行政院國家科學委員會專題研究計畫　成果報告

木犀草素對肝細胞生長因子誘發肝癌細胞伸散及遷移作用之影響及其機制之研究

計畫類別：個別型計畫
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計畫主持人：曾翠華

計畫參與人員：吳蘭鳳　李維駿

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行政院國家科學委員會補助專題研究計畫 **V 成果報告** / **□期中進度報告**

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處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
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執行單位：中山醫學大學

中　華　民　國　九十四 年　八　月　　十 日

壹 前言

木犀草素(Luteolin)，屬於黃酮類(flavones)化合物，除了本研究室主要研究對象—兔兒菜之外，在大部分的菊科植物以及蜂膠中均可分析出該成分。從文獻中發現Luteolin具有多種生物活性如 anti-allergy、anti-inflammation，並且具有 neuroprotection 的能力可避免 oxidative stress 所誘發的 SH-SY5Y 細胞死亡，而在抗癌活性方面 Luteolin 可抑制 TPA 所誘發的 mice skin papillomas、抑制人類肝癌細胞 HepG2 proliferation、藉由 Block EGF receptor tyrosine kinase activity 造成 pancreatic tumor cells 生長抑制並走向細胞凋亡，Luteolin 對於其他人類癌細胞包含(renal A-549,ovary SK-OV-3,melanoma SK-MEL-2,XF-498,HCT-15,gastric HGC-27)也都有抑制作用。由這些文獻資料確知，Luteolin 在抗癌活性及抗癌機轉方面的確有深入研究以及開發的價值 。

肝癌是國人十大死因之一，肝癌初期增生很緩慢，但進入惡化階段則肝腫大速度非常快且極易轉移，約34～78%的肝癌患者，在解剖時發現有肝癌轉移的現象，肝癌轉移大多數發生於肺、淋巴結及骨骼等，而且肝癌一旦轉移則預後奇差。因此，如何有效抑制因肝癌細胞轉移所造成的病情急速惡化以及預後差、低存活率等問題是目前研究人員極力探尋及研究的方向。肝細胞生長因子(HGF/SF)及其接受器c-Met(tyrosine kinase receptor)的細胞訊息傳遞促使細胞走向proliferation(mitosis)、 scattering (motility)以及branching morphogenesis。而經由HGF/SF刺激c-Met活化的訊息傳遞流程也參與了embryological development、wound healing、angiogenesis tissue regeneration、growth、invasion以及morphogenic differentiation等過程。但近年來研究發現，不正常之HGF/SF—c-Met訊息傳遞在許多腫瘤的之進展與惡化中扮演著重要的角色，例如肝癌、乳癌、結腸癌等。臨床上的統計資料顯示，肝癌病患血清中的HGF濃度通常比一般人高且其濃度高低與其預後好壞具有相關性，HGF濃度越高則癌細胞惡化轉移之機率也越高。HGF透過c-Met啟動細胞產生形態上的改變，細胞藉由片狀觸手(lamellipodia)及釘狀偽足(filopodia)開始伸展(scattering)，同時它也破壞細胞與細胞間正常的排列與黏附，進而使細胞可以開始移行(migration)甚至侵襲(invasion)其他組織，因而造成癌症之惡化轉移。

貳、研究目的

癌症的預防及治療一直是醫界與科學界學者致力研究的目標。然而行政院衛生署近年來的統計資料仍顯示惡性腫瘤依然是國人十大死因榜首，尤以肝癌對國人的殺傷力最高。近年來已發現許多天然物中存在著具生物活性的化學物質，而且當中的化學物質有些甚至具有抑制癌細胞生長的功效。天然植物-兔兒菜(*Ixeris chinensis*)，在民間常用於解熱、消炎、腫瘍、乳癌、毒蛇咬傷等用途，由本研究室先前之研究已發現兔兒菜萃取物具有抗氧化、抗發炎等功效，並且更進一步分離出兔兒菜主要活性成分—Luteolin，發現Luteolin可抑制肝癌細胞株生長甚至具有誘發肝癌細胞凋亡之能力。然而在肝癌的治療上最令醫療人員困擾的即是其惡化轉移的特性，不但造成癌細胞擴散至其他組織難以控制更使得治療的預後極差。因此，本研究即深入探討兔兒菜主要活性成分—Luteolin抑制肝癌細胞擴散轉移之作用及機制。

三、研究方法

(一)、細胞毒性分析(MTT Assay)：

根據 Alley 等人發表於 1988 年的 *Cancer research* 期刊(40)，其原理是利用活細胞能經由粒腺體 dehydrogenase 的作用，將 MTT 代謝還原成紫色的 formazan crystal，並在波長 563nm 有特殊吸光值。

(二)、細胞發散性測定(Cell Scattering)：

步驟：

1. 將細胞培養後，以 trypsin-EDTA 將細胞由培養皿 trypsinize 下來，用 PBS 沖洗後，以培養基將細胞濃度調整成 2×10^4 cells/ml。
2. 各取 1ml 細胞液分別培養於 6-Well 培養皿中。
3. 待細胞貼壁後，以 serum-free DMEM Medium 進行 Starvation overnight。
4. 更換新的培養基（內含不同 sample 濃度）

   Sample 為→ Luteolin

   Sample 為→ 0.2% DMSO（對照組）
5. 於加藥處理 2 小時後，加入 HGF(40ng/ml)。
6. 放入 5% CO_2，37℃培養箱中，培養 24 小時。
7. 以 Microscope 進行觀察。

(三)、細胞移動性分析(Cell Migration Assay)：

根據 Falk W 於 1980 年發表於 *J Immunol Methods* 期刊之方法，在癌細胞擴散轉移的過程中癌細胞接觸或穿透 basement membranes 是一個關鍵性的步驟，因此相較於傳統的 Boyden chemotaxis chamber，Falk W 等人利用一個 48-well chemotaxis chamber 將操作所需的物料及時間降到最低，以利快速評估癌細胞 migration 的能力。

(四)、細胞侵襲性(Cell Invasion Assay)：

根據 Falk W 於 1980 年發表於 J Immunol Methods 期刊之方法 ，在癌細胞擴散轉移的過程中癌細胞是否可分解並穿透 basement membranes 是一個關鍵性的步驟，因此相較於傳統的 Boyden chemotaxis chamber，Falk W 等人利用一個 48-well chemotaxis chamber 將操作所需的物料及時間降到最低，以利快速評估癌細胞 invasion 的能力。

(五)、反轉錄聚合酶鏈鎖反應 Reverse Transcriptase-Polymerase Chain Reaction(RT-PCR)：

在進行 RNA 層次的分析實驗時由於 RNA 極易因外在環境因素影響而分解，因此利用 RT-PCR 方法以 RNA 為起始材料經逆轉錄反應產生 cDNA。cDNA 的穩定較佳也比較容易保存，最後再以 cDNA 為範本進行 PCR 擴增，而獲取目的基因或檢測基因表達。

(六)、西方墨點法(Western blot analysis)：

實驗原理為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。

四、結果與討論

一、Luteolin對HepG2細胞的毒性測試：

本研究針對 Luteolin 進行一連串與 Metastasis 具有相關性的預試驗。首先利用 MTT assay 來進行 Luteolin 對肝癌細胞株(HepG2)之毒殺能力分析。由(Figure 1)結果顯示 Luteolin 濃度分別為 20μM、40μM 及 80μM 時，HepG2 細胞存活率分別為 92.9355%、82.7846%及 62.0713%，由於本研究方向著重於肝癌細胞 Metastasis 的研究，因此根據(Figure 4)結果選擇 40μM 以下之無毒劑量進行接下來的實驗。

二、Luteolin 可抑制 HepG2 Cell Scattering：

由(Figure 2)結果可知，Luteolin 濃度分別為 10μM、20μM 及 40μM 時，由圖中結果可以發現 Luteolin 對 HepG2 Cell Scattering 的抑制效果具有 dose-dependent 的情形。相較於處理 HGF/SF 組，當 Luteolin 濃度為 10μM 時由細胞形態可發現呈現擴張及伸出偽足的細胞數目較 HGF/SF 組少，而細胞向外發散的情形也比較不嚴重(細胞與細胞間的距離較近)，當 Luteolin 濃度為 20μM 時即可明顯發現細胞形態呈現擴張及伸出偽足的細胞僅剩少數並且細胞與細胞間的距離又更為接近，當 Luteolin 濃度達到 40μM 時細胞則呈現一團一團島狀無明顯發散之情形。

三、Luteolin 可抑制 HepG2 Cell Migration：

本實驗模擬 HGF/SF 誘發人類肝癌細胞 HepG2 移行，以觀察 Luteolin 對 HepG2 細胞 Migration 是否具有抑制效果？利用 modified Boyden Chamber 進行細胞移動性的分析，low chamber 置入含有 HGF(20ng/ml)的 conditioned medium 當 inducer，並在 low chamber 放上一層 polycarbonate filter (8μl) 而 up chamber 則是處理不同 sample 濃度(Luteolin＝5μM 、10μM、20μM、30μM 、40μM)的 HepG2 細胞(5×10^4 cells/ml)，放入 5% CO_2，37℃培養箱中，培養 24 小時後觀察細胞 migration 的情形。由(Figure 3)結果可發現隨著 Luteolin 處理濃度增加其對 HepG2 Cell Migration 的抑制效果呈現 dose-dependent 的情形，且相較於處理 HGF/SF 組，Luteolin 濃度為 20μM 即可達到抑制 HepG2 細胞 Migration 程度達 51.25%的效果。而 Luteolin 濃度為 40μM 時更可以抑制 HepG2 細胞 Migration 高達 78.5%。綜合(Figure 2、3)的結果可證實 Luteolin 的確可抑制 HGF/SF 所誘發人類肝癌細胞 HepG2 的轉移。

