

行政院國家科學委員會專題研究計畫 成果報告

黃酮類化合物抑制不同腎臟癌細胞株(Caki-1, Caki-2, 786-0)轉移能力、誘導細胞凋亡及輔助抗癌藥之研究(第3年)

研究成果報告(完整版)

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中華民國 101 年 10 月 30 日

中文摘要：黃酮類(flavonoids)屬於天然多酚類(polyphenol)廣泛存在於日常生活蔬果之中。具有抗過敏、抗發炎、抗氧化、強化毛細血管、抗癌化以及調節酵素活性等生物活性。黃酮化合物(flavones)是黃酮類的主要基本結構，在自然界中具有許多不同取代基之衍生物，其中以氫氧基(-OH)、甲基(-CH₃)及糖苷基(-glycoside, -rutoside)的取代最為常見。在抗氧化的研究中，有文獻指出黃酮化合物可有效抑制 LPS 或 LTA 所誘導之 NO 活性，且結構上之氫氧基數目越多其抗氧化能力越強。而在細胞癌化的化學預防方面，有文獻指出黃酮化合物在細胞癌化的化學預防方面可促使癌細胞的凋亡並抑制其增生能力及抑制正常細胞的癌化，且對於抑制癌瘤細胞侵襲轉移能力方面的研究，則尚未明瞭。

本實驗室初步以十種不同的黃酮類化合物(flavone, flavonol, 5-hydroxyflavone, 7-hydroxyflavone, chrysin, baicalein, apigenin, luteolin, quercetin 及 kaempferol) 處理腎臟癌細胞株 786-0，觀察侵襲、轉移和存活率的影響，結果發現 apigenin, chrysin, baicalein, quercetin, luteolin 可有效降低腎臟癌細胞 786-0 存活率；而 flavone 和 kaempferol 則不具細胞毒性，但對癌細胞之 invasion 及 migration 能力則有顯著抑制。而有些黃酮化合物也會抑制 786-0 分泌 MMP-9 及 MMP-2 的能力。第二年的實驗中則使用 MTT assay、Trypan blue dye exclusion assay、DAPI staining 及 Measurement of mitochondrial membrane potential assay 方式分析 quercetin 及 baicalein 降低腎臟癌細胞株 786-0 存活率的影響。結果發現 quercetin 和 baicalein 可以造成腎臟癌細胞株 786-0 的染色質濃染 (chromosome condensation)，顯示 quercetin 及 baicalein 可誘導 786-0 細胞走向凋亡。此外發現 quercetin 和 baicalein 誘導 786-0 細胞凋亡會經由粒線體的傷害來造成。第三年實驗以 western blot 探討對腎臟癌細胞株 786-0 細胞轉移之相關蛋白表現。發現在 kaempferol 處理下，腎臟癌細胞株 786-0 轉移相關蛋白 MMP-2、p-Akt 和 p-FAK Tyr925 會產生變化。動物實驗則以免疫缺陷的小鼠 (C. B17/Icr-Prkdcscid/Cr1Narl) 以尾靜脈注射的模式接種人類腎臟癌細胞株 786-0，處理 kaempferol，進一步觀察動物肺部組織，發現 kaempferol 會抑制癌細胞轉移情形。綜合以上結果，黃酮類中 quercetin 和 baicalein 可以抑制腎臟癌細胞株 786-0 的增生，kaempferol 對於腎臟癌的侵襲轉移則具有抑制的效果。

中文關鍵詞： 癌細胞轉移； 黃酮類； 腎臟癌； 凋亡

英文摘要： Flavonoids are a group of polyphonic compounds that widely distributed in dietary foods of vegetables and fruits. Documented biologic effects of dietary flavonoids include anti-inflammatory, anti-allergic, antioxidant, capillary strengthening, and anti-cancer potency and modulation of enzyme activities, among others. In the antioxidant aspect, several previous studies indicated that flavones possess an effective inhibitory effect on NO activation induced by LPA or LTA, and the number of hydroxyl (OH) substitutions was a critical factor in the reactive oxygen species (ROS) scavenging activity. In the anti-cancer potency aspect, flavones might be able to influence processes that are dysregulated during cancer development, and possess the propensity to anti-proliferation and induce apoptosis. Compared to the abovementioned aspects, studies on the inhibitory effect of flavones on cancer cell invasion behavior have been relatively less and warrant a further study.

In our preliminary study, a renal carcinoma cell 786-0 has been treated with flavones and then subjected to assays for cell viability, invasion and migration. The results showed that flavone and kaempferol significantly inhibited invasive and migration potential through a decrease of MMP-2 and MMP-9 expressions in 786-0 cell. And apigenin, chrysin, baicalein, quercetin, luteolin were decrease cell viability of renal carcinoma cells 786-0 using MTT assay.

In this study, we showed quercetin and baicalein induced apoptotic death in renal carcinoma cells 786-0 via dose-dependent manner. In addition, quercetin and baicalein also induced cell death by increasing of chromosome condensation. The result showed that quercetin and baicalein induced apoptotic death in 786-0 cells via dose-dependent manner as well as increase of chromosome condensation. Further analysis demonstrated the involvement disruption of mitochondrial membrane potential in quercetin and baicalein apoptosis.

Western blot demonstrate the effect of kaempferol on metastasis related proteins. Kaempferol could decrease MMP-2, p-Erk, p-Akt and p-FAK Tyr925 in renal carcinoma cells (786-0). Analyze the effect of kaempferol on migration/invasion in BALB/c nude mice model. And kaempferol were inhibition 786-0 migration/invasion in mice. Our results suggest that flavonoids exerted a fine anti-cancer activity, such as anti-invasion, anti-angiogenesis, and anti-proliferation.

英文關鍵詞： metastasis； flavonoids； renal carcinoma； apoptosis

行政院國家科學委員會補助專題研究計畫

成果報告

期中進度報告

黃酮類化合物抑制不同腎臟癌細胞株(Caki-1, Caki-2, 786-O)

轉移能力、誘導細胞凋亡及輔助抗癌藥之研究

計畫類別： 個別型計畫 整合型計畫

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計畫主持人：張浚榮 中山醫學大學 醫研所

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執行單位：中山醫學大學

中華民國 101年 7月 31日

中英文摘要

癌細胞轉移 (metastasis) 是癌症導致死亡以及治療複雜度提昇的主要原因; 而癌細胞轉移已知與多種細胞生理改變密切相關, 如改變癌細胞與胞外基質 (extracellular matrix, ECM) 間及基底膜 (basement membrane) 的貼附 (adhesion) 能力, 破壞細胞間交互作用 (intercellular interaction) 的力量等。癌細胞之所以能轉移主要是透過癌細胞分泌蛋白分解酵素, 如: serine proteinase、matrix metalloproteinases (MMPs)、cathepsins 以及 plasminogen activator (PA) 分解胞外基質, 最後導致 intercellular matrix 分離和基底膜的分解, 進而使癌細胞 migration 及 invasion 提昇。而在這些水解酵素中, MMP-2、MMP-9 及 urokinase-type PA (u-PA) 在癌細胞 migration 及 invasion 過程中扮演著最重要的角色。

腎臟腫瘤是發源於腎臟細胞失調所形成的腫瘤。腎臟腫瘤有時因為發病部位位於腎臟的內襯會被歸類為腎上腺瘤。腎臟腫瘤中大約 85% 為惡性腫瘤, 亦即腎臟癌。腎臟癌在任何年齡均可發生, 但最常發生在 50 至 70 歲間, 男性與女性比例為 1.5 : 1, 像其它癌症一樣, 腎臟癌可分為偶發性及遺傳性。

黃酮類 (flavonoids) 屬於天然多酚類 (polyphenol) 廣泛存在於日常生活蔬果之中。具有抗過敏、抗發炎、抗氧化、強化毛細血管、抗癌化以及調節酵素活性等生物活性。黃酮化合物 (flavones) 是黃酮類的主要基本結構, 在自然界中具有許多不同取代基之衍生物, 其中以氫氧基 (-OH)、甲基 (-CH₃) 及糖苷基 (-glycoside, -rutinoside) 的取代最為常見。在抗氧化的研究中, 有文獻指出黃酮化

合物可有效抑制 LPS 或 LTA 所誘導之 NO 活性，且結構上之氫氧基數目越多其抗氧化能力越強。而在細胞癌化的化學預防方面，有文獻指出黃酮化合物在細胞癌化的化學預防方面可促使癌細胞的凋亡並抑制其增生能力及抑制正常細胞的癌化，且對於抑制癌細胞侵襲轉移能力方面的研究，則尚未明瞭。

