第三章 結果

第一節 RAW264.7 細胞培養條件的建立

一 LPS刺激PGE_2生成的時程

以 100ng/mL的LPS培養RAW264.7 細胞，分別於 0、12、18 及 24 小時收集培養液，進行PGE_2測定。結果如圖 3-1 所示：RAW264.7 細胞於LPS (100ng/mL)刺激 12 小時後，PGE_2大量生成，並於 18 小時達到最高峰。因此，乃選擇培養 18 小時為後續培養細胞的條件。

二 LPS刺激PGE_2生成的劑量

以不同濃度的LPS (1、5、10、20、50、100、200 及 400 ng/mL)，在有或無 10%FBS存在下，培養RAW264.7 細胞 18 小時後收集培養液，進行PGE_2測定。結果如圖 3-2 所示：無FBS存在下(DMEM組)，PGE_2的生成量隨著濃度增加而增加，並於 100 ng/mL時達到飽和；有FBS存在下(10%FBS+DMEM組)，PGE_2的生成量亦隨著濃度增加而增加，且相同濃度之LPS下，添加FBS組所生成之PGE_2均較無FBS(DMEM組)高。因此，本實驗為測量苦瓜區分物對PGE_2生成的抑制作用，其活化劑LPS的濃度選擇以 100ng/mL為後續實驗之活化濃度。此外，為避免FBS刺激PGE_2生成之效應，後續實驗在以樣品處理細胞時，均以不含FBS之 DMEM稀釋樣品進行培養。

第二節 實驗樣品的處理對RAW264.7 細胞生成PGE_2的影響

一 樣品對細胞存活的影響

待測樣品以培養液(DMEM)稀釋為不同濃度進行細胞實驗，培養 18 小時後，以MTT測細胞存活率，若實驗組之細胞數低於僅以培養液培養的控制組，則表示該濃度之樣品對細胞生長或存活具有影響。因此，在決定培養之樣品濃度範圍以不影響細胞生長為原則，以排除樣品濃度對細胞生長存活之干擾。此外，為避免因細胞增生造成PGE_2濃度的差異，經計算細胞數後，將每well所測得之PGE_2生成量除以 1×10^5單位細胞數表示之。

二 山苦瓜水溶性及非水溶性部分

山苦瓜粗萃取物分為：水萃物(WE)及凍乾粉末的乙酸乙酯萃取物(EAE)。分別以不同濃度添加於含或不含LPS (100ng/mL)的培養液中，培養 18 小時後，測PGE_2生成量。結果顯示：在LPS誘發RAW264.7 細胞大量生成PGE_2下，水萃物(WE)具有抑制PGE_2生成的作用，Max inhibition=66.85%(圖 3-3)，但當處理濃度增加至 400μg/mL，細胞存活率則顯著下降。而僅以水萃物單獨處理下，則與空白組無顯

著差異。

此外，乙酸乙酯萃取物(EAE)與LPS同時處理下，亦具有抑制PGE_2生成的作用(圖 3-4)，IC_{50}=14.25μg/mL，Max inhibition=99.12%。而僅以乙酸乙酯萃取物單獨處理下，亦與控制組無顯著差異。整體而言，乙酸乙酯萃取物(EAE)的抑制效果較於水萃物(WE)強。

三 山苦瓜乙酸乙酯萃物(EAE)對萃分離

將山苦瓜全果凍乾之乙酸乙酯萃物(EAE)以n-hexane與 90%MeOH/H_2O對萃分離後，水層再以乙酸乙酯進行對萃，所得區分物極性由高至低為：水萃物 > EAE-2 > Hexane萃取物。Hexane層區分物分別以各種不同濃度添加於含或不含LPS (100ng/mL)的培養液中，培養 18 小時後，測PGE_2生成量。結果如圖 3-5 所示：Hexane萃取物與LPS同時處理細胞時，當濃度大於 100 μg/mL，具有抑制PGE_2生成的作用。

水層區分物部分(H_2O)，以各種不同濃度添加於含LPS (100ng/mL)的培養液中，培養 18 小時後，測PGE_2生成量。結果如圖 3-6 所示：PGE_2生成量顯著下降，但其Max inhibition僅有 53.54%。

EAE-2 區分物部分，當以濃度大於 50 μg/mL處理細胞時，則會引起細胞毒性，造成細胞死亡，故將濃度調整於 50 μg/mL以下，以 20 μg/mL以下濃度處理細胞。結果如圖 3-7 所示：EAE-2 可顯著抑制PGE_2的生成，IC_{50} =6.99 μg/mL，由於提高濃度至 50 μg/mL會造成細胞存活率下降，因此其Max inhibition僅達 73.3%。

三者分別單獨處理細胞，不加 LPS 時，均與空白組無顯著差異。

四 山苦瓜 Hexane 區分物以矽膠管柱層析分離

Hexane區分物以silica gel chromatography依極性最低的 100% n-hexane開始流洗分離，逐漸提高沖提液之極性至 100%EA，共收集 7 個區分物(圖 2-4)。分別以不同濃度添加於含LPS (100ng/mL)的培養液中，培養 18 小時後，測PGE_2生成量。結果如圖 3-8 所示：對LPS活化RAW264.7 細胞大量生成PGE_2之效應，第 7 個區分物 (fraction58-62)具有顯著的抑制效果。

將fraction58-62 進行劑量反應實驗，結果如圖 3-9 所示：fraction58-62 隨劑量增加(1-100 μg/mL)，抑制活化後RAW264.7 細胞生成PGE_2的效應逐漸增強，於 25-50 μg/mL 即幾乎達到完全抑制之效果，IC_{50} = 7.16 μg/mL，Max inhibition=98.01%。但當濃度大於 100 μg/mL，細胞存活率有下降的趨勢。

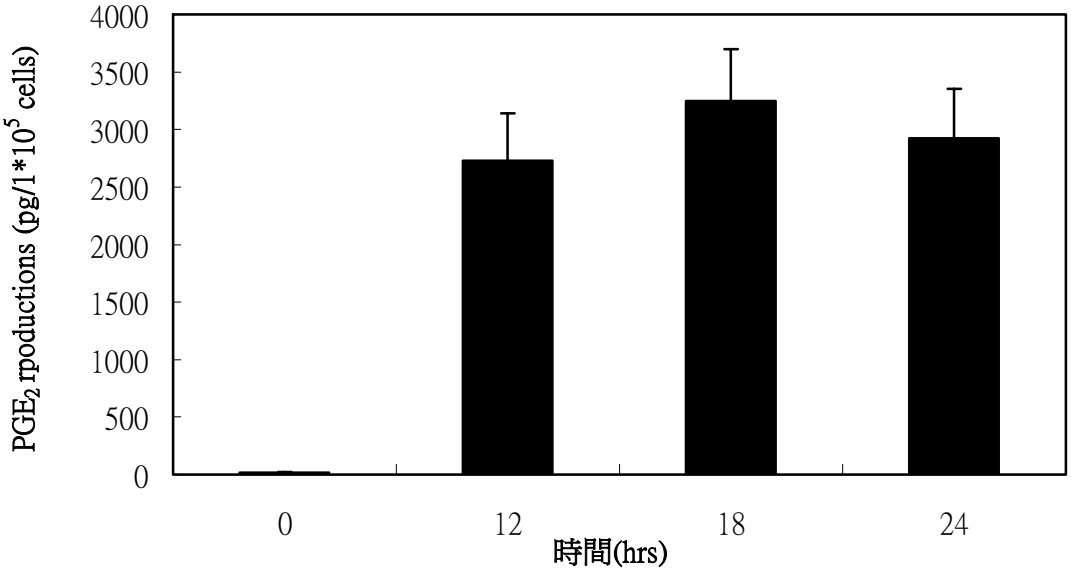


圖 3-1 LPS (100 ng/mL)刺激RAW264.7 細胞生成PGE_2之時程反應

Fig3-1 Time course of the PGE_2 production induced by LPS (100 ng/mL) in RAW264.7cells .The cells were treated with LPS for 0、12、18 or 24 hrs and medium was collected for PGE_2 analysis using an EIA assay. At least three batches of separate experiments were carried out with similar results.The values are Mean ± SD of triplicates in a representative experiment .

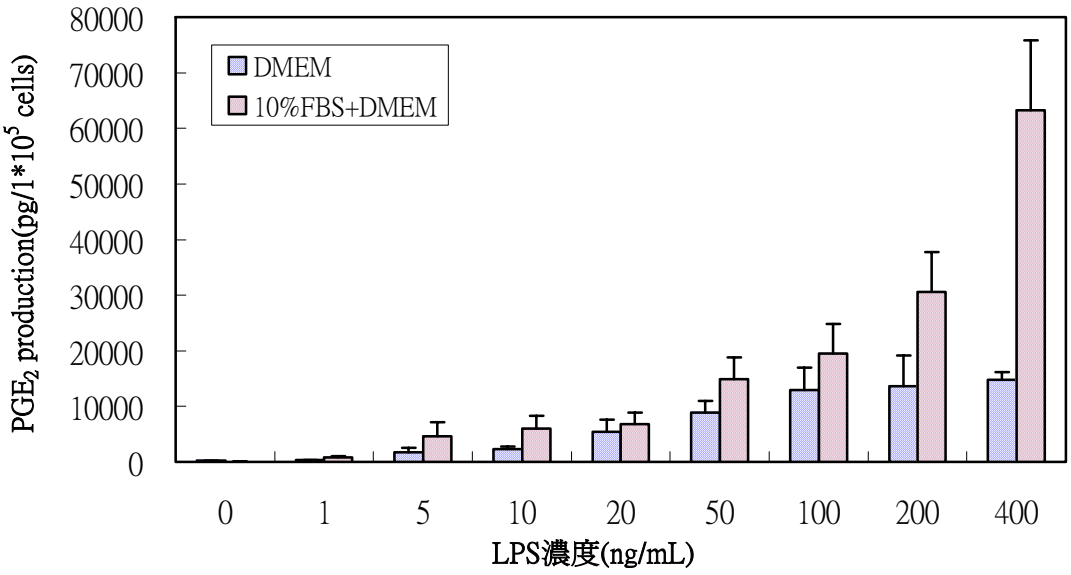


圖 3-2 以不同濃度之LPS刺激RAW264.7 細胞生成PGE_2之劑量反應

Fig3-2 The PGE_2 production induced by various concentrations of LPS in RAW264.7 cells .The cells were treated with various concentrations of LPS in the presence or absence of FBS (10%) for 18 hrs and medium was collected for PGE_2 analysis using an EIA assay. At least three batches of separate experiments were carried out with similar results. The values are Mean ± SD of triplicates in a representative experiment .

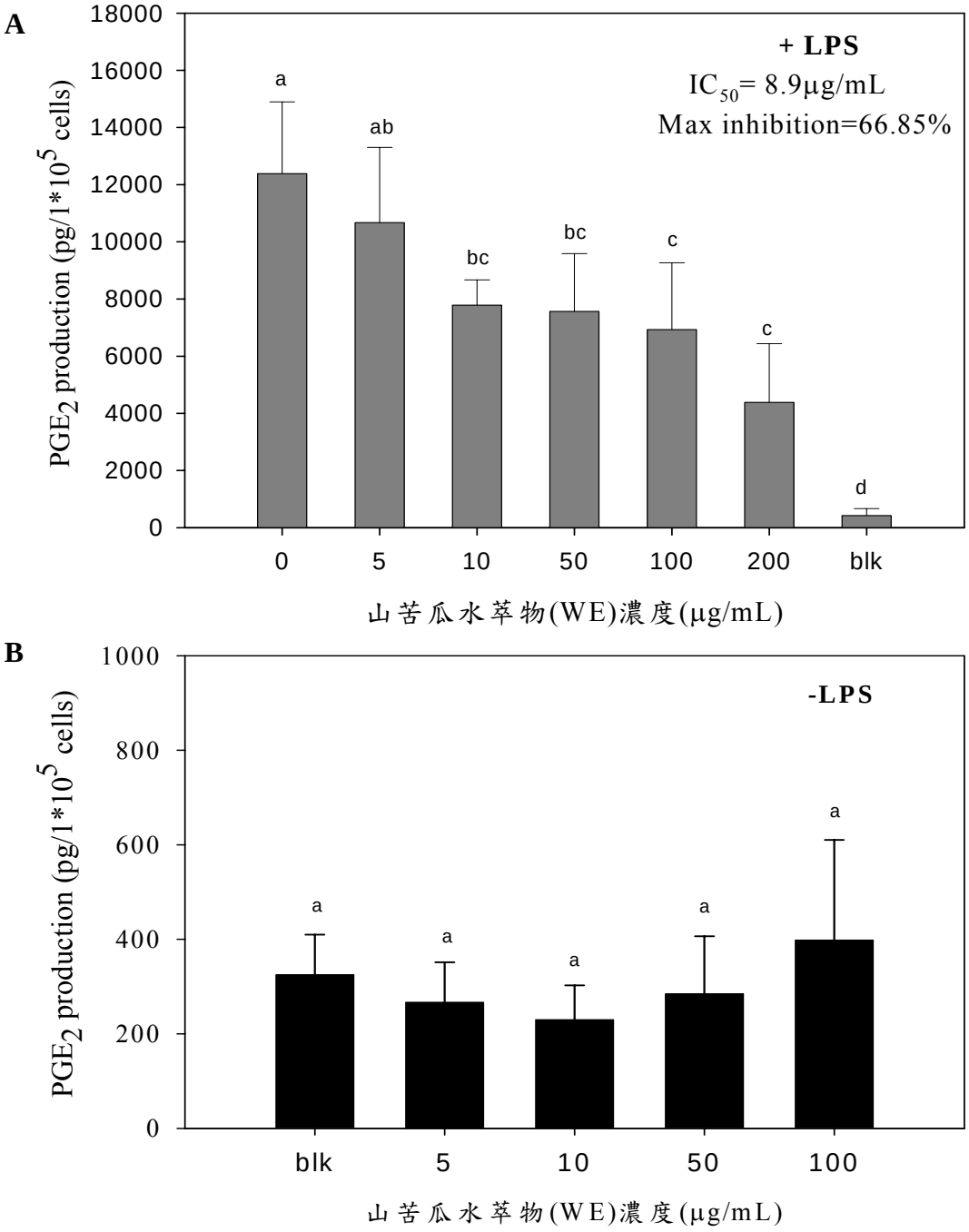


圖 3-3 山苦瓜水萃物對RAW264.7 細胞生成PGE_2之影響 (流程圖 2-1)

Fig3-3 Effects of water extract of *Momordica charantia* L. on the PGE_2production in RAW264.7 cells.The cells were treated with various concentrations of the extract in the presence (+LPS) or absence (-LPS) of LPS (100 ng/mL) for 18 hrs and medium was collected for PGE_2 analysis using an EIA assay. At least three batches of separate experiments were carried out with similar results. The values are Mean ± SD of triplicates in a representative experiment .

blk：「blank」RAW264.7 cells were incubated with medium only for 18 hrs,

0: RAW264.7 cells were incubated with medium containing 100ng/mL LPS only for 18 hrs.