四、Luteolin 可抑制 HepG2 Cell Invasion：

在癌細胞Metastasis的過程中，癌細胞會透過分泌matrix metalloproteinases(MMPs)來分解 extracellular matrix(ECM)使得癌細胞能隨著血管轉移及侵襲其它組織。因此，癌細胞是否具有侵襲其它組織的能力我們可利用 Modified Boyden Chamber 來進行 Invasion Assay。本實驗模擬 HGF/SF 誘發人類肝癌細胞 HepG2 invasion 的情形，來觀察 Luteolin 對 HGF/SF 誘發人類肝癌細胞 HepG2 invassion 的情形是否具有抑制效果？首先將 low chamber 置入含有 HGF(20ng/ml)的 conditioned medium 當 inducer，並在 low chamber 放上一層 polycarbonate filter (8μl)，polycarbonate filter 表面 coated 一層 40μg/cm^2 的 Matrigel basement membrane matrix。並且在 而 up chamber 則是處理不同 Luteolin 濃度(10μM、20μM、40μM)的 HepG2 細胞(5×10^4cells/ml)，放入 5% CO_2，37℃培養箱中，培養 24 小時後觀察細胞 invassion 的情形。由(Figure 7)結果可發現隨著 Luteolin 處理濃度增加，其對 HepG2 cell

invasion 的抑制效果呈現 dose-dependent 的情形，且相較於處理 HGF/SF 組，當 Luteolin 處理濃度為 10μM 時即可達到抑制 HepG2 細胞 invassion 程度達 59.75%的效果。而在 Luteolin 處理濃度為 40μM 時其抑制 HepG2 細胞 invassion 程度更高達 80.75%。

五、Luteolin 對 HepG2 Cell Morphology 的影響：

由(Figure 5~7)的結果我們已經證實 Luteolin 具有抑制細胞 Scattering、Migration 及 Invassion 這些癌細胞 Metastasis 所必經過程的能力，然而 Luteolin 是否也對癌細胞 Metastasis 時細胞產生片狀或釘狀偽足這些形態上的變化也同樣具有影響力呢？首先將培養的細胞(2×10^2cells/ml)處理不同濃度 Luteolin＝10μM、20μM、40μM)，於加藥處理 2 小時後，加入 HGF(40ng/ml)當 inducer，接著放入 5% CO_2 ，37℃培養箱中，培養 4 小時後以 TRITC-phalloidin 進行染色之後於 Fluorescence Microscopy 進行觀察。由(Figure 5)結果我們發現，相對於 control 組在 HGF(40ng/ml)處理之下細胞形態呈現拉伸的情形並且在細胞邊緣形成片狀(Ruffles)及釘(Spike)偽足(Lamellipodia、Filopodia 等)。然而在處理 Luteolin＝10μM、20μM、40μM 各組中我們可發現，隨著 Luteolin 濃度增加細胞擴張拉伸的情形逐漸消失並且片狀及釘狀偽足的數目也越來越少而細胞邊緣也呈現如 control 組一般圓滑。因此由(Figure 5)的結果我們證實 Luteolin 可抑制 HGF/SF 誘發肝癌細胞藉由 cytoskeleton organization 所形成的細胞形態變化。

六、Luteolin抑制HGF/SF所誘發之HepG2細胞c-Met之RNA level及磷酸化證據：

由(Figure 6、7)的結果我們已經證實 Luteolin 具有抑制 HGF/SF 所誘發之人類肝癌細胞 HepG2 metastasis 的能力，我們針對 c-Met 進行 RNA level 的驗證。首先將培養的細胞(2×10^5 cells/ml)處理不同濃度 Luteolin(10μM、20μM、40μM)，於加藥處理 2 小時後，加入 HGF(40ng/ml)當 inducer，接著放入 5% CO_2 ，37℃培養箱中，培養 4 小時後萃取細胞 RNA 以 RT-PCR 分析 c-Met RNA 表現量。(Figure 6)的結果我們發現，相較於處理 HGF(40ng/ml)組在處理 Luteolin(10μM、20μM、40μM)情況下 c-met RNA 表現量隨著 Luteolin 濃度增加而下降，而且在 Luteolin 濃度為 10μM 時即可看出明顯差異。接著我們利用 Immunoprecipitation 及 Western blotting 方法進行 c-Met 蛋白磷酸化測定，由(Figure 7)的結果可發現相較於 HGF(20ng/ml)組，在處理 Luteolin(10μM、20μM、40μM)情況下隨著 Luteolin 濃度增加 c-Met 蛋白磷酸化情形反而下降。綜合以上結果，我們證實 Luteolin 不但能抑制 c-met RNA level 表現，更可以抑制 c-Met 活化。

七、Luteolin 抑制 HGF/SF 所誘發之 HepG2 細胞 MMP9 之活化證據

文獻指出人類肝癌細胞中 MMP-9 有過度表達的現象，而這種情形與癌細胞的 invasion 及 metastasis 具有相關性，因此我們利用 Western blotting 進行 MMP-9 表現量的分析。由(Figure8)的結果發現在處理 Luteolin 濃度為 10μM 情形之下即可有效抑制活化形式的 MMP-9 表現，，因此我們推斷 Luteolin 抑制 HGF 所誘發之人類肝癌細胞 HepG2 invasion 可能是透過抑制 MMP-9 活化使 MMP-9 無法分解 ECM 而達到抑制 HepG2 invasion 的效果。

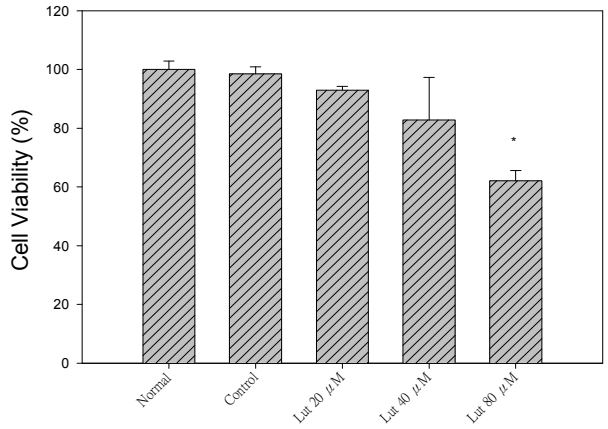

FIGURE 1.Cell Viability of Luteolin-treated HepG2 cells. HepG2 cells were treated with Luteolin(20 μM、40 μM、80 μM) for 24 h, and the viability of the cells were determined by MTT assay. *$p < 0.05$, compared with normal group.

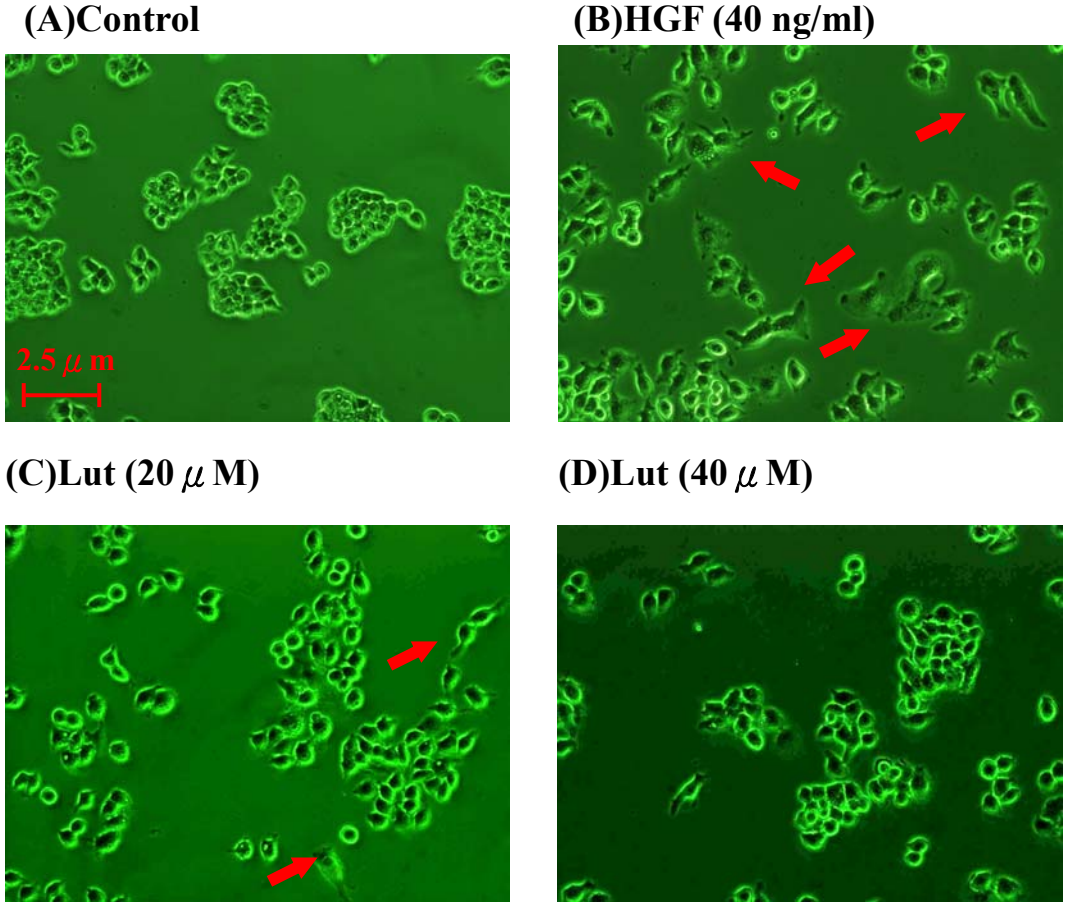

FIGURE 2. The effect of Luteolin on HGF-induced cell Scattering of HepG2 cells. (A)Control cells. (B) Cells treated with HGF (40 ng/ml) for 24 h. (C、D、) Cells treated with HGF plus Luteolin (20 μM、40 μM) for 24 h. (The inhibitors were added to the cells 2 h before the addition of HGF). (observed with 100 X microscope).The cell treated with HGF were scattering and formation lamellipodia and filopodia(fig.B),treated with Luteolin inhibited cell scattering and cell morphology change.

