行政院國家科學委員會專題研究計畫　成果報告

石鹼樹皮中之皂素成分抑制人類前列腺素癌細胞生長與誘發細胞凋亡之機制探討

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摘要

皂素（saponins）是存在於植物界的一類比較複雜的苷類化合物，具有特殊的生理活性。如大豆皂素、人蔘皂素、絞股藍皂素和柴胡皂素等的生理功能及藥用價值等方面已有深入研究。本研究選用石鹼樹樹皮的皂素，是屬於三帖類植物皂素，其結構似類固醇，具有表面活性之特性。利用石鹼樹皮中的皂素對一些在台灣有著高發生率和高死亡率的腫瘤細胞，包括肝癌細胞(HepG2、Hep3b)、乳癌細胞(MCF7)、前列腺癌(PC-3)進行藥物細胞毒性試驗，從初步結果觀察到此種來自石鹼樹樹皮之皂素，以抑制前列腺癌細胞(PC-3)生長的效果最佳。此外，若以較高濃度劑量的 Saponin 處理前列腺癌細胞(PC-3)，會對細胞引起急性的毒性，在三小時的處理下觀察到細胞膜呈現萎縮的外型，甚至有漂浮的死細胞。以 trypan blue assay 再次確認 Quillaja saponin 會抑制人類前列腺癌細胞(PC-3)的生長。在兩株不同類型(androgen dependent- LNCap 及 androgen indenpent- PC-3)的前列腺癌細胞處理 Quillaja saponin 時，發現 androgen indenpent- 的 PC-3 細胞對於 Quillaja saponin 會有高度的藥物敏感毒性。進一步探討造成前列腺癌細胞死亡之機制，以 20μg/ml 劑量的 Quillaja saponin 處理下，subploid 的細胞群落會隨時間增加而增加、染色質濃縮，處理 9 小時後出現 DNA 斷裂等細胞凋亡型態上的特徵。處理 6 小時測得 caspase-3 的活性，並從蛋白質電泳圖看到活化態 caspase-3 蛋白質出現，其下游的受質也就是具有修補受損 DNA 功能的 PARP 蛋白質也會受到活化態 caspase-3 的切割作用而出現 85Kda 的蛋白質片段。

接著偵測細胞凋亡時，細胞內的氧化壓力是否增加及粒線體膜電位功能是否喪失，但實驗結果並沒有偵測到細胞內有過量的活性氧物質及膜電位的下降，因此細胞凋亡的過程並不是透過細胞氧化的增加所致。

關鍵詞：皂素、石鹼樹皮、前列腺癌細胞(PC-3)、細胞凋亡

Triterpenoid Saponins from Quillaja bark inhibit PC-3 human prostate cancer cells proliferation and induce apoptosis

Abstract

Saponin, which are present in plants, have been suggested as possible anticarcinogens. As we know, soybeans are one of the most important source of dietary saponins and ginseng saponins have been demonstrated that also can induce apoptosis in many tumor cell lines. Apoptosis play a critical role in both the normal development and the pathology of a wide variety of tissues and can be characterized by cytoplasmic shrinkage, nuclear condensation, and DNA fragmentation. Initiation of apoptosis is controlled by regulation the balance between the death and life signals perceived by cell. In recent years, the regulation of cell cycle and apoptosis has received much attention as possible means of eliminating excessively proliferatiing cells.

Quillaja bark saponins, one kind of triterpenoids, have a common steroid-like structure and possess surface-active characteristics that are due to their chemical structure. In this preliminary study, we use MTT assay to evaluate the cell viability of several different kinds of tumor cells and find that Quillaja bark saponins can inhibit tumor cels proliferation especially prostate cancer cell line, PC-3, and also induce PC-3 cells death through apoptosis, which is examined by sub-G1, nuclear condensation, and DNA ladder.

Excessively oxidative stress may trigger cell death through mitochondrial dependent apoptosis. By means of flow cytometry, we find that Quillaja bark saponins don’t cause oxidative stress in PC-3 cell. As the result, Quillaja bark saponins inducing PC-3 cells death may go through the mitochondrial-independently apoptotic pathway.

key word: saponins、Quillaja bark、prostate cancer cell line (PC-3)、apoptosis

Introduction

生藥是我國傳統國粹，國人用來治病已有數千年歷史，而目前有許多文獻顯示自由基和許多疾病有關，本實驗室一向致力於自由基的研究，而自由基又與許多疾病有關，因此大部分著重於生藥抗自由基的研究，尤其是超氧陰離子及脂質過氧化作用。

在超氧陰離子實驗中，先以非酵素性產生的超氧離子系統(NADH-PMS-NBT 系統)來產生超氧陰離子，並加入不同生藥於反應系統看生藥清除超氧陰離子的情形。結果發現所測試的生藥皆可減弱該反應所產生的自由基。

由於自由基所引起的細胞毒性反應很多，而其中重要也容易測得的是脂質過氧化，我們做了生藥抑制鼠肝微粒體脂質過氧化的初步實驗。在由微粒體酵素系統引起脂質過氧化的實驗中，發現脂質過氧化的過程含有多種自由基參與，倘若生藥可以直接去除超氧陰離子，則可以阻斷H_2O_2的生成，以致於$^{\bullet}HO$也不能產生而抑制脂質過氧化的起始反應，因為無法使--CH_2--形成自由基阻斷了脂質過氧化作用。

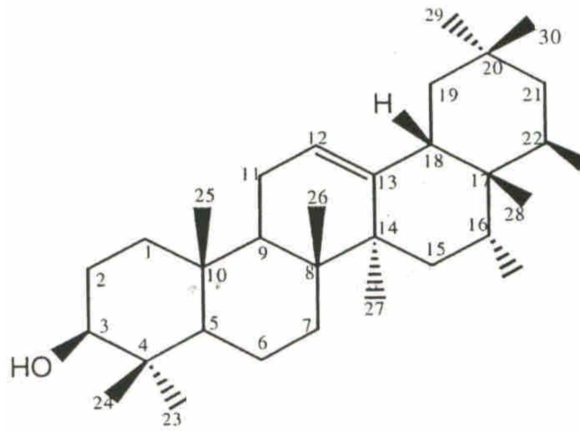
除了生藥抗自由基的研究外，近年來更致力於抗癌物的研發，有別於傳統研究上對於治療癌症藥物只著重於對細胞壞死與細胞毒性之研究，而在殺死癌細胞的同時亦造成了許多正常的死亡，導致病患在治療過程中造成個體極大的傷害。因而利用計畫性細胞凋亡的機轉作為治療癌症藥物的發展方向，並著重於天然物對抗癌細胞脂生長甚至殺死癌細胞。由石鹼樹皮的實驗中，我們發現石鹼樹皮中的皂素能抑制前列腺癌細胞的生長，並希望進一步研究石鹼樹皮的皂素透過哪一種途徑抑制癌細胞的生長。

由上面的結果可知生藥能減少自由基引起的反應，藉由生藥抗自由基的分析有助於了解生藥如何去除自由基及脂質過氧化的作用。天然植物中的萃取物含有抗癌的作用，藉由計畫性凋亡來抑制甚至殺死癌細胞。並期能找到優良的抗癌藥物應用於臨床上。

前列腺癌(prostate carcinoma)位列歐美癌症發病率的第一位，近年來，其發病率和死亡率仍有較明顯的上升。雖然中國人前列腺癌的發病率比歐美低得多，但由於近 20 年來飲食習慣的西化趨勢，前列腺癌的發病率也有所增加。根據研究文獻得知，前列腺癌為美國男性癌症診斷率中的第一位，同時也是致死率第二位的癌症疾病(Wingo et al., 1997; Greenlee et al., 2001)。就台灣而言，1991 年台灣地區前列腺癌占男性癌症發生率的第九位、死亡率第十一位；到了 2002 年其發生率與死亡率接爬升至第六位。雖然就發生率而言，前列腺癌對台灣男性不若美國或西方男性之嚴重。但就台灣而言，期逐年升高的發生率與死亡率對台灣男性造成的生命威脅是不容忽視的。

目前的臨床治療方法多以荷爾蒙移除(hormone ablation)為第一線的治療，但大部分的病患最後會對荷爾蒙移除治療出現無效反應，並在臨床上進展成荷爾蒙無效反應之前列腺癌(hormone refractory carcinoma, HRPC)，一旦進展為 HRPC，大部分的病患其平均存活時間(survival mediam)約只有一年的時間(Kantoff et al., 1999)。除了荷爾蒙移除治療外，尚有其他治療策略及藥物可用，然而這些治療並無太大助益。因此開發治療癌症的新藥為目前刻不容緩的目標，以往的癌症用藥以造成細胞壞死的途徑來殺死癌細胞，結果亦使正常的細胞大量死亡，導致病患在治療的過程中身體極大的不適。近年來計畫性細胞凋亡機轉是研究治療癌症藥物的發展方向，若能由天然植物中純化出有效抗癌化合物，研究其是否可經由計畫性細胞死亡之途徑來抑制細胞之生長甚至殺死癌細胞，則將有別於傳統研究治療癌症之藥物只有著重於對細胞壞死與細胞毒性的研究。

在許多植物中都含有皂素(saponin)的成分，皂素對於植物本身所扮演的作用並不完全清楚，已知能作用於動物細胞及黴菌、細菌等，具有抗微生物、抑制黴菌生長，以及保護植物免於昆蟲的接觸，因此皂素也被認為是植物的防禦系統，因為這些分子的保護作用而使皂素有另一個名稱為”phytoanticipins” 或”phytoprotectants”(Morrissey & Osbourn, 1999)。皂素是由 reiterpenoids 或是 steroids 和各種醣類所形成的配醣體，其非醣體的部分稱為 sapogenin。皂素多為無晶形，若形成溶液震盪後可產生持續性的泡沫，有苦味跟辛辣味，對冷血動物的毒性較強。Triterpenoids 有 $C_{30}H_{50}$ 的基本骨架，由六個單位的 isopprene 結合而形成三環到五環的化合物，以 hydrocarbon、alcohol、aldehyde、ketone、acid 等衍生物形態存在於植物中。Triterpenoids 大部分在 C_3 的位置上具有氫氧基，以 triterpene alcohol 游離存在或和醣類結合成 saponin，若 saponin 水解後所得到的 triterpene alcohol 稱為 sapogenol(即為 sapogenin 部分)。



Triterpenoid structure from plant

所以由於本身化學結構上具有表面活性的特性，使其具有抗癌作用(Rao et al., 1995)。大多具有抑制癌細胞生長的效果(Kuo et al., 2000; Hsu et al., 2000; Mujoo et al., 2001; Oh et al., 2001)。不同種類植物的皂素成分因其官能基的不同，表現的功能也不盡相同，例如在中國被譽為百藥之王的人蔘，其萃取物在體內試驗研究上具有抑制多種癌症生長(Ha and Lee 1985；Kenarova et al., 1990； Lee and Huemer 1971；Odashima et al., 1985； Yun et al., 1983)，之後的研究對於其內所含的皂素成分對於前列腺癌 LNCaP 癌細胞有抑制增生的作用(Liu et al., 2000)、也具有毒殺腫瘤細胞的能力(Lee et al., 1999；Park et al., 1997)；日常飲食中的黃豆也含有皂素成分，其皂素的組成具有中性官能基，是以非細胞毒殺作用方式來抑制腫瘤細胞的生長；反之從 gypsophilla 萃取的皂素，其 aglycone 部位具有酸性官能基，因此能誘發腫瘤細胞毒殺作用(Sung[1] et al., 1995；Sung[2] et al., 1995)；另外大豆的皂素對乳癌細胞也具有抑制生長的作用並能誘發人類結腸癌細胞發生計劃性凋亡計劃(Oh et al., 2001)。洋槐(Acacia victoriae, Bentham)所含的皂素成分對於人類腫瘤細胞如 Jurkat cells (人類 T 細胞白血病細胞)有抑制生長及誘發計劃性細胞凋亡，及抑制 PI3K/Akt 途徑，對 Ras/mitogen-activated protein kinase cascade 並無作用(Mujoo et al., 2001)。本研究使用的石鹼樹樹皮(Quillaja Bark)的皂素萃取物具有免疫刺激作用(Chavali[1], et al., 1987；Chavali[2], et al., 1987) ，並能誘發 IL-6 的產生(Behboudi et al., 1997)，石鹼樹皮的皂素成分屬於 pentacyclic triterpenoid(含有五環的 triterpenoid)，其生物活性在基礎研究仍需要積極的探討。因此，本研究的初步研究結果顯示石鹼樹皮的皂素在幾種本研究的目標是為探討 Quillaja saponin 是否也具有抑制腫瘤細胞生長，甚至誘發癌細胞凋亡的潛力。透過本研究的努力，找出對抗不同種類癌症的有效方法。