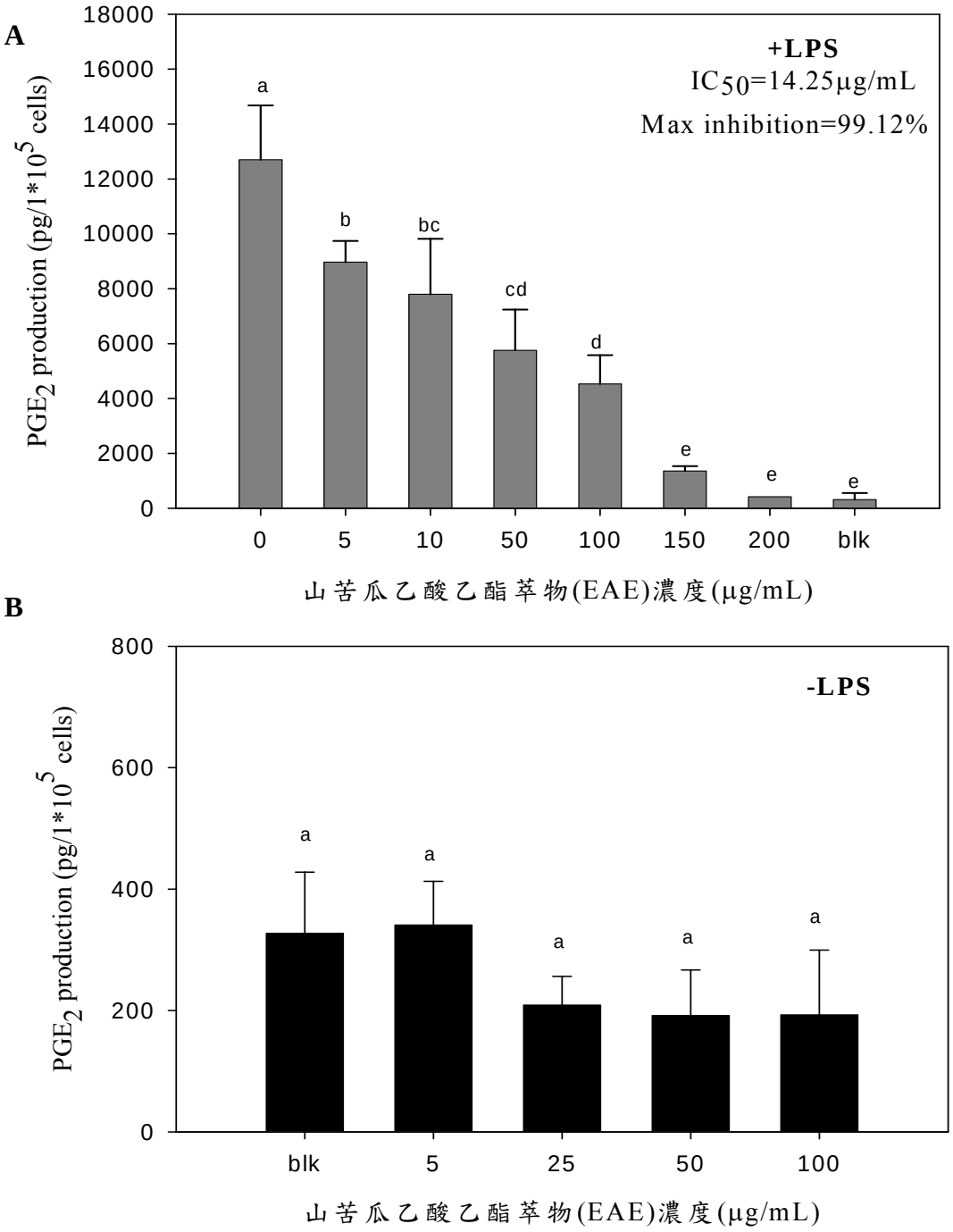


圖 3-4 山苦瓜乙酸乙酯萃取物(EAE)對RAW264.7 細胞生成PGE_2之影響(流程圖 2-2)

Fig3-4 Effects of ethyl acetate extract of *Momordica charantia* L. on the PGE_2 production in RAW264.7 cells.The cells were treated with various concentrations of the extract in the presence (+LPS) or absence (-LPS) of LPS (100 ng/mL) for 18 hrs and medium was collected for PGE_2 analysis using an EIA assay. At least three batches of separate experiments were carried out with similar results. The values are Mean ± SD of triplicates in a representative experiment .

blk：「blank」RAW264.7 cells were incubated with medium only for 18 hrs,

0: RAW264.7 cells were incubated with medium containing 100ng/mL LPS only for 18 hrs.

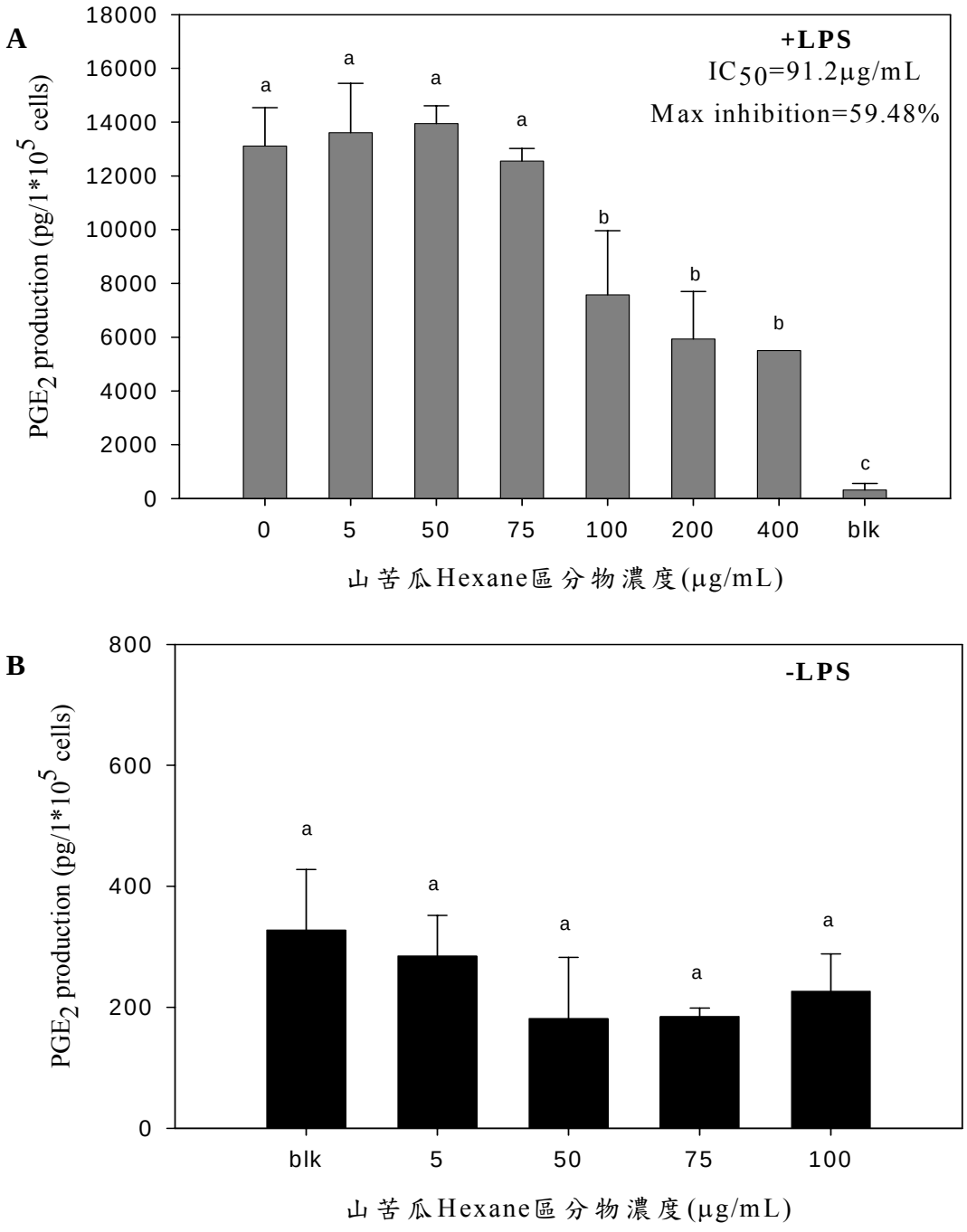


圖 3-5 山苦瓜Hexane區分物對RAW264.7 細胞生成PGE_2之影響(流程圖 2-3)

Fig3-5 Effects of Hexane partitioned fractions of *Momordica charantia* L. on the PGE_2 production in RAW264.7 cells.The cells were treated with various concentrations of the fraction in the presence (+LPS) or absence (-LPS) of LPS (100 ng/mL) for 18 hrs and medium was collected for PGE_2 analysis using an EIA assay. At least three batches of separate experiments were carried out with similar results. The values are Mean ± SD of triplicates in a representative experiment .

blk：「blank」RAW264.7 cells were incubated with medium only for 18 hrs,

0: RAW264.7 cells were incubated with medium containing 100ng/mL LPS only for 18 hrs.

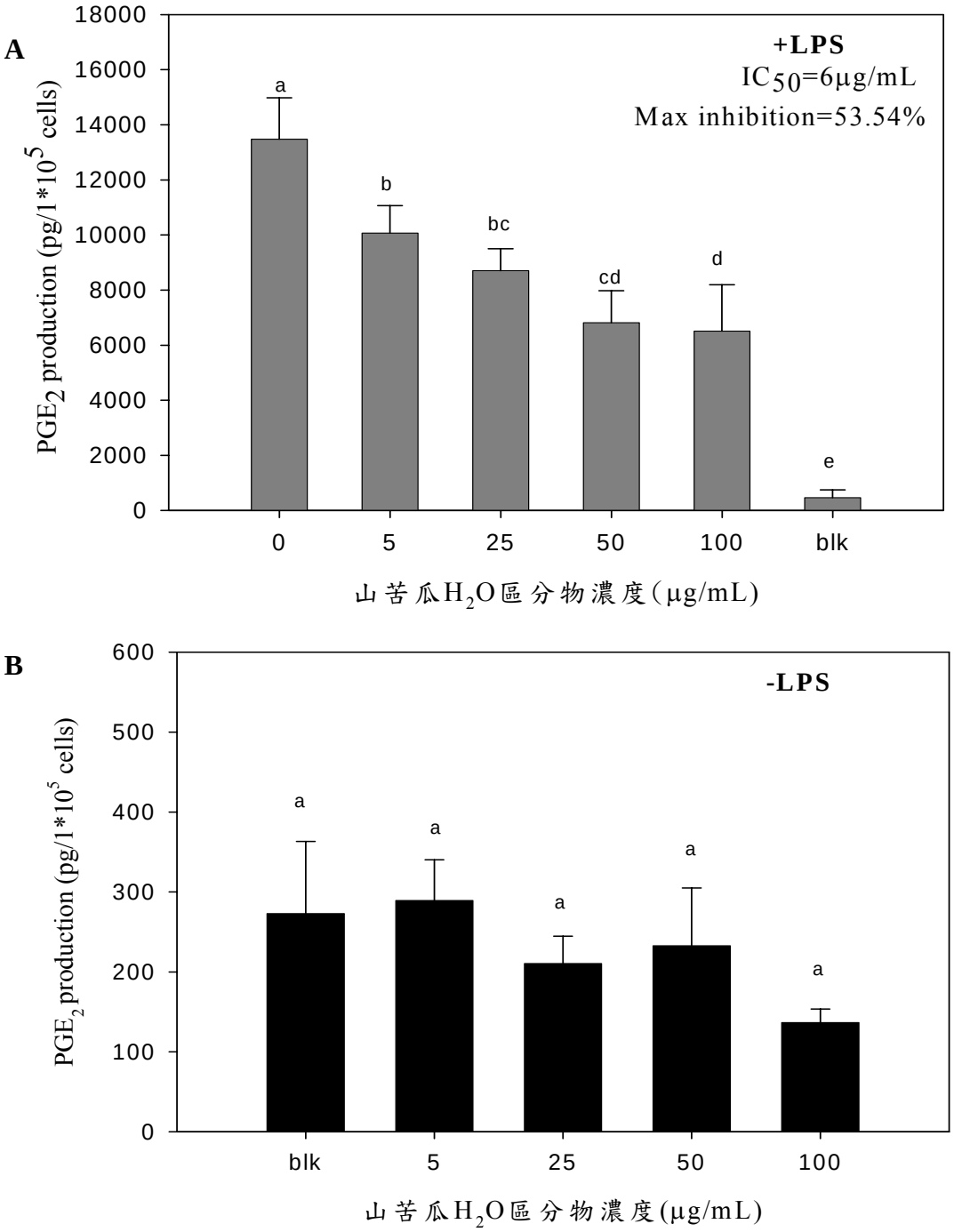


圖 3-6 山苦瓜H_2O區分物對RAW264.7 細胞生成PGE_2之影響(流程圖 2-3)

Fig3-6 Effects of 90%MeOH/H_2O partitioned fractions of *Momordica charantia* L. on the PGE_2 production in RAW264.7 cells.The cells were treated with various concentrations of the fraction in the presence (+LPS) or absence (-LPS) of LPS (100 ng/mL) for 18 hrs and medium was collected for PGE_2 analysis using an EIA assay. At least three batches of separate experiments were carried out with similar results. The values are Mean ± SD of triplicates in a representative experiment .

blk：「blank」RAW264.7 cells were incubated with medium only for 18 hrs,

0: RAW264.7 cells were incubated with medium containing 100ng/mL LPS only for 18 hrs.