本實驗室初步以十種不同的黃酮類化合物 (flavone, flavonol, 5-hydroxyflavone, 7-hydroxyflavone, chrysin, baicalein, apigenin, luteolin, quercetin 及 kaempferol) 處理腎臟癌細胞株 786-O，觀察侵襲、轉移和存活率的影響，結果發現 apigenin, chrysin, baicalein, quercetin, luteolin 可有效降低腎臟癌細胞 786-O 存活率；而 flavone 和 kaempferol 則不具細胞毒性，但對癌細胞之 invasion 及 migration 能力則有顯著抑制。而有些黃酮化合物也會抑制 786-O 分泌 MMP-9 及 MMP-2 的能力。第二年的實驗中則使用 MTT assay、Trypan blue dye exclusion assay、DAPI staining 及 Measurement of mitochondrial membrane potential assay 方式分析 quercetin 及 baicalein 降低腎臟癌細胞株 786-O 存活率的影響。結果發現 quercetin 和 baicalein 可以造成腎臟癌細胞株 786-O 的染色質濃染 (chromosome condensation)，顯示 quercetin 及 baicalein 可誘導 786-O 細胞走向凋亡。此外發現 quercetin 和 baicalein 誘導 786-O 細胞凋亡會經由粒線體的傷害來造成。第三年實驗以 western blot 探討對腎臟癌細胞株 786-O 細胞轉移之相關蛋白表現。發現在 kaempferol 處理下，腎臟癌細胞株 786-O 轉移相關蛋白 MMP-2、p-Akt 和 p-FAK Tyr925 會產生變化。動物實驗

則以免疫缺陷的小鼠 (C.B17/Icr-*Prkdc*^{scid}/CrINarl) 以尾靜脈注射的模式接種人類腎臟癌細胞株 786-O，處理 kaempferol，進一步觀察動物肺部組織，發現 kaempferol 會抑制癌細胞轉移情形。綜合以上結果，黃酮類中 quercetin 和 baicalein 可以抑制腎臟癌細胞株 786-O 的增生，kaempferol 對於腎臟癌的侵襲轉移則具有抑制的效果。

關鍵詞：癌細胞轉移；黃酮類；腎臟癌；凋亡

Metastasis of cancer cells, a primary cause of cancer death and a multiple and intricate processes, may complicate the clinical management and lead to a poor prognosis for cancer patients and has tremendous physical or economical impact to patients or communities. In general, metastasis of cancer cells involves multiple processes and various cytophysiological changes, including changed adhesive capability between cells and extracellular matrix (ECM) and damaged intercellular interaction. Degradation of ECM by cancer cells via protease, such as serine proteinase, matrix metalloproteinases (MMPs), cathepsins, and plasminogen activator (PA), may lead to the separation of intercellular matrix to promote the mobility of cancer cells and eventually lead to metastasis. Among these involved proteases, MMP-2, MMP-9 and u-PA are the most vital ones for degradation of base membrane

and therefore deeply involved in cancer invasion and metastasis.

Kidney cancer is a disease in which the cells in certain tissues of the kidney start to grow uncontrollably and form tumors. Renal cell carcinoma, sometimes referred to as hypernephroma, occurs in the cells lining the kidneys (epithelial cells). It is the most common type of kidney cancer. Eighty-five percent of all kidney tumors are renal cell carcinomas. Wilms' tumor is a rapidly developing cancer of the kidney most often found in children under the four years of age.

Flavonoids are a group of polyphonic compounds that widely distributed in dietary foods of vegetables and fruits. Documented biologic effects of dietary flavonoids include anti-inflammatory, anti-allergic, antioxidant, capillary strengthening, and anti-cancer potency and modulation of enzyme activities, among others. In the antioxidant aspect, several previous studies indicated that flavones possess an effective inhibitory effect on NO activation induced by LPA or LTA, and the number of hydroxyl (OH) substitutions was a critical factor in the reactive oxygen species (ROS) scavenging activity. In the anti-cancer potency aspect, flavones might be able to influence processes that are dysregulated during cancer development, and possess the propensity to anti-proliferation and induce apoptosis. Compared to the abovementioned aspects, studies on the inhibitory effect of flavones on cancer cell invasion behavior have been relatively less and warrant a further study.

In our preliminary study, a renal carcinoma cell 786-O has been treated with flavones and then subjected to assays for cell viability, invasion and migration. The results showed that flavone and kaempferol significantly inhibited invasive and migration potential through a decrease of MMP-2 and MMP-9 expressions in 786-O cell. And apigenin, chrysin, baicalein, quercetin, luteolin were decrease cell viability of renal carcinoma cells 786-O using MTT assay.

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Western blot demonstrate the effect of kaempferol on metastasis related proteins. Kaempferol could decrease MMP-2, p-Erk, p-Akt and p-FAK Tyr925 in renal carcinoma cells (786-O). Analyze the effect of kaempferol on migration/invasion in BALB/c nude mice model. And kaempferol were inhibition 786-O migration/invasion in mice. Our results suggest that flavonoids exerted a fine anti-cancer activity, such as anti-invasion, anti-angiogenesis, and anti-proliferation.

Keyword : metastasis; flavonoids; renal carcinoma; apoptosis

報告內容

前言及研究目的

惡性腎臟腫瘤細胞的侵襲及轉移

惡性腫瘤細胞的擴散轉移（metastasis）往往是癌症病人在臨床治療上預後的一個重要指標；在癌症的分期上，惡性腫瘤細胞的擴散轉移與否也是一個重要的分界。當惡性腫瘤細胞僅生長發病於局部位置，稱為局部癌；若惡性腫瘤細胞尚未破壞基底膜（basement membrane）穿出，稱為原位癌（carcinoma in situ）。如果能在此階段進行治療，則治癒率最高。當惡性腫瘤細胞藉由血管、淋巴管等方式轉移到人體其他部位生長成續發性惡性腫瘤，就稱為轉移性癌。大部分的原發性的病灶可以由外科手術及其他治療方式將其完全移除，但是轉移性癌因已轉移至身體各處，進而造成治療上的困難。

腎臟癌是由腎臟內之腎小管上皮細胞長出來的癌瘤，它通常會形成一個圓形或橢圓形的腫塊。若腫瘤小於 7 公分，尚屬早期，通常尚未轉移。晚期腎臟癌，會向外侵犯到腎臟外圍的組織或到局部的淋巴結等，遠處轉移的部位包括：淋巴結、肺部、肝臟、骨骼等[1-2]。在各種人類的癌症當中，腎臟癌是屬必須經由化學治療及手術完全切除腫瘤來根治[3]。然而，化學療法不但對人體具有相當大的副作用與傷害，在殺死癌細胞的同時也會造成正常細胞的傷害，而且放射線治療的效果對於腎臟癌而言並不能達到良好的效果。另一方面，切除手術則必須切除整個腎臟及腎臟癌，一段輸尿管，腎上腺及包圍著腎臟之脂肪。因此目前許多

研究趨向以天然物成份如多酚酸或黃酮類等天然物合併抗癌藥使用，期望能減少化療對人體造成之傷害，達到輔助治療的效果，易致癌細胞的惡化。

細胞外基質的相關蛋白

惡性腫瘤細胞會產生細胞外基質分解酵素，包括有 serine proteinase、matrix metalloproteinases (MMPs)、cathepsins、plasminogen activator (PA)，其中 MMP-2、MMP-9 及 urokinase-type PA (u-PA) 在基底膜的破壞扮演著重要角色，與癌症的侵襲及轉移能力最有關。惡性腫瘤細胞本身會製造 MMPs，在癌症等級增加時某些 MMPs 亦會相對的增加，而 MMPs 具有蛋白分解活性可分解膠原蛋白，在腫瘤破裂的基底膜處可測得大量的 MMPs，現已證實是用來作局部侵襲和遠處轉移，主要在於助長癌細胞穿透基底膜，由血管或淋巴管滲入組織而達轉移之目的[4-11]。

Matrix metalloproteinases (MMPs) 為一群含鋅之蛋白分解酵素，依其作用之受質特性及作用方式，共可分為五大類，分別是 collagenases，gelatinases，stromlyns，matrilysins 和 membrane type-metalloproteinases (MT-MMPs)，分別參與 ECM 的合成與分解；於正常生理情況下，參與如：胚胎的發育，胚胎的著床，血管生成和組織重建等。自組織分泌出來的 MMPs 為 latent proenzymes，需經過蛋白分解酵素，如 PA 轉換成活化態之後才具有酵素活性；另外，許多證據顯示這些活化後 MMPs 可受內生性組織抑制劑 (tissue inhibitor of metalloproteinases, TIMPs) 調節而改變其活性，也就是說 MMPs 與 TIMPs 之

