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洛神花萃取物及其功能性成分抗癌作用之研究

研 究 報 告

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目　錄

摘　要

癌症預防已為一世界潮流，因此，尋找一化學預防物質（chemopreventive agent）為一重要工作。花青素（Anthocyanins）及多酚（Polyphenol）廣存於蔬菜、水果等植物中，已有許多報告證明花青素及多酚具抗氧化活性，降低血脂肪及防癌等作用，本研究室也發現分離自洛神花之花青素(*Hibiscus* anthocyanins, HAs)有抗氧化及保肝之功能(Wang et.al, Fd. Chem. Toxicol. 35, 1159-1164, 1997；38, 411-416, 2000)。最近我們的研究顯示此類 HAs 及 Hibiscus polyphenol（HPE）會促進胃癌（AGS），肝癌（3B, G2），口腔上皮癌(KB)，血癌(HL-60)，乳癌(MCF-7)，腸癌(Caco-2)等細胞凋謝死亡，因此，本研究擬深入探討 HAs, HPE 之抗癌作用及其作用之分子機制，並加以建立 HAs 及 HPE 之分析。

第一年的工作探討洛神花萃取物（Hibiscus Sabdaffra extracts, HSE）及其功能性成份 HAs 及 HPE 抗癌之作用，工作之項目包括（1）利用 flow cytometer 及 DNA electrophoresis 分析 HSE, HAs, HPE 促進各種癌細胞凋謝死亡;(2)探討 apoptosis-related gene expression，包括 bcl-2 family, caspase, cyt c;(3)探討 HSE, HAs, HPE 對癌細胞 cell cycle 的影響，包括各種 Cyclins 及 CDKs 及它們的 association, Rb phosphorylation 及與 E2F 之 association, cell cycle 分佈等，由以上的結果我們發現 HSE, HAs, HPE 可促進癌細胞凋謝死亡及抑制 cell cycle progression，其機轉為活化 p38/FasL 及 p53/Bax 路

徑。

從以上的結果，我們可能提出 HSE, HAs 及 HPE 可以預防及治療癌症，並說明其作用機轉，由於洛神花在國內容易栽培，產率高，而 HSE, HAs 及 HPE 也容易分離，因此，本研究成果將可應用於發展新藥。

關鍵詞：洛神花，花青素，多酚，抗癌作用，分離及分析

Abstract

Cancer prevention has been a world trend. Because of this, searching for chemopreventive agents is a very important thing. Anthocyanins and polyphenol are abundant in vegetables and fruits. Many reports indicated they have function in anti-oxidation, reducing cholesterol, and preventing cancers. Our laboratory explores anthocyanins extracted from *Hibiscus sabdariffa Linne*, *H*ibiscus anthocyanins (HAs), have antioxidant activity and liver protection (Wang et al., Fd. Chem. Toxicol. 35, 1159-1164, 1997 ; 38, 411-416, 2000). Recent data also shown that HAs and HPE promote AGS, 3B, G2, KB, HL-60, MCF-7, Caco-2 cells apoptosis. Thus, this research investigated that HAs, HPE anticancer function and its molecular mechanism. The first year, we analyzed HSE's function and and its functional component HAs and HPE work in anticancer. The working items: (1) using flow cytometery and DNA electrophoresis analyzed HSE, HAs and HPE promote all kinds of human cancer cells apoptosis; (2) exploring apoptosis-related gene expression, including bcl-2 family, caspase, cytochrome c et al.; (3) testing HSE, HAs and HPE affect cancer cells in cell cycle, including several cyclins, CDKs and their associatation, Rb phosphorylation, with E2F association, cell cycle distribution. Take together, we expect that HSE, HAs and HPE promote cancer cell apoptosis and inhibit cell cycle progression. The mechanisms were through activation of p38/FasL and p53/Bax cascade.

From above, we suppose HSE, HAs and HPE could prevent and treat cancer, furthermore maybe explain this mechanism. Due to *Hibiscus sabdariffa Linne* is planted easily in Taiwan and has high producing rate. HSE, HAs and HPE are also isolated simply. The result of this research will apply in developing new

agents.

Keywords: *Hibiscus Sabdariffa* Linnaeus, Anthocyanins, Polyphenol, anti-tumorgenesis, dissociation and analysis

前　言

洛神花(*Hibiscus* Sabdariffa Linnaeus) 為錦葵科(Malvaceae)植物，原產熱帶地區，分部於印度，馬來西亞及東南亞，台灣東部及南部也盛產。其花成份包含有機酸（如檸檬酸、壞血酸、原兒茶酸等）(1，2)；醣分主要為半乳糖，葡萄糖及果糖（3，4)；Pectin 及類黃酮素（5)，黃酮素包括有 hibiscetin，gossypetin，quercetin。國外之研究，在體外試驗顯示其水萃取物有 anti-spasmodic（6)，降膽固醇（7)，降血壓（8）及抗菌作用（9)。其成份 flavonoids 具有抗氧化及抑制心血管疾病（10，11)；原兒茶酸（protocatechuic acid；PCA）可抑制化學致癌物誘導之癌化（12-16)，本研究室也顯示洛神花中之 PCA 及花青素有強的抗氧化作用（17，18)，我們也發現 PCA 亦有抗 TPA 促腫瘤作用（19）及促進 HL-60 凋謝死亡之作用（20)。

最近我們的研究顯示洛神花水萃取物具有抑制 LDL 氧化作用，抑制 LDL 中 apo-B 的 fragmentation 及抑制 LDL 中膽固醇之 degeneration(21)。進一步的研究顯示洛神花水萃取物有顯著抑制膽固醇及 lard oil 餵食 10 週之兔子之 triglycerides 及膽固醇，並能增加 HDL 及降低 LDL，進一步以病理觀察也顯示洛神花水萃取物抑制 atherosclerosis lesion；以高果糖餵食大白鼠產生之高血脂症，洛神花水萃取物也有相同之功能，能夠抑制血脂肪（22)。

花青素廣泛存在於許多食用的植物果實中，不同的花青素具有不同的顏色，其中包含了：藍色、紫色或紅色等，在植物的果實中通常含有許多不同種類的花青素，並使其具有各種不同的色澤，如：草莓、葡萄及櫻桃等。一些具有醣基的醣化 delphinidin 與 cyanidin 也發現存在許多的植物中。在營養食品中添加入花青素萃取物，可以有效的使人們攝取到數個毫克的花青素。根據統計，在美國每人每天會攝取 180-250 毫克的花青素。現今，由天然植物果實中所萃取出具有高度花青素含量產物也廣泛的被接受。對於花青素的應用方面，被認為對於許多的疾病是具有有效的預防作用，如：糖尿病視網膜病變及的微血管循環疾病等，此外也具有抗發炎及化學預防作用的功效。

洛神花花青素(HAs)萃取自洛神花(*Hibiscus* sabdariffa L.)，在傳統醫學上已被用來治療高血壓、鎮痛及肝炎等，HAs 主要為 cyanidin-3-glucoside 及 delphinidin-3-glucoside（23, 24）此類花青素廣泛存在於蔬菜、水果及穀類等（25）。已有許多報告指出花青素具有抗氧化活性（26-30）抗致突變性（31-33），抗癌作用（34-36）及降低脂質過氧化作用和 DNA 損傷（37）。本研究室也發現分離自洛神花之花青素(HAs)具有抗氧化及保肝之功能（17, 18），且在初步的研究中我們已有數據顯示其具抗癌作用。

多酚（polyphenol）廣泛存於蔬菜、水果中，已有許多報告指出具有抑制癌化的作用（38-41），其作用大部分認為與其他的抗氧化性質有關（42），

洛神花中具有許多種多酚，如 protocatechuic acid（PCA），已被證實具有抗氧化能力（17），對於 diethylnitrosamine 致肝癌、4-nitro-quinoline-1-oxide 致口腔癌、azoxymethane 致腸癌、N-methyl-N-nitrosourea 致胃癌及 N-butyl-N(4-hydroxylbutyl) nitrosamine 致膀胱癌（12-16）均有抑制作用，過去我們也發現 PCA 有抗氧化保護肝臟及抑制腫瘤促進作用（17,19）及促進 HL-60 apoptosis（20），根據商業用品的價值及 cancer chemoprevention 使用 crude extracts 之趨勢(如 tea polyphenol extract, curcuminoid extracts and broccoli extracts 等)，我們已進行 Hibiscus polyphenol（HPE）之分離，發現其有很強之抗氧化能力及殺死癌細胞之作用。因此，本研究擬深入探討 HAs, HPE 之抗癌作用及其作用之分子機制，並加以建立 HAs 及 HPE 之分析。

材 料 與 方 法

一、材料

Tris-base, ethylenediamine-tetraacetic acid (EDTA), sodium hydroxide, MTT (3-[4,5-dimethylthiazol-zyl]-2,5-diphenyltetra- zolium bromide), sodium chloride, sodium citrate, RNase A, propidium iodide, Triton X-100, paraformaldehyde 皆購自美國 Sigma Chemical Co.；DMEM, sodium pyruvate, Trypsin, bovine serum albumin, Penicillin-Streptomycin mixed antibiotics, L-Glutamine, phosphate buffer solution (PBS)皆購自美國 Gibco Co.

二、洛神花萃取物（HSE）之製備

秤取乾燥洛神花 100 g，加入 6 L distilled water，以 100℃煮 2 小時，待冷後過濾，將濾液進行冷凍乾燥，得其粉末即為洛神花粗萃取物。

三、洛神花花青素(HAs)之製備

(1) HAs extracts 製備及定量

將乾燥的洛神花以酸化甲醇(1% HCl)浸泡隔夜後，所得的液體以濾紙過濾後真空乾燥，所得的物質即為洛神花花青素粗萃取物，隨後以 pH 區分法定量總花青素含量。200 μg/ml 的洛神花花青素萃取物溶於 pH1.0 及 4.5 下，在以分光光度儀測其 510 及 700 nm 之吸光值，使用 $A=[(A_{510}-A700)_{pH1.0}-(A_{510}-A700)_{pH4.5}]$ 公式，與 delphindin 標準曲線比照定量。

(2) HPLC 分析

為使 HAs 製備標準化，進一步以 HPLC 分析 cyanidin-3-glucoside 及

delphindin-3-glucoside 之含量是必要的，將洛神花花青素萃取物溶於水中，以高效能液像層析管住(250x4.6 mm，5 μm Hypersil ODS)，mobile phase 為 1.5% H_3PO_4、20% HPAc、MeCN in H_2O、流速為 0.5ml/min。

四、洛神花多酚酸（HPE）之製備

(1) HPE extracts 製備及定量

將 100 克攪拌之乾燥洛神花，以 300 ml 甲醇在 50℃ 中萃取 3 小時過濾，連續三次，收集濾液，於減壓濃縮乾燥溶解於 50℃ 500 ml 水，再以 200 ml 己烷移除色素，水層再以 180 ml 乙酸乙酯萃取三次，然後冷凍乾燥，即得 HPAs。

(2) HPLC 分析

為使 HPE 製備固定標準化，我們利用 HPLC 分離多酚酸，以 PCA 為標準品，分析條件為用 C-18 reverse-phase column(5 μm, Hypersil ODS, 200mm X 2.1 mm)，將 HPAs 過濾（0.45 μm）後，取 20 μl 注入 HPLC，mobile phase 為 A：acteonitrile；B：0.5% glacial acetic acid in water；C：isopropanol；0-5 min 為 100% B；5-15 min 為 5% A, 95% B；15-22 min 為 1% A, 96 % B, 3% C；22-24 min, 5% A, 90% B, 5% C；24-30 min 為 100% A，monitored at 273 nm。

五、MTT 分析

調整細胞（Hep G2, Hep 3B, AGS, MCF-7, KB）濃度為 2 x 10^5/ml，以 2 ml

之細胞沖至 microtiter plate，加入 HSE 或 HAs 或 HPE，培養 20 hr 後，以離心去培養基，加入 2 ml 新鮮無血清之培養基 與 200 µl MTT (5 mg/ml) 繼續培養 4 hr，去除培養液後，加入 1.5 ml isopropanol，均勻分散細胞後離心，取 1 ml 上清液在 563 nm 測其吸光，分別繪圖計算出 IC_{50}，篩選出 HSE, HAs 及 HPE 作用最強之癌細胞。

六、Cell cycle 分析

經 HSE 或 HAs 或 HPE 處理 0-24 hr, 以 FACScan 分析，以緩衝液洗滌 2 次，在 400 x g 離心 5 min, 去除上清液後加 250 µl solution A (trypsin buffer)與細胞混合均勻，在室溫作用 10 min, 再加入 solution B (trypsin 抑制劑及 RNase buffer), 作用 10 min 後以 solution C (propidium iodide stain solution) 在暗處 0℃ 染色 10 min 後以 flow cytometry 分析。

七、細胞凋謝死亡 (apoptosis) 分析

(1)DNA 斷裂片段之測定

收集經 HSE 或 HAs 或 HPE 處理之細胞，經 800 x g, 4℃離心 10min 後，以 .05% SDS 和 100 mM EDTA 溶解，加入 20 µg/ml RNase A，37℃, 1 hr 後，再加入 100 µg/ml proteinase K 於 50℃處理 3 小時，再用 phenol, chloroform 和 isoamylalcohol (25:24:1)萃取後，加入 2 倍體積之 alcohol 及 0.1 倍體積之 3 M sodium acetate (pH7.2)，置於 –20℃ 過夜，沉澱物以 14700 rpm 離心 30 min，再以 DNase-free RNase 在 37℃ 作用 1 hr，溶於

TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH8.0)，以 1.5% agarose gel eletrophoresis.

(2) Leukostate 染色法

實驗原理：Leukostate 染色法，對於正常細胞會出現完整細胞型態出現；凋謝死亡細胞則較少細胞質，反而看到明顯細胞核濃染的現象；而壞死的細胞只會出現暈開的細胞影像(ghost)。細胞經過不同濃度洛神花花青素處理特定時間後，再以 PBS 清洗三次，然後以固定液(Methanol)作用 10 秒後，加入染劑 I 染 10 秒，在衛生紙上扣乾，隨即加入染劑 II 再染 10 秒，最後沖洗多餘的染劑，並風乾即可於顯微鏡下觀察。

相關溶液的配製:

染劑 I : 0.1% eosin Y, 0.1% formaldehyde, 0.4% sodium phosphate dibasic, 0.5% potassium phosphate monobasic.

染劑 II : 0.04% methyleneblue, 0.04% azure A, 0.4% sodium phosphate dibasic, 0.5% potassium phosphate monobasic.