Material and method

1. 細胞培養

將人類腫瘤細胞如 PC3 等以 Dulbecco's modified Eagle's medium (DMEM)培養液培養於 5%CO_2 的 37℃的細胞培養箱中，保持濕度 90%。細胞長滿後，將培養液抽乾，以 PBS 溶液清洗細胞，加入 3ml 0.05%trypsin-EDTA，至於 37℃2 分鐘後，加入新鮮的培養液將細胞收集，離心 1000rpm5 分鐘後吸掉培養液，在加入新鮮培養液，將細胞均勻沖散，依實驗目的的不同，將細胞均勻分配到各種大小的培養皿中。

2. 藥物處理細胞的方法

每次實驗，細胞都經重新換代培養，經過 24 小時的培養後，先吸除原來就的培養液，藥物以培養液充分混合，加入細胞培養皿，依各種實驗情況在培養箱中放不同時間。若因藥物作用而懸浮於培養液之細胞仍和固定於培養皿的細胞一起收集分析。

3. Trypan blue exclusion assay[Freshney, 1994]

細胞以 PBS 洗滌二次後，再以 trypsin-EDTA 處理 2 分鐘，加入培養液，將細胞收集以 1000rpm 離心 10 分鐘，去除培養液，再加入新鮮的培養液，抽吸細胞使細胞均勻分布再培養液中，取出 20ml 的細胞液與 20ml 的 0.2% trypan blue 混合均勻，取出適量的體積置於 hemacytometer 在顯微鏡下觀察，照相並計算呈黑色的死細胞與成明亮的活細胞的數目。

4. MTT 反應分析[Mosmann,] 1983

細胞培養於 12well 的培養皿上，每個 well 含 50000 個細胞，培養液函各種不同濃度石鹼樹皮的皂素成分，經各種時間的培養後，將含藥物的培養液吸除，以一倍濃度的 HBSS 緩衝液洗滌細胞,加入以 PBS 緩衝液配置的 MTT 溶液濃度為 0.5mg/ml,每個 well 體積 0.35ml，於 37℃培養 5 小時，若細胞粒線體的呼吸作用仍在進行，則粒線體內的 dehydrogenase 酵素會將 MTT 轉化成紫色的 formazan 化合物結晶，細胞越健康，粒線體呼吸作用越旺盛，其 dehydrogenase 活性越高，則所形成的紫色結晶越多，再於培養皿中加入 10%SDS，於 37℃培養 12 小時，將結晶溶解，從每個 well 取出 0.2ml 至 96well 培養皿中，於 570-630nm 的 microtitre plate reader 測定吸光值，互相做比較以決定各種濃度的皂素處理後細胞的活性。

5. Flow cytometry 細胞週期分析[Coligan et al., 1992]

細胞以 4℃的 PBS 洗二次以後，加入 3ml0.1% trypsin-EDTA 置於 37℃，5 分鐘後，輕拍培養皿底部，加入 7ml 新鮮的培養液以中和掉 trypsin 的作用，以 pipet 小心吸放使細胞皆成為單一顆粒，以 PBS 清洗並離心 1000rpm 二次，使細胞懸浮於 1ml 的 PBS，取適量的細胞以 hemacytometer 計算細胞數目，以 PBS 調整細胞濃度為一百萬個/ml，取出 1ml 的細胞置於離心管，1000rpm 離心 5 分鐘，直接倒掉上清液，輕彈離心管以打散細胞沉澱塊，以 2ml 的 70%酒精一滴一滴加入離心管使細胞均勻懸浮於酒精中置於 4℃至少 30 分鐘以固定細胞。將細胞以 1000rpm 離心 10 分鐘，將酒精倒掉，輕震離心管使細胞沉澱成為單一細胞懸浮，加入 0.5ml 的 propidium iodide(PI; 50mg/ml)染色液，再加入 0.5ml 以回溫的 Rnase-Dnase free 溶液(最終濃度 100units/ml)，小心震動離心管使細胞均勻懸浮，將離心管置於室溫，保持避光狀態 40 分鐘後，以 35μm nylon mesh 過濾樣品，以 FACStar flow cytometer 分析每個細胞樣品的細胞週期。

6. Flow cytometry 偵測癌細胞早期的計劃性凋亡[Coligan et al., 1992]

細胞以 4℃的 PBS 洗二次後，加入 3ml 0.1% trypsin-EDTA 置於 37℃，5 分鐘後，輕拍培養皿底部，加入 7ml 新鮮的培養液以中和掉 trypsin-EDTA 的作用，以 pipet 小心吸收使細胞皆成為單一顆粒，以 PBS 清洗並離心 1000rpm 二次，使細胞懸浮於 1ml 的 PBS，取適量的細胞以 hemacytometer 計算細胞數目，以 PBS 調整細胞濃度為 10^6 個/ml，取出 1ml 的細胞置於離心管，加入適量的 FITC-conjugated annexin 與存在於細胞膜表面的 phosphotidyl serine 結合，在 4℃作用 30-45 分鐘，用 4℃的 PBS 清洗細胞二次，1000rpm 離心，去除上清液，約留 50μl 的溶液，避免碰到細胞團塊，加入 0.5-3ml 的 0.5% paraformaldehyde，並立即輕搖將細胞團塊打散，並一邊震盪混合，同時一滴一滴地加入 1ml-20℃的 50%ethanol in PBS 溶液。置於冰箱中 30 分鐘以上以固定細胞，固定之後，離心細胞並吸除酒精溶液，約留 0.5ml 酒精溶液，加入 0.5ml PBS，並打散細胞團塊，然後加入 0.2ml RNase (125 units/ml)，並在 4℃下作用 30 分鐘，以 PBS 洗滌細胞二次，離心去除上清液，將細胞團塊打散，並加入 1ml propidium iodide(20μg/ml)，在 4℃溫度下暗室作用至少 1 小時，然後以 FASCtar flow cytometer 分析早期的計劃性細胞凋亡。

7. DNA 分析[Sambrool et al., 1989]

去除細胞的培養液，以 PBS 洗滌細胞二次，加入 Molecular Research Center 公司出品的 DNAzol 1 ml，以 rubbor policeman 刮取細胞，放置於 50℃，2 小時後加入 Dnase free-Rnase A，使其最終濃度為 0.5 mg/ml，繼續放於 50℃ 2 小時，再加入量的 phenol、chloroform、isoamyl alcohol (比例為 25：24：1)，充分混合 3 分鐘後，離心 12000rpm 4 分鐘，取出上清液置另一新的離心管內，加入等量體積的 isopropanol 與十分之一量體積的 3M NaOAc，溫和搖晃數下，冰於 4℃，15 分鐘使 DNA 析出，取出離心管於 12000rpm 離心 15 分鐘，去除上清液，沉澱物為 DNA，將沉澱物於空氣中乾燥，加入 50μl 的 TE 緩衝液溶解 DNA，以 260 及 280nm UV 波長的 spectrophotometer 測 DNA 濃度，各個實驗組細胞 DNA 取等量 2ml 的 DNA loading dye 混合後，加至一倍 TAE 電泳液製備的 2% agarose 電泳凝膠的孔洞內，以 100 伏特的電壓在一倍 TAE 電泳液中，電泳至 DNA loading dye 移至電泳凝膠的底部，將電泳凝膠取出放置於含 ethium bromide 的溶液中 20 分鐘，再以二次去離子水清洗電泳凝膠 20 分鐘後，將電泳凝膠放在 UV 放射盒上，以短波長的 UV 照射，並照相保存。

8. Western blotting 分析細胞蛋白質的表現[Sambrook et al., 1989]

將細胞以 4℃的 PBS 洗滌二次，加入 lysis buffer (2.5 mM Tris-HCl, pH 7.2, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 0.15M NaCl, 1mM EDTA, 1mM PMSF, 10mg/ml aprotinin, 1mM sodium orthovanadate 5 mg/ml leupeptin)，以 rubbor policeman 刮碎並溶解細胞，將此細胞溶解液取至 1.5 ml 的微量離心管中，震盪 1 分鐘，再於 12000rpm 離心 10 分鐘，取上清液，以 Bio-Rad protein assay kit 來測定蛋白質的濃度，並將剩餘的蛋白質萃取液置於-70℃保存。

將蛋白電泳片裝置在電泳槽上，並填滿 Tris-glycine 電泳緩衝液，等待蛋白質樣品的填入。取 100μg 的蛋白質溶液，加入等體積的二倍濃度之 SDS/protein loading buffer，於 100℃煮沸 10 分鐘，冷卻後將蛋白質樣品放置每個 well 中，先固定電壓在 50 伏特，待蛋白質樣品跑到距離底部約 0.5 公分時，停止電泳並進行蛋白質電泳轉印。先剪取一張 nitrocellulose paper，先以甲醇浸濕，在用水將 paper 洗淨，連同二張 3 M paper 放入 protein transfer buffer 中浸濕。操作時的順序由下而上依次為 3M paper，nitrocellulose paper，SDS-PAGE gel，3M paper，輕微壓除夾在其中的氣泡，放入轉印槽內，將轉印槽置於 4℃冰櫃中，以 600 微安培電流進行蛋白質的轉移，4 小時後，將 nitrocellulose paper 取出，放置於含 1% BSA 的 PBS blocking

solution 中，於室溫緩慢搖晃 2 小時，再放到含一次抗體的 blocking solution 中，於室溫緩慢搖晃 1 小時，以 washing buffer(10mMTris-base, pH7.5, 100mM NaCl, 0.1% Tween 20)洗滌 nitrocellulose paper 3 次，每次 20 分鐘，加入以 blocking buffer 稀釋含有 peroxidase 的 2 次抗體，在室溫培養 1 小時，再以 washing buffer 洗滌 nitrocellulose paper 3 次，每次 20 分鐘，將 nitrocellulose paper 置於含有 0.05%的 insoluble chromogen diaminobenzedine (DAB)與 0.02% H_2O_2 的 PBS 中呈色。此外，nitrocellulose paper 可用 Coomassie Brilliant blue R250 溶液染色 10 分鐘後，再以含 30%的甲醇和 10%的醋酸退染液退染，可使 nitrocellulose paper 上蛋白質被染色而可檢視每個實驗組的蛋白質是否一致。

9. 免疫墨點法

轉印好蛋白質的 PVDF membrane 先置於 blocking solution 中，在 4℃下用軌道式震盪器均勻擺動 12-18 小時，以競爭掉非特異性蛋白質的結合反應。之後用 blocking solution 做為溶劑，將欲測蛋白質的一次抗體稀釋到適當的作用濃度，再加到 blocking 好的 PDVF membrane，使其均勻擺動一小時，此時欲測蛋白質已接上一次抗體，再以 0.1% PBST 溶液清洗四次，每次 15 分鐘，以去除未接上、多餘的一次抗體。之後依照一次抗體的種類，加入特異性的二次抗體，在室溫下使用軌道式震盪器均勻擺動一小時，之後在同樣再以 0.1% PBST 溶液清洗四次，每次 15 分鐘，以去除未接上一次抗體的二次抗體，同時也減少非特異性的結合的出現。由於使用的二次抗體上所接的式 horse-radish peroxidase 可以催化 ECL (enhanced-chemiluminescence)發出化學冷光，由此系統可偵測蛋白質訊息表現強弱。首先將 ECL 溶液中 luminol reagent 與 oxidizing reagent 以體積 1：1 等比例混合，再將含有蛋白質之 PVDF membrane 正面沾染混合好的 ECL 溶液，並確定 PVDF membrane 正面完全沾到 ECL 溶液之後，將 PVDF membrane 夾在兩片投影片之中，並確定沒有氣泡產生，於暗房內使用 Kodak BioMax light film 壓片、洗片，視蛋白質訊息所表現出的化學冷光強弱來決定壓片時間的長短，整個動作最好在 20 分鐘內完成，最後透過底片即可檢視細胞內欲測定蛋白質的變化量。

10. 免疫墨點法分析蛋白質的變化量

將轉印好蛋白質的 PVDF membrane 先置於 blocking solution 中，在 4℃下用軌道式震盪氣均勻擺動 12-18 小時，以競爭掉非特異性蛋白質的結合反應。之後用

blocking solution 作為溶劑，將預測蛋白質的一次抗體稀釋到適當的作用濃度，再加到 blocking 好的 PVDF membrane，使其均勻擺動一小時，此時欲測蛋白質已接上一次抗體，再以 0.1%PBST 溶液清洗四次，每次 15 分鐘，已去除未接上、多餘的一次抗體。之後依照一次抗體的種類，加入特異性的二次抗體再室溫下使用軌道式震盪氣均勻擺動一小時，之後同樣再以 0.1%PBST 溶液清洗四次，每次 15 分鐘，已去除未接上一次抗體的二次抗體，同時也減少非特異性的結合的出現。由於使用的二次抗體上所接的式 horse-radish peroxidase (HRP)，可以催化 ECL (enhanced-chemiluminescence)發出化學冷光，由此系統可偵測蛋白質訊息表現強弱。首先將 ECL 溶液以體積 1：1 等比例混合。再將含有蛋白質之 PVDF membrane 正面沾染混合的 ECL 溶液，並確定 PVDF membrane 正面完全沾到 ECL 溶液之後，將 PVDF membrane 夾在兩片投影片之中，並確定無氣泡產生，之後在暗房使用 Kodak BioMax light film 壓片、洗片，視蛋白質訊息所表現出的化學冷光強弱來決定壓片時間的長短，整個動作將於 20 分鐘內完成，最後透過底片即可檢式細胞內預測蛋白質的變化量。

