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行政院衛生署 九十三 年度委託研究計畫

成果半年報 (期末報告)

(自 95 年 1 月 至 95 年 7 月止)

計畫名稱：桑椹萃取物抑制心血管病變之研究

計畫編號：DOH93-TD-1012 (DOH95-TD-1012)

研究起訖：(年月日) 92 年 8 月 1 日至 95 年 7 月 31 日

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一、預定完成工作項目及實際執行情形

預定完成工作項目	實際執行情形
92 年 8 月-10 月: 桑椹萃取物分離	已完成桑椹萃取物之製備，獲得水萃取物與花青素萃取物。
92 年 10 月-12 月:桑椹萃取物抑制 LDL 氧化之效力分析	已確認桑椹萃取物對由銅離子誘導的 LDL 氧化有抑制作用。(fig. 1~3)
93 年 1 月-3 月: 桑椹萃取物抑制 LDL 氧化之效力分析	已確認桑椹萃取物具自由基清除效力而達抑制 LDL 氧化之作用。(fig. 4)
93 年 1 月-5 月:開始進行動物試驗	完成動物實驗之餵食模式，並成功誘導高脂血兔。
93 年 3 月-6 月: 動物試驗血脂肪分析	（1.）完成兔子實驗各組之血脂肪分析與病理切片。(fig. 5~7,table1) （2.）完成倉鼠動物實驗之餵食模式，並成功誘導高脂血倉鼠及血脂肪分析。(fig. 8~9)
93 年 7 月-12 月: 桑椹萃取物抑制 ox-LDL 引起之動脈粥狀硬化生成之細胞試驗	（1.）完成抑制 ox-LDL 引起之巨噬細胞死亡與泡沫細胞(Foam cell)形成之作用。(fig. 10~11) （2.）證實 ox-LDL 引起巨噬細胞死亡是透過 apoptosis 的方式，且桑椹萃取物有效抑制此現象。(fig. 12~13)
94 年 1 月-94 年 7 月: （1.）桑椹萃取物抑制 ox-LDL 引起巨噬細胞凋亡分子機轉分析 （2.）桑椹萃取物抑制血清刺激下 ASMC 之增生作用	（1.）利用西方墨點法分析 apoptosis 相關蛋白（PARP、caspase-3、P53、Bax）以及 anti-apoptosis（bcl-2、MCL-1、Ras、AKT-p）相關蛋白確認桑椹萃取物能抑制 ox-LDL 所刺激巨噬細胞 apoptosis 的訊息傳遞。(fig. 14~16) （2.）已確認桑椹多酚萃取物具有效抑制 ASMC 之增生係經由促進 ASMC 凋亡、DNA 斷裂及細胞週期停滯於 G0/G1。(fig17~19)

94 年 8 月-94 年 12 月: 桑椹萃取物抑制血清刺激下 ASMC 之移動作用	已確認桑椹多酚萃取物透過抑制 MMP 的分泌與表而有效抑制 ASMC 之移動。(fig28-30)
95 年 1 月-95 年 7 月: **(1.)桑椹萃取物誘導 ASMC 細胞凋亡之機轉分析** **(2.)桑椹萃取物誘導 ASMC 細胞週期停滯之機轉分析** **(3.)桑椹萃取物抑制 ASMC 移動之機轉分析**	**(1.)先前已確認桑椹多酚萃取物具有效抑制 ASMC 之增生係經由促進 ASMC 凋亡。本次報告已證實促進 ASMC 凋亡的機制係透過活化 JNK 與 p38 的磷酸化，活化下游路徑以增加 Fas-L 以活化 caspase 路徑；另外也發現 p53 的磷酸化增加 Bax、Myc 的表達、Bad 磷酸化減少與 Bcl-2、Mcl-1 的增加，都會增加粒腺體膜電位的不穩定，同樣也會活化 caspase 路徑造成 DNA 的斷裂。(fig20~23)** **(2.)進一步探討細胞週期停滯的積轉發現，桑椹多酚萃取物使 CDK 與 cyclin 的結合情形減弱，並且觀察到 Rb 磷酸化隨著時間減弱；此外，細胞週期抑制蛋白：p16、p21 與 p27 都呈現遞增的趨勢，推測桑椹多酚萃取物透過這些蛋白的表現使得 ASMC 細胞週期停滯於 G0/G1。(fig24~27)** **(3.)上次報告證實桑椹多酚萃取物透過抑制 MMP 的分泌(fig30)，本次研究證實除了透過抑制 MMP 蛋白的表達外，調控 migration 相關的 FAK /PI3K /Akt 路徑也被抑制，調控細胞骨架組裝的 small G proteins 表現也被抑制。推測桑椹多酚萃取物透過多種路徑來引響 VSMC migration 的能力。(fig30~fig33)**

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二、初步成果

95 年 1 月-95 年 7 月

1. 桑椹萃取物抑制動脈血管平滑肌細胞(ASMC) 細胞凋亡之機轉分析

a. **桑椹萃取物（MWEs、MPEs）誘導動脈血管平滑肌細胞(ASMC)DNA 斷裂的情形（figure 19）：**

運用與 DAPI 染色實驗結果懷疑動脈血管平滑肌細胞(ASMC)有可能在萃取物誘導下有 apoptosis 的情行，所以進一步萃取細胞的 DNA 分析，發現在劑量 MWEs 3~5mg/ml、MPEs 1.5~2mg/ml 的時候就偵測到 DNA 斷裂的現象（figure 19）。

b. **桑椹萃取物（MWEs、MPEs）誘導動脈血管平滑肌細胞(ASMC) caspase 的活化（figure 20）：**

因為在 Figure19 發現有 DNA 斷裂的現象，因此抽取細胞蛋白質利用西方墨點法分析 caspase 活化的情形，發現隨著萃取物的濃度增加，caspase 切割的活化態（active form）逐漸增多，尤其是桑椹多酚萃取物的組別更為明顯（figure 20）。

c. **進一步分析誘導動脈血管平滑肌細胞(ASMC) apoptosis 的相關蛋白表現（figure 21）：**

【1】在西方墨點法分析下，發現 p53 在短時間內就有活化的情形，並發現下游 Bax 與 Myc 也隨萃取物濃度增加而表達增加。(figure 21)

【2】並觀察 Death receptor（Fas）與其 ligand（Fas-L）的表現，結果證實萃取物並吳法增加 Death receptor（Fas）的表現，但是 Fas-L 的表現卻在 9 小時後增加了。(figure 21)

【3】先前觀察到 caspase 3、9 具有活化，表示萃取物可能透過影響粒線體膜電位，使 cytochrome C 從粒線體釋出，西方墨點法分析後證實了我們的推論，cytochrome C 在粒線體中的量減少，而在細胞質中的量變多。(figure 21)

d. **分析誘導動脈血管平滑肌細胞(ASMC) MAPK pathway 的相關蛋白表現（figure 22）：**

【1】利用方墨點法分析，證實萃取物的刺激下會活化 JNK 與 p38 的路徑，並觀察 JNK 與 p38 下游調控因子 AP-1 轉錄因子在 3~6 小時表達達到最高（Jun 的磷酸化與 Fos 的表現增加）。(figure 22)

【2】ERK 的磷酸化卻隨著劑量的增加而有遞減的趨勢，以多酚萃取物 MPEs 組別最為明顯，在 15 分鐘後 ERK 的磷酸化馬上就降低；水萃取物 MWEs 則在 3~6 小時才有漸少的趨勢，表示 ERK 調控細胞生長的功能在很短的時間內就會被桑椹萃取物所阻斷進而走向細胞凋亡。(figure 22)

e.**進一步分析誘導動脈血管平滑肌細胞(ASMC) anti-apoptosis 的相關蛋白表現（figure 23）：**

接著分析細胞 anti-apoptosis 蛋白的表現，經西方墨點法分析後，發現 Bcl-2、Mcl-1、磷酸化 Bad 都呈現遞減的趨勢，以最後 36 以及 48 小時的時候最明顯，也是細胞凋亡小體出現最多的時間，表示桑椹萃取物除了誘導細胞凋亡機制外也會阻斷細胞抵抗凋亡的系統。（figure 23）