(3) DAPI 染色法

DAPI (4', 6-Diamidino-2-phenylindole dihydrochloride hydrate) 是常用的一種與 DNA 結合的螢光染劑。可以進入細胞核內，插入雙股 DNA minor groove 中 adenine 及 thymine rich 的位置，利用 DAPI 染色後於螢光顯微鏡 (340/380nm) 下觀察，可看到細胞核的形態變化，作為判定細胞凋亡的依據。本實驗之方法為：將 AGS cells 培養後，以 trypsin-EDTA 將細胞由培

養皿中打下，以 1500 rpm 離心 5 分鐘，去除培養液，將細胞調整成 2×10^5 cells/well 培養於 6 公分的培養皿中，待 10~12 小時細胞貼壁，更換新的培養液同時加入不同濃度的 HSE (1.0、2.0、3.0mg/ml)反應 18 h，移除舊 medium，以 PBS 洗 3 次。將細胞以 4% paraformaldehyde 室溫固定細胞 30 分鐘，以 DAPI (1ug/mL)染色 30 分鐘，再以 PBS 清洗細胞 3 次，螢光顯微鏡下觀察細胞型態。

(4) RT-PCR

首先，萃取 total RNA，使用 guanidium chloride 的操作步驟，然後進行 cDNA 的合成及 PCR 的放大。

a) Reverse transcription reaction：取 4 g 的 total cellular RNA，加入 20 μl reaction buffer，包含 4 μl dNTPs (2.5 mM), 2.5 μl Oligo dT (10 pmole/μl), and RTase (200 U/μl)，在 42℃反應 1 小時，再以 99℃作用 5 分鐘後保存於 4℃。

b) Primer synthesis：取 5 μl cDNA，加入 primer-5'和 primer-3'各 5 μl，再加入 3.2 μl dNTPs (2.5 mM), 5 μl 的 10X PCR buffer 及 DNA polymerase 5 μl (2 U/μl)，各個基因的 primer 序列及條件如下表。

c) DNA electrophoresis：預先配置 2% agarose gels，取 5 μl 的 PCR 產物進行電泳分析，以 ethidium bromide 染色，觀察結果。

Enzyme	Sequence	Size	Temp. (℃)/ Time (min)	Cycle
Fas	Forward: 5'-CAAGTGACTGACATCAACTCC-3' Reverse: 5'-CTATTTTGGCTTCATTGACACC-3'	727 bp	94℃ (denature)/ 1 min 60℃ (anneal)/ 1 min 72℃ (elongate)/ 2 min	30
FasL	Forward: 5'-GGATTGGGCCTGGGGATGTTT-3' Reverse: 5'-TTGTGGCTCAGGGGCAGGTTGT-3'	344 bp	94℃ (denature)/ 1 min 63℃ (anneal)/ 1 min 72℃ (elongate)/ 2 min	25
GAPDH	Forward: 5'-CGGAGTCAACGGATTTGGTCGT-3' Reverse: 5'-AGCCTTCTCCATGGTTGGTGAA-3'	304 bp	94℃ (denature)/ 1 min 63℃ (anneal)/ 1 min 72℃ (elongate)/ 2 min	25

(5) Apoptosis-related proteins 分析

癌細胞經處理後，其 lysate 經 Western blotting 分析 Bcl-2 family（包括 Bcl-2, Bax, Bcl-xL 及 Mcl-1），CPP 32，Cytochrome c 等蛋白表現之影響。西方點墨法（Western blotting）方法如下：

a) 蛋白質抽取及定量

細胞經過處理後以 PBS 清洗，在 4℃下離心 1500 rpm 後，置於 buffer 中 (20 mM Tris-HCl, pH7.4, 0.03 mM sodium orthovonadate, 2 mM EDTA, 2 mM magnesium chloride, 1 mM DTT, 250 mM sucrose, 2 mM PMSF 及 10 μg/ml leupeptin) 以震盪器震動 1 小時，在 800 rpm 下離心 10 分鐘，取上清液後作蛋白質定量。此法乃利用蛋白質可與 Commassie Billiant blue G-250 形成藍色的複合物質，而這個藍色的複合物在 595 nm 有一個較大吸光值的原理來測定。首先製備以 BSA (Bovine Serum Albumin)為標準液的蛋白質溶液數個濃度，做出標準曲線後再以此換算各個樣本的蛋白質濃度。

b) 蛋白質電泳

取 50 μg 總蛋白含量之細胞萃取液，加入 5 倍之 protein loading buffer 混合均勻，loading 至 10% gel SDS-PAGE。

c) 西方墨點法

電泳完成後，以 4℃，100 mA 轉移蛋白至 NC membrane，16 小時後，將 membrane 浸入含 5%脫脂奶粉之 TBS-T 緩衝液內，於 4℃至少搖動 6 小時，加入一次抗體於 4℃作用 3 小時以上，再以 TBS-T 清洗三次，每次各 10 分鐘，而後與二次抗體 (0.2 μg/ml)反應 1 小時，以 TBS-T 清洗三次，每次 10 分鐘，最後將 membrane 置入高透明度之塑膠袋中，加入 enhanced chemilluscence substrate，進行反應 1 分鐘後，放至壓片匣中以 X-ray film 曝光後加入顯影劑顯影。

(註: TBS-T buffer 為 Tris-base 20 mM, NaCl 138 mM, Tween-20 0.1%)

結　果

Ⅰ. 洛神花水萃取物 (HSE)

1.洛神花水萃取物之萃取

秤取乾燥洛神花 100 g，加入 6 liter distilled water，以 100℃煮 2 小時，待冷後過濾，將濾液進行冷凍乾燥，得其粉末即為洛神花粗萃取物。

2.洛神花水萃取物對 AGS cell（人類胃癌細胞）致死率之敏感度

由 Figure 1 顯示：HSE 的毒性分析是以 0, 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3 mg/ml 等不同濃度，處理 AGS 癌細胞，在 37℃下作用 24 小時後，觀察細胞存活狀態。結果，在 HSE 濃度提高至 2.49 mg/ml，即達到抑制 50%的細胞存活 (IC_{50}: 2.49 mg/ml)。

3.以 DAPI 染色法判斷 HSE 所引起細胞死亡是否為 apoptosis

Figure 2A-E 結果顯示：當 AGS 處理 HSE 濃度為 0.2 mg/ml 時，細胞開始出現細胞質減少及核濃染的 apoptosis 現象；隨著 HSE 濃度逐漸地提高，細胞所出現的 apoptosis 現象愈趨明顯。且在 Figure 2F 中發現 AGS 處理 HSE 濃度達 2.0 mg/ml 時，我們可以觀察到的 DNA ladder 現象，當濃度為 3.0 mg/ml 時，DNA ladder 現象更為顯著。

4.處理 HSE 對 AGS 所造成 cell cycle 之影響

Figure 3 是流式細胞儀分析的結果，當 AGS 處理 HSE 濃度為 2.0 mg/ml 時，分析 sub-G1 phase 有明顯 apoptotic peak 的高峰出現，約為 18.54%，當濃度處理至 3.0 mg/ml 時，pro-apoptotic body 更為顯著有 21.23%。另外，G1 phase 亦發現有些許增加的趨勢。

5.HSE 引起 AGS 細胞凋亡之機制

Figure 4 和 Figure 5 結果顯示：隨著 AGS 處理 HSE 濃度增加，細胞內凋亡蛋白 t-Bid、Bax、Cytochrome c、caspase 8, 9, 3 的表現都有增加的現象；而抗凋亡蛋白 Bcl-2 和 Mcl-1 的表現是降低的。但 phospho-p53 的表現不受到處理 HSE 的影響。這些結果推測 HSE 所誘導的 apoptosis 是透過 p53-independent 的路徑調節 Bcl-2 家族，而啟動粒腺體細胞凋亡路徑（Bax/caspase9, 3）的活化。AGS 細胞預先處理 MAPK 抑制劑：SB203580、PD98059、wortmannin、SP600125，分別是抑制 p38、ERK1/2、PI-3-kinase、JNK 訊息路徑，使用 Flow cytometry 分析 apoptosis， SB203580（p38 抑制劑）和 SP600125（JNK 抑制劑）濃度皆為 30 uM 能都有效降低 HSE 所誘導 AGS 細胞凋亡的現象，特別是 SP600125 達到抑制效果 50%（Figure 6）。為確認 HPE 是透過 p38/JNK 路徑造成細胞 apoptosis，進行 p38/JNK 下游訊息蛋白電泳分析。結果 AGS 細胞內 phospho-Jun、FasL、Fas 以及 Bcl-2、Bax 蛋白質表現在單獨處理 HSE 濃度 3.0 mg/ml 於 24 小時後是被大量活化

的，預先處理 p38 和 JNK 抑制劑單獨或同時則皆可阻斷其表現(Figure 7)，並且觀察到同時處理 p38 和 JNK 兩者抑制劑具有加成的抑制作用。一旦將其抑制劑移除，則 phospho-p38、phospho-JNK 和 phospho-Jun 在細胞內的蛋白表現量又重新回復（Figure 8）。

Ⅱ. 洛神花花青素 (HAs)

1.洛神花花青素之分離與鑑定

a. 洛神花花青素之分離: 乾燥洛神花秤取 20 g，以 0.1% 鹽酸甲醇溶液於 4℃下浸泡，隔夜，將果實過濾後，以真空減壓濃縮機濃縮。濃縮後的溶液加入 250 ml 二次水溶解，再利用 Diaion HP-20 樹脂充填之管柱浸泡 24 h，然後，以 0.1%鹽酸水溶液清洗雜色素，再以甲醇將花青素沖提出後以真空濃縮，所得產物取二次水 250 ml 溶解再經真空冷凍乾燥機乾燥為粉末。

b. 花青素之純度測定: 由 spectrophotometer 分析本實驗室所萃取出的洛神花花青素(HAs)，其吸光值再經公式換算，而換算出所萃取出的花青素純度大約有 85~95%。

c. Delphinidine 之含量(Figure 9)測定: 經 HPLC 分析，Dephinidine 的滯留時間(retention time; RT)在 18.83 min；Sample，也就是本實驗室所萃取出的洛神花花青素酸水溶液 RT 為 18.42 min；而 sample+sandard 就是兩者所合

併，其 RT 在 18.36 min。從結果可得，本實驗室所萃取出的洛神花花青素其 Dephinidine 含量大約有 2.1%。

2.洛神花花青素對數種癌細胞致死率之敏感度

MTT assay: 以 0, 0.05, 0.1, 0.2, 0.5, 1, 3, 4 mg/ml 等不同濃度的洛神花花青素，處理 24 小時，以及在 0, 12, 24, 48 小時等不同時間點，處理 3 mg/ml 的花青素，在 37℃下作用 24 小時後，觀察其存活狀態，結果發現 HL-60 cell 對洛神花花青素最為敏感，且其 IC_{50} 為 2.49 mg/ml (Figure 10A)，且細胞依花青素時間或濃度的增加而增加死亡的現象(Figure 10B)。

3.洛神花花青素(HAs)會引起 HL-60 細胞的凋亡現象

a. Flow cytometric analysis (Figure 11): 為了進一步確認洛神花花青素(HAs)會造成 HL-60 細胞的凋謝死亡，利用流式細胞儀 (flow cytomemery) 觀察細胞內細胞週期分佈，以洛神花花青素(HAs) 3 mg/ml 處理 0, 12, 24, 48 小時；0, 1, 2, 3 mg/ml 處理 24 小時，結果發現在 HAs 處理超過 12 小時及濃度 3 mg/ml 以上，細胞在 sub-G1 期有明顯的 pro-apoptotic body 產生，結果與 DNA fragmentation 互相作印證，可以證實洛神花花青素會引起 HL-60 細胞的凋謝死亡。另外，為了研究 HAs 造成 HL-60 細胞的凋謝死亡是透過哪一條路徑，所以分別預先加入四種抑制劑 SB203580 (50 μM), PD98059 (25 μM), SP600125 (20 μM), wortmannin (1 μM) 24 小時，再處理 HAs (3

mg/ml, 24 hr) 觀察。結果只有 SB203580 對 HAs 造成 HL-60 細胞的凋謝死亡有回復作用，回復率大約有 50%，其餘三種抑制劑皆沒有影響(Figure 12)。

4.HAs 引起 HL-60 細胞凋亡之機制

Western blot analysis (Figure 13):首先，Figure 13 已知可能是透過 MAPK 路徑，所以，利用 Western blot 分析方法，探討 HAs 造成 HL-60 細胞凋謝死亡的機制。發現 MAPK family 蛋白 phospho-p38, phospho-c-Jun 隨著時間的增加，表現量亦增加，反之 phospho-ERK1/2 則沒有影響。Figure 14A, B 也觀察到 Fas, FasL 隨著 HAs 處理的濃度愈高，無論在 protein level 或 mRNA level 的表現量皆有上升的趨勢。另外，HAs 以 0, 1, 2, 3 mg/ml 處理 HL-60 細胞 6 小時，結果 cleaved-caspase-3 及 cleaved-caspase-8 皆有顯著的表現 (Figure 15)。研究 Bcl-2 family 蛋白(Bcl-2, Bax, Bid, tBid)，發現 Bcl-2 及 Bax 無作用，而 Bid 活化形式的蛋白 tBid，在處理 HAs 後，表現量呈現 time- 及 dose-dependent (FigureA, B)。同樣的，在 HAs 的刺激下，Cytochrome c 大量由 mitochondrial 轉位到 cytosol (Figure 16)。在圖已知只有 SB203580 顯著抑制 HAs-mediated apoptosis，結果推測 HAs 可能透過 p38 路徑，而使這些蛋白活化，導致細胞死亡(Figure 17)，因此加入 SB203580 於 HAs 處理之細胞中，顯著的減少 Fas, FasL 之表現及抑制 c-Jun 磷酸化。從圖可以得

到一個結論，HAs 是透過 p38/FasL 的路徑，致使 HL-60 細胞走向凋謝死亡。

Ⅲ. 洛神花多酚 (HPE)

1.洛神花多酚之萃取

將 100 g 攪拌之乾燥洛神花，以 300 ml 甲醇在 50℃ 中萃取 3 小時過濾，連續三次，收集濾液，於減壓濃縮乾燥後溶解於 50℃ 500 ml 水，再以 200 ml 己烷移除色素，水層再以 180 ml 乙酸乙酯萃取三次，然後冷凍乾燥，即得 HPE。

2.八種細胞加入不同濃度之 HPE 24 h 後之存活情形

由 Figure 18 結果顯示：HPE 對癌細胞的毒性分析是以 0.1-2.0 mg/ml 等不同濃度的 HPE 各別處理 NIH/3T3, Hep G2, MCF-7, KB, Caco-2, Hep 3B, HL-60 和 AGS 細胞株，在 37 ℃下作用 24 小時後，觀察細胞存活狀態(Fig. 18A)。綜合 Figure 18B 比較結果顯示：HPE 對 AGS（人類胃癌細胞）具有最強的細胞毒性。而處理不同濃度 HPE 之 AGS 細胞，在 HPE 濃度提高到 0.95 mg/ml 即達到抑制細胞存活 50%，相較於其他株細胞是為最低（IC_{50} 為最低），即 AGS 對於 HPE 抑制細胞存活的敏感度最高，而且呈現 dose-dependent 的情形。

