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馬栗樹皮素抑制人類肝癌細胞株 HepG2 cells 生長分子機制之探討

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目 錄

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Abstract

Esculetin is a phenolic compound of coumarin derivative containing in many plants such as Atemisiae capillaris Flos (Compositae), leaves of Citrus limonia (Rutaceae), Digitalis purpurea L. (Scropulariaceae), Euphorbia lathyris L. (Eupho-biaceae), Atropa belladonna (Solanaceae), Datura Stramonium L., Hyoscyamus niger L. In the past decade esculetin has been reported to have antiplatelet, antioxdant, anti-inflammatory and anticarcinogenic effects. Recently, it has also been reported to decrease the survival of human leukemia and breast cancer cells. However, the antiproliferation mechanism of esculetin on cancer is not well understood. In this study, the preliminary assay showed that esculetin inhibited the proliferation of human HepG2 hepatoma cells. By flowcytometric assay, it demostrated that esculetin inhibited the cell cycle progression of HepG2 hepatoma cells. In addition, we examined the expression of cell cycle-related proteins including cyclins, cdks, and CKIs. It showed that (1) esculetin delayed Rb phosphorylation compared with control group in HepG2, （2） attenuating the interaction of E2F1/DP-1 and c-Myc/Max. （3） Esculetin also decreased of cyclin E, CDC25A, and increased expression of p16, p53, p21, p27 significantly in HepG2 cells. Furthermore, （4） esculetin blocked the Ras/MAPK, p38 and Akt signal transduction resulting of cell growth arrest or apoptosis. Take together, esculetin presented antiproliferation effect involving modulating the expression of cell cycle related proteins and the MAPK signal pathway.

中 文 摘 要

馬栗樹皮素（esculetin, 6,7-dihydroxycoumarin）是香豆精衍生物，屬於一種多酚類的化合物。在一些藥用植物例如菊科的茵陳蒿、芸香科的橙柚、玄參科的毛地黃、大戟科的續隨、茄科的顛茄以及蔓陀羅等都可發現 esculetin 的存在。早期文獻指出 esculetin 具有抗氧化、抗凝血、抗發炎以及降低腫瘤生成等藥理活性。最近的研究發現，esculetin 能有效的抑制一些人類血癌與乳癌細胞株的增生，然而其中的詳細分子機制未明。本實驗以人類肝癌細胞株 HepG2 cells 為材料，並以 esculetin 刺激後做了一連串的實驗來研究其對肝癌細胞增生的影響。由流式細胞儀分析結果顯示 esculetin 能抑制 HepG2 細胞週期的進行；進一步以西方點墨法觀察調控細胞週期進行的分子表現 (cyclins, CDKs, CKI et. al.)，結果發現 esculetin 能（1）延遲 HepG2 細胞中 Rb 的磷酸化現象而降低 E2F-1/DP-1 間的結合；（2）促使 p16、p53、p21、p27 等 CKI 的蛋白表現上升，抑制細胞週期的進行；（3）降低 Cyclin E 和 CDC25A 的蛋白表現，同時此二蛋白的上游轉錄因子 c-myc/Max 間的交互作用力亦受到 esculetin 的抑制。（4）Esculetin 能阻斷 HepG2 細胞中 Ras/MAPK、p38 以及 Akt 的訊息傳遞路徑影響細胞增生，並誘導 apoptosis 的發生。綜合以上結果 esculetin 抑制細胞增生乃藉 Ras/MAPK 以及 Akt 這兩條訊息傳遞路徑而影響調節細胞週期分子的表現。

緒　　論

據估計全美國有三分之一人口會發生腫瘤，每年的總死亡數中有五分之一是腫瘤引起。在台灣，根據行政院衛生署 89 年發佈的統計資料，國人十大死因也是以惡性腫瘤(癌症)為首，佔總死亡人數的 20％以上，換句話說癌症在一年中就奪去 25,700 多條寶貴的生命。惡性腫瘤前三名分別為肺癌、肝癌與結直腸癌，其中又以肝癌為國人們最防不勝防的夢魘。肝腫瘤發生的致病因子很多，例如過量的酒精和吸菸將加速外來致癌性化學品與標的細胞的接觸而致癌；其他像黴菌毒素（如黃麴毒素）、病毒（如 C 型肝炎病毒、B 型肝炎病毒）亦與肝癌的引起有關。今日雖然很多疾病都能被妥善治療，但惡性腫瘤卻仍是不治之症。即使手術、合成藥物及化學放射療法對癌症可以產生積極的治療效果，但卻經常伴隨驚人的副作用或是產生抗藥性。

早在數千年前人們就已經開始使用藥草來治病而且效果還非常好，古代西伯萊人有句俗諺：「*上帝自土地創造出藥方，智者必不輕視它...*」。近代臨床上許多天然西藥都是屬於植物的活性成分，例如最近很熱門的新聞焦點-太平洋紫杉醇(Taxol)，可有效的治療卵巢癌和乳癌；含有多醣體的靈芝和化學抗癌藥併用，能降低罹患腫瘤老鼠的死亡率等等。在預防醫學盛行的今天，中草藥的傳統療效將成為下一世紀重要的研究熱潮，所

以本實驗室乃致力於研究天然植物中的防癌抗癌活性成分。此篇以天然草藥中之馬栗樹皮素（6,7-dihydroxycoumarin，esculetin）為研究材料，探討其抑制肝癌細胞生長的詳細分子機制。

馬栗樹皮素 esculetin（EST）是香豆精（coumarin）衍生物的一種，主要發現於中藥材中菊科的茵陳蒿、芸香科的橙柚、玄參科的毛地黃、大戟科的續隨、茄科的顛茄和曼陀羅等 (1)，為一種多酚類的天然抗氧化物 (2-4)，傳統上常被作為鎮痛及抗發炎的藥材。Esculetin 結構上苯環位置具有 6、7 兩處的 hydroxy group，所以具有良好的捕捉超氧陰離子(O_2^-)的能力以及抑制 xanthin oxidase 的作用(1,5)。1994 年 Chang 等人研究發現一些肝癌和腦部腫瘤的患者其 xanthin oxidase 的濃度均較正常人為高，由此立下 esculetin 或許在將來可當成抗癌藥使用(6)。

近代有越來越多關於 esculetin 在生物及生化方面的活性研究被提出，例如其具有抑制花生四烯酸代謝過程中 5- 和 12-lipoxigenase 的作用(7)以及抗腫瘤的形成(8)。1998 年 Matsunaga 等人提出 esculetin 對於 N-methyl-N-nitrosourea (MNU)誘發 Sprague-Dawley 品系雌性大白鼠乳癌的形成具有抑制效果(9)；1999 年 Stephen 等人也指出，利用香菸中致癌物質 benzo[*a*]pyrene plus 4-(methylnitrosamino)-1- (3-pyridyl)

-1-butanone 誘導雌性 A/J 小白鼠肺臟腫瘤生成，在投予 esculetin 處理的老鼠其腫瘤發生的比例遠小於實驗組(10)。也有研究證實 esculetin 具有 Anti-proliferation 的藥理活性，主要是透過下列幾種不同的機轉使細胞生長受到抑制：1.較早期的研究報告指出，利用 esculetin 的抗氧化活性，降低細胞中 Reactive oxygen species [ROS] 的濃度，使得老鼠大腸黏膜及上皮細胞生長受阻(11,12)，B 淋巴球的生長及分化亦受影響(13)。2.透過阻斷 5-和 12-lipoxigenase 這條代謝路徑，使得人類乳癌細胞株 MDA-MB-231、HS578T、U937 生長受到抑制(14,15)；稍後的研究證實，處理 linoleic acid 後的人類乳癌細胞株 MDA-MB-231, 當 esculetin 存在下將會造成 leukotriene B 的分泌顯著降低，進而導致細胞的生長受到抑制(16)。3. esculetin 也可以透過影響 Protein tyrosin kinase 和 Protein kinase C 的量，降低 platelet-derived growth factor-A(PDGF-A)轉錄層次的表現而抑制人類正常 T 淋巴球細胞以及 T 細胞淋巴癌 CEM 細胞的生長(17,18)。4.Esculetin 減少花生四烯酸代謝成 Prostaglandin E2 (PGE2)，使得下游的 Protein kinase A 活性降低，影響大白鼠腎臟細胞的正常成長(19)。5.處理 Esculetin 後，花生四烯酸的下游代謝產物 12-HETE、PGE2、6-keto-prostaglandin F1-α減少，同時其 Protein tyrosin kinase 活性降低，導致兔子血管平滑肌細胞生長受到抑制(20)。6.透過調節 transforming growth factor

β(TGF- β)，使得 esculetin 造成肺癌細胞株 CCL64 停滯在 G1 phase，DNA 合成降低(21)。

綜合以上的研究可知, esculetin 在抑制某些癌細胞生長有良好的效果，但對於其中詳細的分子機制了解的並不多。由過去文獻我們已知，細胞的生長受到一定的週期性蛋白調控，當這些分子調節失序將引起細胞的生長停滯、死亡或癌化。這些維護細胞週期正確進行的分子包括讓 Cell cycle 往前進行的 Cyclin 及其催化受質 cyclin-dependent kinases(CDKs)；而透過 cyclin-dependent kinase inhibitor(CKI)和 check-point 的路徑使細胞生長得以維持一個平衡。Cell cycle 分為 G0/G1(細胞成長期)、S(DNA 複製期)、以及 G2/M(細胞有絲分裂期)。首先讓細胞通過 cell cycle 第一個限制點進入 S 期是由 cyclin-depented kinases(CDK2/4/6)以及他們的調控蛋白 cyclins D、E 及 A 負責。當 restricted point retinoblastoma tumor suppressor protin (pRb) 受到 G1 早期表現蛋白 cyclin D-dependent kinases(cycD-K4/K6)的磷酸化會釋出轉錄因子 E2F-1，此時 E2F-1 再和 DP-1 形成 heterodimers，活化下游 cyclin E，A 及其本身等蛋白的基因轉錄作用；而在 G1 末期，cyclin E-CDK2 能促使 pRb 完全的磷酸化，使的細胞進入 S 期，並導致 cyclin A-CDK2 的活化(22)。相對的，細胞也會透過一些 CKI，如 p27，以及由 p53 誘導產生的 p21 和 p16 抑制 cyclin D-,, E-，和 A-dependent kinases 的作用(23,24)，導致細胞

生長停滯在 G1 期。另外一個對於 S phase entry 相當重要且必須的蛋白-cell division cycle 25A(CDC25A)是一種去磷酸脢，主要在 G1 期被 Myc 和 E2F 誘導產生，藉由去除 cdk2 上 threonine14 和 tyrosine15 位置的磷酸根, 而讓 cdk2 完全活化(25-27)。

許多文獻指出 cyclin D、E、A、Cdk2、Cdk4、和 CDC25 等過度表現將導致腫瘤的發生，例如一些乳癌病例中發現 cyclin E 過度表現(28)；cyclin D1 也被指出和 retina 及 乳癌的發生有關 (29)。Testis 和一些 Leukemic cell lines 表達大量的 Cyclin A(30)； Cdk-2 及 Cdk-4 的 overexpression 則和子宮內膜異位癌有關(31)。1995 年 Galaktionov, K.等學者指出 CDC25A 扮演誘發裸鼠腫瘤形成的致癌角色(32)，近期的一篇研究也指出人類乳癌的病例中 CDC25A 過度表現，造成 cdk-2 活性的上升(33-36)。

由此可知當調節細胞週期的蛋白如果過度表現，將使得細胞不斷的增生、分裂而癌化，所以這些蛋白的上游轉錄因子 E2F-1、c-myc 等的調控相形之下便顯得相當重要。Myc 屬於 c-myc proto-oncogene 的產物，在人類的癌症包括肺癌、乳癌和一些大腸癌中，該基因會被放大導致 Myc overexpression(36,37)。 正常下 c-myc 透過 Wnt 這條訊息傳遞路徑中β-catenin 與轉錄因子 Tcf 結合而表現(38)。透過

Ras/Raf/ERK 以及 Cdk-2/4 的活化，使 c-myc 與 Max 形成 heterodimer 而啟動其標的基因的蛋白表現(39)，包括 CDC25A、cyclin A、cyclin E、ARF 、eIF4E、eIF2α等與細胞週期或蛋白合成有關的分子(40)。

一般情況下當細胞受到外界的刺激，例如一些生長因子、cytokines、輻射線或藥物處理等等，會透過 signal pathway，將外來的訊息層層的往細胞內送，而引起一連串的生物反應，調控細胞的增生、分化、壓力反應以及細胞凋亡。許多報告指出 Ras/Raf/MAPK 以及 PI3K/Akt 這兩條路徑對於 cell cycle G1 期的進行以及細胞增生有關(41)。PI3K/Akt 的活化能抑制 GSK3β表現，穩定 Cyclin D 蛋白，同時能阻斷 p27 的轉錄作用，使得 CyclinE/Cdk-2 活性不被抑制而促使細胞週期的進行。另外 Akt 具有磷酸化 Bad 的能力， 使之無法誘發轉接到粒腺體的膜上，引發 apoptosis 的發生(42)。MAPK 主要可分為三種：stress-activated protein kinases(SAPK)/Jun N-terminal kinases(JNK)；p38 kinases；extracellular signal-regulated kinases(ERK1/2)。這些 MAPK members 藉著磷酸化下游蛋白，一般大多是一些轉錄因子，來調控細胞週期的進行(43)，例如 p38 活化 Max、ATF-2；JNK 活化 p53、c-Jun；ERK 活化 STATs、Myc 等。MAPK 除了影響轉錄活性而調控 cell cycle，其中 ERK 和 p38 也會去活化轉譯因子 eIF4E，促使蛋白合成(44,45)。

近年來有許多的研究指出一些天然多酚類的抗氧化物例如 Hibiscus protocatechuic acid (PCA)， Ellagic acid 以及 tea catechins 等對於癌細胞生長有抑制作用，並且導致細胞的凋亡(46-48)。同樣屬於多酚類的 coumarin 或其衍生物 8-nitro-7hydroxycoumarine 也被指出能促使癌細胞走向 Apoptosis(49,50)。本實驗室已經證實 esculetin 對於 HL-60 能促使 cytochrome c 由粒腺體釋放到細胞質，並且活化 CPP32 的活性，誘導 apoptosis 的發生(51)。所以在此我們除了研究 esculetin 對於調控細胞週期蛋白的影響外，同時也想了解 esculetin 對於肝癌細胞株 HepG2 是不是也能造成細胞的凋亡。

目前臨床上使用的抗癌藥常藉由引起癌細胞的程序式死亡或改變癌細胞的生長週期，來達到抑制腫瘤的增生與惡化。Paclitaxel 與 Cisplatin 已廣泛的被醫界用來治療某些癌症，其作用機制是讓癌細胞生長停滯在 G2/M 期，並促使 Apoptosis 的發生（54-55)。然而此一治療癌症的過程冗長，為病患帶來不少副作用，所以我們希望 esculetin 能有助於提高這些抗癌藥的效率，縮短療程，以期能在抗癌這場戰役中貢獻一己之力。

研 究 目 的

本實驗室及其他文獻報告顯示，馬栗樹皮素（esculetin ：6,7-dihydrooxycoumarin）除了具有良好的抗氧化、發炎以及捕捉自由基的能力外，在動物實驗中對於抑制致癌物誘發腫瘤生成方面也有顯著的效果。自 80 年代中期直至今日，已有許多研究證實 esculetin 對於一些人類惡性腫瘤細胞株能阻斷其增生，並誘導細胞發生凋零性死亡，然而其中的詳細分子機制未明。所以本實驗以 esculetin 為材料，從四個方向去研究其對人類肝癌細胞株 HepG2 cells 的生化活性：1. 對細胞增生抑制的作用 2. 影響 cell cycle 調控分子的表現 3. 訊息傳遞的路徑 4.誘導細胞凋零性死亡。

材 料 與 方 法

一.材 料

(一) 化 學 試 劑

[1] **購自美國** GIBCO BRL **公司**:

名稱	**英文全名**
DMEM:	Dulbecco’ s Modified eagle medium
PS:	Penicillin–Streptomycin
NEAA:	MEM non-essential amino acids solution
FBS:	Fetal bovine serum
PBS:	Phosphate buffer saline
Trypsin-EDTA	

[2] **購自美國** Sigma **公司**:

名稱	**英文全名**
Esculetin:	6,7-Dihydroxycoumarin
SDS:	Sodium dodecyl sulfate
Tris-base:	Tris(hydroxymethyl)-aminomethane
RNase A:	Ribonuclease A
PI:	Propidium iodide
MTT:	3-[4,5–Dimethylthiazol–2-yl]-2,5–diphenyl tetrazolium bromide
Tris-HCl:	Tris(hydroxymethyl aminoethan hydrochloride
Leupeptin:	Acetyl–Leu–Leu–Arg-al

PMSF:	Phenyl-methylsulfonyl fluoride
Sodium ortho-vanadate	
β-mercaptoethanol	
Paclitaxel	
Cisplatin	
RNase A	Ribonuclease A

[3] **購自美國** Bio-Rad **公司**:

名稱	**英文全名**
Bis:	Bis N,N' –methylene–bis-acrylamide
BSA:	Lyophilized Bovine Serum Albumin
TEMED:	N' ,N' ,N' ,N' ,-Tetramethylethylenediamine
Glycine	
Acrylamide	

[4] **購自德國** Boehringer Mannheim **公司**:

名稱

Cellular DNA Fragmentation ELISA kit

[5] **購自美國** Kodak **公司**:

名稱

Kodak GBX fixer and replenisher

Kodak GBX developer and replenisher

Kodak BioMax light film

[6] **購自** Santa Cruz Biotechnology **公司**:

名稱

Anti-p14 rabbit polyclonal antibody

Anti-p16 mouse monoclonal antibody

Anti-p21 mouse monoclonal antibody

Anti-p27 mouse monoclonal antibody

Anti-CDC25A mouse monoclonal antibody

Anti-cdk2 mouse monoclonal antibody

Anti-cdk4 rabbit polyclonal antibody

Anti-cdk6 mouse monoclonal antibody

Anti-cyclin D1 mouse monoclonal antibody

Anti-cyclin E mouse monoclonal antibody

Anti-β-catenin mouse monoclonal antibody

Anti-E2F-1 mouse monoclonal antibody

Anti-eIF4E mouse monoclonal antibody

Anti-c-Myc mouse monoclonal antibody

Anti-Rb mouse monoclonal antibody

Anti-Shc mouse monoclonal antibody

Anti-Max rabbit polyclonal antibody

Anti-Raf mouse monoclonal antibody

Anti-p-Tyr(PY20) mouse monoclonal antibody

[7] **購自** Promega **公司**:

名稱

Anti–active–JNK(pTPpY) rabbit monoclonal antibody

Anti–active–MAPK(pTEpY)rabbit monocloal antibody

Anti–active-p38 mouse monocloal antibody

[8] **購自** Oncogene **公司**:

名稱

Anti-cyclin A mouse monoclonal antibody

Anti-PARP mouse monoclonal antibody

Anti-α-tubulin mouse monoclonal antibody

Anti-DP-1 mouse monoclonal antibody

Protein A-agarose

[9] **購自** CALBIOCHEM **公司**:

名稱

Anti-actin antibody

Anti-phosphoserine mouse monoclonal antibody

[10] **購自** NEN **公司**:

名稱

Amersham ECL reagent kit

[11] **其他**:

名稱

CPP32/Caspase-3 Colorimetric kit

Methanol

Ethanol

Isopropanol

sodium chloride

Tween-20#

Bromophenol blue

glycerol

Amonium persulfate

deoxycholic acid

Coomassie blue

（二）器 材

[1] **購自** FALCON **公司**:

名稱

Tissue culture Dish

Tissue culture Flask

Microtest culture plates

Polypropylene conical tubes

[2] **購自** Millipore **公司**:

名稱

Nitrocellulose membrane

Sterivex - GV 0.22μm filter unit

[3] **其他**:

名稱

Filtertips

Micro test tubes

Disposable syringe

（三）儀 器

中文名稱	**英文名稱/廠商型號**
二氧化碳培養箱:	NUATR CO_2 water-Jacketed incubator
冷藏冰箱:	Whirlpoor
無菌操作台:	NUATR Biological Safety Cabinots
分光光譜儀:	UV and Visble Spectrophotometer HITACHI/U2000
倒立式顯微鏡:	Nikon/Diaphot 300
流式細胞分析儀:	Flowcytometer/FACS Calibur
數位影像處理儀:	AlphaImager 2000
閃爍偵檢器:	Liquid Scitillation Analyzer/Packard

	2100TR
蛋白電泳槽:	Bio-Rad Mini protein II cell
	Bio-Rad Mini protein III cel
蛋白質轉漬槽:	Hoefer pharmacia Biotech
電泳電源供應器:	Bio-Rad
震盪器:	Scientific industries vortex genie 2
桌上型離心機:	KUBOTA 2010
微量離心機:	Shelton VS 15
冷凍離心機:	Universal 32/32R
乾浴槽:	T hermolyne
水浴槽:	Techne TE 8J
電子天秤:	Sartorius analytic
電磁攪拌加熱器:	CORNING
酸鹼值測定儀:	JENCO electronics
三度空間震盪器:	pippet shaker
酵素免疫分析儀:	ELISA reader

二.實 驗 方 法

(一) 細 胞 培 養

人類肝癌細胞株 Hepatomablastoma G2 (**含有** Rb **和** p53) (from The

NHRI Cell Bank) 培養於含有 10% heat - inactivated fetal bovine serum (FBS: Gibco BRL)、1% nonessential amino acid (NEAA: Gibco)、1% Penicillin – Streptomycin (PS: Gibco) 的 Dulbecco's Modified eagle medium (DMEM: Gibco)，培養箱設定 5% CO_2，37℃恆溫的環境，細胞密度維持在 2×10^5 ~ 1×10^6，每週更換培養基 2~3 次。

人類肝癌細胞株 Hepatocellular carcinoma 3B (不含 Rb 和 p53) (from The NHRI Cell Bank) 培養於含有 10% heat - inactivated fetal bovine serum(FBS: Gibco BRL) 、 1% Sodium pyruvate (Gibco) 、1% (Gibco) Nonessential amino acid(NEAA: Gibco) 、 1% Penicillin- Strepto -mycin(PS: Gibco)的 Dulbecco's Modified eagle medium(DMEM: Gibco)，培養箱設定 5% CO_2，37℃恆溫的環境，細胞密度維持在 2×10^5~1×10^6，每週更換培養基 2~3 次。

人類血癌細胞株 HL-60(from acute promyelocytic leukemia patient，含有 Rb 但不含 p53) 培養於含有 10% heat - inactivated fetal bovine serum(FBS: Gibco BRL)、1% Penicillin-Strepto- mycin (PS: Gibco)、1% glutamine 的 RPMI 1640(RPMI: Gibco)，培養箱設定 5% CO_2，37℃恆溫的環境，細胞密度維持在 2×10^5~1×10^6，每週更換培養基 2~3 次。

(二)細胞毒性分析(MTT assay)

實驗原理：根據 Alley 等人發表於 1988 年的 Cancer research 期刊(54)，其原理是利用活細胞能經由粒腺體 dehydrogenase 的作用，將(3 -[4,5 – Dimethylthiazol -2-yl]-2,5-diphenyl-tetrazolium bromide)(MTT)代謝還原成紫色的 formazan crystal，並在波長 563nm 有特殊吸光。

實驗方法: **將細胞培養後，以** trypsin-EDTA **將細胞由培養皿打下，用** PBS **沖洗後，以培養基將細胞濃度調整成** 2×10^4 cell／ml, **各取** 1ml **的細胞液分別培養於** 24-well **的培養皿中。待細胞貼壁，更換新的培養基，同時加入不同濃度的** esculetin（0、10、20、50、100μM）**作用** 24、48、72 **小時；另一組實驗設計乃以** 100μM esculetin（**實驗組**）**或** 0.2% DMSO（**對照組**），**分別培養** 0、24、36、48 **小時後，分別更換新的培養基，同時加入** 100μl MTT (5mg/ml)**反應４小時，除去培養基後，最後以** 1ml **的** isopropanol **將紫色的** formazan **結晶溶解，並於波長** 563nm **下測定吸光值**(O.D.)**。比較實驗組與對照組吸光值的差異，以反映出** esculetin **對** HepG2 cells **的細胞毒性。**

（三）細胞存活率分析 (trypan blue dye exclusion assay)

實驗原理：利用活細胞細胞膜具有調控物質進出之功能，而得以防止染劑通透進入活細胞，進而判斷細胞的存活率。

實驗方法: **將細胞培養後，以** trypsin-EDTA **將細胞由培養皿打下，用** PBS **沖洗，加培養基將細胞濃度調整成** 2×10^5 cell／ml，**培養於** 10 **公分的培養皿中。待細胞貼壁，更換新的培養基，同時加入** 100μM esculetin（**實驗組**）**或** 0.2% DMSO（**對照組**），**分別培養** 0、24、36、48 **小時後，以** trypsin -EDTA **將細胞由培養皿打下，**PBS **沖洗後，以** 1000rpm **離心** 5 **分鐘，取沉澱細胞，以** 2ml **培養基使細胞懸浮，再從中取** 100μl **細胞液與等量的** trypan blue **混勻，在顯微鏡下觀察，以計數器計算活細胞數。**

（四）細胞增生之分析

實驗原理: **將** DNA **合成的原料**(A, T, C, G)**以放射線物質標定，並與細胞培養一段時間後偵測細胞中放射線強度，以反映出該細胞新合成** DNA **的效率。**

實驗方法: **將細胞培養後**, **以** trypsin - EDTA **將細胞由培養皿打下，** PBS **沖洗後，以** 1000rpm **離心** 5 **分鐘，取沉澱細胞，加培養基使細胞懸浮，將細胞濃度調整成** 2×10^4 cell／ml**，培養於** 24-well **的培養皿中。待細胞貼壁，更換不含血清的培養基培養** 24 **小時，再改以一般培養基(** **含** 10% FBS)**，同時加入** 100μM esculetin**（實驗組）或** 0.2% DMSO(**對照組**)**以及**[^{3}H]-methyl-thymidine 0.5μCi／well **分別培養** 24、48 **小時。將培養後的細胞倒除上層液，並以** PBS **洗三次，倒除上層液，最後每一個** well **加入** 0.4ml **之** 0.4M NaOH **溶出細胞內含物，取溶出液，加** 2-3ml **閃爍液，以閃爍偵檢器偵測**[^{3}H]**之放射強度。**

（五）細胞週期分析

實驗原理：利用特殊染劑（ex. PI**）嵌入** DNA **的特性，得以分辨細胞生長狀態：**G0/G1 DNA **套數為** 2n**，**G2/M **期** DNA **套數複製一倍，成為** 4n**，比較細胞** DNA **含有** 2n **及** 4n **的量，以反映出** cell **的生長的速度。**

實驗方法: **將細胞培養後，以** trypsin-EDTA **將細胞由培養皿打下，** PBS **沖洗後，以** 1000rpm **離心** 5 **分鐘，取沉澱細胞，加培養基使細胞懸浮，將細胞濃度調整成** 2×10^5 cell／ml**，培養於** 10 **公分的培養皿中。待細**

胞貼壁，更換新的培養基，同時加入 100μM esculetin（實驗組）或 0.2% DMSO（對照組），培養 24 小時後，以 trypsin - EDTA 將細胞由培養皿打下，PBS 沖洗，調整細胞濃度成 2×10^{6}/ml，以 1000rpm 離心，倒除上層液，加入 80%酒精置入-20℃冰箱固定細胞至少 30 分鐘以上。接著以 400ｇ離心 5 分鐘，倒除上層液，並加 0.1% tritonX-100 0.5ml 於暗室中反應 30 分鐘。再次離心，倒除上層液，加 1m PI solution（10μg/ml，內含 40μg/ml Rnase A），於 37℃水浴槽中避光反應 30 分鐘，最後置於冰上，以流式細胞儀測定。

（六）西方點墨法

實驗原理：利用電流將帶有負電荷的不同分子量的蛋白分離，並以專一抗體與標的蛋白結合，放大並顯示該蛋白表現的變化情形。

實驗方法: 將細胞培養後, 以 trypsin - EDTA 將細胞由培養皿打下, PBS 沖洗後, 以 1000rpm 離心 5 分鐘，取沉澱細胞，加培養基使細胞懸浮，將細胞濃度調整成 2×10^{5} cell／ml，培養於 10 公分的培養皿中。待細胞貼壁，更換新的培養基，同時加入 100μM esculetin 培養 0、12、24、36、48 小時後，以 trypsin-EDTA 將細胞由培養皿打下，PBS 沖洗離心後，倒除上層液，加入 RIPA buffer【(150mM NaCl, 1% NP-40，0.5%Deoxycholic acid，0.1% SDS，50mM Tris – base，PH=7.5)，內含 1mM sodium orthovanadate, 100μg/ml PMSF, 170μg/ml leupeptin】，於冰上震盪 20 分鐘後在 4℃下以 10000ｇ離心 10 分鐘，即得 Total cell lysate，取上層液定量蛋白濃度。