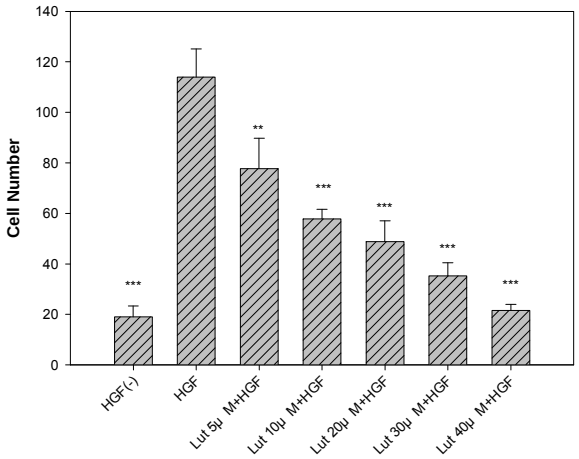

FIGURE 3. The effect of Luteolin on HGF-induced cell migration of HepG2 cells. HepG2 cells were seeded onto the upper chamber consisting of 8μm pore-size filters. The agent added to the lower chamber was HGF (20ng/ml) or none. The upper chamber seeding (5 $\times 10^4$ HepG2 cells) treated with or without Luteolin (10 μM、20 μM、30 μM and 40 μM). After24-hour incubation, cells that migrate the filter were counted as described in Materials and Methods. **p<0.05 , **p<0.005, ***p<0.0001*,compared with HGF treatment.

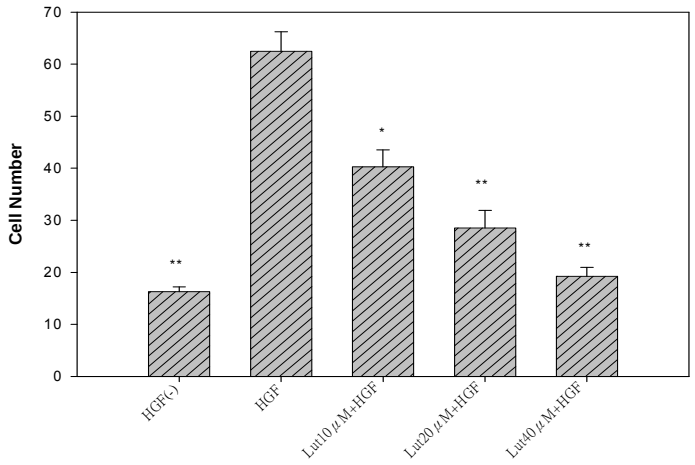

FIGURE 4. The effect of Luteolin on HGF-induced cell Invasion of HepG2 cells. HepG2 cells were seeded onto the upper chamber consisting of 8μm pore-size filters coated with Matrigel basement membrane matrix. The agent added to the lower chamber was HGF (20ng/ml) or none. The upper chamber seeding (5 $\times 10^4$ HepG2 cells) treated with or without Luteolin (10μM、20μM、30μM and 40μM). After24-hour incubation, cells that migrate the filter were counted as described in Materials and Methods. **p<0.05 , **p<0.005* compared with HGF treatment.

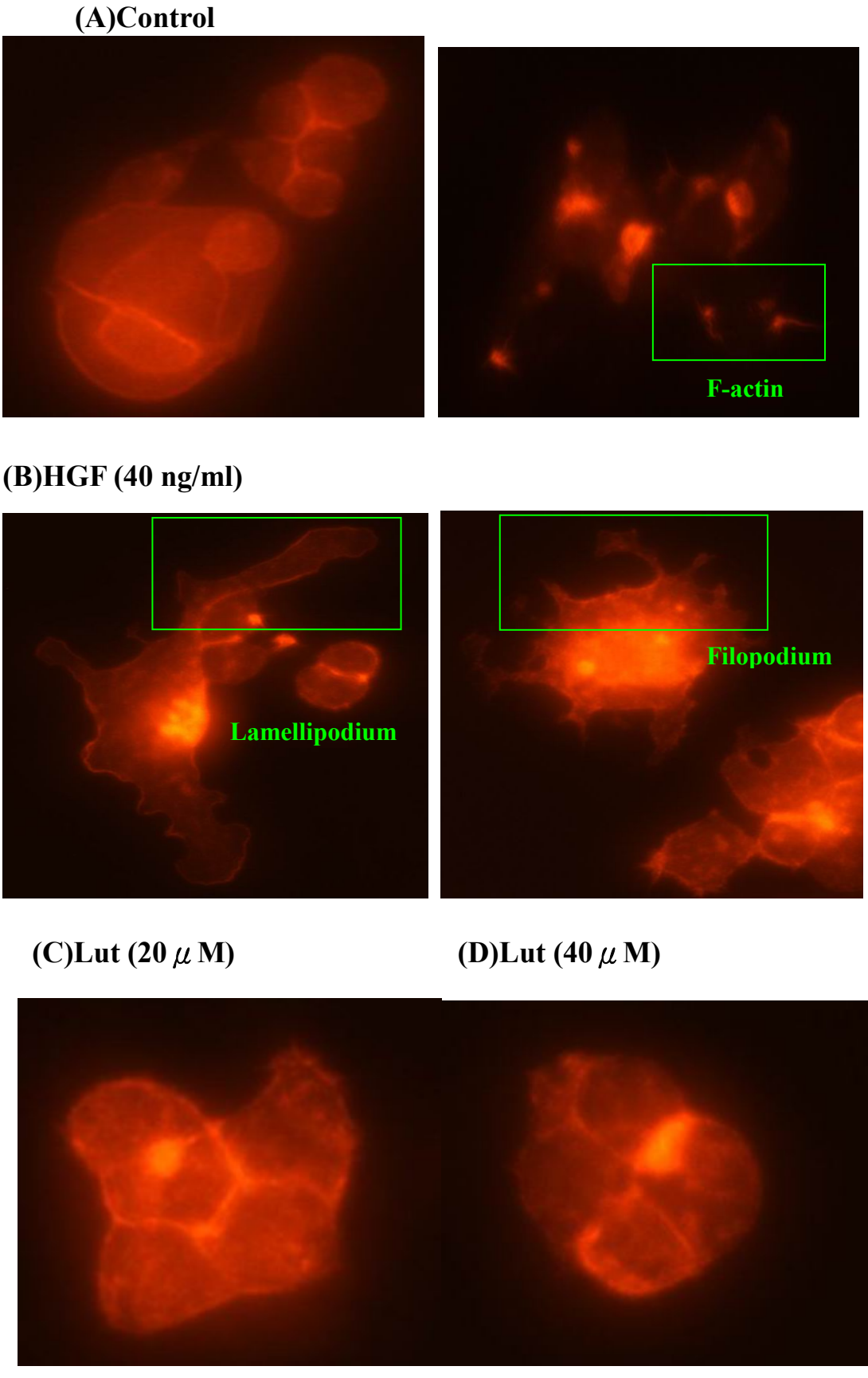

FIGURE 5. The effect of Luteolin on HGF-induced HepG2 cell morphological change and formation of Lamellipodium、Filopodium. HepG2 cells were treated with HGF（40 ng/ml）with or without Luteolin（10、20、40 μM）for 4 h, and F-actin polymerization was visualized by TRITC-conjugated phalloidin.（200X microscope）The cell treated with HGF were formation lamellipodia and filopodia(fig.B),treated with Luteolin inhibited cell morphology change.

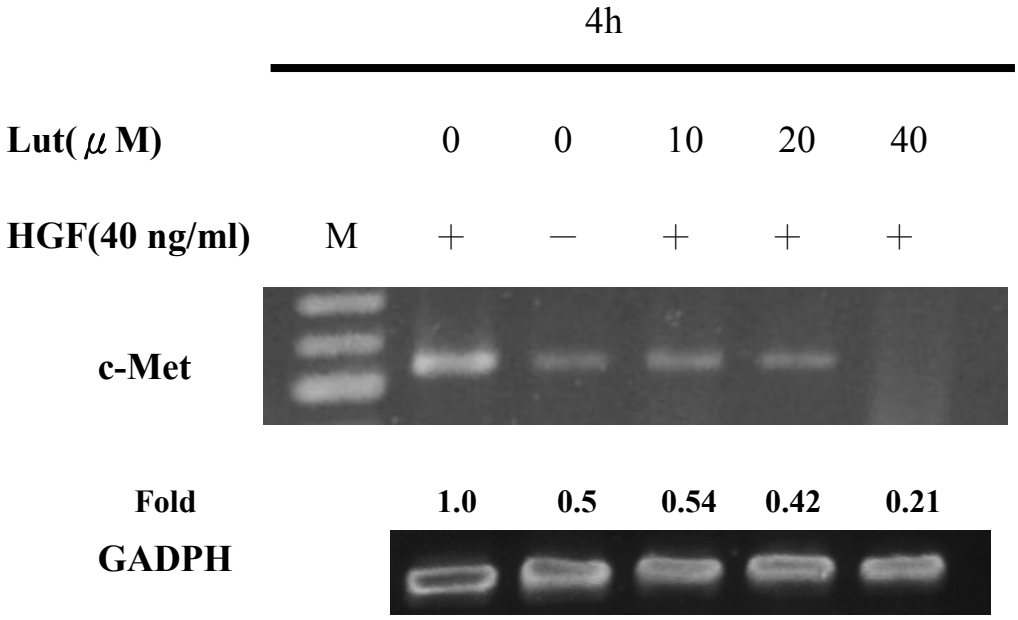

FIGURE 6. The expression of c-met mRNA in human hepatoma cell lines. Cells were cultured in medium containing HGF (40 ng/ml) with or without Luteolin (10 μM、20 μM and 40 μM) for 4h, following treatment cell were harvested. Total RNA were extracted from hepatoma cells. 4.0 μg total RNA was used as template in RT-PCR . GADPH was used as an internal control.