2. 桑椹萃取物抑制動脈血管平滑肌細胞(ASMC) 細胞週期停滯之機轉分析

a.**桑椹萃取物（MWEs、MPEs）抑制動脈血管平滑肌細胞(ASMC) CDKs / cyclins 的結合（figure 25）：**

先以 CDK4 與 CDK2 做 immunoprecipitation，再用西方墨點法分析 CDKs 與 cyclins 結合的多寡，結果顯示，CDKs 與 cyclins 結合都在 24 小時開始明顯的遞減，而調控進入 S phase 的 CDK2 與 cyclin A 在 9~12 小時就有減少，使得細胞無法進入 DNA 合成的階段而停留在 G0/G1 phase。

b. **桑椹萃取物（MWEs、MPEs）抑制動脈血管平滑肌細胞(ASMC) Rb 磷酸化（figure 26）：**

先以西方墨點法分析發現 Rb 的磷酸化程度隨著處理萃取物時間增加而減弱，以 E2F 做 immunoprecipitation 在分析 Rb 與 E2F 結合的多寡，結果顯示，未磷酸化的 Rb 與 E2F 結合變多，顯示 E2F 無法釋放，所以細胞無法進入 S phase。

c.**桑椹萃取物（MWEs、MPEs）誘導動脈血管平滑肌細胞(ASMC) cell cycle inhibitors 蛋白的表現（figure 27）：**

cell cycle inhibitors 蛋白當中 p16、p21、p27 調控細胞週期由 G0/G1 進入 S phase，在 figure 24 結果知道萃取物會誘導動脈平滑肌細胞（ASMC）停滯於 G0/G1 phase，因此進一步以西方墨點法分析 p16、p21、p27，發現 MWEs 3mg/ml 在 3 個小時後使這些蛋白增加；而 MPEs 0.5mg/ml 再短短 15 分鐘 就大量表達這些 cell cycle inhibitors。（figure 27）

3. 桑椹萃取物抑制動脈血管平滑肌細胞(ASMC) 移動之機轉分析

a. **桑椹萃取物（MWEs、MPEs）抑制動脈血管平滑肌細胞(ASMC) Ras、PI3K/Akt 路徑，與抑制 NFκB 活化（figure 32）：**

對於細胞的移動 Ras/Raf 路徑與 PI3K/Akt 路徑被證實是很重要的關係，以西方墨點法分析這些蛋白發現，MWEs 1mg/ml、MPEs 0.5mg/ml 處理 24 小時後有明顯的降低，在 figure30~31 証實過 NFκB 轉錄 *MMP2/9* 基因活性下降，因此進一步分析 NFκB 與 IκB 的表現，結果發現 NFκB 只有在 48 小時左右有下降一點，但是 IκB 明顯增多，IκB 的增多表示 NFκB 並無被活化，這樣的結果與 figure30~31 所証實的相符合。（figure 32）

b. **桑椹萃取物（MWEs、MPEs）抑制動脈血管平滑肌細胞(ASMC) small G**

proteins 的表現（figure 33）：

small G proteins 是調控細胞骨架重要的一群蛋白，在萃取物：MWEs 1mg/ml 與 MPEs 0.2mg/ml 的作用下 cdc42, Ras, RhoA, Rac1 就有明顯的抑制，表示萃取物可能透過抑制這群蛋白來抑制動脈血管平滑肌細胞(ASMC)的移動。(figure 33）

目前之成果已接受或正審稿於 SCI 期刊，特此提供予以參考：

1.Chang-Che Chen, Li-Kaung Liu, Jeng-Dong Hsu, Hui-Pei Huang, Mon-Yuan Yang, Chau-Jong Wang, 2004, **Mulberry extract inhibits the development of atherosclerosis in cholesterol- fed rabbits.**
Food Chem. 91, (2005), 601~607.

2.Li-Kang Liu, Miao-Jane Lee, Chang-Che Chen, Chau-Jong Wang, 2004, **Antioxidantion of low-density lipoprotein and inhibition of macrophage to atherogenic stimuli by mulberry extracts.**
Food Chem.（submitted; FOCH MS 6605）

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三、研究中所遭遇之問題與困難，並請自評是否符合進度。
進度超前 V 符合進度 落後　(　　　　)月 所遭遇之問題與困難

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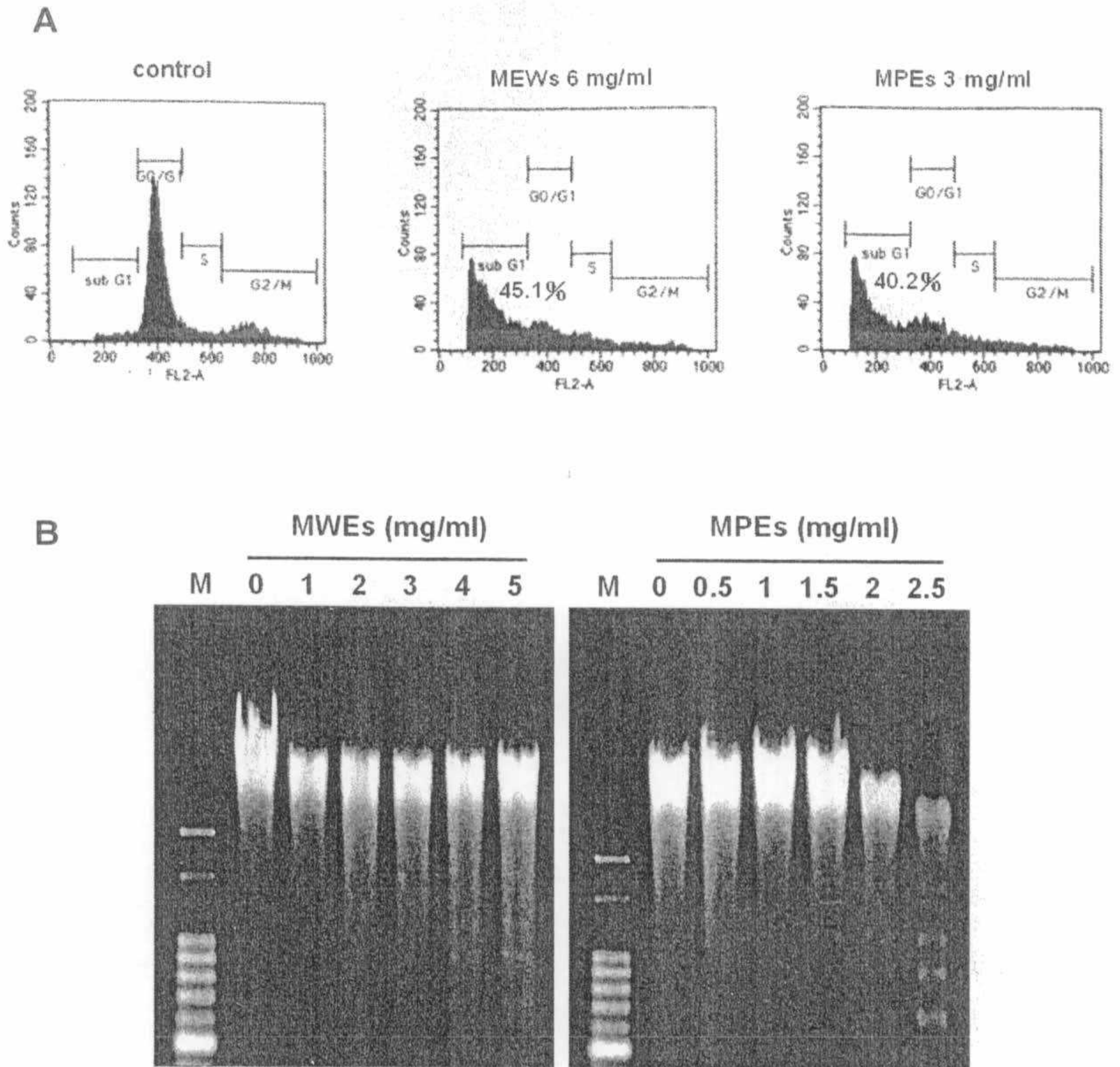


Fig 19. Apoptosis of A7r5 cells treatment with MWEs and MPEs. Cells were treated with or without MWEs and MPEs and incubated for 48 hr, the apoptotic cells were analysis by **flow cytometry (A).** (control, MWEs 6.0mg/mL, MPEs 3.0mg/mL) **Electrophoresis of fragment DNA of A7r5 cells treatment with MWEs and MPEs (B).** Genomic DNA was isolated from A7r5 cells, either untreated as control or treated with MWEs and MPEs for 48 hr.DNA fragmentation was evaluated by electrophoresis on agarose gel containing ethidium bromide which was photographed under UV light.