3.以 Leukostat 染色法判斷 HPE 所引起細胞死亡是否為 apoptosis

由 Figure 19 結果顯示：當 AGS 處理 HPE 濃度為 0.1 mg/ml 時，細胞開始出現細胞質減少及核濃染的 apoptosis 現象；隨著 HPE 濃度逐漸地提高，細胞所出現的 apoptosis 現象愈趨明顯。

4.處理 HPE 對 AGS 所造成 DNA 斷裂之影響

在 Figure 20 中發現 AGS 處理 HPE 濃度高達 2.0 mg/ml 時，我們可以觀察到明顯的 DNA ladder 現象。

5.處理 HPE 對 AGS 所造成 cell cycle 之影響

Figure 21 是流式細胞儀分析的結果，當 AGS 處理 HPE 濃度為 2.0 mg/ml 時，分析 sub-G1 phase 有明顯 apoptotic peak 的高峰出現；另外，G1 phase 也發現有些許增多的趨勢。

6.HPE 引起 AGS 細胞凋亡之機制

Figure 22 和 Figure 23 結果顯示：隨著 AGS 處理 HPE 濃度增加，細胞內凋亡蛋白 t-Bid、Bax、phospho-p53、Cytochrome c、caspase 8, 9, 3 的表現都有增加的現象；而抗凋亡蛋白 Bcl-2、Mcl-1 和 phospho-Bad 的表現是降低的。這些結果推測 HPE 所誘導的 apoptosis 是透過促進 p53 磷酸化以及調節 Bcl-2 家族，啟動粒腺體細胞凋亡路徑（p53/Bax/caspase9, 3）。AGS 細

胞預先處理 MAPK 抑制劑：SB203580、PD98059、wortmanin、SP600125，分別是抑制 p38、ERK1/2、PI-3-kinase、JNK 訊息路徑，使用 Flow cytometry 分析 apoptosis，只有 SB203580（p38 抑制劑）濃度 20 μM 能有效降低 HPE 所誘導 AGS 細胞凋亡的現象（Figure 24）。為確認 HPE 是透過 p38 路徑造成細胞 apoptosis，進行 p38 下游訊息蛋白電泳分析。結果 AGS 細胞內 phospho-p53、phospho-Jun、FasL、Fas 蛋白質表現在單獨處理 HPE 濃度 2.0 mg/ml 於 24 小時後是被大量活化的，預先處理 p38 抑制劑則可以阻斷其表現（Figure 25）。一旦將其抑制劑移除，則 phospho-p38、phospho-Jun 和 FasL 在細胞內的蛋白表現量又重新回復（Figure 26）。

綜合本研究的結果，可得知 HSE、HAs 及 HPE 皆顯著地造成細胞 DNA 斷裂，且導致細胞的凋謝死亡(apoptosis)，我們推論洛神花具有治療癌症的高度潛力。

討　論

首先，本研究結果顯示該洛神花水萃取物(HSE)可能藉由活化 p38/JNK 路徑，磷酸化 c-Jun，造成 FasL 大量被表達，啟動 Fas 這個 receptor 下游訊號傳遞(p38 和 JNK/c-Jun/ FasL/ Fas/ caspase 8/ t-Bid/ Bax/caspase9, 3)，促使 AGS 細胞走向凋謝死亡（Figure 7, 8）。顯示該洛神花多酚可能藉由活化 p38 路徑，磷酸化 c-Jun，造成 FasL 大量被表達，啟動 Fas 這個 receptor 下游訊號傳遞(p38/ c-Jun/ FasL/ Fas/ caspase 8/ t-Bid/ caspase9, 3)；另一方面，也結合 p53 調控 Bax，促使 AGS 細胞走向凋謝死亡（Figure 27），以減緩癌症發生的進程。

洛神花的甲醇萃取物，花青素(HAs)，會經由引起細胞的凋謝死亡，致使癌細胞生長受抑制。利用細胞毒性試驗，發現以人類血癌細胞(HL-60)最具效力，然而，NIH3T3 (正常老鼠纖維母細胞)則對花青素較不敏感，故本篇以 HL-60 為研究模式。細胞內存在某些蛋白酵素，可能在起始細胞凋亡作用中扮演一重要角色，例如 caspase。Caspase 是一 cysteine protease 的家族，此家族蛋白會活化細胞凋亡的程式(39, 40)，然而，caspase 的活化路徑目前尚未非常明確，且某些 caspase 同時具有上游及下游分子之功能。在本篇研究中，發現處理 HAs 後 caspase-3 會活化，從結果得知，HAs 所引起的細胞凋亡，caspase-3 為主要下游分子。根據上述，我們已得知 HAs 的確會引起癌細胞的凋謝死亡。接下來，當細胞預處理 SB203580

(p38-MAPK 抑制劑)、PD98059(MAP Kinase 抑制劑)、SP600125(JNK 抑制劑)、Wortmannin(PI-3K 抑制劑)，結果只有 SB203580 可以有效的抑制由 HAs 所引起的細胞死亡，由此我們可以推測 HAs 所造成的細胞死亡可能主要是透過 p38-MAPK 這條訊息傳遞路徑(Figure 17)，不過由於處理 SB203580 尚無法完全的抑制由 HAs 所引起的細胞死亡，是否還有其他的路徑還有待進一步的釐清。另外，透過 p38 的活化會磷酸化 c-jun 而引起細胞凋亡(41, 42)。在本篇研究中，亦觀察到 c-jun 磷酸化的表現量上升且導致下游 FasL 的活化，然後致使 caspase-8 及 tBid 被切割，證實了 apoptosis 與 p38/FasL 路徑是有相關的。綜合以上結論，可得知 HAs 明顯造成 HL-60 細胞 DNA 斷裂，而導致細胞的凋謝死亡(apoptosis)，其詳細機制透過四種抑制劑的使用，結果推測可能經由 p38 pathway 抑制癌細胞生長。細胞經由 HAs 的處理，活化 p38/JNK 造成 c-jun 的磷酸化，而活化下游的 FasL/Fas 使得 Bid 變成 tBid，可能造成 Bax 會在粒腺體膜上聚集在一起而形成孔洞，使得 cytochrome c 釋放出來跑到細胞質活化 caspase-3 造成細胞的死亡(Figure 28)。

多酚是許多植物、蔬果和茶的重要成份之一。近年來有相關的研究指出：多酚具有抗氧化 (43, 44) 以及抗腫瘤促進作用 (45, 46) 之功效。本研究室近年來投入洛神花之研究，也陸續發現洛神花 PCA 及花青素有強的抗氧化作用，另外 PCA 亦有抗 TPA 促腫瘤作用及促進 HL-60 凋謝死亡之作

用。在本實驗研究中，我們發現以較高濃度（2.0 mg/ml）HPE 處理人類胃癌細胞，結果發現嚴重的細胞死亡，並且死亡是以 apoptosis 的方式進行（Figure 18-21）。Figure 24 的實驗中，我們篩選阻擋 HPE 所造成之 apoptosis 最敏感的抑制劑。結果顯示 SB203580 以不毒殺細胞為最高限的濃度 20 μM 所造成的抑制 apoptosis 效果可達 70%，遠高於 PD98059、wortmannin 和 SP600125 的影響力。在預先處理抑制劑 SB203580 後，這種由 HPE 所誘導的死亡現象將有效被抑制，其含義是 HPE 透過 p38 MAPK 路徑誘導 AGS 細胞走向 apoptosis（Figure 29）。

本研究新發現 HAs 及 HPE 經由 JNK/p38 MAPK signals 增加 FasL 表現，進而促進癌細胞凋謝死亡。

目前世界上許多研究單位發現，癌細胞與正常細胞差別之一為無法進行一般細胞之 apoptosis，因此生長無法控制。HSE 及其成份 HAS, HPE 發現會透過 p38/FasL 或 p53/Bax 路徑，促進癌細胞凋謝死亡及停止細胞週期，之後，進一步以動物模式探討其致癌作用及作用之機轉，俾以發展為抗癌物質。

結 論 與 建 議

本研究發現洛神花含有豐富的各種多酚及花青素，且洛神花具有抗氧化、保肝及抗癌作用等，可能是歸功於其主要成份多酚及花青素。本研究計劃之結果確認洛神花及其成份花青素(HAs), 多酚(HPE)具有促進癌細胞凋謝死亡及抑制 cell cycle progression 之功能，其作用機轉可能為兩條路徑：1) HSE 及 HAs 活化 p38/JNK 造成 c-jun 的磷酸化，而活化下游的 FasL/Fas 使得 Bid 變成 tBid。2) HPE 會活化 p53 及 Bax，而 Bax 會在粒線體表面上造成孔洞，使得 cytochrome c 釋放出來跑到細胞質，導致 caspase-3 cleavage，最後造成細胞的死亡。進一步的動物試驗在九十四年度衛生署計畫申請中，希望能獲得經費補助繼續完成。

因此，本研究計劃建議將洛神花及其成份花青素、多酚推廣為預防癌症之保健食品。本研究符合發展中草藥醫學研究，由於洛神花在台灣甚易栽植，因此本研究亦符合經濟發展，對國人健康及經濟效應均有極大的意義，未來或許可進一步進入中草藥臨床試驗而發展為新藥。

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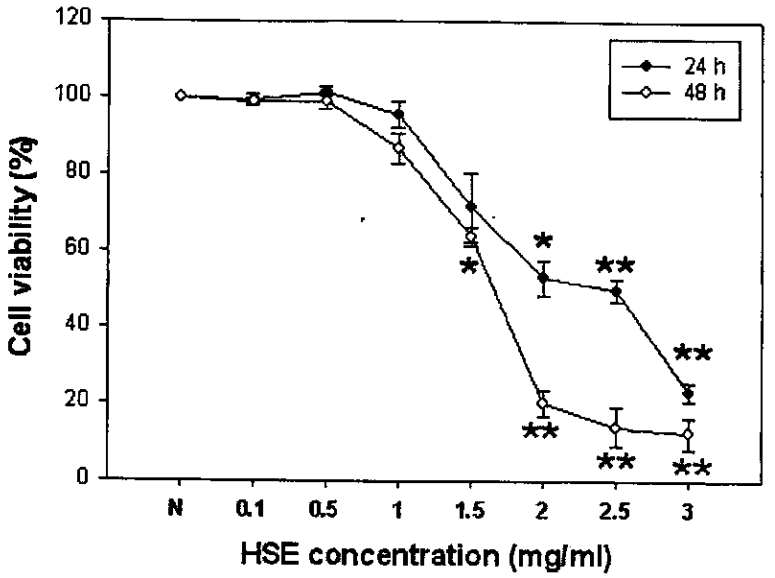

120
100
80
60
40
20
0
Cell viability (%)
24 h
48 h
*
**
N
0.1
0.5
1
1.5
2
2.5
3
HSE concentration (mg/ml)

Fig. 1.

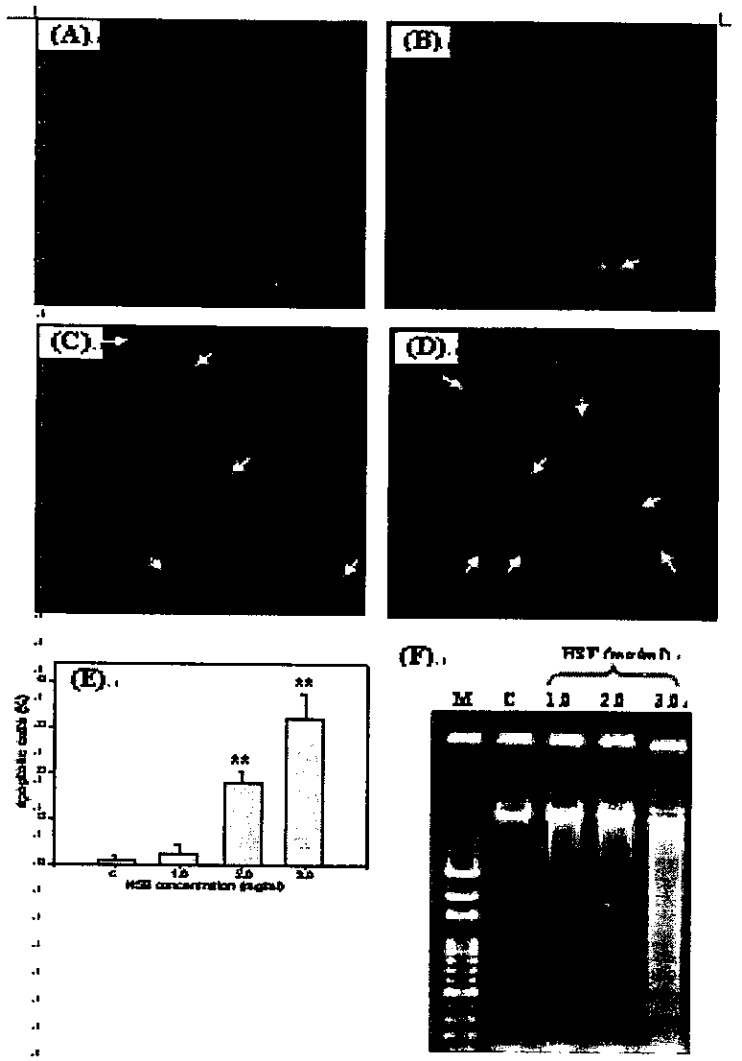

(A)
(B)
(C)
(D)
(E)
(F)
M
C

Fig. 2.

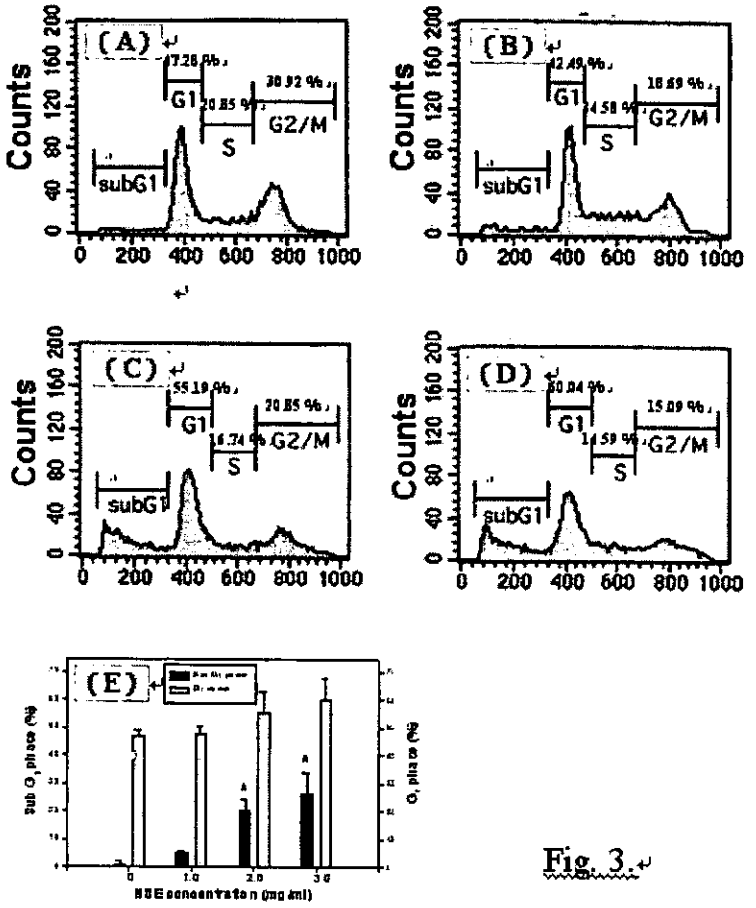

(A)
Counts
G1
S
G2/M
subG1
(B)
(C)
(D)
(E)

Fig. 3.