將定量後之蛋白質取 60μg，加入等量的 Sample Buffer【2ml 0.5M Tris-HCl（PH=6.8），1.6ml Glycerol, 3.2ml 10%SDS, 0.8ml 2-β-mercaptoethanol, 0.4ml 0.5% bromophenol blue】，以 95℃加熱 3 分鐘，並快速置入冰中冷卻。以小型離心機將 Sample spin down 後再 loading 至每個 well 中。上層膠以 70 伏特，下層 130 伏特跑電泳。待電泳結束後跟著進行蛋白質的轉漬，也就是將膠上已分層的蛋白質轉漬到 Nitrocellular paper 上。以 5%脫脂牛奶【PBS-non-fat milk powder】於室溫下進行 blocking 1小時，稍稍 wash 後，將 NC paper 置於 4 度冰箱中與一級抗體反應 overnight，之後用 washing buffer【PBS with 0.5% tween-20】以 5’ -5’ -5’ -5’ -5’ ，175rpm 的方式清洗 NC-paper，再以 Horseradish peroxidase conjugated goat anti-mouse/rabbit antibody 反應 50-60 分鐘，以相同的方式 wash NC-paper，最後加入 Western Blot Chemiluminescence Reagent Plus 反應１分鐘，並於暗房中以 KODAK FILM 曝光 1 - 10 分鐘，以顯影劑及定影劑沖片觀察。

(七)免疫沉降法

實驗原理：以專一抗體與標的蛋白結合的特性將之沈澱純化出來。

實驗方法: 將細胞培養後, 以 trypsin - EDTA 將細胞由培養皿打下，PBS 沖洗後，以 1000rpm 離心 5 分鐘，取沉澱細胞，加培養基使細胞懸浮，將細胞濃度調整成 2×10^5 cell／ml，培養於 10 公分的培養皿中。待細胞貼壁，更換新的培養基，同時加入 100μM esculetin 培養 0、12、24、36、48 小時後，以 trypsin-EDTA 將細胞由培養皿打下，PBS 沖

洗離心後，倒除上層液，加入 RIPA buffer【(150mM NaCl, 1% NP-40, 0.5%Deoxycholic acid, 0.1%SDS, 50mM Tris-base, PH=7.5)，內含 1mM sodium orthovanadate, 100μg/ml PMSF, 170μg/ml leupeptin】，於冰上震盪 20 分鐘後在 4℃下以 10000ｇ離心 10 分鐘，即得 Total cell lysate。取上層液定量蛋白濃度。

將定量後之蛋白質取 0.5mg，加入 1μg 一級抗體在 4℃冰箱中反應 overnight。隔日將 sample 和 15μl protein A -agarose beads 在４度下繼續作用 2-6 小時。之後以 2500rpm 離心 10 分鐘，並以冰的 PBS 洗，如此重複三次，最後離心取沉澱物，加 20-30μl sample buffer 使之懸浮，跑 SDS-PAGE，並以免疫點墨法看結果。

（八）細胞凋亡之測定

實驗原理:當細胞凋亡的時候，DNA 會發生斷裂，偵測這些 DNA 片段的量，以反映出細胞發生 apoptosis 的程度。

實驗方法: 將細胞培養後, 以 trypsin - EDTA 將細胞由培養皿打下，PBS 沖洗後，以 1000rpm 離心 5 分鐘，取沉澱細胞，加培養基使細胞懸浮，將細胞濃度調整成 2×10^5 cell／ml，培養於 10 公分的培養皿中。待細胞貼壁，加入 Thymidine 的相似物 BrdU 當作 DNA 合成原料之一。將細胞培養 18-20 小時後，除去上層液，以 trypsin-EDTA 將細胞由培養皿打下，PBS 沖洗後，以 1000rpm 離心 5 分鐘，取沉澱細胞，加培養基使細胞懸浮，調整細胞濃度成 1×10^5 cell／ml，取 100μl 的細胞液移種至 96-well 的培養皿中，加入 100μl 含有 esculetin 的培

養基（esculetin 終濃度=100μM）分別培養 24，48 小時。依指定培養時間，去上層液，以 200μl 之 incubation solution 室溫下作用 30 分鐘，以將細胞水解，跟著以 250g 離心 10 分鐘，取上層液 100μl 加到已 coated anti-DNA antibody 的 96-well 培養皿中，在室溫下避光反應 90 分鐘。之後以 250-300μl 的 washing solution 洗三次，每次 2-3 分鐘。將最後一次的 washing solution 留下，送入微波爐中以 500W 加熱 5 分鐘後立即送入－20℃冰箱中冷卻 10-20 分鐘。之後完全移除 well 中的液體，加入 100μl 之 anti – BrdU – POD（0.2ml anti – BrdU - peroxidase solution + 9.8ml incubation solution）室溫下避光反應 90 分鐘，之後同樣以 washing solution 洗三次，移除上層液，加入 100μl substrate solution，避光輕搖反應直到呈色。最後加入 25μl stop solution（7% H_2SO_4）1 分鐘，並於波長 450nm 下測定吸光值。

（九）CPP32 活性之分析

實驗原理：當細胞發生計劃性的自然凋亡，常伴隨著 CPP32 活性的增高。測定 CPP32 活性，以反映出細胞 apoptosis 的狀況。本組 kit 利用 CPP32 具有切割蛋白的能力，當其受質經 CPP32 作用切割反應後將產生某一特定的吸光值，偵測此一吸光值的變化即可得知 CPP32 的活性。

實驗方法: 將細胞培養後，以 trypsin - EDTA 將細胞由培養皿打下，PBS 沖洗後，以 1000rpm 離心 5 分鐘，取沉澱細胞，加培養基使細胞懸浮，將細胞濃度調整成 2×10^5/ml，培養於 5 公分培養皿中。待細胞

貼壁，更新培養基，同時依實驗需要加入 esculetin，paclitaxel 或 0.2% DMSO（對照組）。培養 24 小時後以 trypsin - EDTA 將細胞由培養皿打下，PBS 沖洗後以 1000rpm 離心 5 分鐘，取沉澱細胞，加冰的 lysis buffer 於冰上震盪 10 分鐘後，以 10000g 離心 1 分鐘。取上層液，並測定蛋白濃度，調整濃度至 1mg/ml。將以上製備好的 sample 取 12.5 μl 加上等量的 reaction buffer（內含 10mM DTT），再加 1.25 μl 之 4mM DEVD-ρNA substrate，於 37℃下避光反應 1.5 - 2 小時，最後以波長 405nm 測定吸光值。

結　　果

一. Esculetin 對於人肝癌細胞株 HepG2 cells 具有細胞毒殺的效果，且能有效的抑制細胞增生。

【Fig. 1】Structure of esculetin

Esculetin 為香豆精 coumarin 的衍生物之一，其苯環上 6,7 兩處被 hydroxy group 取代，屬於一種天然的多酚類抗氧化物。

【Fig. 2】Cytotoxicity of esculetin in HepG2 cells

研究天然物 esculetin 對細胞毒性的實驗中，將人類肝癌細胞株 HepG2 cells 處理不同濃度的 esculetin（0，10，20，50，100μM）48 小時後，以 MTT 分析法發現在 50μM 及 100μM 濃度下細胞存活率分別為 73%和 70%。

【Fig. 3】Cell viability of esculetin in HepG2 cells

由前一個實驗發現處理 esculetin 100μM 48 小時對 HepG2 即有顯著細胞毒性，將細胞暴露在此劑量 0、12、24、36、48 小時後以 MTT 分析法發現，隨著加藥時間增長，細胞存活率隨之降低。

【Fig. 4】Effect of esculetin on HepG2 cells proliferation

過去文獻指出 esculetin 能抑制一些癌細胞的生長，所以實驗一開始先研究此一天然物對於本人類肝癌細胞株 HepG2 是

不是也有相同的效果。處理 esculetin 100μM 12、24、36、48 小時後，在顯微鏡下分別計算各組的細胞數，結果顯示加了藥的實驗組 24 小時其細胞生長率只有同期細胞的 73%，隨著加藥時間的增長，esculetin 能有效的抑制 HepG2 cell proliferation。

【Fig. 5】Effect of esculetin on DNA content of HepG2 cells

將 HepG2 cell 培養在含有 100μM esculetin 的培養基 24 小時後收集細胞，並以 flow cytometry 分析該細胞週期的變化情形。結果發現，esculetin 能導致肝癌細胞 HepG2 DNA replicatiion phase（S）減少，細胞停滯在 G0/G1。

【Fig. 6】 Effect of esculetin on HepG2 cells DNA synthesis

肝細胞增生（proliferation） 是造成肝細胞癌（hepatocellular carcinoma）的主要原因之一。本實驗培養人類肝癌細胞株 HepG2 cells 以[^{3}H] thymidine 攝入（incorporation into）DNA 的程度來決定 esculetin 對細胞增生之影響，結果顯示處理 100μM esculetin 24 小時的 HepG2 細胞，新生的 DNA 合成的效率只有正常組的 85%，48 小時後整個細胞合成 DNA 的能力只有 60%

二. Esculetin 影響調控細胞週期進行中 G0/G1 phase 某些蛋白的表現，使得 HepG2 cells 生長停滯。

由細胞流式儀的測定顯示，處理 esculetin 會導致肝癌細胞 HepG2 細胞週期停滯在 G1/S，在 [^{3}H]-methyl-thymidine incorporation assay 也證明 esculetin 能阻斷 HepG2 細胞進入 S 期去合成 DNA，所以我們進一步去研究調控 G1/S phase 的分子表現情形。

【Fig. 7】Effect of esculetin on pRb protein of HepG2 cells

Cell cycle 進行之初，首先 Rb 要被磷酸化，釋放出 E2F-1，進而活化下游基因的表現。由實驗結果發現，esculetin 造成 Rb 的磷酸化程度的下降。

【Fig. 8】Effect of esculetin on E2F-1 protein of HepG2 cells

E2F-1 能誘導一些 G1 晚期調節分子之蛋白表現, 例如 cyclin E、A 以及 E2F-1 本身。在處理 esculetin24 小時後發現 E2F-1 的蛋白表現開始減少。

【Fig. 9】Effect of esculetin on DP-1 of HepG2 cells

由於 Rb 的磷酸化程度被 esculetin 抑制，將使得 free form 的 E2F-1 下降，而和 DP-1 形成 heterodimer 的機會降低，影響轉錄效率，使的細胞生長受到抑制。由實驗證實，DP-1 和 E2F-1 之間的交互作用的確受到 Rb 的磷酸化的降低而減少。

【Fig. 10】Effect of esculetin on cyclin D1 of HepG2 cells

負責 G1 早期的另一個分子 D-type cyclins 對於 Rb 的磷酸

化相當重要，當其過度表達將使得 Rb 的磷酸化調節失序，導致腫瘤的形成（55)。在本實驗中雖然 esculetin 能使 Rb 的磷酸化下降，但對於 cyclin D1 的蛋白表現沒有影響。

【Fig. 11】【Fig. 12】Effect of esculetin on Cdk-4/6 of HepG2 cells

Cyclin D1 要與 cdk4/6 形成複合體才具有活性，雖然前一個實驗指出 esculetin 對 cyclin D1 沒有影響, 那麼是不是 cdk4/6 的表現受到抑制？由實驗結果發現無論是 cdk-4 或 cdk-6，他們的蛋白表現都不受影響。

【Fig. 13】Effect of esculetin on Cyclin E of HepG2 cells

由先前的結果已知 esculetin 能抑制 E2F-1 的轉錄效率，則其下游的蛋白 cyclin E 的表現是否也受到影響？Cyclin E 在 G1 末期表達，能和 cdk-2 形成一複合體，促使 pRb 的完全磷酸化（full-phosphorylated pRb)，驅動細胞週期由 G1 進入 S 期（56)。結果顯示 cyclin E 在 24 小時即有顯著的降低。

【Fig. 14】Effect of esculetin on cyclin A of HepG2 cells

另一個 E2F-1 的下游蛋白 cyclin A 主要調節 cell cycle S 期的進行，並協助細胞的有絲分裂。實驗結果顯示 esculetin 對於 cyclin A 並不會造成任何影響。

【Fig. 15】Effect of esculetin on cdk-2 of HepG2 cells

Cdk-2 能透過 cyclin E 或 cyclin A 的協助而被 CAK 催活，調

節 G1-S 的進行。經過 esculetin 處理，細胞中活化態的 cdk-2 在 24 小時左右開始下降，到 48 小時此一現象更為明顯。

【Fig. 16】Effect of esculetin on CDC25A of HepG2 cells

Cdk-2 活性的調控除了受到一些 CKI 的抑制外（例如 p27），CDC25A 也佔有相當重要的角色。當 cdk-2 和 cyclin E 形成一複合體而被 CAK 磷酸化，必須再藉由 CDC25A dephosphorylation 的作用去掉 cdk-2 上 Thr14 以及 tyr15 兩處的磷酸根，才能使之完全活化（57)。實驗結果顯示，處理 esculetin24 小時 CDC25A 即顯著的下降，而到了 48 小時則完全抑制該蛋白表現。

【Fig. 17】Effect of esculetin on CDC25A of H3B cells

Esculetin 在抑制 CDC25A 蛋白表現的效果顯著, 我們便感興趣的想知道，esculetin 對於其他腫瘤細胞株中的 CDC25A 是否也有相同的結果。所以本實驗以不表現 p53 和 Rb 的肝癌細胞 H3B 為材料，發現處理 esculetin 24 小時後即能有效的抑制 CDC25A 的表現。

【Fig. 18】Effect of esculetin on CDC25A of HL-60 cells

除了肝癌細胞株外，我們也比較了 esculetin 對血癌細胞株 HL-60（有 Rb，但不表現 p53）中 CDC25A 的影響情形。結果顯示雖然 CDC25A 在 36 和 48 小時有下降，但效果並不顯著。

【Fig. 19】Effect of esculetin on cdk-2 activity of HepG2 cells

Esculetin 能有效的抑制 HepG2 細胞中 CDC25A 的表現，而 CDC25A 是活化 cdk-2 的要角之一，所以我們利用免疫沈降法的方式來看 cdk-2 tyrosine 磷酸化的程度，以反應出 cdk-2 活性的變化。結果顯示，處理 esculetin 36 小時後 cdk-2 在 tyrosine 位置上磷酸化的程度上升。以此推測 cdk-2 因著 CDC25A 減少，導致活性的下降。

三. Esculetin 對於細胞週期中 restriction point regulators 能刺激他們的蛋白表現，進而抑制 cell cycle 由 G0/G1 進入 S phase，導致肝癌細胞 HepG2 生長停滯。

細胞週期的進行除了受 cyclins/CDKs 的正向調節外，也會藉由細胞週期的〝checkpoints〞作一負向調控，使細胞不致過度增生而癌化；這個〝checkpoint〞包括一些 cyclin-dependent knase inhibitors（CKI）以及 p53。Esculetin 能有效的促使肝癌細胞株 HepG2 cells 生長停滯在 G1/S 期，所以接下來我們所探討的主題是 esculetin 對這些（CKI）以及 p53 的影響情形。

【Fig. 20】Effect of esculetin on p53 protein of HepG2 cells

當細胞受到外來刺激例如輻射線、化學物質及環境壓力等，會誘導 p53 的表現，進而促使其下游基因的蛋白表現，使的細胞生長停滯以利 DNA 修復，或是讓細胞走向凋亡（58）。

由實驗結果得知 esculetin 能讓 HepG2 cells 在 12 小時後 p53 的表現增加。

【Fig. 21】Effect of esculetin on p16 of HepG2 cells

CKI 中的 p16 能專一性的抑制 cdk-4，造成細胞週期 G0/G1 的停滯。當本實驗發現 HepG2 cells 處理 esculetin 24 小時後，p16 的蛋白表現增加。

【Fig. 22】Effect of esculetin on p21 of HepG2 cells

$p21^{WAF}$ 能抑制 D-type cyclins 的作用，而造成 cell cycle arrest。處理 esculetin 12 小時後，HepG2 cells 中 $p21^{WAF}$ 的表現上升。

【Fig. 23】Effect of esculetin on p27 of HepG2 cells

p27 具有廣泛性的抑制 cell cycle G1/S 中所有的 cyclins/cdks 的活性，在這裡我們也發現 esculetin 也能增加 HepG2 cells 中 p27 蛋白，而且在 24-36 小時有最大表現量。

四. Esculetin 會阻止轉錄因子 c-myc 與 Max 形成 heterodimer 的形式，使之無法 translocate 到細胞核中，影響下游一些調控 cell cycle 的蛋白表現。

由前面的實驗證據得知，esculetin 能有效的抑制 cyclin E 和 CDC25A 的表達，所以我們想瞭解 esculetin 是否影響在基因的轉錄層次？於是進一步探討其上游轉錄因子的表現情

形。

【Fig. 24】Effect of esculetin on β-catenin of HepG2 cells

由過去文獻已知，cyclin E 和 CDC25A 的上游轉錄因子是 c-myc（40），其表現乃透過 Wnt 這條訊息路徑中的β-catenin 而被調控。本實驗結果顯示，HepG2 在 esculetin 的存在下，對β-catenin 的影響只有輕微減少其蛋白的表現。

【Fig. 25】Effect of esculetin on c-Myc of HepG2 cells

直接看 HepG2 cells 中 c-myc 蛋白的變化，結果也顯示 esculetin 對於 c-myc 的蛋白表現沒有影響。有趣的是在電泳圖上 12 小時處發現活化型的 c-myc 的表現有降低的情形。

【Fig. 26】Effect of esculetin on c-myc transcription activity of HepG2 cells

c-myc 要成為一個有效用的轉錄因子，必須先和 Max 形成 heterodimer 的形式。由前一個實驗結果我們推測 esculetin 是否影響 c-myc 的活化進而阻斷 c-myc/Max 複合體的形成，降低下游蛋白表現。利用免疫沈降法以及西方點墨法來看二者結合的情形，結果顯示 esculetin 確實會阻止 HepG2 cells 5 中 c-myc/Max heterodimer 的形成，影響轉錄效率。

五. Esculetin 透過 Ras/MAPK 這條訊息傳遞路徑影響肝癌細胞 HepG2 的基因轉錄及蛋白轉譯的活性，造成生長停滯的現

象。

一般細胞從 G0 進到 S 期需要生長因子（EGF）的刺激（59），當 EGF 接到 EGF receptor 將活化一連串的 kinases 及 proteases 而調節 cell cycle 的進行。目前被研究較多的包括 Ras/Raf/MAPK 以及 PI3K/Akt 這兩條訊息傳遞路徑。所以研究其中訊息傳遞分子變化，以利我們瞭解 esculetin 是如何透過 signal transduction pathway 而影響了調控細胞週期分子的表現。

【Fig. 27】Effect of esculetin on tyrosine phosphorylation of HepG2 cells

當 mitogen 刺激細胞後會活化 receptor tyrosine kinase 及其下游的 protein tyrosine kinase 使細胞生長。由細胞流式儀分析等一連串的實驗證實 esculetin 能使 HepG2 cells 細胞細胞週期停滯，抑制 cell proliferation。所以在這個實驗中我們想知道 esculetin 是否影響這條 pathway，導致細胞的生長停滯。利用 anti-PY-20 antibody 來看 esculetin 對 HepG2 cells 中 protein tyrosine kinase 的影響。結果顯示，在箭頭所指的位置，其 tyrosine phosphorylation 的程度較未處理 esculetin 組為低。

【Fig. 28】Effect of esculetin on Shc activity of HepG2 cells

由上一個實驗結果我們推測第一、二個箭頭所指分子量介於

29-81kDa 間的蛋白為 Shc。當 EGF 接到 EGFR 上會使之發生 autophosphorylation，此時 Shc 會被吸引到細胞膜上而在 tyrosine 的位置接上磷酸根而活化（60)。結果顯示 esculetin 對於 Shc 的活性沒有影響。

【Fig. 29】Effect of esculetin on Raf activity of HepG2 cells

根據【Fig. 27】，分子量介於 49-81kDa 間箭頭所指處推測為 Raf 蛋白，屬於 mitogen-activated protein kinase ，透過 GTP-bound Ras 的結合而活化下游的 MAPK pathway（61)。實驗結果顯示 esculetin 處理後 9-12 小時能顯著的降低 HepG2 cells 中 Raf tyrosine phosphorylation 的現象。

【Fig. 30】Effect of esculetin on p-ERK of HepG2 cells

Raf tyrosine phosphorylation 程度的下降是否會影響其下游 MAPK/ERK 的活化？ERK 對於由調節細胞的增生以及存活相當重要。由實驗結果指出 esculetin 能抑制 MAPK/ERK 的磷酸化而降低活性。

【Fig. 31】Effect of esculetin on p-p38 of HepG2 cells

另一個 MAPK members-p38 也是屬於 tyr/thr kinase，會因加入 mitogen 而活化，調節 cell cycle 的進行。當細胞遭受到壓力因子的刺激，p38 也能藉由活化下游的 ATF-2 而增進細胞的存活率（62)。由實驗結果發現 esculetin 刺激 HepG2 cells 9-12 小時，p38 的活性降低。

【Fig. 32】Effect of esculetin on p-JNK of HepG2 cells

和 p38 一樣, JNK 也是屬於 stress-activated group of mitogen activated protein（SAPK)。活化的 JNK 會促使下游轉錄因子 c-Jun 磷酸化而讓細胞免除一些外來壓力刺激所誘導的 death signal pathway(62)。實驗結果顯示 esculetin 對於 HepG2 cells 中 JNK 的活性沒有影響。

【Fig. 33】Effect of esculetin on eIF4E of HepG2 cells

轉譯因子 eIF4E 是 c-myc target gene 之一，Esculetin 能降低 c-myc 的轉錄效率，但在這裡結果顯示 eIF4E 蛋白並不受 c-myc/Max 降低所影響。

【Fig. 34】Effect of esculetin on eIF4E translation activity of HepG2 cells

Esculetin 對於 c-myc 下游的 eIF4E 沒有影響。根據文獻指出 ERK 和 p38 能透過磷酸化轉譯因子 eIF4E，調節蛋白合成。由實驗結果發現，eIF4E 在處理 esculetin9-12 小時後，serine phosphorylation 的程度下項。

六. Esculetin 本身能造成肝癌細胞 HepG2 某個程度的自然凋亡，且能增強 paclitaxel 對 HepG2 細胞的毒殺效率。

本實驗室已經證實 esculetin 能導致血癌細胞株 HL-60 走向 apoptosis（51)。所以我們進一步研究 esculetin 在人類肝癌

細胞株 HepG2 能否誘導自然凋亡的發生。

【Fig. 35】Induction of apoptosis in HepG2 cells by esculetin

Apoptosis 一個重要的特徵是 DNA 會發生斷裂的情形。所以我們以一種能偵測 DNA 斷裂的 ELISA KIT 來分析 esculetin 誘發 HepG2 cells apoptosis 的情形。結果顯示，以 100μM esculetin 刺激 HepG2 cells 24 小時後即能促使細胞凋亡，此一現象隨著劑量的增高以及作用時間的加長而呈現一正相關性。

【Fig. 36】Effect of esculetin on p-Akt of HepG2 cells

正常情況下 EGF 除了透過 Ras/Raf/MAPK 來調控 cell cycle 的進行，另外一條 signal pathway-PI3K/Akt 對於 G1/S transition 以及抗凋亡的能力也相當重要（63)。過去文獻指出，PI3K/Akt 能抑制 p27 的表現，使得 cyclins/cdks 活性不被抑制，使細胞由 G1 進入 S 期(64)。先前的實驗結果發現，在 esculetin 刺激下 p27 的蛋白表現增加，所以在本實驗我們做了一個假設：esculetin 能否影響 PI3K/AKT 這條路徑來誘導 apoptosis 的發生。初步結果顯示，HepG2 cells 中 Akt 的活性的確會因著加入 esculetin 而被抑制。

【Fig. 37】Effect of esculetin on pBAD of HepG2 cells

進一步我們再去研究 Akt 下游的 BAD 蛋白表現情形。當 Akt 無法磷酸化 BAD 時，其能取代掉 Bax 而和 Bcl-xl 結合，導

致細胞發生 apoptosis（65)。**結果顯示，**BAD **磷酸化的程度確實因著** esculetin **的刺激而增高。**

【Fig. 38】【Fig. 39】Enhance cytotoxicity of chemotherapeutic drugs in HepG2 cells

綜合以上的結果, esculetin **能抑制人類肝癌細胞** HepG2 **的增生，並誘導** apoptosis **的現象。實驗最後我們將** esculetin **和現今廣泛使用在臨床上的抗癌藥** paclitaxel **與** cisplatin **合用，期盼有助於這些** anticancer drugs **藥效的提升。實驗設計上我們以單獨處理** paclitaxel **與** cisplatin **或個別加入** esculetin 100μM **共同作用** 24、48 **小時，並以** MTT **法分析細胞存活的情形。結果顯示在** 24 **小時，**esculetin **即能顯著的增強** paclitaxel **對** HepG2 cells **的毒殺作用。**

【Fig. 40】Induction of apoptosis in HepG2 cells by esculetin plus chemotherapeutic drugs

根據過去的研究指出 paclitaxel **與** cisplatins **能透過誘導癌細胞發生** apoptosis **的作用達到抗癌的效果（**54,55)。**當加入** esculetin **與這些抗癌藥一同處理** 24 **小時後發現，**esculetin **能促使** paclitaxel induced HepG2 cells apoptosis **的程度上升，對於** cisplatin **並沒有顯著的影響。**

【Fig. 41】Effect of CPP32 activity in HepG2 cells by esculetuin plus paclitaxel

由先前的實驗數據得知 esculetin 能促使 HepG2 cells 發生自然凋亡的發生，而 caspase 3（CPP32）的活化與 apoptosis 有關(66)，所以在這個實驗中我們發現處理 100μM esculetin 24 小時，CPP32 活性增高，且隨劑量的增加而上升。另外 esculetin 也能 enhance paclitaxel 誘導 CPP32 的活性。

【Fig. 42】Induction of apoptosis in HepG2 cells by cisplatin

由【Fig. 40】結果顯示，cisplatin 處理 24 小時後，誘導人類肝癌細胞走向 apoptosis 的效率不高，所以在此我們進一步將 HepG2 處理 cisplatin 48 小時後發現其 apoptosis 的程度與過去所報告的文獻有相似的結果（55)。以此來證明本次所用的藥物是具效用的。

討　　論

本篇研究指出馬栗樹皮素(esculetin)抑制人類肝癌細胞 HepG2 cells 的增生乃是透過影響許多分子的所調控（附件 1)。當 HepG2 細胞以 esculetin 刺激後使得 Rb 在早期（12-24 小時）磷酸化的程度降低，雖然 esculetin 對 cyclin D1 或 cdk4/6 沒有影響，但 p16 及 p21 在 12 小時表達的蛋白量很高，由此推測 p16 和 p21 抑制 cyc D_1-cdk4/6 的活性，導致 Rb 的磷酸化降低。當 Rb 處在一個 hypophosphorylation 的程度會緊緊的抓住 E2F-1，此時 E2F-1 無法以 free-form 的形式和 DP-1 結合，降低下游蛋白的表達。

p27 在 12-36 小時上升，除了降低 cyclin D_1/cdk4/6 活性，同時抑制了 cyclin E/cdk-2 的活性，然而細胞要由 G1 完全進入 S 期，必須藉由cyclin E/cdk-2 使 Rb full-phosphorylation 以釋出更多的 E2F-1 供 cell cycle 進行所需的蛋白表達（67)。在此我們做了一個推論：當 HepG2 以 esculetin 處理的前期（12-24 小時）會很快的誘導大量 CKI 的產生而抑制 cyclin D_1/cdk4/6 和 cyclin E/cdk-2，進而讓 Rb/E2F-1 穩定結合降低轉錄效率，導致後期（24-48 小時）cyc E 表現降低，使得細胞生長停滯在 G1 期。

另外有報告指出 cyclin E 的蛋白表現也受到 c-myc 所調節（68)。由

實驗結果發現 esculein 對 c-myc 的蛋白表現沒有影響但卻能抑制 c-myc/Max 的形成，使得轉錄效率下降。c-myc 能被 cdk2/4 以及 ERK1 所磷酸化，而 Max 則由 p38 所調節。此一磷酸化的作用有助於 c-myc/Max 穩定的結合到 DNA E-box motif（69）。實驗中證實了 esculetin 能抑制 Raf/ERK 以及 p38 的活性而影響 c-myc/Max heterodimer 的形成。除了 cyclin E 外，c-myc 調節 G1 期進行的另一個下游蛋白 CDC25A 也顯著性的降低。當 cdk-2 受到 CAK 的催化後，能在 ^{14}Thr、^{15}Tyr 以及 ^{160}Thr 接上磷酸根，此時的 cdk-2 必須再藉由 CDC25A phosphatase 去掉 ^{14}Thr 和 ^{15}Tyr 位置的磷酸化而被完全的活化（70）。當 c-myc 轉錄活性降低，CDC25A 蛋白表現減少，使得 cdk-2 不活化。或許這也是 esculetin 導致 G1/S arrest 的原因之一。在未來的研究上可以直接利用 in vitro kinase assay 來分析 cdk2 及 cdk-4 的活性是否因著 CKI 上升或 CDC25A 減少而被抑制。

在這裡我們提出一個問題，esculetin 如何造成 CKIs 的增加？過去文獻指出，當細胞遭受外界刺激會誘發 p53 的表現，p53 能直接或間接誘導其他蛋白的表現而促進 p21 或穩定 p16（71,72）。由實驗結果證實，esculetin 能有效的刺激 HepG2 cells 中 p53 的表現而造成 p16 與 p21 的上升。另外廣泛性的 G1 cyclins/cdks inhibitor p27 的增高，我們認為可能是

