由苦瓜中篩選可改善胰島素抗性之活性天然物

鄭雪玲　助理教授

國立屏東科技大學生物科技研究所

摘　要

第二型糖尿病發生的機制目前認為與細胞胰島素抗性（insulin resistance）的形成有關。因此目前醫學界認為若能治療胰島素抗性，將能預防第二型糖尿病的發生或改善已患該病者之病情。本研究模仿動物體內胰島素抗性發生的機制，測試以 TNF-α（tumor necrosis factor-α）刺激，誘導小鼠 FL83B 肝臟細胞產生胰島素抗性，以建立一糖尿病藥物篩選平台，並利用此平台進行苦瓜降血糖活性成分之篩選。以此平台檢測白蓮苦瓜各部份之甲醇粗萃物，發現白蓮苦瓜莖材、果實、種子之粗萃物，皆有改善細胞胰島素抗性、促進葡萄糖吸收之活性。將白蓮苦瓜莖材之甲醇粗萃物進一步萃取、分層，發現其中正己烷層具有活性。再將正己烷層進一步分成 22 個分液（fractions），發現其中 3 個分液有改善細胞胰島素抗性的降血糖活性。分液 13 及分液 18 之成分已被純化鑑定。其中，純物質 CH10、CH63 及 CH93 被證實對胰島素抗性細胞的葡萄糖吸收及 IRS-1 的酪胺酸磷酸化均有明顯提高作用。同時，這些物質亦被證明可活化 AMP-activated protein kinase。以相同的方法對新品種山苦瓜，花蓮 2 號，進行篩選工作，目前至少發現了一種三萜類化合物具有相同的活性。這些結果建議苦瓜含有改善胰島素抗性的成分，具有開發為第二型糖尿病保健食品或治療藥物之潛力。

關鍵詞：第二型糖尿病、胰島素抗性、白蓮苦瓜、花蓮 2 號山苦瓜、AMP-activated protein kinase, FL83B 細胞

前言

糖尿病是目前世界各國最普遍的多病因代謝疾病（Gerich, 2001）。台灣糖尿病人口不僅逐年增加，且為十大死亡原因排名第四位。台灣地區的糖尿病型態以非胰島素依賴型糖尿病為主，亦即第二型糖尿病，約佔罹患率的 95%左右。根據統計資料顯示，罹患有第二型糖尿病者，在患病之前身體會先出現胰島素抗性（又稱代謝症候群），而此症狀若沒有及時治療或改善，將來可能就會發展成為第二型糖尿病，亦即胰島素抗性症狀患者為罹患第二型糖尿病的高危險群。因此目前認為若能對此高危險群者採取預防措施，盡快矯正其胰島素抗性的問題，則可預防將來繼續發展為糖尿病，並能節省醫療資源。

世界各地的民俗草藥治療糖尿病已有十分悠久的歷史。根據本草綱目的記載及民間流傳，葫蘆科植物包括苦瓜、南瓜及冬瓜等食用植物，經常食用有降血糖之功效。然而這些草藥雖民間流傳為有效用，卻往往因為缺乏系統性的研究與比較，所以仍停留於民間草藥或秘方，實在可惜。以苦瓜為例，於中國、日本、印度、南美洲等地，苦瓜不僅長期被使用為食用性蔬菜，亦被使用為傳統民俗藥物，包括用於治療糖尿病（Grover and Yadav, 2004)，但是其降血糖之天然成分卻一直不清楚，且沒有被系統性地分析與研究。缺乏一適當的分析、篩選工具是主要原因之一。

植物品系眾多，且所含天然物相當複雜，若欲分析其活性成分，所需進行的篩選工作相當龐大，無法直接以實驗動物如老鼠等進行篩選工作。因此，本研究開發以細胞為工具，將細胞株誘導使具有胰島素抗性，以此為一平台篩選出可克服細胞胰島素抗性、促進細胞葡萄糖吸收的活性天然物。

胰島素抗性是指血液循環中之胰島素調節體內能量代謝的能力降低(Zick, 2001)。亦即，在正常濃度的胰島素生理濃度下，身體細胞對胰島素的敏感度下降甚或對之毫無反應。研究資料顯示，除了糖尿病，胰島素抗性也會導致其他代謝相關的疾病，包括高血壓(hypertension)、血脂異常(dyslipidemia)、動脈硬化(atherosclerosis)、微蛋白尿(microalbuminuria)、血液易凝固(hypercoagulability)、中央性肥胖(central obesity)及其他心血管疾病等(Weyer et al., 1999; Isomaa et al., 2001; Hanley et al., 2002)，因此胰島素抗性又被稱為代謝症候群(the metabolic syndrome) (Lebovitz, 2001)。研究資料指出，第二型糖尿病患者在患病前之 10-20 年間會先出現胰島素抗性，

