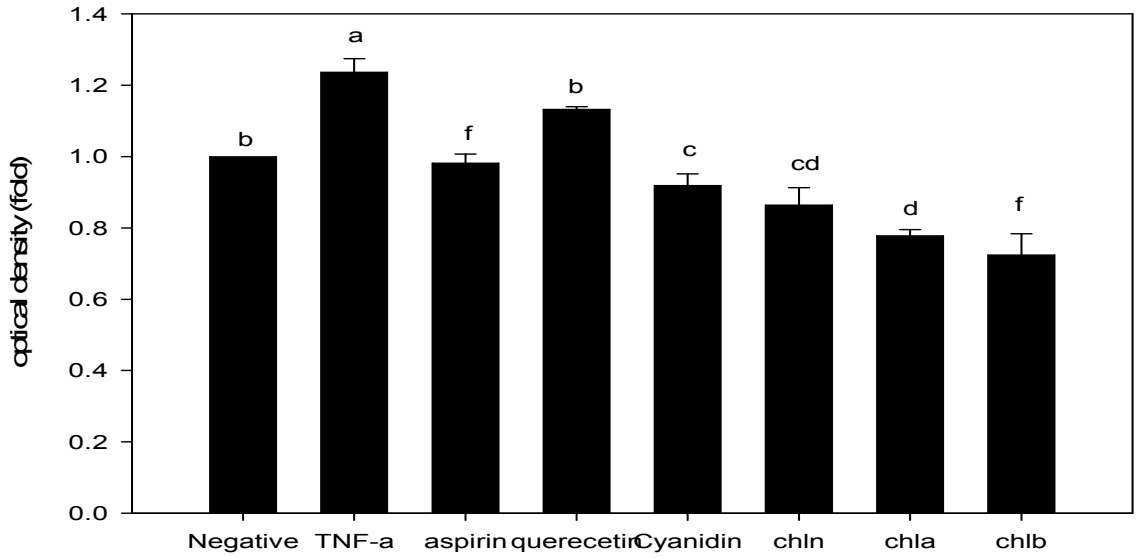
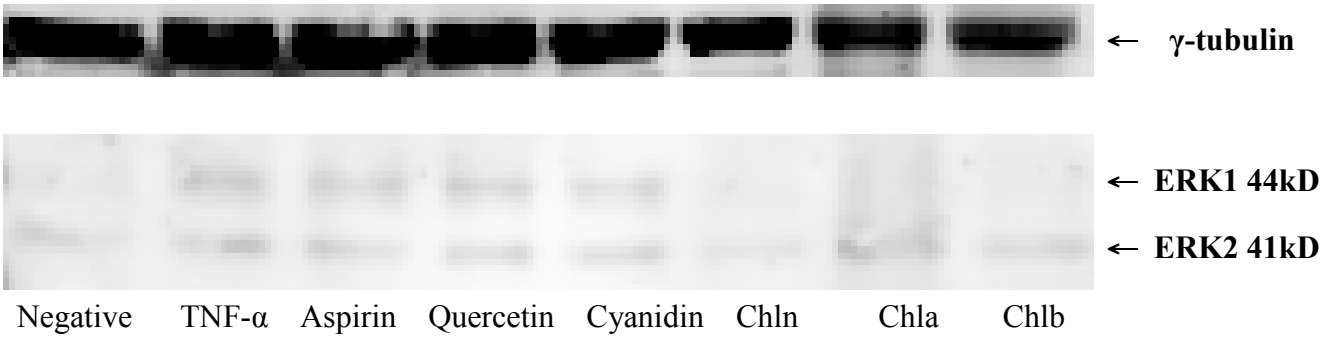


Fig 5. 此為西方點墨法測定 PI-3kinase 之結果。以 10μM 之 chlorophyll a、chlorophyll b、chlorophyllin、quercetin、cyanidin and aspirin 處理細胞 18 小時後，以 2ng/mL TNF-α 誘導細胞 6 小時後、萃取細胞質蛋白與抗體反應測定 PI-3kinase 所產生之結果。

Fig 5. HAEC pretreated with chlorophyll related compounds and aspirin for 18h then with TNF-α 2ng mL-1 for 6h, and the expression of PI-3kinase in sytosol compartment was shown by Western Blot.