間imbalance 為決定 ECM 分解或堆積的，甚至惡性腫瘤細胞貼附、移動、侵襲和轉移的主要因素[12-23]。

黃酮類

黃酮類是屬於天然多酚類(polyphenol)並廣泛存在於日常生活蔬果之中，主要存在於柑橘類植物(citrus)、豆類(soy bean)、蔬菜及藥草類植物之中。在傳統療法中，常被用作抗發炎藥物的成分。槲皮酮(querletin)、山茶酚(kaempferol)、芸香醇(rutin)、芹菜素(apigenin)等都是常見的黃酮類。其中已有不少成員被認為擁有各種顯著的生物活性，包括：抗過敏(antiallergic)、抗發炎(anti-inflammatory)、抗氧化(antioxidant)、抗突變(antimutagenic)、抗致癌化(anticarcinogenic)以及調節酵素活性(enzyme modulation)等[24]。其中較廣為人知的是槲皮酮(querletin)及芸香醇(rutin)，這兩種黃酮類能有效強化毛細血管，並調節毛細血管的通透性，有預防心血管疾病及腦中風的效用，也能降低糖尿糖性白內障及高血壓[25-29]。近來也有許多關於抑制癌化的研究，而參與其中的主要分子機轉，可分為：【一】 Preventing carcinogen metabolic activation、【二】 Anti-proliferation、【三】 Cell cycle arrest、【四】 Induction of apoptosis、【五】 Promotion of differentiation、【六】 Antioxidative activity、【七】 Inhibition of angiogenic process、【八】 Modulation of multidrug resistance [30-41]。

黃酮類實際上是一個龐大的家族，在化學結構上都具有由 15 個碳原子以

C₆-C₃-C₆ 所構成的三個環狀構造(phenylbenzopyrone structure)，然而黃酮類的化學分類極為複雜，其中包括：黃酮(flavone)、黃烷酮(flavanone)、黃烷醇(flavanol)、異黃酮(isoflavone)、花青素(anthocyanidin)等[42]。

黃酮化合物(flavones)是黃酮類(flavonoids)的主要基本結構，在自然界中具有許多不同取代基的衍生物，其中以氫氧基(-OH)、甲基(-CH₃)及醣苷基(-glycoside, -rutinoside)的取代最為常見，而黃酮由 15 個碳原子以 C₆-C₃-C₆ 所構成的三個環狀構造具有十個可供取代的位置，其中常見的取代位置為 A 環上 3, 5, 7 號碳原子(R₃, R₅, R₇)及 B 環上 3, 4, 5 號碳原子(R_{3'}, R_{4'}, R_{5'})。在抗氧化的研究中，有文獻指出黃酮可有效抑制 LPS 或 LTA 所誘導之 NO 表現，且實驗結果顯示第 3 個碳原子(R₃)上之氫氧基對抑制 NO 產生活性而言是很重要的，而且黃酮化合物結構上之氫氧基數目越多其抗氧化能力越強[43-45]。而在抑制癌症的研究方面，則是主要作用於抑制正常細胞的癌化[46-49]，及抑制癌瘤細胞的增生[50-53]，誘發細胞凋亡等作用[54-57]。對於抑制癌瘤細胞轉移能力的研究則尚未明瞭。最近研究顯示，綠茶及大豆中黃酮類成分 EGCG (Epigallocatechingallat) 及 Genistein 具有顯著的抑制癌瘤細胞轉移之能力[58-63]。雖然黃酮化合物可抑制惡性腫瘤細胞生長能力，甚至高劑量時可促使惡性腫瘤細胞凋亡的作用已經相當明確。但在化學防癌作用之角色上，如低劑量下黃酮化合物可否影響 MMPs family、plasmin、u-PA、tissue-type PA (t-PA) 或其抑制劑 TIMPs、PA inhibitors (PAIs) 之表現，除了本實驗室近年來的相關研究之外[64-77]，仍極少被研究。

二、研究方法

1. 細胞培養及處理

786-O 以 RPMI 培養基培養，加入適量 antibiotics 及 10% heat-inactivated FBS；以不同濃度之各種黃酮類化合物（0, 5, 10, 20, 40 μM ）於細胞培養箱中培育 24、48、72 小時後，進行 MTT、DAPI、JC-1 以及 Cell cycle assay。

2. MTT(Microculture Tetrazolium) 分析

將腎臟癌細胞株 786-O 以 3×10^4 細胞數分至 24 well 中， 37°C 培養 16 小時後，處理不同濃度(0, 5, 10, 20, 40 μM)的各種黃酮類化合物，培養 48、72 小時後，去除加藥的細胞培養液，再加入 1 ml 的 MTT reagent (0.5 mg/ml)，作用 4 小時之後，以異丙醇將結晶溶出，於 O.D. 565 nm 下測定溶液吸光，由吸光強度可得知存活的細胞數多寡。

3. 死活細胞計數

將腎臟癌細胞株 786-O 以 3×10^4 細胞數分至 24 well 中， 37°C 培養 16 小時後，處理不同濃度(0, 5, 10, 20, 40 μM)的各種黃酮類化合物，培養 72 小時後，使用 Trypan blue exclusion assay。

4. DAPI stain

細胞經過不同的黃酮類化合物處理後，以 PBS 清洗兩次，然後以 4% para-formaldehyde 固定作用 30 min 後，以 PBS 清洗，加入染劑 (DAPI) 染 30 min，以 PBS 清洗，於螢光顯微鏡下觀察 (UV 461nm)。

5. 粒線體膜電位測定

60 mm 培養皿培養內皮細胞處理不同濃度(0, 5, 10, 20, 40 μM)的各種黃酮類化合物，培養 72 小時後，以 PBS 清洗兩次，加入染劑 JC-1 (1 μM)染 30 min，再以 PBS 清洗，於螢光顯微鏡下觀察 (red fluorescence light at 590 nm; green fluorescence light at 527 nm)。在低膜電位的狀態下，JC-1 染劑會以 monomer 的形式發出綠色螢光，而高膜電位下，則會形成 J-aggregates 發出紅色螢光。

6. Cell cycle 分析

以 60 mm 培養皿培養細胞添加不同的黃酮類化合物，並處理 72 小時後，將細胞打下，加入 1 ml 的 70% cold ethanol 以固定細胞，置於 4°C 中隔夜。移除上清液並分別加入 1 ml 之 propidium iodide mixture (PI stain) 靜置室溫 30 分鐘。用 40 m nylon mesh 過濾，使用 Fluorescence-Activated Cell Sorter (FACS) 系統，以流式細胞儀 (FACSCalibur, BECTON DICKINSON) 作分析。

7. AVO stain

細胞經過不同的黃酮類化合物處理後，吸去培養液，以 PBS 清洗三次，加入 1 $\mu\text{g/ml}$ AVO，放置無菌操作台，避光 15 分鐘後，PBS 洗三次，加入培養液於螢光顯微鏡下觀察 (UV 461 nm)。

8. MDC stain

細胞經過不同的黃酮類化合物處理後，吸去培養液，以 PBS 清洗三次，加

入 1 $\mu\text{g/ml}$ MDC，放置細胞培養箱 10 分鐘後，PBS 洗三次，加入培養液，於螢光顯微鏡下觀察 (UV 461 nm)。

9. Cell motility 分析

利用 48 well Boyden chamber 的分析方法，lower chamber 為含有 10% FBS 的 DMEM，將細胞處理不同的黃酮類化合物 24 小時後，以 0.05% 的 trypsin-EDTA 打下所有細胞並用 trypan blue 計算細胞數，然後注入固定量的細胞 (1.5×10^4 cell/well) 於 upper chamber，待細胞移動 5 小時以後，取下薄膜，以甲醇固定細胞 10 分鐘，風乾 5 分鐘之後，以 Giemsa (1:20) 染色 1 小時，最後固定住薄膜，擦拭掉薄膜之上層細胞，在 $400\times$ 顯微鏡底下每個 well 隨機選取 3 個視野，數 5 個 wells，作移動細胞數之統計。

10. cell invasion 分析

將 cellulose nitrate filters 預先 coating 上 $100 \mu\text{g/cm}^2$ Marix gel (0.5 mg/ml)，在 laminar flow 風乾 3~5 小時，在將細胞處理不同的黃酮類化合物 24 小時後，以 0.05% 的 trypsin-EDTA 打下所有細胞並用 trypan blue 計算細胞數，然後注入固定量的細胞 (10^4 - 1.5×10^4 cell/well) 於 upper chamber，待細胞移動 5 小時以後，取下薄膜，以甲醇固定細胞 10 分鐘，風乾 5 分鐘之後，以 Giemsa (1:20) 染色 1 小時，最後固定住薄膜，擦拭掉薄膜之上層細胞，在 $400\times$ 顯微鏡底下每個 well 隨機選取 3 個視野，數 5 個 wells，作移動細胞數之統計。

11. gelatin-zymography (gelatinase 活性測試)