11. 利用流式細胞儀分析細胞內粒線體膜電位(mitochondrial transmembrane potential) (Johnson LV et al., 1980)

原理：Rhodamine 123 (RH123) 是一種親脂性、陽離子性、穩定性高和缺乏毒性的螢光染劑，而且可以自由通透細胞膜，並專一性的堆積於維持負電荷環境的粒線體中，當粒線體膜電位約-180mV 時，Rh123 再粒線體的濃度可能式細胞質的三次方強，因此可用 Rh123 攝入的情形來偵測活細胞內粒線體內外膜電位的差異。另外 Rh123 只需低濃度即可偵測到或細胞內粒線體的功能極力現體的膜電位。因此可利用流式細胞儀來偵測細胞內膜電位的變化。

實驗步驟：將細胞依 5×10^5 起始細胞濃度培養在 6cm 的 culture dish 中，24 小時後去除原培養液，加入已知不同濃度含有石鹼 saponin 之新鮮培養液，經過不同時間點的培養後，再各時間點的前三十分鐘，於 5ml 樣本液中加入 2.5 μl 10 mM Rh123，使其最終濃度為 5 μM，混合均勻後再置入 37℃、5%CO_2 的培養箱中培養三十分鐘，時間一到，開始收集全部的細胞至 15ml 離心管。首先，先收集樣本液並用一倍的 PBS 緩衝液清洗兩次，洗去細胞表面上的血清分子，再加入 1ml trypsin-EDTA，於 37℃、5%CO_2 的培養箱中培養五分鐘，待細胞完全脫離 culture dishes，形成懸浮太后，再將剛才收集再 15ml 離心管的樣本液加入原先的 culture

dishes，以其中的血清分子中和 trypsin-EDTA 的作用，並將細胞和所有溶液直接收集於 15ml 離心管，在室溫下 1500rpm 離心五分鐘，去除上清液，輕震離心管以打散細胞，並用 5ml 的 PBS 緩衝液清洗細胞，之後將細胞液轉移至含有 2.8μl 的 1 mg/ml PI。因為是偵測活細胞，所以要盡快上機，然後以 FASCtar flow cytometer 分析細胞內粒線體膜電位。

12. 利用流式細胞儀分析細胞內過氧化物的生成量(Johnson I, 1998)

原理：2’, 7’-dichlorodohydrofluorescein diacetate(H_2DCF-DA)是一種穩定的、沒有螢光的化學物質，由於其含有兩個親脂性基團而呈現非極性，固可以自由通透細胞膜而進入細胞內。進入細胞後，會被細胞內的 esterase 進行 deacetylation 去掉兩個乙基，形成具有極性的 2’, 7’-dichlorodihydrofluorescein (H_2DCF)，因此無法穿透細胞膜而留在細胞內，一般可以穩定存在細胞內數個小時。當細胞產生 hydrogen peroxide 時，其會快速的氧化 H_2DCF 丟掉一個氫原子，形成了能夠發螢光的 2’, 7’-dichlorofluorescein (DCF)，因此可利用流式細胞儀來偵測細胞內過氧化物的生成量，雷射激發波長為 488nm，DCF 的放射螢光波長為 529nm。實驗步驟與過程如第十一點所述，其中不同的是：在各時間點的前三十分鐘，於 5ml 樣本液中加入 5μl 10mM H_2DCF-DA，使其最終濃度為 10μM，混合均勻後再置入 37℃、5%CO_2 的培養箱中培養三十分鐘。

13. 利用流式細胞儀分析細胞內超氧陰離子的生成量(Johnson I, 1998)

原理：dihydroethidium (dihydroethidine, HE)是一種經化學方式還原螢光染劑，而且可以自由通透細胞膜，在細胞質呈現藍色螢光，一但細胞內產生 superoxide anion。便會將 HE 反應成 ethidium，並會嵌入在 DNA 中而且呈現紅色螢光。因此可利用流式細胞儀來偵測細胞內超氧陰離子的生成量，雷射激發光波波長 488nm，ethidium 的放射螢光波長 605nm。此外，當氧化物是 superoxide anion，而不是 O_2、H_2O_2、HOCl 或 $ONOO^-$時便會快速的將 HE 氧化成 Ethidium，因此，HE 可用來當作細胞內超氧陰離子生成的指標。實驗步驟與過程如第十一點所述，其中不同的是：在細胞液轉移至康試管中時，不需要在康試管事先加入 PI，因為 ethidium 和 PI 的放射螢光之主要波長範圍重複，容易造成干擾，而無法判讀出正確的結果。另外 HE 是以 99.9%absolute ethanol 溶劑配製成 10mM，並保存於-20℃冰箱備用。

Result

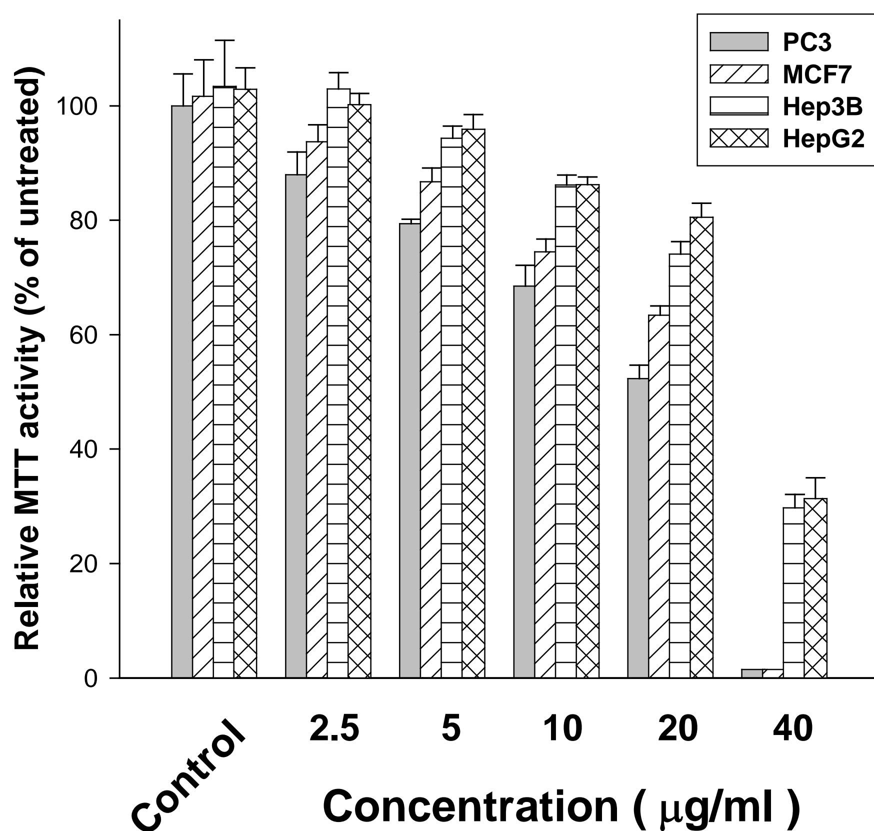
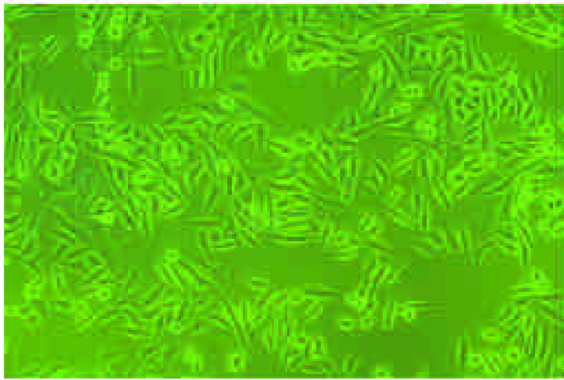
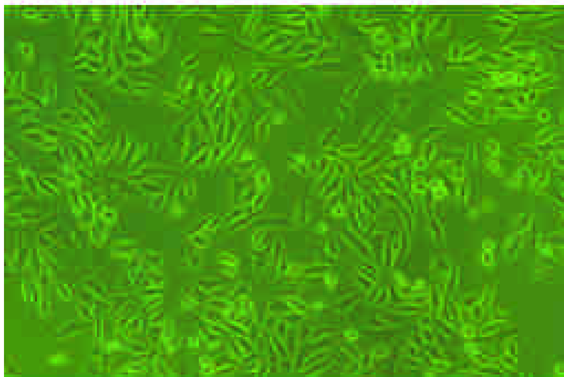


Fig. 1 Effects of Saponin in the viability of different cell lines (PC-3; MCF7; Hepa3B; HepaG2). The cells were treated with indicated doses of Saponin for 48 hrs. The viability was measured by the MTT assay. Medium served as the vehicle of Saponin. The data were compared with the untreated groups of each cell line. Each bar represents the mean ± standard deviation (n=4).

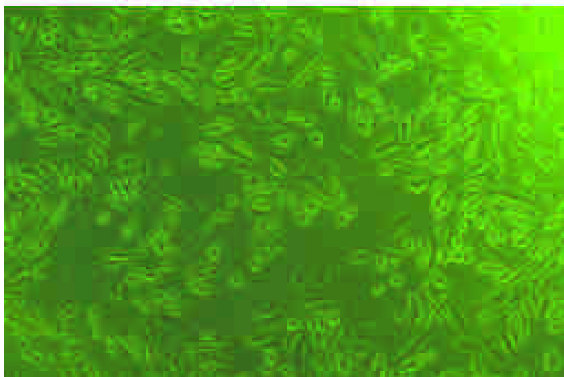
3 h



Untreated



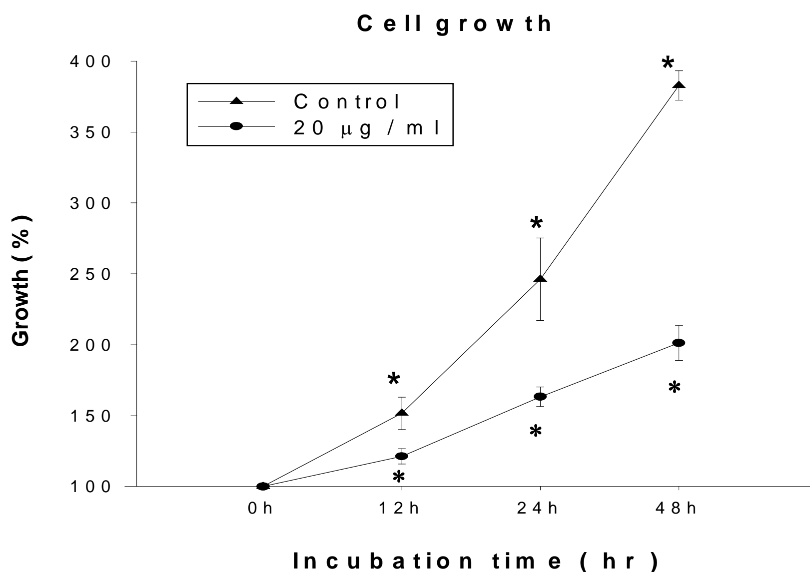
20 μg/ml



40 μg/ml

Fig. 2 Morphalogy of Saponin treated- and untreated- PC3 cells. The untreated- and Saponin (20 μg/ml; 40μg/ml) treated- PC 3 cells at 3 h were viewed under a light microscope. Morphological difference between the untreated- and saponin (20 μg/ml; 40μg/ml) treated- PC 3 cells. The round and suspended cells were observed in the sapnin (40 μg/ml; 3h) treated- cells. Results are from one experiment that is representative of three similar experiments. (3h: magnification × 100)

A.



B.