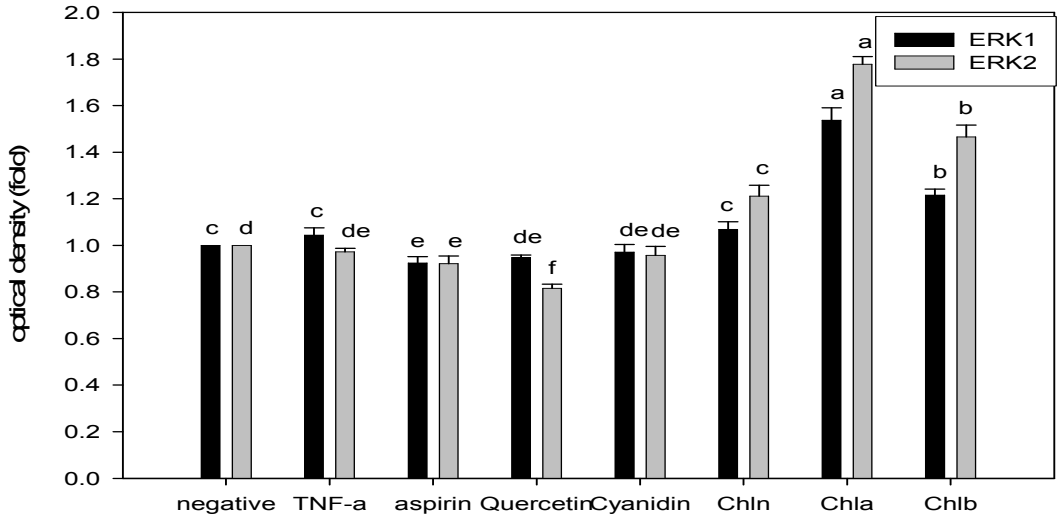


Fig 6. 此為西方點墨法(western blotting)測定 MAP-kinase 1/2 之結果。以 10μM 之 chlorophyll a、chlorophyll b、chlorophyllin、quercetin、cyanidin and aspirin 處理細胞 18 小時後，以 2ng/mL TNF-α 誘導細胞 6 小時後、萃取細胞質與抗體反應測定 ERK-1 與 ERK-2 所產生之結果。

Fig 6. HAEC pretreated with chlorophyll related compounds and aspirin for 18h then with TNF-α 2ng mL-1 for 6h, and the expression of ERk-1 and ERK-2 in sytosol compartment was shown by Western Blot.

Negative TNF-α Aspirin Quercetin Cyanidin Chln Chla Chlb

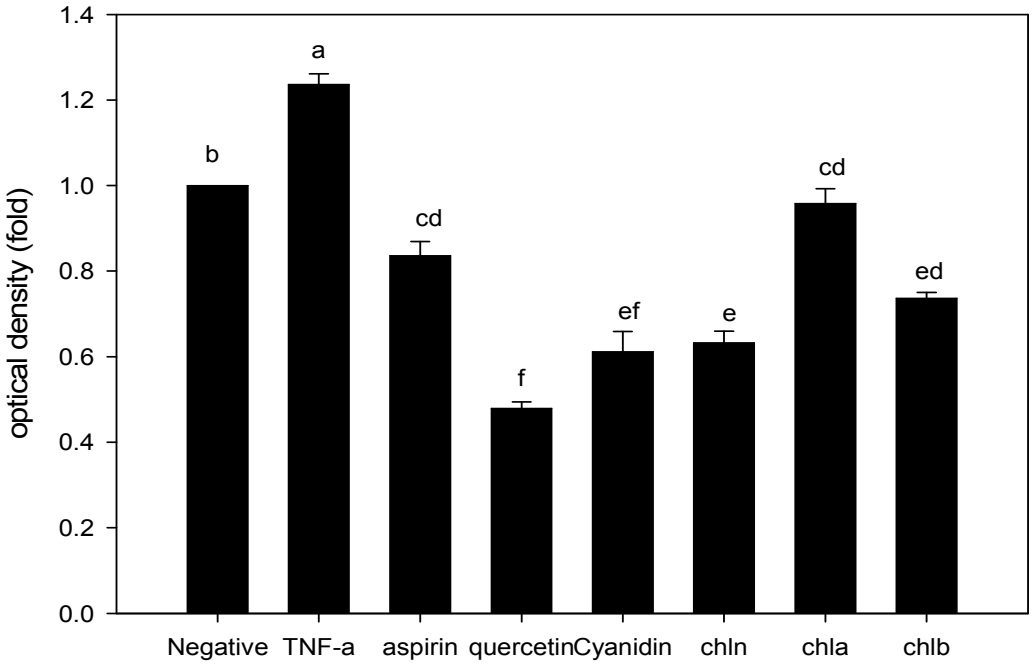


Fig 7. 此為西方點墨法測定 P38 MAP Kinase 之結果。以 10μM 之 chlorophyll a、chlorophyll b、chlorophyllin、quercetin、cyanidin and aspirin 處理細胞 18 小時後，以 2ng/mL TNF-α 誘導細胞 6 小時後、萃取細胞質與抗體反應測定 p38MAPK 所產生之結果。

Fig 7. HAEC pretreated with chlorophyll related compounds and aspirin for 18h then with TNF-α 2ng mL-1 for 6h, and the expression of p38MAPK in sytosol compartment was shown by Western Blot.

Self-evaluation

In the present study, the effects of pytochemicals on TNF-α-induced NF-κB activation were analyzed along with the NF-κB downstream target genes of IL-8 and CD40. Our results show that pytochemicals treatment of HAEC inhibits the expression of these genes via suppression of the NF-κB signaling pathway, p38MAPK and support the notion of the potential for developing pytochemicals as an anti-inflammatory for therapeutic use particularly in cytokine-induced vascular disorders. The methodology developed up today may apply for the coming year project and to get more data regarding to the upstream regulation mechanisms and the aspect regarding to the prevention signaling pathways.