透過增加其蛋白新合成以及分解降效率低所致。1999 Alvaro J. 等人所寫的一篇 review 指出 c-myc 能誘導一假想的蛋白產生，並與受 G1 期 cyclins/cdks 所磷酸化的 p27 結合, 導致 p27 走向 ubiquitination- dependent proteasome degradation（73)。另外實驗數據指出 esculetin 能阻斷 PI3K/Akt 的作用，由過去研究我們已知 PI3K/Akt 這條路徑會抑制 AFX/FKHR 的轉錄作用，使得下游的 p27 蛋白表現降低（74)。所以當 PI3K/Akt 訊息傳遞受阻，將導致 p27 合成增加。

Esculetin 除了能透過抑制基因的轉錄作用調控蛋白的表現，也能影響在蛋白轉譯層次。由實驗數據顯示，同樣是 c-myc 下游的轉譯因子 eIF4E 並沒有因著 c-myc/Max 作用的減少而影響蛋白表現。由文獻指出當 eIF4E 上游的 MNK1 被 Ras/ERK 或 p38 活化將使得 eIF4E 在 serine209 的位置接上磷酸根而能與其他轉譯因子（eIF4G/eIF4A）形成轉譯複合體，促使蛋白的合成（75)。由實驗結果顯示 esculetin 藉著阻斷 Ras/ERK 以及 p38 的作用降低了 eIF4E 的活性。而 PI3K/Akt 能抑制 4E-BP1 這個 Translation inhibitor，對轉譯效率的提升扮演一正向的調控。所以猜測 esculetin 或許也能透過 Akt 活性的降低而影響蛋白的轉譯。

在實驗最後我們對於 esculetin 是否能造成 HepG2 細胞的 apoptosis

也做了研究。由 cell apoptosis ELISA assay 中指出 esculetin 能誘導細胞凋亡的發生，而且可能是透過 Akt/BAD 這條路徑造成。另外 esculetin 能增加 paclitaxel 引發 HepG2 cell apoptosis 的程度，文獻指出 paclitaxel 乃是透過影響 mitochondria 釋出 cytochrome c，活化 caspase 3 所造成（76)。所以是不是除了 Akt/Bad 這條 mitochondrial-death pathway，esculetin 還能誘導其他 death signal 而 induce cell apoptosis？由稍前的實驗證明，處理 esculetin 的 HepG2 cells 其 p53 的表現明顯的上升，或許此一增高的 p53 會去誘導一些 membrane death receptor 的增加，而加強 paclitaxel 的效用。所以在未來我們除了進一步研究 esculetin 對粒腺體膜電位的影響以及 cytochrome c 釋放到細胞質的情形，也可以往 receptor-mediated caspase cascades 來探討 esculetin 誘發 apoptosis 的詳細機制。

綜合以上的討論我們在這裡做一個總結：加入 esculetin 初期（0-12 小時）能很快的誘發 CKI 的表現，抑制 cyclins/cdks 的活性，造成 Rb 磷酸化的減少，E2F-1 轉錄效率降低；同時，esculetin 阻斷了 Ras/ERK 和 p38 活性，影響 c-myc 和 eIF4E 的作用。在較晚期（24-48 小時），一些正向調節 G1 phase 進行的蛋白開始降低，這使得一些 CKI 的穩定性增加，c-myc/Max 結合能力減少。所以 esculetin 對於 HepG2 cells 的影響向

是透過一種循環式的放大抑制而造成 cell cycle arrest。

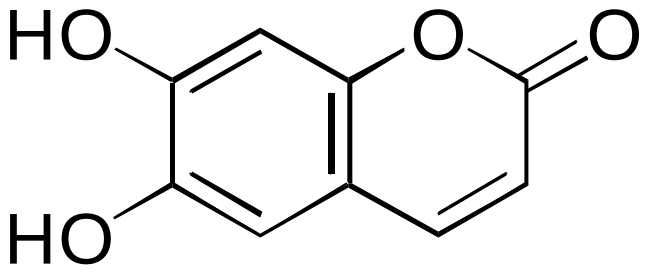

Figure 1 Structure of esculetin

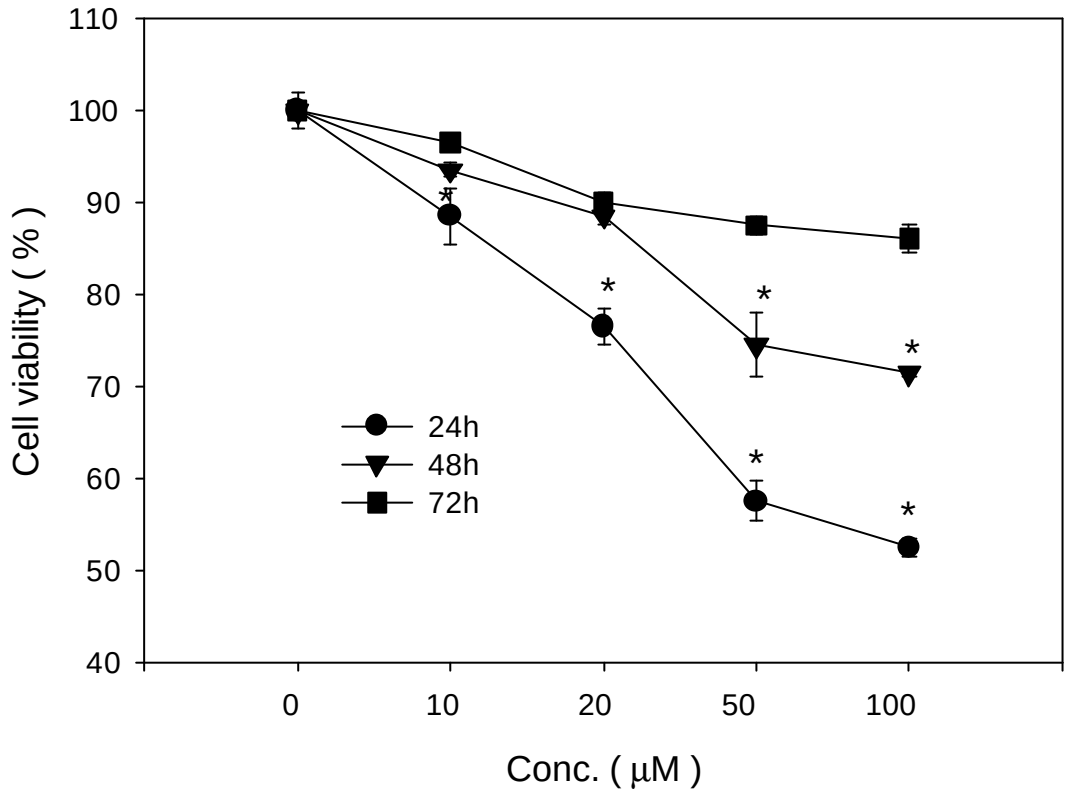

Figure 2. Cytotoxicity of esculetin in HepG2. Cells were cultured with various concentration of esculetin for the indicated times following treatment cells were then incubated with MTT for 4 hours. The optical density is then read at 560nm by a spectrophotometer. Data presented as means ± S.D. of three independent experiments. *$P < 0.05$, compared with control group (0.2% DMSO)

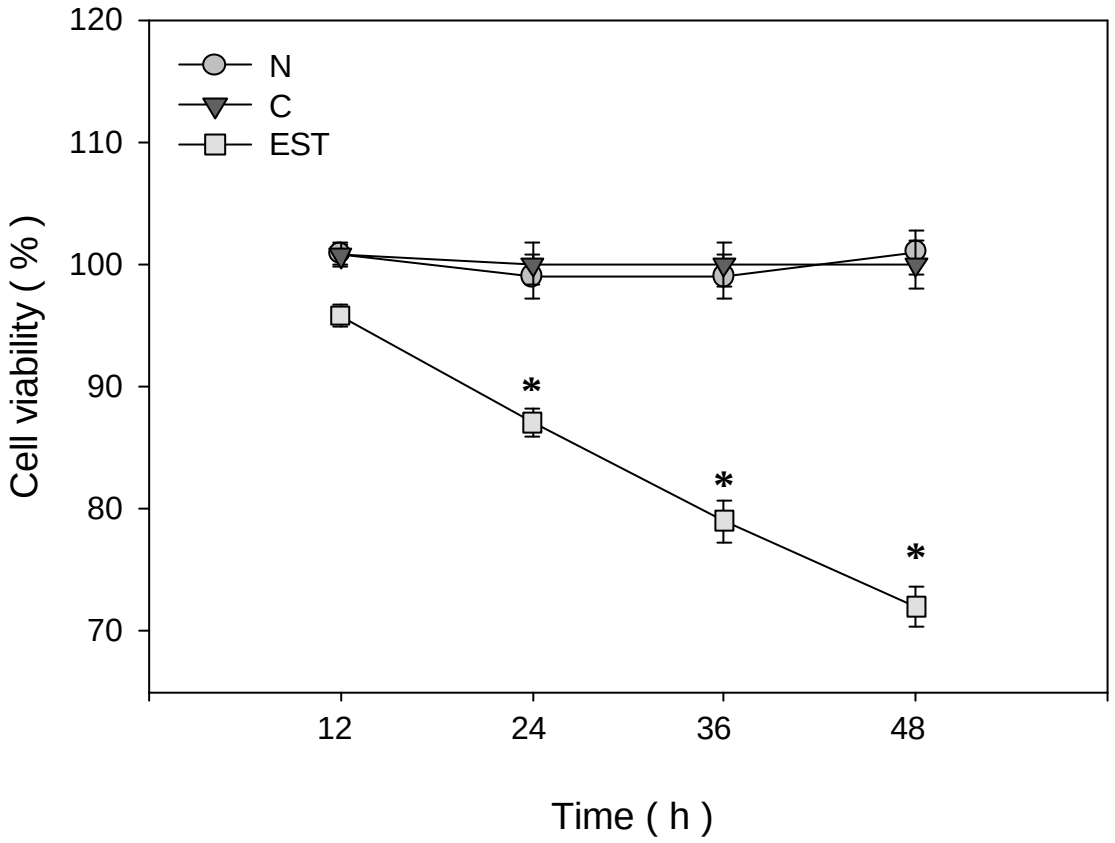

Figure 3. Cytotoxicity of esculetin in HepG2. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were then incubated with MTT for 4 hours. The optical density is then read at 560nm by a spectrophotometer. Data presented as means ± S.D. of three independent experiments. *$P < 0.05$, compared with control group (0.2% DMSO)

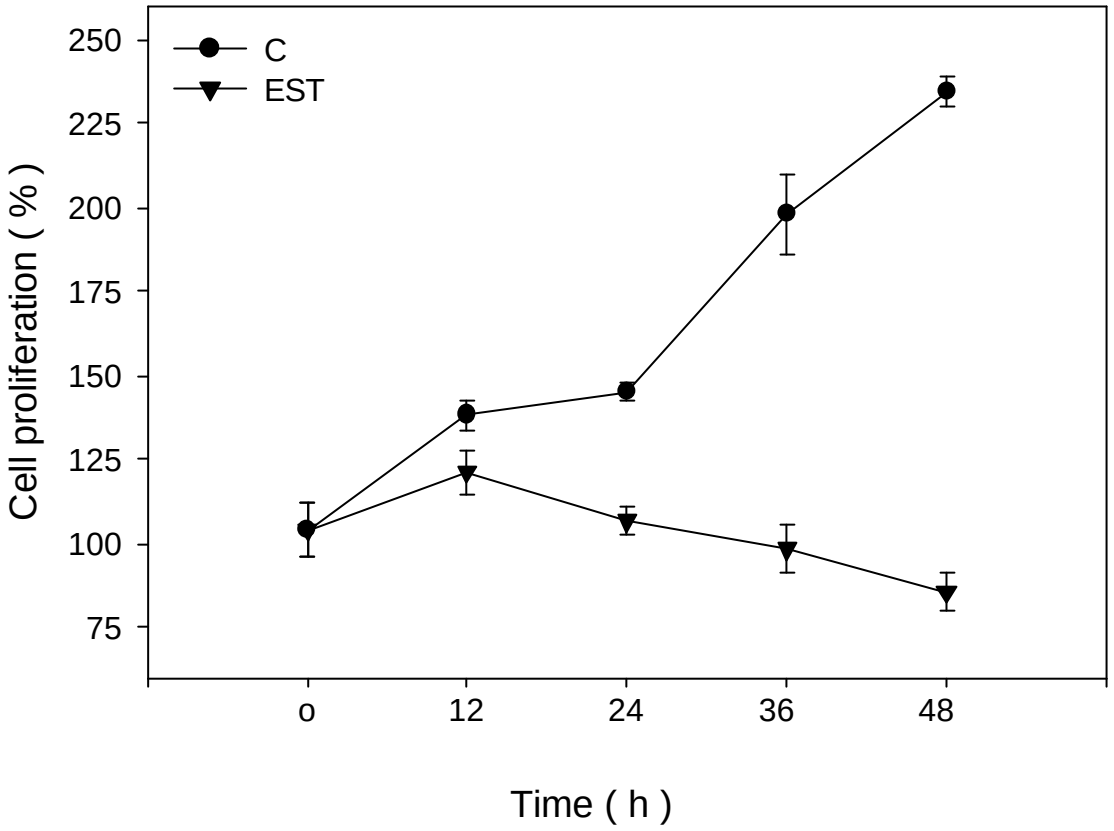

Figure 4. Effects of esculetin in HepG2. Cells were cultured with or without 100μM esculetin in the indicated times. The percentage of viable cells was determined using trypan blue dye exclusion assays. Data presented as means ± S.D. of three independent experiments.

Cell cycle	Control	Esculetin
G0/G1	62.9 %	73.2 %
S	10.8 %	4.8 %
G2/M	26.2 %	15.8 %

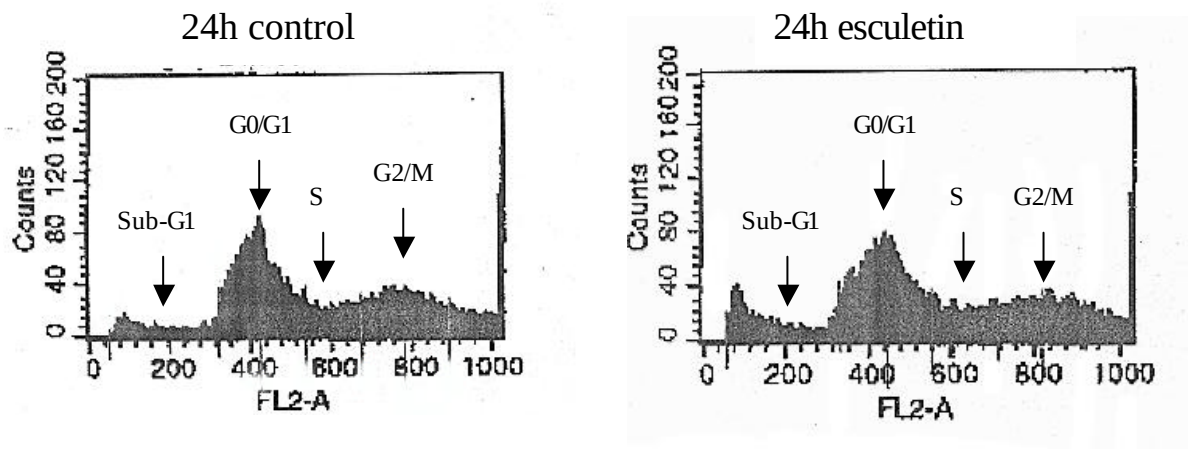

Figure5. Redistribution of cell cycle analysis of HepG2. Cells were treated with or without 100μM esculetin. After 24 hours cells were prepared for cell cycle analysis and assessed by flow cytometry.

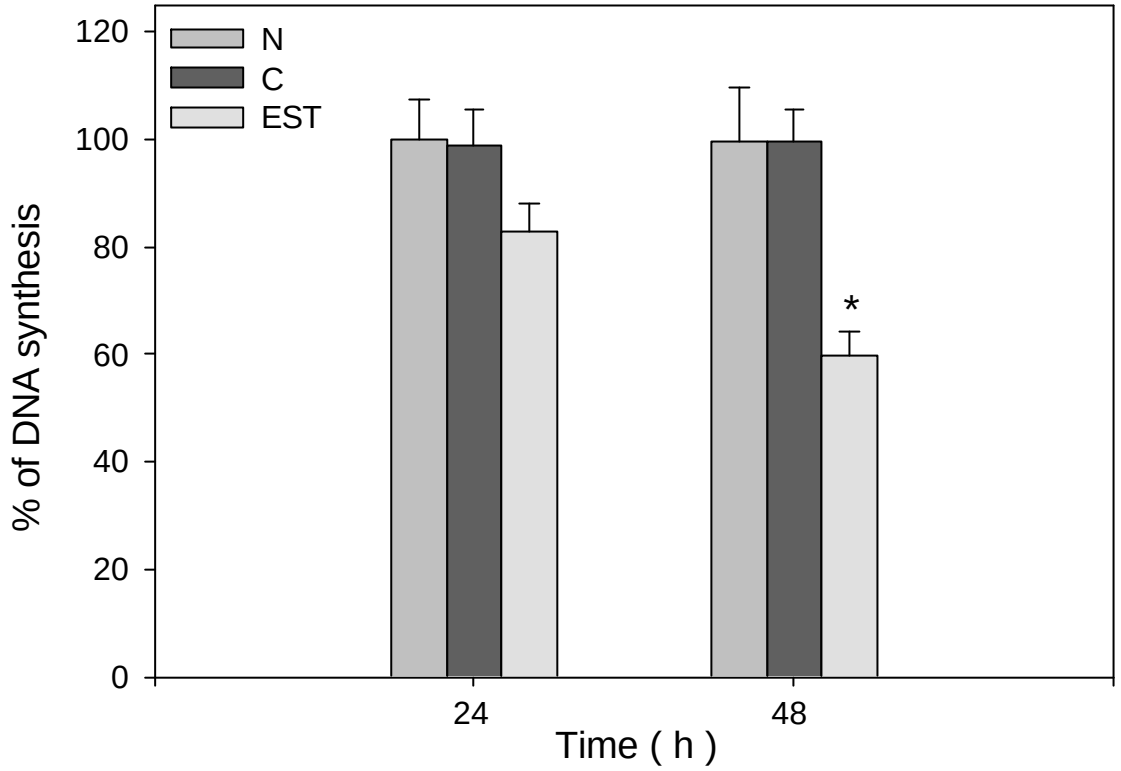

Figure 6. Effect of esculetin on [^{3}H] – thymidine incorporation in HepG2. Cells were incubated with 100μM esculetin and 0.5Ci/ml of [^{3}H] –thymidine for indicated times. The incorporation of [^{3}H] –thymidine in HepG2 was measured by a Scintillation Counter. Data presented as means ± S.D. of three independent experiments. *$P < 0.05$, compared with control group (0.2% DMSO).

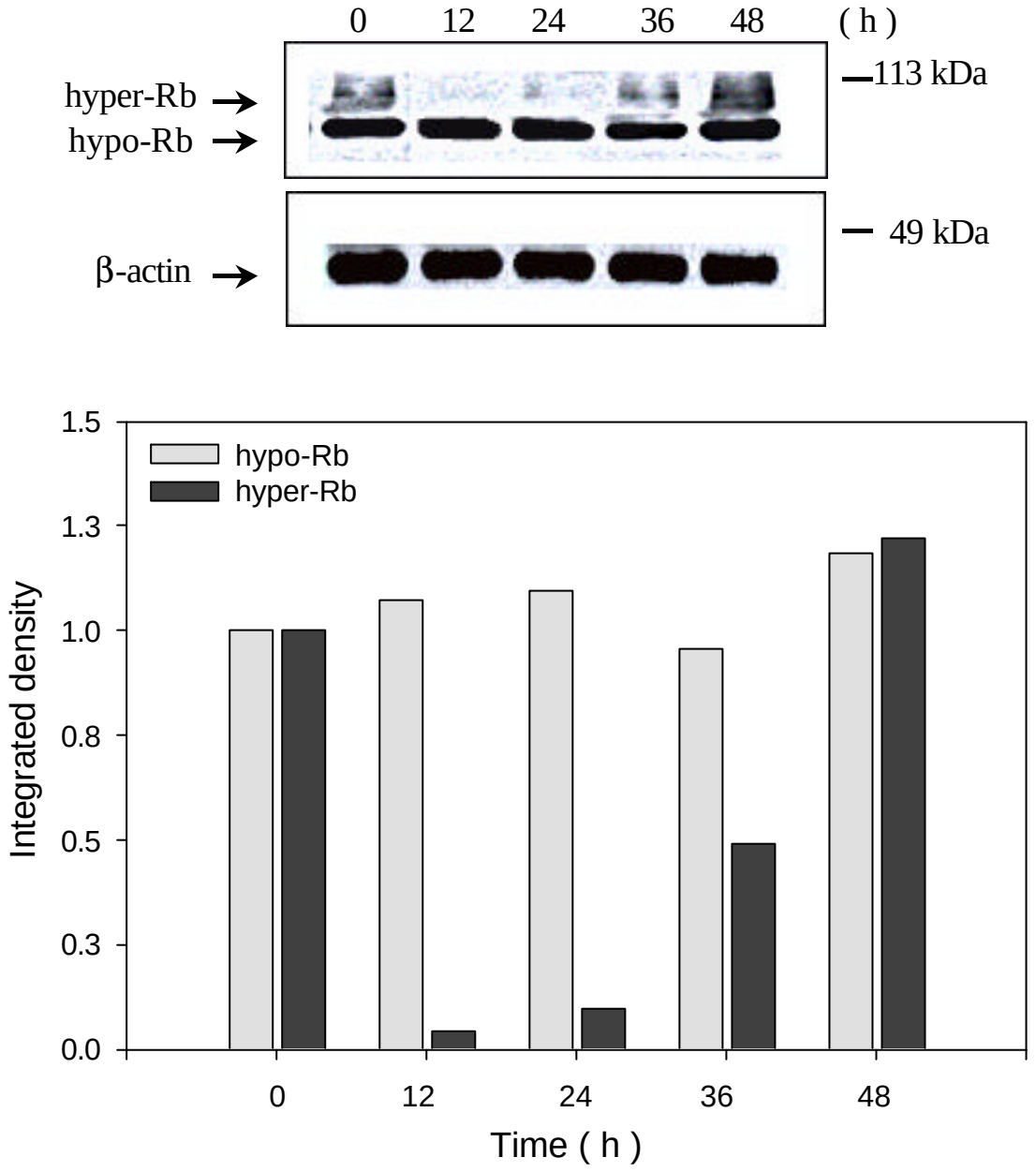

Figure 7. Time course effect of esculetin on Rb protein expression in HepG2. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and subjected to western blot analysis: 60μg of protein extract from each condition was electrophoresed in each lane of 8% SDS-PAGE and analysed by immunoblot with the anti-Rb antibody or actin used for equal loading.

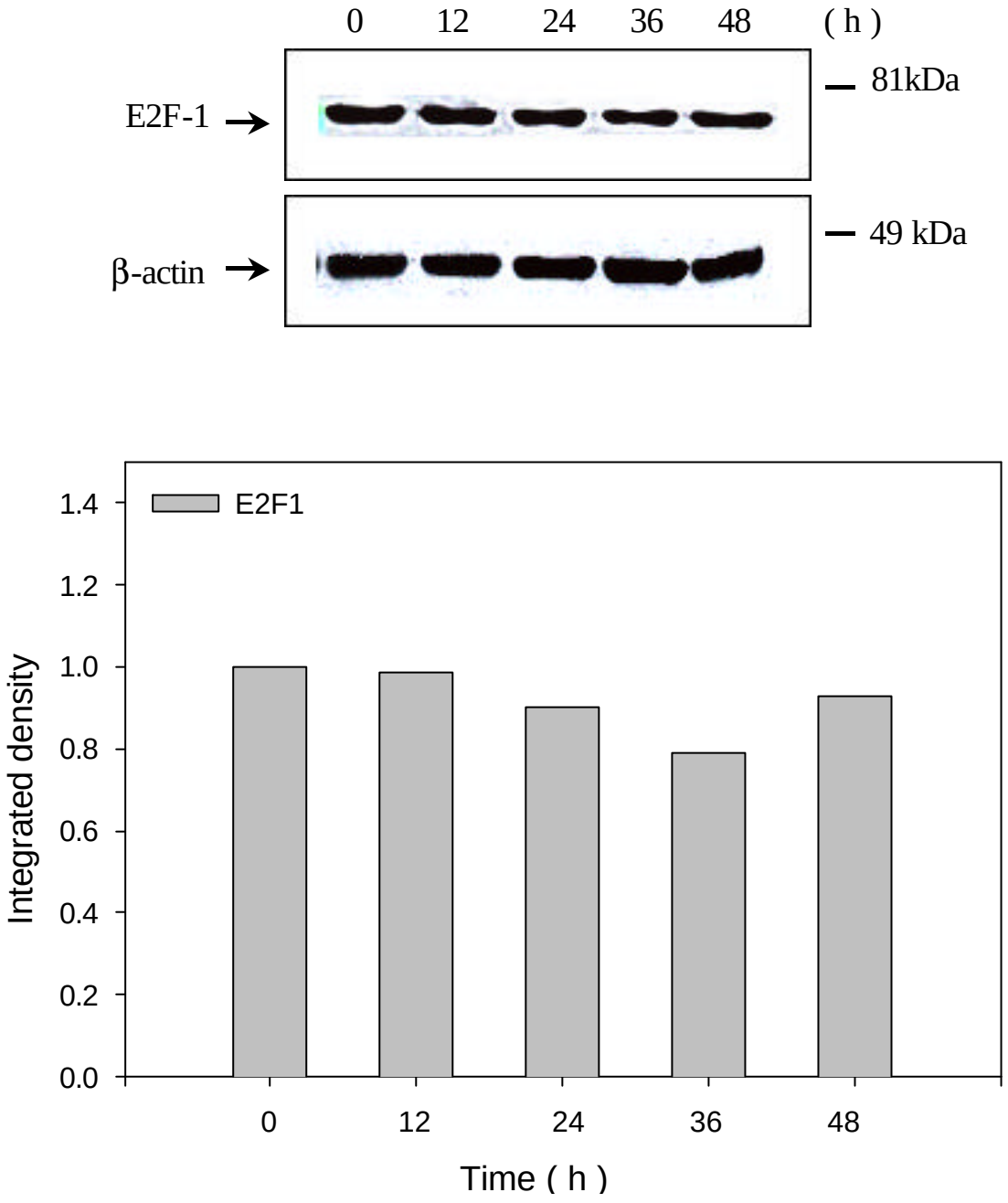

Figure 8. Time course effect of esculetin on E2F-1 protein expression in HepG2. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and subjected to western blot analysis: 60μg of protein extract from each condition was electrophoresed in each lane of 10% SDS-PAGE and analysed by immunoblot with the anti-E2F-1 antibody or actin used for equal loading.

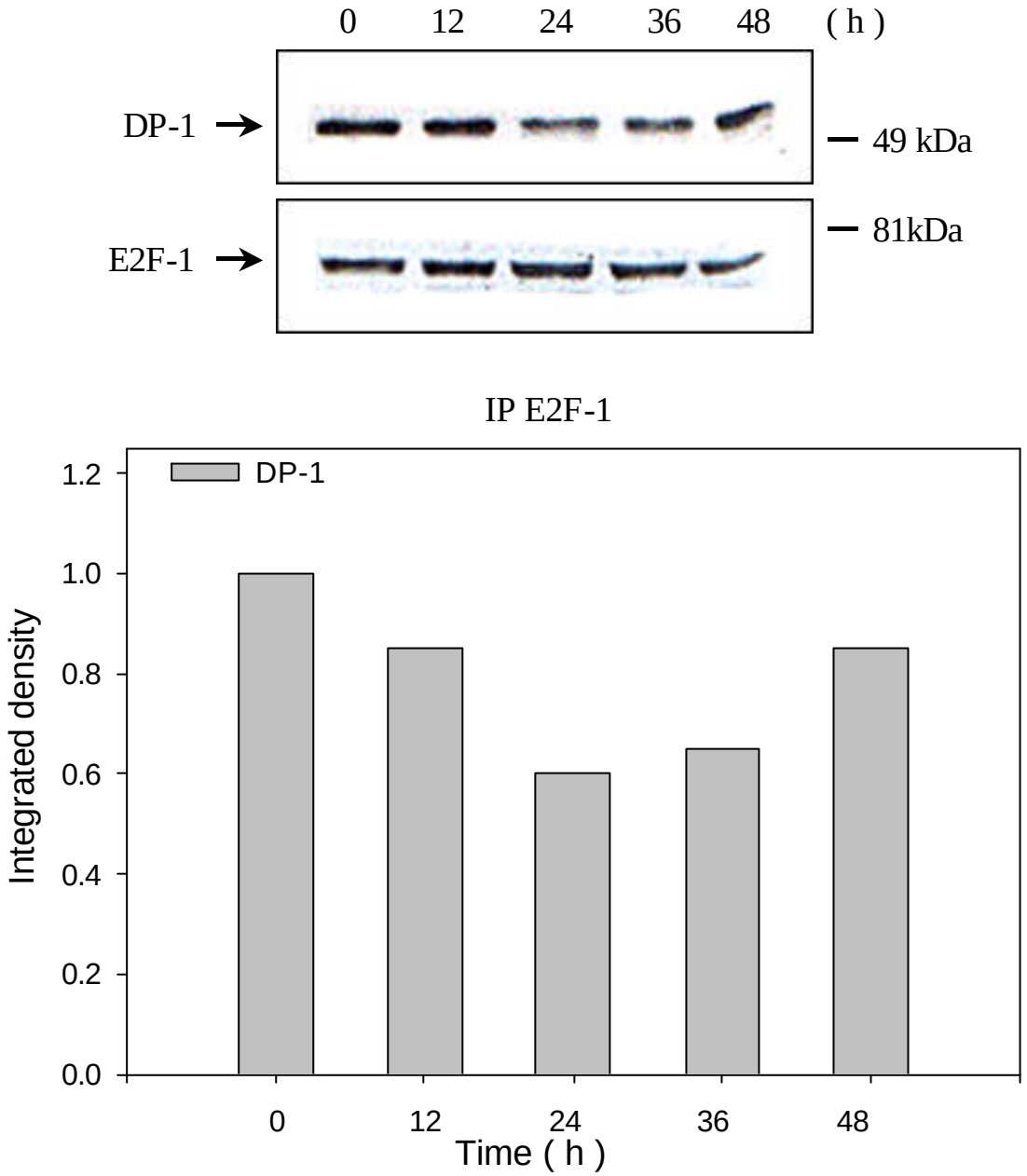

Figure 9. Time course effect of esculetin on DP-1 protein expression in HepG2. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and immunoprecipitation were performed with specific antibodies against E2F-1. E2F-1 immunoprecipitates were subjected to western blot analysis : 10% SDS-PAGE and analysed by immunoblot with the anti-DP-1 antibody or E2F-1 used for equal loading.