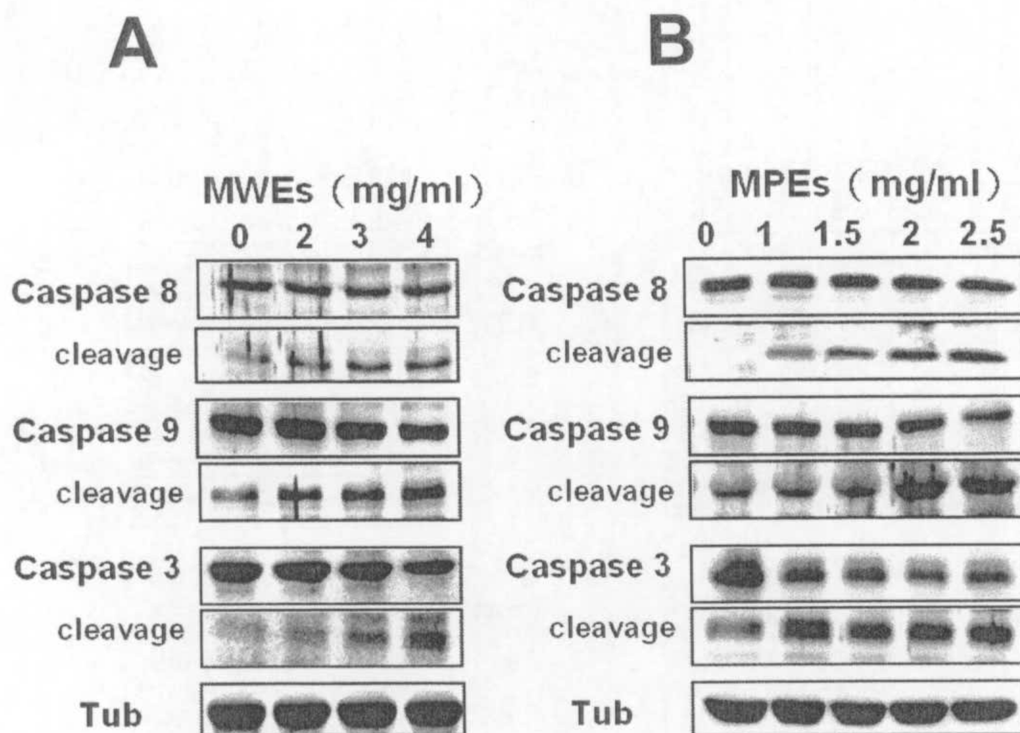


Figure 20. Induced the cleavage of caspase family proteins in A7r5 cells after treat with MWEs and MPEs. Immunoblot analysis the proteins expression of apoptosis in A7r5 cells treatment with MWEs **(A)** and MPEs **(B)**. Cultured cells were treated with or without MWEs (3 mg/ml) and MPEs (1.5 mg/ml) for different time until 48hr. Cell lysates (50 μg) prepared and subjected to western blot analysis. Proteins were dected by specific antibodies (caspase 3.8.9) or α-tubulin used for equal loading.

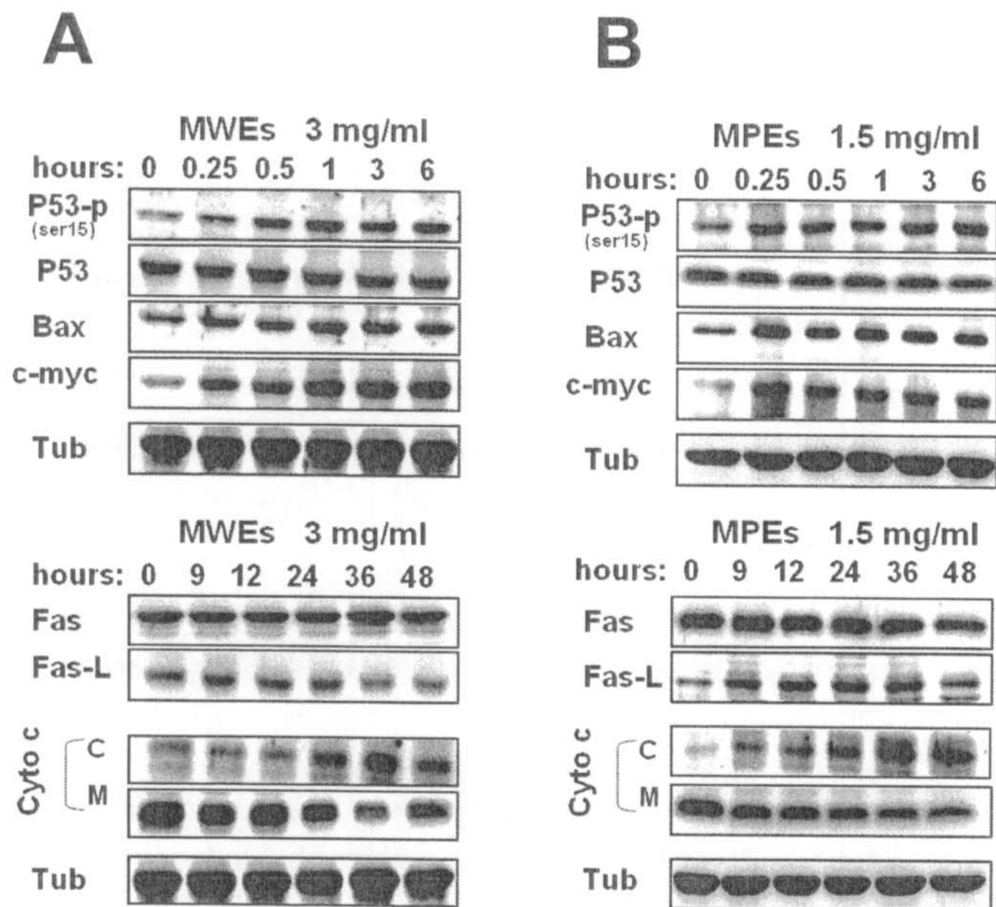


Figure 21. Induced the expression of apoptosis related proteins in A7r5 cells treatment with MWEs and MPEs. Immunoblot analysis the proteins expression of apoptosis in A7r5 cells treatment with MWEs **(A)** and MPEs **(B)**. Cultured cells were treated with or without MWEs (3 mg/ml) and MPEs (1.5 mg/ml) for different time until 48hr. Cell lysates (50 μ g) prepared and subjected to western blot analysis. Proteins were dected by specific antibodies (phospho-P53, Bax, c-Myc, Fas, Fas-L, cytochrom c) or α -tubulin used for equal loading.

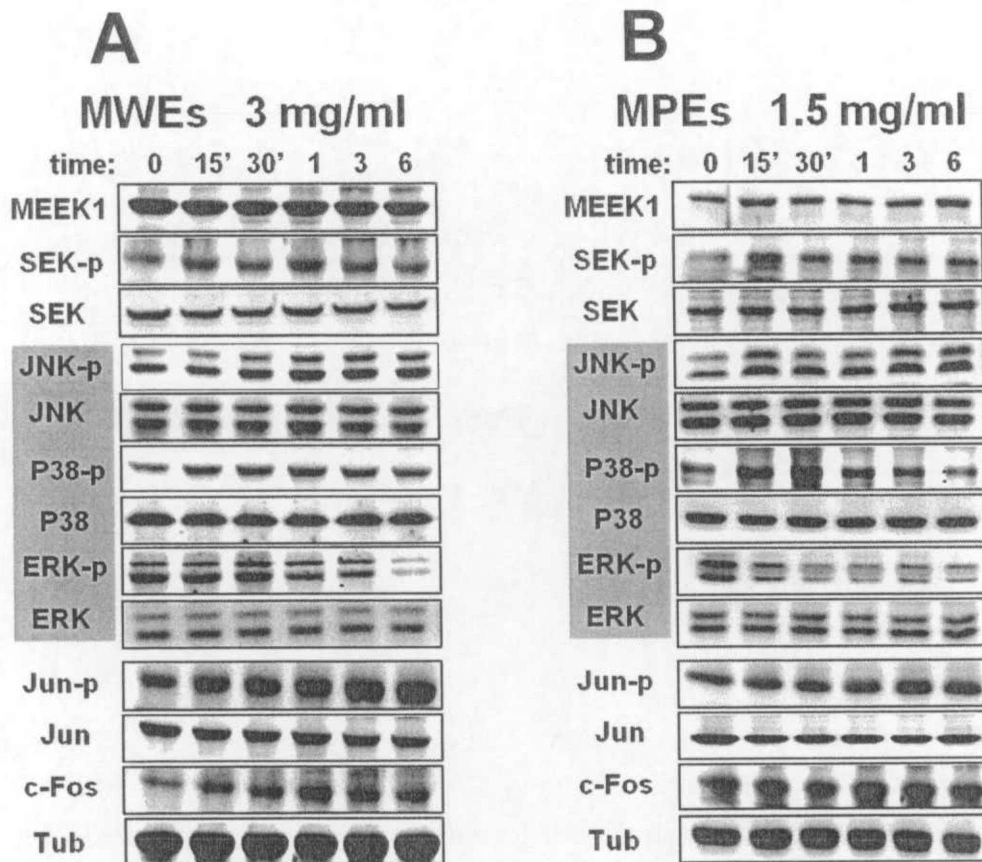


Figure 22. Induced the expression of MAPK pathway proteins in A7r5 cells treatment with MWEs and MPEs. Cultured A7r5 cells were treated with MWEs （2,3 and 4 mg/ml）**(A)** and MPEs（1,1.5,2 and 2.5 mg/ml）**(B)** for different time. Cell lysates（50 μ g） prepared and subjected to western blot analysis. Proteins were dected by specific antibodies （MEKK-1,phospho-SEK, phospho-JNK, phospho-P38, phospho-ERK, phospho-Jun and c-Fos） or α -tubulin used for equal loading.