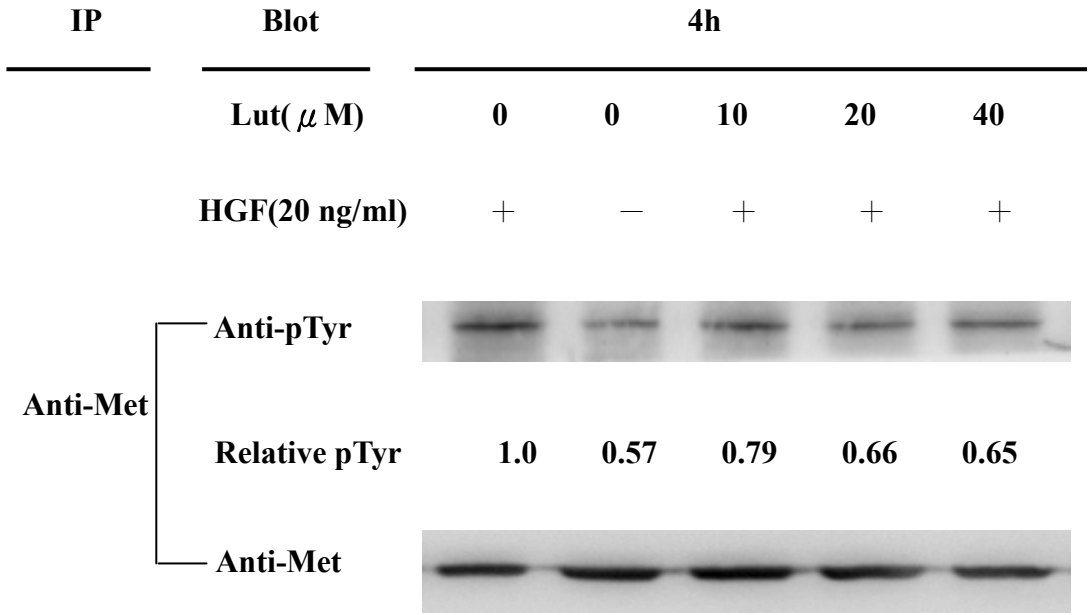

FIGURE 7. c-Met protein level and tyrosine phosphorylation. Cells were cultured in medium containing HGF (20 ng/ml) with or without Luteolin (10 μ M、20 μ M and 40 μ M) for 4h, following treatment cell were harvested. Met was immunoprecipitated (IP) from HepG2 cell lysates with a specific anti-human Met polyclonal antibody, and the resulting immune complexes were separated by SDS–8% polyacrylamide gel electrophoresis.The blots were probed sequentially with antibodies against the phosphotyrosine epitope (anti-pTyr) and Met β subunit (anti-Met).

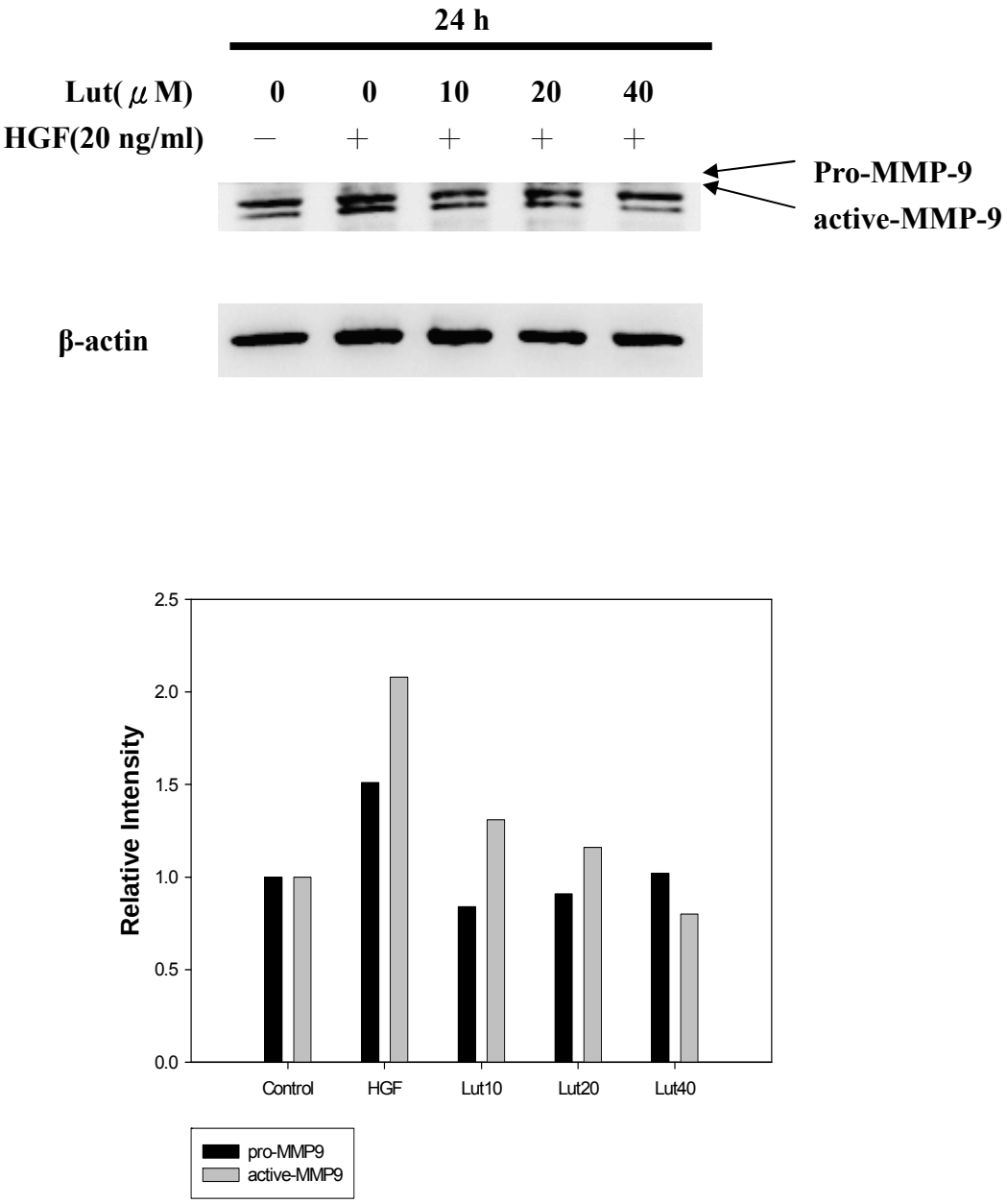

FIGURE 8. The effect of Luteolin on HGF-induced MMP-9 expression in HepG2 cells. Cells were cultured in medium containing HGF (20 ng/ml) with or without Luteolin (10 μM、20 μM and 40 μM) for 24h, following treatment cell were harvested. Cell lysates from HepG2 cells were analyzed by Western blot analysis：50μg of protein extract from each condition was electrophoresed in each lane of 8% SDS-PAGE and analysed by immunoblot with the anti-MMP-9 antibody or actin used for equal loading.

五、自評

本研究首次發現木犀草素對肝細胞生長因子誘發肝細胞伸散及遷移作用具有抑制之作用，有達預定探討之目標，以後將發展動物模式做進一步之研究。

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Inhibitory effect of luteolin on hepatocyte growth factor/scatter factor-induced HepG2 cell invasion involving both MAPK/ERKs and PI3K–Akt pathways

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Abstract

Hepatocyte growth factor (HGF), also known as scatter factor (SF), and its receptor, the c-Met tyrosine kinase, play roles in cancer invasion and metastasis in a wide variety of tumor cells. Clinical observations suggest that HGF can promote metastasis of hepatoma cells while stimulating tumor invasiveness. We use HGF as an invasive inducer of human hepatoma HepG2 cells to investigate the effect of flavonoids on anti-invasion. In our preliminary study, we investigated the effect of flavonoids including luteolin, quercetin, baicalein, genistein, taxifolin and catechin on HGF-mediated migration and invasion of HepG2 cells. We found that luteolin presented the most potent potential on anti-migration and anti-invasion by Boyden chamber assay. Furthermore, luteolin inhibited HGF-induced cell scattering and cytoskeleton change such as filopodia and lamellipodia was determined by both phase-contrast and fluorescence microscopy studies. In addition, Western blotting and immunoprecipitation were performed to confirm luteolin suppressed the phosphorylation of c-Met, the membrane receptor of HGF, as well as ERK1/2 and Akt, but not JNK1/2, which is activated by HGF. Our investigation demonstrated that luteolin similar to PD98059, which acts as a specific inhibitor of MEK, an up stream kinase regulating ERK1/2, and wortmannin, a PI3K inhibitor, inhibited the invasiveness induced by HGF. In conclusion, the luteolin inhibited HGF-induced HepG2 cell invasion involving both MAPK/ERKs and PI3K–Akt pathways.

Keywords: Luteolin; Invasion; HGF; HepG2; ERK; Akt

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors seen in the tropics and the Far East, including Taiwan. During the early stage of hepatocarcinogenesis, HCC is predominantly well differentiated, and the HCCs proliferate slowly [1]. However, HCC becomes progressively dedifferentiated with tumor enlargement, and most advanced HCCs have high proliferation activity. During this stage, the tumors progress to give rise to intrahepatic metastasis as well as extrahepatic metastasis [2]. Many factors have been reported to be involved in metastasis including hepatocyte growth factor (HGF). HGF is produced by nonparenchymal liver cells, and serum levels of HGF are elevated in patients

Abbreviations: ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; MAPK, mitogen activator protein kinase; HGF, hepatocyte growth factor

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with acute and chronic liver diseases such as hepatocellular carcinoma. HGF is a pleiotropic factor, inducing mitogenesis, morphogenesis and metastagenesis on a variety of epithelial cells [3]. These pleiotropic effects of HGF could contribute to tumor invasion and metastasis. The receptor for HGF is a receptor type tyrosine kinase encoded by the c-Met proto-oncogene. Increased c-Met and HGF expression by human tumor cells is often associated with high tumor grade and poor prognosis [4]. While normal HGF-Met signaling is involved in many aspects of embryogenesis, abnormal HGF-Met signaling has been shown to play a significant role in promoting tumor invasion, metastasis and angiogenesis. Cell invasion is a complicated process that involves partial detachment from intercellular adhesions, and from cell–ECM interaction mediated by integrins, reorganization of the actin cytoskeleton and movement through the ECM [5,6]. Cell scattering is an important component of several physiological and pathological processes such as embryonic morphogenesis, tissue regeneration and tumor invasion. In addition, directed cell migration, a fundamental characteristic of tumor cell invasion, involves protrusive activities at the leading edge of the cell driven by a regulated cycle of polymerization and depolymerization of actin filaments. The initial protrusive structure of filopodia and lamellipodia, for example, contains dense arrays of actin filaments.