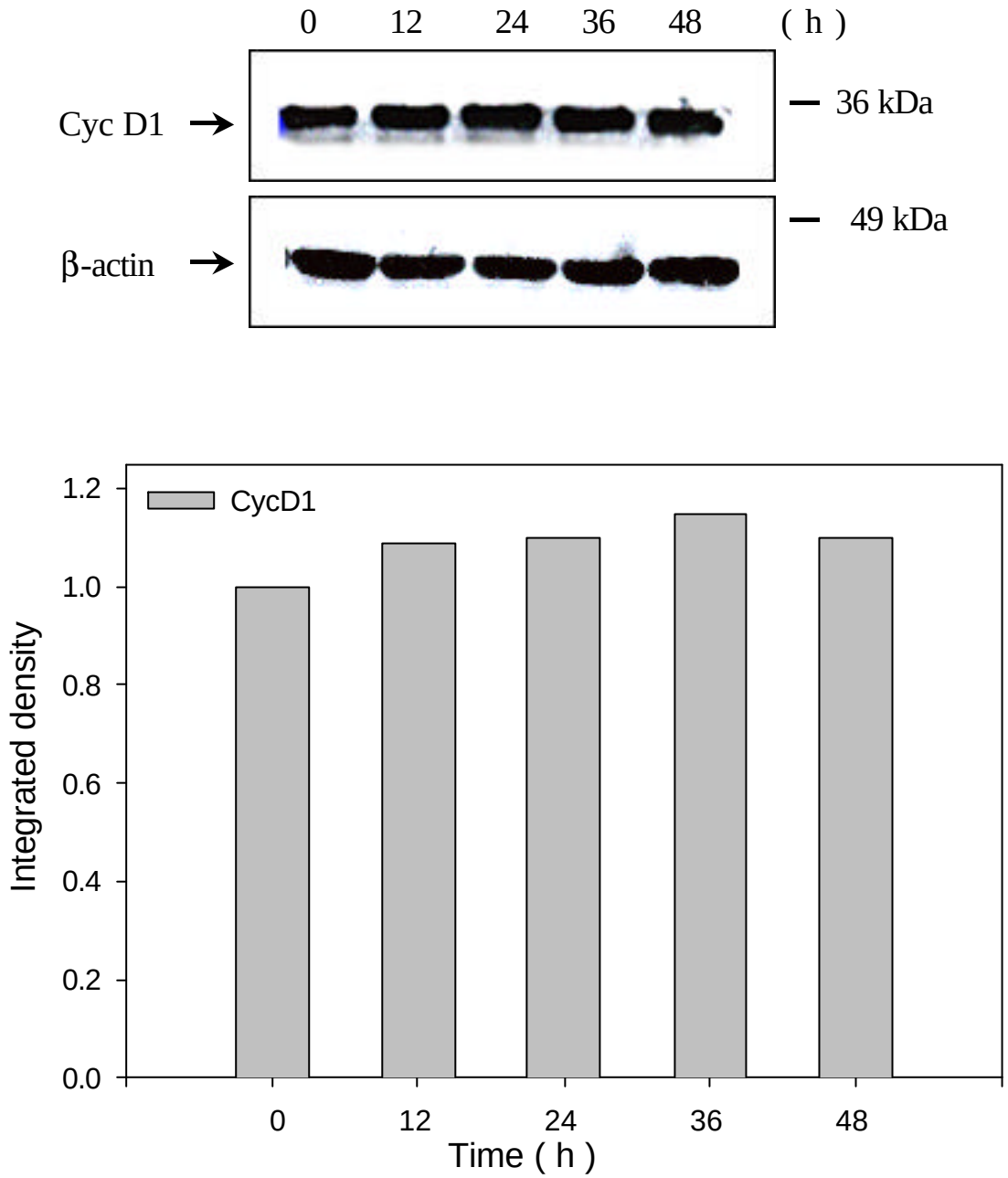

Figure 10. Time course effect of esculetin on cyc D1 protein expression in HepG2. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and subjected to western blot analysis: 60μg of protein extract from each condition was electrophoresed in each lane of 12% SDS-PAGE and analysed by immunoblot with the anti-cyc D1 antibody or actin used for equal loading.

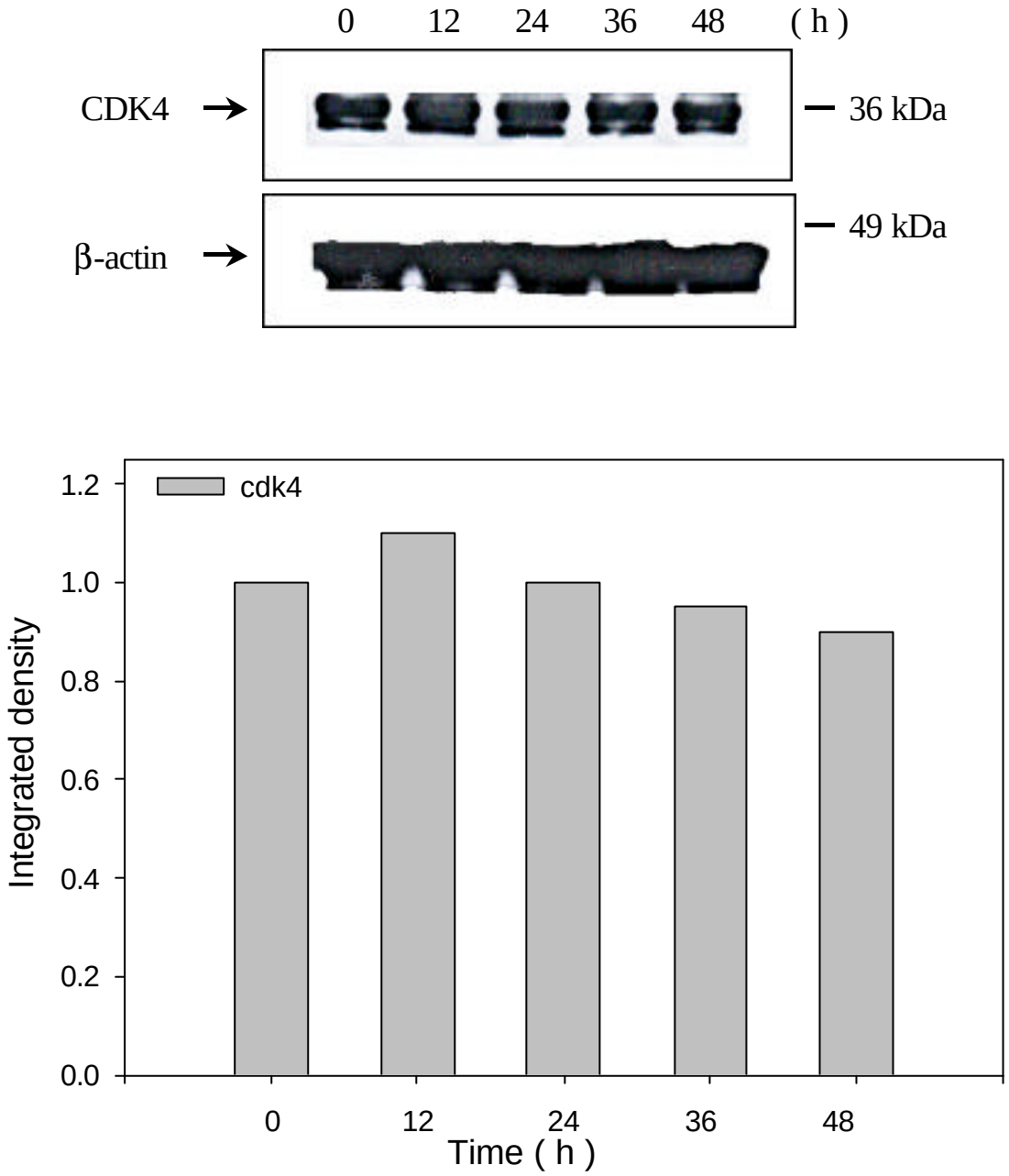

Figure 11. Time course effect of esculetin on CDK4 protein expression in HepG2. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and subjected to western blot analysis: 60μg of protein extract from each condition was electrophoresed in each lane of 12% SDS-PAGE and analysed by immunoblot with the anti-cdk4 antibody or actin used for equal loading.

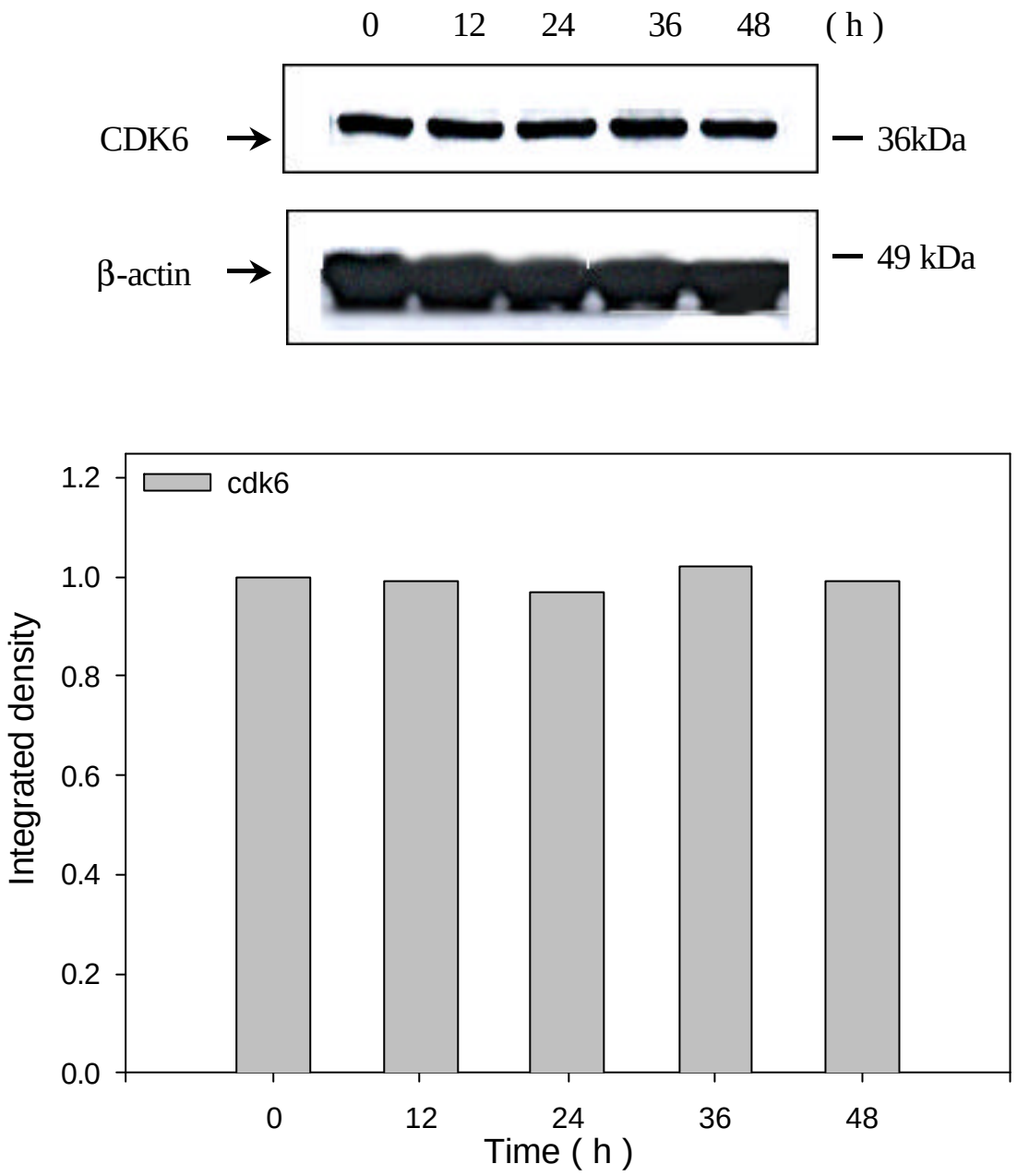

Figure 12. Time course effect of esculetin on CDK6 protein expression in HepG2. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and subjected to western blot analysis: 60μg of protein extract from each condition was electrophoresed in each lane of 12% SDS-PAGE and analysed by immunoblot with the anti-cdk6 antibody or actin used for equal loading.

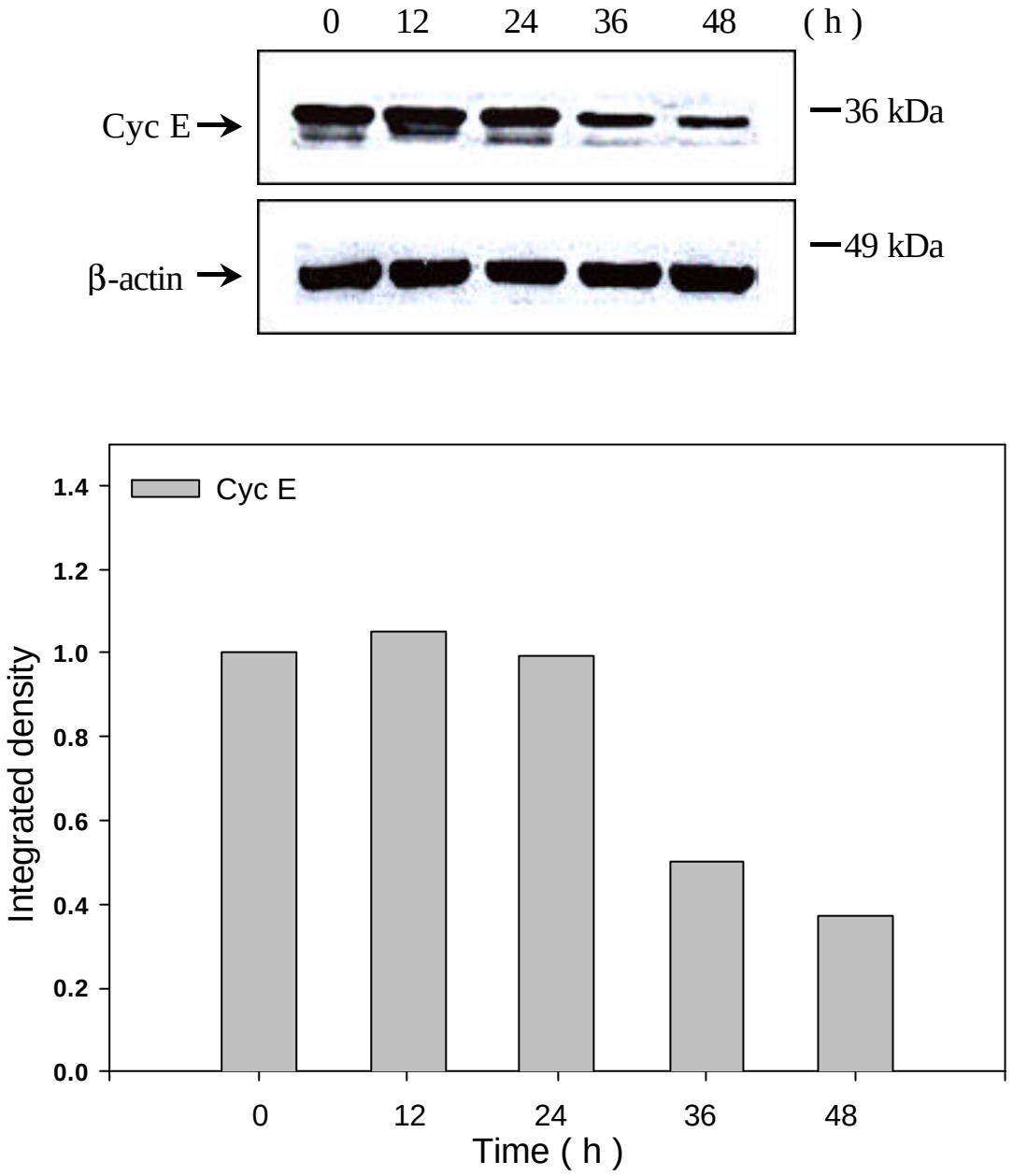

Figure 13. Time course effect of esculetin on cyc E protein expression in HepG2. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and subjected to western blot analysis: 60μg of protein extract from each condition was electrophoresed in each lane of 12% SDS-PAGE and analysed by immunoblot with the anti-cycE antibody or actin used for equal loading.

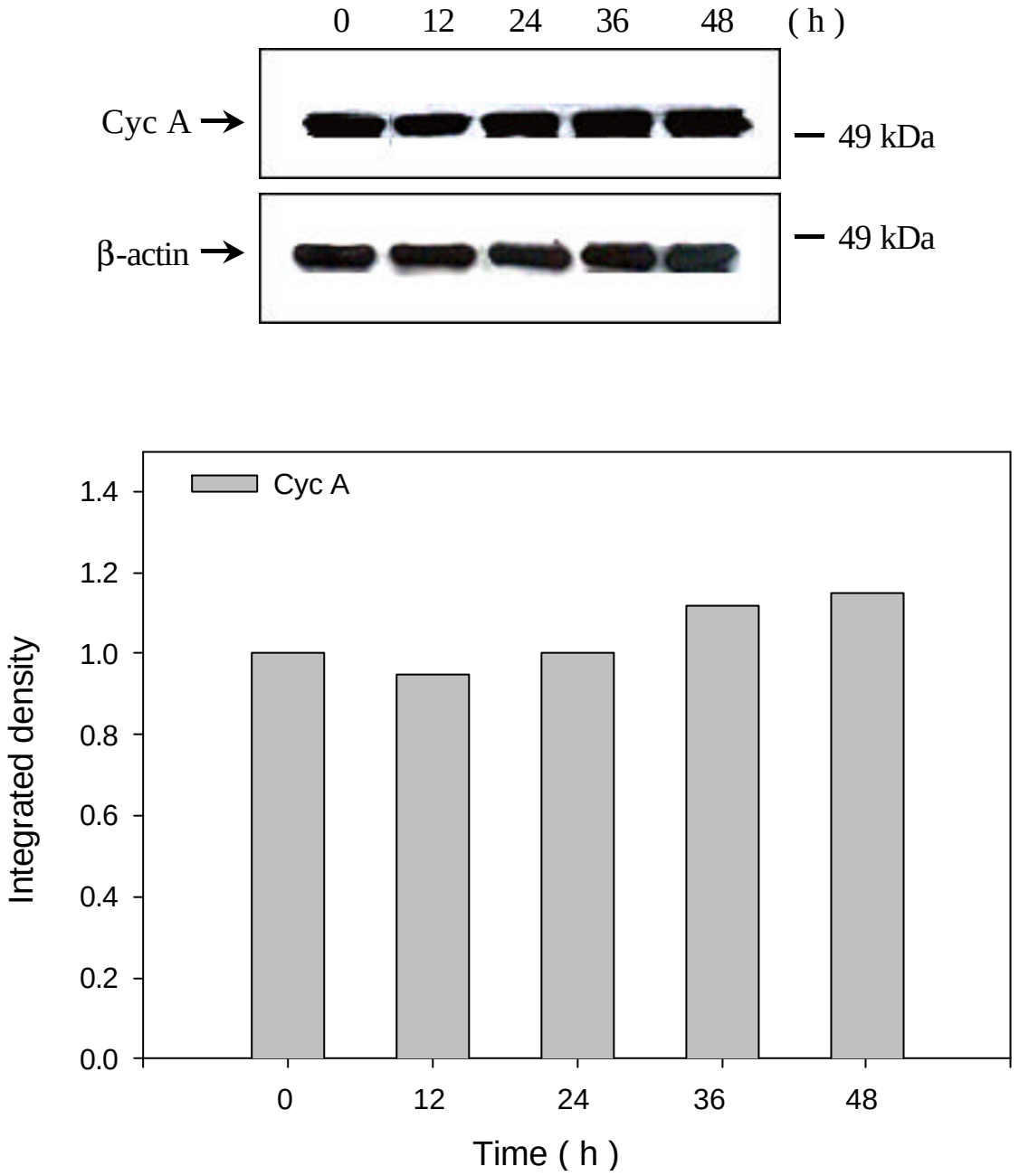

Figure 14. Time course effect of esculetin on cyc A protein expression in HepG2. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and subjected to western blot analysis: 60μg of protein extract from each condition was electrophoresed in each lane of 12% SDS-PAGE and analysed by immunoblot with the anti-cyc A antibody or actin used for equal loading.

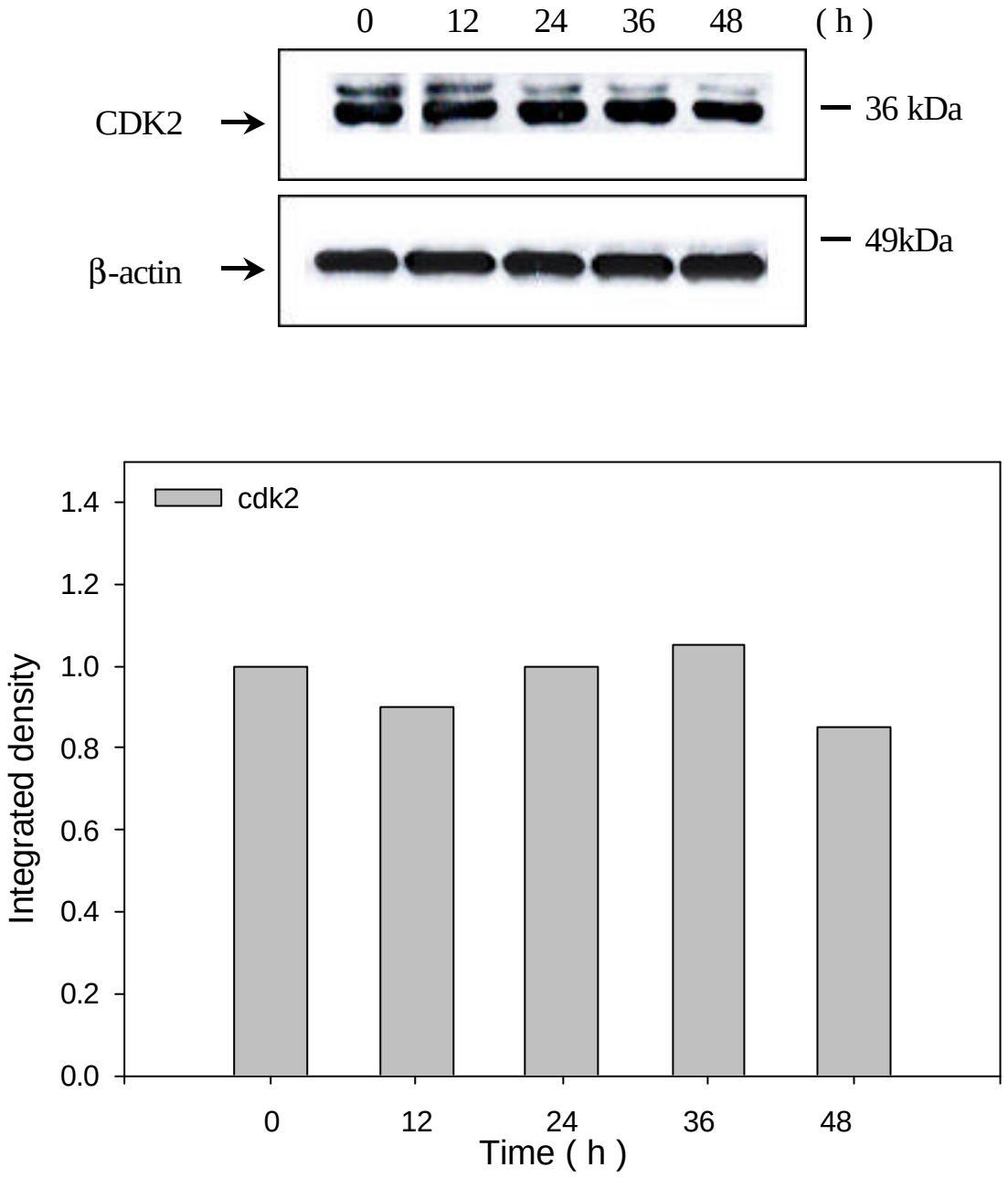

Figure 15. Time course effect of esculetin on CDK2 protein expression in HepG2. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and subjected to western blot analysis: 60μg of protein extract from each condition was electrophoresed in each lane of 12% SDS-PAGE and analysed by immunoblot with the anti-cdk2 antibody or actin used for equal loading.

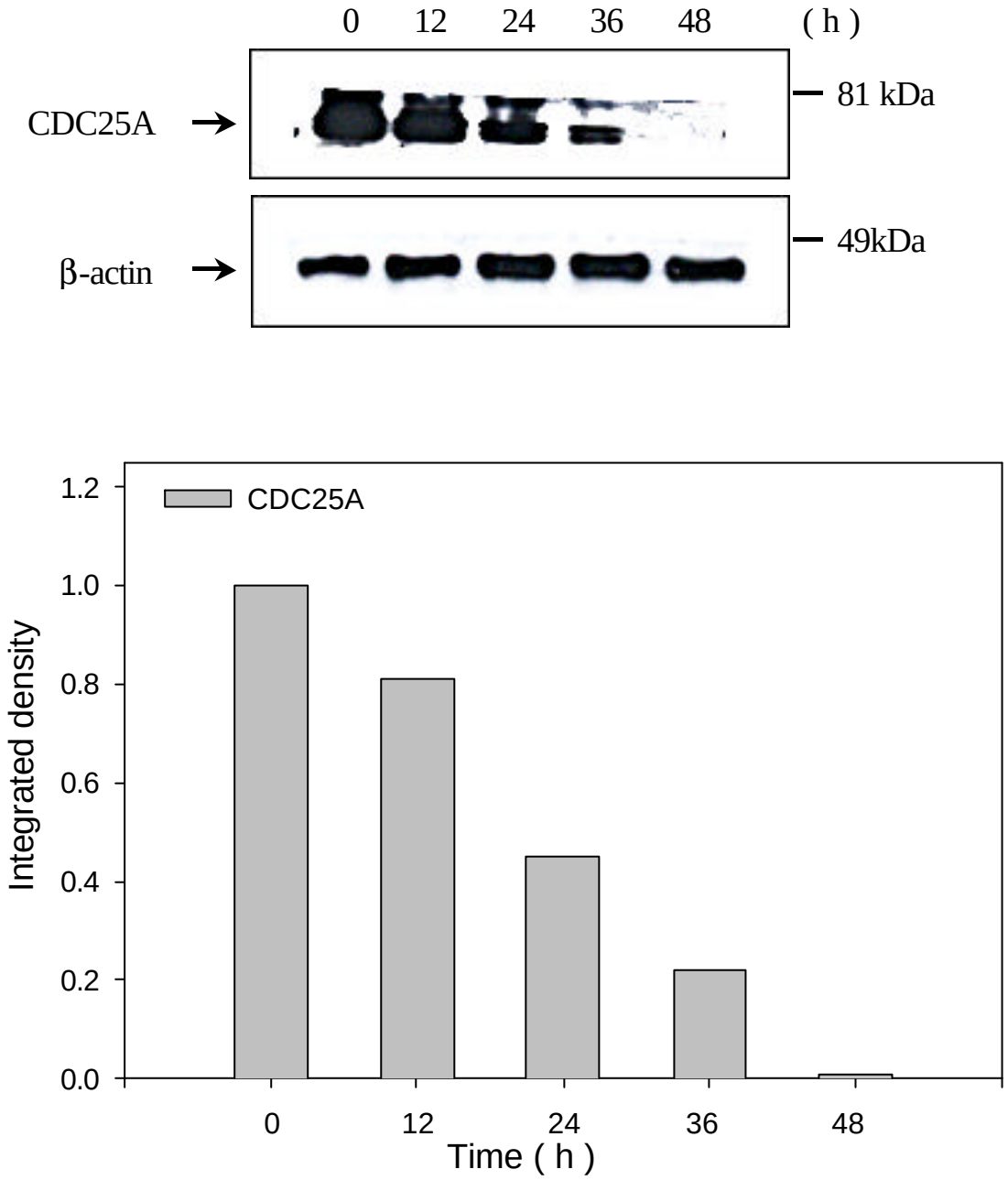

Figure 16. Time course effect of esculetin on CDC25A protein expression in HepG2. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and subjected to western blot analysis: 60μg of protein extract from each condition was electrophoresed in each lane of 10% SDS-PAGE and analysed by immunoblot with the anti-CDC25A antibody or β-actin used for equal loading.