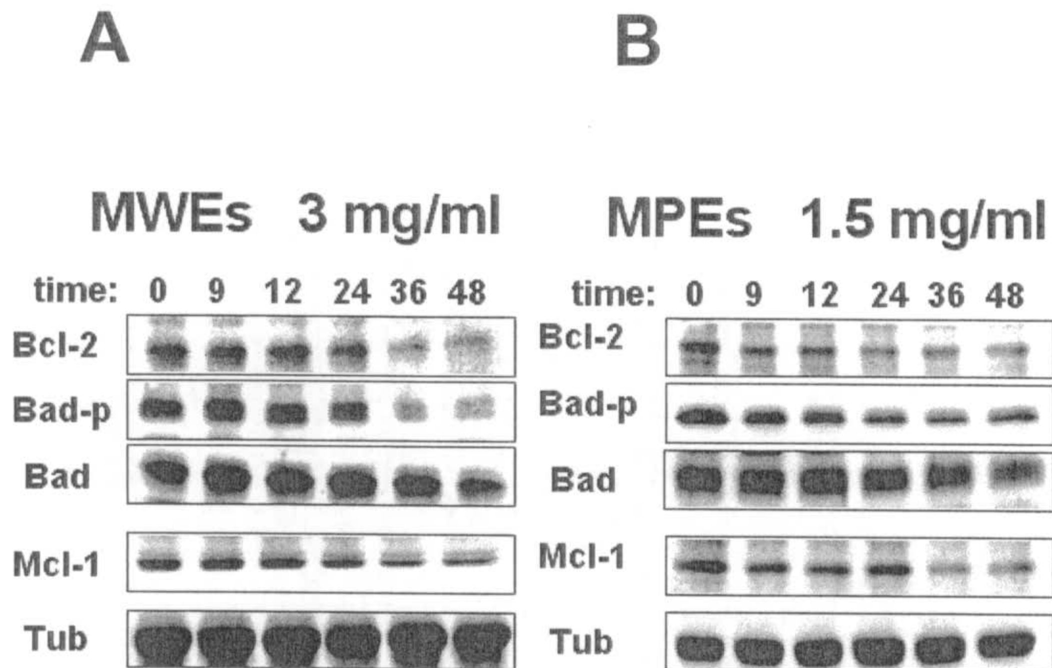
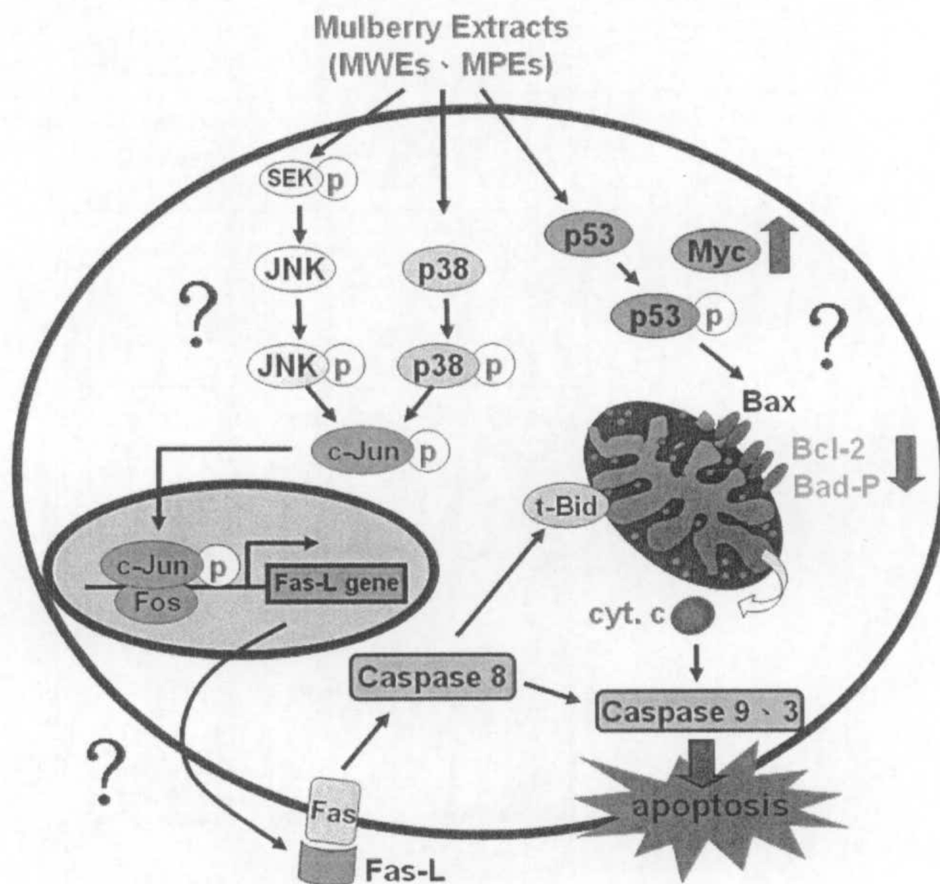


Figure 23. Reduced the expression of anti-apoptosis proteins in A7r5 cells treatment with MWEs and MPEs. Cultured A7r5 cells were treated with MWEs (3 mg/ml) **(A)** and MPEs (1.5 mg/ml) **(B)** for different time. Cell lysates (50 μg) prepared and subjected to western blot analysis. Proteins were dected by specific antibodies (Bcl-2,phospho-Bad, non-phospho-Bad, and Mcl-1) or α-tubulin used for equal loading.

summary



We surmise possible signal transduction machine as below figure (figure17~23)

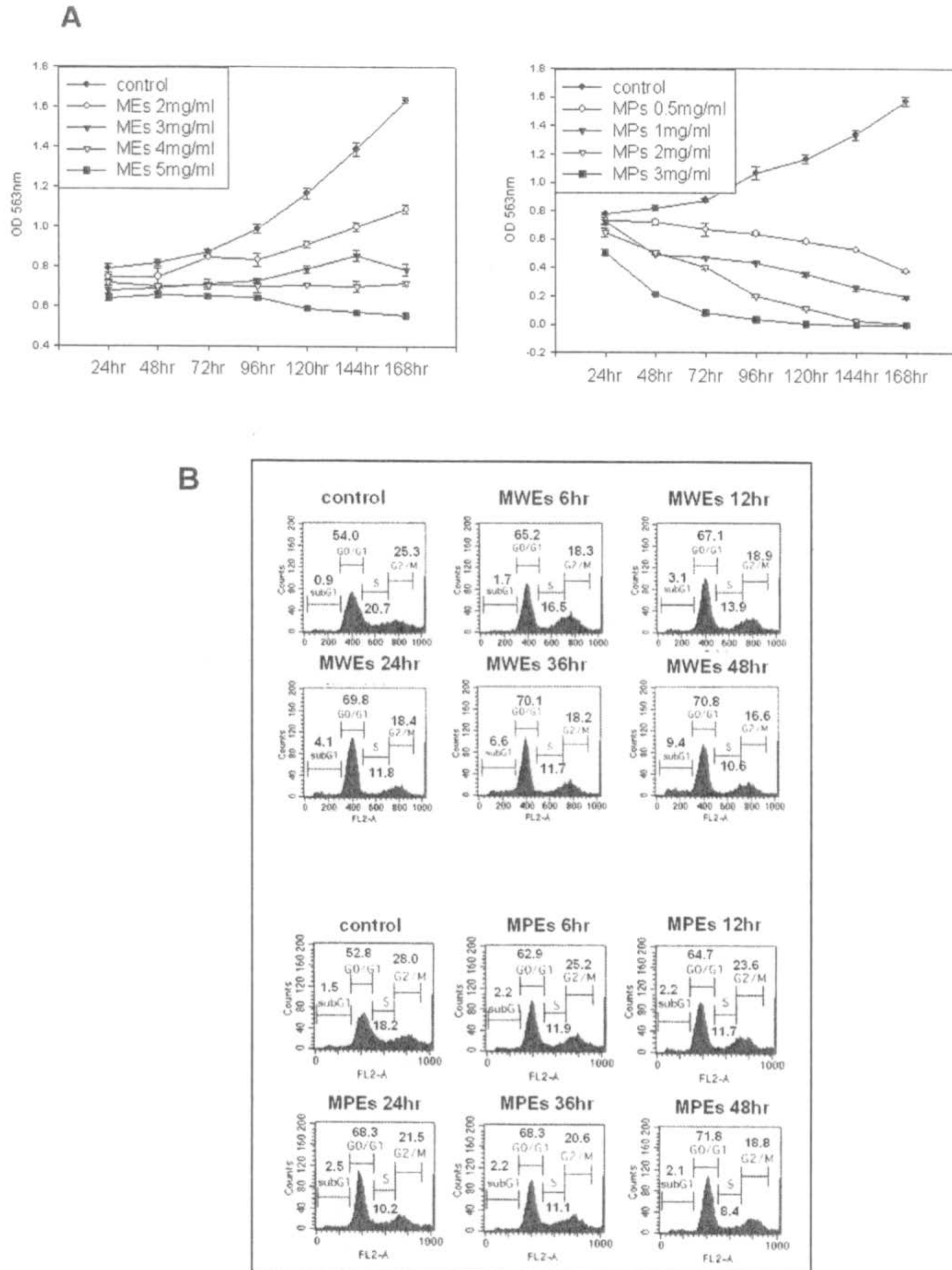


Fig 24.(A) MWEs and MPEs inhibits cell proliferation in A7r5 cells by MTT assay for time-dependent . Cultured cells were treated with or withiut MWEs (2.0~5.0 mg/mL) and MPEs (0.5~3.0 mg/ml) for 7 days. Cell viability was analyzed by MTT assay in different time course. The data were means ± SD from 3 samples for each group. **(B) Low does of MWEs and MPEs inhibits cell cycle entry in A7r5 cells by flow cytometry assay with time-dependent.** PI-stained DNA histograms of MWEs(4mg/ml) 、MPEs(0.5mg/ml)-treated cells´ are shown. Cells were untreated control or treated with different concentration MWEs and MPEs for 48 hr in 10% FBS medium.