首先製備 0.1 % gelatin-8 % SDS-PAGE 電泳膠片，置於電泳槽中，並加入電泳緩衝液。取 16 μ l sample (蛋白總量 20 μ g)，加入 4 μ l loading buffer，將 sample loading 到電泳片中，以 140 V 進行電泳分離。大約 3 小時之後，將膠拆下，加入 50 ml 的 washing buffer，在室溫下洗 30 分鐘，共兩次。倒掉 washing buffer 之後，加入 50 ml 的 reaction buffer，於 37°C 恆溫箱下反應 12 個小時。將反應完後的 gel，以 staining buffer 染色 30 min，之後再以退色液退染觀看結果，並以 densitometer (AlphaImage 2000, AlphaImage comp) 量化結果。

12. 蛋白質 (protein lysate) 製備

試驗細胞經 PBS 洗淨，以刮杓自 culture dish 刮離，接著加入適量緩衝液 A (20 mM Tris-HCl、5 mM EDTA、0.5 mM EGTA、0.3 M sucrose、2 mM PMSF、10 μ g/ml leupeptin、25 μ g/ml soybean trypsin inhibitor typ I-S 和 50 mM β -mercaptoethanol, pH 7.5)，以攪拌器研磨細胞，先將所得萃取液經 4°C 下離心 (800 g) 10 min 移除細胞核後，再將上清液經超高速離心在 4°C 下離心 (40 Krpm) 1 小時，分出上清液 (即細胞質部份) 及沉澱物 (含胞膜及胞核之部份)。將細胞質部份置於 -80°C 貯存；沉澱物以 2 ml 緩衝液 B (含有 0.1% Triton-X 100 的緩衝液 A) 處理之，混合完全後靜置 1 小時，在 1 小時內每 15 分鐘混合一次 (重覆四次)，在 4°C 下離心 (40 Krpm) 1 小時，取上清液即為微粒部

份，分裝於 -80°C 貯存。

13. 蛋白濃度測定

蛋白質的定量採用 Bradford's protein assay 方法，其原理為蛋白質可與 Coomassie brilliant blue G-250 形成藍色複合物。測定方法：首先以一系列已知濃度 BSA，加入五分之一體積的 Bradford protein dye 以波長 595 nm 之吸光度作一標準曲線，再以同樣的測定方法測得樣品之 O.D.值，即可對照標準曲線求得樣品蛋白之濃度。

14. western blotting 分析

利用 western blotting 的方法測定 MMPs、u-PA、t-PA、plasmin、TIMPs、PAIs 及 u-PAR 的蛋白量及與其相關的訊息傳遞蛋白如：MAPK pathway (p-ERK1/2, p-P38 及 p-JNK 1/2)、PI3K-Akt pathway (p-Akt)、Rho family (Rho-A)、FAK pathway; 首先製備 12.5% SDS-PAGE 電泳膠片，置於電泳槽中，並加入電泳緩衝液，取 16 μl sample (蛋白總量 20 μg)，加入 4 μl loading buffer，將 sample denature (95°C , 10 min) 之後再 loading 到電泳片中，以 140 V 進行電泳分離。大約 3 小時之後，將膠拆下後進行蛋白轉漬，將膠體置入冰冷之 transfer buffer，將預先浸濕的 NC paper 蓋在膠體上面後裝入 transfer holder，於 4°C 下，以 100 V 進行轉漬 1 小時之後，取出 NC paper 加入 blocking buffer，在室溫下搖動 1 小時。然後加入一級抗體於 TBS buffer，在 4°C 下反應 overnight，之後以 washing buffer (TBS + 0.05% Tween 20) 清洗三次，

每一次 10 分鐘。接著再加入二級抗體於 TBS buffer，於室溫作用二個小時後以 washing buffer 清洗三次，每一次 10 分鐘。最後加入 25 ml substrate buffer 進行呈色反應（或以 ECL 冷光系統紀錄）。以 densitometer（AlphaImage 2000，AlphaImage comp）量化結果。

15. 癌瘤細胞轉移之動物模式

在腎臟癌動物模式方面，我們將 $5 \times 10^6/100 \mu\text{l}$ PBS 洗淨的腎臟癌細胞株 (786-O)，打入免疫缺陷的小鼠 (C.B17/Icr-Prkdc^{scid}/CrINarl) 的尾靜脈，7 天後將接種癌瘤細胞的老鼠隨機分組，進一步餵食適量不同的黃酮類化合物，17 週後犧牲老鼠，取出肺部以觀察其組織、重量及其動物存活率。

三、結果與討論

第一部分 探討黃酮類促進腎臟癌細胞凋亡之能力

我們發現十種黃酮類化合物 (flavone, flavonol, 5-hydroxyflavone, 7-hydroxyflavone, chrysin, baicalein, apigenin, luteolin, quercetin 及 kaempferol) 中 quercetin 和 baicalein 具有誘導 786-O 細胞株凋亡的效果 (**Fig. 1**)。其中在腎臟癌細胞株 786-O 的實驗中，發現以 quercetin 和 baicalein 處理 48 小時後，在 20 μM 的濃度下即能有效降低細胞存活率，而處理 72 小時的組別中，有效降低細胞存活率的濃度則降到 10 μM (**Fig. 2**)。在細胞死活計數方面，以 quercetin 和 baicalein 處理 72 小時可以看到 786-O 細胞死亡的結果 (**Fig. 3**)，並且在

細胞型態上也有受到傷害的明顯改變 (Fig. 4)。之後，以 DAPI 染色進一步分析 quercetin 和 baicalein 是否造成 786-O 細胞的細胞凋亡。證實 quercetin 和 baicalein 的處理會造成 786-O 細胞的 chromosome condensation (Fig. 5)。另外，以 JC-1 染色再以螢光顯微鏡觀察 786-O 細胞的粒線體膜電位，發現 quercetin 和 baicalein 會造成 786-O 細胞的粒線體膜電位降低 (Fig. 6)。實驗證實 quercetin 和 baicalein 在腎臟癌細胞株誘導細胞凋亡的效果。然而，以 Flow cytometry 分析的結果中卻發現，quercetin 和 baicalein 的處理不會造成 786-O 細胞的 subG1 phase 增加 (Fig. 7)。處理 quercetin 的 796-O 細胞在 AVO 和 MDC 染色下則顯示出酸性泡和細胞自噬體的增加 (Fig. 8)。此外，以與 Caki-1、Caki-2 同為人類腎小管上皮細胞株的 HK-2 測試的毒性分析，顯示 quercetin 和 baicalein 對於正常細胞不具有毒殺性 (Fig. 9)。這或許表示在 quercetin 和 baicalein 處理下會使 786-O 細胞走向 autophagy 的現象，而其中相關機制尚待進一步分析。

第二部分 探討黃酮類抑制腎臟癌細胞轉移之能力

以黃酮類化合物處理腎臟癌細胞株 786-O 細胞 24 hrs 後，以相同細胞數進行 *in vitro* migration assay，觀察其轉移能力(migration)的改變，證實baicalen, apigenin, luteolin 和 kaempferol 具有不同程度之抑制效果(Fig. 10)。進一步以黃酮類化合物處理腎臟癌細胞株 786-O 細胞 24 hrs 後，收集其培養液，利用 gelatin zymography 的分析方式，觀察其水解酵素 MMP-2 及 MMP-9 的改變，

我們發現 flavonol, chrysin, baicalein, apigenin, luteolin, quercetin 及 kaempferol 具有不同程度之抑制效果(Fig. 11)。而 luteolin 為例，實驗中還發現低濃度的 luteolin 可以增強 taxol 這一個抗癌藥其誘導腎臟癌細胞凋亡的能力(Fig. 12)。接著使用 boyden chamber 分析中則觀察到 kaempferol 在處理 786-O 後，細胞的 Invasion 和 Migration 能力都會降低 (Fig. 13)。而 kaempferol 抑制 786-O 細胞爬行能力的蛋白調控路徑分析，得知是藉由 MMP-2、p-FAK Tyr925 和 p-Akt 的改變促成抑制反應 (Fig. 14)。在 *in vivo* 的動物實驗方面，結果顯示給予小鼠 kaempferol 後會導致體重減輕 (Fig. 15)，但因為 786-O 腫瘤轉移所造成的肺部型態變形與腫脹狀態則會因為有餵食 kaempferol 而獲得減緩 (Fig. 16)。

四、計畫成果及自評

實驗結果中證明，屬於黃酮類的 quercetin 和 baicalein 可以誘導腎臟癌細胞的凋亡甚至可能產生細胞自噬反應。而 luteolin 則可以與 抗癌藥物合併，增強 taxol 的癌細胞抑制效果。另一方面，kaempferol 則可以藉由 MMP-2、p-Akt 和 p-FAK Tyr925 來抑制腎臟癌細胞的轉移。在動物實驗方面也獲得證實。證實這些黃酮類化合物對於腎臟癌細胞的影響。未來 quercetin、baicalein、luteolin 和 kaempferol 將有可能被賦予腎臟癌治療上的應用價值。