Flavonoids are the most abundant polyphenols in our diet and are found in soybeans, tea, fruits and vegetables. They have been suggested to possess anticancer and chemopreventive property in numerous epidemiological studies and to inhibit the proliferation of tumor cells including breast, prostate and lung cancer cells in vitro [7,8]. However, their anti-metastasis or anti-invasiveness properties remain unclear. Luteolin (3′4′5′7′-tetrahydroxyflavone), an important member of the flavonoid family, is present in various fruits and vegetable. It exhibits a wide spectrum of pharmacological properties including anti-inflammatory and anti-allergic properties [9]. Much attention has been recently paid to its antioxidant properties and to its anti-proliferative effects [10,11]. It was reported that luteolin has potential for anticancer therapy through inhibiting DNA topoisom erase I and II [12,13]. Ko et al. reported that luteolin demonstrated inhibition of proliferation and induction of apoptosis in human myeloid leukemia cells [14]. In addition, our previous study showed that luteolin induced apoptosis via a mechanism involving mitochondria translocation of Bax/Bak and activation of JNK [15]. The aim of this study is to evaluate the anti-migration and anti-invasion potential of luteolin and to investigate the signal pathway involved.

2. Materials and methods

2.1. Materials

Luteolin and recombinant human HGF (rhHGF) (Sigma, St. Louis, MO, USA), Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY), Matrigel (Collaborative Biomedical Products, Bedford, MA), wortmannin (PI3K inhibitor; Tocris Cookson, Bristol, UK), PD98059 (MEK inhibitor; Promega, WI, USA), SP600125 (JNK inhibitor; Promega), anti-phospho-ERKs (Thr^{202}/Tyr^{204}), anti-phospho-JNKs (Thr^{183}/Tyr^{185}), anti-phospho-p38 (Thr^{180}/Tyr^{182}), anti-phospho-Akt (Ser^{473}) and anti-phospho-tyrosine antibodies were purchased from Cell Signaling Technology (Beverly, MA), ERK, JNK, p38, Akt and c-Met antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and anti-β-actin was obtained from Sigma.

2.2. Cell culture

Human hepatoma HepG2 cells were maintained in Dulbecco's modified Eagle's medium and in a humidified atmosphere of 5% CO_2/95% air at 37 °C. The medium was supplemented with 10% (v/v) fetal calf serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Before the addition of HGF, the cells were incubated overnight without serum.

2.3. Assessment of cell viability

The effect of flavonoids on cell viability was estimated by the MTT assay. In brief, the cells were seeded at 5×10^4 cells/ml density and incubated with flavonoids (40 μM) including luteolin, quercetin, baicalein, genistein, taxifolin and catechin for 24 h. Thereafter the medium was changed and incubated with MTT (0.5 mg/ml) for 4 h. The number of viable cells was directly proportional to the production of formazan, which was then solubilized with isopropanol, and measured spectrophotometrically at 563 nm.

2.4. Morphologic study

HepG2 cells were cultured at 2×10^4 cells/ml in 10% FBS–DMEM in a six-well plate for 12 h, followed by starvation overnight. After pretreatment with various concentrations of luteolin for 2 h, HGF (40 ng/ml) was added and the morphologic changes of HepG2 for 24 h were observed under a phase-contrast microscope. In addition, cells plated onto the six-well plate for 24 h with a density of 2×10^2 cells/ml were starved overnight.

After pretreatment with various concentrations of luteolin for 2 h, HGF (40 ng/ml) was applied for 4 h in 5% CO_2 37 °C incubator, fixed in 4% formaldehyde for 10 min and washed with PBS. The cells were stained with TRITC-phalloidin (500 ng/ml) for 1 h and washed with PBS. The actin stress fibers in the cells were observed under fluorescence microscopy.

2.5. In vitro assays of cell migration and invasion

Cell invasion assays were performed using Boyden chemotaxis chamber obtained from Neuro Probe Inc. The upper culture chamber consisted of an 8-μm pore size polycarbonate filter coated with a uniform layer of 40 μg/cm^2 of Matrigel basement membrane matrix and was placed on the top of the lower culture chamber. HepG2 cells (5×10^4) suspended in DMEM medium were placed in the upper compartment of the chemotaxis chamber in the presence of flavonoids (40 μM) or various concentrations of luteolin. In the lower chamber, serum-free DMEM medium containing 20 ng/ml HGF served as a source of chemoattractants. After incubation for 24 h, the cells on the upper surface of the filter were wiped with a cotton swab. The cells on the lower surface of the filters were fixed for 10 min with methanol and stained with Giemsa for 1 h, and the cells that invaded the lower surface of the filter were then counted under a microscope. For each replicate, the HepG2 cells in five randomly selected fields were determined, and the counts were averaged. Migration assays were done by the same procedure, except that the polycarbonate filters were not coated with Matrigel.

2.6. Preparation of total cell extracts and immunoblots analysis

To prepare the whole-cell extract, cells were washed with PBS plus zinc ion (1 mM) and suspended in a lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 1% NP 40, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, 81 μg/ml aprotinine, 170 μg/ml leupeptin, 100 μg/ml PMSF; pH 7.5). After mixing for 30 min at 4 °C, the mixtures were centrifuged (10,000 × *g*) for 10 min, and the supernatants were collected as whole-cell extracts. The protein content was determined with Bio-Rad protein assay reagent using bovine serum albumin as a standard. The ECL Western blotting was performed as follows. An equal protein content of total cell lysates from HGF-treated samples with pretreatment of luteolin (0–40 μM) was resolved on 10–12% SDS-PAGE gels along with prestained protein molecular weight standard (Bio-Rad). Proteins were then blotted onto NC membrane (Sartorious), and probed with ERKs, JNKs, p38 and Akt. The total and phosphorylated proteins and β-actin were detected with each specific antibody for 2 h, and then with an appropriate peroxidase-conjugated secondary antibody for 1 h. After binding, the bands were examined by enhanced chemiluminescence using the ECL commercial kit and relative photographic density was quantitated by scanning the photographic negatives on a gel documentation and analysis system (Alpha Imager 2000, Alpha Innotech Corporation). All the data are the representative of three independent experiments.

2.7. Immunoprecipitation assay

Approximately 0.5 mg of the lysate protein was immunoprecipitated using monoclonal antiserum of c-Met (1 μg) and 15 μl of protein A/G-agarose (Santa Cruz). Pellet beads were incubated at 4 °C for overnight. After centrifugation at 2500 rpm, the eluates were analyzed by immunoblotting against phospho-tyrosine antibodies.

2.8. Characterization of effect of luteolin on signal pathway mediated by HGF

First, the effect of HGF on MAPKs and PI3K–Akt pathways was investigated by Western blotting analysis. Then, HepG2 cells were pretreated with MEK inhibitor PD98059, JNK inhibitor SP600125 or PI3K inhibitor wortmannin for 2 h, followed by a treatment with 20 ng/ml of HGF for 30 min to analyze the p-ERKs, p-JNKs and p-Akt. In addition, to evaluate the effect of cell invasion, HepG2 cells were pretreated with MEK inhibitor PD98059, JNK inhibitor SP600125 or PI3K inhibitor wortmannin HGF and combination with 40 μM of luteolin for 2 h, followed by a treatment with 20 ng/ml of HGF for 24 h.

2.9. Statistical analysis

Data were reported as the mean ± standard deviation of three independent experiments and evaluated by one-way ANOVA. Significant differences were established at $p < 0.05$.

3. Results

3.1. Cytotoxicity of flavonoids to HepG2 cells

Fig. 1 illustrates the results of MTT assay performed with the logarithmically growing HepG2 cells treated with 40 μM of various flavonoids including luteolin,

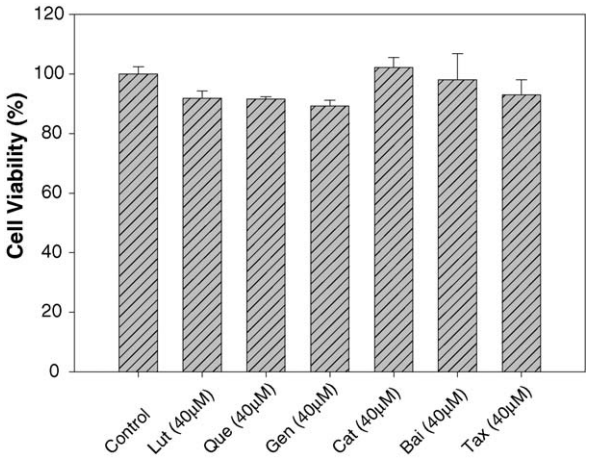

Fig. 1. Effect of flavonoids on viability of HepG2 cells. Cells were treated with 40 μM of flavonoids including luteolin (Lut), baicalein (Bai), quercetin (Que), genistein (Gen), catechin (Cat) and taxifolin (Tax) for 24 h. Viability of HepG2 cells was measured by MTT assays as described in Section 2. The results were presented as means ± S.D. of three independent experiments.

quercetin, baicalein, genistein, taxifolin and catechin. The result showed that there was no significant difference in cell viability. It was therefore clear that all the flavonoids, at a concentration ranging from 0 to 40 μM, pose no cytotoxicity to HepG2 cells.