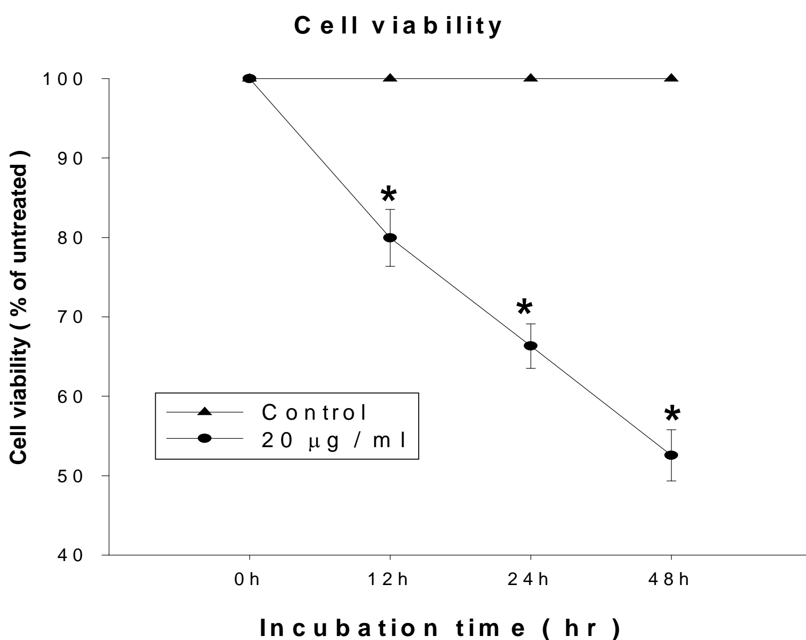


Fig. 3. Effects of *Quillaja* saponin on the proliferation (A) and cell viability (B) of PC 3 cells. Cells were seeded in DMEM with 10% FBS at 2.5´105 cells per well in 6-well culture plates. After 24 hours, the cells were treated with indicated doses of *Quillaja* saponin for 12, 24, and 48h. Subsequently, the cells were enumerated using a hemocytometry, and the number of viable cells was determined by trypan blue assay. Cell viability was calculated as the ratio between the number of the treated cells and the number of untreated cells. Values are means ± SD for two independent experiments performed in duplicate.

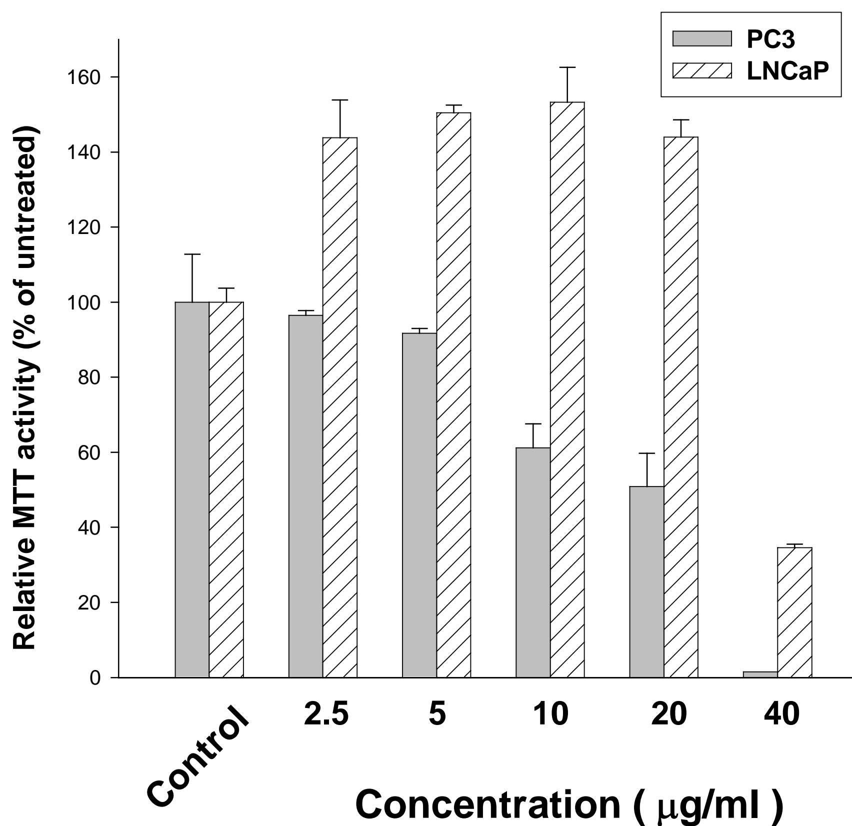


Fig. 4. Effects of Saponin on the viability of different cell lines (PC3 ; LNCaP). The cells were treated with indicated doses of Saponin for 48 h. The viability was measured by the MTT assay. Medium served as the vehicle of Saponin. The data were compared with the untreated groups of each cell line. Each bar represents the mean±standard deviation(n=4).

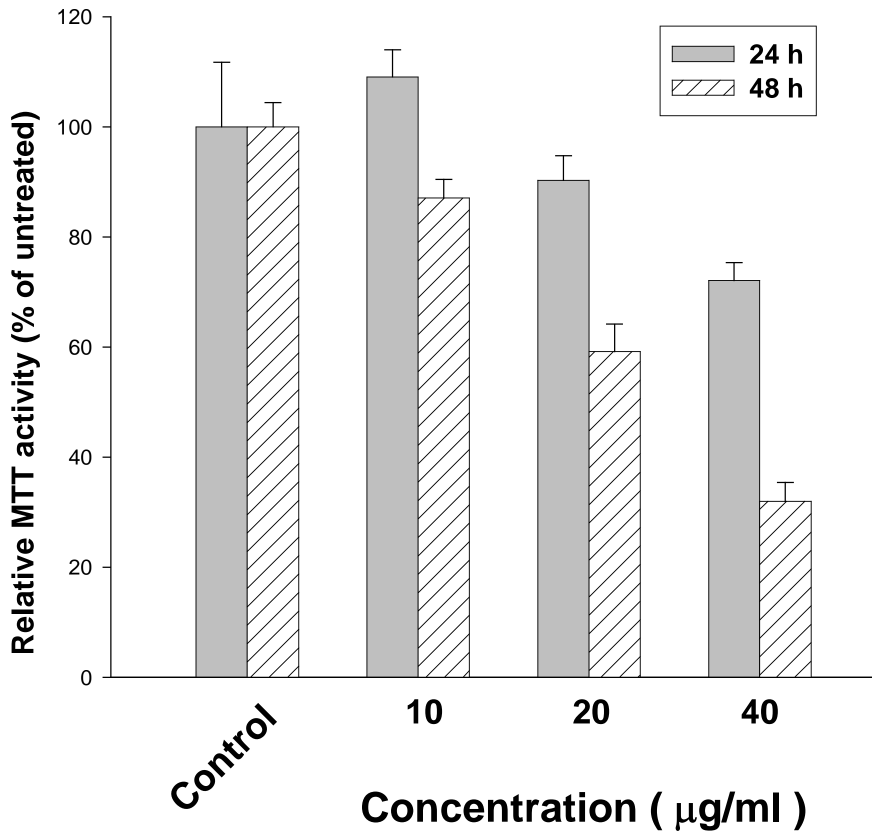


Fig. 5. Effects of *Quillaja* Saponin on the viability of human normal gingival fibroblasts. The cells were treated with indicated doses of *Quillaja* saponin for 48 h. The viability was measured by the MTT assay. Medium served as the vehicle of *Quillaja* saponin. The data were compared with the untreated groups of each cell line. Each bar represents the mean ± standard deviation(n=4).

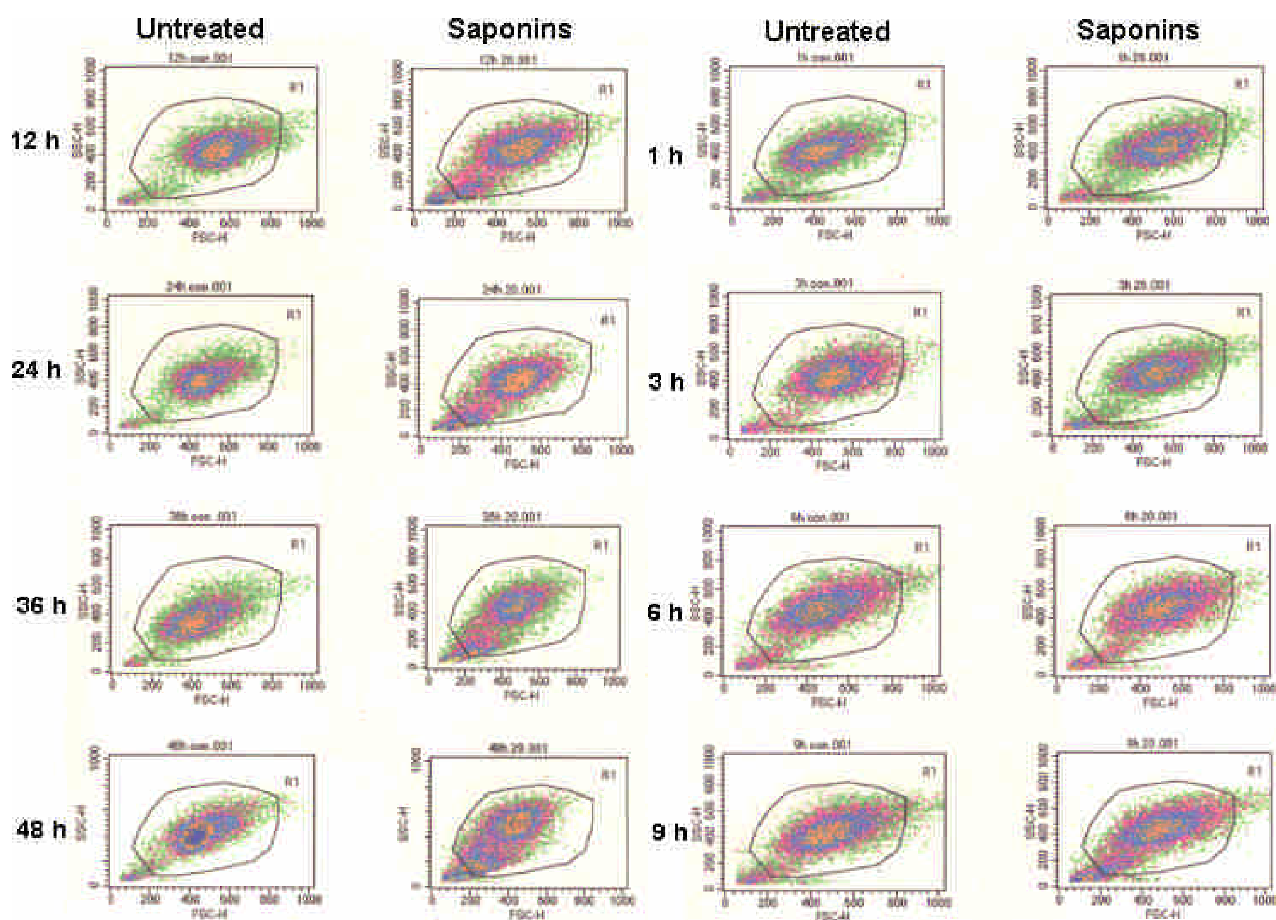


Fig. 6. Effects of *Quillaja* saponin on cell size and granularity of PC 3 cells. The untreated- and *Quillaja* saponin treated- PC 3 cells at 1-48 h were collected and stained with PI as described in the Materials and Methods and analyzed by flow cytometry. Results are from one experiment that is representative of three similar experiments.(*Quillaja* saponin：20 mg/ml)

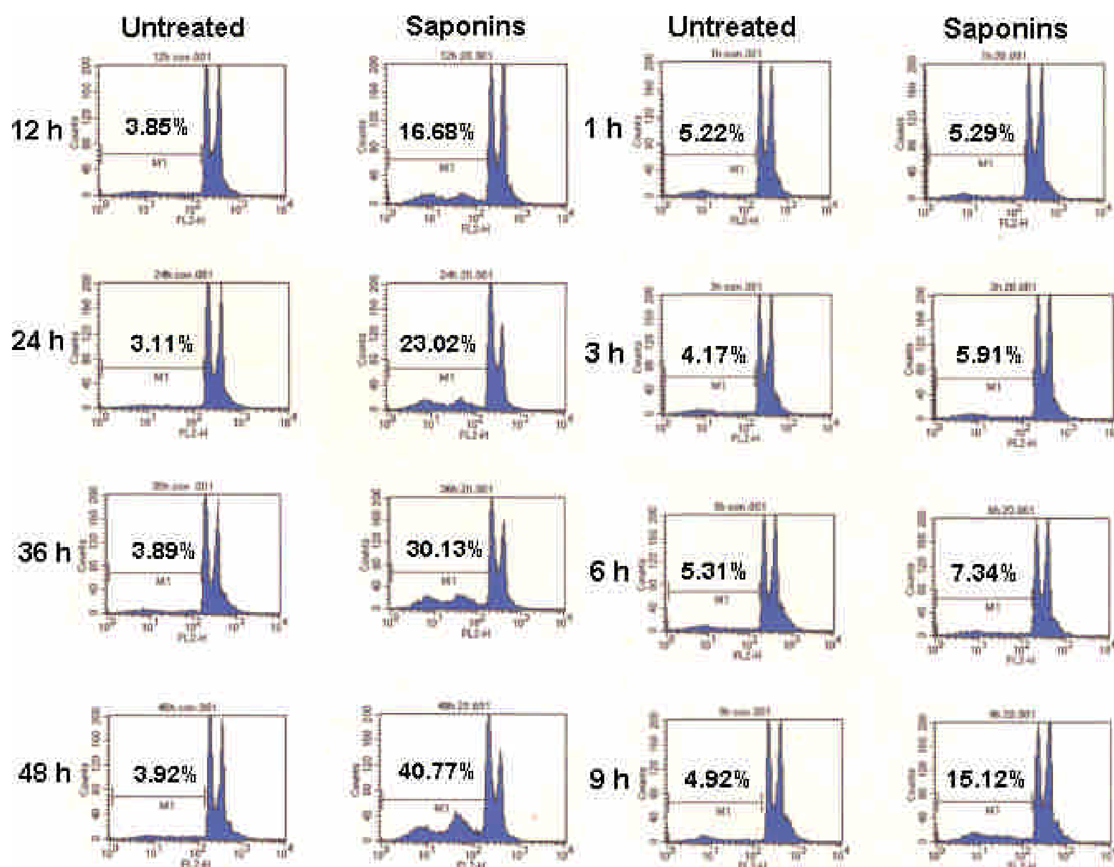


Fig. 7. Effects of *Quillaja* saponin on the cellular DNA content. The untreated- and *Quillaja* saponin treated- PC 3 cells at 1-9 h were collected and stained with PI as described in the Materials and Methods. Following flow cytometric analysis, cellular DNA profile was further analyzed by the CellQuest software. Data represent the percentage of cell counts displaying a hypoploid DNA population.Results are from one experiment that is representative of three similar experiments.(*Quillaja* saponin：20 mg/ml)