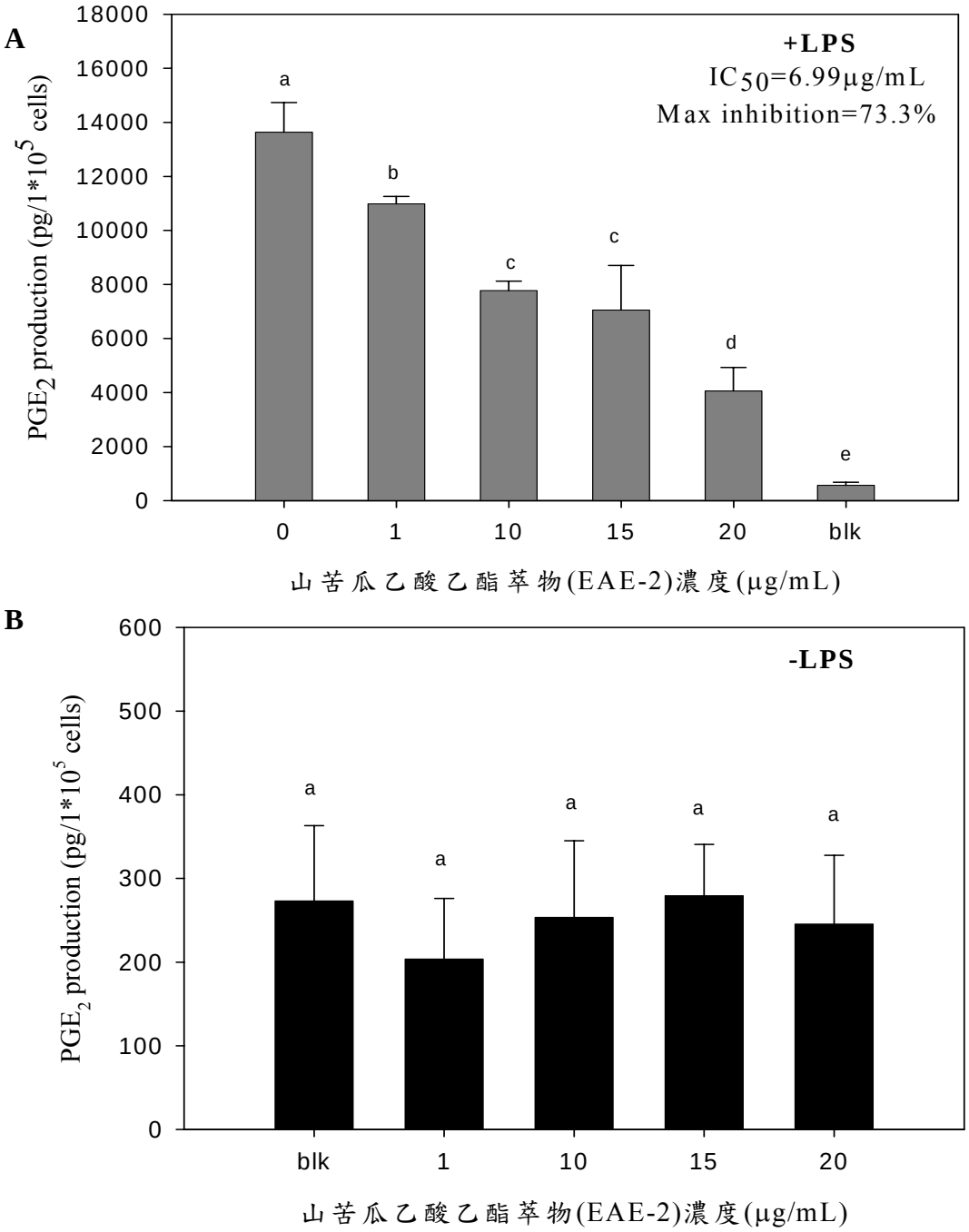


圖 3-7 山苦瓜乙酸乙酯區分物(EAE-2)對RAW264.7 細胞生成PGE_2之影響(流程圖 2-3)

Fig3-7 Effects of EA extract partitioned from *Momordica charantia* L. on the PGE_2 production in RAW264.7 cells.The cells were treated with various concentrations of the fraction in the presence(+LPS) or absence (-LPS) of LPS (100 ng/mL) for 18 hrs and medium was collected for PGE_2 analysis using an EIA assay. At least three batches of separate experiments were carried out with similar results. The values are Mean ± SD of triplicates in a representative experiment .

blk：「blank」RAW264.7 cells were incubated with medium only for 18 hrs,

0: RAW264.7 cells were incubated with medium containing 100ng/mL LPS only for 18 hrs.

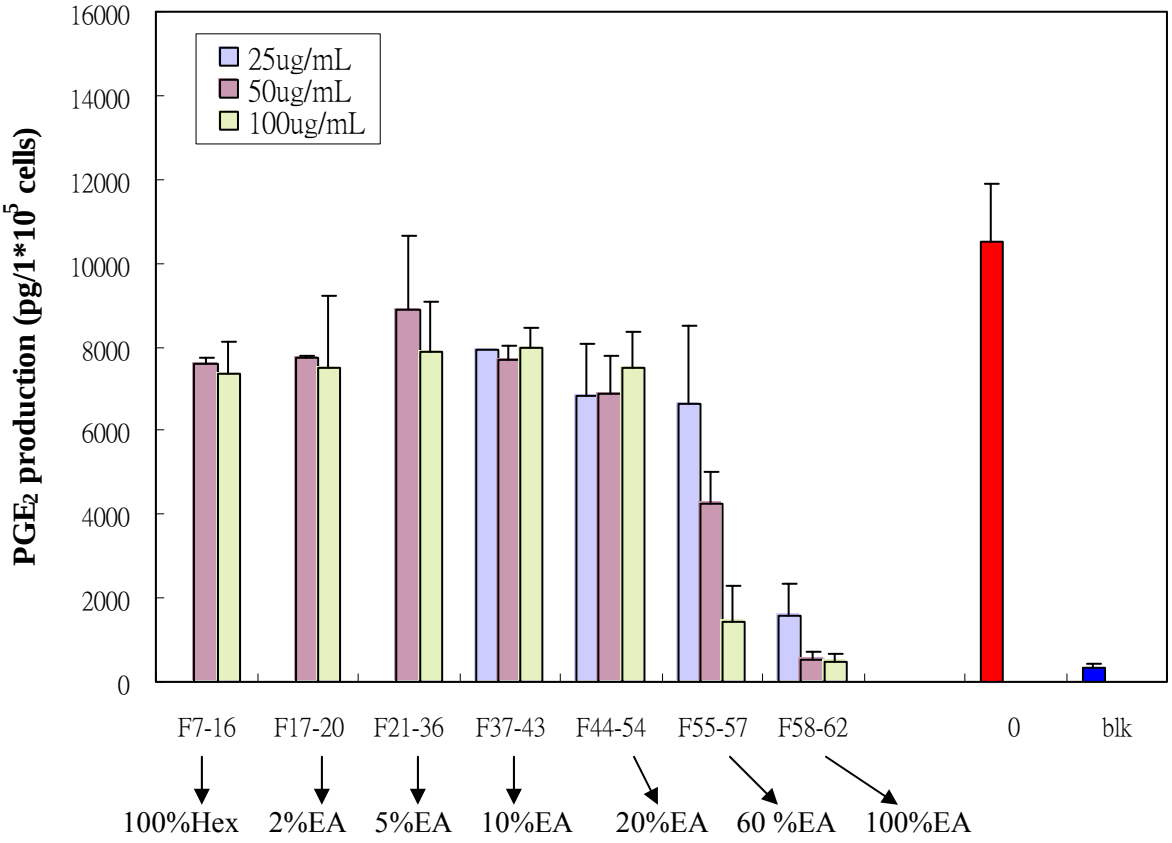


圖 3-8 山苦瓜Hexane區分物經矽膠管柱層析分離後各區分物對RAW264.7 細胞生成PGE_2之影響 (流程圖 2-4)

Fig3-8 Effects of fractions separated by silica gel chromatography of *Momordica charantia* L. hexane partitioned fraction on the PGE_2 production in LPS activated RAW264.7 cells.The cells were treated with various concentrations of fractions in the presence of LPS (100 ng/mL) for 18 hrs and medium was collected for PGE_2 analysis using an EIA assay. At least three batches of separate experiments were carried out with similar results. The values are Mean ± SD of triplicates in a representative experiment .Fractionation procedure was shown in Fig2-4.

blk：「blank」RAW264.7 cells were incubated with medium only for 18 hrs,

0: RAW264.7 cells were incubated with medium containing 100ng/mL LPS only for 18 hrs.

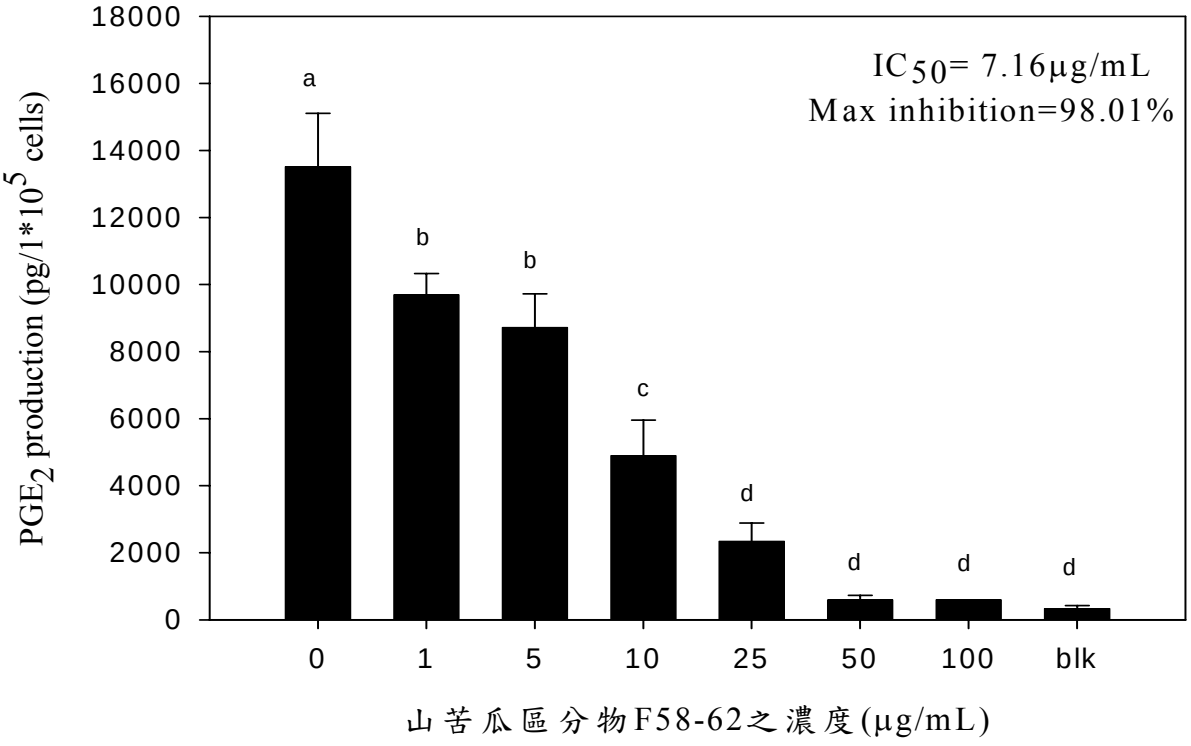


圖 3-9 山苦瓜區分物F58-62 抑制LPS誘發RAW264.7 細胞生成PGE_2之劑量效應

Fig3-9 Dose-dependent inhibition of F58-62 on the PGE_2 production in LPS activated RAW264.7 cells. The cells were treated with various concentrations of the fraction in the presence of LPS (100 ng/mL) for 18 hrs and medium was collected for PGE_2 analysis using an EIA assay. At least three batches of separate experiments were carried out with similar results. The values are Mean ± SD of triplicates in a representative experiment.

blk：「blank」RAW264.7 cells were incubated with medium only for 18 hrs,

0: RAW264.7 cells were incubated with medium containing 100ng/mL LPS only for 18 hrs.