而且第二型糖尿病患者皆患有胰島素抗性(Lillioja, 1988)。因此胰島素抗性的出現被認為是一個個體可能會發展成第二型糖尿病之先兆(Lillioja, 1988; Warram et al., 1990; Shulman, 2000)。研究證實，胰島素抗性之分子機制與胰島素訊息傳遞路徑中之 IRS(insulin receptor substrate)的酪胺酸磷酸化(tyrosine phosphorylation)有關。胰島素訊息傳遞之過程，IRS 因接受酪胺酸磷酸化而被活化，進而繼續活化下游之胰島素訊息傳遞路徑。但證據顯示，具有胰島素抗性的細胞，其 IRS 的酪胺酸磷酸化受到干擾，所以無法活化下游的路徑，使胰島素訊息傳遞路徑之活動中斷(Dresner et al., 1999; Lebovitz and Banerji, 2004)。

體重過重的患者是罹患第二型糖尿病的高危險群。研究其機制，發現可能與脂肪細胞所分泌的 adipokines 有關。例如，研究發現，肥胖者的脂肪細胞會分泌較多 TNF-α，而 TNF-α 會抑制 IRS-1（為 IRS 的主要 isoform 之一）的酪胺酸磷酸化而導致胰島素抗性的產生(Zick, 2001)。此時，若不及時治療，最終即可能形成第二型糖尿病。故本研究意圖發展一個篩選系統，以具有胰島素抗性的細胞為平台，篩選出可改善或克服細胞胰島素抗性的物質，幫助預防或改善第二型糖尿病。

胰島素於動物體內主要作用於肝臟細胞、肌肉細胞及脂肪細胞。已有實驗證實以 TNF-α 處理肌肉細胞株(del Aguila et al., 1999)及脂肪細胞株(Iwata et al., 2001)，會使細胞產生胰島素抗性。但是常用的肌肉細胞株及脂肪細胞株，如 C2C12 及 3T3L1，皆須先經過分化才具有胰島素敏感性。如此，增加實驗操作的複雜度，使之要發展成為一個需要篩選大量物質的篩選系統較不利。而且不同盤或不同批次的細胞之間，分化的程度可能不相同，使細胞對胰島素的敏感程度不同，造成實驗誤差，使不同物質之間效果的比較會有差異，降低篩選工作的準確性。因此，本研究另外發展以肝臟細胞株作為篩選平台的方法。所選用之細胞株為小鼠正常肝臟細胞株 FL83B。此細胞株不必經過分化就有胰島素敏感性。本研究更發現 FL83B 細胞株經過 TNF-α 處理亦會產生胰島素抗性。目前已利用此平台由白蓮苦瓜及花蓮 2 號山苦瓜中篩選出數種可克服細胞胰島素抗性的活性天然物。並對這些天然物之作用機轉進行初步的探討。

內容

一、以TNF-α誘導FL83B細胞產生胰島素抗性

將FL83B以不同濃度之TNF-α(10 ng/ml、20 ng/m)處理細胞5小時，接著再培養於無血清培養基2小時，最後再改用MEM培養基加100 nM胰島素，刺激細胞的葡萄糖吸收作用。分別於加入胰島素後第0、1、2、3、4、5小時取30μl培養基檢驗其葡萄糖濃度。如圖1所示。X軸代表加入胰島素後的時間，Y軸之500 nm吸光值與葡萄糖濃度成正比，吸光值愈高代表葡萄糖濃度愈高。Control爲正常的FL83B細胞沒有胰島素刺激；control+insulin爲接受胰島素刺激的FL83B細胞；TNF-α指經TNF-α處理5小時後再添加胰島素刺激的細胞。資料顯示，control+insulin與control相較，果然其培養基中之葡萄糖濃度有更明顯的減少，顯示FL83B細胞具有胰島素敏感性，在胰島素刺激下葡萄糖吸收速率明顯增加，但以10 ng/ml或20 ng/ml的TNF-α處理過的FL83B細胞，雖以胰島素刺激之，其培養基中葡萄糖濃度的降低明顯趨緩，其葡萄糖吸收速率接近control，暗示這些細胞對胰島素的敏感性降低，亦即細胞產生胰島素抗性。這些結果顯示以10 ng/ml或20 ng/ml的TNF-α進行前處理，均能誘導FL83B細胞產生胰島素抗性，其中以20 ng/ml的TNF-α具有更明顯的誘導效果。