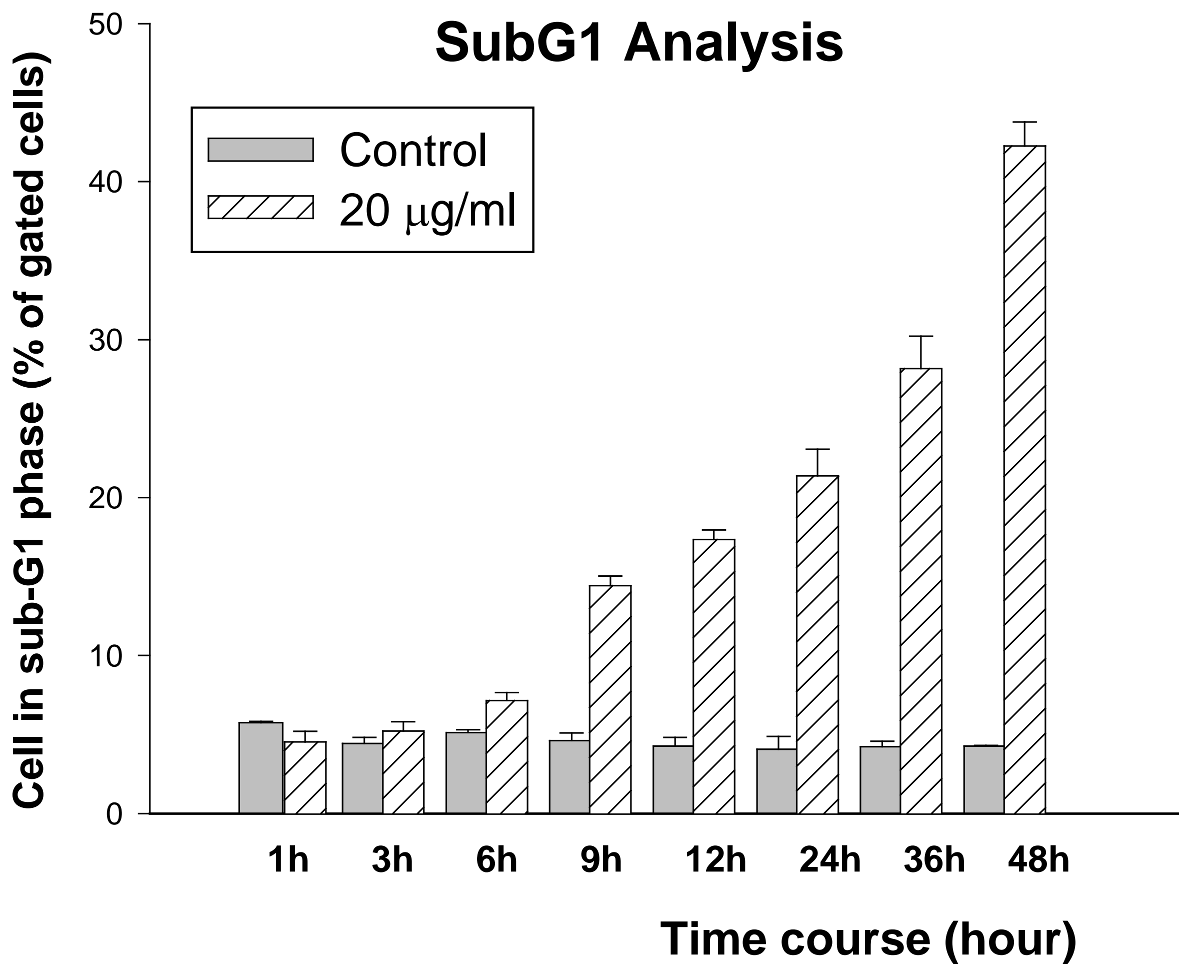


Fig. 8. Effects of *Quillaja* saponin on the percentage of apoptosis population in PC 3 cells. The untreated- and *Quillaja* saponin treated- PC 3 cells at 12-48 h were collected and stained with PI as described in the Materials and Methods,and analyzed by flow cytometry. Data are presented as percentage of gated cells in sub-G1 phase. Values are means ± SD for one of three similar experiments

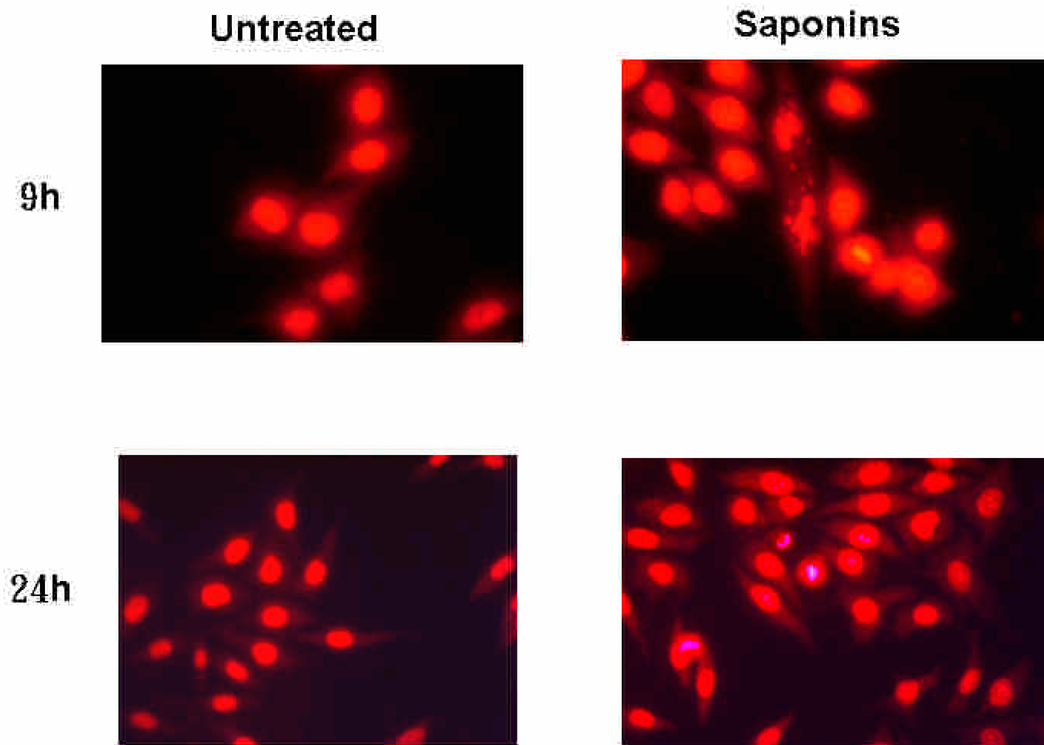
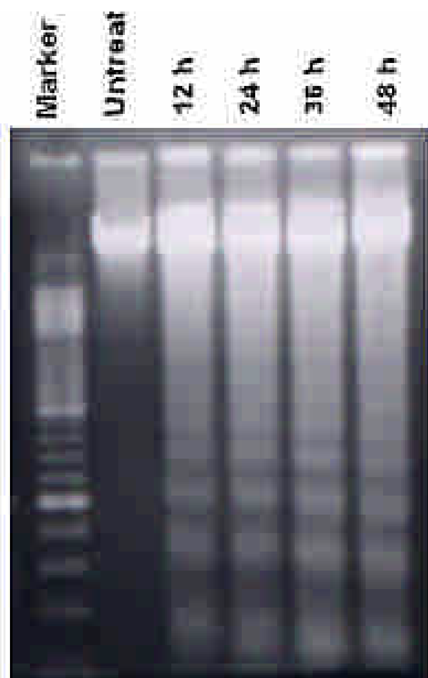


Fig. 9. Effects of *Quillaja* saponin on the morphology of nuclear chromatin. The untreated- and *Quillaja* saponin (20 mg/ml) treated-PC 3 cells at 9 h and 12 h were fixed and stained as described in the Materials and Methods. Morphological changes of nuclear chromatin were then viewed under a fluorescence microscope. The nuclei feature of control cells showed the round and homogeneous nuclei ; whereas apoptotic cells (arrows) showing condensed and fragmented nuclei were observed in the *Quillaja* saponin-treated cells. Results are from one experiment that is representative of three similar experiments. (9 h : magnification ´ 200 ; 24 h : magnification ´ 150)

A



B

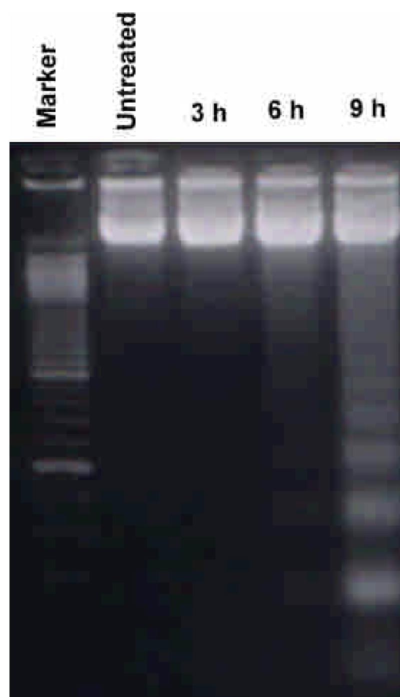
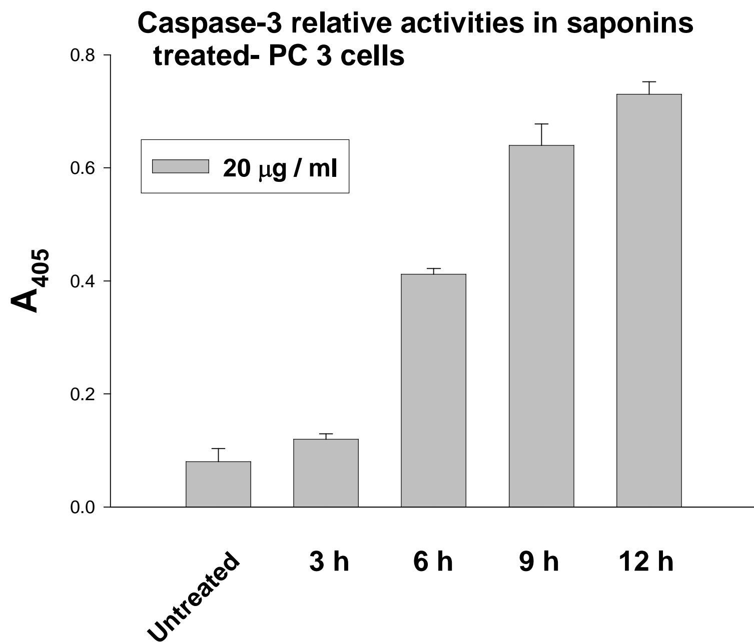


Fig. 10. The expression of DNA ladder in *Quillaja* saponin-treated PC3 cells were characterized by agarose gel electrophoresis. The untreated- and *Quillaja* saponin (20 mg/ml)treated- PC 3 cells at （A）：12- 48 h; （B）：3- 9 h, were collected, and the DNA fraction were prepared as described in Materials and Methods. The formation of DNA ladder induced by *Quillaja* saponin in PC 3 cells. Results are from one experiment that is representative of three similar experiments.