五 山苦瓜大量萃取分離

Hexane 萃取物經矽膠管柱層析

大量山苦瓜冷凍乾燥後之乙酸乙酯萃物(EAE)以n-hexane與 90%MeOH/H_2O分離，所得之Hexane區分物，以矽膠管柱層析分離(圖 2-5)。依不同流洗極性，分別取具有最大收量的一瓶進行細胞活性試驗。結果如圖 3-10 所示：由90%EA+10%MeOH所流洗出的F190 抑制效果最顯著，其次為 40%EA+60%Hex所流洗出的F152。分別進行劑量反應實驗，兩者皆具明顯的劑量反應。如圖 3-11 所示：F190 在 25 μg/mL時，幾乎達到完全抑制效果，IC_{50}=3.04 μg/mL；而F152 在 50 μg/mL時，幾乎達到完全抑制效果，IC_{50}=13.6 μg/mL。

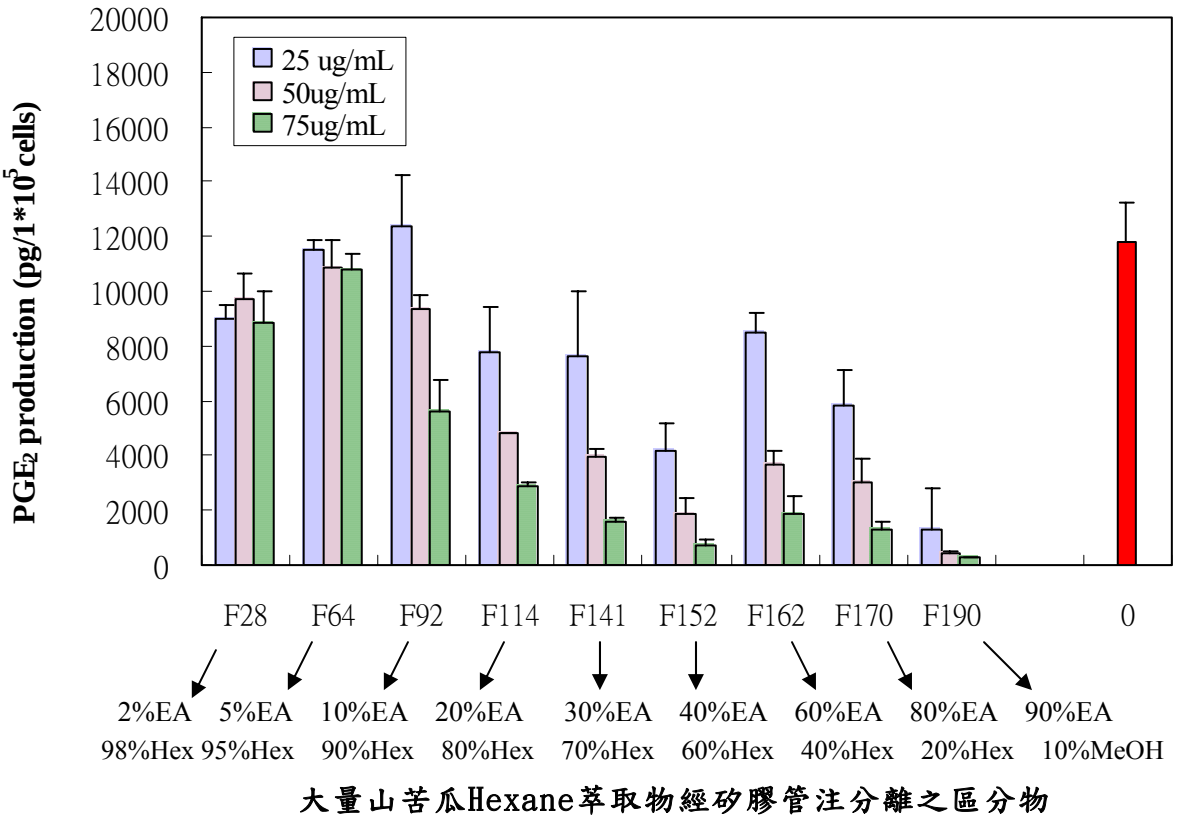


圖 3-10 大量山苦瓜Hexane區分物經矽膠管柱層析分離後各區分物對RAW264.7 細胞生成PGE_2之影響 (流程圖 2-5)

Fig3-10 Effects of fractions obtained by separating hexane partitioned fraction using silica gel chromatography on the PGE_2 production in LPS actived RAW264.7 cells. The cells were treated with three concentrations of fractions in the presence of LPS (100 ng/mL) for 18 hrs and medium was collected for PGE_2 analysis using an EIA assay. At least three batches of separate experiments were carried out with similar results. The values are Mean ± SD of triplicates in a representative experiment .Fractionation procedure was shown in Fig2-5.

0: RAW264.7 cells were incubated with medium containing 100ng/mL LPS only for 18 hrs.

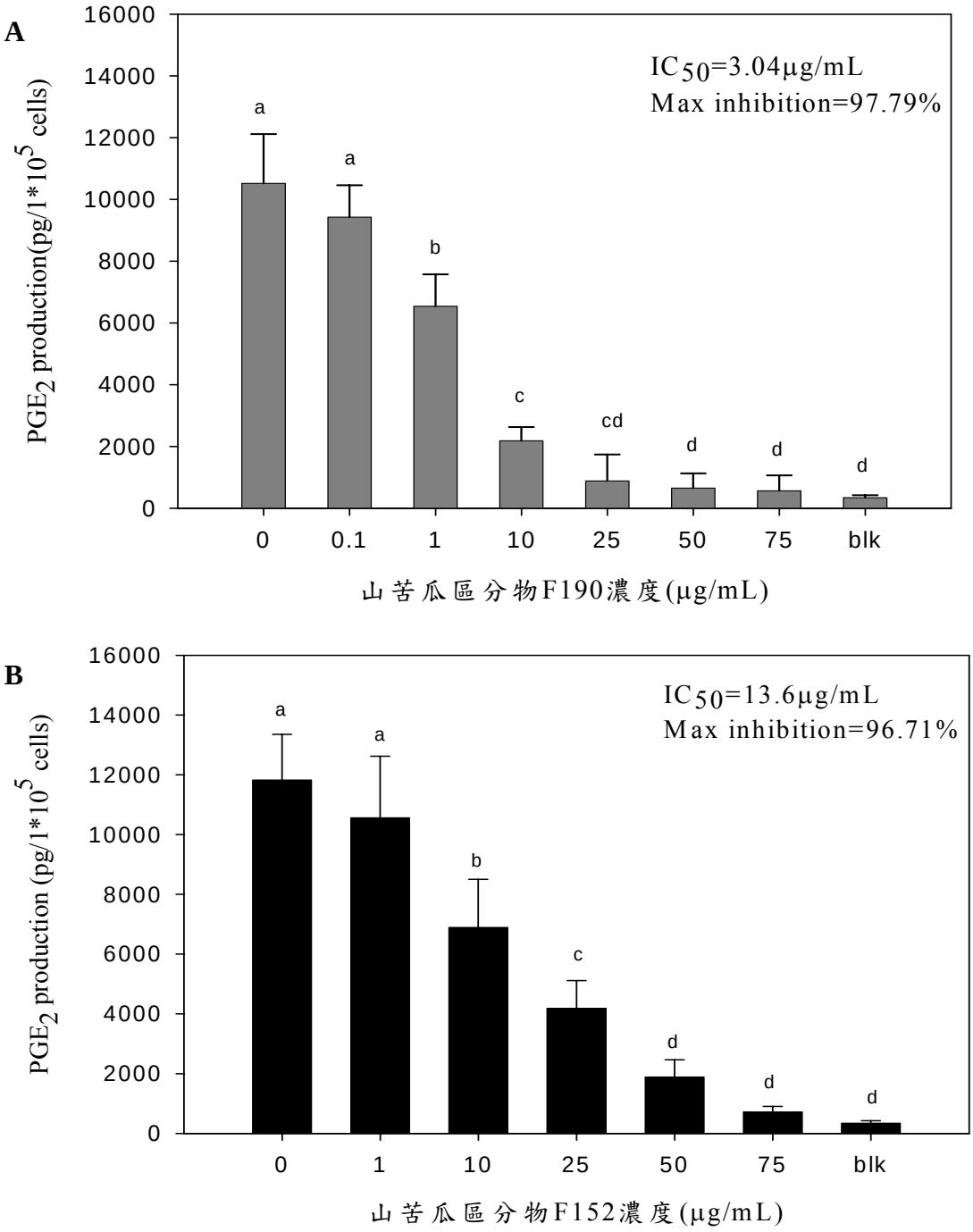


圖 3-11 山苦瓜區分物 F190 (A)及F152 (B)抑制RAW264.7 細胞生成PGE_2之劑量效應

Fig3-11 Dose-dependent inhibition of Hexane fraction F190(A) and F152(B) on the PGE_2 production in LPS activated RAW264.7 cells. The cells were treated with various concentrations of the fraction in the presence of LPS (100 ng/mL) for 18 hrs and medium was collected for PGE_2 analysis using an EIA assay. At least three batches of separate experiments were carried out with similar results. The values are Mean ± SD of triplicates in a representative experiment .Fractionation procedure was shown in Fig2-5.

blk：「blank」RAW264.7 cells were incubated with medium only for 18 hrs,

0: RAW264.7 cells were incubated with medium containing 100ng/mL LPS only for 18 hrs.

六 活性區分物再層析分離

(一) Fraction189 ~ 191 純化分離

山苦瓜經大量萃取分離後所得之Hexane區分物，以矽膠管柱分離，於90%EA+10%MeOH流洗出具有抑制活性的區分物F190(圖 3-11A)。因此，將相同極性的Fraction189.190.191 合併後，再以矽膠管柱流洗分離成F1-6、F8-12 及F13-21三個區分(圖 2-6)。分別與LPS共同處理細胞後發現：F1-6 及F8-12 均具有顯著抑制效果(圖 3-12)。其中，F1-6 進行劑量反應實驗，結果如圖 3-13 所示：F1-6 在 50 μg/mL時，幾乎達到完全抑制效果，IC_{50}=7.05 μg/mL。此外，從^{1}H-NMR可發現這兩個區分物的特徵相似，均包括：存在脂肪酸，有雙鍵，OH-gourp及酯化的特徵。由於F1-6 的量較多，因此決定由F1-6 再進一步純化分離。

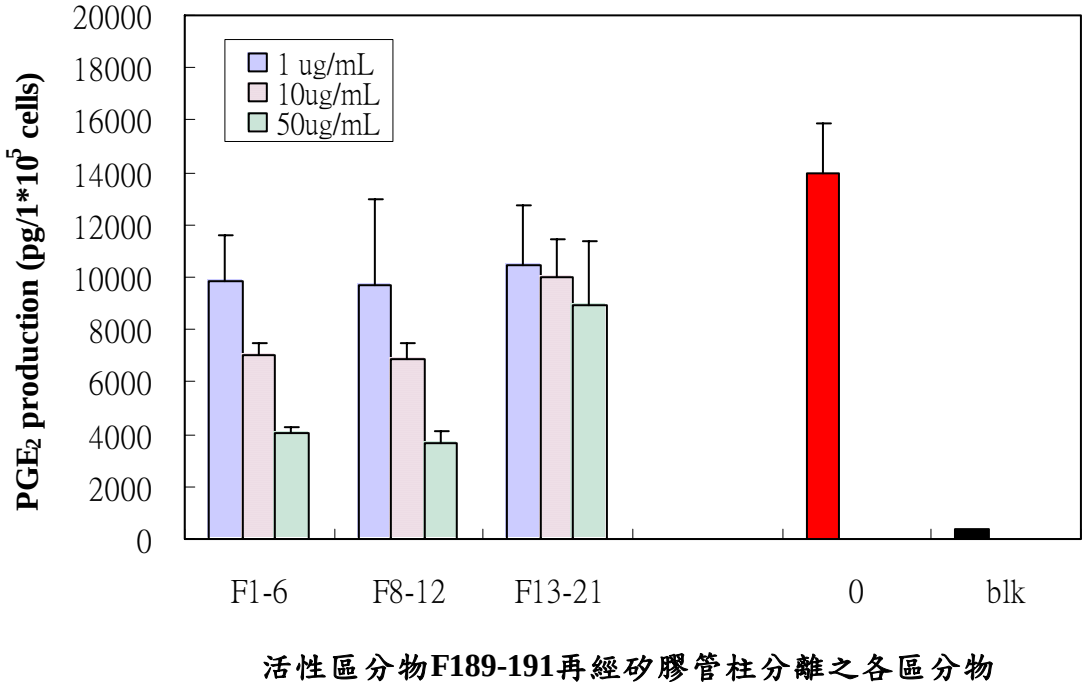


圖 3-12 活性區分物 F189-191 再經矽膠管柱分離之各區分物對RAW264.7 細胞生成 PGE_2之影響 (流程圖 2-6)

Fig3-12 Effects of the fractions obtained by separating F189-191 using silica gel chromatography on the PGE_2 production in LPS activated RAW264.7 cells. The cells were treated with three concentrations of these fractions in the presence of LPS (100 ng/mL) for 18 hrs and medium was collected for PGE_2 analysis using an EIA assay. At least three batches of separate experiments were carried out with similar results. The values are Mean ± SD of triplicates in a representative experiment .Fractionation procedure was shown in Fig2-6.

blk：「blank」RAW264.7 cells were incubated with medium only for 18 hrs,

0: RAW264.7 cells were incubated with medium containing 100ng/mL LPS only for 18 hrs.