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附表及附圖

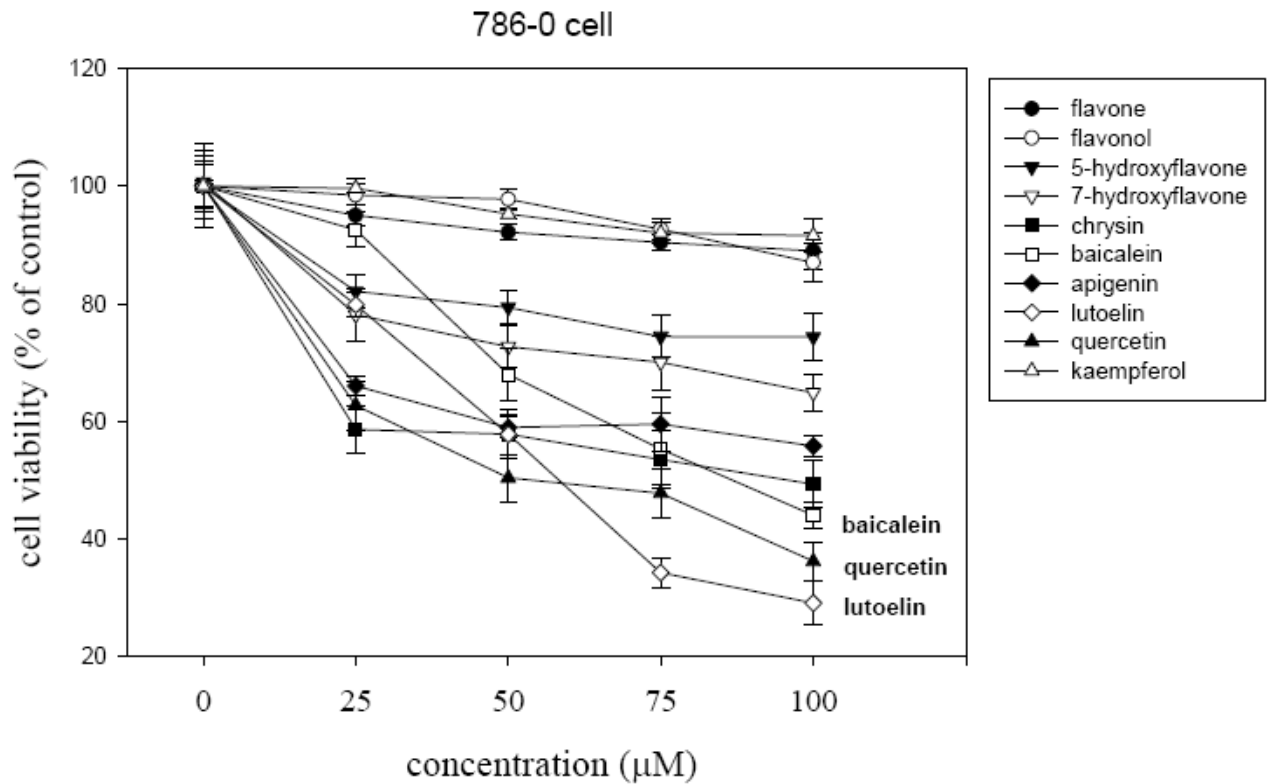


Figure 1. The effect of ten flavonoids (flavones, flavonol, 5-hydroxyflavone, 7-hydroxyflavone, chrysin, baicalein, apigenin, lutoelin, quercetin, kaempferol) on the cell viability in 786-O cells. In concentration assays, cell were treated with ten flavonoids (flavones, flavonol, 5-hydroxyflavone, 7-hydroxyflavone, chrysin, baicalein, apigenin, lutoelin, quercetin, kaempferol) at a concentration of 25, 50, 75, and 100 µM for 24 h., and then cell viability was detected using MTT test. The data show were mean \pm SD of three independent experiments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

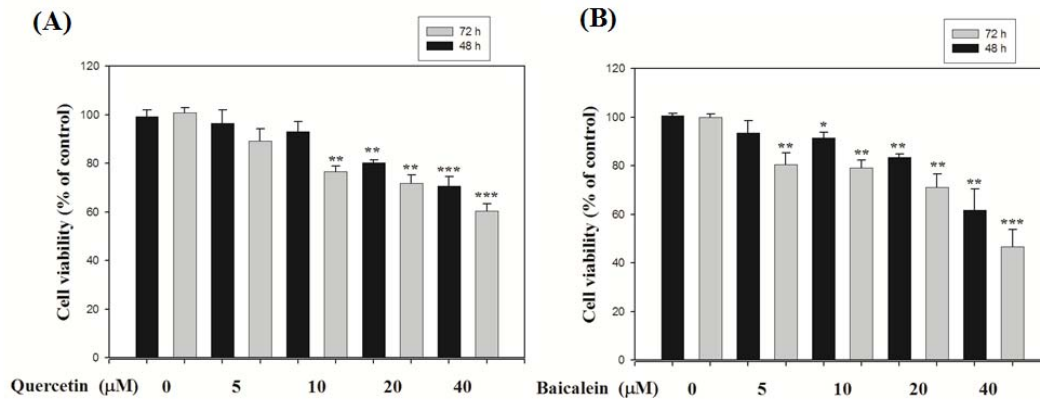


Figure 2. The effect of quercetin or baicalein on the cell viability in 786-O cells.

In concentration- and time-dependent assays, cells were treated with (A) quercetin or (B) baicalein at a concentration of 5, 10, 20, and 40 μM for 48 and 72 h., and then cell viability was detected using MTT test. The data shown were mean \pm SD of three independent experiments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

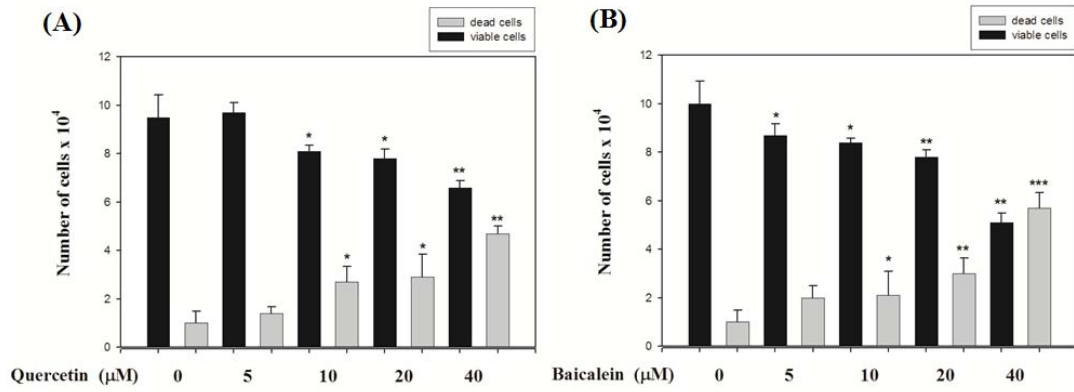


Figure 3. The effect of quercetin or baicalein on the cell death in 786-O cells. In concentration- and time-dependent assays, cell were treated with (A) quercetin or (B) baicalein at a concentration of 5, 10, 20, and 40 μM for 72 h., and then cell viability was detected using Trypan blue exclusion assay. The data show were mean \pm SD of three independent experiments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

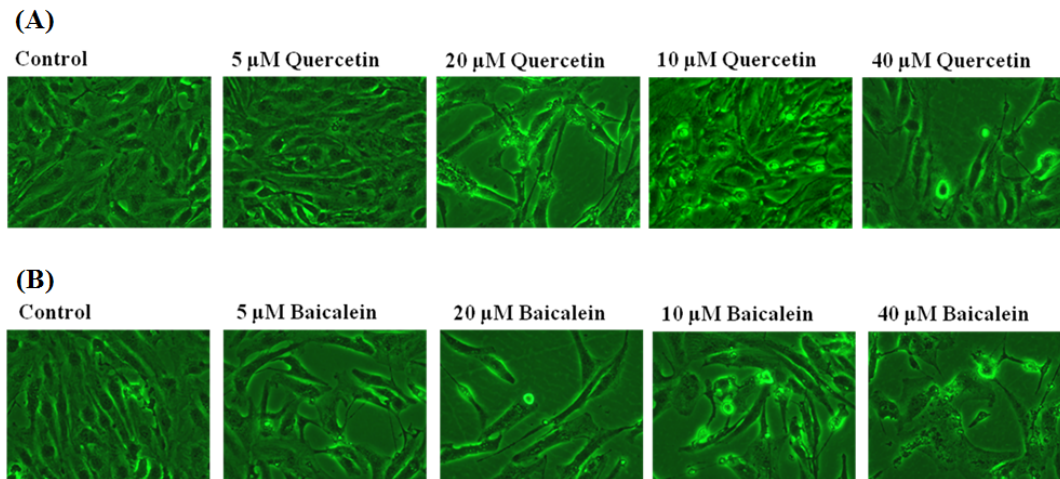


Figure 4. The effect of quercetin or baicalein on the cell morphology change in 786-O cells. 786-O were treated with (A) quercetin or (B) baicalein (5, 10, 20, and 40 μ M) for 72 h. Photomicrographs of the treated 786-O cells were observed by using phase-contrast microscopy.