3.2. Effect of flavonoids and luteolin on HGF-induced cell migration and invasion

By Boyden chamber assay, 20 ng/ml of HGF induced in vitro migration and invasion of HepG2 cells (Fig. 2A and B). The effect of flavonoids on HGF-induced migration and invasion was evaluated. It was found that all the flavonoids (40 μM) exhibit significant inhibitory effect on HGF-induced migration and invasion and that luteolin is the most potent. The effect of luteolin and mode of action involved were then investigated. Luteolin resulted in a dose-dependent inhibition of HGF-induced cell migration and invasion (Fig. 3A and B). Comparison with the effect of luteolin on cell viability at 40 μM within 24 h treatment (Fig. 1) shows that inhibitory effect of luteolin on cell migration and invasion is independent of its cellular cytotoxicity.

3.3. Effect of luteolin on HGF-induced cell scattering and cytoskeleton change

Cell scattering is an important component of several physiological and pathological processes such as embryonic morphogenesis, tissue regeneration and tumor invasion. As seen in Fig. 4, when the HepG2 cells were

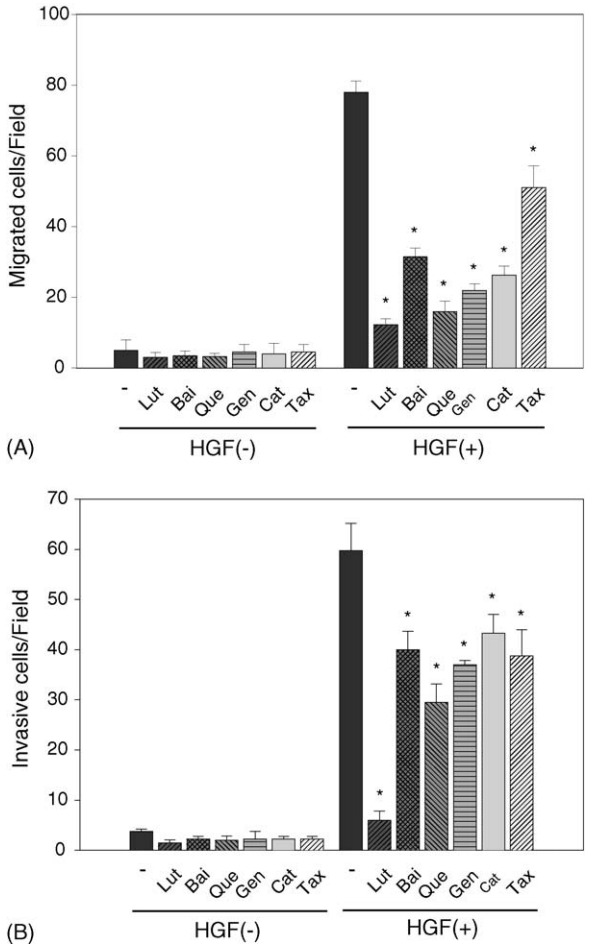

Fig. 2. Effect of flavonoids on HGF-induced HepG2 cell migration and invasion. The HGF (20 ng/ml) was applied to the lower chamber. HepG2 cells (5×10^4 cells/ml) were seeded onto the upper chamber consisting of 8 μm pore-size filters coated without (A) and with (B) Matrigel basement membrane matrix, then treated with 40 μM of luteolin (Lut), baicalein (Bai), quercetin (Que), genistein (Gen), catechin (Cat) and taxifolin (Tax) for 24 h and with or without 20 ng/ml HGF/SF as a chemoattractive agent in the lower chamber. Cells that migrated or invaded the filter were counted as described in Section 2. $^{*}p < 0.01$, compared with HGF treatment alone.

cultured, they showed a cobblestone shape and tight junction of the cells (Fig. 4A). After treatment with HGF for 24 h, the cells showed a remarkable scattering (Fig. 4B and C), and pretreatment with 10 μM of luteolin was partially effective in blocking HGF-stimulated cell scattering of HepG2 (Fig. 4D). In addition, pretreatment with 20 and 40 μM luteolin increased the cobblestone shape cells of HepG2 (Fig. 4E and F). Because modulation of cytoskeleton proteins has been linked with cell growth and invasion, we proceeded to investigate the cytoskeleton change by staining with TRICT-conjugated phalloidin. In Fig. 5, to enhance the HGF-

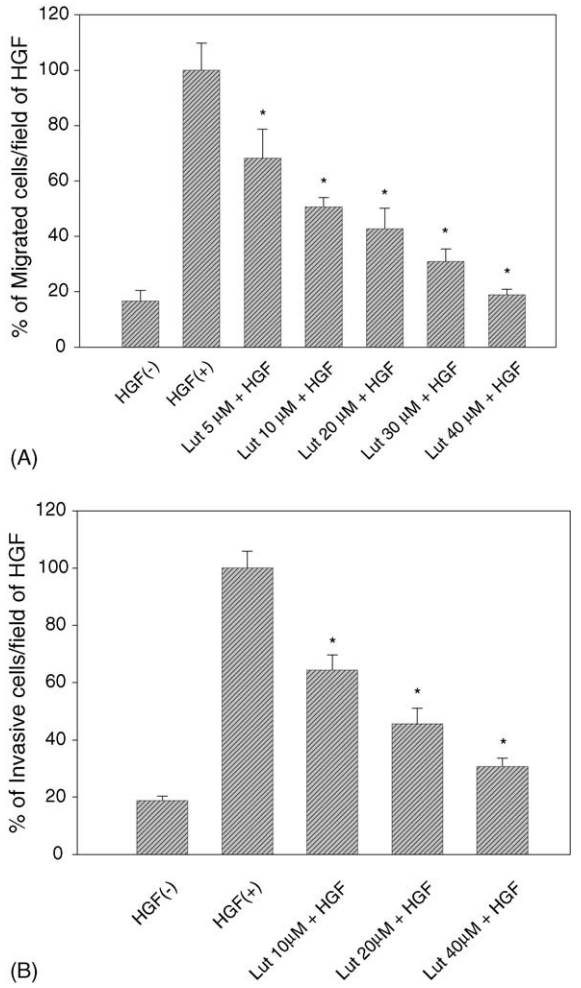

Fig. 3. Dose effect of luteolin on HGF-induced HepG2 cell migration and invasion. The HGF (20 ng/ml) was applied to the lower chamber. HepG2 cells (5×10^4 cells/ml) were seeded onto the upper chamber consisting of 8 μm pore-size filters coated without (A) and with (B) Matrigel basement membrane matrix, then treated with various concentrations of luteolin (Lut) for 24 h. Cells that migrated or invaded the filter were counted as described in Section 2. $^{*}p < 0.01$, compared with HGF treatment alone.

mediated cytoskeleton change in short-duration treatment, we used the concentration of 40 ng/ml. The results demonstrate that filamentous actin in serum-starved cells is mainly seen in the more peripheral regions. However, HGF-induced membrane protrusions such as lamellipodia (Fig. 5B) and filopodia (Fig. 5C), which provide attachment and movement of cytoplasmic components that are responsible for cell moving, were seen obviously in HGF-treated cells. With the pretreatment of luteolin HGF-induced cytoskeleton change was inhibited (Fig. 5D–F).

3.4. Effect of luteolin on HGF-mediated c-Met phosphorylation

c-Met is a member of the tyrosine kinase-type receptor family and binding of HGF induces autophosphorylation of tyrosine residues in c-Met [16]. HGF-induced c-Met tyrosine phosphorylation was detected by Western blot analysis with anti-phospho-tyrosine monoclonal antibody after immunoprecipitation with anti-c-Met antibody. The c-Met of HepG2 cells were strongly phosphorylated in response to stimulation with 20 ng/ml HGF for 30 min (Fig. 6). Pretreatment with luteolin inhibited the HGF-mediated c-Met phosphorylation.

3.5. Effect of luteolin on HGF-mediated signal activation

The activation of several signaling molecules has been observed in HGF-stimulated cells that may play a role in mediating cell motility and metastasis. HGF-induced activation of PI3K, which is often associated with the stimulation of cell motility, results in the production of inositol 3,4,5-triphospate, which activates multiple downstream targets including Akt [17,18]. HGF induces phosphorylation of ERKs, which are phosphorylated and activated by MEK involving cell scattering [19]. When HepG2 cells were stimulated with HGF, the phosphorylated forms of ERK, JNK/SAPK and Akt were increased. Western blot analysis showed that the activation of these signaling molecules occurred 30 min after treatment, whereas there was no change in total ERK, JNK/SAPK and Akt expression. However, p38 was not activated by treatment with HGF, although this kinase was present in the cells (Fig. 7A). We then evaluated the effect of luteolin on the phosphorylation of ERK1/2, JNK1/2 and Akt induced by HGF and compared it with the application of MEK inhibitor PD98059, JNK inhibitor SP600125 or PI3K inhibitor wortmannin. The results revealed that luteolin similar to PD98059 and wortmannin suppresses HGF-induced ERK1/2 and Akt activation (Fig. 7B).