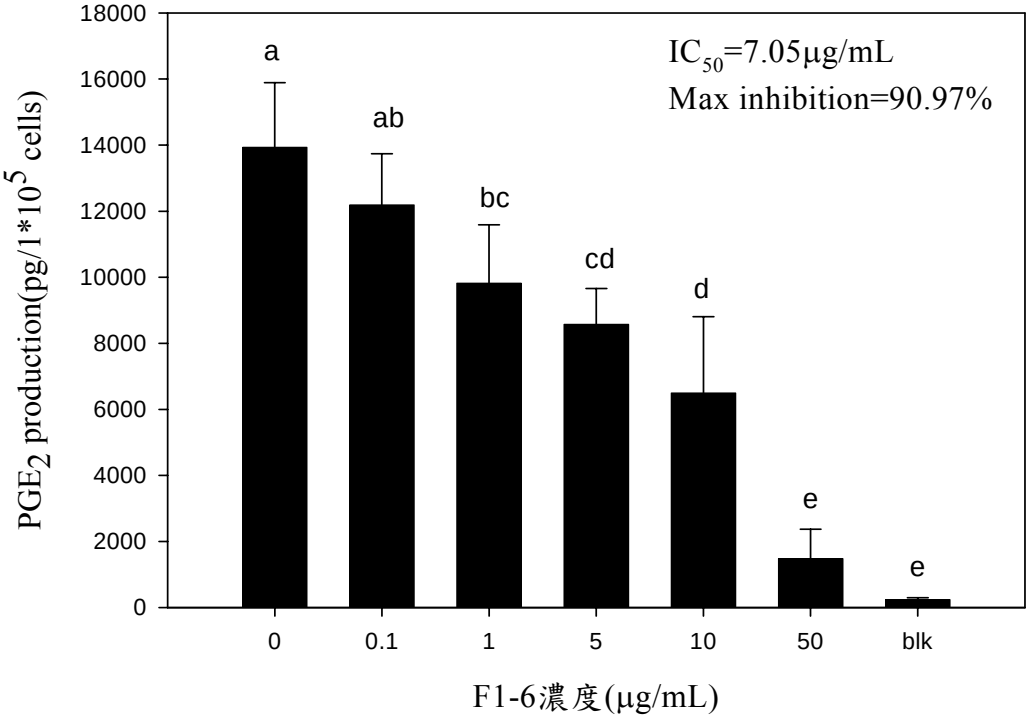


圖 3-13 活性區分物F1-6 抑制LPS誘發RAW264.7 細胞生成PGE_2之劑量效應

Fig3-13 Dose-dependent inhibition of the fraction F1-6 on the PGE_2 production in LPS activated RAW264.7 cells. The cells were treated with various concentrations of the fraction in the presence of LPS (100 ng/mL) for 18 hrs and medium was collected for PGE_2 analysis using an EIA assay. At least three batches of separate experiments were carried out with similar results. The values are Mean ± SD of triplicates in a representative experiment

blk：「blank」RAW264.7 cells were incubated with medium only for 18 hrs,

0: RAW264.7 cells were incubated with medium containing 100ng/mL LPS only for 18 hrs.

1. F1-6 甲基乙醯化 (圖 2-7) (第一次實驗)

因 F1-6 中存在脂肪酸，容易在分離時出現拖曳的現象，且此區分物極性偏高，可能因含有 COOH-及 OH-gourp 所致，故加以甲基化以避免酸所產生分離拖曳的現象，並加以乙醯化降低極性。

經甲基乙醯化的 F1-6 再以矽膠管柱分離，區分合併後得 3 個區分，分別為：A8-22、A23-59 及 A60-76。其中 A8-22 以 TLC 點片結果發現許多明顯的點，但因為極性相近，故決定以製備式 HPLC 再分離。而 A23-59 為明顯綠色點，可能是色素。A60-76 則出現連續拖曳的現象，無明顯的點。

A8-22 經製備式HPLC分離後，共得到 8 個區分(H1-H8)，分別測^{1}H-NMR後，發現各區分仍為混合物。其中，H3 主要為Triglycerides (TG)的特徵。但因為分離後樣品的量過少，無法再次進行分離，且不足以將其甲基乙醯基切除進行細胞實驗。決定由F1-6 重新分離。

2. F1-6 以 RP-18(Reverse-phase)管柱進行分離 (第二次實驗)

將具有抑制活性的F1-6 以RP-18 管柱分離後(圖 2-8)，對照TLC的結果，選擇R6、R13、R24、R41 及R55 進行抑制PGE_2生成之細胞活性試驗，結果如圖 3-14 所示：80% MeOH/H_2O所流洗出的R13 及 100% MeOH 所流洗出的R24 均有活性。其中R13 在濃度大於 20μg/mL後，細胞存活率會顯著下降。因此，乃選擇與R24 極性相近的區分物合併，進行進一步的分離。然而，陸續分離後的樣品可能因：於室溫光照下氧化或樣品溶於*d*-Chloroform中存放而改變，故決定再由F1-6 重新分離。

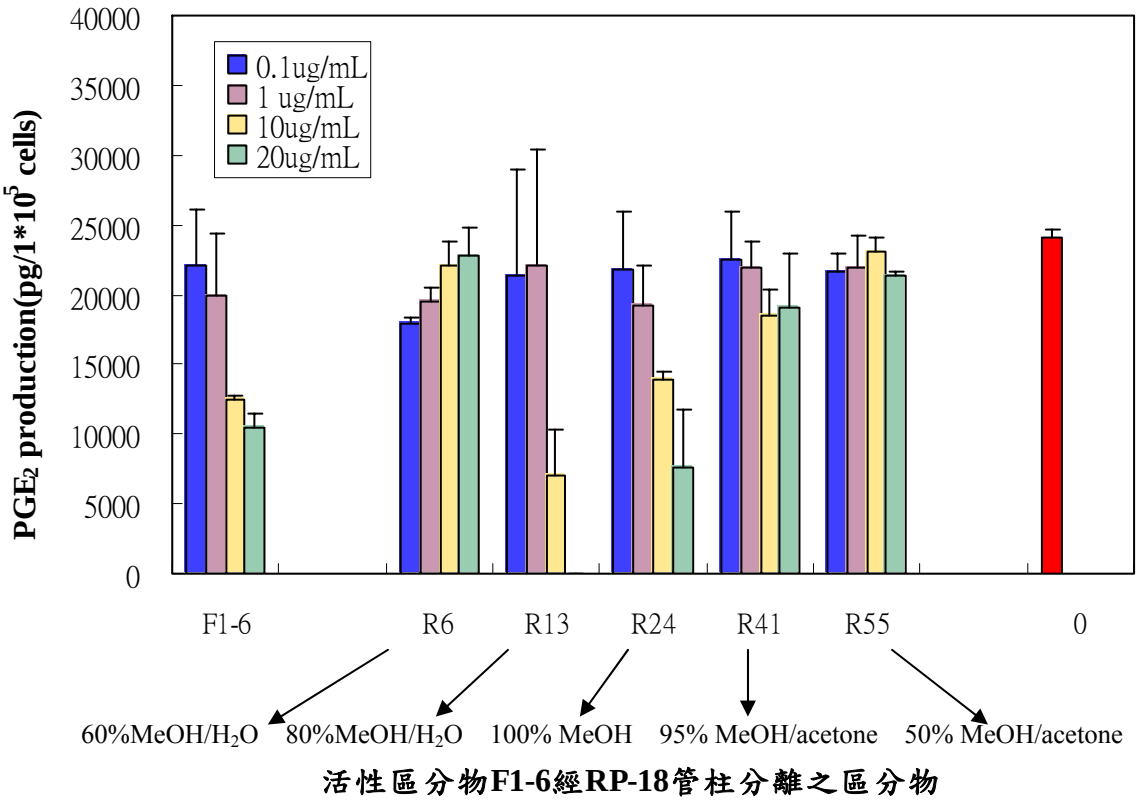


圖 3-14 活性區分物 F1-6 經RP-18 管柱分離之區分物對RAW264.7 細胞生成PGE_2之影響 (流程圖 2-8)

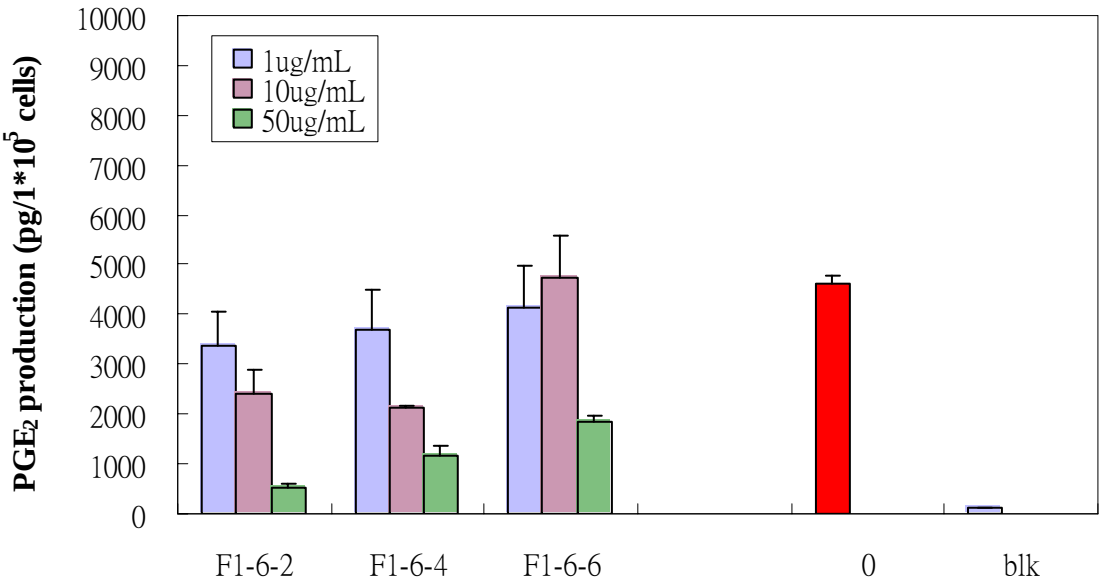
Fig3-14 Effects of the fractions obtained by separating F1-6 using reverse phase chromatography on the PGE_2 production in LPS activated RAW264.7 cells. The cells were treated with various concentrations of these fractions in the presence of LPS (100 ng/mL) for 18 hrs and medium was collected for PGE_2 analysis using an EIA assay. At least three batches of separate experiments were carried out with similar results. The values are Mean ± SD of triplicates in a representative experiment .Fractionation procedure was shown in Fig2-8.

0: RAW264.7 cells were incubated with medium containing 100ng/mL LPS only for 18 hrs.

3. F1-6 重新分離 (第三次實驗)

在重新分離F1-6 的過程中，選擇先以矽膠管柱將其大致分離成三個區分物 (圖2-9)。依照TLC追蹤的結果，F1-6-2、F1-6-4 及F1-6-6 呈現 3 個極性不同的區分，因此將此三個區分進行細胞活性試驗，結果如圖 3-15 所示：F1-6-2 及F1-6-4 具有抑制活性。因此，將F1-6-2、F1-6-3 及F1-6-4 合併，再續以矽膠管柱及RP-18 管柱分離，得到^{1}H-NMR 光譜結構與第二次實驗所分離出具有活性的R24 相近的RP-10。並再進行細胞活性實驗，以確定其具有抑制PGE_2合成的作用，結果如圖3-16 所示(n=1)：RP-10 具有抑制PGE_2生成的活性，且呈劑量效應，並於 25 μg/mL 達完全抑制，IC_{50} = 2.31 μg/mL，Max inhibition=96.3%。

活性區分物RP-10 經由^{1}H-NMR 及H-H COSY的鑑定結果(附錄圖一，圖二)，推測其組成主要為TG接中、短鏈飽和脂肪酸為主。



活性區分物F1-6以矽膠管柱分離之區分物

圖 3-15 活性區分物 F1-6 經矽膠管柱分離之區分物對RAW264.7 細胞生成PGE_2 之影響(流程圖 2-9)

Fig3-15 Effects of the fractions obtained by separating F1-6 using silica gel chromatography on the PGE_2 production in LPS activated RAW264.7 cells. The cells were treated with three concentrations of these fractions in the presence of LPS (100 ng/mL) for 18 hrs and medium was collected for PGE_2 analysis using an EIA assay. At least three batches of separate experiments were carried out with similar results. The values are Mean ± SD of triplicates in a representative experiment .Fractionation procedure was shown in Fig2-9.

blk：「blank」RAW264.7 cells were incubated with medium only for 18 hrs,

0: RAW264.7 cells were incubated with medium containing 100ng/mL LPS only for 18 hrs.

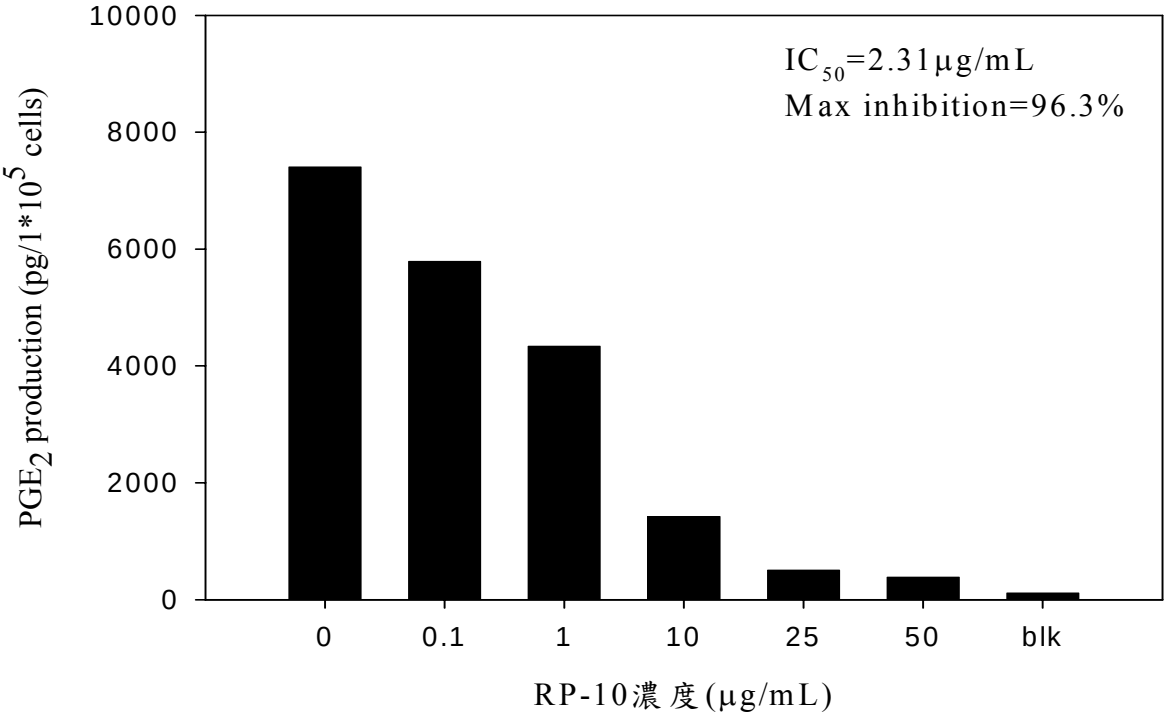


圖 3-16 活性區分物RP-10 抑制LPS誘發RAW264.7 細胞生成PGE_2之劑量效應

Fig3-16 Dose-dependent inhibition of the fraction RP-10 on the PGE_2 production in LPS activated RAW264.7 cells. The cells were treated with various concentrations of the fraction in the presence of LPS (100 ng/mL) for 18 hrs and medium was collected for PGE_2 analysis using an EIA assay.RP-10 was obtained in the procedure was shown in Fig2-9.

blk：「blank」RAW264.7 cells were incubated with medium only for 18 hrs,

0: RAW264.7 cells were incubated with medium containing 100ng/mL LPS only for 18 hrs.