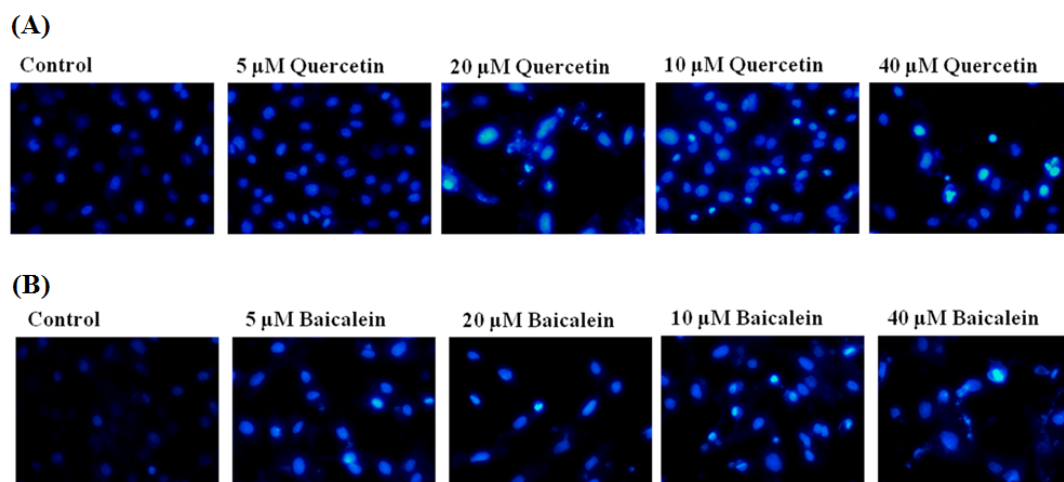


Figure 5. Apoptotic effect of quercetin or baicalein in 786-O cells was assessed by DAPI stain. After a 24-hour treatment of 786-O cells with the indicated concentrations of (A) quercetin or (B) baicalein, the nuclear morphology of the treated cells was observed by fluorescence microscopy using DAPI stain (at a magnification of 200 \times).

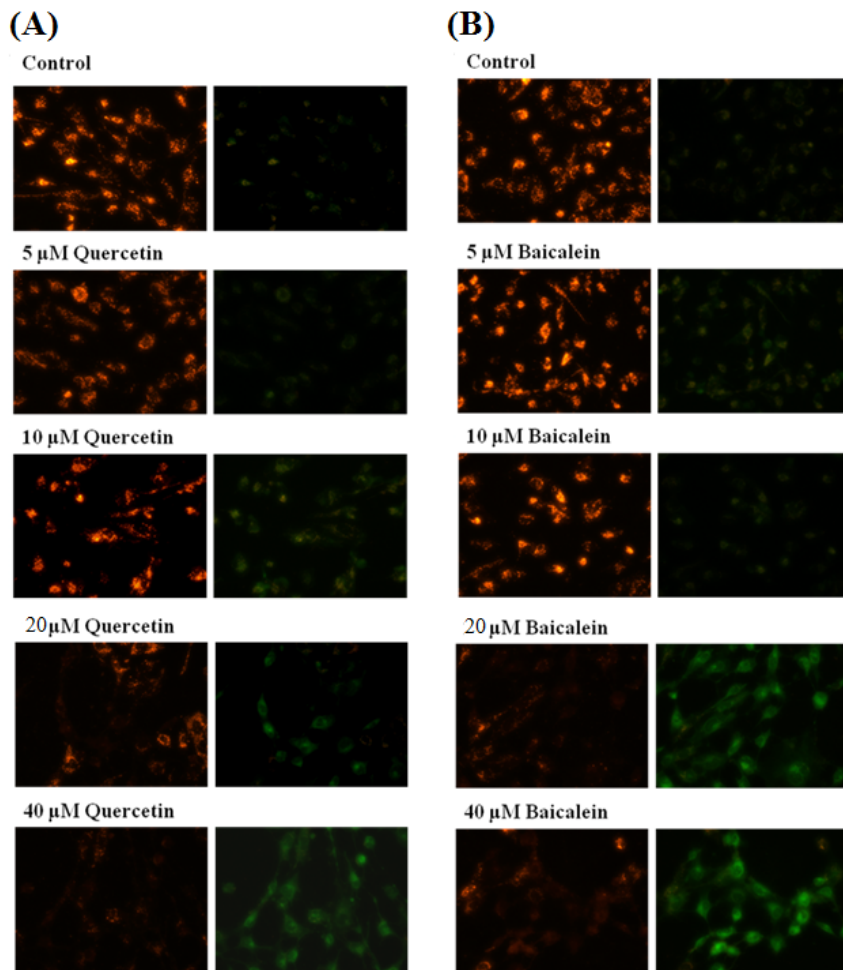


Figure 6. Effects of quercetin or baicalein in mitochondrial membrane potential in 786-O cells. 786-O were treated with (A) **quercetin** or (B) **baicalein** (5, 10, 20, and 40 μM) for 24 h. The changes of mitochondrial membrane potential ($\Delta\Psi_m$) were assessed by using fluorescent lipophilic cationic JC-1 dye. JC-1 is selectively accumulated within intact mitochondria to form multimer J-aggregates emitting fluorescence light at 590 nm (red) at a higher membrane potential, *left*, and monomeric JC-1 emits light at 527 nm (green) at a low membrane potential, *right*.

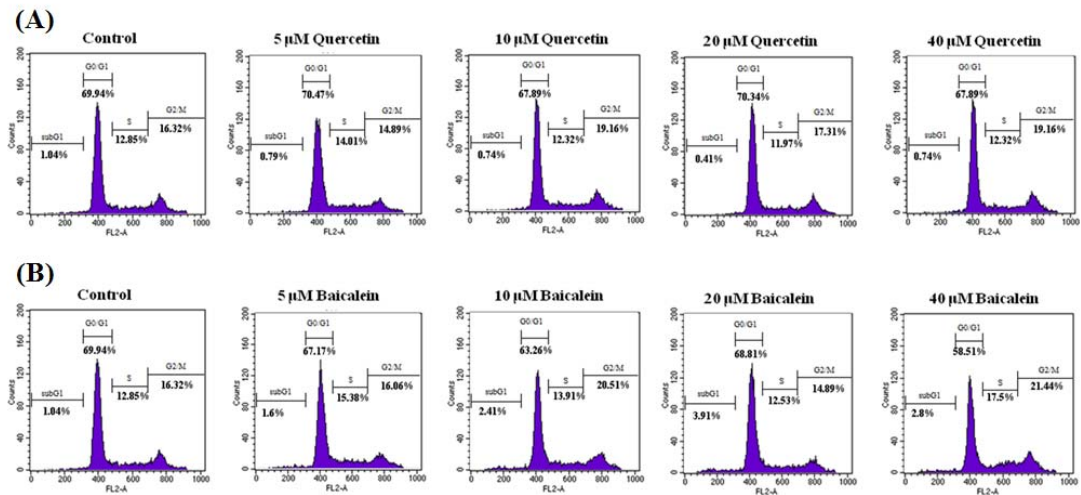


Figure 7. The effect of quercetin or baicalein on the cell apoptosis in 786-O cells.

786-O cells were treated with (A) quercetin or (B) baicalein (5, 10, 20, and 40 μ M) for 72 h. Hypodiploid cells population (sub G1 phase) of the treated HUVEC cells were analyzed by flow cytometry using PI stain and at last 10,000 event of total cells were analyzed for each experimental treatment.

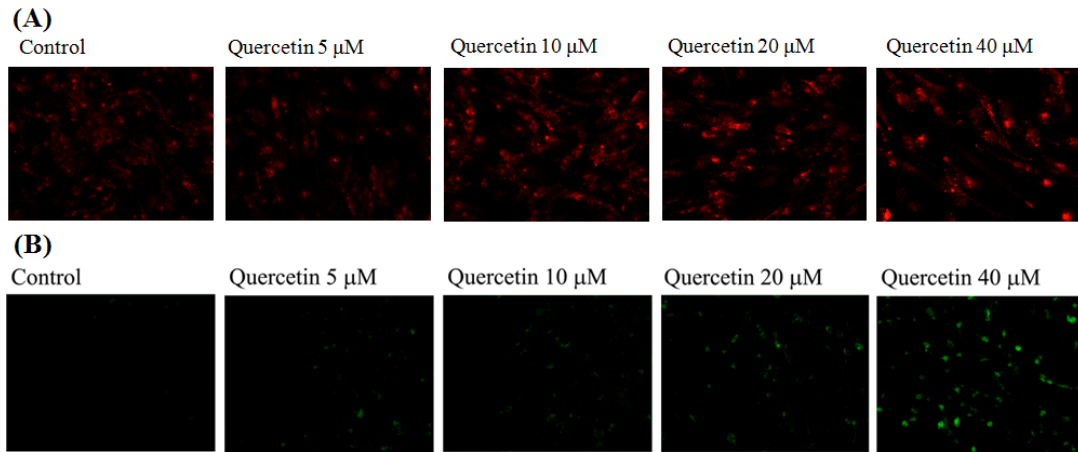


Figure 8. Autophagy effect of quercetin in 786-O cells was assessed by AVO and MDC stain. After a 76-hour treatment of 786-O cells with the indicated concentrations of quercetin. (A) AVO or (B) MDC (at a magnification of 200 \times).