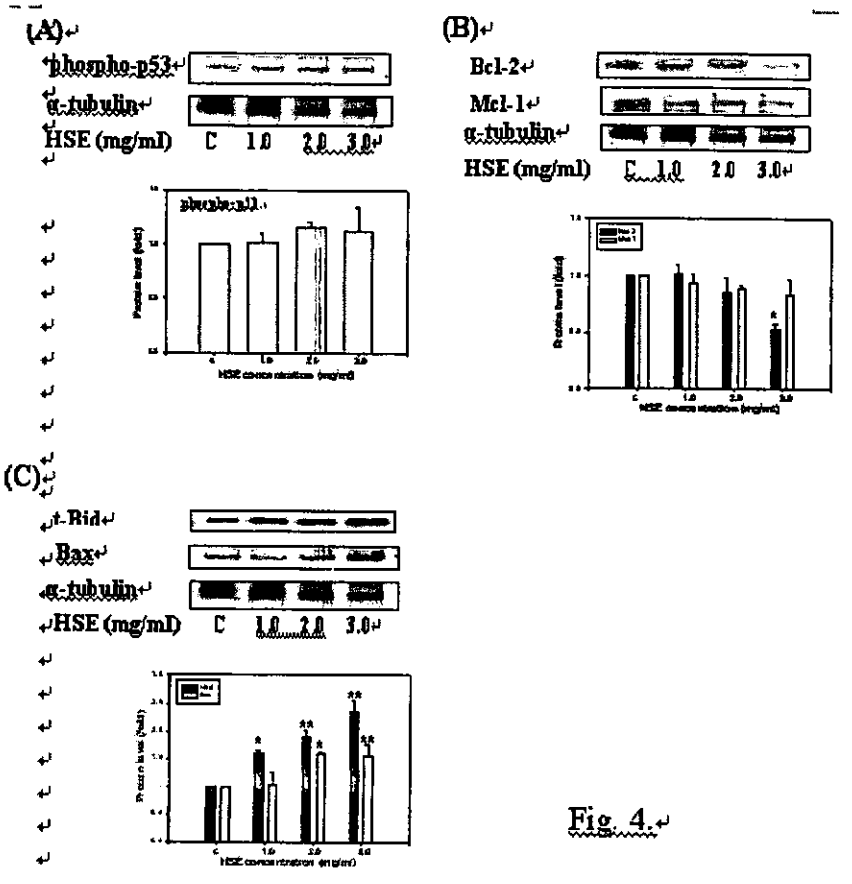

(A)
phospho-p53
α-tubulin
HSE (mg/ml) C 1.0 2.0 3.0
(B)
Bcl-2
Mcl-1
α-tubulin
HSE (mg/ml) C 1.0 2.0 3.0
(C)
t-Bid
Bax
α-tubulin
HSE (mg/ml) C 1.0 2.0 3.0

Fig. 4.

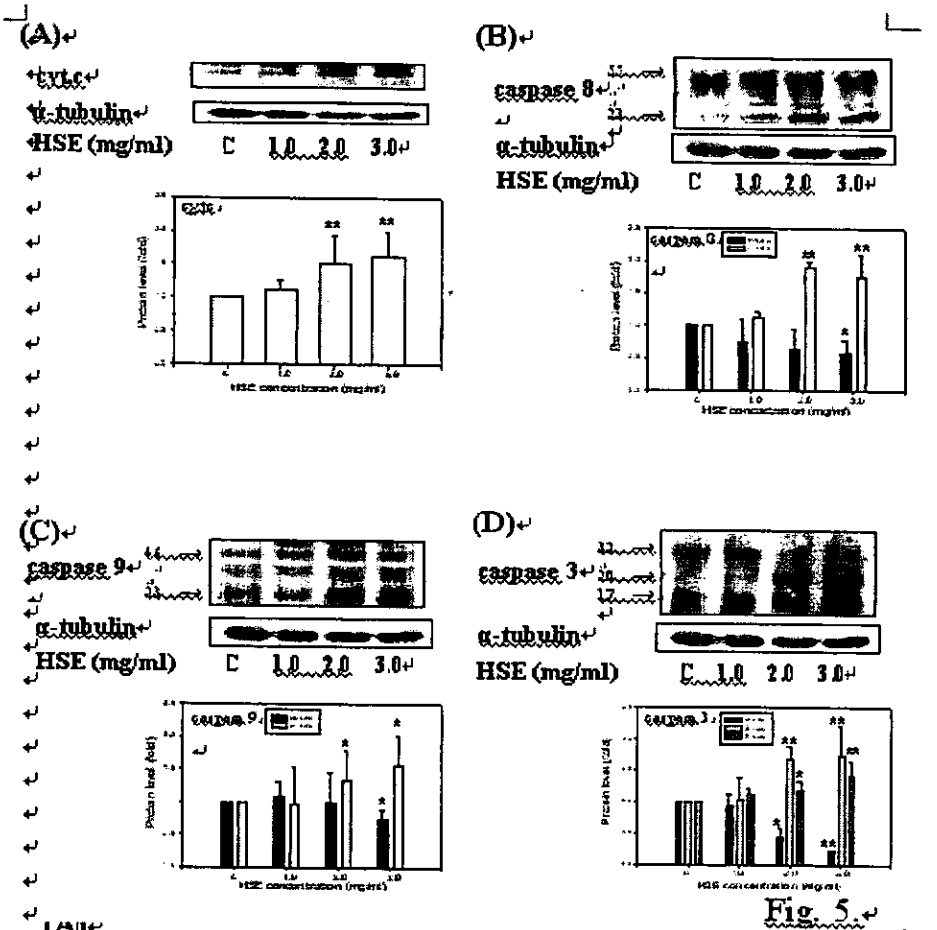

(A)
cyt.c
α-tubulin
HSE (mg/ml)
C 1.0 2.0 3.0
(B)
caspase 8
α-tubulin
HSE (mg/ml)
C 1.0 2.0 3.0
(C)
caspase 9
α-tubulin
HSE (mg/ml)
C 1.0 2.0 3.0
(D)
caspase 3
α-tubulin
HSE (mg/ml)
C 1.0 2.0 3.0

Fig. 5.

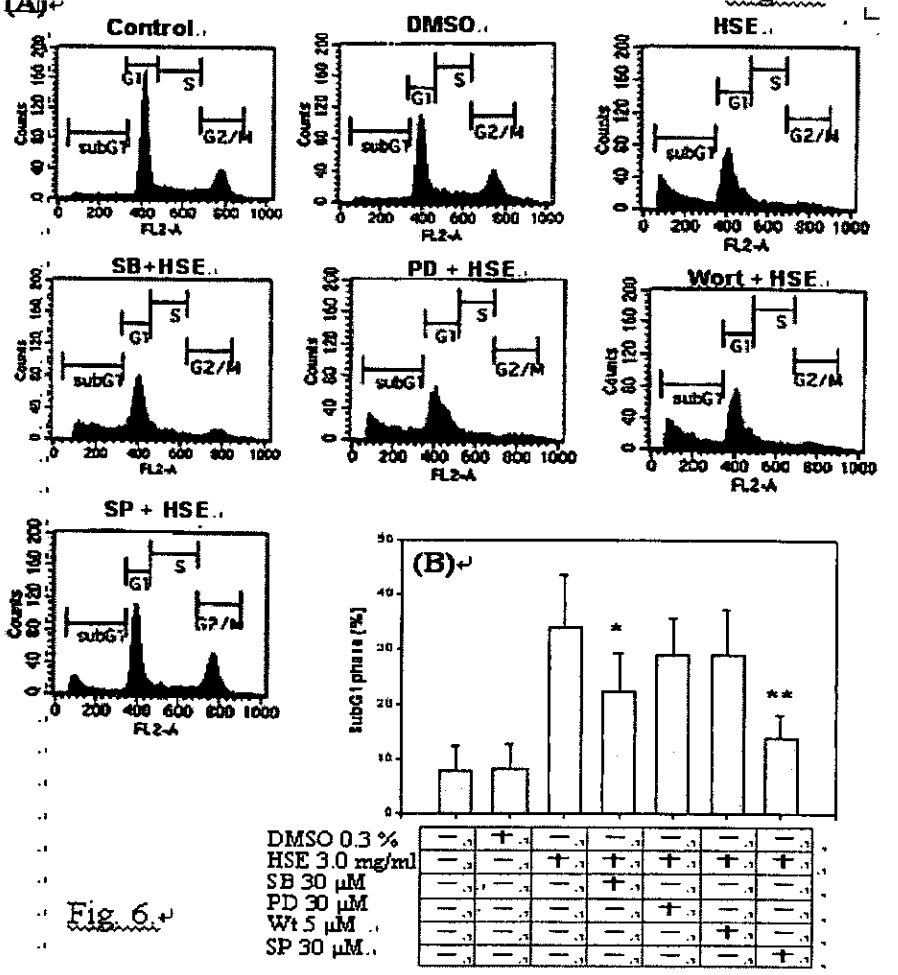

(A)
Control
DMSO
HSE
SB+HSE
PD + HSE
Wort + HSE
SP + HSE
subG1
G1
S
G2/M
Counts
FL2-A
(B)
subG1 phase (%)
DMSO 0.3 %
HSE 3.0 mg/ml
SB 30 μM
PD 30 μM
Wt 5 μM
SP 30 μM

Fig. 6.

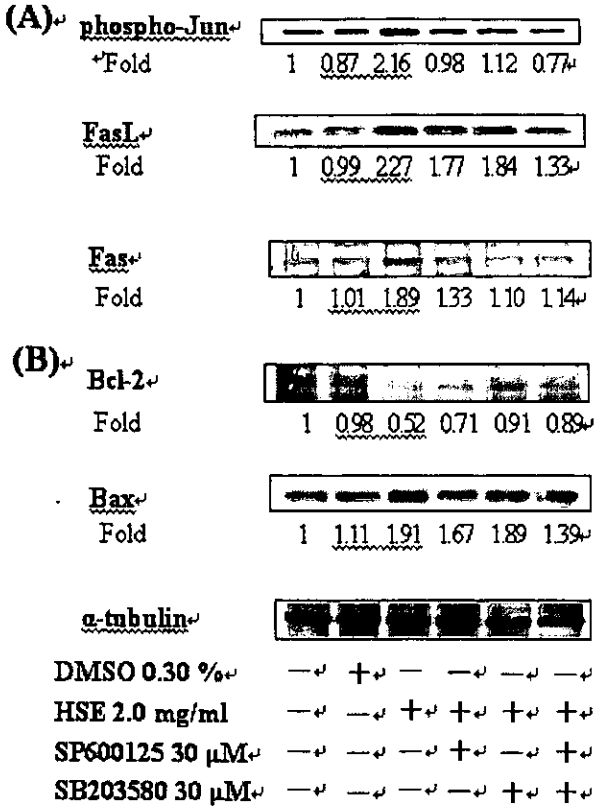

(A)
phospho-Jun
Fold
1 0.87 2.16 0.98 1.12 0.77
FasL
Fold
1 0.99 2.27 1.77 1.84 1.33
Fas
Fold
1 1.01 1.89 1.33 1.10 1.14
(B)
Bcl-2
Fold
1 0.98 0.52 0.71 0.91 0.89
Bax
Fold
1 1.11 1.91 1.67 1.89 1.39
α-tubulin
DMSO 0.30 %
HSE 2.0 mg/ml
SP600125 30 μM
SB203580 30 μM

Fig. 7.

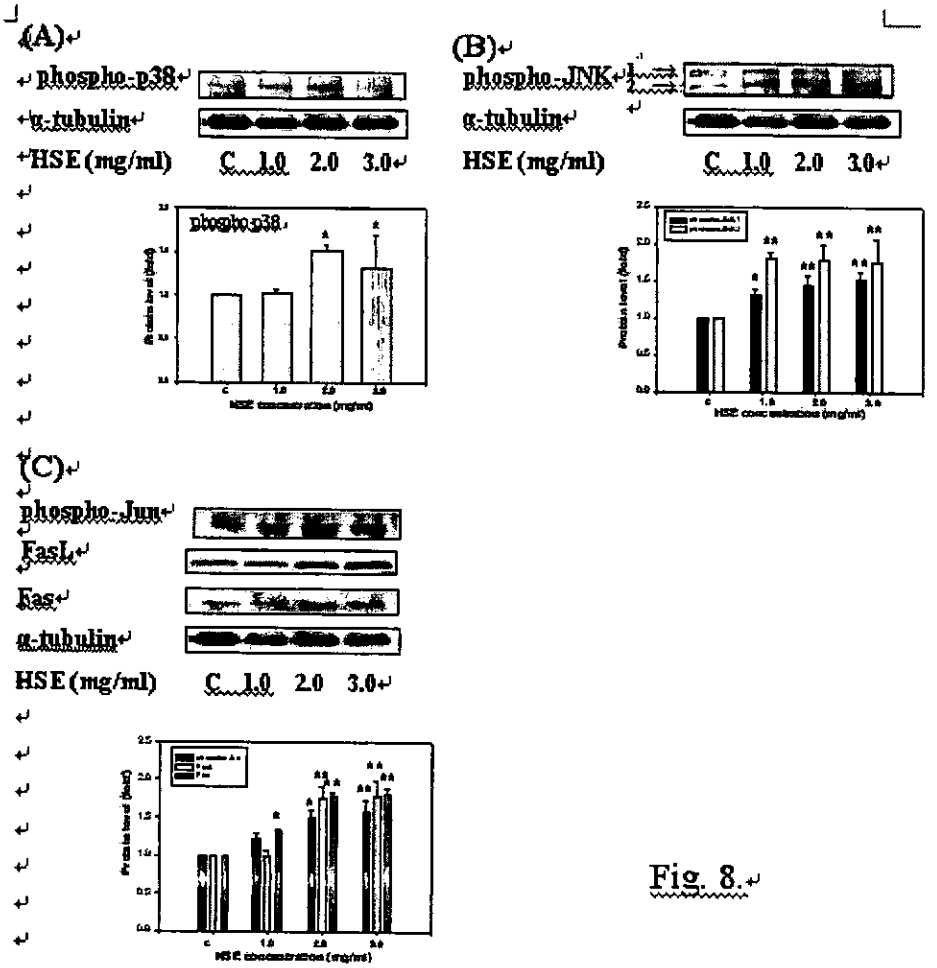

(A)
phospho-p38
α-tubulin
HSE (mg/ml) C 1.0 2.0 3.0
phospho-p38
(B)
phospho-JNK
α-tubulin
HSE (mg/ml) C 1.0 2.0 3.0
(C)
phospho-Jun
FasL
Fas
α-tubulin
HSE (mg/ml) C 1.0 2.0 3.0

Fig. 8.