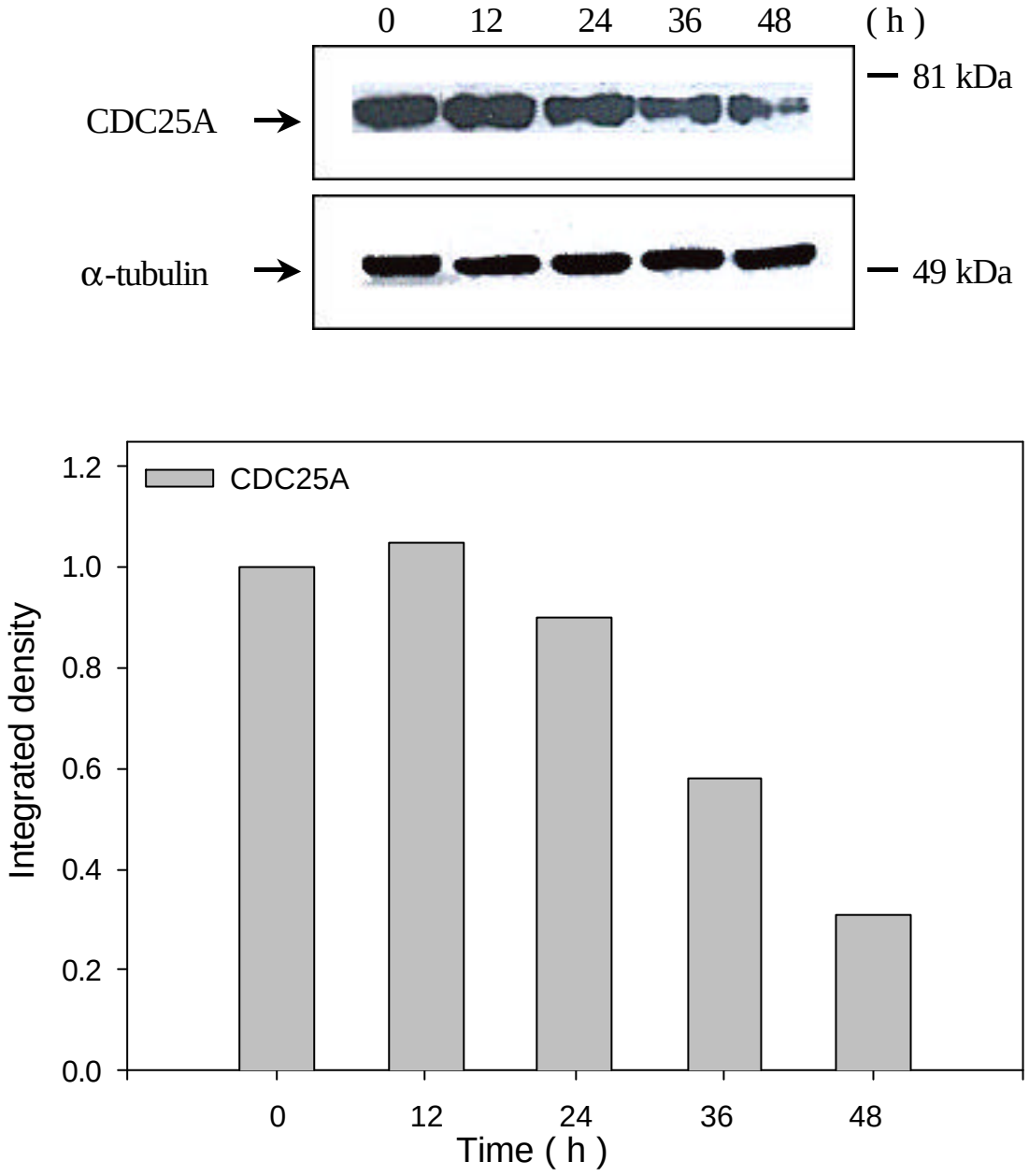

Figure 17. Time course effect of esculetin on CDC25A protein expression in H3B. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and subjected to western blot analysis: 60μg of protein extract from each condition was electrophoresed in each lane of 10% SDS-PAGE and analysed by immunoblot with the anti-CDC25A antibody or tubulin used for equal loading.

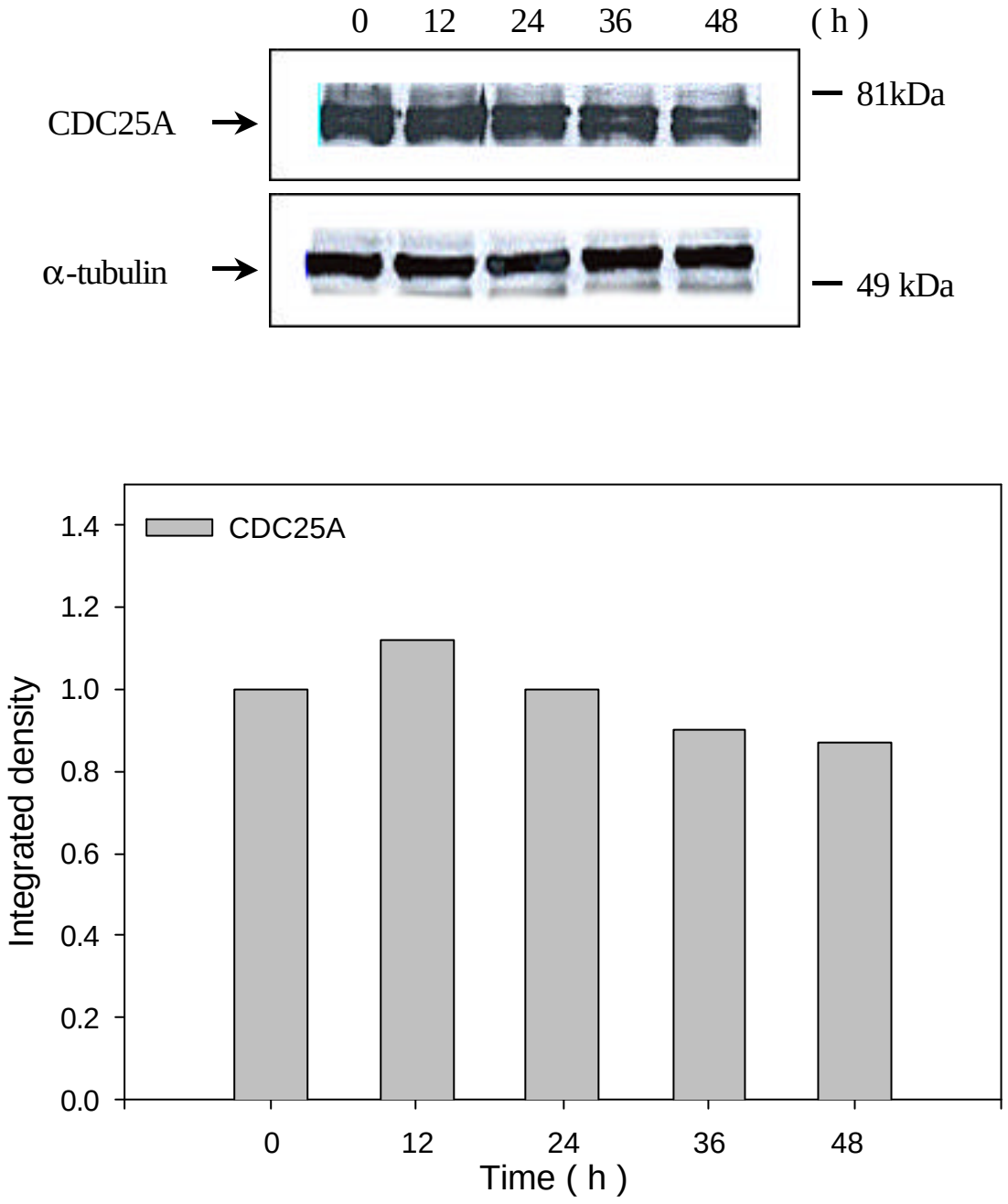

Figure 18. Time course effect of esculetin on CDC25A protein expression in HL-60. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and subjected to western blot analysis: 60μg of protein extract from each condition was electrophoresed in each lane of 10% SDS-PAGE and analysed by immunoblot with the anti-CDC25A antibody or tubulin used for equal loading.

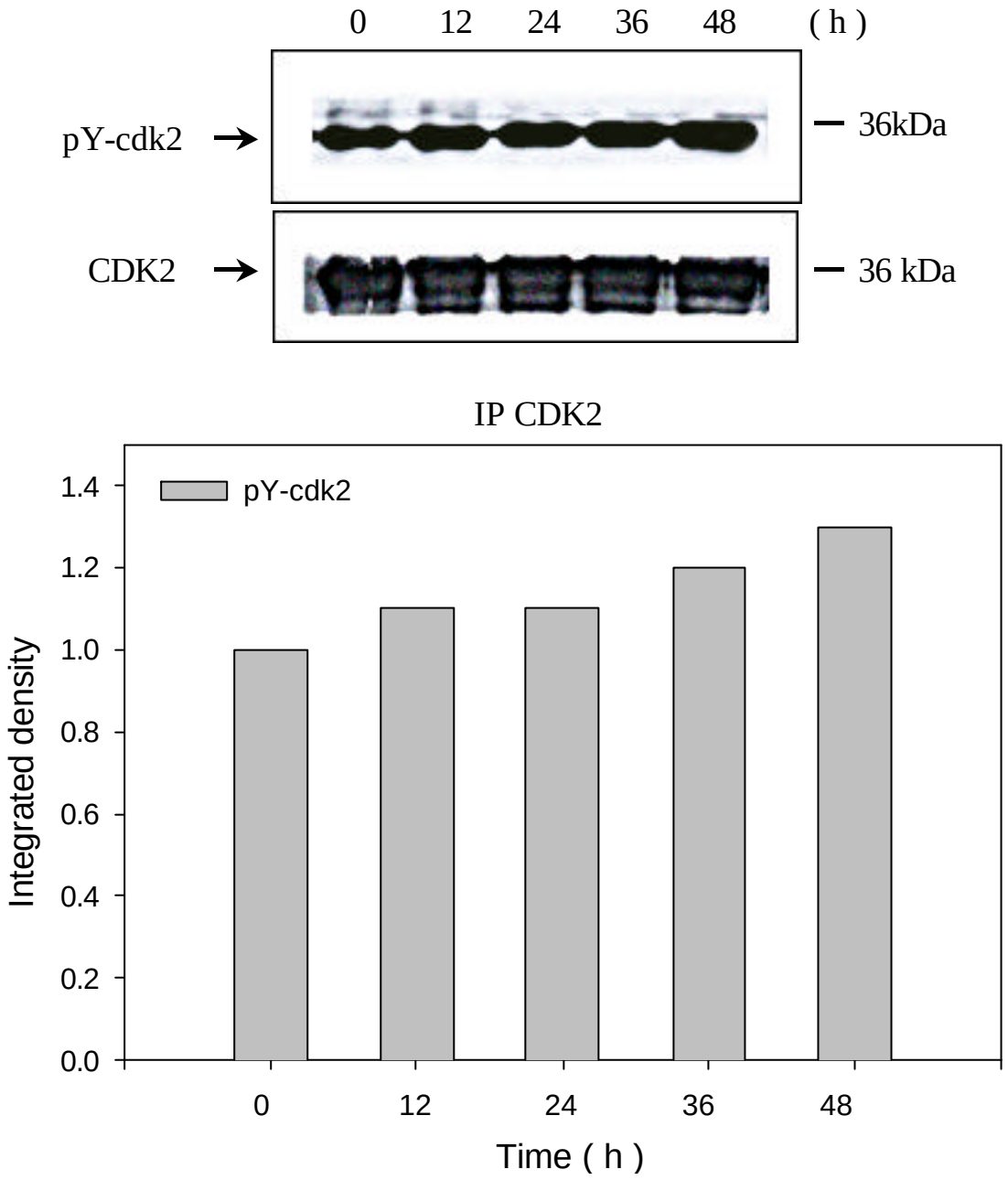

Figure 19. Time course effect of esculetin on pY-CDK2 protein expression in HepG2. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and immunoprecipitation were performed with specific antibodies against CDK2. CDK2 immunoprecipitates were subjected to Western blot analysis : 12% SDS-PAGE and analysed by immunoblot with the anti-py20 antibody or CDK2 used for equal loading.

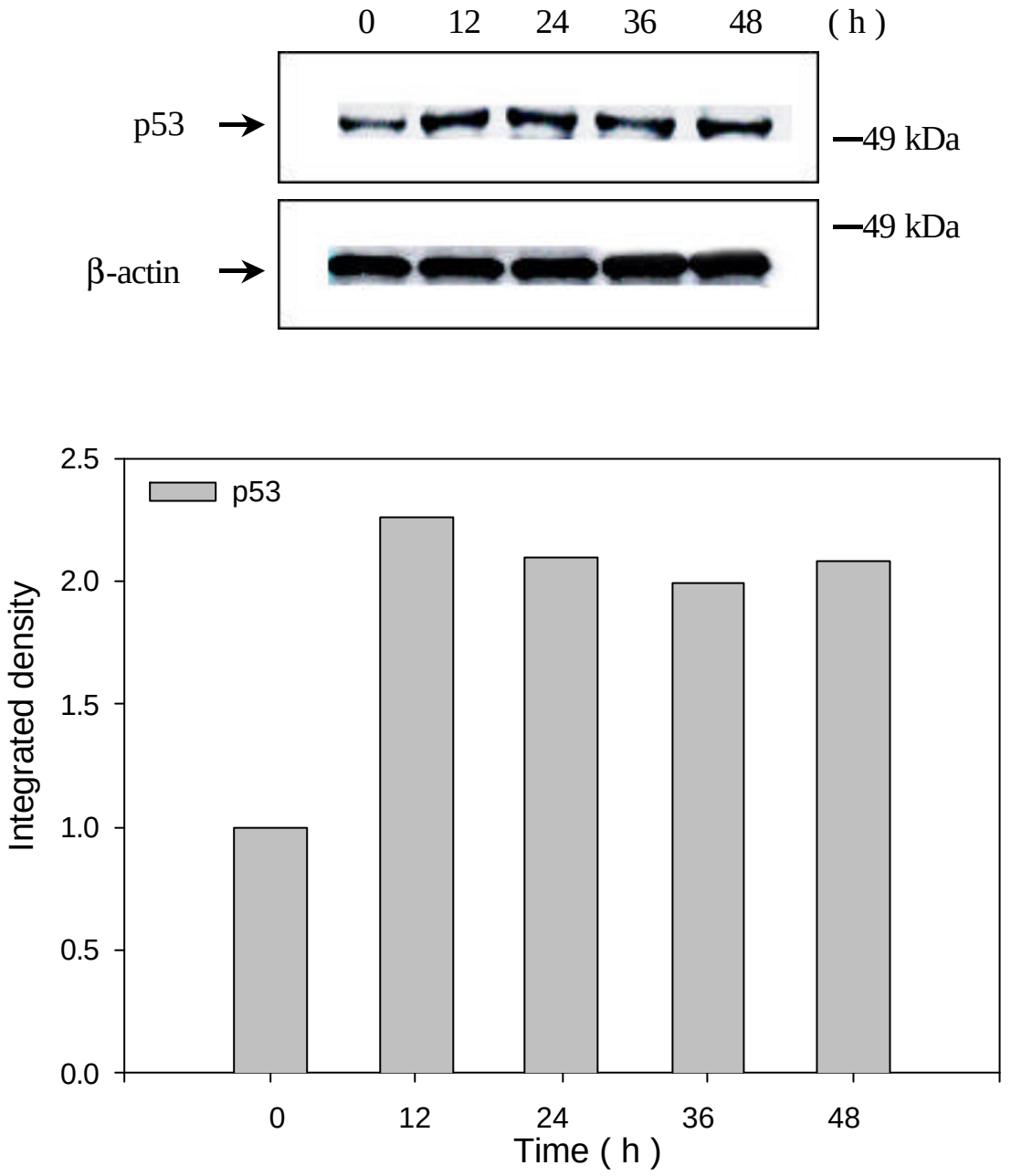

Figure 20. Time course effect of esculetin on p53 protein expression in HepG2. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and subjected to western blot analysis: 60μg of protein extract from each condition was electrophoresed in each lane of 12% SDS-PAGE and analysed by immunoblot with the anti-p53 antibody or actin used for equal loading.

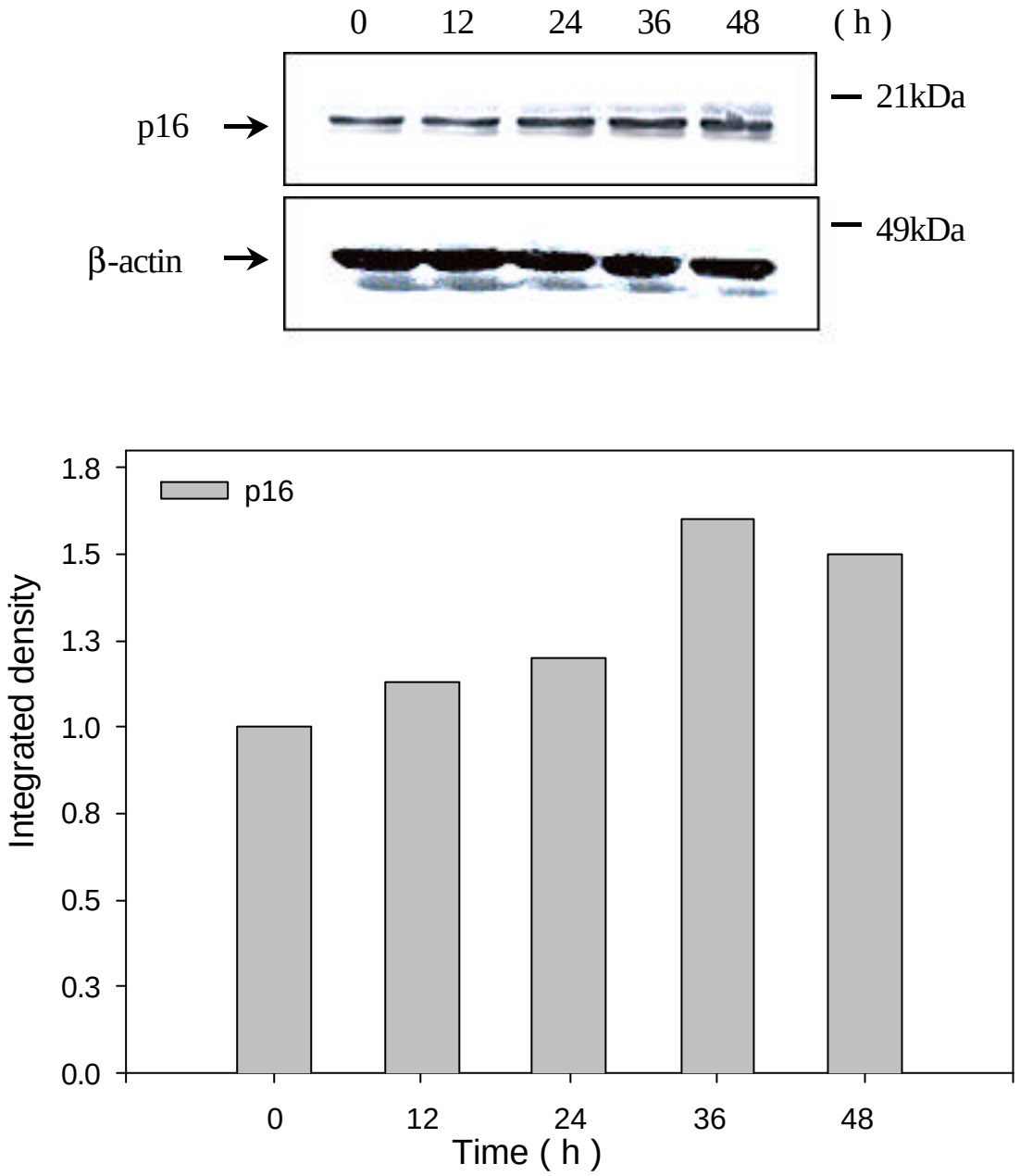

Figure 21. Time course effect of esculetin on p16 protein expression in HepG2. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and subjected to western blot analysis: 60μg of protein extract from each condition was electrophoresed in each lane of 15% SDS-PAGE and analysed by immunoblot with the anti-p16 antibody or actin used for equal loading.

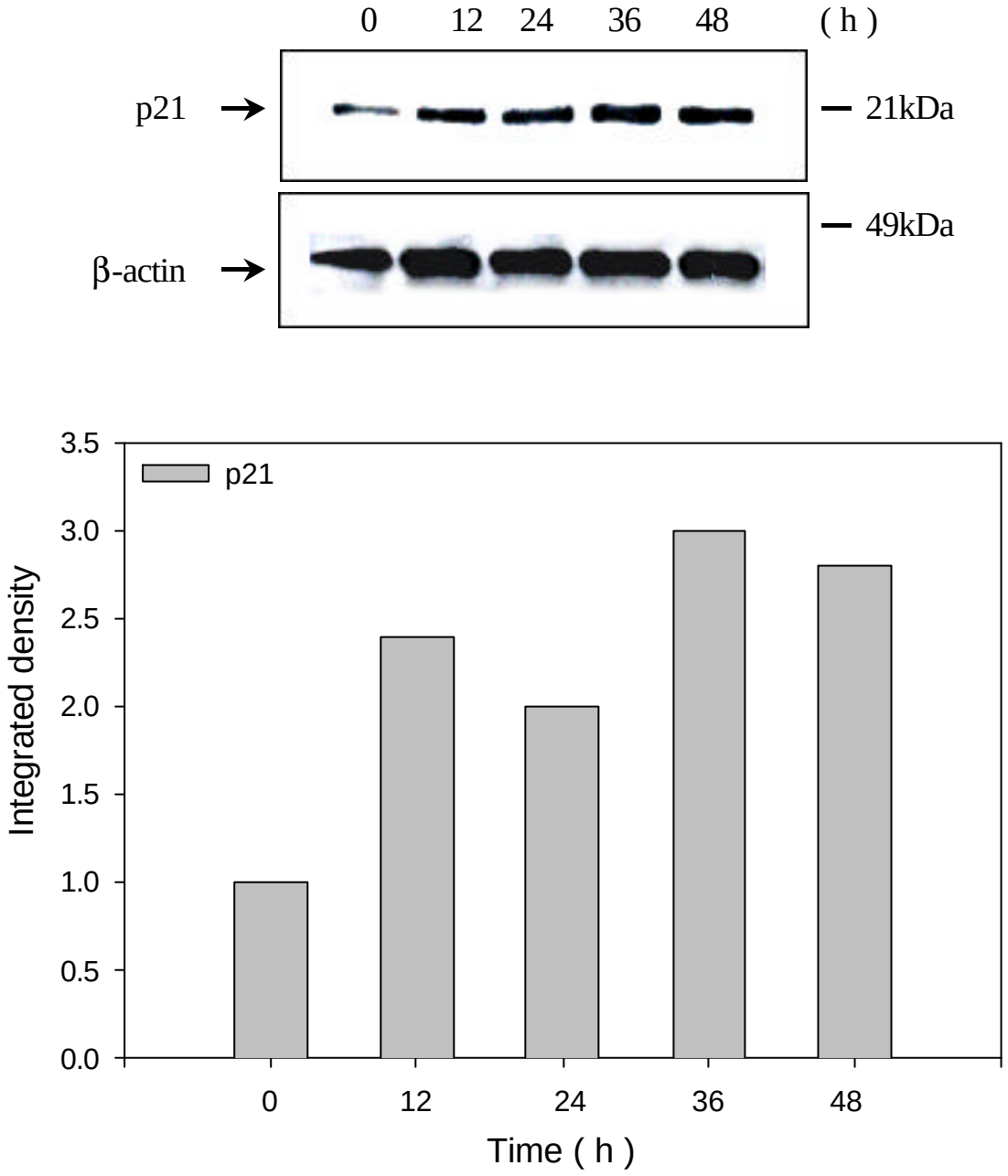

Figure 22. Time course effect of esculetin on p21 protein expression in HepG2. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and subjected to western blot analysis: 60μg of protein extract from each condition was electrophoresed in each lane of 15% SDS-PAGE and analysed by immunoblot with the anti-p21 antibody or actin used for equal loading.

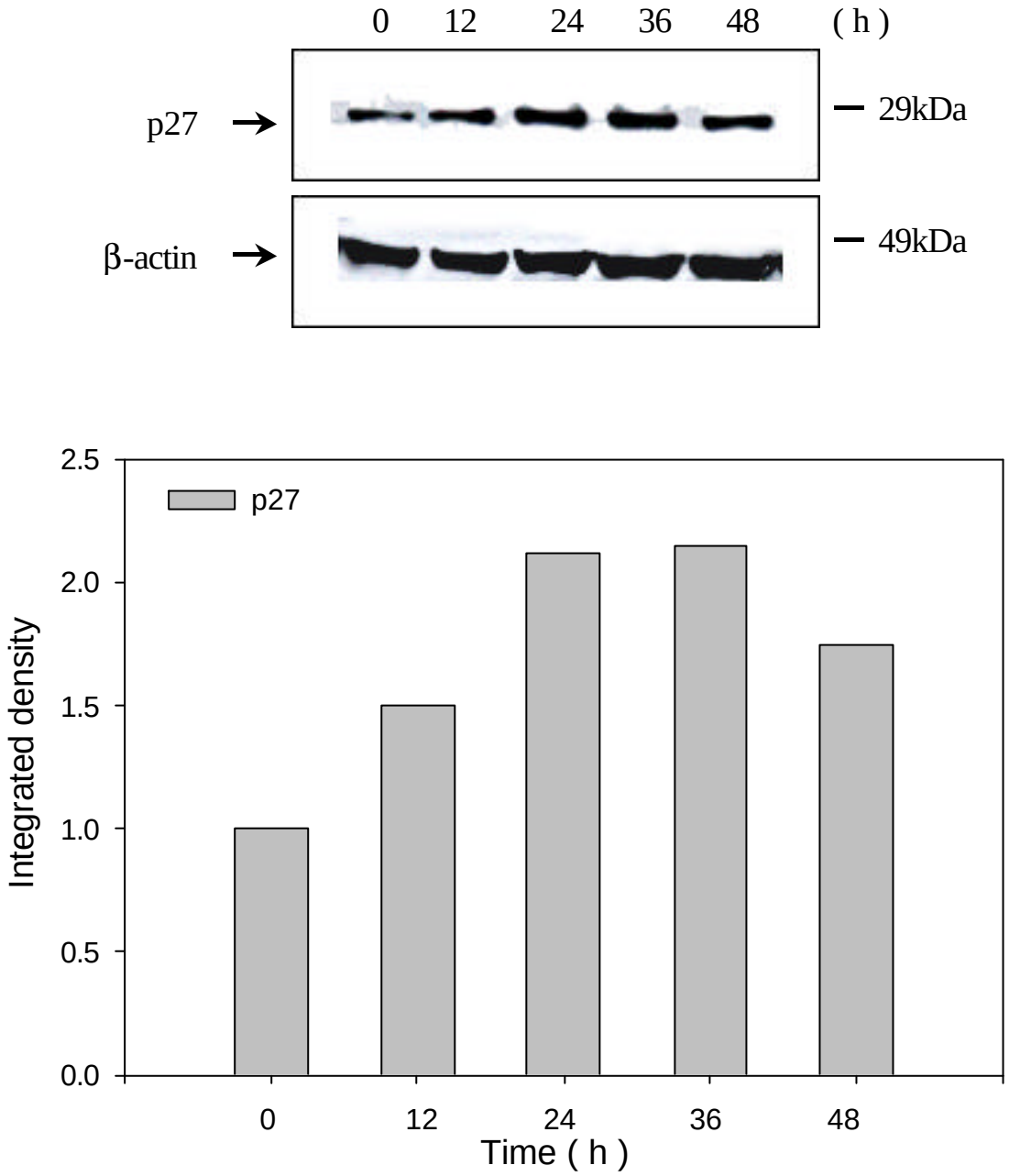

Figure 23. Time course effect of esculetin on p27 protein expression in HepG2. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and subjected to western blot analysis: 60μg of protein extract from each condition was electrophoresed in each lane of 12% SDS-PAGE and analysed by immunoblot with the anti-p27 antibody or actin used for equal loading.