3.6. Inhibitory effect of luteolin on HGF-induced invasion involving ERKs and Akt

To determine whether the inhibition of HGF-induced cell invasion by luteolin involved mainly the ERKs and Akt signal pathways, we examined the effect of these signal inhibitors on in vitro invasion assay. It showed that treatment with PD98059 or wortmannin similar to luteolin decreases the number of invasive cells stimulated by HGF. Combination treatment of luteolin with PD98059 or wortmannin promoted the inhibitory effect (Fig. 8A

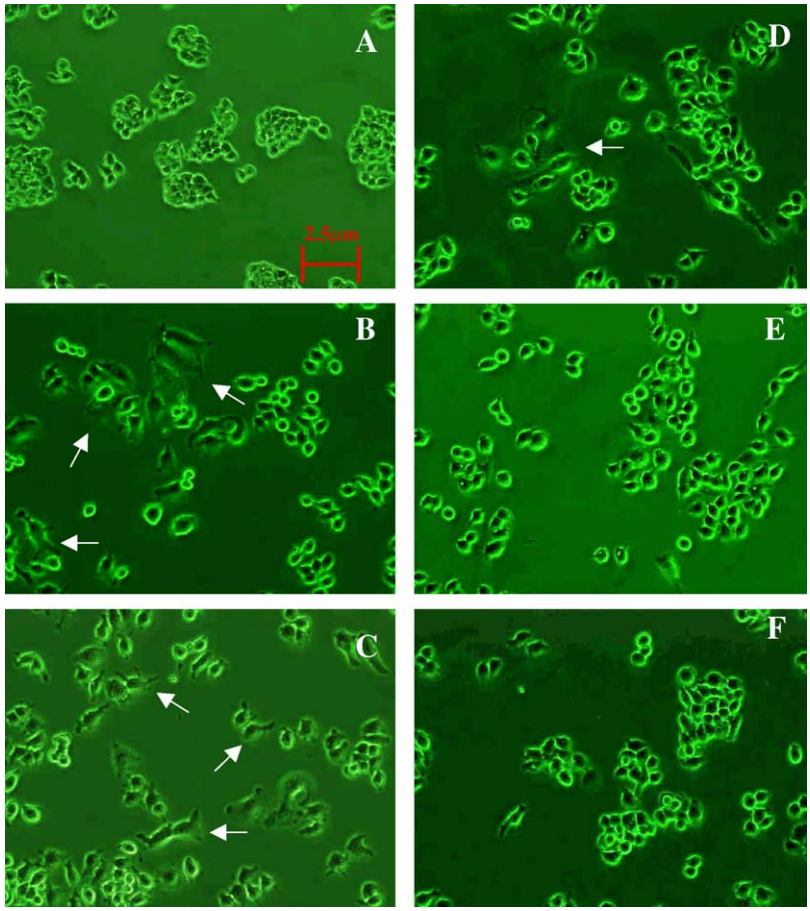

Fig. 4. Effect of luteolin on HGF-induced morphologic change of HepG2 cell. HepG2 cells (2×10^4 cells/ml) were seeded on six-well plate in serum-free medium overnight and then cultured in medium containing without (A) or with HGF (B and C) for 24 h. In addition, cells pretreated with various concentrations of luteolin: (D) 10 μM, (E) 20 μM and (F) 40 μM for 2 h, and then treated with HGF for 24 h. The cells were observed under phase contrast microscope 100× and arrow bar indicated cell scattering.

and B). Therefore, we suggest that both MAPK/ERKs and PI3K–Akt signaling pathways were involved in the anti-invasion effect of luteolin induced by HGF in HepG2 cells.

4. Discussion

It is suggested that HGF is a multifunctional modulator of biological activities in a variety of cell types and it influences the growth motility, differentiation and morphogenesis through the c-Met tyrosine kinase receptor of its target cells [20]. Following ligand binding and autophosphorylation, Met transmits intercellular signals using a unique multisubstrate docking site present within the c-terminal end of the receptor. The multisubstrate docking site mediates the binding of several adapter proteins such as Grb2, Srk, Crk/CRKL, and the large adapter protein Gab1. These adapter proteins in turn recruit several signal transducing proteins to form an intricate signaling complex which leads to more substantial models of HGF-Met signal transduction that mediates tumor cell invasion and metastasis [21]. It has been reported that divergent signaling pathways initiated by PI3K, Ras and Rac are involved in scattering, morphologic changes and migration induced by HGF in different cell types [22,23] and MAPK pathway plays some other roles in cell scattering [24]. In addition, the ETS1 transcription factor is activated by HGF through RAS–RAF–MEK–ERK signaling pathway [25] and that promotes the expression of matrix metalloproteinases, which can facilitate cell motility [26]. In primary cultures of rat hepatocytes it has been reported that JNK is activated by HGF and that it plays a role in mediating their proliferation [27]. In addition, it was found that the oncogenic form of c-Met, TPR-MET, activates JNK in FR3T3 fibroblast cells, an activation that seemed to be required for their transfor-

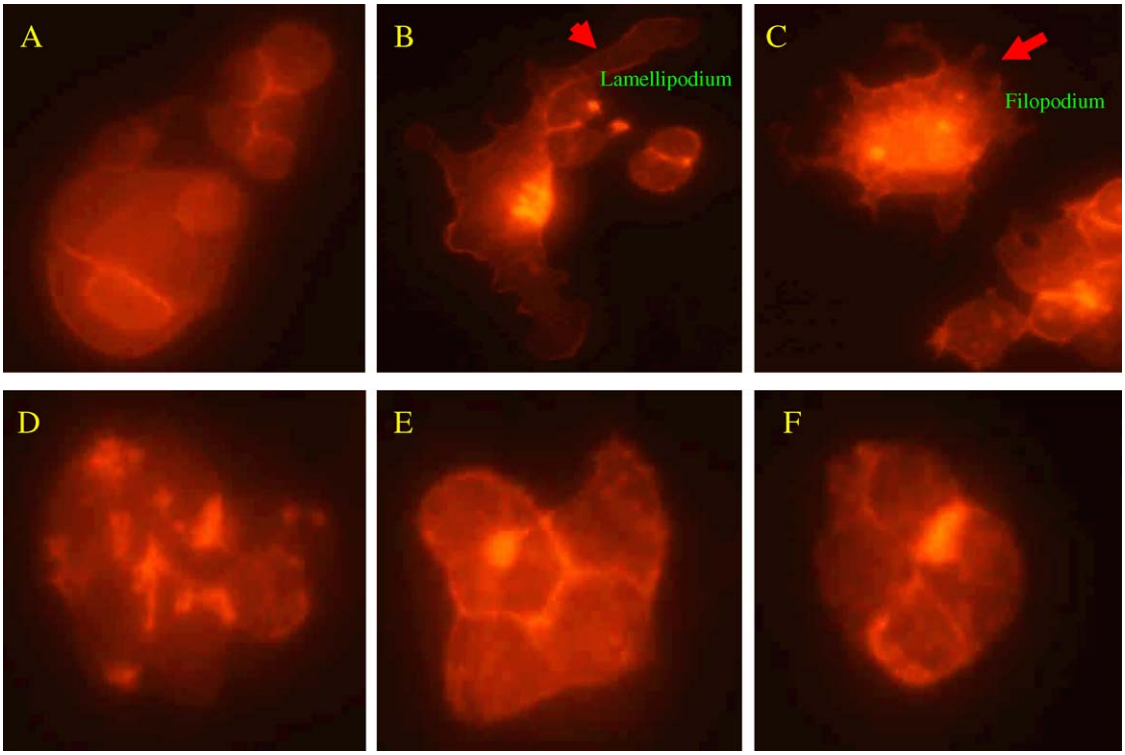

Fig. 5. Effect of luteolin on HGF-induced cytoskeleton change of HepG2 cell. HepG2 cells were treated without (A) or with HGF (40 ng/ml) (B and C) or in the presence of luteolin: (D) 10 μM, (E) 20 μM and (F) 40 μM for 4 h, and F-actin polymerization was visualized by TRITC-conjugated phalloidin and observed under fluorescence microscope (200×).

mation [28]. From Figs. 6 and 7, we find that luteolin inhibits the HGF-activated c-Met and MAPK/ERKs and PI3K–Akt signaling pathways, but not JNK.

The lethality of malignant tumors is attributable largely to the metastasis of neoplastic cells [29]. Several agents, including flavonoids, have been reported to inhibit invasion and metastasis [30–34]. Up to now, no established anti-metastatic agents are available for clinical use. Therefore, there is an urgent need for compounds capable of interfering successfully with one or more steps of the metastatic process [35]. c-Met and HGF are dysregulated in human cancer and are also believed to contribute to dysregulation of cell growth and tumor invasion during disease progression and metastasis. c-Met and HGF are highly expressed relative to the surrounding tissue in numerous cancers, and their expression correlates with poor patient prognosis [20]. Therefore, c-Met and HGF may be attractive candidates for targeted chemoprevention or cancer therapy. Selective small molecular inhibitor of c-Met kinase has recently been developed [36]. In this study, luteolin presents inhibitory effect on HGF–c-Met signaling pathway resulting in blocking cell invasion. However, metastasis involves matrix remoulding of organ tissues, which thus allow cells to migrate away from its origin and through circulation to a distant organ.

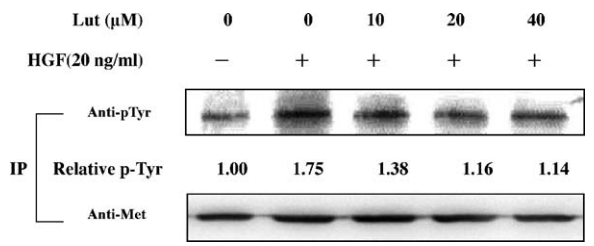

Fig. 6. Effect of luteolin on HGF-induced c-Met phosphorylation. Cells were cultured in medium containing HGF (20 ng/ml) with or without luteolin (10, 20 and 40 μM) for 4 h, and treatment cells were then harvested. Met was immunoprecipitated (IP) from HepG2 cell lysates with a specific anti-human Met polyclonal antibody, and the resulting immune complexes were separated by SDS-8% polyacrylamide gel electrophoresis. The blots were probed sequentially with antibodies against the phospho-tyrosine and Met β subunit.