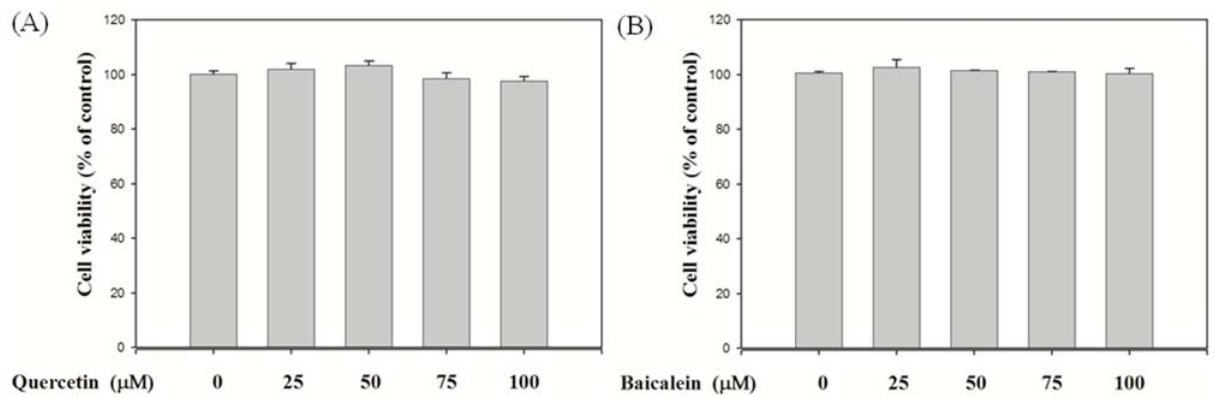
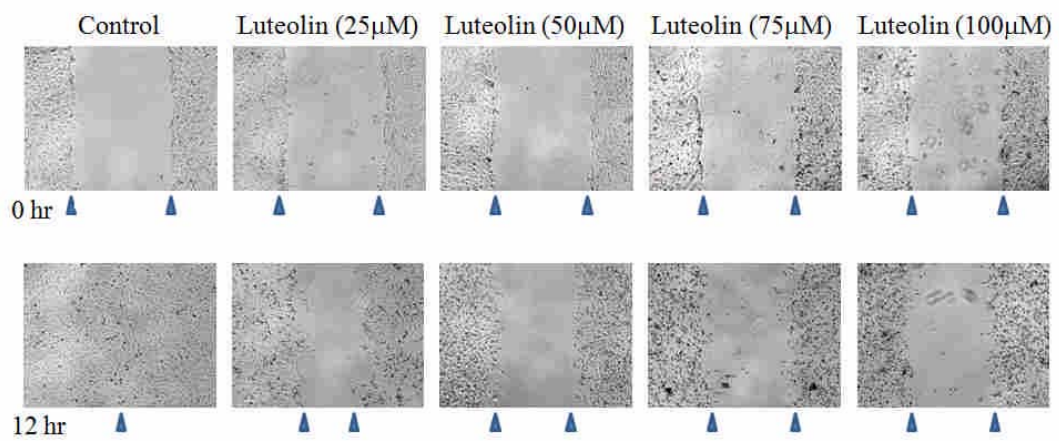
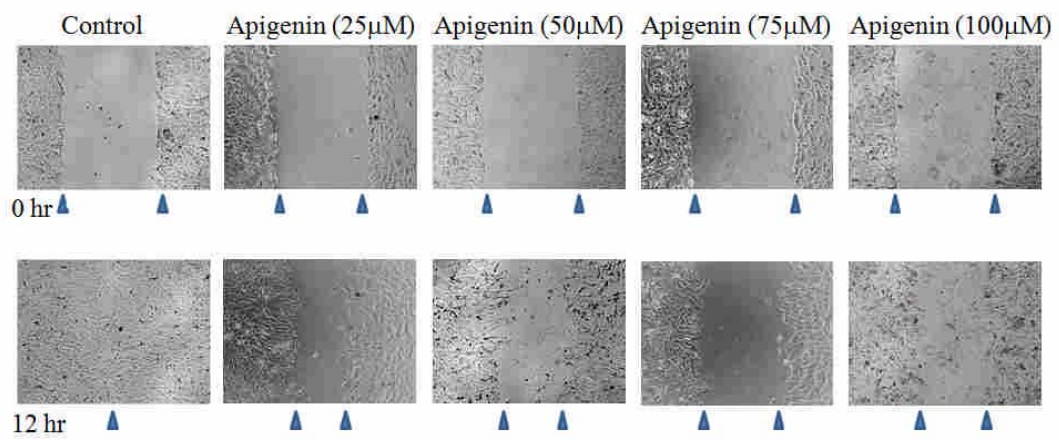
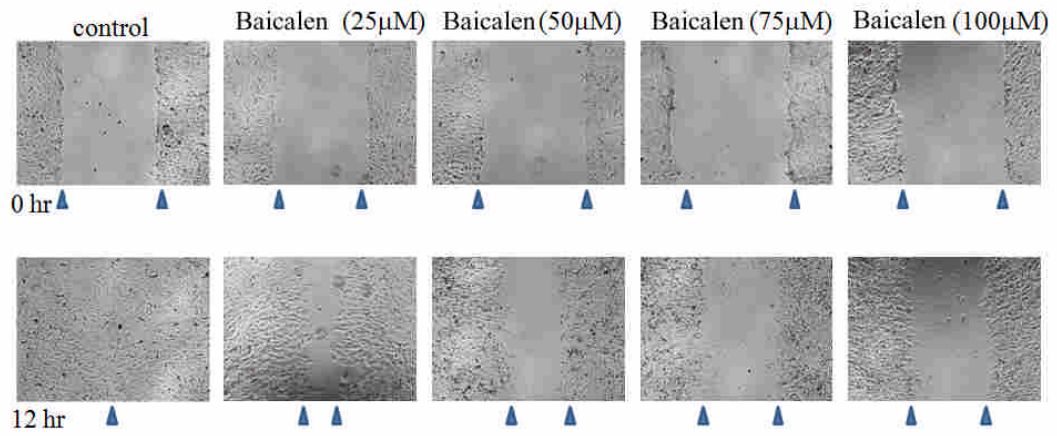


Figure 9. The effect of quercetin and baicalein on the cell viability in HK-2 cells.

In concentration assays, cell were treated with (A) quercetin (B) baicalein at a concentration of 25, 50, 75, and 100 μM for 24 h., and then cell viability was detected using MTT test. The data show were mean \pm SD of three independent experiments.



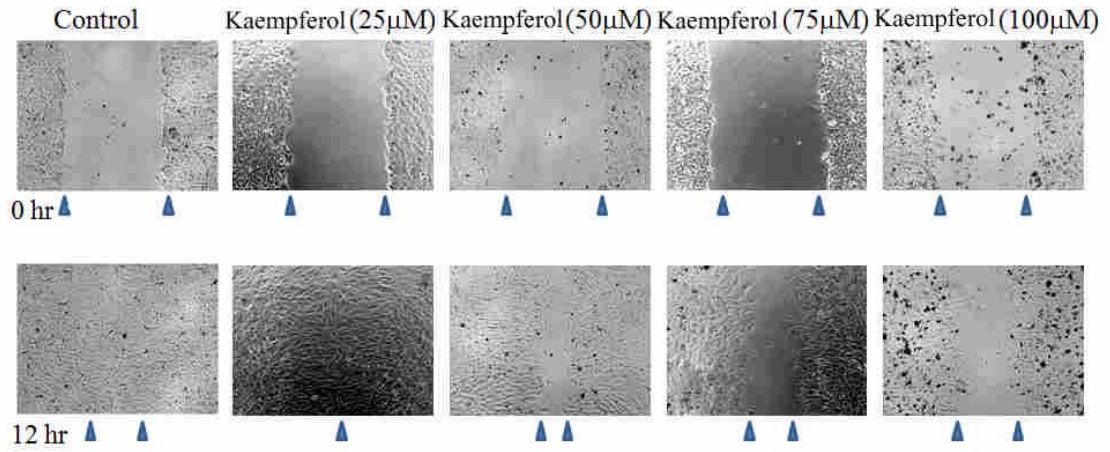


Figure 10. Photographs show wound closure of 786-O treat the flavonoids.