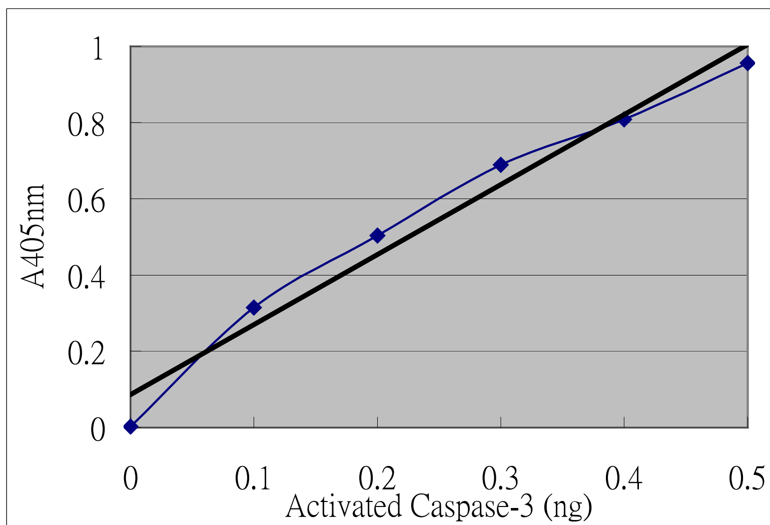


Fig. 11. Effects of *Quillaja* saponin on the caspase 3 activity in PC 3 cells. The untreated- and *Quillaja* saponin (20 mg/ml) treated- PC 3 cells at 3-12 h were collected and the proteins were prepared as described in Materials and Methods. The activities of caspase-3 were assessed by chromogenic caspase-3 assay kit. The relative activities of different time courses. The total protein (100 mg) were used to measure the caspase activity. The caspase activity was measured by ELISA reader.

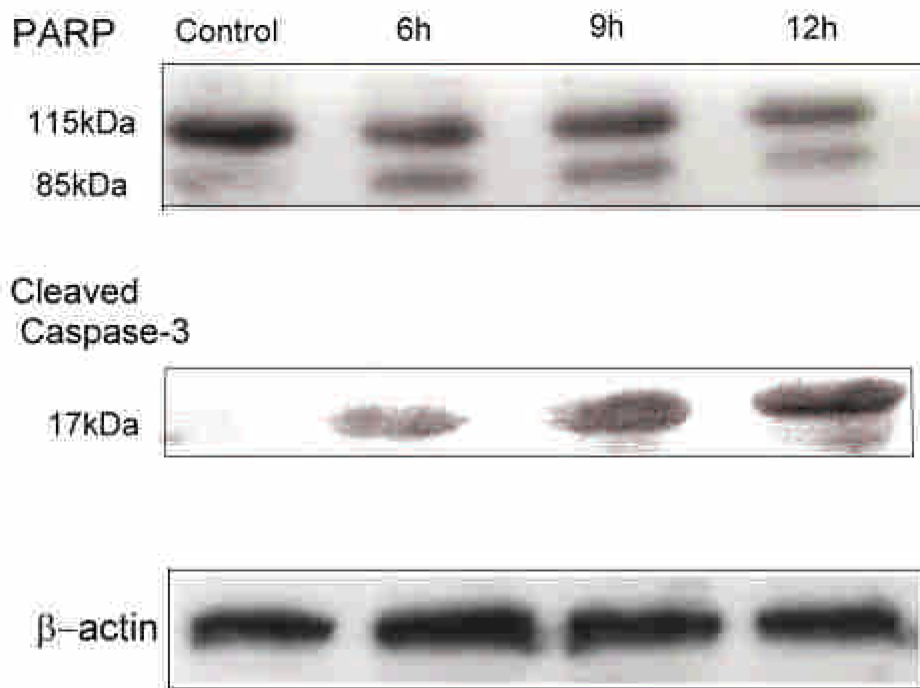


Fig. 12. Effects of Saponin on the expression of PARP and cleaved caspase-3 protein in PC 3 cells. The untreated- and saponin treated- PC 3 cells at 6-12 h were collected, the proteins were prepared as described in materials and Methods. Fifty micrograms of protein per lane were separated by 10 % polyacrylamide gel electrophoresis. PARP and cleaved caspase-3 proteins were determined individually by western blotting using specific antibody, and were visualized on X-ray film by chemiluminescence. Results are from one experiment that is representative of three similar experiments.

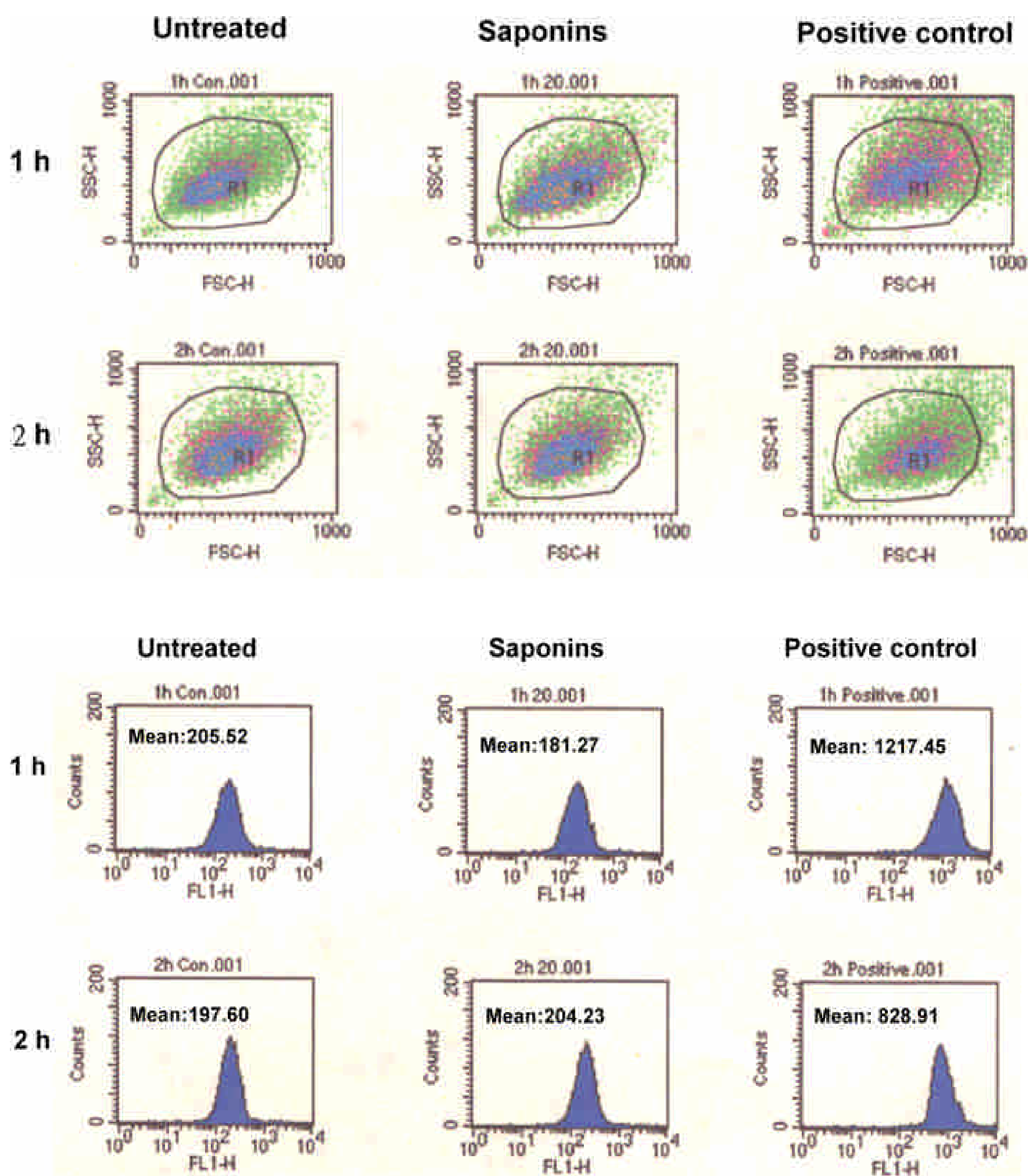


Fig. 13. Effects of *Quillaja* saponin on generation of intracellular peroxides in PC 3 cells. The untreated- and *Quillaja* saponin (20 g/ml) treated- PC 3 cells at 1-2 h were collected and stained in Materials and Methods. Following flow cytometric analysis, the generation of intracellular peroxides was further estimated by the CellQuest software. (A) Cell size and granularity of PC 3 cells ; FSC : forward angle light scatter ; SSC : side angle light scatter. (B) The average fluorescence intensity of cells. The mean values reflect the generation of intracellular peroxides. Results are from one experiment that is representative of three similar experiments. (Positive control : H_2O_2 0.5 mM)

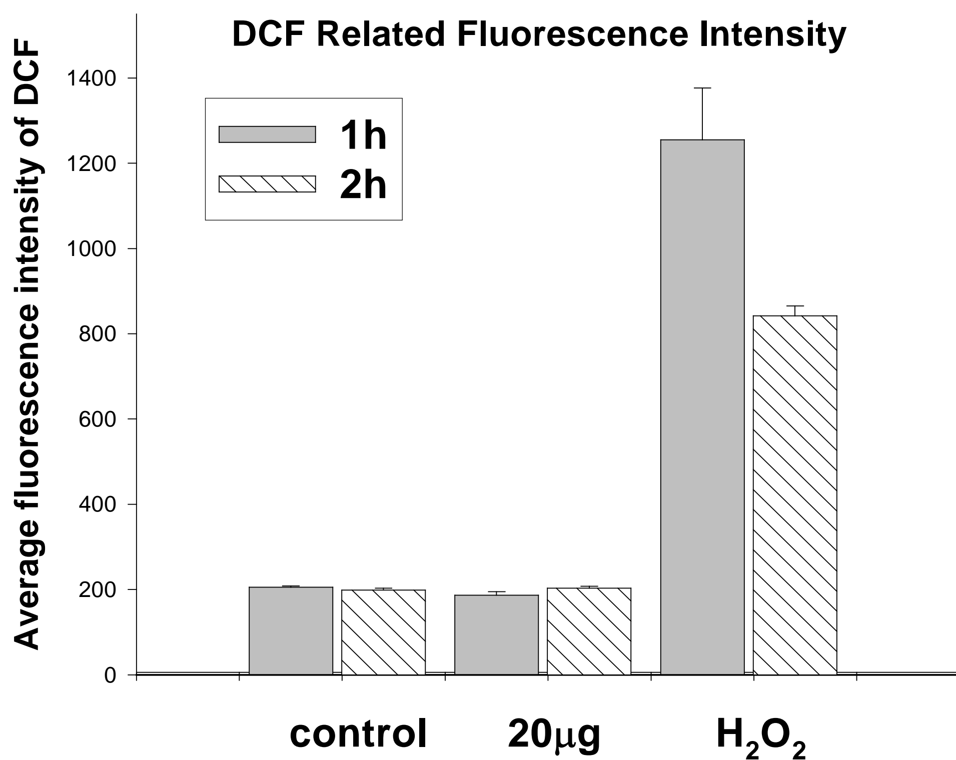


Fig. 14. Effects of *Quillaja* saponin on generation of intracellular peroxides in PC 3 cells. The untreated- and *Quillaja* saponin (20 μg/ml) treated- PC 3 cells at 1-2 h were collected and stained in Materials and Methods. Following flow cytometric analysis, the generation of intracellular peroxides was further estimated by the CellQuest software. The average fluorescence intensity of cells. The mean values reflect the generation of intracellular peroxides. Each plot is shown as means ± SD of results from one of three similar experiments. (Positive control：H_2O_2 0.5 mM)