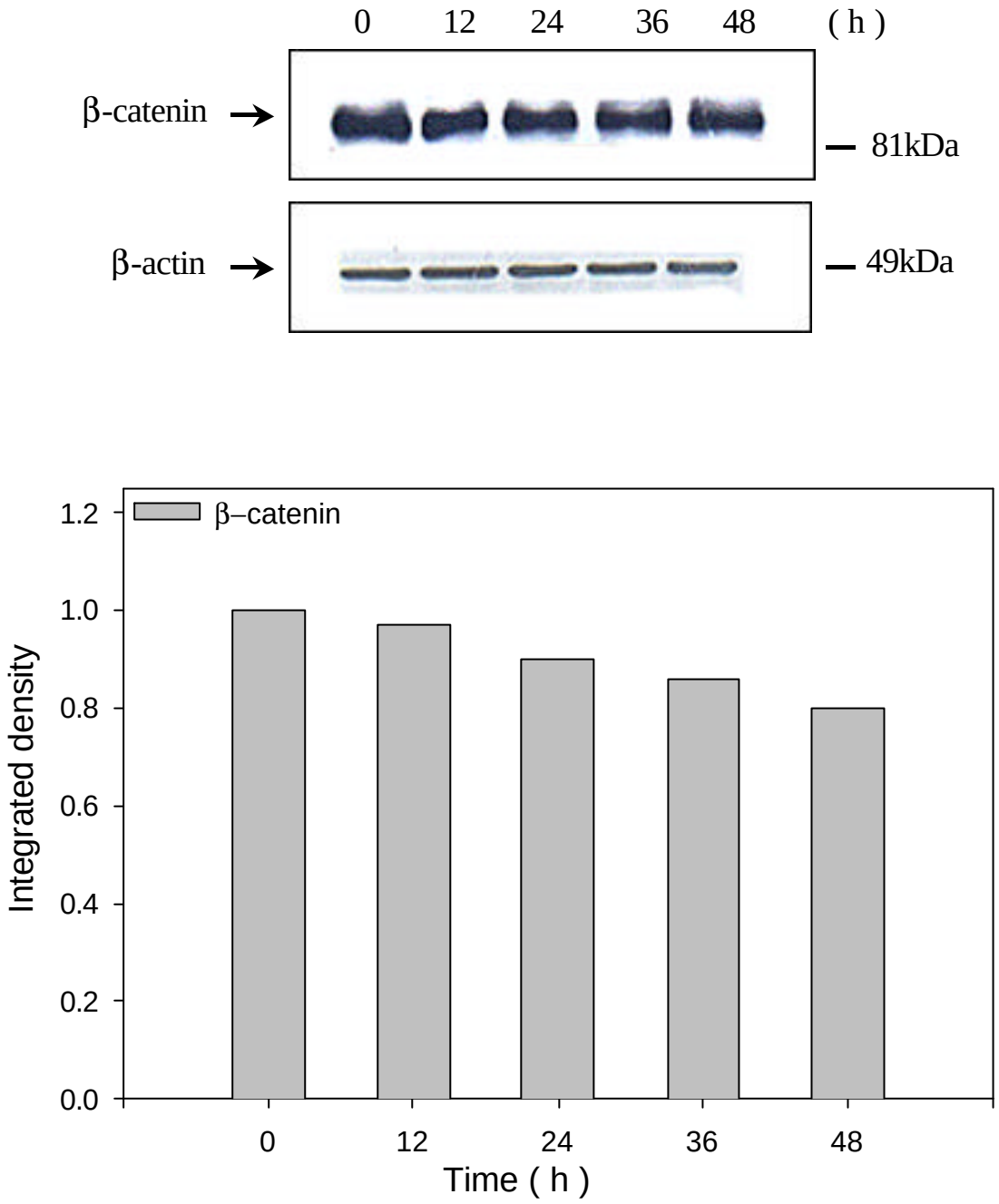

Figure 24. Time course effect of esculetin on β-catenin protein expression in HepG2. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and subjected to western blot analysis: 60μg of protein extract from each condition was electrophoresed in each lane of 10% SDS-PAGE and analysed by immunoblot with the anti-β-catenin antibody or actin used for equal loading.

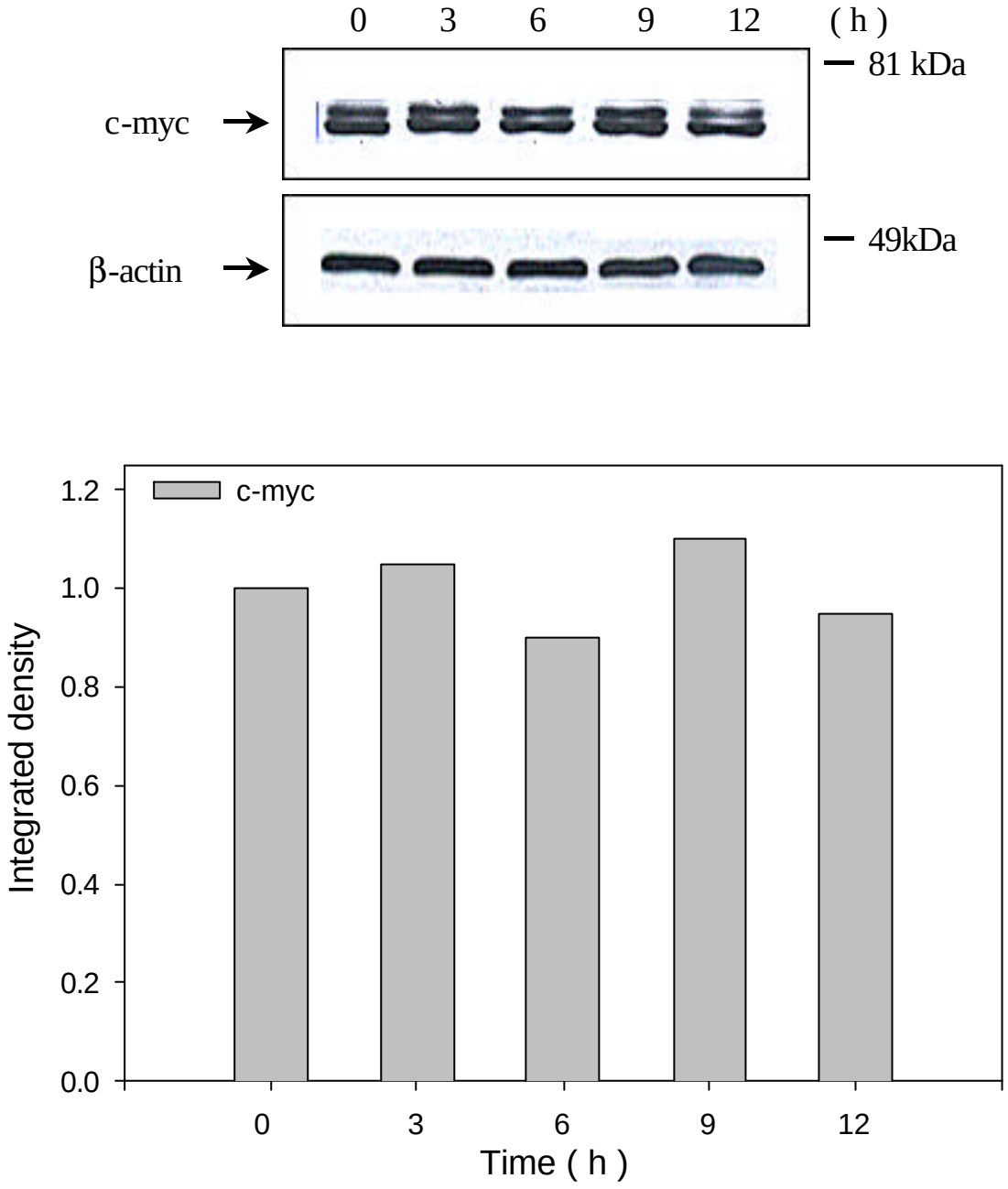

Figure 25. Time course effect of esculetin on c-myc protein expression in HepG2. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and subjected to western blot analysis: 60μg of protein extract from each condition was electrophoresed in each lane of 10% SDS-PAGE and analysed by immunoblot with the anti-c-myc antibody or actin used for equal loading.

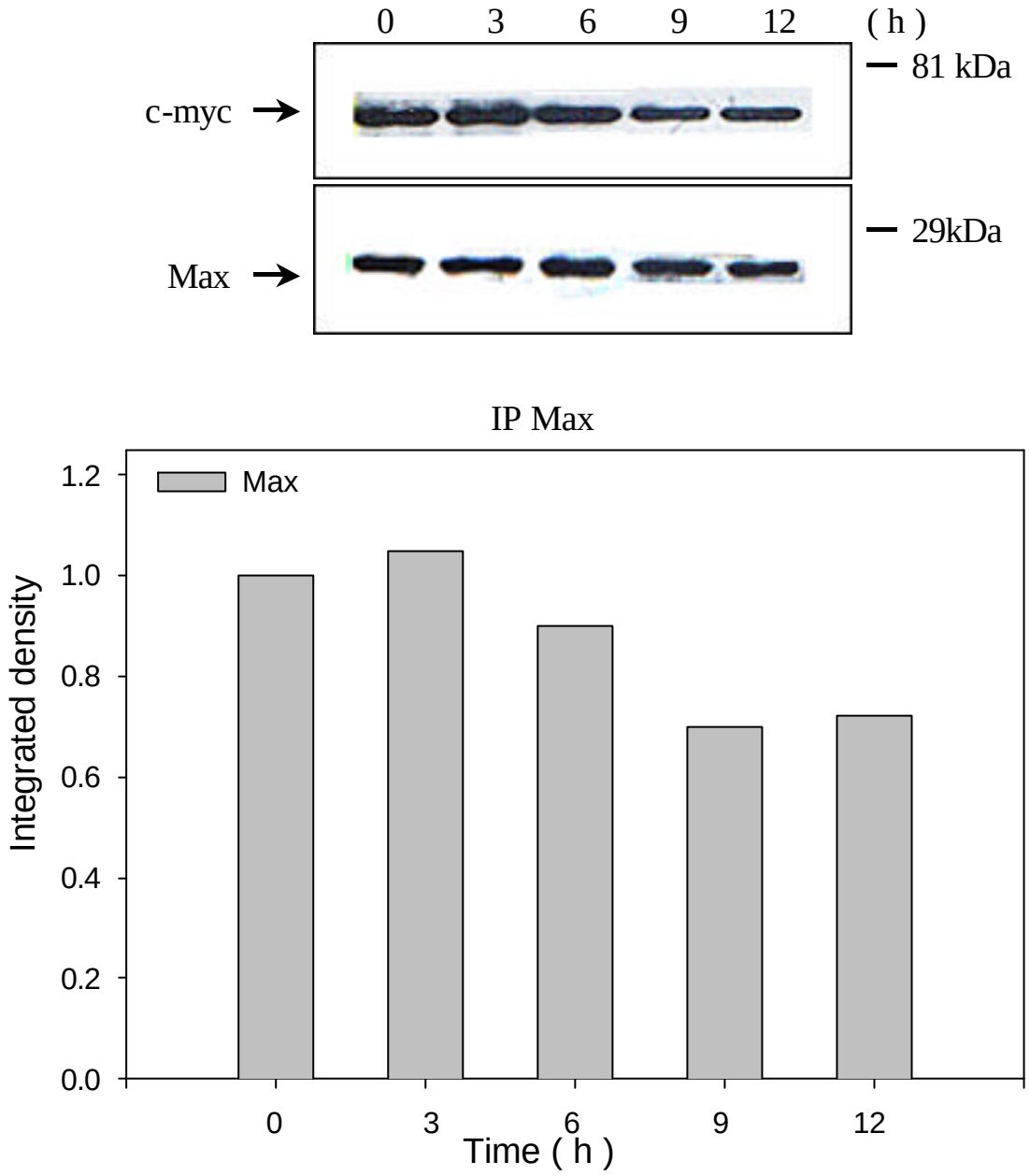

Figure 26. Time course effect of esculetin on c-myc protein expression in HepG2. Cells were cultured in medium containing 100 μM esculetin. At the indicated times following treatment cells were harvested. cell lysates prepared and immunoprecipitation were performed with specific antibodies against Max. Max immuno -precipitates were subjected to Western blot analysis : 12% SDS-PAGE and analysed by immunoblot with the antic-myc antibody or Max used for equal loading.

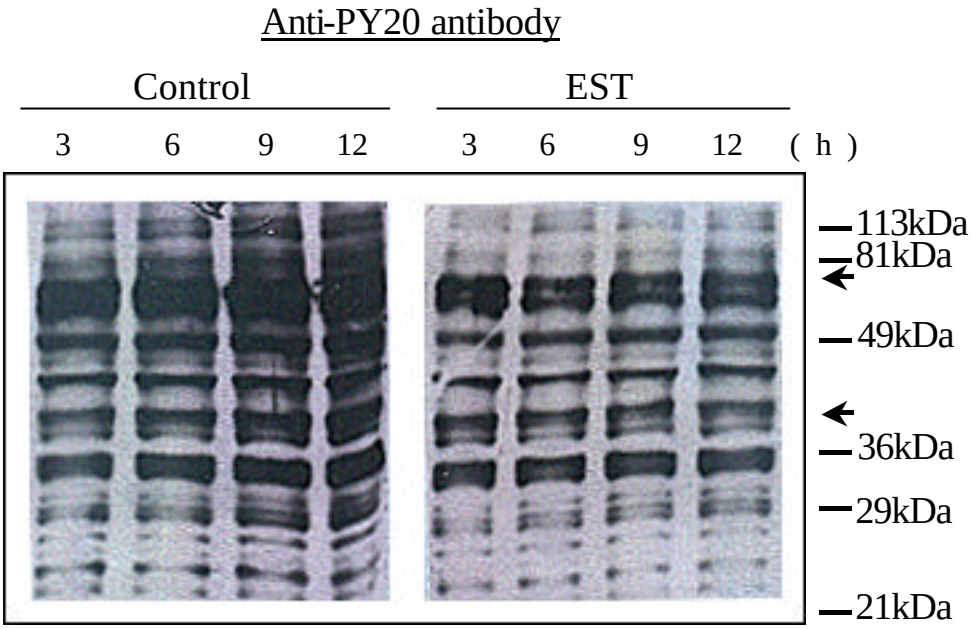

Figure 27. Time course effect of esculetin on phospho-tyrosin protein expression in HepG2. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and subjected to western blot analysis: 60μg of protein extract from each condition was electrophoresed in each lane of 12% SDS-PAGE and analysed by immunoblot with the anti-py20 antibody.

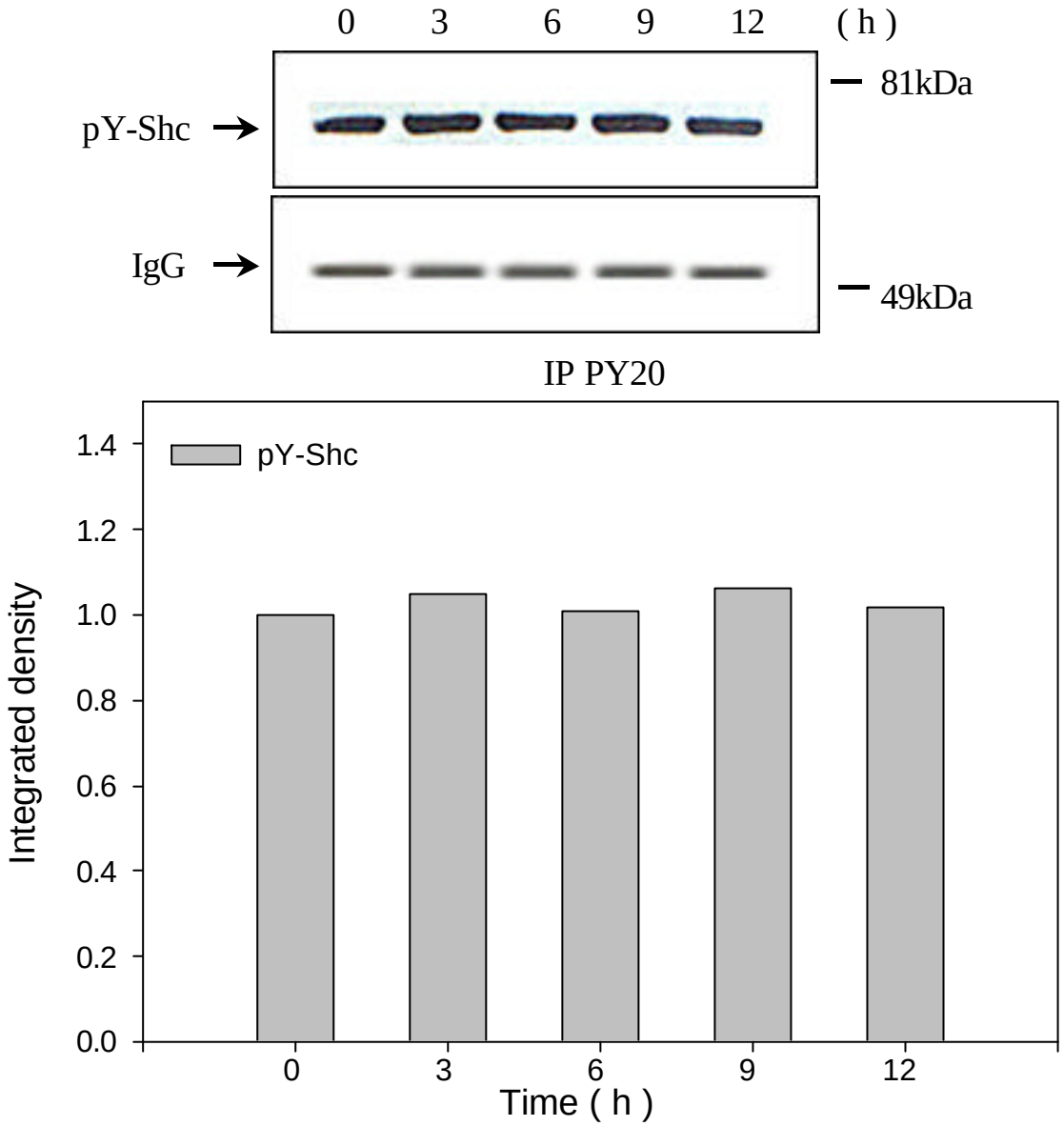

Figure 28. Time course effect of esculetin on pY-Shc protein expression in HepG2. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and immunoprecipitation were performed with specific antibodies against PY-20. PY-20 immunoprecipitates were subjected to western blot analysis : 12% SDS-PAGE and analysed by immunoblot with the anti-Shc antibody or IgG used for equal loading.

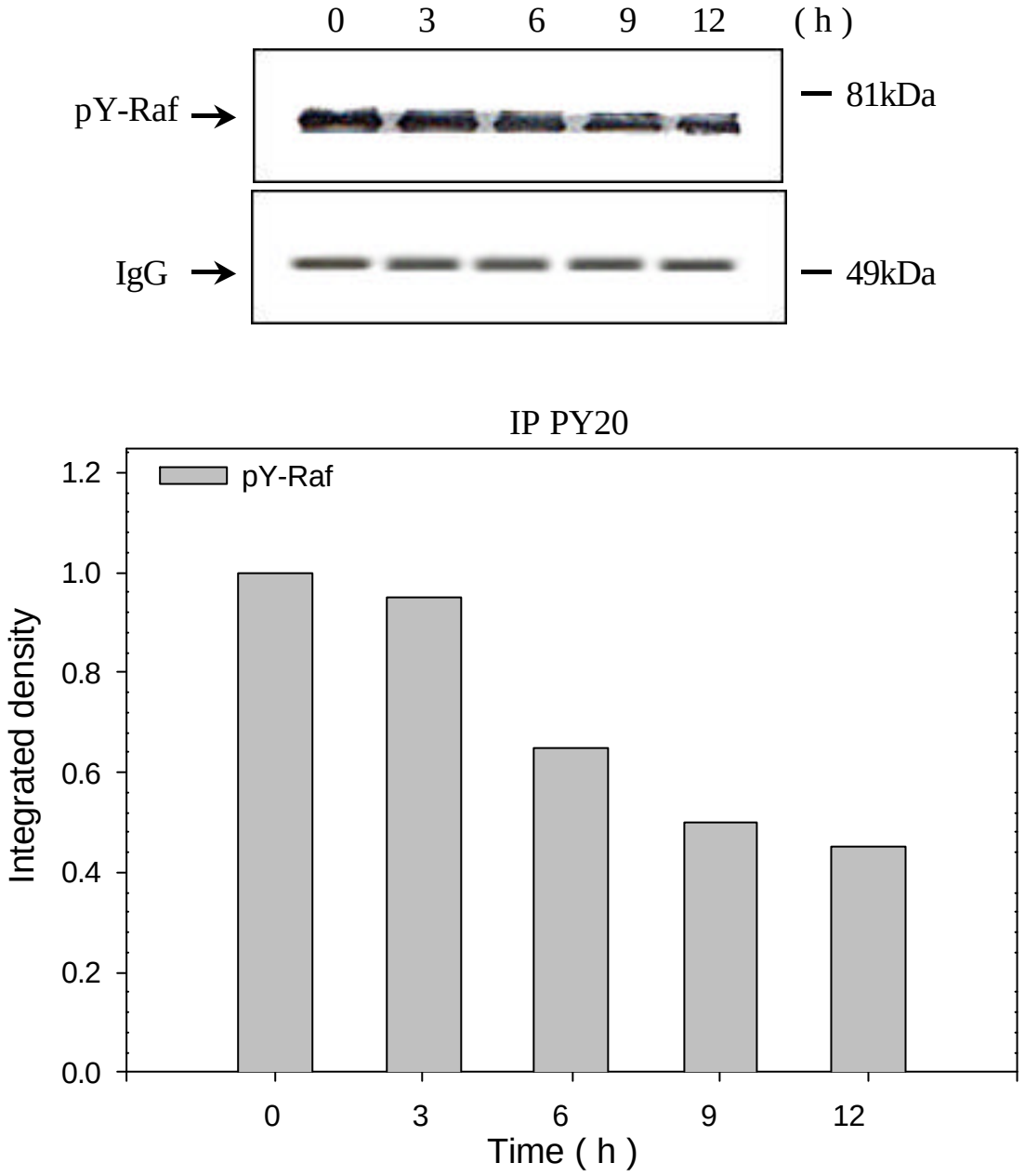

Figure 29. Time course effect of esculetin on pY-Raf protein expression in HepG2. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and immunoprecipitation were performed with specific antibodies against PY-20. PY-20 immunoprecipitates were subjected to western blot analysis : 12% SDS-PAGE and analysed by immunoblot with the anti-Raf antibody or IgG used for equal loading.

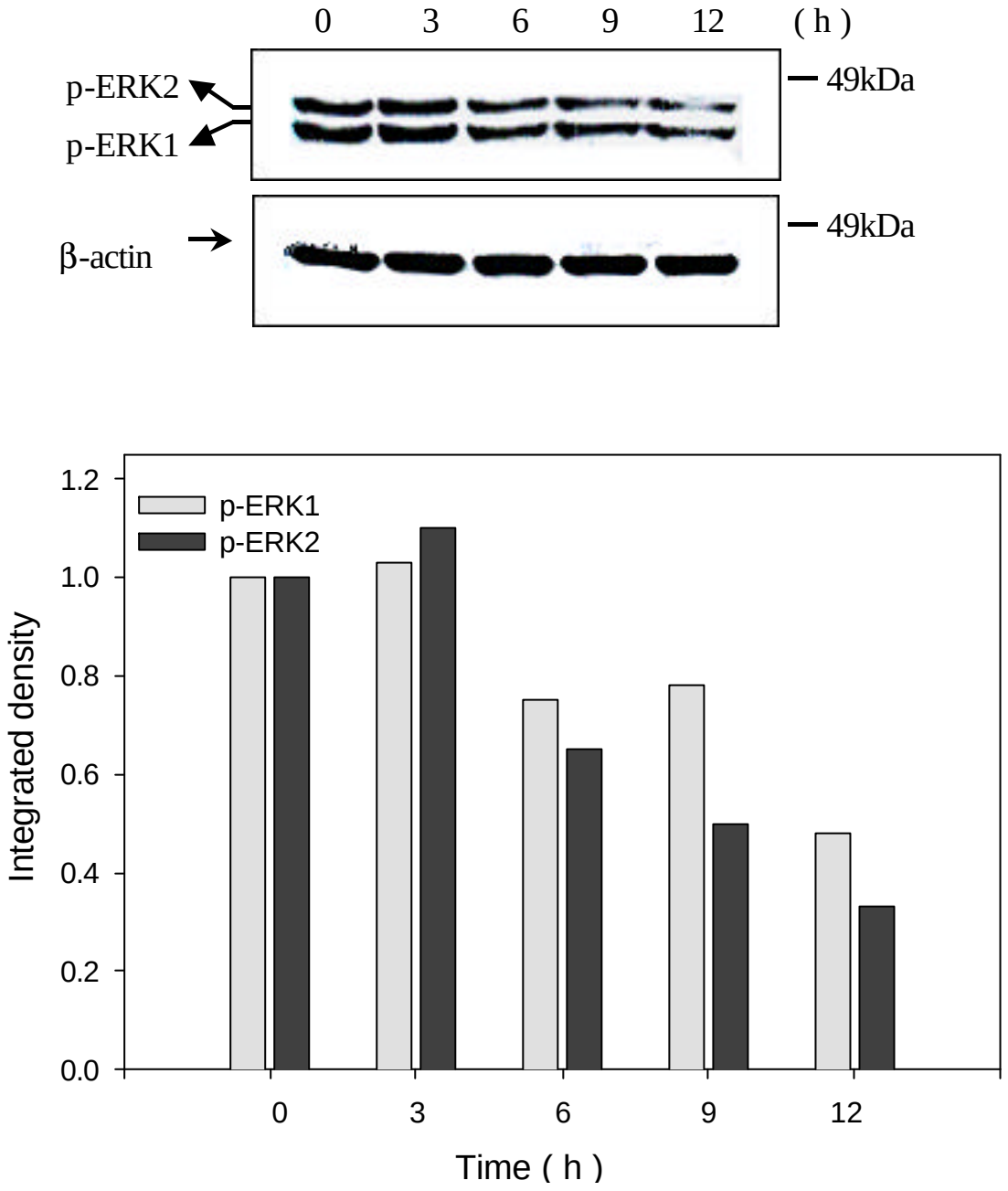

Figure 30. Time course effect of esculetin on phospho-ERK protein expression in HepG2. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and subjected to western blot analysis: 60μg of protein extract from each condition was electrophoresed in each lane of 12% SDS-PAGE and analysed by immunoblot with the anti-pMAPK antibody or actin used for equal loading.

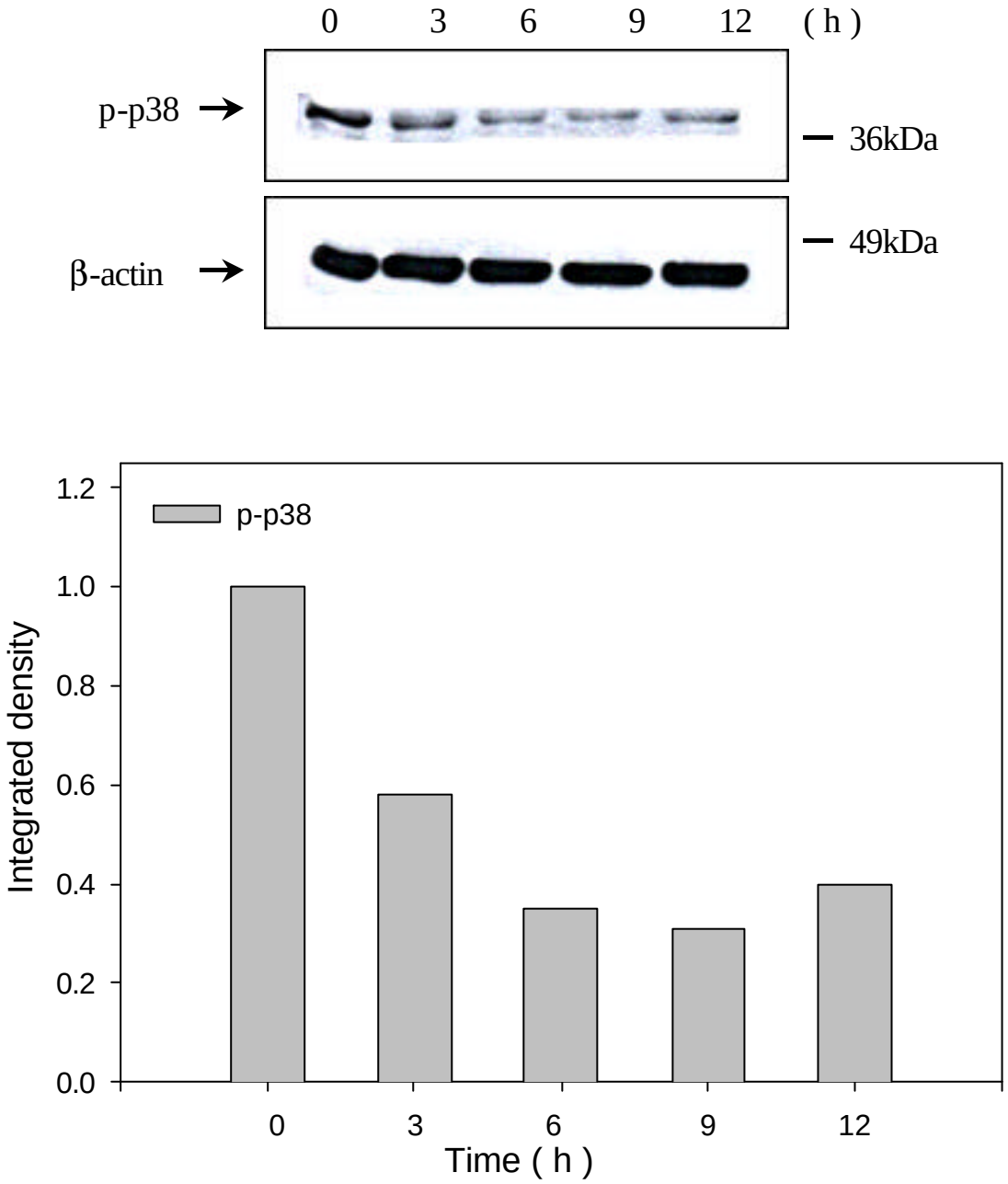

Figure 31. Time course effect of esculetin on phospho-p38 protein expression in HepG2. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and subjected to western blot analysis: 60μg of protein extract from each condition was electrophoresed in each lane of 12% SDS-PAGE and analysed by immunoblot with the anti-p-p38 antibody or actin used for equal loading.