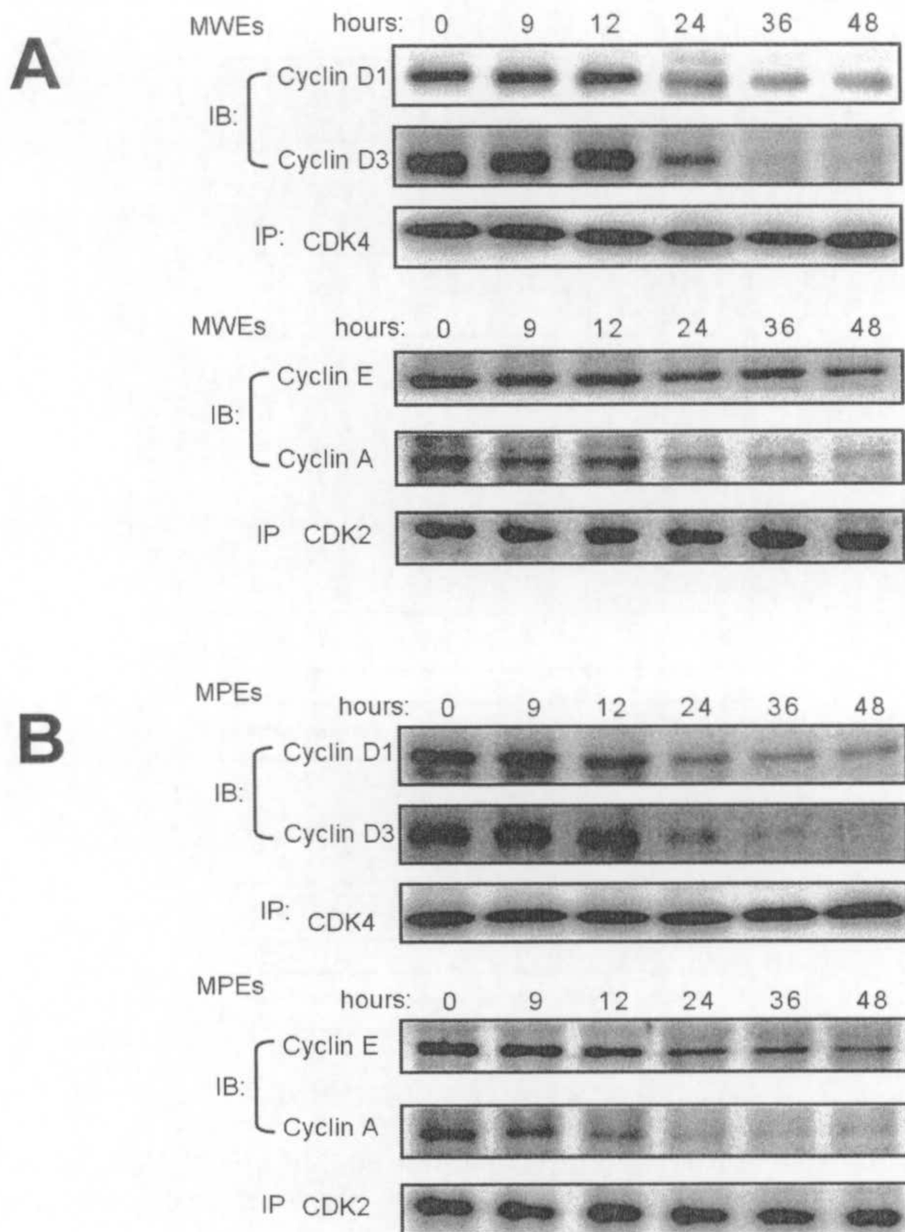


Figure 25. Reduced the expression of cell cycle regulators in A7r5 cells treatment with MWEs and MPEs. Cultured A7r5 cells were treated with MWEs (3 mg/ml) **(A)** and MPEs (1 mg/ml) **(B)** for different time. Cell lysates (50 μg) prepared and subjected to western blot analysis. Proteins were immunoprecipitated with CDK4 and CDK2 antibodies. The precipitated complexes were examined for immunoblotting using Cyclins and CDKs antibodies (Cyclin D1, Cyclin D3, Cyclin E, Cyclin A , CDK4 and CDK2) .

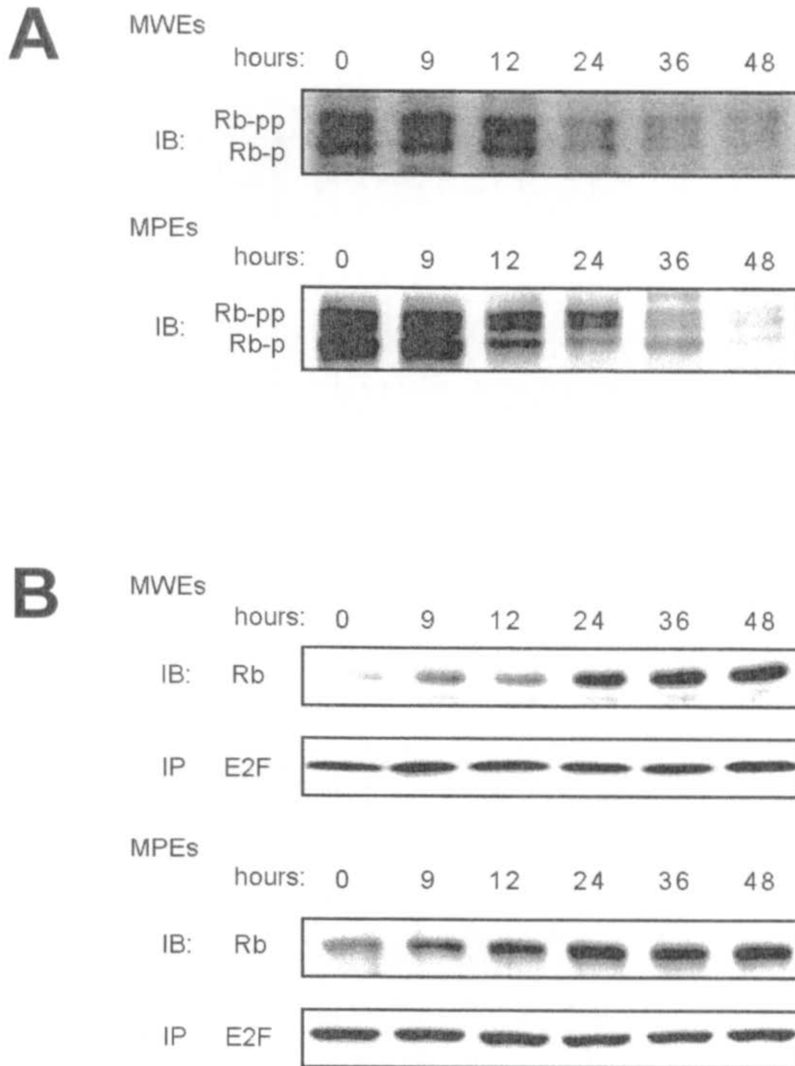
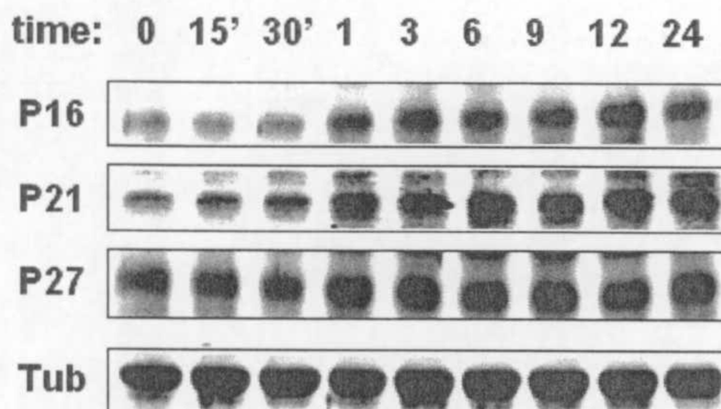


Figure 26. Expression of Rb phosphorylation was inhibited by MWEs and MPEs in A7r5 cells. Cell extracts prepared from A7r5 cells indicated following treatment for different time with MWEs 3 mg/ml and MPEs 1mg/ml, Cell lysates（50 μg）were immunoblotted with phospho-Rb antibody（**A**） and immunoprecipitated with E2F antibody（**B**）. The precipitated complexes were examined for immunoblotting using non-phospho-Rb and E2F antibodies.