二、檢驗白蓮苦瓜萃取物對胰島素抗性細胞葡萄糖吸收之影響

將分別來自白蓮苦瓜之果實、種子、莖部的甲醇萃取物，處理上述具有胰島素抗性的FL83B細胞，再檢驗這些細胞的葡萄糖吸收。結果如圖2所示[resistant+insulin爲胰島素抗性細胞（經20 ng/ml TNF-α處理之FL83B細胞）接受胰島素刺激]，白蓮苦瓜果實、種子、莖材萃取物之效果與50μM troglitazone（爲一胰島素增敏劑，臨床上用以治療胰島素抗性，這裡作爲陽性對照組）的效果相似，均具有促進胰島素抗性細胞葡萄糖吸收的能力 ($p < 0.05$ versus resistant+insulin)，顯示具有改善細胞胰島素抗性之效果。

將白蓮苦瓜莖材的甲醇萃取物進一步分層萃取（如圖3所示），再一一檢驗各層之活性。如圖4所示，100μg/ml甲醇粗萃物及10μg/ml正己烷層[hexane；圖3中標示爲(2)者]萃取物具有促進胰島素抗性細胞葡萄糖吸收的效果，顯示具有改善胰島素抗性之功效。其餘的萃取層，有的沒有明顯差異，有的則甚至較resistant+insulin的葡萄糖吸收速率還慢[100μg/ml乙酸乙酯層，圖3中標示爲(3)者；100μg/ml正丁醇層，圖3中標示爲(4)者]，暗示不但沒有改善胰島素抗性之功效，且對葡萄糖吸收可能有抑制效果。

接著將正己烷層萃取物進一步分為22個分液(fractions；如圖3所示)，並一一檢驗這些分液的活性。結果發現分液13、18及22之活性最高、最明顯。將分析分液10~22之結果分示於圖5A~C。

三、檢驗白蓮苦瓜單一天然物之活性及初步分子機制之探討

將分液13及18進一步純化。由分液13共獲得7種化合物，皆為三萜類(triterpenes)；由分液18共獲得5種化合物，一種三萜類，三種固醇類(steroids)，一種芳香類化合物。分液13所含的最主要兩種化合物分別為(23E)-cucurbita-5,23,25-triene-3β,7β-diol (以下簡稱為CH10) 及3β,25-dihydroxy-7β-methoxycucurbita-5,23(E)-diene (以下簡稱為CH63)。分液18所含的最主要化合物是 3β,7β,25-trihydroxycucurbita-5,23(E)-dien-19-al (以下簡稱為 CH93) ，是一種三萜類化合物。三者的結構如圖5D所示。將三者分別以胰島素抗性細胞測試，發現皆可克服胰島素抗性而促進細胞葡萄糖吸收（圖6A）。

將CH10、CH63及CH93所處理過之胰島素抗性細胞分析其IRS-1酪胺酸磷酸化情形，如圖6B所顯示，三者皆使胰島素抗性細胞內之IRS-1酪胺酸磷酸化提高，顯示三者可活化IRS-1。再者，分析亦發現，此三種化合物皆能於胰島素抗性細胞內活化AMPK (AMP-activated protein kinase)（如圖6C所示）。因為前人已證實AMPK可提高IRS-1之酪胺酸磷酸化而活化胰島素訊息傳遞路徑(Jakobsen et al., 2001)，所以，這些結果暗示，CH10、CH63及CH93可能是經由活化AMPK而提昇IRS-1之酪胺酸磷酸化，進而活化胰島素訊息傳遞路徑，所以可以克服細胞的胰島素抗性而改善細胞對胞外葡萄糖的吸收。目前這三種天然物之詳細作用機制正積極探討中。

四、檢驗花蓮2號山苦瓜單一天然物的活性

以與上述相似的方法對新品種苦瓜，花蓮2號山苦瓜，進行活性天然物之篩選。於過程中發現有數個分液具有活性，包括分液16。目前分液16已被進一步純化而得到3個化合物，分別簡稱為RA2-10、RA2-11及RA2-19，皆為三萜類化合物。將三者分別以胰島素抗性細胞測試，發現RA2-19可克服胰島素抗性而促進細胞葡萄糖吸收，但是另外兩種化合物的活性則較不明顯（圖7）。所以，目前亦正積極探討RA2-19之作用機制。

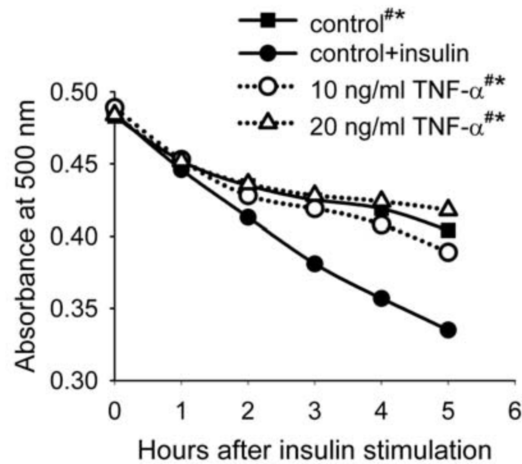


圖 1. 以 TNF-α 誘導 FL83B 細胞產生胰島素抗性。Control 爲正常的 FL83B 細胞沒有胰島素刺激；control+insulin 爲正常 FL83B 細胞受胰島素刺激；TNF-α 代表經 TNF-α 處理後再添加胰島素刺激的細胞。所顯示結果爲三次重複實驗之平均值±標準差(mean±SD)。所有資料皆以 two-way ANOVA 與“control+insulin”分析比較。#代表 p between groups < 0.05; *代表 p of interaction < 0.05.