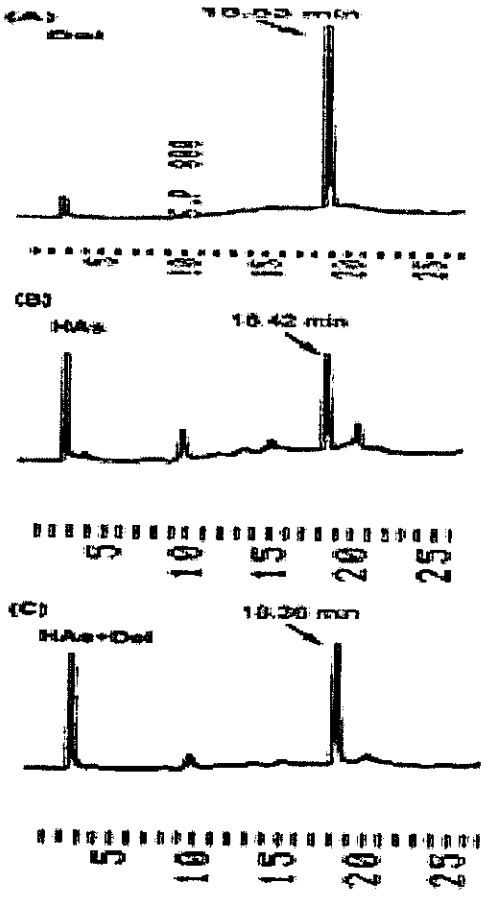
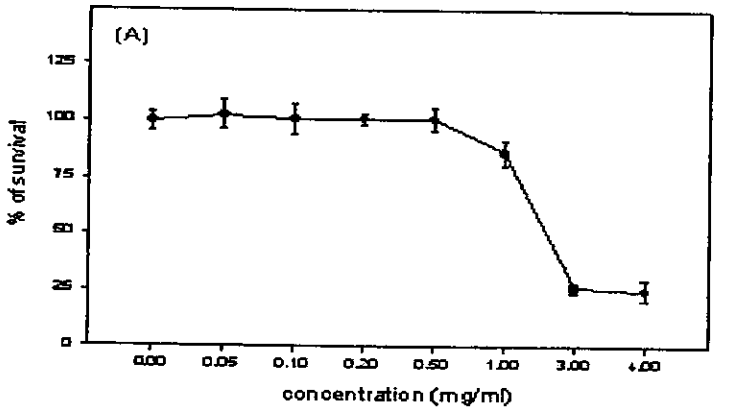

(B)
HAs
18.42 min
5
10
15
20
25
(C)
HAs+Del
18.20 min
5
10
15
20
25

Fig. 9


(A)
% of survival
0
25
50
75
100
125
0.00
0.05
0.10
0.20
0.50
1.00
3.00
4.00
concentration (mg/ml)

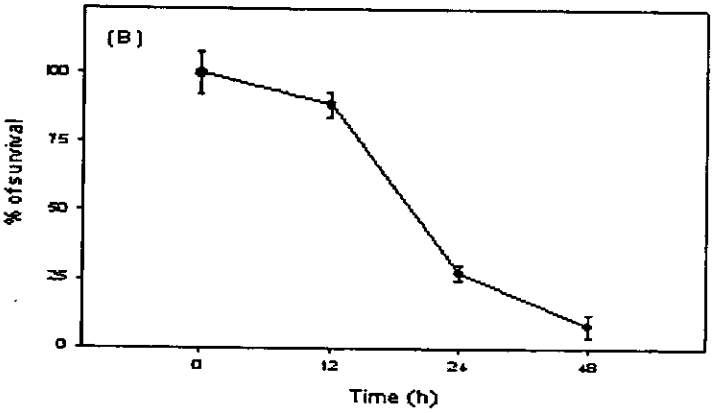

(B)
% of survival
0
25
50
75
100
0
12
24
48
Time (h)

Fig. 10

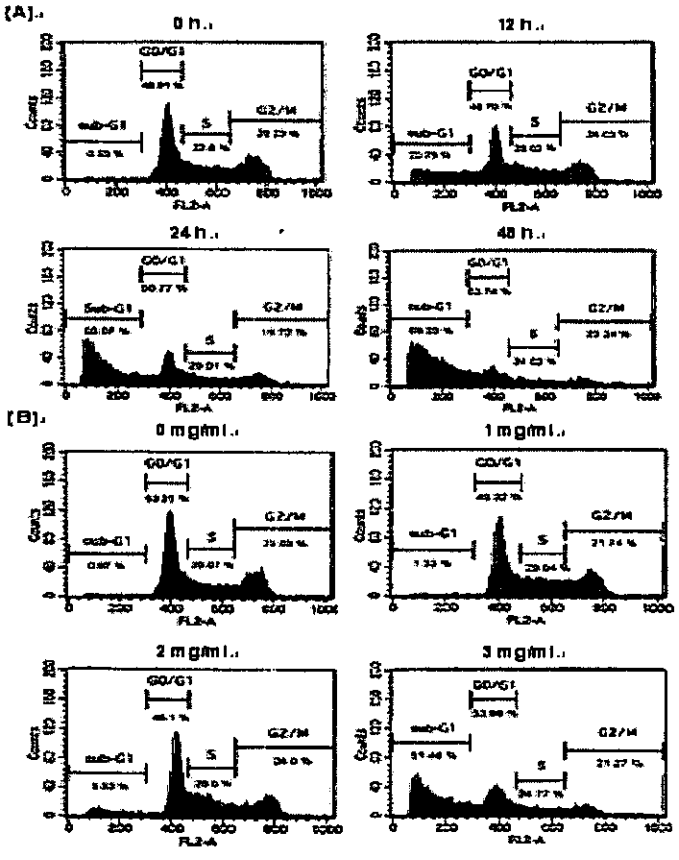

(A)
0 h
12 h
24 h
48 h
(B)
0 mg/ml
1 mg/ml
2 mg/ml
3 mg/ml

Fig. 11

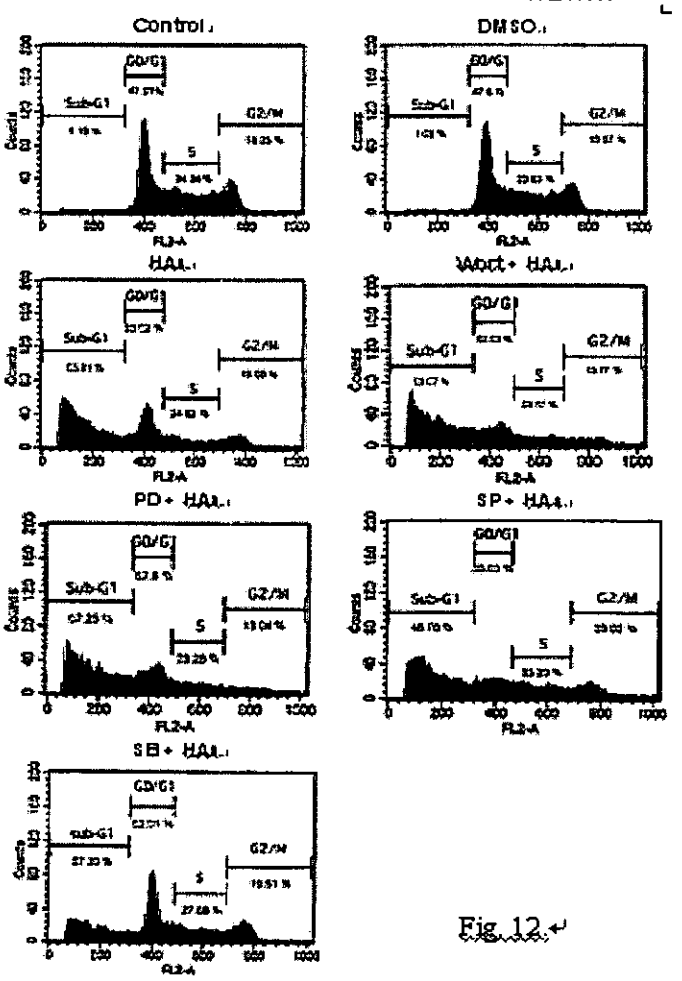
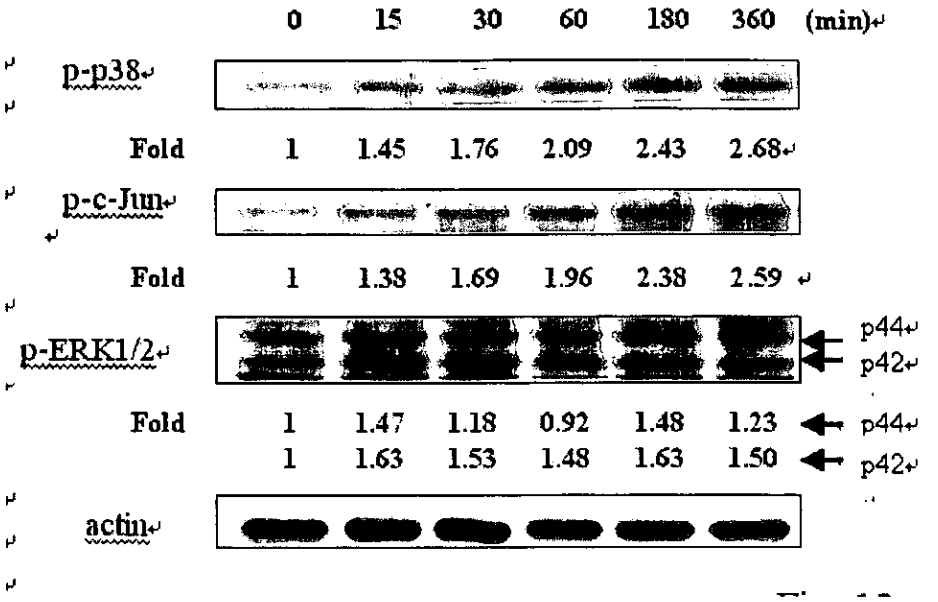
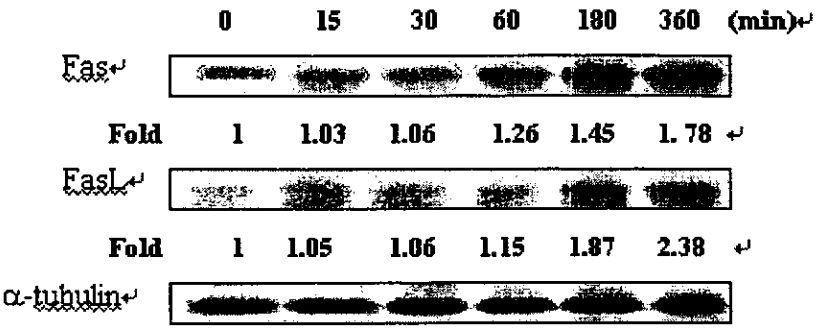

Control
DMSO
HAA
Wort + HAA
PD + HAA
SP + HAA
SB + HAA

Fig. 12


0 15 30 60 180 360 (min)
p-p38
Fold 1 1.45 1.76 2.09 2.43 2.68
p-c-Jun
Fold 1 1.38 1.69 1.96 2.38 2.59
p-ERK1/2
p44
p42
Fold 1 1.47 1.18 0.92 1.48 1.23 p44
1 1.63 1.53 1.48 1.63 1.50 p42
actin

(A)


0 15 30 60 180 360 (min)
Fas
Fold 1 1.03 1.06 1.26 1.45 1.78
FasL
Fold 1 1.05 1.06 1.15 1.87 2.38
α-tubulin

(B)

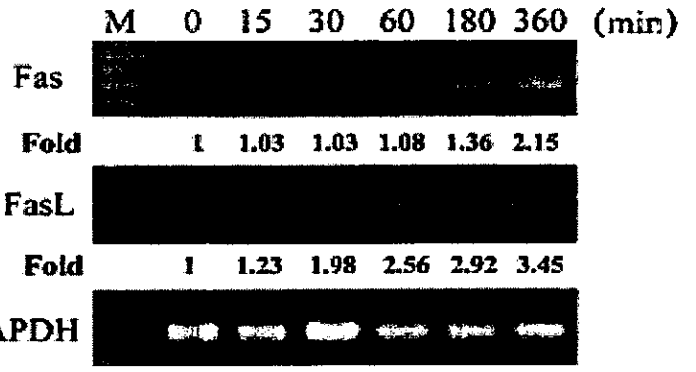

M 0 15 30 60 180 360 (min)
Fas
Fold 1 1.03 1.03 1.08 1.36 2.15
FasL
Fold 1 1.23 1.98 2.56 2.92 3.45
GAPDH

Fig. 14.

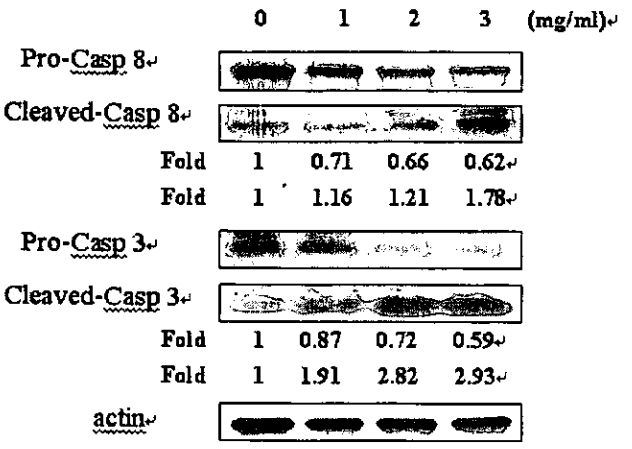

0 1 2 3 (mg/ml)
Pro-Casp 8
Cleaved-Casp 8
Fold 1 0.71 0.66 0.62
Fold 1 1.16 1.21 1.78
Pro-Casp 3
Cleaved-Casp 3
Fold 1 0.87 0.72 0.59
Fold 1 1.91 2.82 2.93
actin

Fig. 15.