MWEs



MPEs

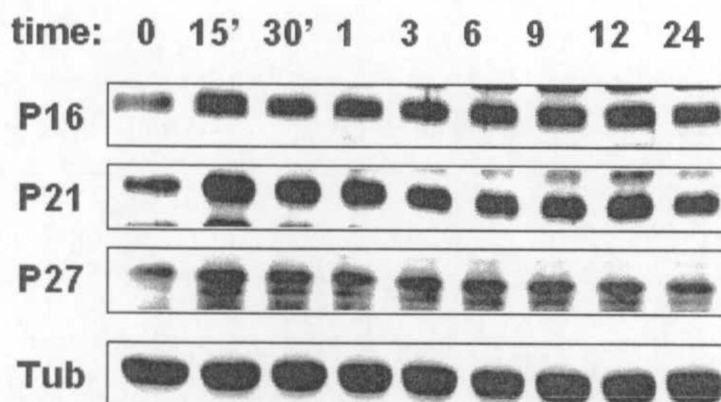
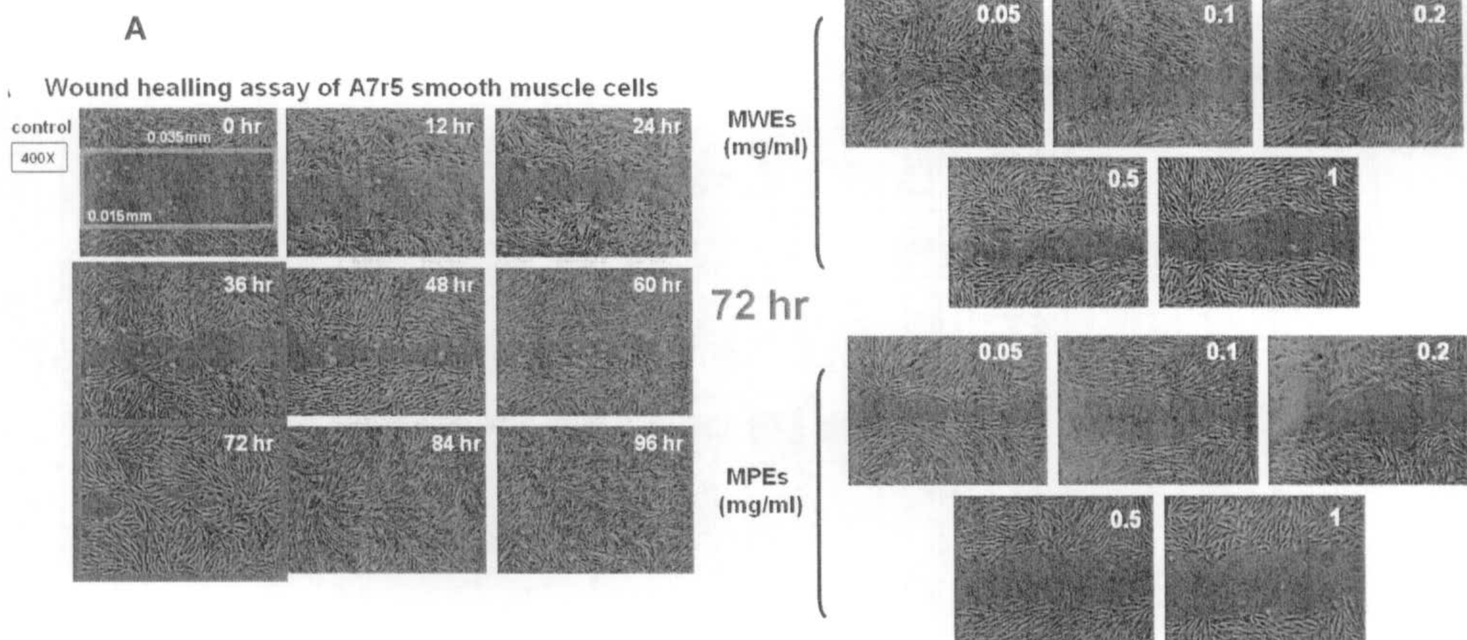


Figure 27. Expression of cell cycle negative regulators was induced by MWEs and MPEs in A7r5 cells. Cell extracts prepared from A7r5 cells indicated following treatment for different time with MWEs 3 mg/ml (**A**) and MPEs 1 mg/ml (**B**), Cell lysates(50 μ g)were immunoblotted with p16, p21, and p27 specific antibodies or α -tubulin used for equal loading.



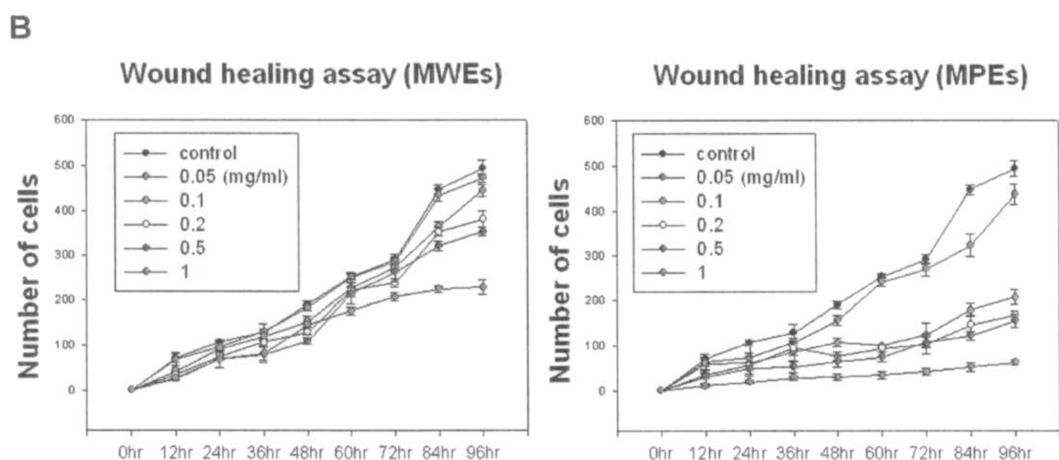


Figure 28.(A) Wound healling assay of A7r5 smooth muscle cells treates with MWEs and MPEs. After starvation 48 hr ,VSMC treated with MWEs and MPEs for 96 hr and Migrated cells were analyzed using a Wound healling assay .(B) Motility was quantified by counting the number of cells that migrate into and fill the wounded area under microscopy (100X). Results are shown as mean ± SD from three independent experiments.