4. F1-6 鹼水解 EA 萃取物 (第四次實驗)

由RP-10 所測得^{1}H-NMR 及H-H COSY之光譜結果，可知該活性成分為TG，且其組成的脂肪酸為中、短鏈飽和的型式。因此，藉由將F1-6 鹼水解，分離並以EA萃取其中極性的脂肪酸(圖 2-10)，進行細胞活性的實驗，以評估此活性區分物經水解後是否仍具有抑制PGE_2的活性。結果如圖 3-17 所示：此活性區分物F1-6 即使經過水解後，仍有活性，且於 50 μg/mL達完全抑制，IC_{50} = 14.99 μg/mL，Max inhibition=99.35%。並將水解後含中極性脂肪酸的EA萃取物，進行甲基化，送測GC-Mass。結果得知(附錄圖三)：當中含有短鏈雙酸的型式包括：octanedioic acid (八碳雙酸)、nonanedioic acid(九碳雙酸)及decanedioic acid(十碳雙酸)。其中，以nonanedioic acid(九碳雙酸)的含量較多

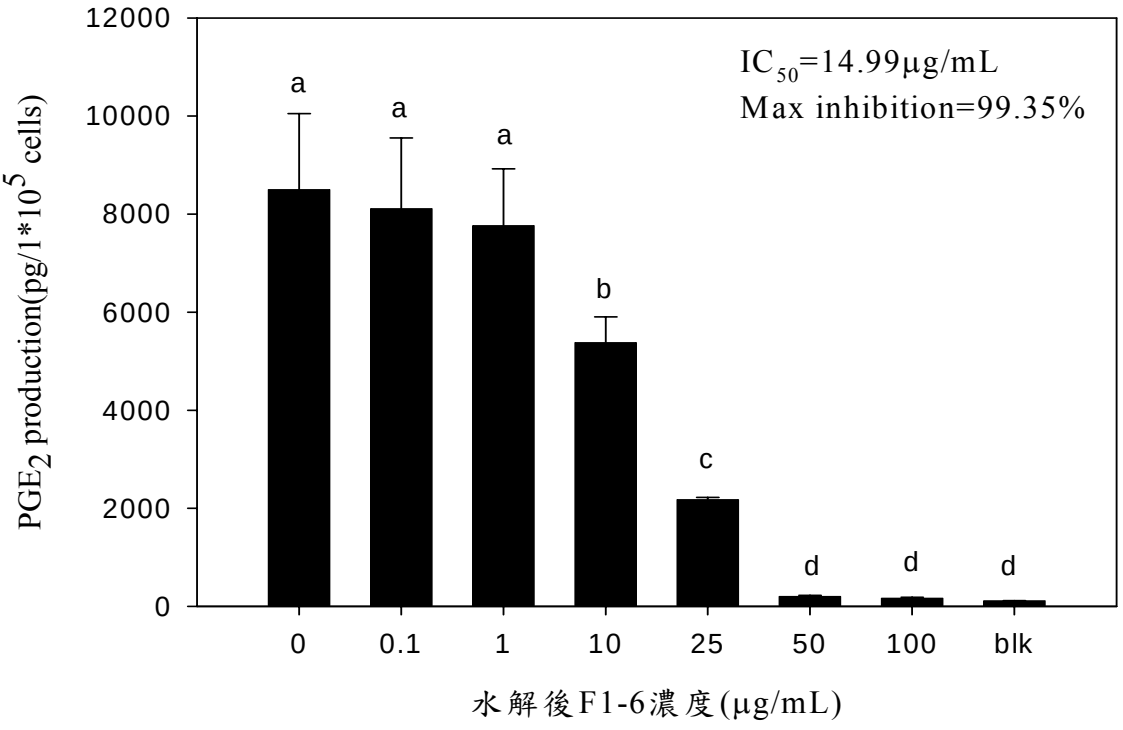


圖 3-17 活性區分物F1-6 水解後抑制LPS誘發RAW264.7 細胞生成PGE_2 之劑量效應(流程圖 2-10)

Fig3-17 Dose-dependent inhibition of EA extract of hydrolyzed F1-6 on the PGE_2 production in LPS activated RAW264.7 cells. The cells were treated with various concentrations of the product in the presence of LPS (100 ng/mL) for 18 hrs and medium was collected for PGE_2 analysis using an EIA assay. The values are Mean ± SD of triplicates in a representative experiment .Hydrolysis and extraction procedure was shown in Fig2-10.

blk：「blank」RAW264.7 cells were incubated with medium only for 18 hrs

0: RAW264.7 cells were incubated with medium containing 100ng/mL LPS only for 18 hrs.

(二) Fraction151 ~ 158 純化分離

山苦瓜經大量萃取分離所得之Hexane區分物，以矽膠管柱分離所得之九個區分物(圖 2-5)，進行細胞活性實驗後發現：40%EA/Hex所流洗出來極性較低的F152亦具有抑制細胞PGE_2合成的活性(圖 3-10)。因此，將F152 再以矽膠管柱層析分離得到 3 個區分物(圖 2-11)，進行細胞活性試驗，結果如圖 3-18 所示：B3-5 及B7-12 均具有抑制效果。此兩個區分物從^{1}H-NMR及IR中，發現的特徵有：

B3-5：存在脂肪酸，有雙鍵、ω-3 雙鍵、OH-group 及酯化的特徵

B7-12：存在 steroids 及脂肪酸，有雙鍵、多組 OH-group 及酯化的特徵

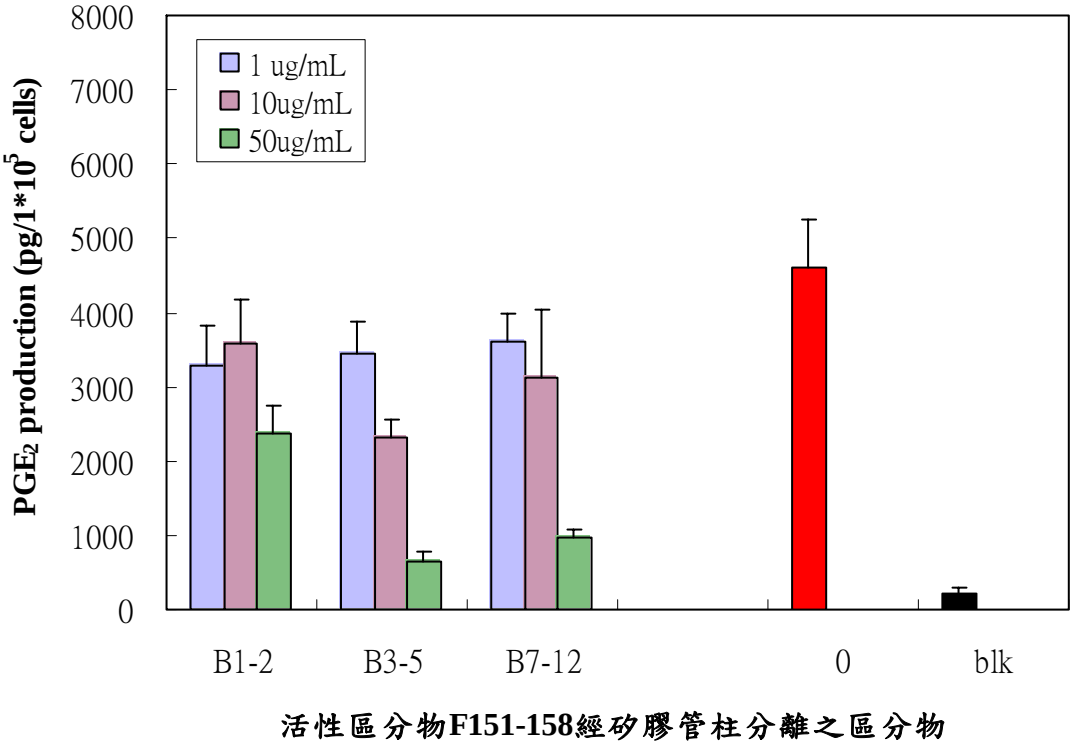


圖 3-18 活性區分物 F151-158 經矽膠管柱分離之區分物對RAW264.7 細胞生成PGE_2之影響 (流程圖 2-11)

Fig3-18 Effects of the fractions obtained by separating F151-158 using silica gel chromatography on the PGE_2 production in LPS activated RAW264.7 cells. The cells were treated with three concentrations of these fractions in the presence of LPS (100 ng/mL) for 18 hrs and medium was collected for PGE_2 analysis using an EIA assay. The values are Mean ± SD of triplicates in a representative experiment .Separation procedure was shown in Fig2-11.

blk：「blank」RAW264.7 cells were incubated with medium only for 18 hrs

0: RAW264.7 cells were incubated with medium containing 100ng/mL LPS only for 18 hrs.

1. B7-12 的純化分離

由於 B7-12 在 TLC 的結果為明確的點，且可能含有 steroid 的結構，因此決定先由 B7-12 著手分離。將具有抑制活性的 B7-12 進行甲基化，並以矽膠管柱層析分離，得 M16-24 及 M58-71(實驗一:圖 2-12)。結果發現：M16-24 為甲基化後的產物，而 M58-71 為起始物，於 40%EA/Hex 中靜置約一週後，出現透明針狀結晶。由於甲基化後仍為混合物。因此，決定改以其他方式分離 (實驗二:圖 2-13)。

而將M58-71 在 40%EA/Hex的溶劑中靜置約一週後，溶劑揮發，析出透明針狀結晶並由光譜鑑定出其結構(附錄圖四、五、六)。並以此結晶進行細胞活性實驗，結果如圖 3-19 所示：並無抑制PGE_2合成的作用 。

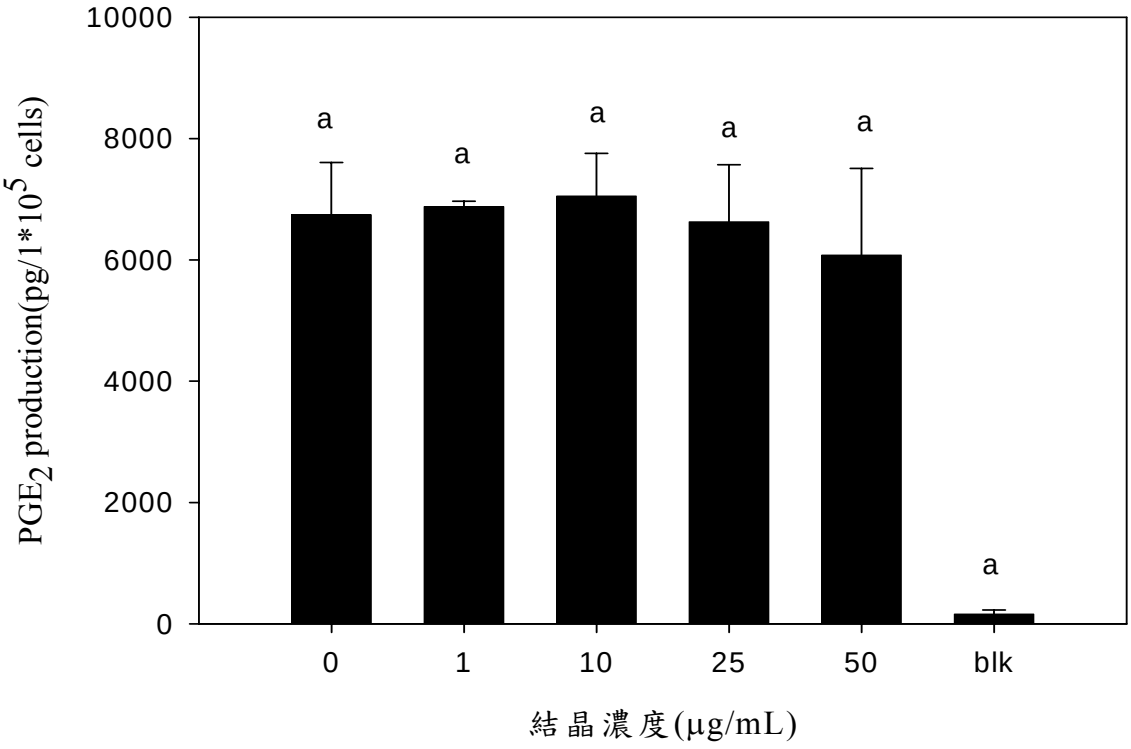


圖 3-19　B7-12 所析出的結晶對RAW264.7 細胞生成PGE_2無抑制作用

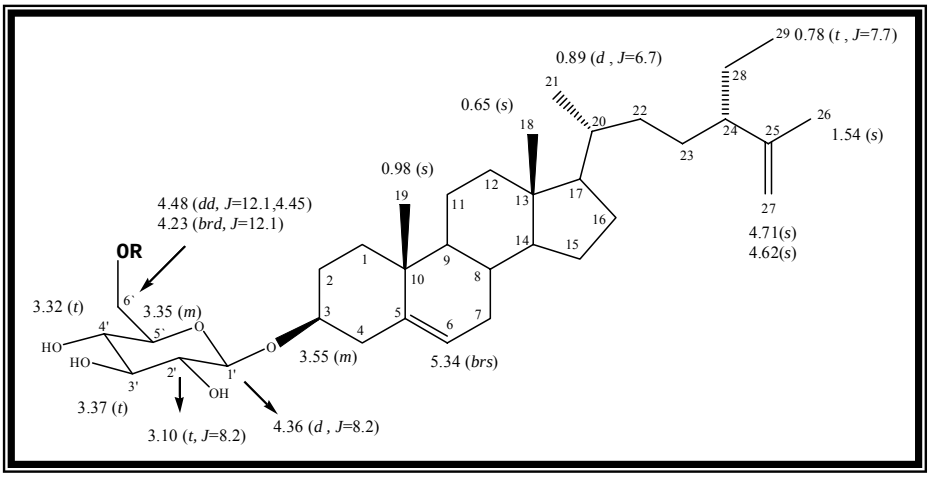
Fig3-19　No inhibition of the compound recrystallized from B7-12 on the PGE_2 production in LPS activated RAW264.7 cells. The cells were treated with various concentrations of the compound in the presence of LPS (100 ng/mL) for 18 hrs and medium was collected for PGE_2 analysis using an EIA assay. The values are Mean ± SD of triplicates in a representative experiment .The structure was shown below.