Fig. 1. Induction of insulin resistance in FL83B cells by TNF-α. Control was normal cells without insulin stimulation; control+insulin was normal cells stimulated with insulin; other groups of cells were pre-incubated for 5 hours in medium containing either 10 ng/ml or 20 ng/ml of TNF-α, then stimulated with insulin. Glucose concentrations of the media were assayed at the time points indicated on the x-axis. Glucose concentrations were presented as absorbance at 500 nm and plotted as the y-axis. Each experiment was carried out in triplets. Data represent mean ± SD. Dataset of each cell group was statistically analyzed versus that of “control+insulin”by two-way ANOVA. #p between groups < 0.05; * p of interaction < 0.05.

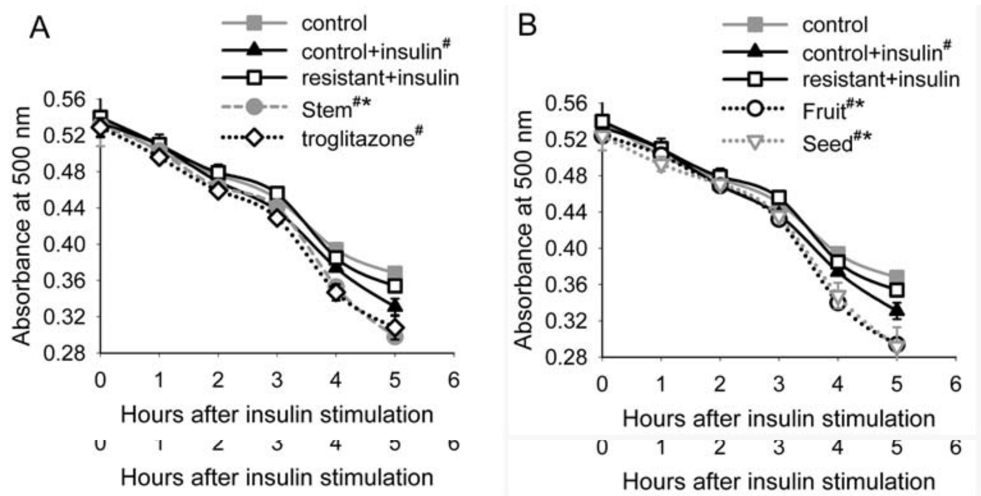


圖 2. 分析白蓮苦瓜各部甲醇萃取物對具胰島素抗性之 FL83B 細胞葡萄糖吸收之影響。Control 與 control+insulin 的意義與圖 1 同。Resistant+insulin 爲以 20 ng/ml TNF-α 處理過之胰島素抗性細胞，以胰島素刺激之。其他組細胞則是分別以 50μg/ml 白蓮苦瓜之果實（fruit）、種子（seed）、或莖部（stem）的甲醇萃取物處理胰島素抗性細胞，同時以胰島素刺激之。所顯示結果爲二重複實驗之平均值±標準差（mean±SD）。資料皆以 two-way ANOVA 與“resistant +insulin”分析比較。#代表 p between groups < 0.05; *代表 p of interaction < 0.05.

Fig. 2. Glucose uptake assays for cells treated with the crude extracts from different parts of *M. charantia*. Control and control+insulin were identical to those in Figure 1. Resistant+insulin was cells incubated in 20 ng/ml of TNF-αfor 5 hours (i.e. insulin-resistant cells) before stimulated by insulin. Other groups of cells were insulin-resistant cells treated with 50μg/ml of the methanol extract of either the stem, fruit, or seed of *M. charantia*, or treated with 50μM troglitazone, then stimulated by insulin. All groups of cells were assayed simultaneously, but due to the overlapping of curves, crude extract-treated groups were presented separately in two figures to make their curves more observable. The assays were carried out in duplicates. Data represent mean ± SD. Dataset of each cell group was statistically analyzed versus that of “resistant+insulin”by two-way ANOVA. #p between groups < 0.05; * p of interaction < 0.05.