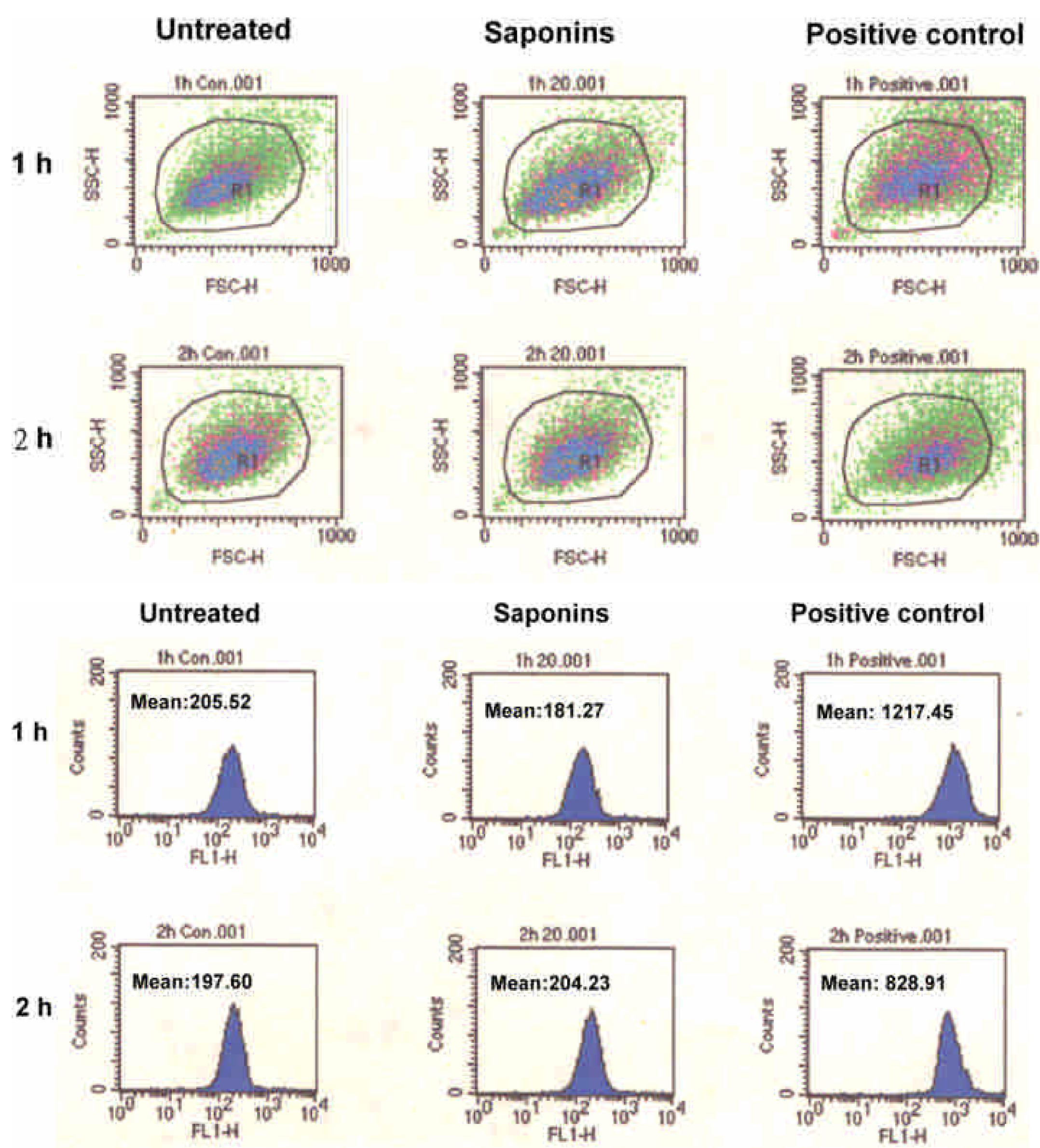


Fig. 13. Effects of *Quillaja* saponin on generation of intracellular peroxides in PC 3 cells. The untreated- and *Quillaja*
saponin (20 g/ml) treated- PC 3 cells at 1-2 h were collected and stained in Materials and Methods. Following flow cytometric analysis, the generation of intracellular peroxides was further estimated by the CellQuest software. (A) Cell size and granularity of PC 3 cells ; FSC：forward angle light scatter ; SSC：side angle light scatter. (B) The average fluorescence intensity of cells. The mean values reflect the generation of intracellular peroxides. Results are from one experiment that is representative of three similar experiments. (Positive control：H_2O_2 0.5 mM)

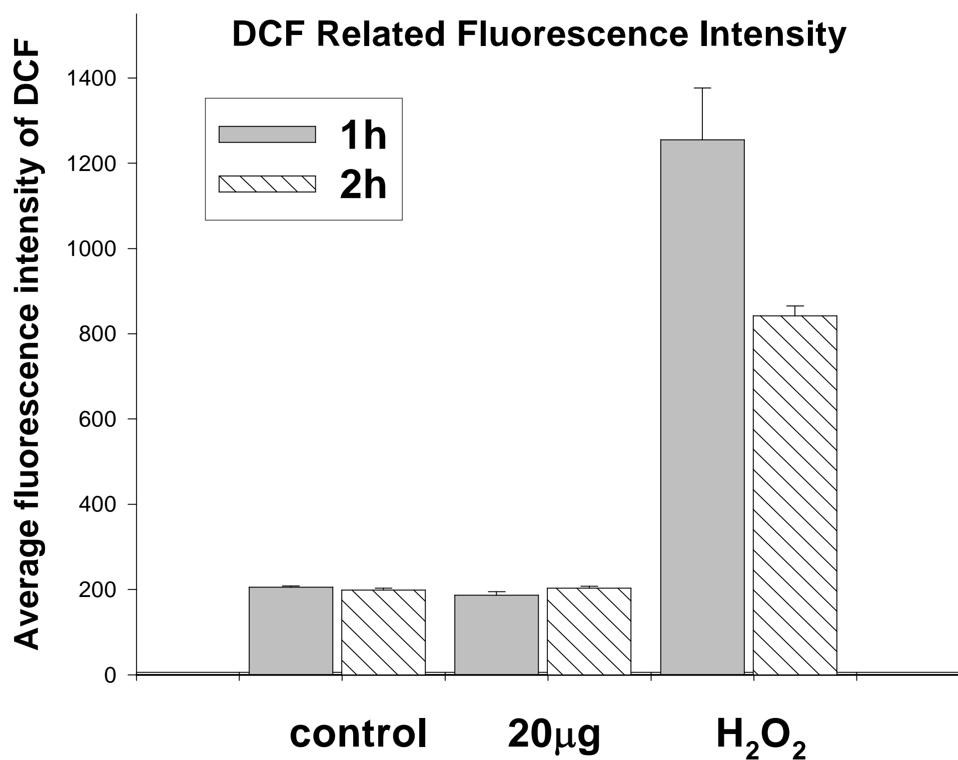


Fig. 14. Effects of *Quillaja* saponin on generation of intracellular peroxides in PC 3 cells. The untreated- and *Quillaja* saponin (20 g/ml) treated- PC 3 cells at 1-2 h were collected and stained in Materials and Methods. Following flow cytometric analysis, the generation of intracellular peroxides was further estimated by the CellQuest software. The average fluorescence intensity of cells. The mean values reflect the generation of intracellular peroxides. Each plot is shown as means ± SD of results from one of three similar experiments. (Positive control：H_2O_2 0.5 mM)

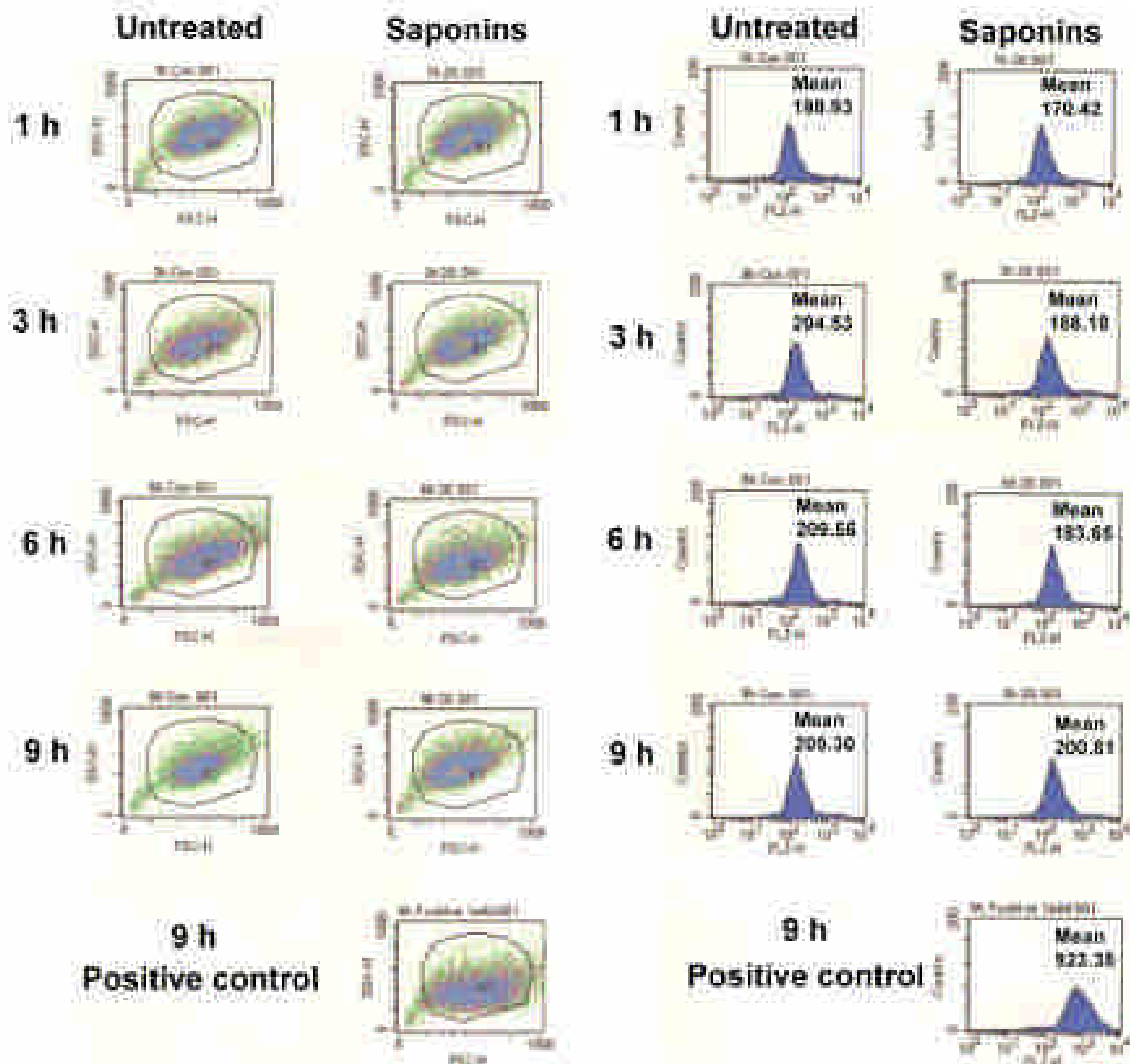


Fig.15. Effects of *Quillaja* saponin on generation of intracellular superoxide anion in PC 3 cells. The untreated- and *Quillaja* saponin (20 mg/ml) treated- PC 3 cells at 1-9 h were collected and stained in Materials and Methods. Following flow cytometric analysis, the generation of superoxide anion was further estimated by the CellQuest software. (A) Cell size and granularity of PC 3 cells ; FSC：forward angle light scatter ; SSC：side angle light scatter. (B) The average fluorescence intensity of cells. The mean values reflect the generation of intracellular superoxide anion. Results are from one experiment that is representative of three similar experiments. (Positive control: Menadione 1 mM)

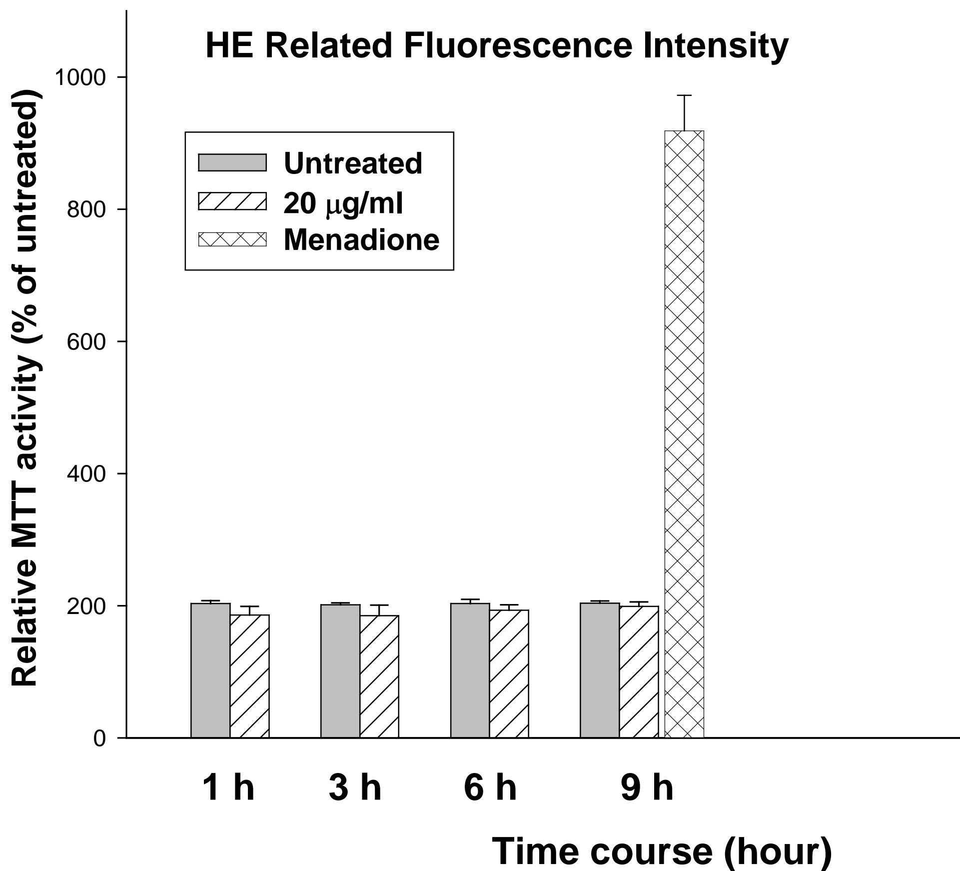


Fig. 16. Effects of *Quillaja* saponin on generation of intracellular superoxide anion in PC 3 cells. The untreated- and *Quillaja* saponin (20 mg/ml) treated- PC 3 cells at 1-2 h were collected and stained in Materials and Methods. Following flow cytometric analysis, the generation of intracellular superoxide anion was further estimated by the CellQuest software. The average fluorescence intensity of cells. The mean values reflect the generation of intracellular superoxide anion. Each plot is shown as means ± SD of results from one of three similar experiments. (Positive control: Menadione 1 mM)