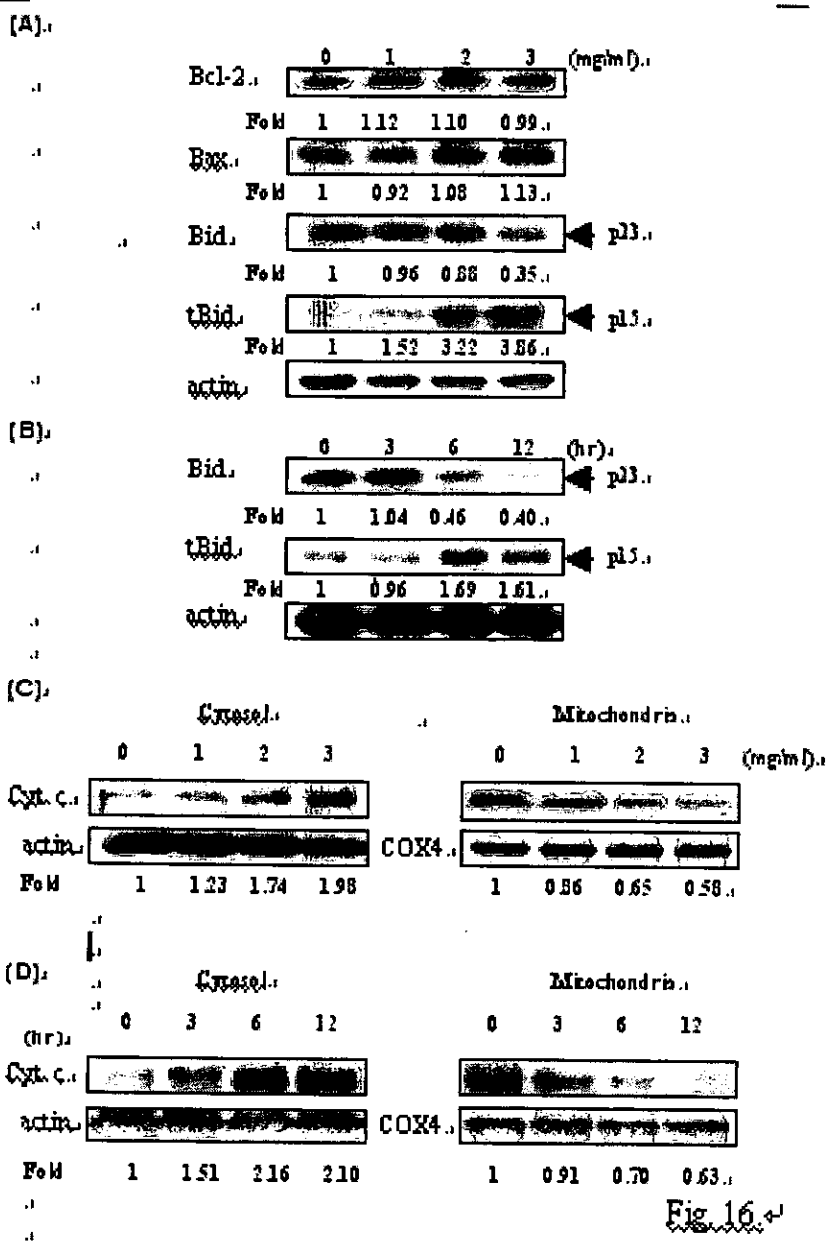

(A)
0 1 2 3 (mg/ml)
Bcl-2
Fold 1 1.12 1.10 0.99
Bax
Fold 1 0.92 1.08 1.13
Bid
p13
Fold 1 0.96 0.88 0.35
tBid
p15
Fold 1 1.53 3.22 3.86
actin
(B)
0 3 6 12 (hr)
Bid
p13
Fold 1 1.04 0.46 0.40
tBid
p15
Fold 1 0.96 1.69 1.61
actin
(C)
Cytosol
Mitochondria
0 1 2 3
0 1 2 3 (mg/ml)
Cyt. c
actin
COX4
Fold 1 1.23 1.74 1.98
1 0.86 0.65 0.58
(D)
Cytosol
Mitochondria
(hr)
0 3 6 12
0 3 6 12
Cyt. c
actin
COX4
Fold 1 1.51 2.16 2.10
1 0.91 0.70 0.63

Fig. 16.

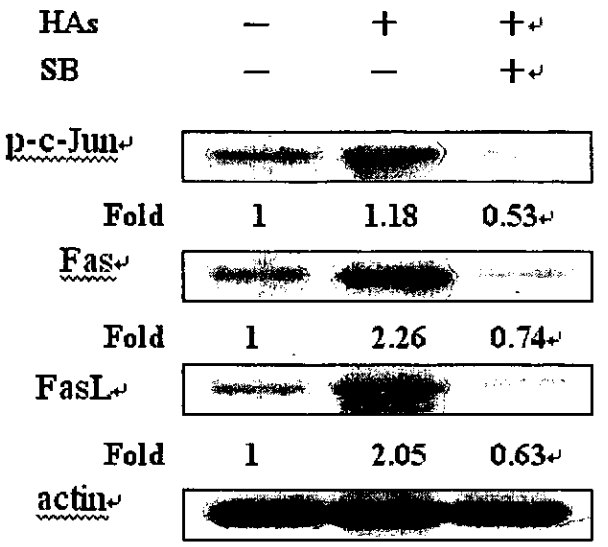
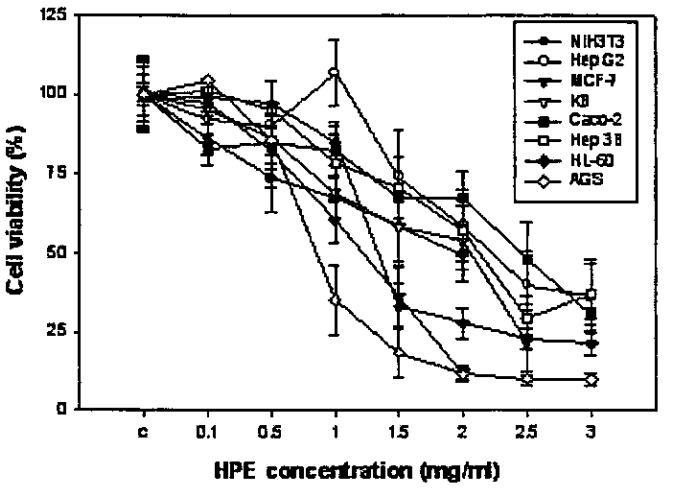


Fig. 17.



Cell line	IC_{50} (mg/ml)
NIH3T3	2.98
Hep G2	2.72
KB	2.56
Caco-2	2.46
Hep 3B	2.06
MCF-7	1.52
HL-60	1.46
AGS	0.95

Fig. 18.

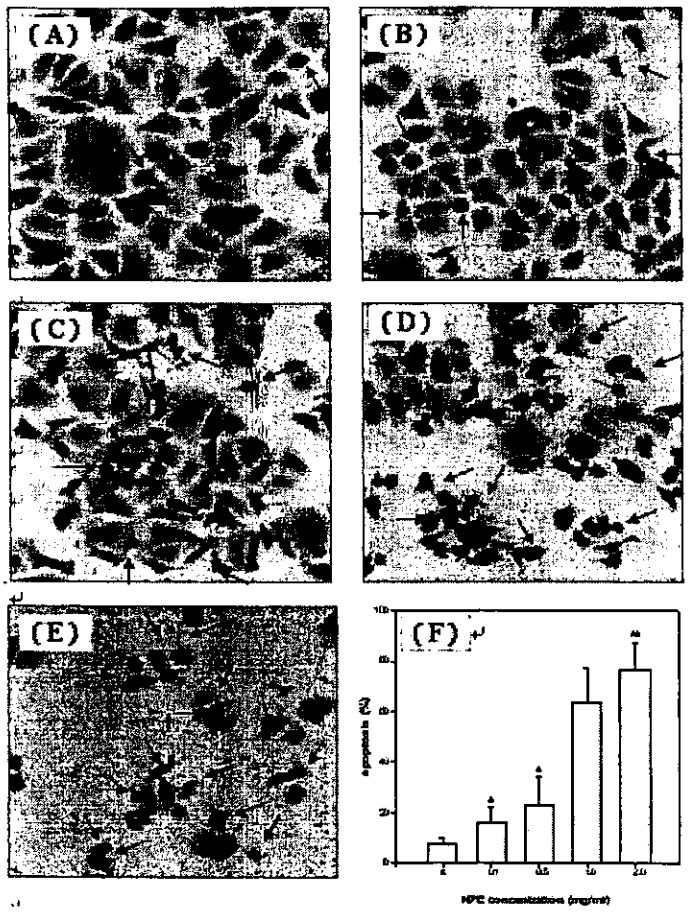


Fig. 19

Fig. 20

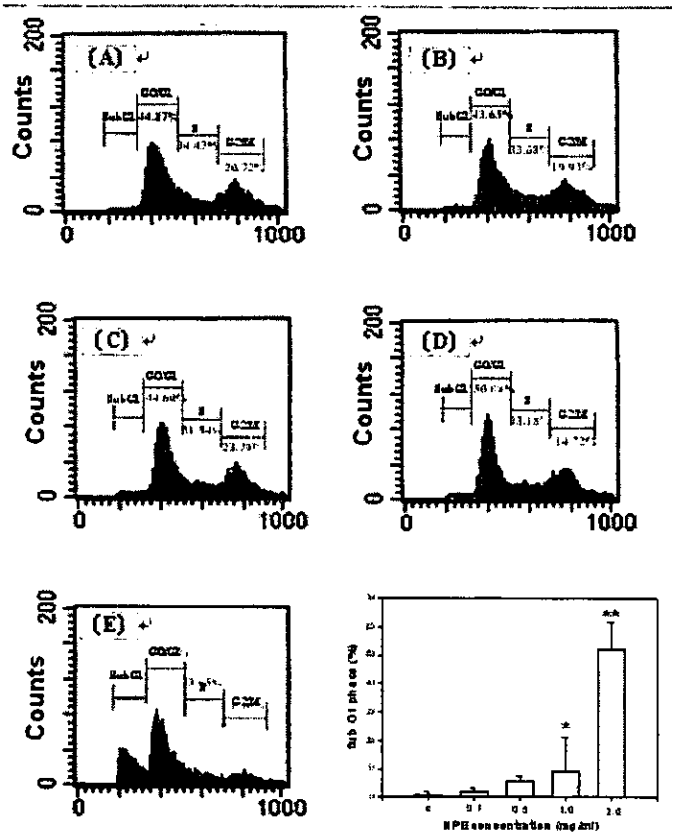

(A)
(B)
(C)
(D)
(E)
Counts
0
200
1000

Fig. 21

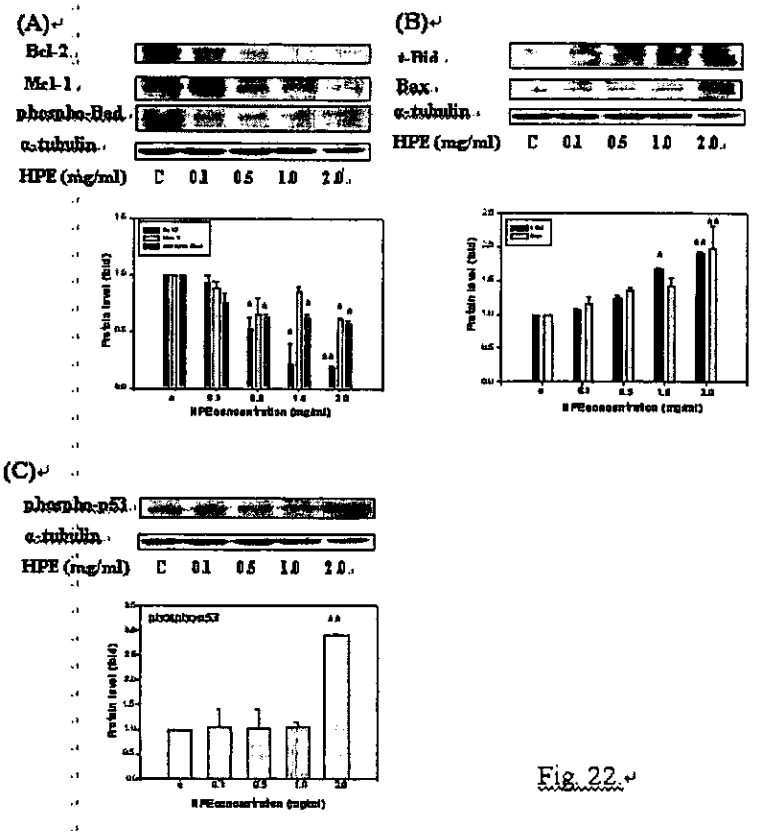

(A)
Bcl-2
Mcl-1
phospho-Bad
α-tubulin
HPE (mg/ml) C 0.1 0.5 1.0 2.0
(B)
t-Bid
Bax
α-tubulin
HPE (mg/ml) C 0.1 0.5 1.0 2.0
(C)
phospho-p53
α-tubulin
HPE (mg/ml) C 0.1 0.5 1.0 2.0

Fig. 22

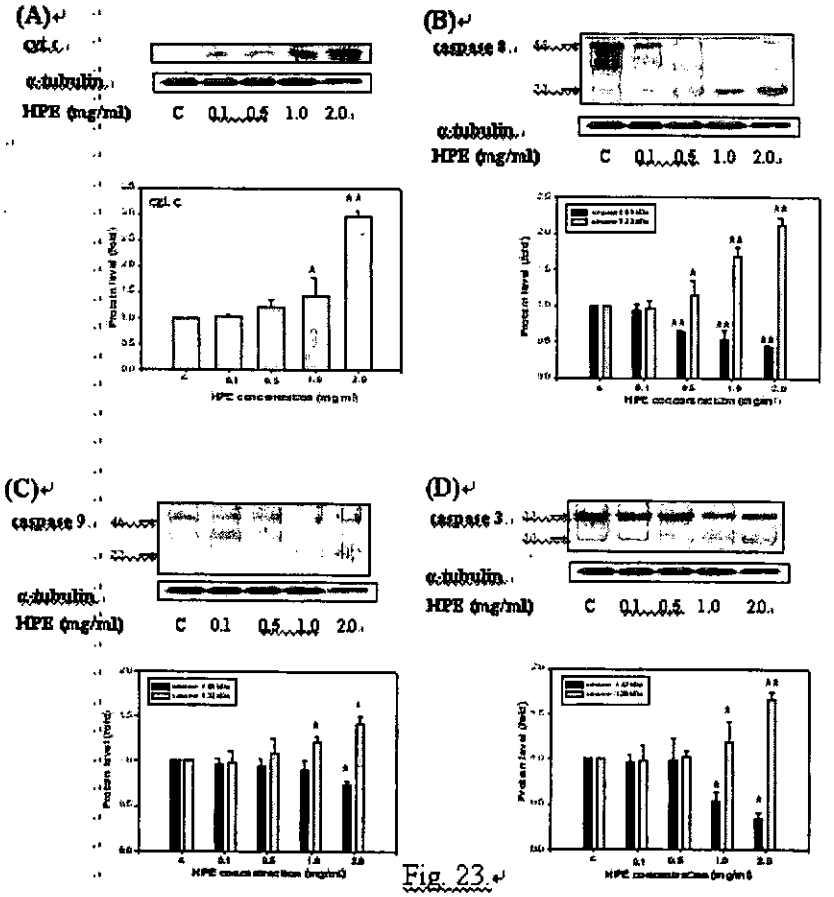
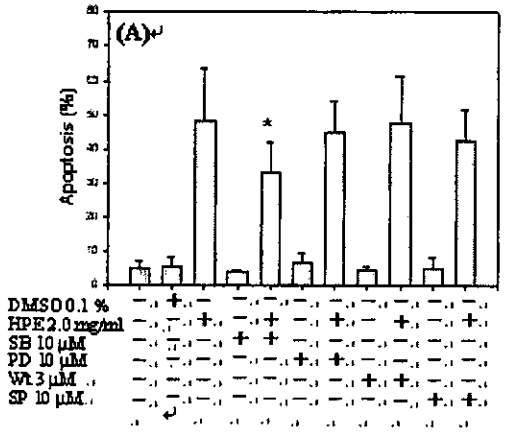

(A)
cyt.c
α-tubulin
HPE (mg/ml) C 0.1 0.5 1.0 2.0
(B)
caspase 8
α-tubulin
HPE (mg/ml) C 0.1 0.5 1.0 2.0
(C)
caspase 9
α-tubulin
HPE (mg/ml) C 0.1 0.5 1.0 2.0
(D)
caspase 3
α-tubulin
HPE (mg/ml) C 0.1 0.5 1.0 2.0

Fig. 23.