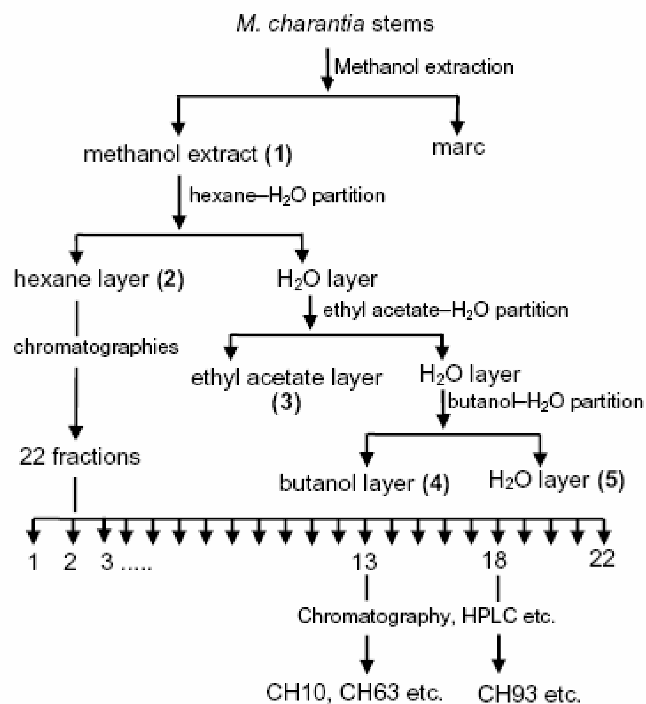


圖 3. 白蓮苦瓜莖材萃取物分離、純化流程圖。

Fig. 3. A flow chart showing the partitioning and purification of components in the methanol extract of the stem of *M. charantia.*

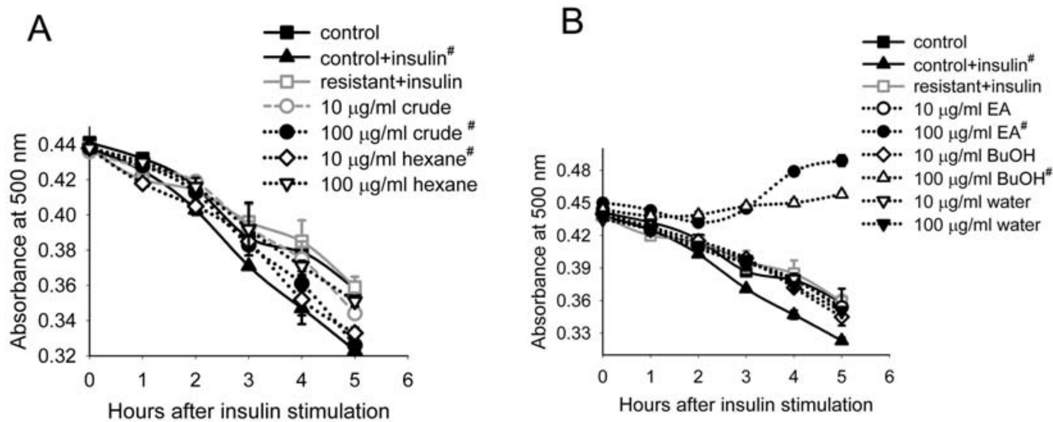
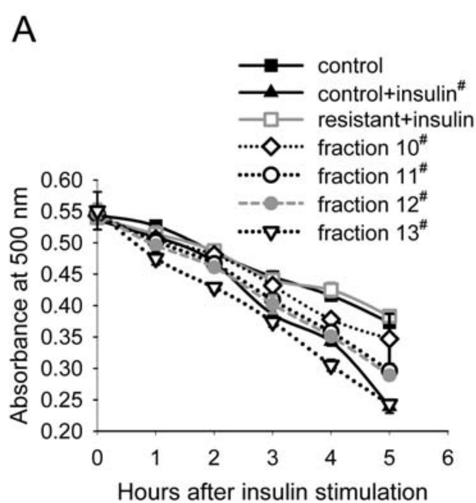
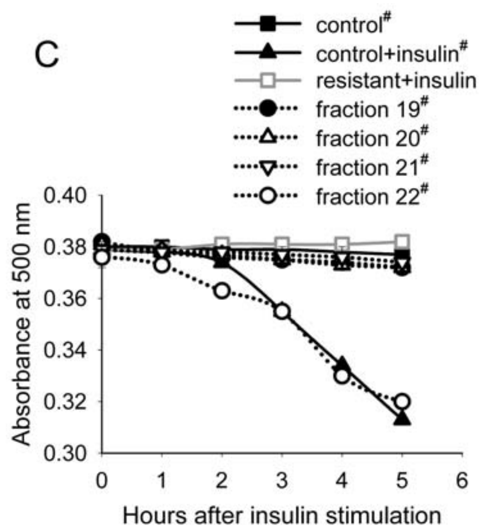
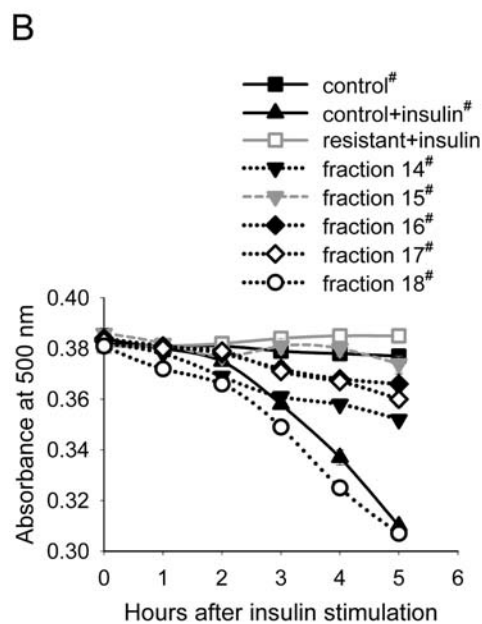