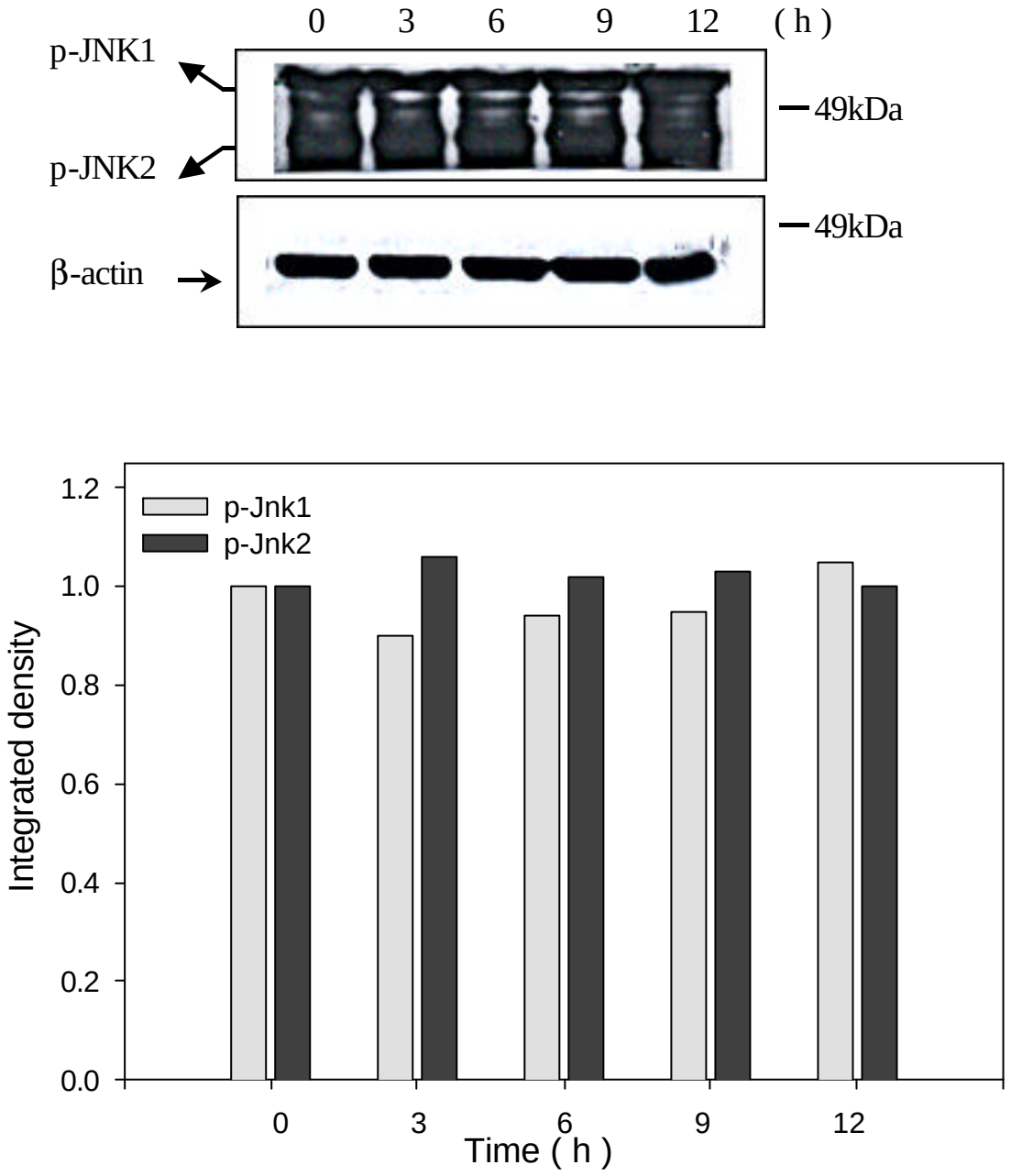

Figure 32. Time course effect of esculetin on phospho-JNK protein expression in HepG2. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and subjected to western blot analysis: 60μg of protein extract from each condition was electrophoresed in each lane of 12% SDS-PAGE and analysed by immunoblot with the anti-pJNK antibody or actin used for equal loading.

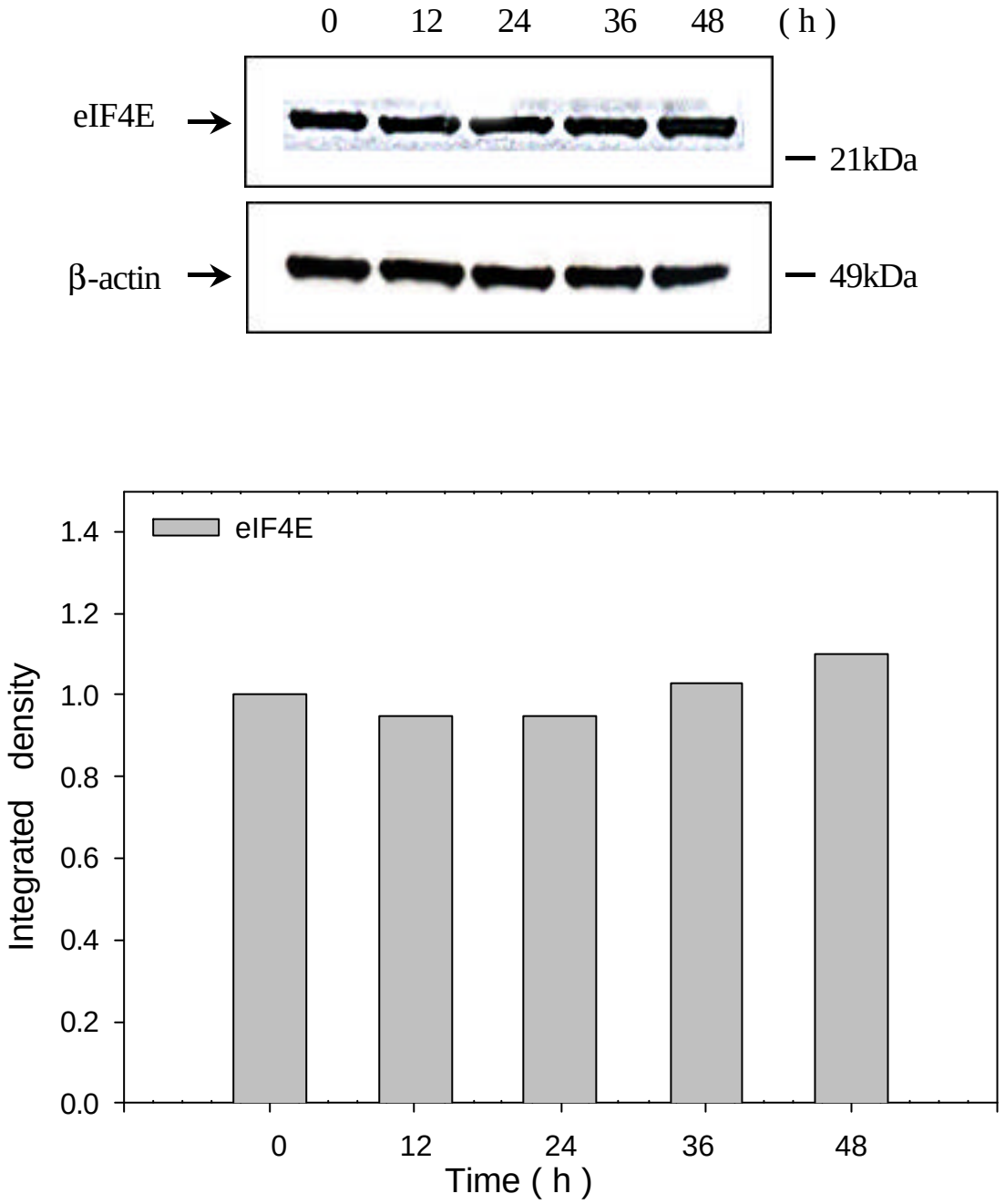

Figure 33. Time course effect of esculetin on eIF4E protein expression in HepG2. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and subjected to western blot analysis: 60μg of protein extract from each condition was electrophoresed in each lane of 15% SDS-PAGE and analysed by immunoblot with the anti-eIF4E antibody or actin used for equal loading.

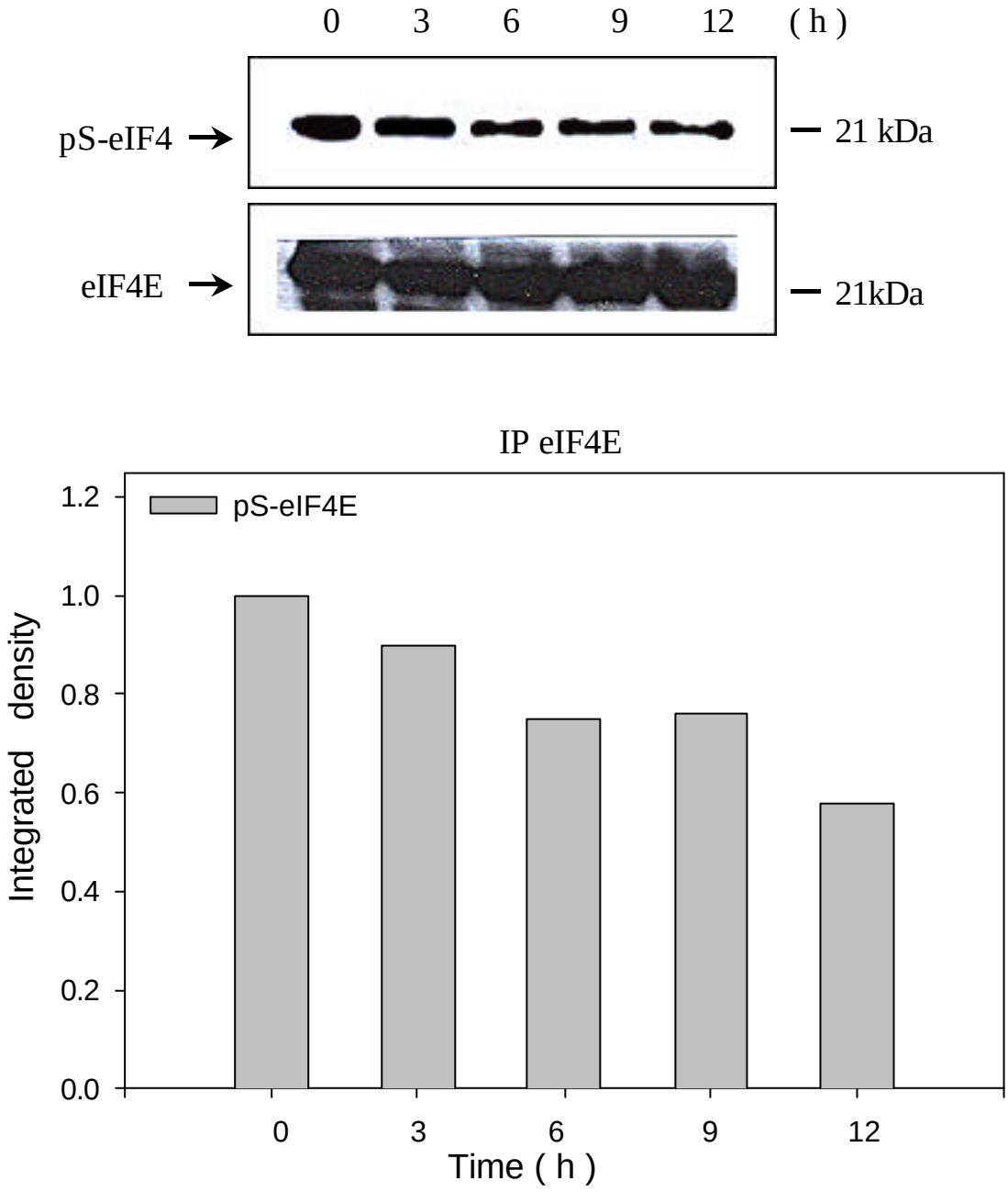

Figure 34. Time course effect of esculetin on pS-eIF4E protein expression in HepG2. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and immunoprecipitation were performed with specific antibodies against eIF4E. eIF4E immunoprecipitates were subjected to western blot analysis : 15% SDS-PAGE and analysed by immunoblot with the anti-phospho-Serine antibody or eIF4E used for equal loading.

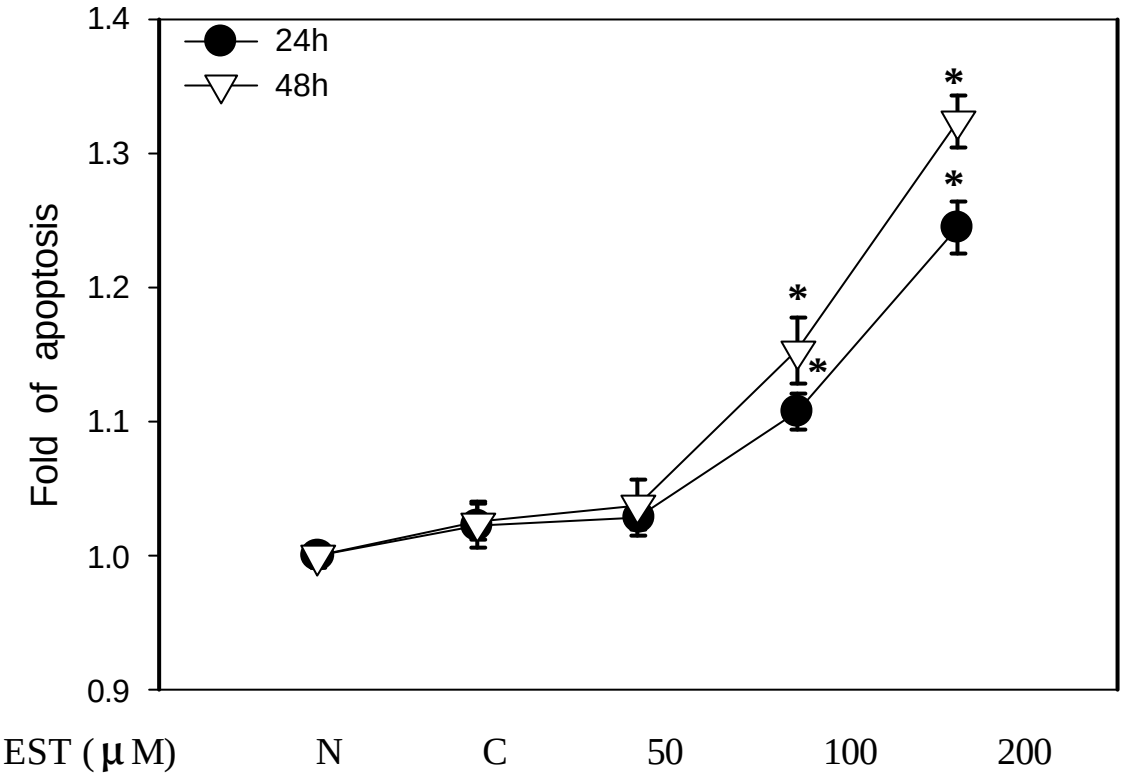

Figure 35. The apoptosis of HepG2 induced by esculetin. HepG2 cells were cultured for indicated times with various concentration of esculetin. The apoptosis was assessed by cell death detection ELISA assays. Data presented as means ± S.D. of two independent experiments. *$P < 0.05$, compared with normal group.

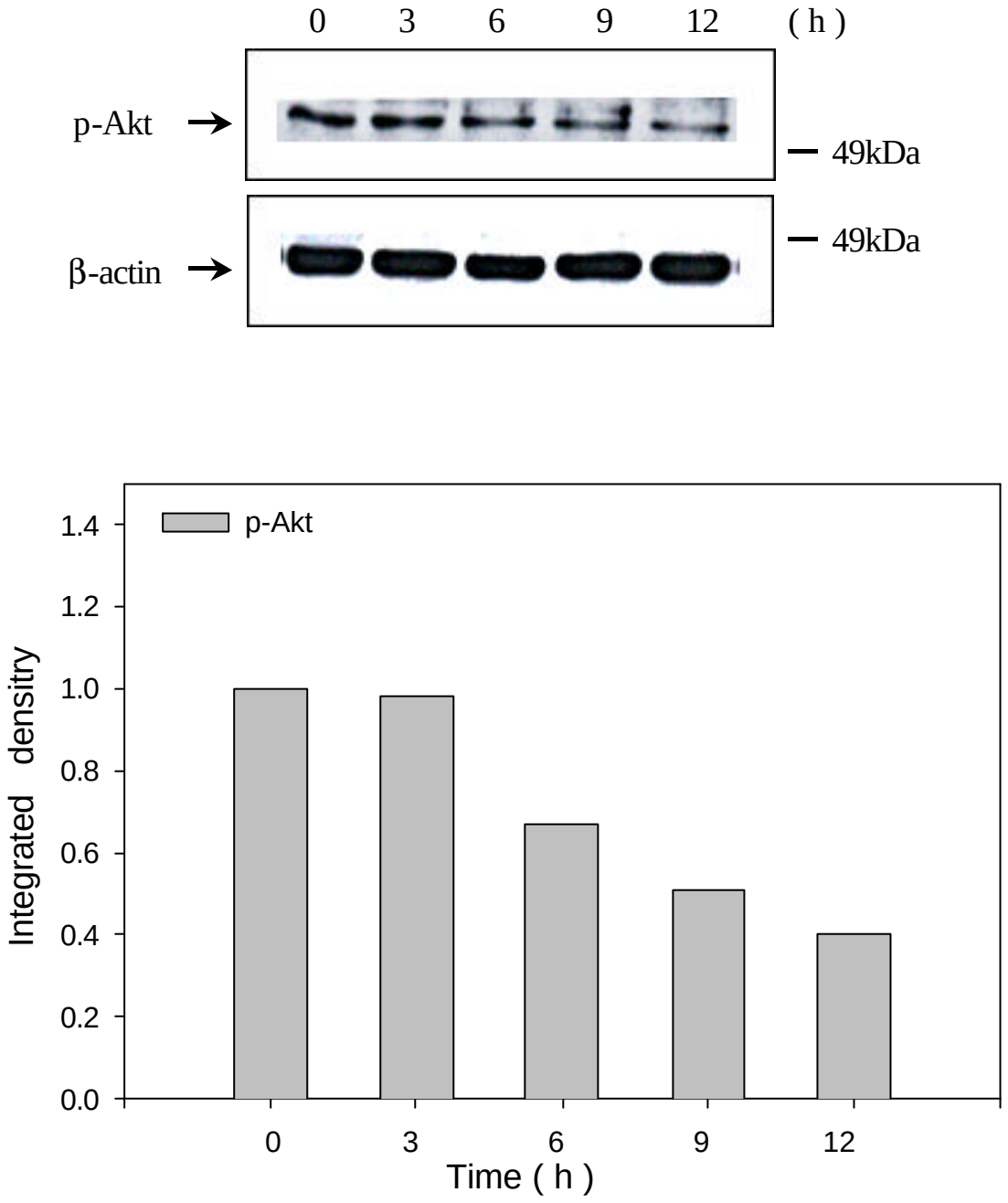

Figure 36. Time course effect of esculetin on phospho-Akt protein expression in HepG2. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and subjected to western blot analysis: 60μg of protein extract from each condition was electrophoresed in each lane of 12% SDS-PAGE and analysed by immunoblot with the anti-pAkt antibody or actin used for equal loading.

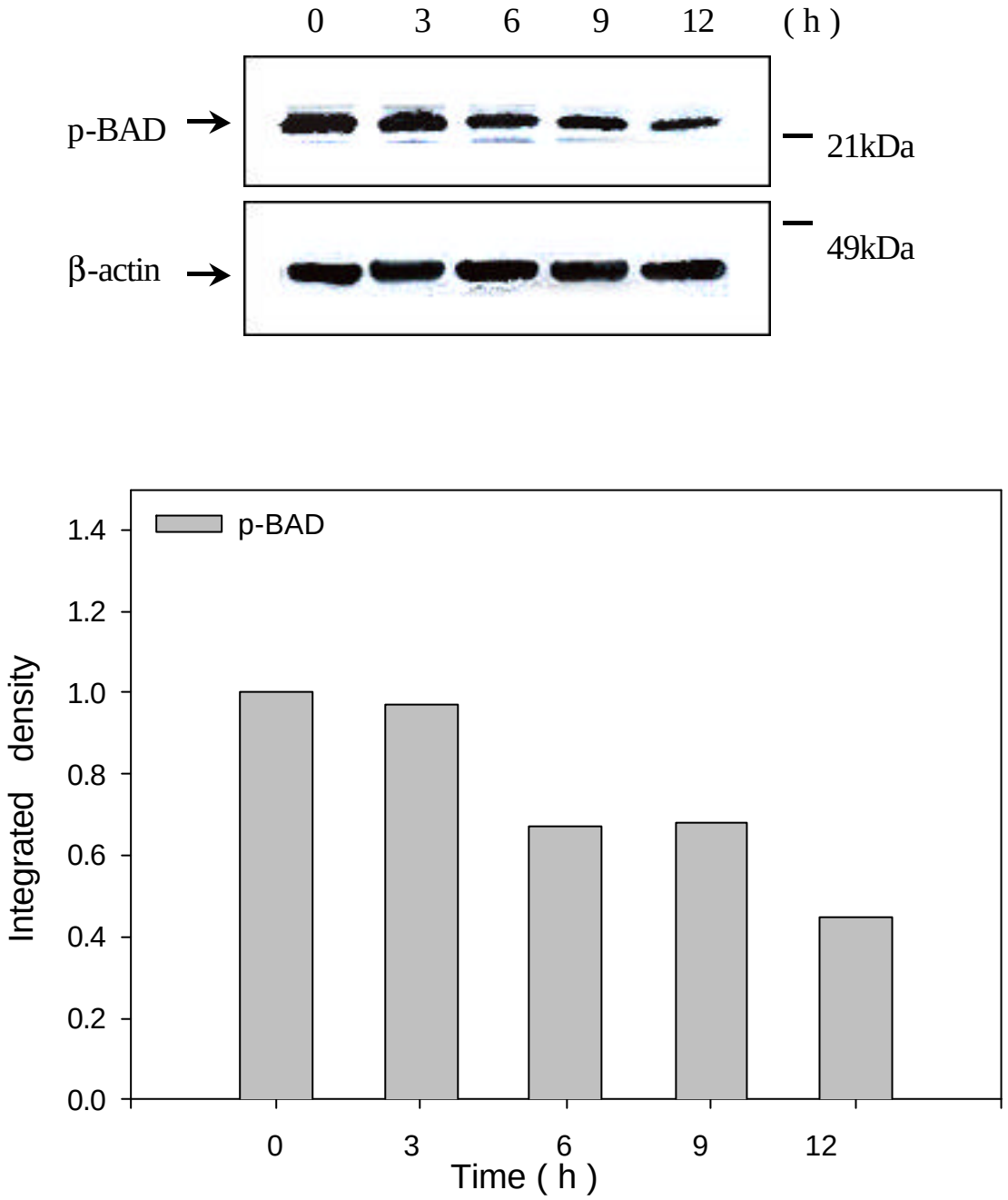

Figure 37. Time course effect of esculetin on phospho-BAD protein expression in HepG2. Cells were cultured in medium containing 100μM esculetin. At the indicated times following treatment cells were harvested. Cell lysates prepared and subjected to western blot analysis: 60μg of protein extract from each condition was electrophoresed in each lane of 15% SDS-PAGE and analysed by immunoblot with the anti-pBAD antibody or actin used for equal loading.

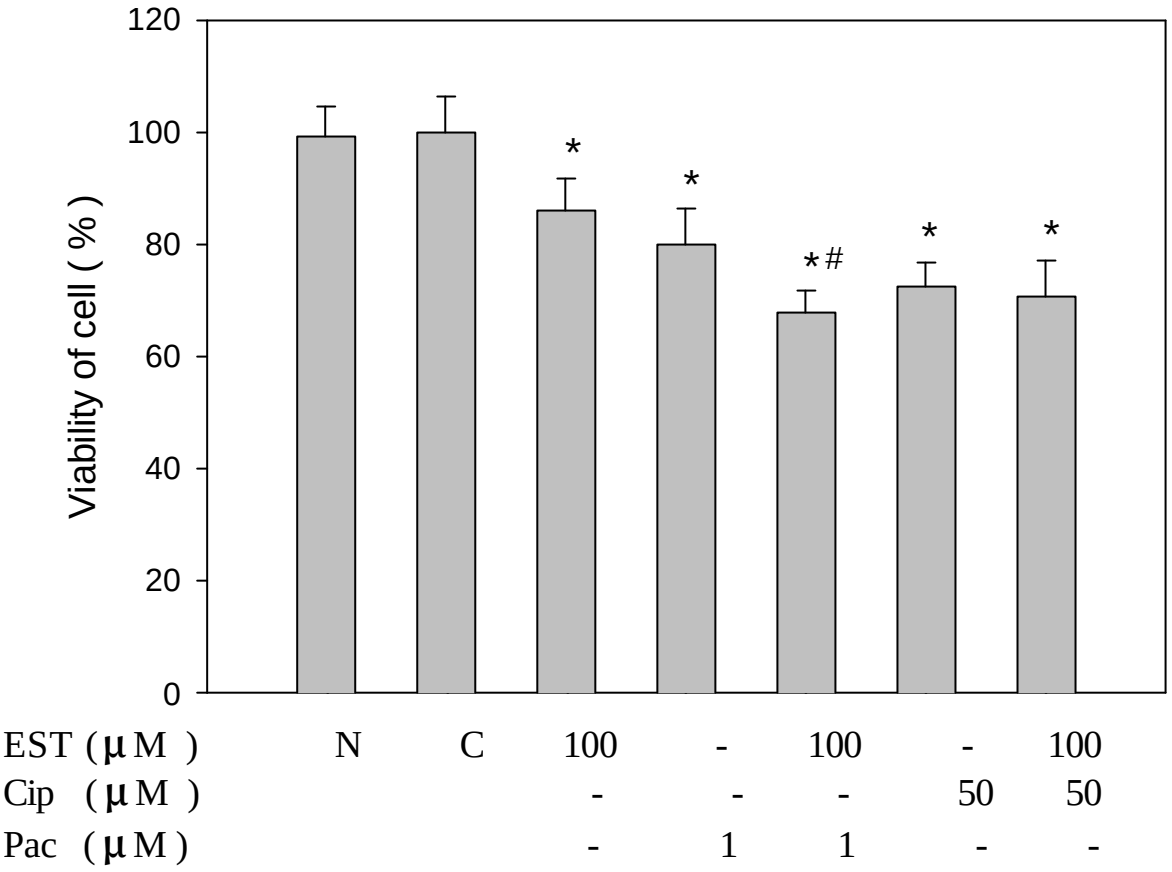

Figure 38. Cytotoxicity of esculetin and anticancer drugs in HepG2. Cells were cultured in medium containing indicated concentration of esculetin, pacliyaxel or cisplatin . At the indicated times (24h) following treatment cells were then incubated with MTT for 4 hours. The optical density is then read at 560nm by a spectrometer. Data presented as means ± S.D. of three independent experiments. *$P < 0.05$, compared with control group (0.2% DMSO) ; $^{\#}P < 0.05$, compared with paclitaxel (1.0μM)