(A)
Apoptosis (%)
DMSO 0.1 %
HPE 2.0 mg/ml
SB 10 μM
PD 10 μM
Wt 3 μM
SP 10 μM

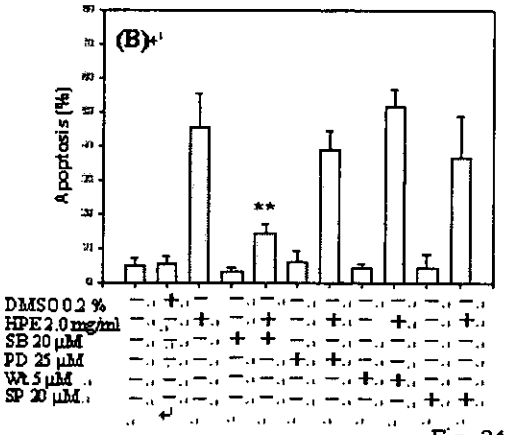
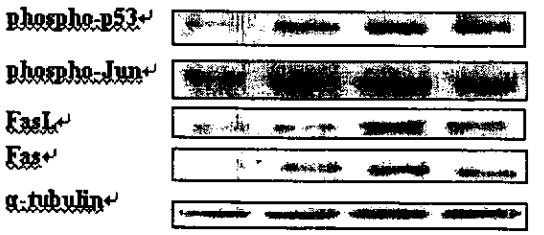

(B)
Apoptosis (%)
DMSO 0.2 %
HPE 2.0 mg/ml
SB 20 μM
PD 25 μM
Wt 5 μM
SP 20 μM

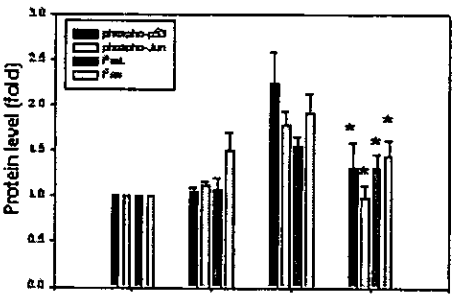
Fig. 24.

(A)



HPE 2.0 mg/ml	−	−	+	+
DMSO 0.2 %	−	+	−	−
SB203580 20 μM	−	−	−	+

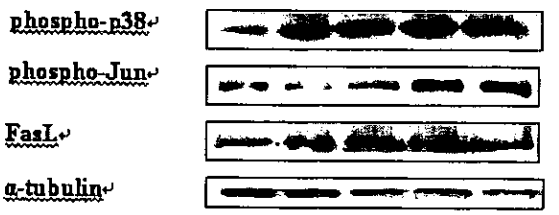
(B)



HPE 2.0 mg/ml	−	−	+	+
DMSO 0.2 %	−	+	−	−
SB203580 20 μM	−	−	−	+

Fig. 25

(A)



HPE (mg/ml)	C	0.1	0.5	1.0	2.0

(B)

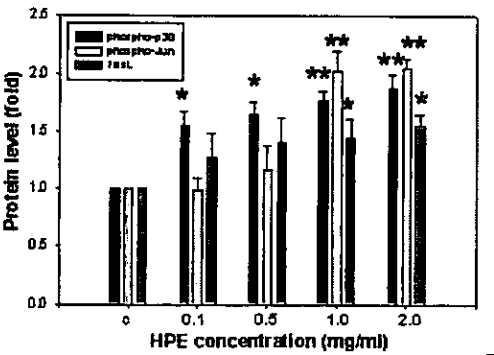


Fig. 26

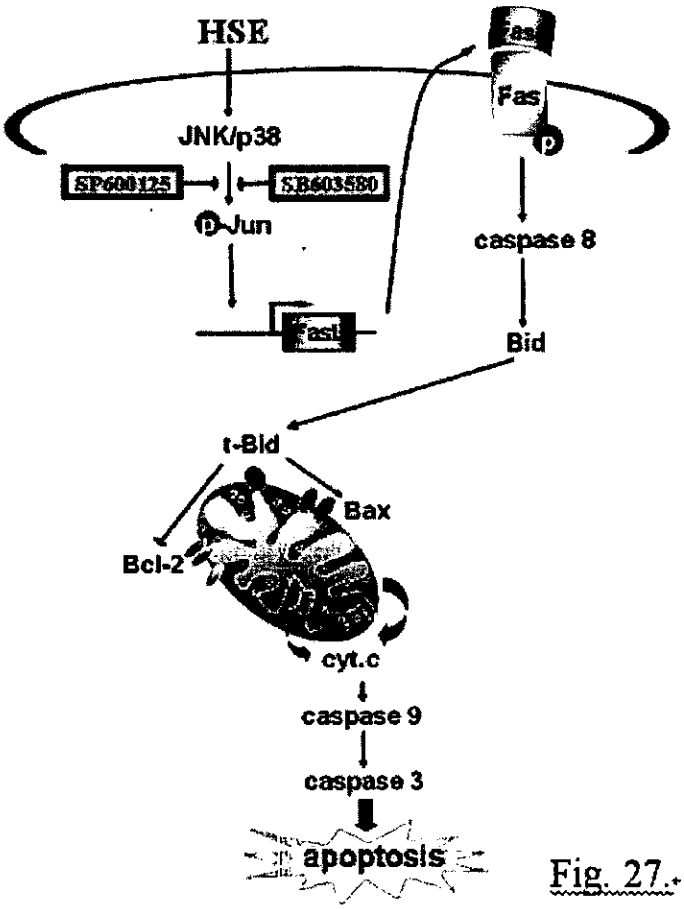

HSE
JNK/p38
SP600125
SB603580
Jun
Fas
caspase 8
Bid
t-Bid
Bax
Bcl-2
cyt.c
caspase 9
caspase 3
apoptosis

Fig. 27.

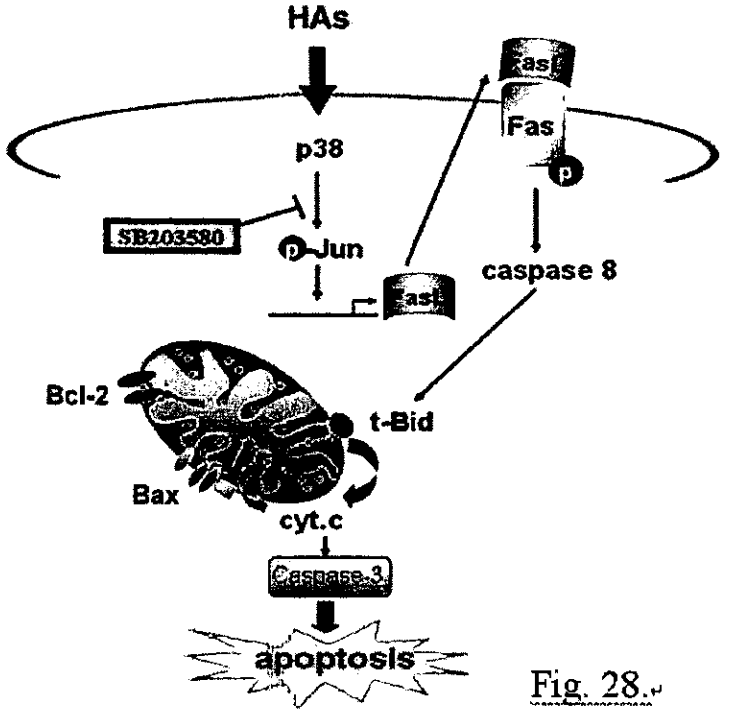
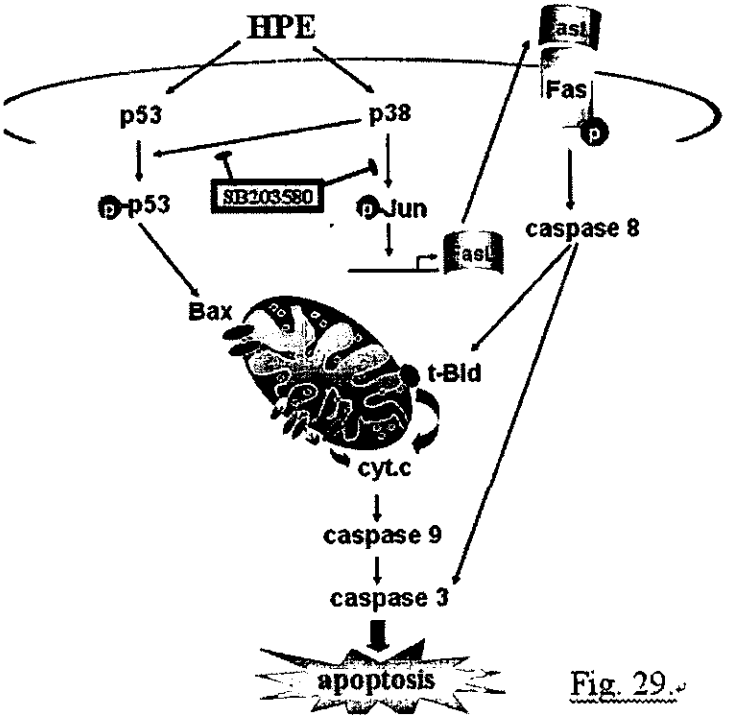

HAs
p38
SB203580
Jun
Fas
caspase 8
Bcl-2
t-Bid
Bax
cyt.c
Caspase 3
apoptosis

Fig. 28.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FasL
Fas
p53
p38
SB203580
p53
Jun
caspase 8
FasL
Bax
t-Bid
cyt.c
caspase 9
caspase 3
apoptosis

Fig. 29.

九十三年度衛生署科技研究計畫成果報告審查意見

計畫名稱	洛神花萃取物及其功能性成份抗癌作用之研究		
計畫編號	DOH-93-TD- F -113- 016	計畫主持人	王朝鐘
執行機構	中山醫學大學生化暨生物科技所		
計畫期程	■一年期計畫 ； □多年期計畫，共_____年，本年度為第____年		
請補充圖表之標題與說明			

Figure legends

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Figure 2. Morphology of AGS treatment with HSE. Cells were treated with or without HPE (A), 0 mg/ml; (B), 1.0 mg/ml; (C), 2.0 mg/ml and (D), 3.0 mg/ml for 24 h, the apoptotic cells were assayed by DAPI stain. The arrow indicated apoptotic cells. Apoptotic values were calculated as the percentage of apoptotic cells relative to the total number of cells in each random field (> 100 cells) (E) and represent the average of three independent experiments ± SD. *p<0.05, **p<0.005 compared with control. (F), Dose response of DNA fragment induced by HPE. AGS cells were treated with various concentrations of HSE for 24 h. After extraction, pure DNAs were electrophoresed in a 1.2% agarose gel, stained with GELSTAR GEL STAIN10000, and photographed under UV illumination. M (marker), C (control).

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Figure 7. Inhibitory effects of SB203580 and SP600125 treatment on the HSE-induced levels of (A), phospho-Jun, FasL, Fas and (B), Bcl-2, Bax in AGS cells. Cultured cells were treated with or without HSE 3.0 mg/ml in the absence or presence of DMSO 0.30%, SB203580 30 μM and SP600125 30 μM for 24 h. These proteins were detected by anti-phospho-Jun, -FasL, -Fas, -Bcl-2 and –Bax antibodies, and the results were represented by using an ECL system, and (B) represents the average of three independent experiments ± SD. *$p < 0.01$ compared with the HSE-treated group.

Figure 8. (A) Immunoblot analysis of the expression of phospho-p38, phospho-JNK and phospho-Jun in AGS cells treated with HSE. Cultured cells were treated with or without HSE 1.0, 2.0 and 3.0 mg/ml for 24 h. These proteins were detected by anti-phospho-p38, -phospho-JNK and -phospho-Jun antibodies, and the results were represented by using an ECL system, and (B) represent the average of three independent experiments ± SD. *$p < 0.05$, **$p < 0.01$ compared with the control.

Figure 9. Delphinidin content of HAs was analyzed by HPLC. (A) Standard is delphindin (0.1 mg/ml; 10 μl); (B) HAs extract (1 mg/ml; 10 μl); (C) HAs (1 mg/ml; 5 μl) + Delphinidin (0.1 mg/ml; 5 μl)

Figure 10. Induction of cell death by HAs. HL-60 cells (5×10^4 cells/well) were treated with various concentrations (0-4 mg/ml) of HAs for 24 h (A), or treated with 3 mg/ml of HAs for the indicated times (B). The survival cell number is directly proportional to formazan which was measured spectrophotometrically at 563 nm. The value are means ± SD, n=3.