圖 4. 分析白蓮苦瓜莖材各萃取層對具胰島素抗性之 FL83B 細胞葡萄糖吸收之影響。Control、control+insulin、resistant+insulin 的意義與圖 2 同。其他組細胞則是分別以 10 或 100μg/ml 白蓮苦瓜莖部甲醇粗萃物（crude）、正己烷層（hexane）、乙酸乙酯層（EA）、正丁醇層（BuOH）、水層(water)處理胰島素抗性細胞，同時以胰島素刺激之。所顯示結果爲三重複實驗之 mean±SD。資料皆以 two-way ANOVA 與“resistant +insulin”分析比較。#代表 p between groups < 0.05 及 p of interaction < 0.05.

Fig. 4. Glucose uptake assays for cells treated with the partitions of the methanol extract of bitter melon stems. Glucose uptake assays were performed as in Figure 2. Crude, hexane, EA (ethyl acetate), BuOH (butanol), and water represent insulin-resistant cells treated with the partition marked as (1), (2), (3), (4), and (5) in Figure 3, respectively, then stimulated with insulin. The assays were carried out in triplets. Data represent mean ± SD. Dataset of each cell group was statistically analyzed versus that of “resistant+insulin”by two-way ANOVA. #p between groups < 0.05 and p of interaction < 0.05.


A
control
control+insulin#
resistant+insulin
fraction 10#
fraction 11#
fraction 12#
fraction 13#
Absorbance at 500 nm
Hours after insulin stimulation


C
control#
control+insulin#
resistant+insulin
fraction 19#
fraction 20#
fraction 21#
fraction 22#
Absorbance at 500 nm
Hours after insulin stimulation


B
control#
control+insulin#
resistant+insulin
fraction 14#
fraction 15#
fraction 16#
fraction 17#
fraction 18#
Absorbance at 500 nm
Hours after insulin stimulation

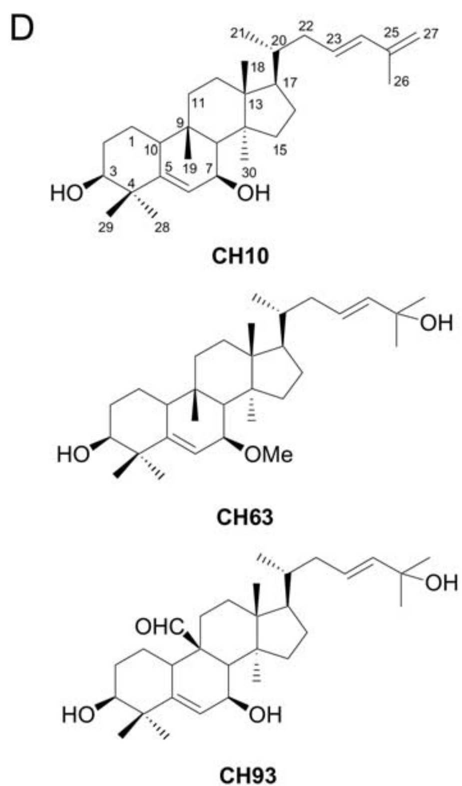

D
HO
OH
CH10
OMe
OH
CH63
OHC
HO
OH
CH93

圖 5. 分析白蓮苦瓜正己烷層各分液對胰島素抗性細胞葡萄糖吸收之影響響。以 10μg/ml 各分液處理胰島素抗性細胞，再分析其葡萄糖吸收情形。分液 10~22 的結果分示於 A~C。所顯示結果爲三重複實驗之 mean±SD。資料皆以 two-way ANOVA 與“resistant +insulin”分析比較。#代表 p between groups < 0.05 及 p of interaction < 0.05. D, CH10, CH63, 及 CH93 的化學結構。

Fig. 5. Glucose uptake assays for cells treated with fractions of the hexane layer and the chemical structures of CH10, CH63, and CH93. A, assays for fractions 10~13; B, assays for fractions 14~18; C, assays for fractions 19~22. All fractions were in 10μg/ml, and all were performed in triplets. Data represent mean ± SD. Dataset of each group was statistically analyzed versus that of “resistant+insulin” by two-way ANOVA. #p between groups and p of interaction both < 0.05. D, the chemical structures of CH10, CH63, and CH93.