Currently, matrix metalloproteinases (MMPs) are able to degrade basement membranes and the stromal connective tissue [37], that are believed to play an important role in cancer metastasis. Therefore, MMPs have been implicated in processes leading to cancer invasion and metastasis [38], and may also play a major

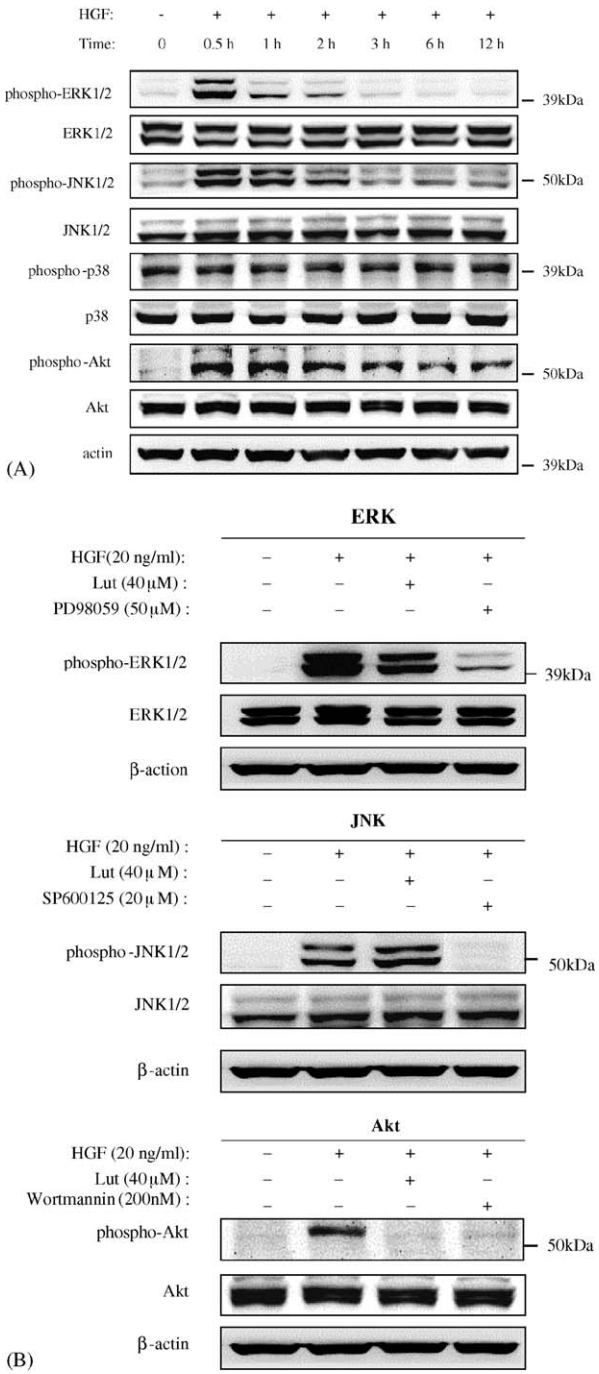

Fig. 7. Effect of luteolin on HGF-mediated activation of MAPKs and PI3K/Akt pathways. (A) The serum-starved HepG2 cells were stimulated with 20 ng/ml of HGF for the indicated time. The cell lysates were subjected to the Western blot analysis using either specific antibodies against the active phosphorylated forms of ERK, JNK/SAPK, p38 and Akt, or ERK, JNK, p38 and Akt, and actin used for equal loading as control. (B) The serum-starved HepG2 was pretreated with luteolin (40 μM) and the specific MEK inhibitor PD98059 (50 μM), JNK inhibitor SP600125 (20 μM) and PI3K inhibitor wortmanin (200 nM) for 2 h, and then incubated in the absence or presence of HGF (20 ng/ml) for an additional 0.5 h. The status of active form of ERK, JNK and Akt was determined by Western blot analysis: 50 μg of protein extract from each condition was electrophoresed in each lane of 10% SDS-PAGE and using the specific antibody against phospho-ERK1/2, phospho-JNK1/2 and Akt and ERK1/2, JNK1/2 and Akt antibody or actin used for equal loading.

role in tumor angiogenesis [39]. It has been reported that flavonoids including genistein, apigenin and 3-hydroxyflavone inhibit VEGF/bFGF-induced angiogenesis [40], in part via preventing the VEGF/bFGF-induced MMP-1, MT1-MMP and uPA expression and the activation of pro-MMP-2. Our study also demonstrates the inhibitory activity of flavonoids on HGF-induced invasion in HepG2 cells. Though luteolin, the most potent anti-invasion potential of flavonoids in our study, is shown to suppress phosphorylation of c-Met, ERK and Akt that involved in HGF-induced invasion, the effects on MMPs need further investigation.

Flavonoids, which are benzo-γ-pyrone (phenylchromone) derivatives, comprise a very large class of naturally occurring, low-molecular-weight polyphenol plant compounds. Previous studies on melanoma lines using several flavonoids including luteolin and quercetin of a *Citrus* origin showed that the presence of the C2–C3 double bond on the C ring [41], conjugated with the 4-oxo function [42], was critical for this biological activity. From a structural point of view, the double bond between C2 and C3 results in ring B and ring C being on the same plane, which might be critical for access to the kinase substrate binding site [43], and the two adjacent polar OH groups on C3 and C4 of ring B are required for suppressing kinase activity [41]. Consisting with our result it demonstrates that flavones such as luteolin exhibit potent anti-invasion bioactivity. However, a more systematic study, employing X-ray crystallography of flavonoids and known kinase data, is required to elucidate the detailed structure–function relationship involved in the anti-invasion biological activity of flavonoids.

Cancer chemoprevention is the use of agents to slow the progression of carcinogenesis, reverse or inhibit it, with the aim of lowering the risk of developing invasion or clinically significant disease. Flavonoids have been suggested to possess chemopreventive property in numerous studies [44]. This paper demonstrates that flavonoids possess anti-invasion potential that stimulated by HGF and luteolin exhibits the most potent potential. Furthermore, luteolin inhibited HGF-induced HepG2 cell invasion involving both MAPK/ERKs and PI3K–Akt signaling pathways. The results shed light

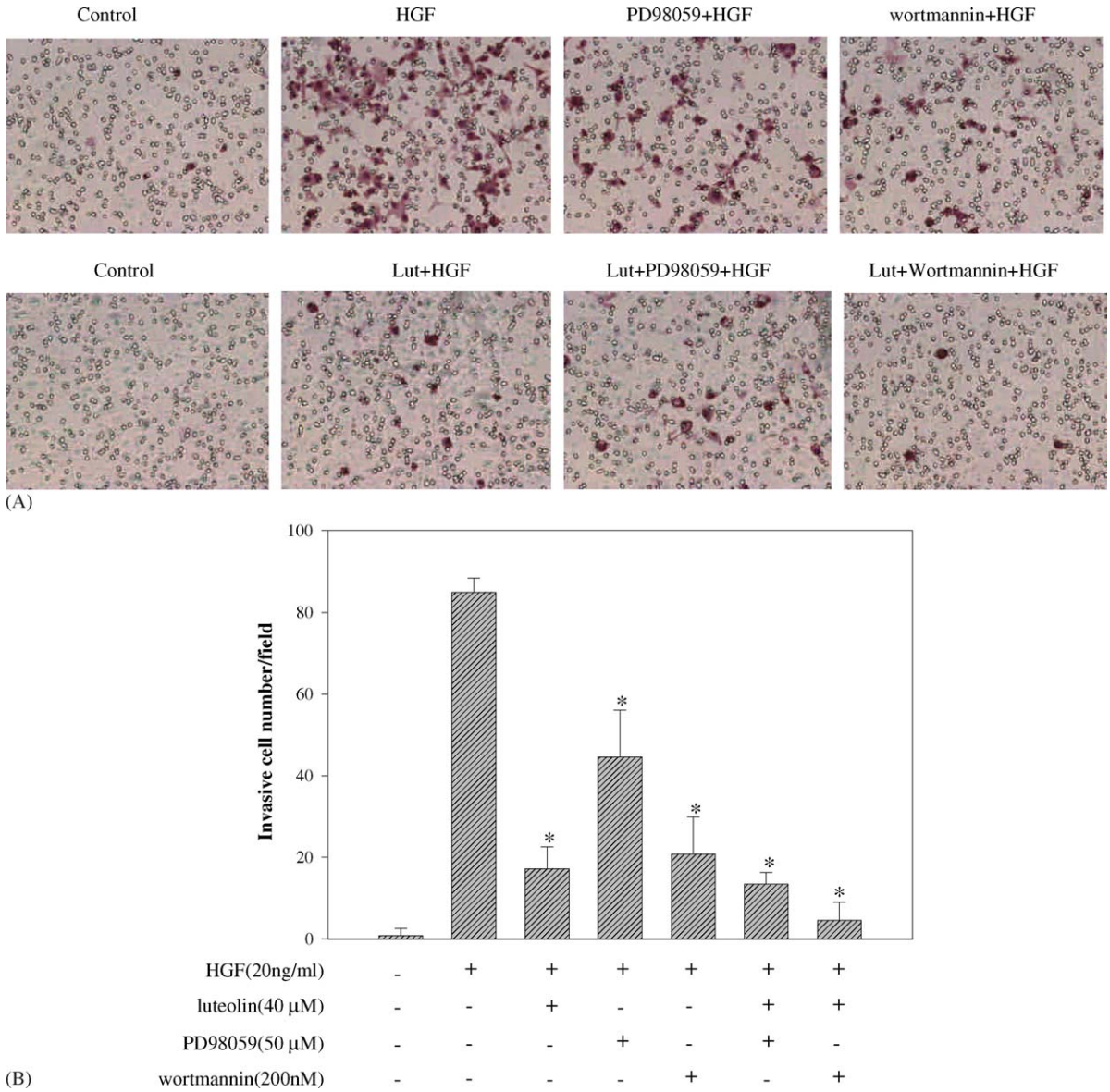

Fig. 8. Effect of luteolin with the combination of PD98059 and wortmannin on HGF-stimulated cell invasion. HepG2 cells (5×10^4 ml^{-1}) were seeded onto transwell plates and then incubated with 40 μM luteolin, 50 μM PD98059 and 200 nM wortmannin alone or in different combinations for 24 h. The lower surfaces of the membranes from the transwell units were fixed with 100% methanol and stained with Giemsa solution. (A) Photographs of HepG2 cells after invasion. (B) Cells (in red color) that had invaded to the lower surface of the membranes were counted in triplicate wells and in three identical experiments under a light microscope, and the data represent mean ± S.D. $^{*}p < 0.01$, compared with the group treated with HGF alone.

on the mechanism of action of phytochemicals, such as flavonoids, which might explain the protective action of plant-based diets on the progression of cancer. Moreover, it might provide the basis of development of more potent synthetic analogs for the inhibition of HGF-induced cell invasion.

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