blk：「blank」RAW264.7 cells were incubated with medium only for 18 hrs

0: RAW264.7 cells were incubated with medium containing 100ng/mL LPS only for 18 hrs.

【結晶】

結晶的部分測^{1}H-NMR、^{13}C-NMR、FAB-MS及熔點測試，以確定其結構(附錄圖四、圖五及圖六)。分析所得之結構為如下：



☞ R= fatty acid (palmitate or C17 or C19)

Molecular Formula：$C_{51}H_{88}O_7$ M^+ *m/z*：813.7

Molecular Formula：$C_{52}H_{92}O_7$ M^+ *m/z*：827.6

Molecular Formula：$C_{54}H_{96}O_7$ M^+ *m/z*：855.6

☞熔點測試結果為：181ºC

☞Chemical Name：

3-O-<6'-O-palmitoyl-β-D-glucosyl>-stigmasta-5,25(27)-diene hexadecanoic acid 6-[17-(4-ethyl-1,5-dimethyl-hex-5-enyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-3-yloxyl]-3,4,5-trihydroxy-tetrahydro-pyran-2-ylmethyl ester.

此結晶所鑑定的結構為接上飽和脂肪酸，是目前已知成分(Leitao *et al*.,1994；Guevara et al.,1989)。此外，由實驗二 (圖 2-13) 乙醯化的結果確知當中含有 3 個 OH-group 存在，也符合結晶結構的結果，為六碳醣上的 3 個 OH-group。

2. B3-5 的純化分離

從B7-12 所分離的結晶並非為具有活性的成分，因此推測當中可能有活性的成分與B3-5 是重疊的。因此，欲從B3-5 再進行分離。以製備式TLC分離得到T1 ~ T5 的區分物(圖 2-14)。目前未測細胞活性。其中，T3 較為大量，測^{1}H-NMR的結果發現：為脂肪酸混合物。特徵包括：含有雙鍵，ω-3 雙鍵、OH-group及酯類。

七 測試脂肪酸標準品之活性

在山苦瓜的分離過程中，發現具有活性的區分物當中，存在脂肪酸或脂肪酸酯化(TG)型式的特徵。而為進一步確知何種脂肪酸具有抑制PGE_2的活性，乃以標準品進行細胞實驗。首先，以一般長鏈脂肪酸、CLN及CLA進行活性分析。結果如圖 3-20 所示：一般長鏈脂肪酸、CLN及CLA並無抑制細胞PGE_2的合成，且Linoleic acid及Arachidonic acid相對的有促進細胞PGE_2合成的作用。

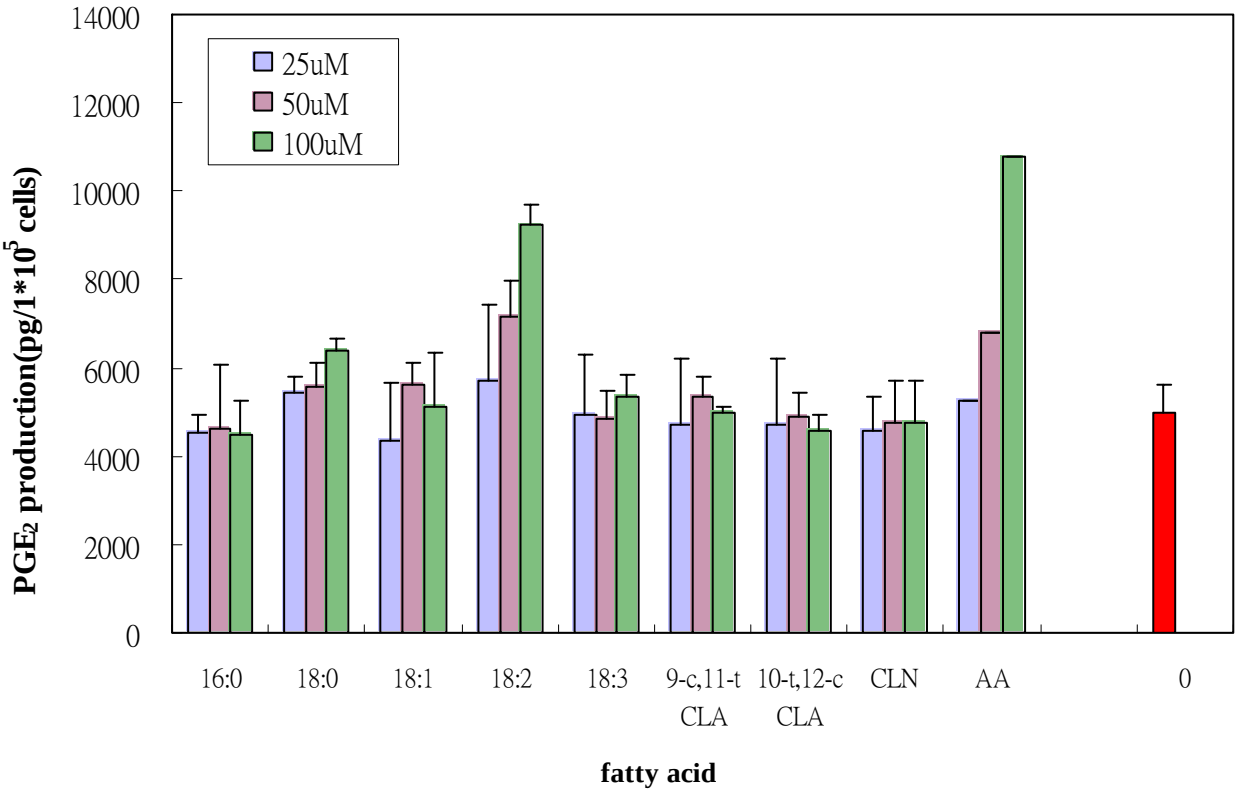


圖 3-20 一般長鏈脂肪酸、CLN及CLA對RAW264.7 細胞生成PGE_2之影響 (表 2-2)

Fig3-20 Effects of common long chain fatty acids , CLN and CLA on the PGE_2 production in LPS activated RAW264.7 cells. The cells were treated with three concentrations of fatty acids in the presence of LPS (100 ng/mL) for 18 hrs and medium was collected for PGE_2 analysis using an EIA assay.

0: RAW264.7 cells were incubated with medium containing 100ng/mL LPS only for 18 hrs.

由於鑑定F1-6 的活性區分物RP10 可能為中、短鏈飽和脂肪酸的TG型式，故

選擇以中、短鏈脂肪酸標準品及MCT oil進行細胞實驗。結果如圖 3-21 所示：Capric acid (10:0)可顯著抑制巨噬細胞PGE_2的合成，而Lauric acid (12:0)於 100 μg/mL亦有抑制的現象。至於MCT oil對細胞PGE_2合成的影響，如圖 3-22 所示發現：MCT oil有部分抑制PGE_2合成的現象，但當MCT oil濃度為 50 μg/mL時，細胞存活率有減少的現象。由圖 3-21 的結果發現：Capric acid可顯著抑制細胞PGE_2的合成，因此試以不同濃度的Capric acid處理細胞，視其劑量效應。結果如圖 3-23 所示：Capric acid於 50 μg/mL幾可完全抑制PGE_2的合成，IC_{50}=6.46 μM (1.216 μg/mL)，Max inhibition=98.69%。

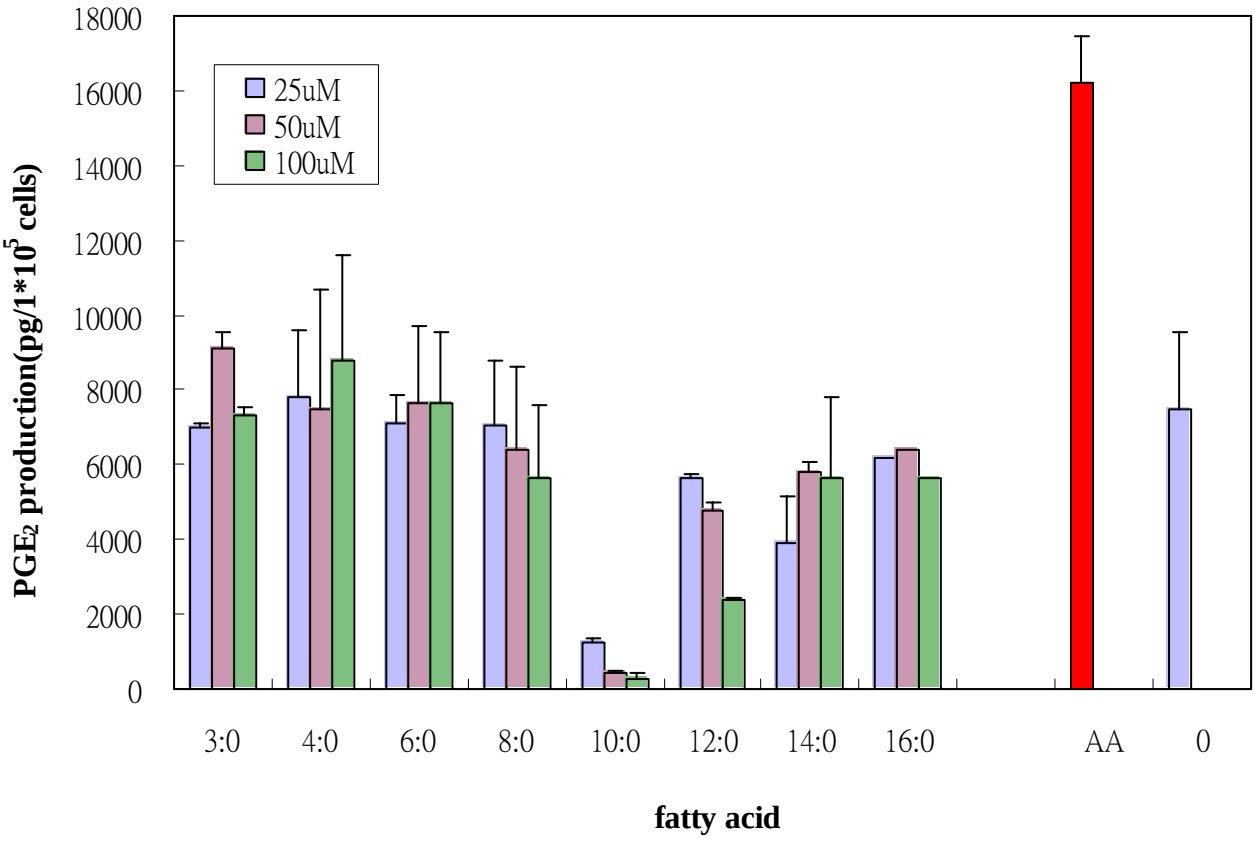


圖 3-21 中、短鏈脂肪酸對RAW264.7 細胞生成PGE_2之影響 (表 2-2)

Fig3-21 Effects of medium and short chain fatty acids on the PGE_2 production in LPS activated RAW264.7 cells. The cells were treated with three concentrations of fatty acids in the presence of LPS (100 ng/mL) for 18 hrs and medium was collected for PGE_2 analysis using an EIA assay. AA；arachidonic acid.

0: RAW264.7 cells were incubated with medium containing 100ng/mL LPS only for 18 hrs.