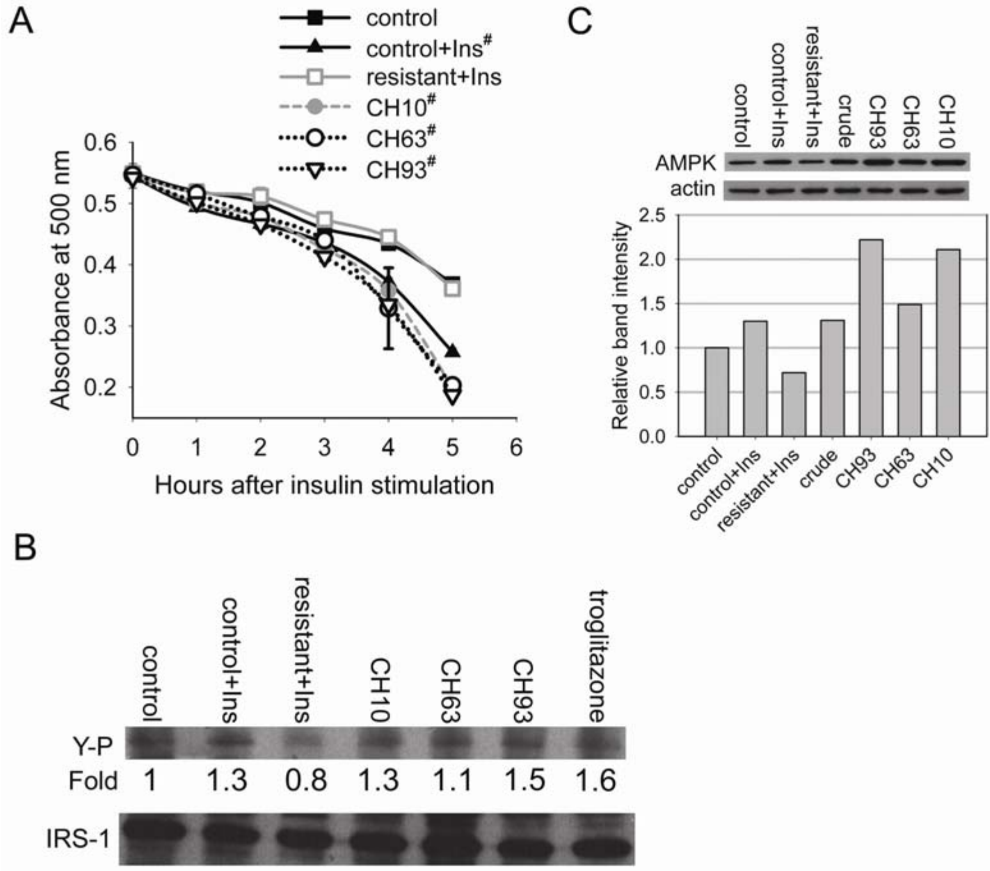

A
control
control+Ins#
resistant+Ins
CH10#
CH63#
CH93#
Absorbance at 500 nm
Hours after insulin stimulation
0.6
0.5
0.4
0.3
0.2
0 1 2 3 4 5 6
B
control
control+Ins
resistant+Ins
CH10
CH63
CH93
troglitazone
Y-P
Fold 1 1.3 0.8 1.3 1.1 1.5 1.6
IRS-1
C
control
control+Ins
resistant+Ins
crude
CH93
CH63
CH10
AMPK
actin
Relative band intensity
2.5
2.0
1.5
1.0
0.5
0.0

圖 6. 分析 CH10、CH63 及 CH93 之活性。以 10μg/ml 之 CH10、CH63 或 CH93 分別處理胰島素抗性細胞。Ins, insulin. A, 分析細胞葡萄糖吸收情形，方法同圖 2。所顯示結果爲三重複實驗之 mean±SD。資料皆以 two-way ANOVA 與“resistant +insulin”分析比較。# 代表 p between groups < 0.05 及 p of interaction < 0.05。B, 以 IRS-1 抗體進行免疫沉澱，再以 phosphotyrosine-specific antibody（Y-P）及 IRS-1 抗體（IRS-1）進行西方墨點法分析。Fold 代表各蛋白質條帶相對於 control 之密度，並以 IRS-1 之條帶密度校正過；C, 以 AMPK 磷酸化狀態（代表 AMPK 之活化）之專一性抗體進行西方墨點法分析。