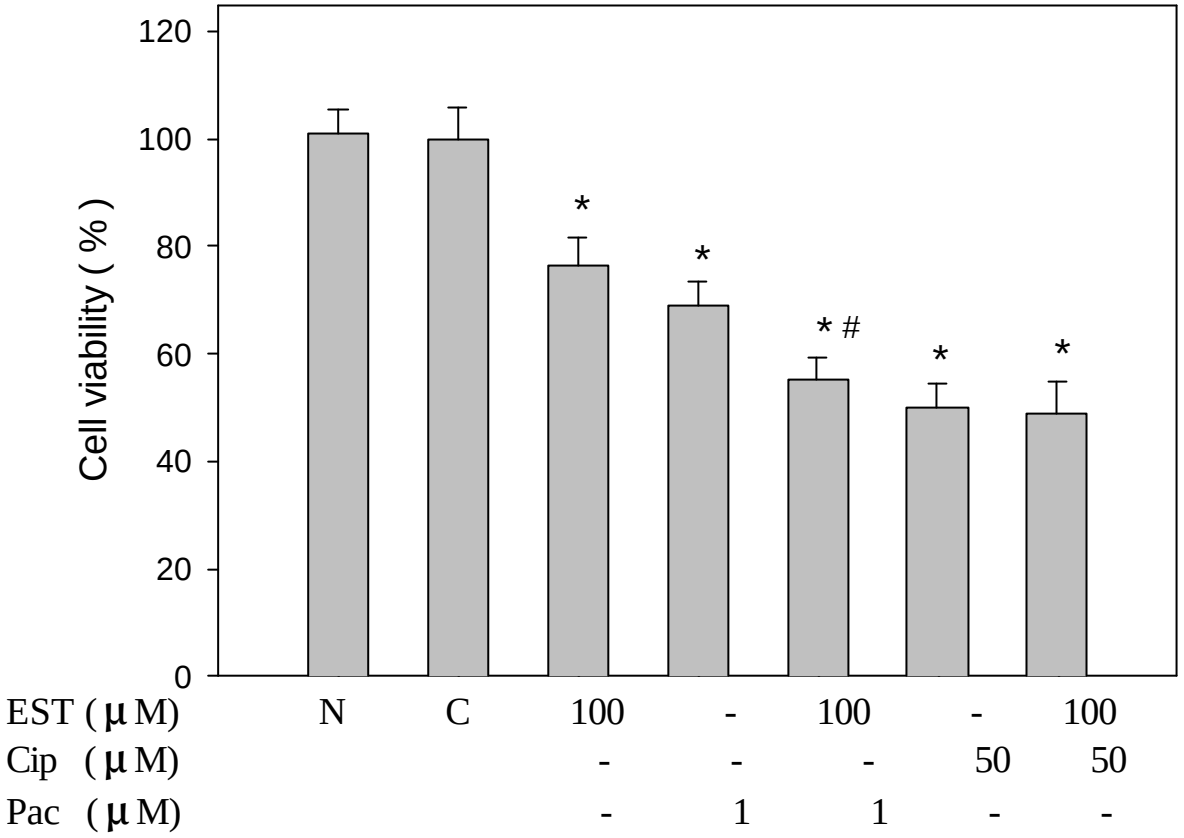

Figure 39. Cytotoxicity of esculetin and anticancer drugs in HepG2. Cells were cultured in medium containing indicated concentration of esculetin, pacliyaxel or cisplatin . At the indicated times (48h)following treatment cells were then incubated with MTT for 4 hours. The optical density is then read at 560nm by a spectrometer. Data presented as means ± S.D. of three independent experiments. *P < 0.05, compared with control group (0.2% DMSO) ; $^{\#}P$ < 0.05, compared with paclitaxel (1.0μM)

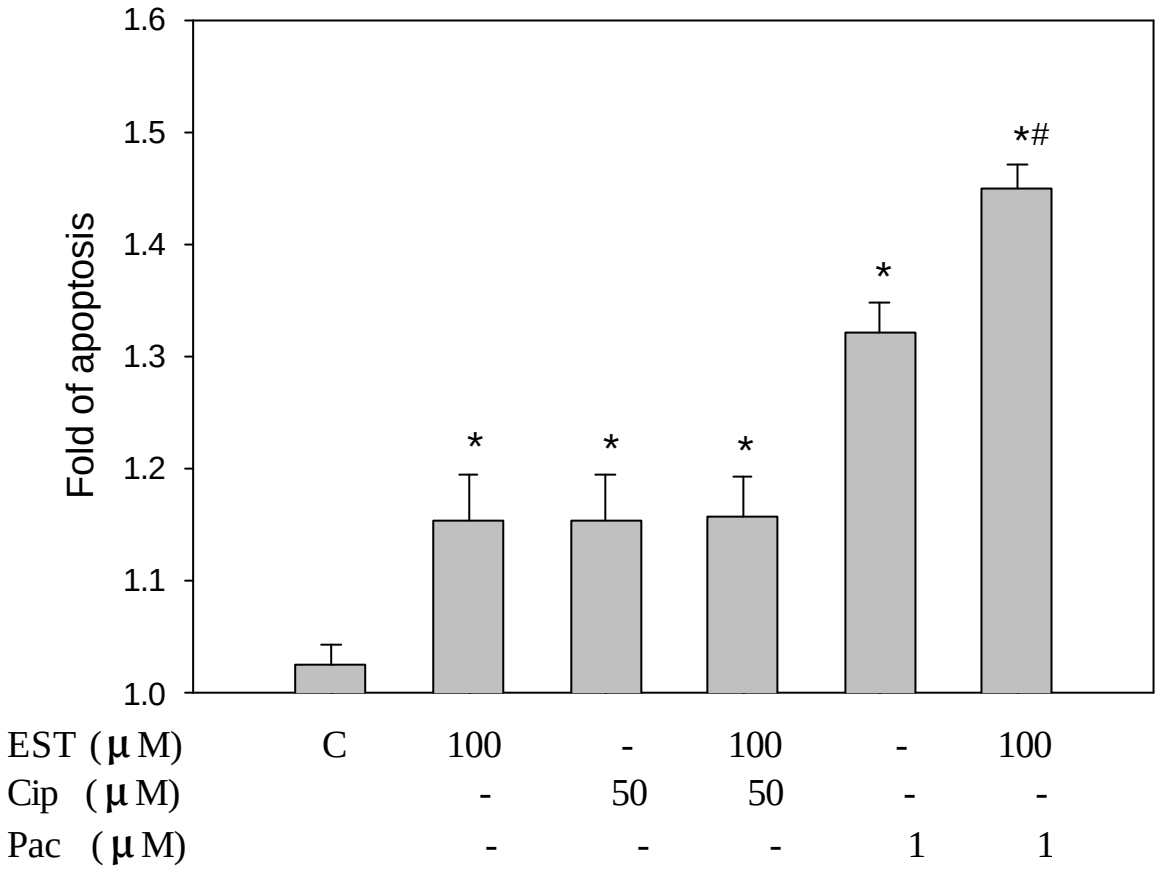

Figure 40. The apoptosis of HepG2 induced by esculetin, cisplatin and paclitaxel. HepG2 cells were cultured for indicated times with various concentration of esculetin , Cisplatin or Paclitaxel. The apoptosis was assessed by cell death detection ELISA assays. Data presented as means ± S.D. of two independent experiments. *$P < 0.05$, compared with normal group ; $^{\#}P < 0.05$, compared with paclitaxel (1.0μM)

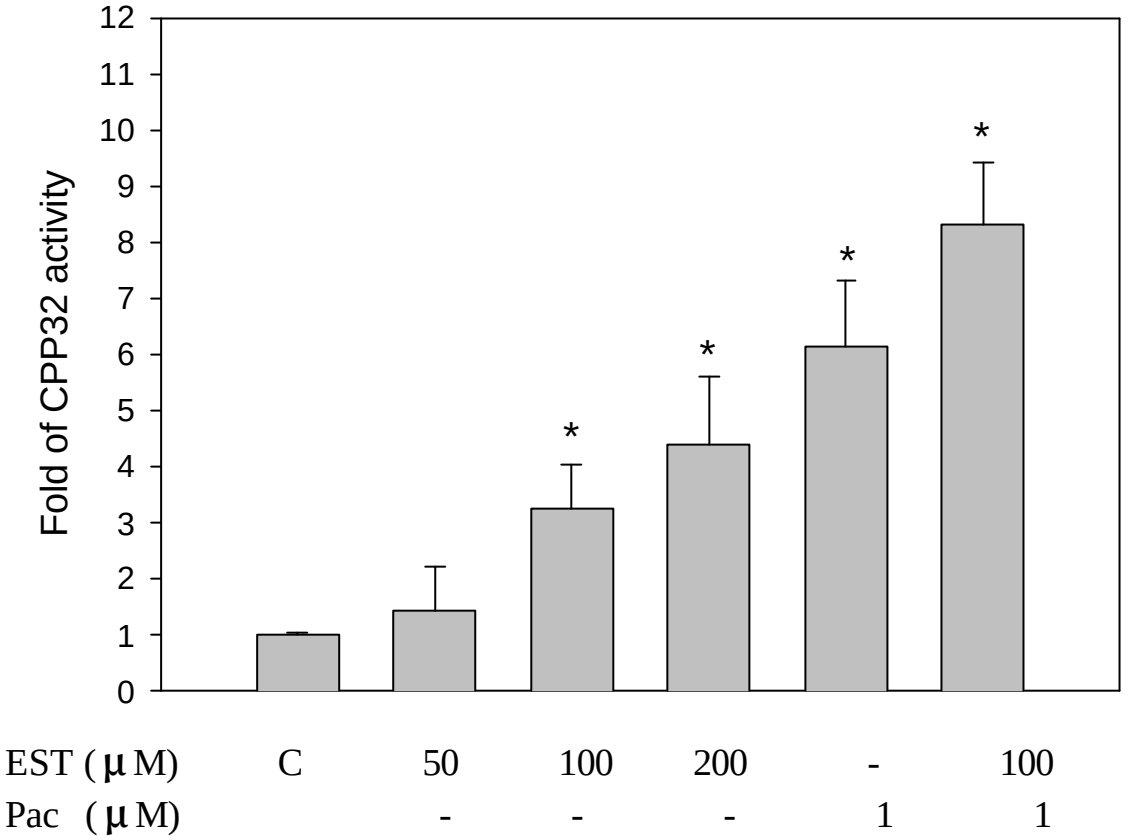

Figure 41. CPP32 activity assay. The CPP32 activity was measured in cell extracts obtained from HepG2 cells cultivated in DMEM with or without 100μM esculetin in 24 hours. The CPP32 activity is then read at 405nm by a ELISA reader. Data presented as means ± S.D. of two independent experiments. *$P < 0.05$, compared with control group (0.2% DMSO).

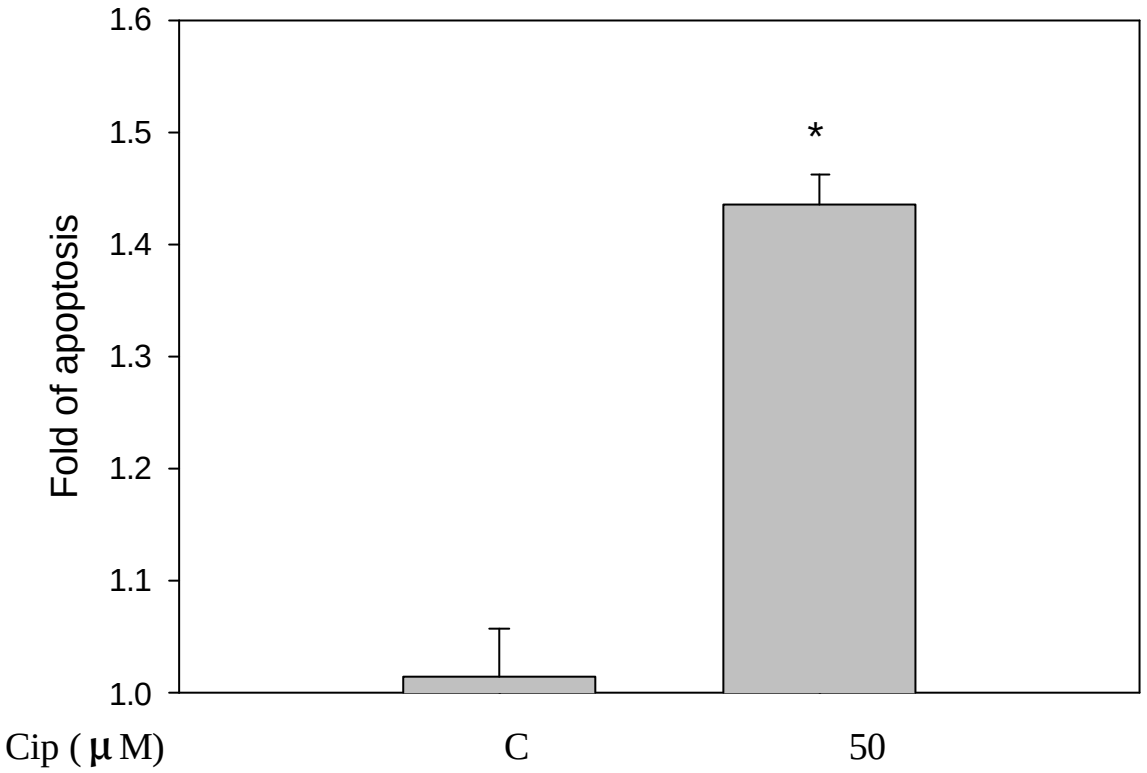

Figure 42. The apoptosis of HepG2 induced by Cisplatin. HepG2 cells were cultured for indicated times with 50μM cisplatin. The apoptosis was assessed by cell death detection ELISA assays. Data presented as means ± S.D. of three independent experiments. *P < 0.05, compared with control group (0.2% DMSO).

參 考 資 料

1. Chang WS, Chiang HC. Structure-activity relationship of coumarins in xanthine oxidase inhibition. Anticancer Research, 15 (5B): 1969-73, 1995.

2. Paya M, Ferrandiz ML, Miralles F, Montesinos C, Ubeda A, Alcaraz MJ. Effects of coumarin derivatives on superoxide anion generation. Arzneimittel-Forschung, 43 (6): 655-8, 1993.

3. Paya M, Halliwell B, Hoult JR. Interactions of a series of coumarins with reactive oxygen species. Scavenging of superoxide, hypochlorous acid and hydroxyl radicals. Biochemical Pharmacology, 44 (2): 205-14, 1992.

4. Hiramoto K, Ojima N, Sako K, Kikugawa K. Effect of plant phenolics on the formation of the spin-adduct of hydroxyl radical and the DNA strand breaking by hydroxyl radical. Biological & Pharmaceutical Bulletin, 19 (4): 558-63, 1996.

5. Chang WS, Lin CC, Chuang SC, Chiang HC. Superoxide anion scavenging effect of coumarins. American Journal of Chinese Medicine, 24 (1): 11-7, 1996.

6. Chang WS, Chang YH, Lu FJ. Chiang HC. Inhibitory effects of phenolics on xanthine oxidase. Anticancer Research, 14 (2A): 501-6, 1994.

7. Neichi T, Koshihara Y, Murota S. Inhibitory effect of esculetin on

5-lipoxygenase and leukotriene biosynthesis. Biochimica et Biophysica Acta, 753 (1): 130-2, 1983.

8. Noguchi M, Kitagawa H, Miyazaki I, Mizukami Y. Influence of esculetin on incidence, proliferation, and cell kinetics of mammary carcinomas induced by 7,12-dimethylbenz[a]anthracene in rats on high- and low-fat diets. Japanese Journal of Cancer Research, 84 (10): 1010-4, 1993.

9. Matsunaga K, Yoshimi N, Yamada Y, Shimizu M, Kawabata K, Ozawa Y, Hara A. Mori H. Inhibitory effects of nabumetone, a cyclooxygenase-2 inhibitor, and esculetin, a lipoxygenase inhibitor, on N- methyl- N-nitrosourea- induced mammary carcinogenesis in rats. Japanese Journal of Cancer Research, 89 (5): 496-501, 1998.

10. Hecht SS, Kenney PM, Wang M, Trushin N, Agarwal S, Rao AV, Upadhyaya P. Evaluation of butylated hydroxyanisole, myo-inositol, curcumin, esculetin, resveratrol and lycopene as inhibitors of benzo[a]pyrene plus 4- (methylnitrosamino)-1-(3-pyridyl)-1-butanone -induced lung tumorigenesis in A/J mice. Cancer Letters. 137 (2): 123-30, 1999.

11. Craven PA, Pfanstiel J, DeRubertis FR. Role of reactive oxygen in bile salt stimulation of colonic epithelial proliferation. Journal of Clinical Investigation. 77(3): 850-9, 1986

12. Morisaki N, Lindsey JA. Stitts JM, Zhang H, Cornwell DG. Fatty acid metabolism and cell proliferation. V. Evaluation of pathways for the generation of lipid peroxides. Lipids. 19 (6): 381-94, 1984.

13. Fedyk ER, Phipps RP. Reactive oxygen species and not lipoxygenase

products are required for mouse B-lymphocyte activation and differentiation. International Journal of Immunopharmacology, 16 (7): 533-46, 1994.

14. Rose DP. Connolly JM. Effects of fatty acids and inhibitors of eicosanoid synthesis on the growth of a human breast cancer cell line in culture. Cancer Research, 50 (22): 7139-44, 1990.

15. Hofmanova J, Musilova E, Kozubik A. Suppression of human cancer cell proliferation by lipoxygenase inhibitors and gamma-radiation in vitro. General Physiology & Biophysics, 15 (4): 317-31, 1996.

16. Earashi M, Noguchi M, Tanaka M. In vitro effects of eicosanoid synthesis inhibitors in the presence of linoleic acid on MDA-MB-231 human breast cancer cells. Breast Cancer Research & Treatment, 37 (1): 29-37, 1996.

17. Papadogiannakis N, Barbieri B. Lipoxygenase inhibitors counteract protein kinase C mediated events in human T lymphocyte proliferation. International Journal of Immuno- pharmacology, 19 (5): 263-75, 1997.

18. Huang HC, Hsieh LM, Chen HW, Lin YS, Chen JS. Effects of baicalein and esculetin on transduction signals and growth factors expression in T-lymphoid leukemia cells. European Journal of Pharmacology, 268 (1): 73-8, 1994.

19. Nakahama K, Morita I, Murota S. Effects of endogenously produced arachidonic acid metabolites on rat mesangial cell proliferation. Prostaglandins Leukotrienes & Essential Fatty Acids, 51 (3): 177-82, 1994.

20. Huang HC, Lai MW, Wang HR, Chung YL. Hsieh LM, Chen CC. Antiproliferative effect of esculetin on vascular smooth muscle cells: possible roles of signal transduction pathways. European Journal of Pharmacology, 237 (1): 39-44, 1993.

21. Kozubik A, Hofmanova J, Dusek L. Eicosanoid inhibitors enhance synergistically the effect of transforming growth factor beta 1 on CCL 64 cell proliferation. European Journal of Pharmacology, 316 (2-3): 349-57, 1996.

22. Lania L, Majello B, Napolitano G. Transcriptional control by cell-cycle regulators. Journal of Cellular Physiology, 179 (2): 134-41, 1999.

23. Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. p21 is a universal inhibitor of cyclin kinases. Nature, 366 (6456): 701-4, 1993.

24. Lee MH, Reynisdottir I, Massague J. Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. Genes & Development, 9 (6): 639-49, 1995.

25. Galaktionov K, Beach D. Specific activation of cdc25 tyrosine phosphatases by B-type cyclins: evidence for multiple roles of mitotic cyclins. Cell, 67(6): 1181-94, 1991

26. Jinno S, Suto K, Nagata A, Igarashi M, Kanaoka Y, Nojima H, Okayama H. Cdc25A is a novel phosphatase functioning early in the cell cycle. EMBO Journal, 13 (7): 1549-56, 1994.

27. Blomberg I, Hoffmann I. Ectopic expression of Cdc25A accelerates the G(1)/S transition and leads to premature activation of cyclin E- and

cyclin A-dependent kinases. Molecular & Cellular Biology, 19 (9): 6183-94, 1999.

28. Yang R, Morosetti R, Koeffler HP. Characterization of a second human cyclin A that is highly expressed in testis and in several leukemic cell lines. Cancer Research, 57 (5): 913-20, 1997.

29. Sicinski P, Donaher JL, Parker SB, Li T, Fazeli A, Gardner H, Haslam SZ, Bronson RT, Elledge SJ, Weinberg RA. Cyclin D1 provides a link between development and oncogenesis in the retina and breast. Cell, 82 (4): 621-30, 1995.

30. Yang R, Morosetti R, Koeffler HP. Characterization of a second human cyclin A that is highly expressed in testis and in several leukemic cell lines. Cancer Research, 57 (5): 913-20, 1997.

31. Ito K, Sasano H, Yoshida Y, Sato S, Yajima A. Immuno- histochemical study of cyclins D and E and cyclin dependent kinase (cdk) 2 and 4 in human endometrial carcinoma. Anticancer Research, 18 (3A): 1661-4, 1998.

32. Galaktionov K, Lee AK, Eckstein J, Draetta G, Meckler J, Loda M, Beach D. CDC25 phosphatases as potential human oncogenes. Science, 269 (5230): 1575-7, 1995.

33. Little CD. Nau MM. Carney DN. Gazdar AF. Minna JD. Amplification and expression of the c-myc oncogene in human lung cancer cell lines. Nature. 306 (5939): 194-6, 1983.

34. Erisman MD, Rothberg PG, Diehl RE, Morse CC. Spandorfer JM. Astrin SM. Deregulation of c-myc gene expression in human colon carcinoma is

not accompanied by amplification or rearrangement of the gene. Molecular & Cellular Biology, 5 (8): 1969-76, 1985.

35. Cangi MG, Cukor B, Soung P, Signoretti S, Moreira G. Jr, Ranashinge M, Cady B, Pagano M, Loda M. Role of the Cdc25A phosphatase in human breast cancer. Journal of Clinical Investigation, 106 (6): 753-61, 2000.

36. Lavoie JN, L'Allemain G, Brunet A, Muller R, Pouyssegur J. Cyclin D1 expression is regulated positively by the p42/p44 MAPK and negatively by the p38/HOGMAPK pathway. Journal of Biological Chemistry, 271 (34): 20608-16, 1996.

37. Escot C, Theillet C, Lidereau R, Spyratos F, Champeme MH, Gest J, Callahan R. Genetic alteration of the c-myc proto- oncogene (MYC) in human primary breast carcinomas. Proceedings of the National Academy of Sciences of the United States of America, 83 (13): 4834-8, 1986.

38. Cadigan KM, Nusse R. Wnt signaling: a common theme in animal development. Genes & Development, 11 (24): 3286-305, 1997.

39. Zou X, Rudchenko S, Wong K, Calame K. Induction of c-myc transcription by the v-Abl tyrosine kinase requires Ras, Raf1, and cyclin-dependent kinases. Genes & Development, 11 (5): 654-62, 1997.

40. Dang CV. c-Myc target genes involved in cell growth, apoptosis, and metabolism. Molecular & Cellular Biology, 19 (1): 1-11, 1999.

41. Peeper DS, Upton TM, Ladha MH, Neuman E, Zalvide J, Bernards R, DeCaprio JA, Ewen ME. Ras signalling linked to the cell-cycle machinery by the retinoblastoma protein. Nature, 386 (6621): 177-81, 1997.

42. Jones SM, Kazlauskas A. Growth factor-dependent signaling and cell cycle progression. FEBS Letters, 490 (3): 110-6, 2001.

43. Brunet A, Roux D, Lenormand P, Dowd S, Keyse S, Pouyssegur J. Nuclear translocation of p42/p44 mitogen- activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. EMBO Journal, 18 (3): 664-74, 1999.

44. Waskiewicz AJ, Flynn A. Proud CG, Cooper JA. Mitogen- activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. EMBO Journal, 16 (8): 1909-20, 1997.

45. Cobb MH, Goldsmith EJ. How MAP kinases are regulated. Journal of Biological Chemistry, 270 (25): 14843-6, 1995.

46. Tseng TH, Kao TW, Chu CY, Chou FP, Lin WL, Wang CJ. Induction of apoptosis by hibiscus protocatechuic acid in human leukemia cells via reduction of retinoblastoma (RB) phosphorylation and Bcl-2 expression. Biochemical Pharmacology, 60 (3): 307-15, 2000.

47. Narayanan BA, Geoffroy O, Willingham MC, Re GG, Nixon DW. p53/p21(WAF1/CIP1) expression and its possible role in G1 arrest and apoptosis in ellagic acid treated cancer cells. Cancer Letters, 136 (2): 215-21, 1999.

48. Saeki K, Sano M, Miyase T, Nakamura Y, Hara Y. Aoyagi Y. Isemura M. Apoptosis- inducing activity of polyphenol compounds derived from tea catechins in human histiolytic lymphoma U937 cells. Bioscience, Biotechnology & Biochemistry, 63 (3): 585-7, 1999.

49. Egan D, James P, Cooke D, O'Kennedy R. Studies on the cytostatic and cytotoxic effects and mode of action of 8- nitro-7-hydroxy coumarin. Cancer Letters, 118 (2): 201-11, 1997.

50. Mahmoud NN, Carothers AM, Grunberger D, Bilinski RT, Churchill MR, Martucci C, Newmark HL, Bertagnolli MM. Plant phenolics decrease intestinal tumors in an animal model of familial adenomatous polyposis. Carcinogenesis, 21 (5): 921-7, 2000.

51. Chu CY, Tsai YY, Wang CJ, Lin WL, Tseng TH. Induction of apoptosis by esculetin in human leukemia cells. European Journal of Pharmacology, 416 (1-2): 25-32, 2001.

52. Blagosklonny MV, Fojo T. Molecular effects of paclitaxel: myths and reality. International Journal of Cancer, 83 (2): 151-6, 1999.

53. Jiang S, Song MJ, Shin EC, Lee MO, Kim SJ, Park JH. Apoptosis in human hepatoma cell lines by chemotherapeutic drugs via Fas-dependent and Fas-independent pathways. Hepatology, 29 (1): 101-10, 1999.

54. Alley MC, Scudiero DA, Monks A, Hursey ML, Czerwinski MJ, Fine DL, Abbott BJ, Mayo JG, Shoemaker RH, Boyd MR. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Research, 48 (3): 589-601, 1988.

55. Jiang S, Song MJ, Shin EC, Lee MO, Kim SJ, Park JH. Apoptosis in human hepatoma cell lines by chemotherapeutic drugs via Fas-dependent and Fas-independent pathways. Hepatology, 29 (1): 101-10, 1999.

56. Dulic V, Lees E, Reed SI. Association of human cyclin E with a periodic G1-S phase protein kinase. Science, 257 (5078): 1958-61, 1992.

57. Sebastian B, Kakizuka A, Hunter T. Cdc25M2 activation of cyclin-dependent kinases by dephosphorylation of threonine-14 and tyrosine-15. Proceedings of the National Academy of Sciences of the United States of America, 90 (8): 3521-4, 1993.

58. Bennett MR, Mechanisms of p53-induced apoptosis. Biochemical Pharmacology, 58 (7): 1089-95, 1999.

59. Kirschner MW, The biochemical nature of the cell cycle. Important Advances in Oncology, : 3-16, 1992.

60. Gotoh N, Tojo A, Shibuya M. A novel pathway from phosphorylation of tyrosine residues 239/240 of Shc, contributing to suppress apoptosis by IL-3. EMBO Journal, 15 (22): 6197-204, 1996.

61. Avruch J, Zhang XF, Kyriakis JM. Raf meets Ras: completing the framework of a signal transduction pathway. Trends in Biochemical Sciences, 19 (7): 279-83, 1994.

62. Roulston A, Reinhard C, Amiri P, Williams LT. Early activation of c-Jun N-terminal kinase and p38 kinase regulate cell survival in response to tumor necrosis factor alpha. Journal of Biological Chemistry, 273 (17): 10232-9, 1998.

63. Cross TG, Scheel-Toellner D, Henriquez NV, Deacon E, Salmon M, Lord

JM. Serine/threonine protein kinases and apoptosis. Experimental Cell Research, 256 (1): 34-41, 2000.

64. Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-zL. Cell, 87 (4): 619-28, 1996.

65. Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell, 86 (1): 147-57, 1996.

66. Weinberg RA. The retinoblastoma protein and cell cycle control. [Review] [80 refs] Cell, 81 (3): 323-30, 1995.

67. Perez-Roger I, Solomon DL, Sewing A, Land H. Myc activation of cyclin E/Cdk2 kinase involves induction of cyclin E gene transcription and inhibition of p27(Kip1) binding to newly formed complexes. Oncogene, 14 (20): 2373-81, 1997.

68. Chang ZL, Lin MQ, Wang MZ, Yao Z. Studies on cell signaling immunomodulated murine peritoneal suppressor macrophages: LPS and PMA mediate the activation of RAF-1, MAPK p44 and MAPK p42 and p38 MAPK. Journal of Experimental Biology, 30 (1): 73-81, 1997.

69. Sexl V, Diehl JA, Sherr CJ, Ashmun R, Beach D, Roussel MF. A rate limiting function of cdc25A for S phase entry inversely correlates with tyrosine dephosphorylation of Cdk2. Oncogene, 18 (3): 573-82, 1999.

70. Bartek J, Lukas J. Pathways governing G1/S transition and their response to DNA damage. FEBS Letters, 490 (3): 117-22, 2001.

71. Liggett WH Jr, Sidransky D. Role of the p16 tumor suppressor gene in cancer. Journal of Clinical Oncology, 16 (3): 1197-206, 1998.

72. Espeso EA, Roncal T, Diez E, Rainbow L, Bignell E, Alvaro J, Suarez T, Denison SH, Tilburn J, Arst HN Jr, Penalva MA. On how a transcription factor can avoid its proteolytic activation in the absence of signal transduction. EMBO Journal, 19 (10): 2391, 2000.

73. 8Collado M, Medema RH, Garcia-Cao I, Dubuisson ML, Barradas M, Glassford J, Rivas C, Burgering BM, Serrano M, Lam EW. Inhibition of the phosphoinositide 3-kinase pathway induces a senescence-like arrest mediated by p27Kip1. Journal of Biological Chemistry, 275 (29): 21960-8, 2000.

74. 9Wang X, Flynn A, Waskiewicz AJ, Webb BL, Vries RG, Baines IA, Cooper JA, Proud CG. The phosphorylation of eukaryotic initiation factor eIF4E in response to phorbol esters, cell stresses, and cytokines is mediated by distinct MAP kinase pathways. Journal of Biological Chemistry, 273 (16): 9373-7, 1998.

75. Weigel TL, Lotze MT, Kim PK, Amoscato AA, Luketich JD, Odoux C. Paclitaxel-induced apoptosis in non-small cell lung cancer cell lines is associated with increased caspase-3 activity. Journal of Thoracic &

Cardiovascular Surgery, 119 (4 Pt 1): 795-803, 2000.

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