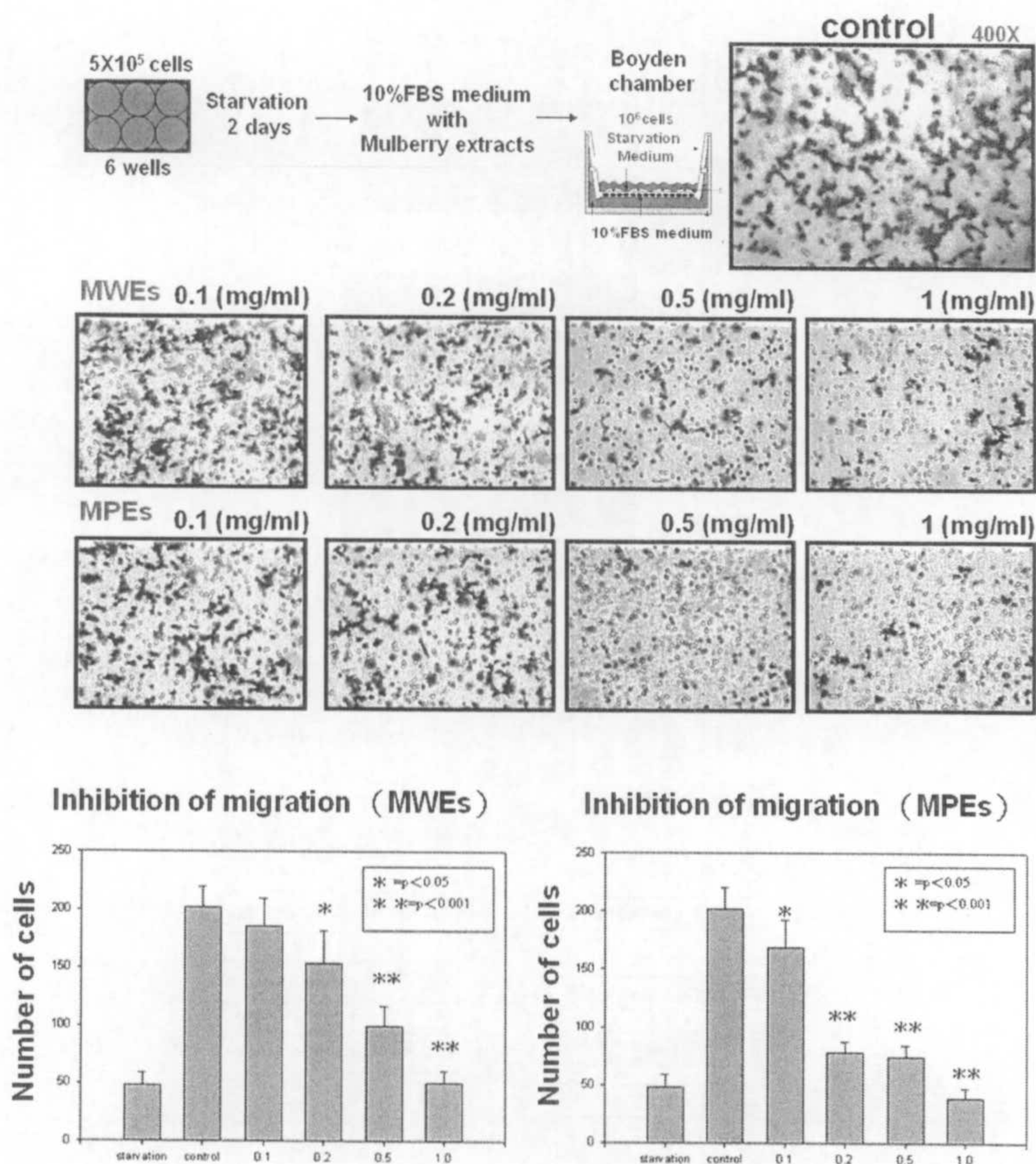
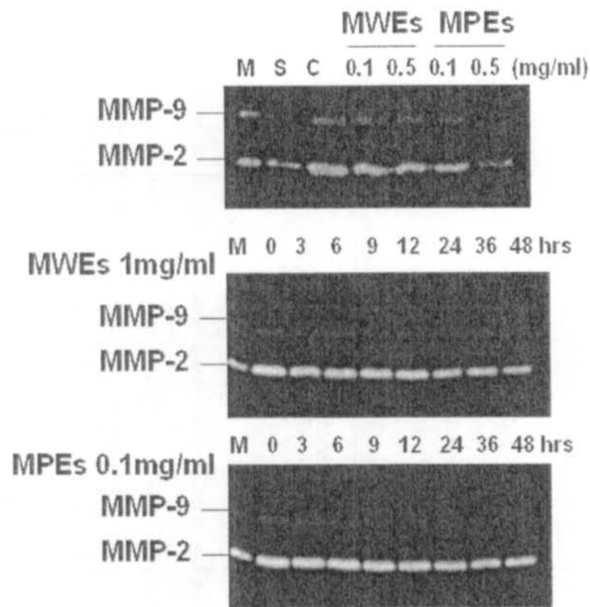


Figure 29. Mulberry extracts inhibit VSMC migration. After starvation 48 hr ,VSMC treated with MWEs and MPEs for 96 hr and Migrated cells were analyzed using using a modified Byden chamber（A）. Cells in serum-free DMEM were added to the upper chamber and allowed to migrate for 6 h thgough 8-μm porous membrane chamber toward the lower chamber to conditioned medium. Motility was quantified by counting the number of cells that migrate to the undersides of the membrane under microscopy (400X) （B）. Results are shown as mean ± SD from three independent experiments.



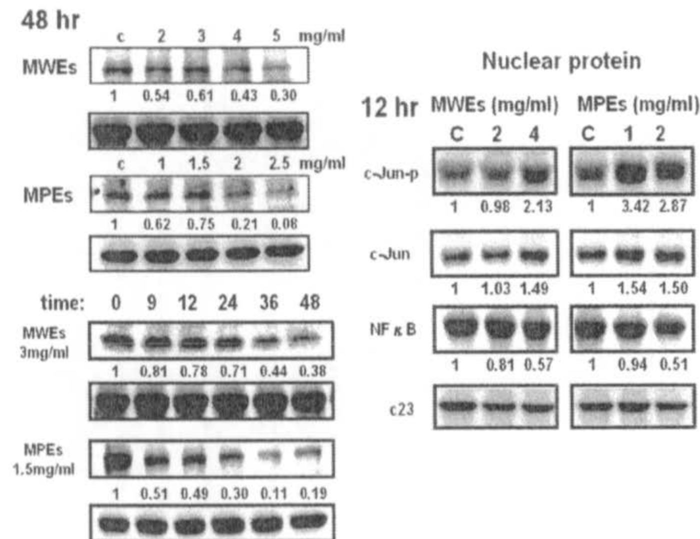


Figure 30.(A) Mulberry extracts inhibit VSMC migration by down regulate the secretion of MMP-2 and MMP-9 . Gelatin zymography assay of A7r5 smooth muscle cells treated with MWEs and MPEs. This figure is representing of three independent experiments with similar results. **(B) Effect of MWEs and MPEs on the level of FAK and nuclear protein in A7r5 cell .** Protein expression were analyzed after the treatment of MWEs and MPEs at the indicated time by Western blot assay using the specific monoclonal antibodies.

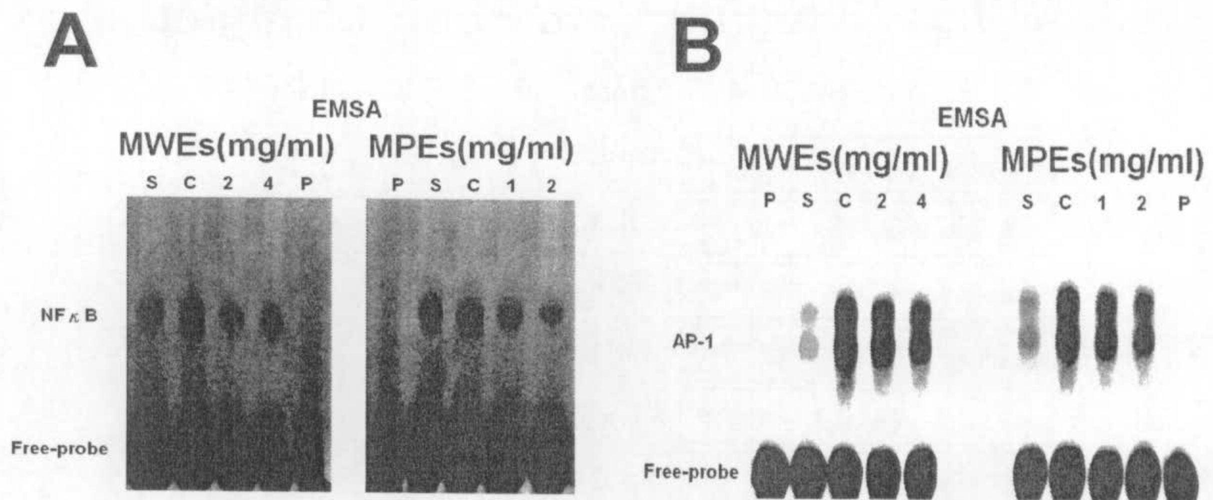


Figure 31. Mulberry extracts inhibit VSMC migration by down regulation of *MMP-2* and *MMP-9* gene expression. A7r5 cell treated with MWEs(2 and 4 mg/ml) and MPEs (1 and 2 mg/ml) for 12hr. Nuclear protein were analyzed by EMSA (probe for NFκB （**A**）and AP-1（**B**）biding site of MMP-2 gene）.