Fig. 6. Assay for the activities of CH10、CH63 and CH93. 10μg/ml of CH10, CH63, or CH93 was used to treat insulin-resistant cells. A, glucose uptake assays as in Figure 2. Data represent mean ± SD. Dataset of each group was statistically analyzed versus that of “resistant+insulin” by two-way ANOVA. #p between groups and p of interaction both < 0.05. B, cells were harvested at 30 minutes after the treatment. Cellular proteins were immunoprecipitated by an IRS-1-specific antibody, then analyzed by Western blotting using either a phosphotyrosine-specific antibody (panel Y-P), or the IRS-1-specific antibody (panel IRS-1). Fold represents the relative band intensities between lanes in panel Y-P that were pre-normalized by the amount of IRS-1. The band intensity of “control” was set as 1. C, Western blotting using an antibody specific for the phosphorylated form of AMPK (panel AMPK) and an antibody forβ-actin (panel actin). Cells were harvested at 30 minutes after insulin stimulation. The histogram shows the relative band intensities of phosphorylated AMPK that were normalized by the levels of actin, with the band intensity of control being set as 1.

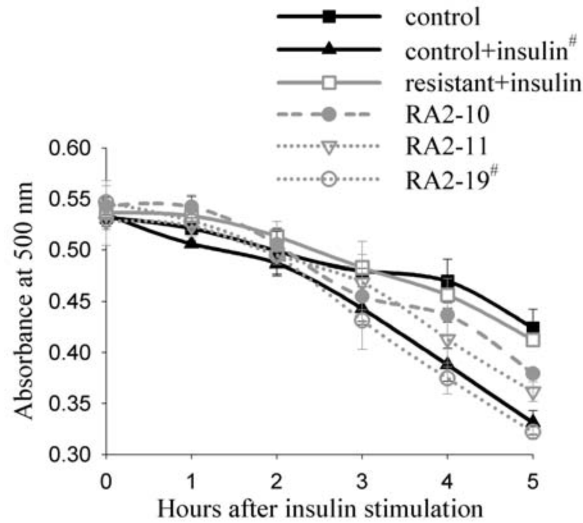


圖 7. 分析 RA2-10、RA2-11 及 RA2-19 對胰島素抗性細胞葡萄糖吸收之影響。以 10μg/ml 之 RA2-10、RA2-11 或 RA2-19 分別處理胰島素抗性細胞，再分析其葡萄糖吸收情形。所顯示結果爲三重複實驗之 mean±SD。資料皆以 two-way ANOVA 與"resistant +insulin"分析比較。#代表 p between groups < 0.05 及 p of interaction < 0.05。

Fig. 7. Glucose uptake assays for insulin-resistant cells treated with RA2-10、RA2-11 or RA2-19. 10μg/ml of RA2-10、RA2-11 or RA2-19 was used to treat insulin-resistant cells, and glucose uptake assays were performed as in Figure 2. Data represent mean ± SD. Dataset of each group was statistically analyzed versus that of "resistant+insulin" by two-way ANOVA. #p between groups and p of interaction both < 0.05.

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ABSTRACT

Insulin resistance plays a major role in the development of type 2 diabetes. Therefore, treatment of insulin resistance is suggested to prevent the occurrence of type 2 diabetes or improve the conditions of diabetic patients. To mimic the molecular mechanism of the development of cellular insulin resistance in vivo, we have treated mouse liver cell line FL83B with tumor necrosis factor-α(TNF-α) for the induction of insulin resistance. This established a system for the screening of hypoglycemic compounds from Momordica charantia. It was revealed that the crude extracts of stem, fruit, seed of M. charantia all contained bioactive compounds that could overcome cellular insulin resistance and improve glucose uptake of the cells. The extract from the stem of bitter gourds was further partitioned that three out of 22 fractions were with obvious hypoglycemic activities. Subsequently, pure compounds CH10, CH63 and CH93 were isolated from fractions 13 and 18. These compounds were confirmed to enhance glucose uptake and tyrosine phosphorylation of IRS-1 in insulin-resistant cells. Moreover, they were shown to activate AMP-activated protein kinase. Similar approaches were utilized to screen hypoglycemic compounds from a new strain of M. charantia , Hualien no. 2 wild bitter melon. Currently, at least one pure compound was isolated and identified to contain a similar effect. These results support that M. charantia contains components able to overcome cellular insulin resistance, suggesting a great potential of M. charantia in the development of new anti-diabetic food supplement or medicine.

Key words：type 2 diabetes, insulin resistance, *Momordica charantia,* Hualien no. 2 wild bitter melon, AMP- activated protein kinase, FL83B cells.