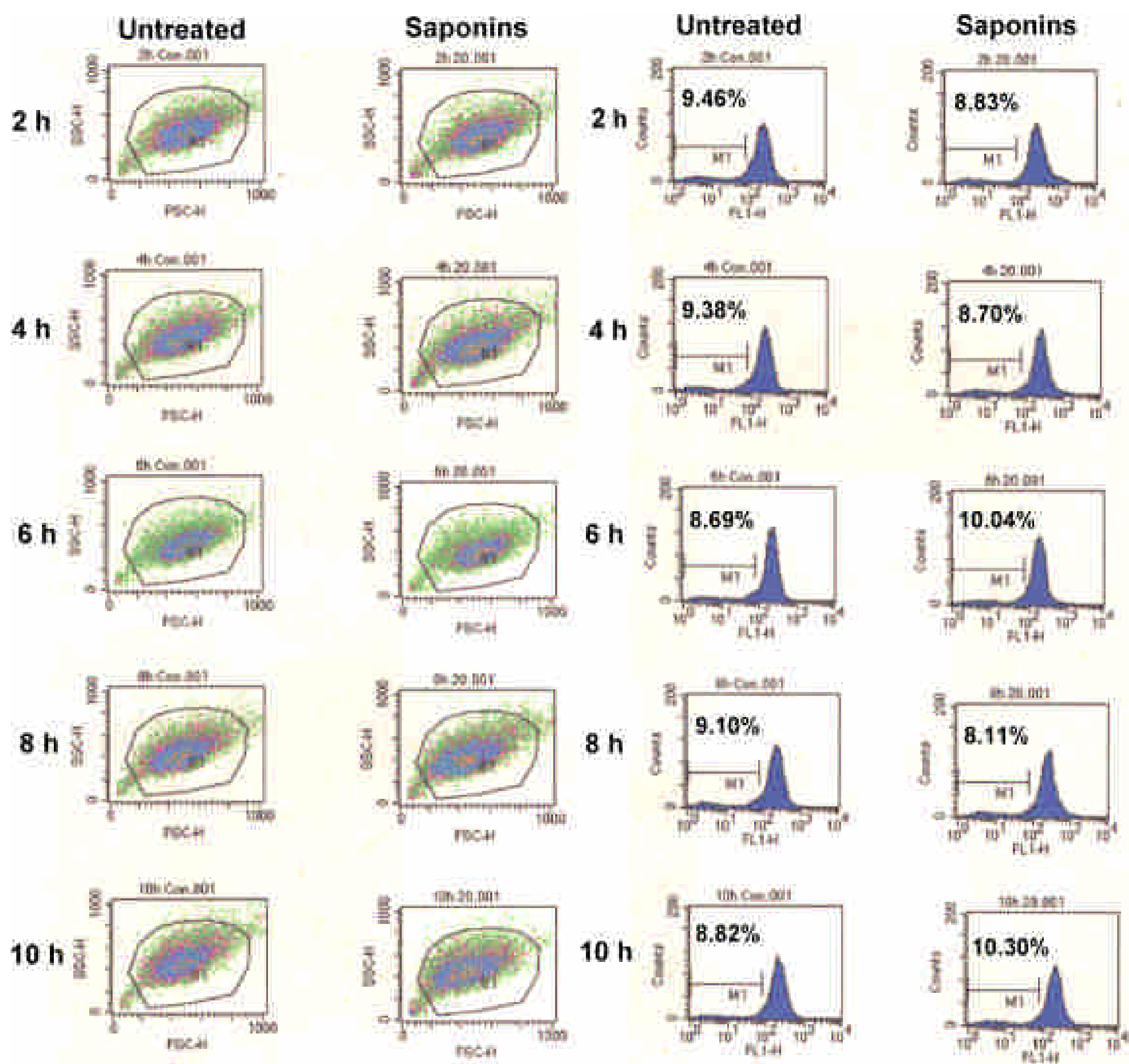


Fig. 17. Effects of *Quillaja* saponin on mitochondrial transmembrane potential ($\Delta\Psi_m$) in PC 3 cells. The untreated- and *Quillaja* saponin (20 g/ml) treated- PC 3 cells at 2-10 h were collected and stained in Materials and Methods. Following flow cytometric analyis, the change of $\Delta\Psi_m$ was further estimated by the CellQuest software. (A) Cell size and granularity of PC 3 cells ; FSC： forward angle light scatter ; SSC： side angle light scatter. (B) Data represent the percentage of cell counts displaying a abnormal $\Delta\Psi_m$. Results are from one experiment that is representative of three similar experiments.

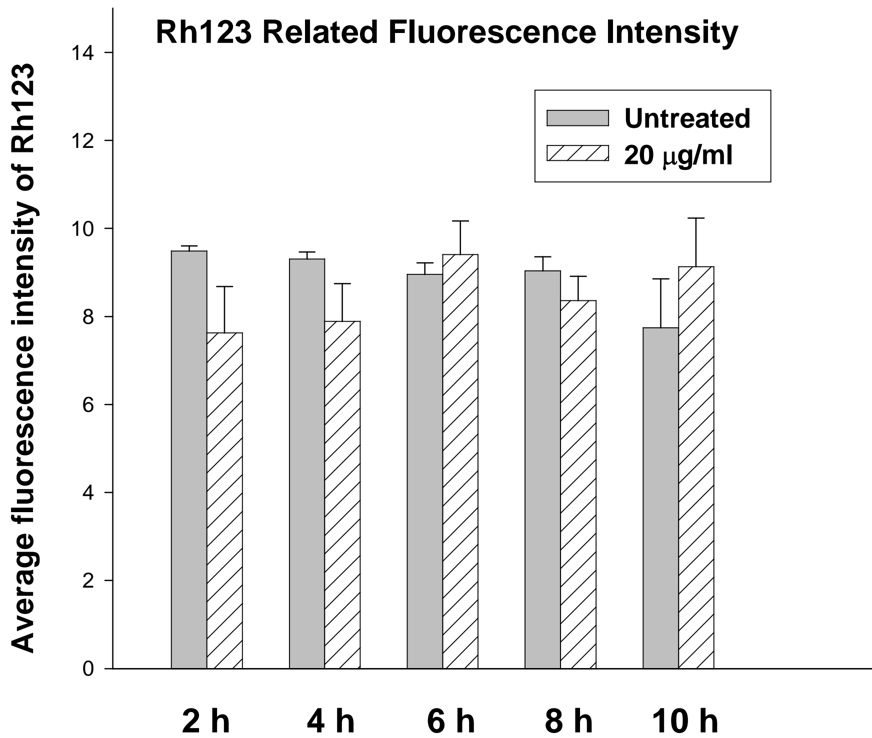


Fig. 18. Effects of Quillaja saponin on generation of mitochondrial transmembrane potential ($\Delta\Psi_m$) in PC 3 cells. The untreated- and Quillaja saponin (20 g/ml) treated- PC 3 cells at 1-9 h were collected and stained in Materials and Methods. Following flow cytometric analysis, the generation of superoxide anion was further estimated by the CellQuest software. The relative fluorescence intensity of cells. Fluorescence intensity of the treated cells is shown as percentage relative to the untreated cells. Each plot is shown as means ± SD of results from one of three similar experiments.

Discussion

在本報告中，使用來自石鹼樹樹皮中的皂素先對一些在台灣有著高發生率及高死亡率的腫瘤細胞，包括肝癌(HepG2, Hep 3b)、乳癌(MCF-7)、前列腺癌(PC-3)進行藥物細胞毒性試驗，結果觀察到此種來自石鹼樹樹皮之皂素，與其他像是人參、大豆、甘草等來源的皂素，具有抑制癌細胞生長的效果(Lee et al., 1999, 2000; Liu et al., 2000; Park et al 1997)，而且又以抑制前列腺癌細胞(PC-3)生長的效果最佳。此外，若以較高濃度劑量的 Saponin 處理(40μg/ml)前列腺癌細胞，對細胞引起急性的毒性，在三小時的處理下觀察到細胞膜呈現萎縮的外型，甚至漂浮的死細胞，之後 trypan blue assay 的結果也再次肯定 Quillaja Saponin 會抑制人類前列腺癌細胞的生長。而在兩株不同類型(androgen dependent-及 androgen-independent-)的前列腺癌細胞皆處理 Quillaja Saponin 時，發現 androgen dependent-的 LnCap 細胞有增生的情況，在劑量高達 40μg/ml 仍會造成細胞死亡，而 androgen independent 的 PC3 對於 Quillaja Saponin 會有高度的藥物敏感毒性。至於較高劑量的 Quillaja Saponin 處理下，屬於 androgen dependent-的 LnCap 細胞，仍會出現細胞生長受到抑制的現象，推測此種結果，可能是由於 androgen dependent-的 LnCap 細胞在其生長上需要有 androgen 此種荷爾蒙，而屬於 triterpenoid 類的 Saponin，結構上類似 sterol，可能是 LnCap 細胞膜上的 androgen 接受器，對於 Quillaja Saponin 有些許的親和力，因此造成較低濃度的處理時會有增生的現象；而高濃度處理下，在兩株前列腺癌細胞均有抑制癌細胞生長的情形，且 PC3 細胞更具有高度的藥物敏感毒性，推測其原因，可能是具有親脂性端的 Saponin，在高濃度下會(1)增加嵌入細胞膜的機率，(2)在細胞膜上聚集形成孔洞，如此一來，細胞膜上的結構受到破壞，因此加速細胞走向死亡的途徑。

在得到 Quillaja Saponin 對於屬於 androgen independent 的前列腺癌細胞具有較高的藥物毒性後，進一步探討其對細胞造成死亡的方式，結果觀察到染色質的濃縮以及 DNA 斷裂的發生等細胞凋亡型態上的特徵，因此可以知道此種藥物毒性引起死亡的方式是細胞進行細胞凋亡的型態。在 Quillaja Saponin 處理 9 小時即會發生細胞凋亡的現象，6 小時可測得 caspase-3 的活性，並從蛋白質電泳圖也可看到活化態 caspase-3 蛋白質的出現，而其下游的受質也就是具有修補受損 DNA 功能的 PARP 蛋白質也受到活化態 caspase-3 的切割作用而出現 85kDa 的蛋白質片段。因而確認引起 PC-3 細胞死亡的方式是經由進行細胞凋亡路徑。

然有越來越多的研究指出大部分的細胞凋亡過程中，粒線體膜電位有下降及粒線體功能喪失的情況產生(Chen et al., 2000; Chang et al., 2002)，並且可能是因為細胞內出現氧化壓力所致(Haridas et al., 2001)。因此我們也進一步偵測，是否由於細胞內氧化壓力的驟增及粒線體膜電位功能喪失，而出現細胞凋亡的情形。但經由流式細胞儀技術和特殊染劑的輔助下，並未偵測到細胞內有過量的活性氧物質產生，包括 peroxides 及 super oxide anion。粒線體膜電位也並未改變。

在此篇結果中，我們得到 Quillaja Saponin 也是具有抑制癌細胞生長的能力，包括肝癌、乳癌、前列腺癌，而且人類前列腺癌 PC-3 對於石鹼樹皮之 Saponin 具有高度藥物敏感毒性。希望藉由初步的研究結果，能提供石鹼皂素作為中草藥在抑制癌細胞生長研究上的科學依據。且對於現今荷爾蒙移除治療失效的 HRPC(hormone refractory prostate cancer)來說，積極尋找及研究新的治療藥物上，Quillaja Saponin 可能是治療上的新研究選擇對象。

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