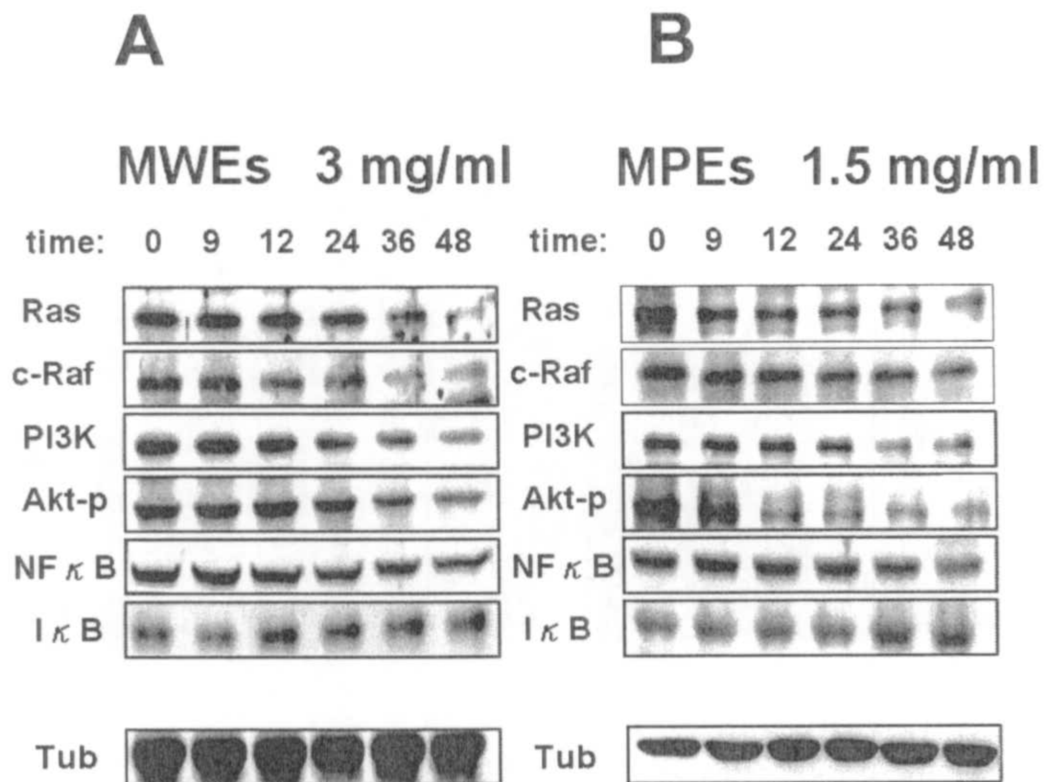


Figure 32. Expression of Ras/Raf/PI3K pathway and NFκB activation were inhibited by MWEs and MPEs in A7r5 cells. Cell extracts prepared from A7r5 cells indicated following treatment for different time with MWEs 1 mg/ml (**A**) and MPEs 0.5mg/ml (**B**), Cell lysates (50 μg) were immunoblotted with specific antibodies or α-tubulin used for equal loading.

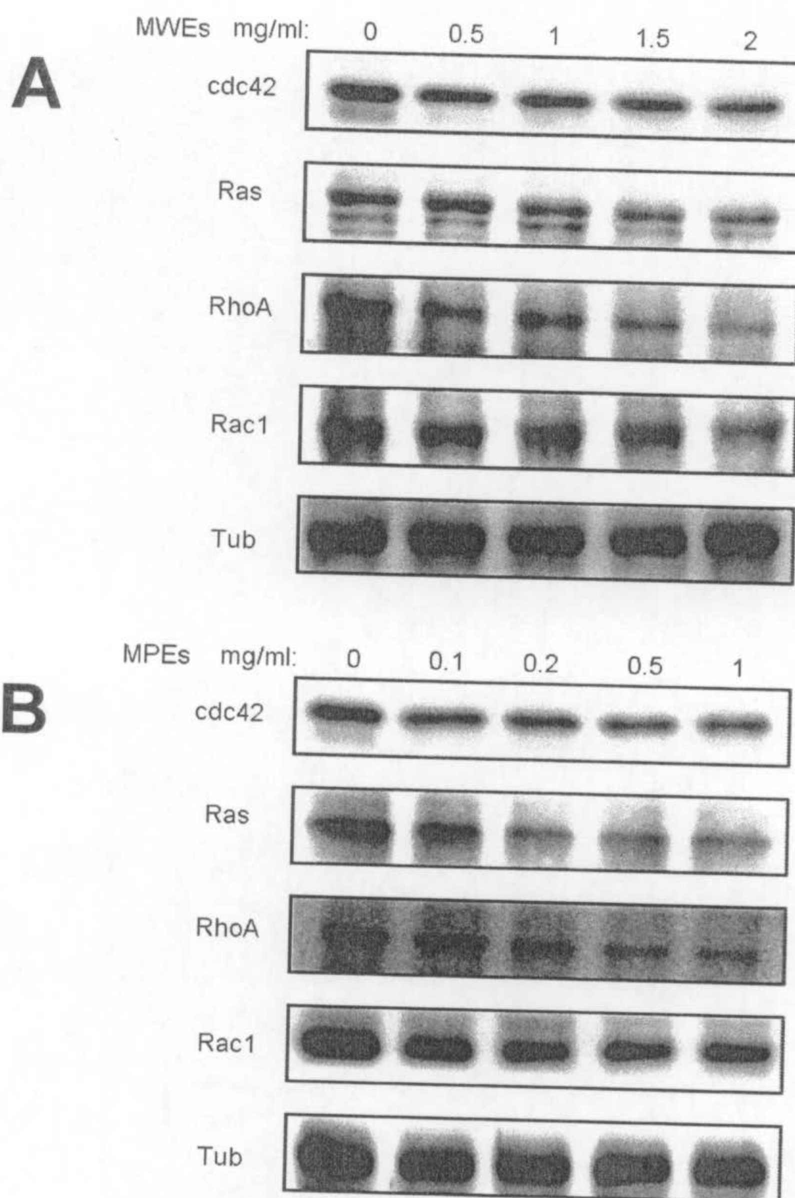
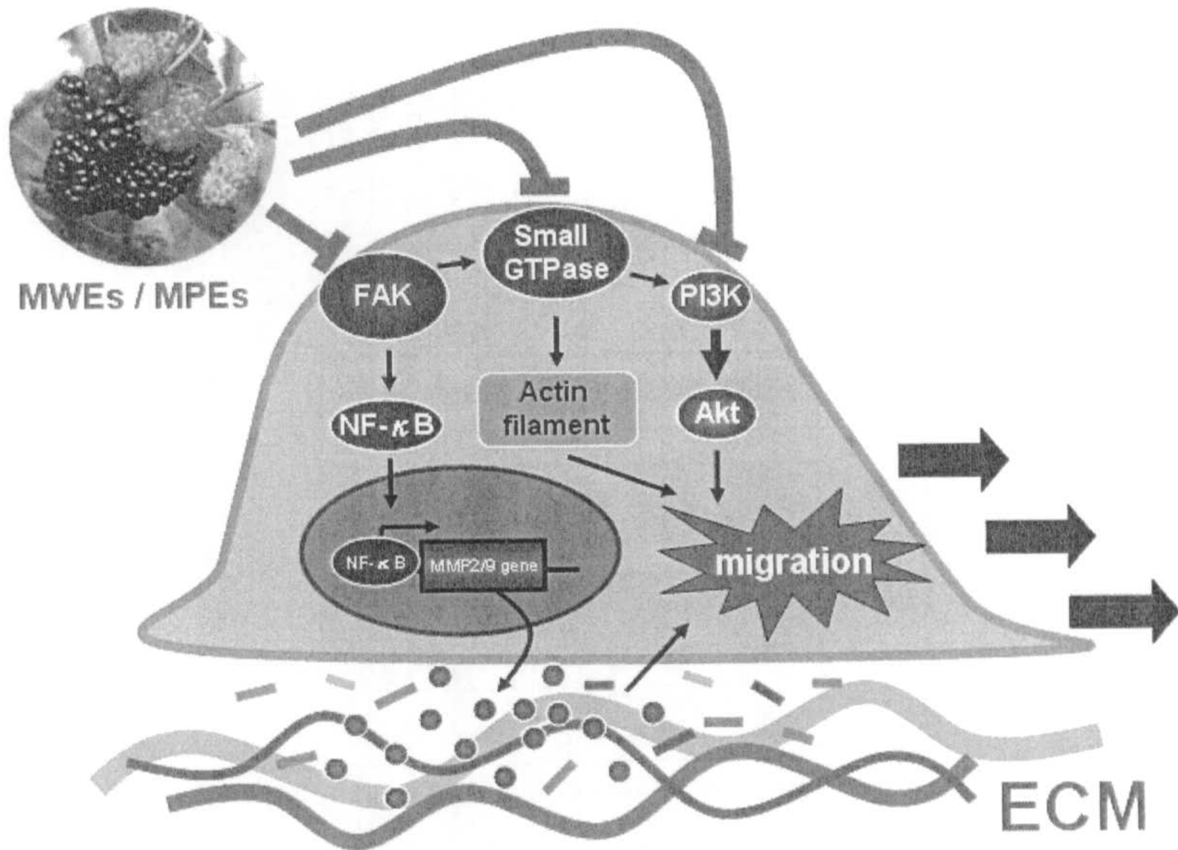


Figure 33. Expression of small G proteins were inhibited by MWEs and MPEs in A7r5 cells. Cell extracts prepared from A7r5 cells indicated following treatment for different time with MWEs 0.5~2 mg/ml(**A**) and MPEs 0.1~1mg/ml(**B**), Cell lysates (50 μg) were immunoblotted with cdc42, Ras, RhoA, Rac1 specific antibodies or α-tubulin used for equal loading.

summary



MWEs and MPEs inhibits growth factor inducing migration in VSMC.
(Figure 30~33)