Figure 11. The effects of HAs on HL-60 cell cycle distribution. HL-60 cells were treated with 3 mg/ml of HAs for indicated time (A), or with various concentrations (0-3 mg/ml) of HAs for 24 h (B). The DNA content was analyzed using fluorescence flow cytometry. The position of the sub-G_1 peak (hypodiploidy), integrated by

apoptotic cells, and the G_0/G_1 and G_2/M peaks are indicated. Quantitative assessment of the percentage of HL-60 cells in the sub-G_1 and G_0/G_1 phases was indicated by propidium iodide (PI). The experiment was repeated three times and the representative histograms are shown.

Figure 12. Induction of apoptosis by HAs and the effects of various MAP kinase inhibitors. HL-60 cells (1×10^6 cells/dish) were preincubated for 24 h with various MAP kinase inhibitors, including SB203580 (SB, 50 μM), SP600125 (SP, 20 μM), PD98059 (PD, 25 μM) and wortmannin (Wort, 1 μM), and then incubated for 24 h with HAs (3 mg/ml). DMSO (0.25 %) served as solvent control. Quantitative assessment of the percentage of HL-60 cells in sub-G_1 and G_0/G_1 phases was indicated by propidium iodide (PI). Quantitative analysis of apoptosis was determined by flow cytometery assay.

Figure 13. HAs activates MAP kinases in a time-dependent manner. HL-60 (1×10^6 cells/dish) was incubated with HAs (3 mg/ml) for various durations (0-360 min). Protein extracts were prepared at the incubated time points to assess the activation of MAP kinases. The levels of phosphorylated MAP kinases (p38, ERK1/2 and c-Jun) were determined by Western blotting using specific antibodies. Actin, load controls. This figure is a representative of three independent experiments with similar results.

Figure 14. Time course of HAs-induced Fas and FasL activation in HL-60 cells. HL-60 cells were treated with HAs (3 mg/ml) for the time indicated and assayed for Fas, FasL activation and mRNA expression. (A) Untreated control cells were run in parallel in the same gel. The proteins were analyzed using Western Blotting. α-Tubulin, load controls. (B) Total RNA was extracted at each time point, Fas and FasL mRNA expression were analyzed by RT-PCR. M, molecular weight marker. GAPDH, loading controls. This figure is a representative of three independent experiments with similar results.

Figure 15. Involvement of caspase-8 and caspase-3 in HAs-induced apoptosis. The levels of cleaved-caspase-8/-3 and pro-caspase-8/-3 proteins in HL-60 cells with HAs treatments were determined. Cells were treated with HAs (360 min) for the indicated concentration and analyzed using immunoblotting with anti-cleaced-caspase-8/-3 and pro-caspase-8/-3 antibody. Actin and α-tubulin, were the loading controls. This figure is a representative of three independent experiments with similar results.

Figure 16. Effect of HAs-induced Bid activation and cytochrome c release. (A)

HL-60 cells were treated with HAs (360 min) for the indicated concentration and analyzed by immunoblotting with anti-Bcl-2, Bax and Bid antibody. The arrows indicate the position of full-length Bid (p23) and the p15 truncated form of active Bid (tBid). Actin was the loading control. (B) HL-60 cells were treated with HAs (3 mg/ml) for the indicated times and analyzed by immunoblotting with anti-Bid antibody. (C) HL-60 cells were treated with HAs (360 min) for the indicated concentration, and the expression of cytochrome c in the cytosol and the mitochondria of the untreated and HAs-treated HL-60 cells was assayed by immunoblotting. (D) HL-60 cells were treated with HAs (3 mg/ml) for the indicated times, and the expression of cytochrome c, cytochrome oxidase subunit Ⅳ(COX4), a mitochondrial marker served as a protein loading control, was determined. This figure is a representative of three independent experiments with similar results.

Figure 17. Effect of p38 MAPK inhibitor (SB203580) on HAs-induced apoptosis in HL-60 cells. Cultured cells were treated in the absence or presence of SB203580 (50 μM, 24 h preincubation) with HAs (3 mg/ml) for 6 h, and phospho-c-Jun, Fas and FasL expression was analyzed by Western Blotting. Actin was used as the loading control. This figure is a representative of three independent experiments with similar results.

Figure 18. (A) Cell viability of various cells (NIH3T3, Hep G2, MCF-7, KB, Caco-2, Hep 3B, HL-60 and AGS cells) treated with HPE. Cultured cells were treated with or without HPE under different concentrations for 24 h. Cell viability was analyzed by MTT assay. The results are expressed as mean ± SD, n=3. (B) Summary of IC_{50} in various cells treated with HPE.

Figure 19. Morphology of AGS treatment with HPE. Cells were treated with or without HPE (A), 0.1 mg/ml (B), 0.5 mg/ml (C), 1.0 mg/ml (D), 2.0 mg/ml (E) for 24 h, the apoptotic cells were stained by Leukostat solutions. The arrow indicated apoptotic cells. Apoptotic values were calculated as the percentage of apoptotic cells relative to the total number of cells in each random field (>100 cells) (F) and represent the average of three independent experiments ± SD. $^{*}p<0.05$, $^{**}p<0.005$ compared with control.

Figure 20. Dose response of DNA fragment induced by HPE. AGS cells were treated with various concentrations of HPE for 24 h. After extraction, pure DNAs were electrophoresed in a 1.2% agarose gel, stained with GELSTAR GEL STAIN10000, and photographed under UV illumination. M (marker), C (control).

Figure 21. Effect of HPE induced apoptosis and cell cycle progress in AGS cells. Cultured cells were treated with or without HPE (A), 0 mg/ml; (B), 0.1 mg/ml; (C), 0.5 mg/ml; (D), 1.0 mg/ml and (E), 2.0 mg/ml. Twenty four hours later, cell cycle was analyzed by flow cytometry. (F), Quantitative assessment of the percentage of AGS cells in Sub G1 phase, as indicated by propidium iodide (PI), and represents the average of three independent experiments ± SD. *p< 0.05, **p< 0.01 compared with the control.

Figure 22. Immunoblot analysis of the expression of Bcl-2 family proteins (Bcl-2, Mcl-1, phospho-Bad, t-Bid and Bax) and phospho-p53 in AGS cells treated with HPE. Cultured cells were treated with or without HPE 0.1, 0.5, 1.0 and 2.0 mg/ml for 24 h. These proteins were detected by -Bcl-2 family proteins and anti-phospho-p53 antibodies, and the results were represented by using an ECL system, and (A)-(C) represent the average of three independent experiments ± SD. *p< 0.05, **p< 0.01 compared with the control.

Figure 23. Immunoblot analysis of the expression of cytochrome c and caspases (caspase 8, caspase 9 and caspase 3) in AGS cells treated with HPE. Cultured cells were treated with or without HPE 0.1, 0.5, 1.0 and 2.0 mg/ml for 24 h. These proteins were detected by anti-cyt. c and -caspases antibodies, and the results were represented by using an ECL system, and (A)-(D) represent the average of three independent experiments ± SD. *p< 0.05, **p< 0.01 compared with the control.

Figure 24. Effect of inhibitors blocking HPE-induced apoptosis in AGS cells. Cultured cells were treated with or without HPE 2.0 mg/ml in the absence or presence of low (A) and high (B) concentrations of SB203580 (SB), PD98059 (PD), wortmannin (Wt) or SP600125 (SP) for 24 h, Apoptosis was analyzed by flow cytometry. Quantitative assessment of the percentage of AGS cells in Sub G1 phase, as indicated by propidium iodide (PI), and represents the average of three independent experiments ± SD. *p< 0.05, **p< 0.01 compared with HPE- treated group.

Figure 25. (A) Inhibitory effect of SB203580 treatment on the HPE-induced levels of phospho-p53, phospho-Jun, FasL and Fas in AGS cells. Cultured cells were treated with or without HPE 2.0 mg/ml in the absence or presence of DMSO 0.20% and SB203580 20 μM for 24 h. These proteins were detected by anti-phospho-p53, -phospho-Jun, -FasL and -Fas antibodies, and the results were represented by using an ECL system, and (B) represents the average of three independent experiments ± SD.

*p< 0.01 compared with the HPE-treated group.

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Figure 27. Model showing pathways that mediate HSE-induced apoptosis in AGS cells. HSE were shown to be capable of inducing AGS cells apoptosis through the mechanisms, activation of p38/JNK signaling activation involves mitochondrial membrane alterations resulting in the release of cytochrome c and caspases activation.

Figure 28. Model showing pathways that mediate HAs-induced apoptosis in HL-60 cells. HAs were been shown to be capable of inducing HL-60 cell apoptosis. p38 signaling activation involves mitochondrial membrane alterations resulting in the release of cytochrome c and caspases activation. See the text for discussion.

Figure 29. Proposed model of HPE-induced signaling pathways leading to apoptosis. HPE was shown to be capable of inducing AGS cell apoptosis through two mechanisms, one was the activation of p38 signaling, which resulted in phosphorylation of c-Jun, activation of FasL and triggered Fas-mediated signaling (Fas/caspase 8/t-Bid signaling module); and the other was the cell apoptosis stimulated by the stabilization of p53, increase in Bax, release of cytochrome c, thus leading to caspase 3 being cleaved.

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Figure 17. Effect of p38 MAPK inhibitor (SB203580) on HAs-induced apoptosis in HL-60 cells. Cultured cells were treated in the absence or presence of SB203580 (50 μM, 24 h preincubation) with HAs (3 mg/ml) for 6 h, and phospho-c-Jun, Fas and FasL expression was analyzed by Western Blotting. Actin was used as the loading control. This figure is a representative of three independent experiments with similar results.

Figure 18. (A) Cell viability of various cells (NIH3T3, Hep G2, MCF-7, KB, Caco-2, Hep 3B, HL-60 and AGS cells) treated with HPE. Cultured cells were treated with or without HPE under different concentrations for 24 h. Cell viability was analyzed by MTT assay. The results are expressed as mean ± SD, n=3. (B) Summary of IC_{50} in various cells treated with HPE.

Figure 19. Morphology of AGS treatment with HPE. Cells were treated with or without HPE (A), 0.1 mg/ml (B), 0.5 mg/ml (C), 1.0 mg/ml (D), 2.0 mg/ml (E) for 24 h, the apoptotic cells were stained by Leukostat solutions. The arrow indicated apoptotic cells. Apoptotic values were calculated as the percentage of apoptotic cells relative to the total number of cells in each random field (>100 cells) (F) and represent the average of three independent experiments ± SD. *$p<0.05$, **$p<0.005$ compared with control.

Figure 20. Dose response of DNA fragment induced by HPE. AGS cells were treated with various concentrations of HPE for 24 h. After extraction, pure DNAs were electrophoresed in a 1.2% agarose gel, stained with GELSTAR GEL STAIN10000, and photographed under UV illumination. M (marker), C (control).

Figure 21. Effect of HPE induced apoptosis and cell cycle progress in AGS cells. Cultured cells were treated with or without HPE (A), 0 mg/ml; (B), 0.1 mg/ml; (C), 0.5 mg/ml; (D), 1.0 mg/ml and (E), 2.0 mg/ml. Twenty four hours later, cell cycle was analyzed by flow cytometry. (F), Quantitative assessment of the percentage of AGS cells in Sub G1 phase, as indicated by propidium iodide (PI), and represents the average of three independent experiments ± SD. *p< 0.05, **p< 0.01 compared with the control.

Figure 22. Immunoblot analysis of the expression of Bcl-2 family proteins (Bcl-2, Mcl-1, phospho-Bad, t-Bid and Bax) and phospho-p53 in AGS cells treated with HPE. Cultured cells were treated with or without HPE 0.1, 0.5, 1.0 and 2.0 mg/ml for 24 h. These proteins were detected by -Bcl-2 family proteins and anti-phospho-p53 antibodies, and the results were represented by using an ECL system, and (A)-(C) represent the average of three independent experiments ± SD. *p< 0.05, **p< 0.01 compared with the control.

Figure 23. Immunoblot analysis of the expression of cytochrome c and caspases (caspase 8, caspase 9 and caspase 3) in AGS cells treated with HPE. Cultured cells were treated with or without HPE 0.1, 0.5, 1.0 and 2.0 mg/ml for 24 h. These proteins were detected by anti-cyt. c and -caspases antibodies, and the results were represented by using an ECL system, and (A)-(D) represent the average of three independent experiments ± SD. *p< 0.05, **p< 0.01 compared with the control.

Figure 24. Effect of inhibitors blocking HPE-induced apoptosis in AGS cells. Cultured cells were treated with or without HPE 2.0 mg/ml in the absence or presence of low (A) and high (B) concentrations of SB203580 (SB), PD98059 (PD), wortmannin (Wt) or SP600125 (SP) for 24 h, Apoptosis was analyzed by flow cytometry. Quantitative assessment of the percentage of AGS cells in Sub G1 phase, as indicated by propidium iodide (PI), and represents the average of three independent experiments ± SD. *p< 0.05, **p< 0.01 compared with HPE- treated group.

Figure 25. (A) Inhibitory effect of SB203580 treatment on the HPE-induced levels of phospho-p53, phospho-Jun, FasL and Fas in AGS cells. Cultured cells were treated with or without HPE 2.0 mg/ml in the absence or presence of DMSO 0.20% and SB203580 20 μM for 24 h. These proteins were detected by anti-phospho-p53, -phospho-Jun, -FasL and -Fas antibodies, and the results were represented by using an ECL system, and (B) represents the average of three independent experiments ± SD.

*p< 0.01 compared with the HPE-treated group.

Figure 26. (A) Immunoblot analysis of the expression of phospho-p38, phospho-Jun and FasL in AGS cells treated with HPE. Cultured cells were treated with or without HPE 0.1, 0.5, 1.0 and 2.0 mg/ml for 24 h. These proteins were detected by anti-phospho-p38, -phospho-Jun and -FasL antibodies, and the results were represented by using an ECL system, and (B) represent the average of three independent experiments ± SD. *p< 0.05, **p< 0.01 compared with the control.

Figure 27. Model showing pathways that mediate HSE-induced apoptosis in AGS cells. HSE were shown to be capable of inducing AGS cells apoptosis through the mechanisms, activation of p38/JNK signaling activation involves mitochondrial membrane alterations resulting in the release of cytochrome c and caspases activation.

Figure 28. Model showing pathways that mediate HAs-induced apoptosis in HL-60 cells. HAs were been shown to be capable of inducing HL-60 cell apoptosis. p38 signaling activation involves mitochondrial membrane alterations resulting in the release of cytochrome c and caspases activation. See the text for discussion.

Figure 29. Proposed model of HPE-induced signaling pathways leading to apoptosis. HPE was shown to be capable of inducing AGS cell apoptosis through two mechanisms, one was the activation of p38 signaling, which resulted in phosphorylation of c-Jun, activation of FasL and triggered Fas-mediated signaling (Fas/caspase 8/t-Bid signaling module); and the other was the cell apoptosis stimulated by the stabilization of p53, increase in Bax, release of cytochrome c, thus leading to caspase 3 being cleaved.