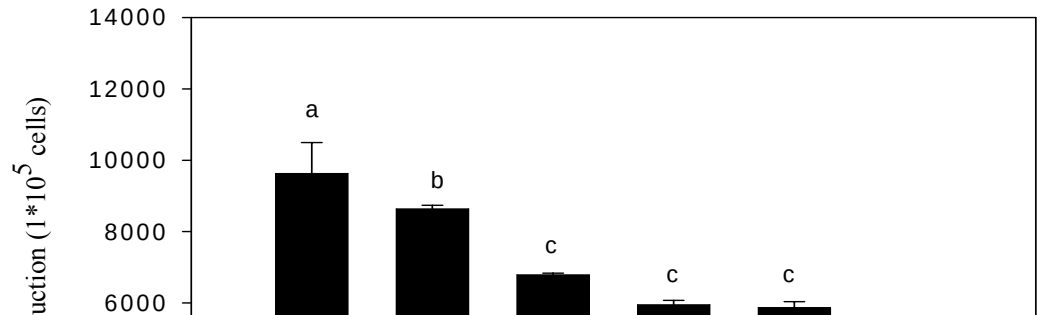


圖 3-22 中鏈三酸甘油酯對RAW264.7 細胞生成PGE_2之抑制作用

Fig3-22 Effects of MCT oil on the PGE_2 production in LPS activated RAW264.7 cells. The cells were treated with various concentrations of MCT oil in the presence of LPS (100 ng/mL) for 18 hrs and medium was collected for PGE_2 analysis using an EIA assay.

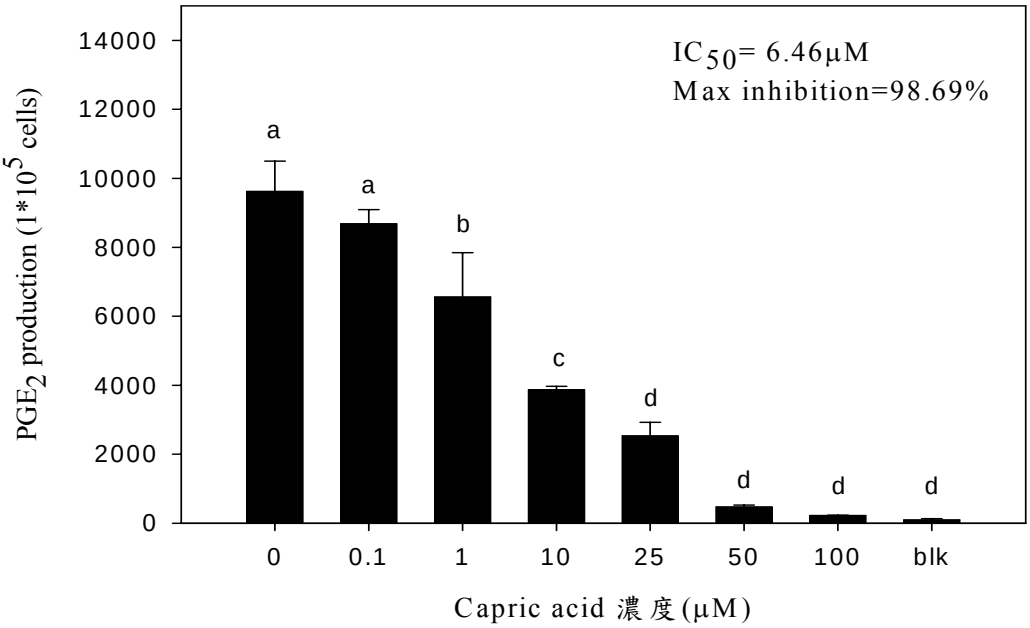


圖 3-23 Capric acid抑制LPS誘發RAW264.7 細胞生成PGE_2之劑量效應

Fig3-23 Dose-dependent inhibition of Capric acid on the PGE_2 production in LPS activated RAW264.7 cells. The cells were treated with various concentrations of Capric acid in the presence of LPS (100 ng/mL) for 18 hrs and medium was collected for PGE_2 analysis using an EIA assay.

blk：「blank」RAW264.7 cells were incubated with medium only for 18 hrs

0: RAW264.7 cells were incubated with medium containing 100ng/mL LPS only for 18 hrs.

而將F1-6鹼水解後，以EA萃取中極性脂肪酸後甲基化(圖 2-10)，送測GC-Mass的結果發現：當中含有短鏈雙酸的型式包括：octanedioic acid (八碳雙酸)、nonanedioic acid(九碳雙酸)及decanedioic acid(十碳雙酸)。其中，以nonanedioic acid(九碳雙酸)的含量較多。而以其標準品進行細胞實驗，結果如圖 3-24 圖 3-25 圖 3-26 所示：八碳及九碳雙酸可部分抑制PGE_2合成。而與Capric acid相較下，雙酸的抑制效果較低。

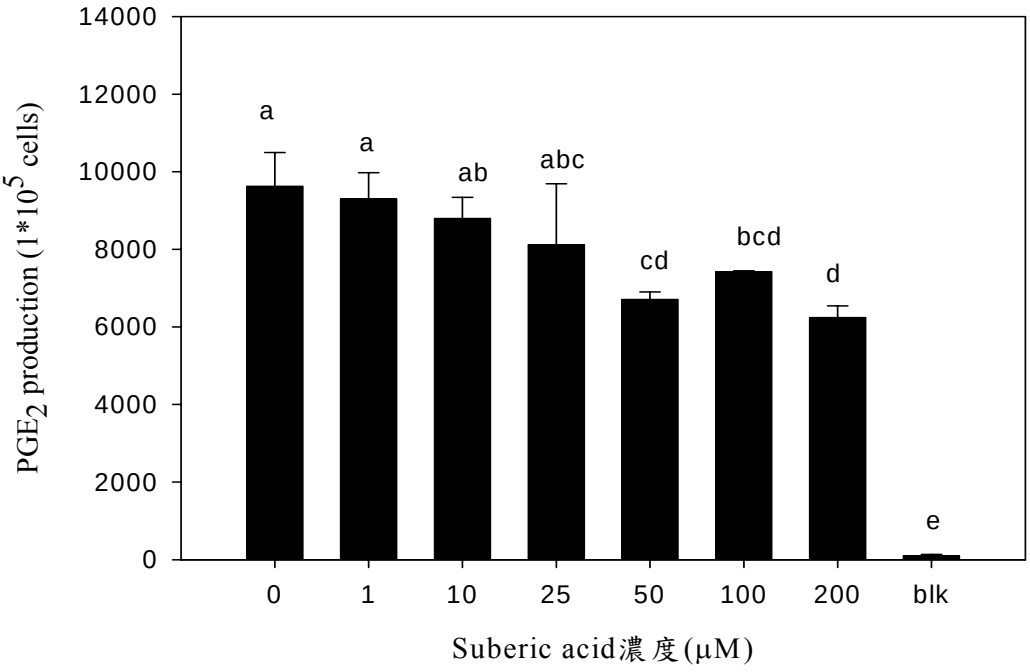


圖 3-24　Suberic acid(Octanedioic acid)對RAW264.7 細胞生成PGE_2之影響

Fig3-24　Effects of Suberic acid on the PGE_2 production in LPS activated RAW264.7 cells. The cells were treated with various concentrations of Suberic acid in the presence of LPS (100 ng/mL) for 18 hrs and medium was collected for PGE_2 analysis using an EIA assay.

blk：「blank」RAW264.7 cells were incubated with medium only for 18 hrs

0: RAW264.7 cells were incubated with medium containing 100ng/mL LPS only for 18 hrs.

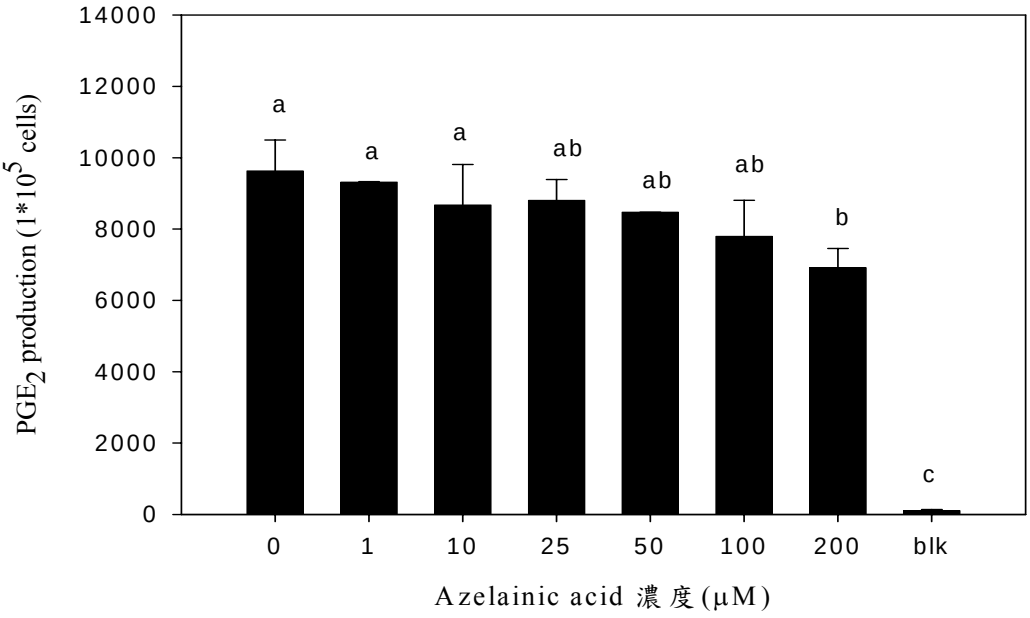


圖 3-25　Azelainic acid (Nonanedioic acid)對RAW264.7 細胞生成PGE_2之影響

Fig3-25　Effects of Azelainic acid on the PGE_2 production in LPS activated RAW264.7 cells. The cells were treated with various concentrations of Azelainic acid in the presence of LPS (100 ng/mL) for 18 hrs and medium was collected for PGE_2 analysis using an EIA assay.

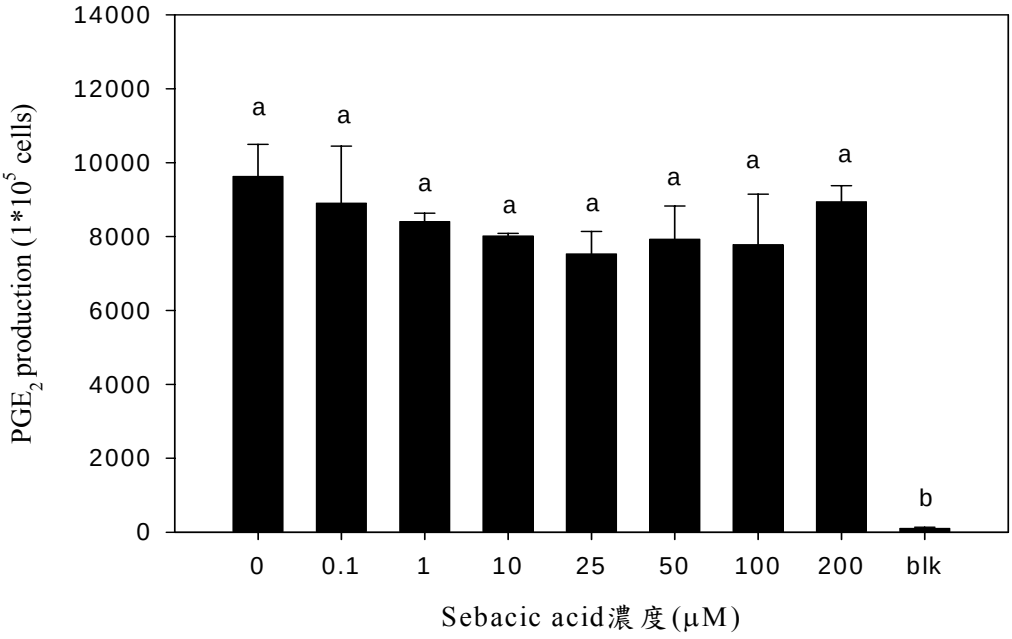


圖 3-26　Sebacic acid (Decanedioic acid)對RAW264.7 細胞生成PGE_2之影響

Fig3-26　Effects of Sebacic acid on the PGE_2 production in LPS activated RAW264.7 cells. The cells were treated with various concentrations of Sebacic acid in the presence of LPS (100 ng/mL) for 18 hrs and medium was collected for PGE_2 analysis using an EIA assay.

表 3-1 山苦瓜活性區分物及標準品之IC_{50}、Max inhibition及最高效應濃度一覽表

山苦瓜處理方式		**IC_{50}**	**Max inhibition**	**最高效應濃度**
山苦瓜全果凍乾粉末	乙酸乙酯萃(EAE)	14.25 μg/mL	99.12 %	200μg/mL
山苦瓜全果凍乾粉末的乙酸乙酯萃物(EAE)經對萃後之區分物	Hexane 區分物	91.2μg/mL	59.48 %	400μg/mL
	EAE-2 區分物	6.99μg/mL	73.3%	20μg/mL*
	H_2O區分物	6μg/mL	53.54 %	100μg/mL
山苦瓜 Hexane 萃取物以矽膠管柱層析分離(少量)	Fraction 58-62	7.16μg/mL	98.01 %	50μg/mL
山苦瓜大量萃取分離	Fraction 190	3.04μg/mL	97.79 %	25μg/mL
Hexane 萃取層以矽膠管柱層析分離	Fraction 152	13.6μg/mL	96.71 %	50μg/mL
Hexane 萃取層之活性區分物 F189-191 以矽膠管柱層析分離	F1-6	7.05μg/mL	90.97 %	50μg/mL
Hexane 萃取層之活性區分物 F1-6 以 RP-18 管柱分離(第三次實驗)	RP10	2.31μg/mL	96.3 %	25μg/mL
Hexane 萃取層之活性區分物 F1-6 經鹼水解 EA 萃取(第四次實驗)	F1-6 水解 (EA 萃取)	14.99μg/mL	99.35 %	50μg/mL
脂肪酸及雙酸樣品之篩選		**IC_{50}**	**Max inhibition**	**最高效應濃度**
Capric acid (C10:0)		6.46 μM (1.216μg/mL)	98.69 %	50 μM

* 當處理細胞之樣品濃度提高，細胞存活率下降。