Representative fields were photographed at 0, 12 hr. (40X) baicalen, apigenin, luteolin and kaempferol.

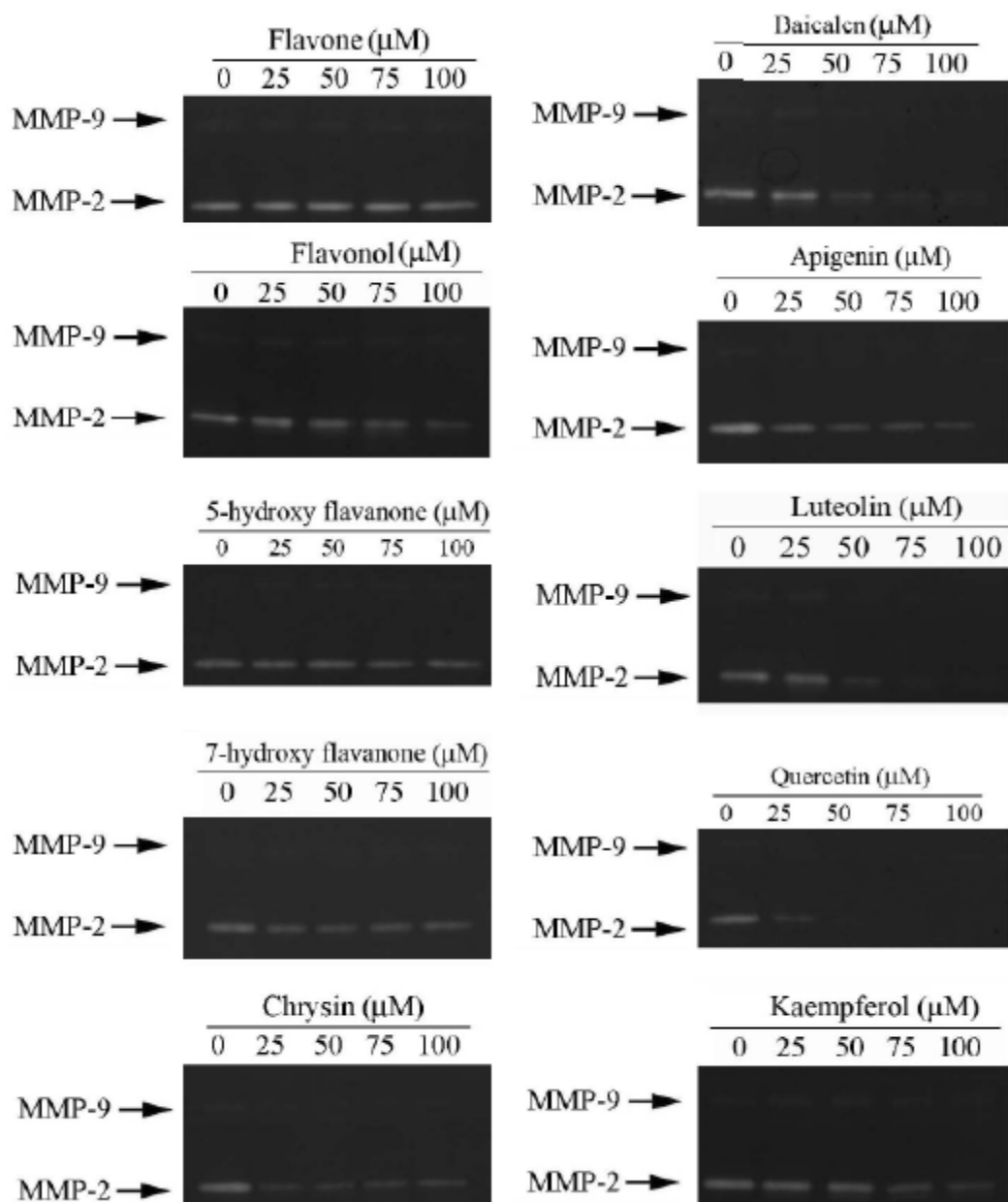


Figure 11. Effect of flavonoids on the activities of MMP-2 and MMP-9. 786-O cells were treated with 0, 25, 50, 75 and 100 μ M of flavone, baicalen, flavonol, apigenin, 5-hydroxy flavanone, luteolin, 7-hydroxy flavanone, quercetin, chrysin and kaempferol for 24 hours and then subjected to gelatin and casein zymography to analyze the activities of MMP-2 and MMP-9 on the 786-O cells, respectively.

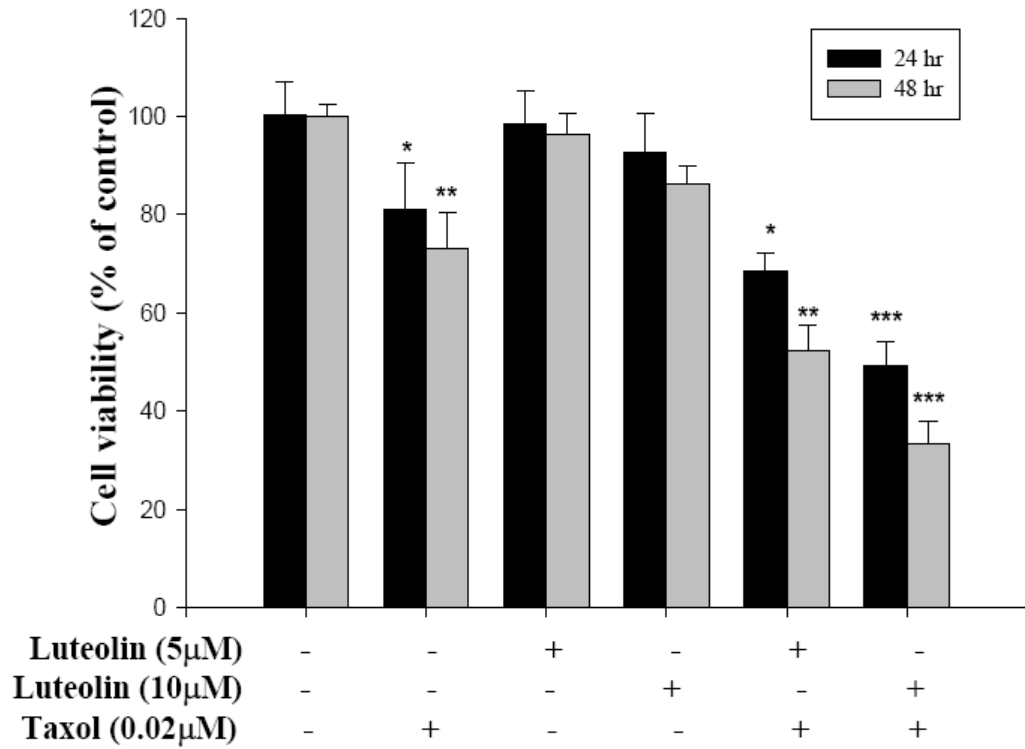


Figure 12. The effect of luteolin combine with taxol on the cell viability in 786-O cells. In concentration- and time-dependent assays, cell were treated with luteolin at a concentration of 5, 10 μM , and combine with taxol 0.02 μM for 48 and 72 h., and then cell viability was detected using MTT test. The data show were mean \pm SD of three independent experiments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

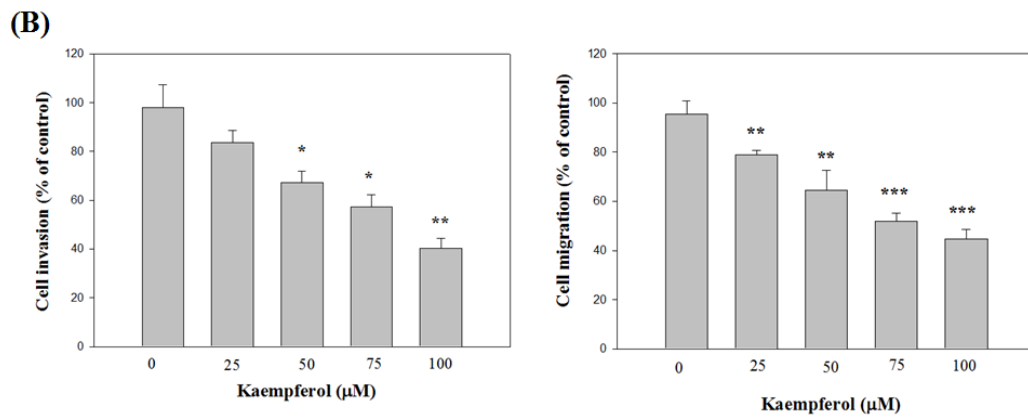
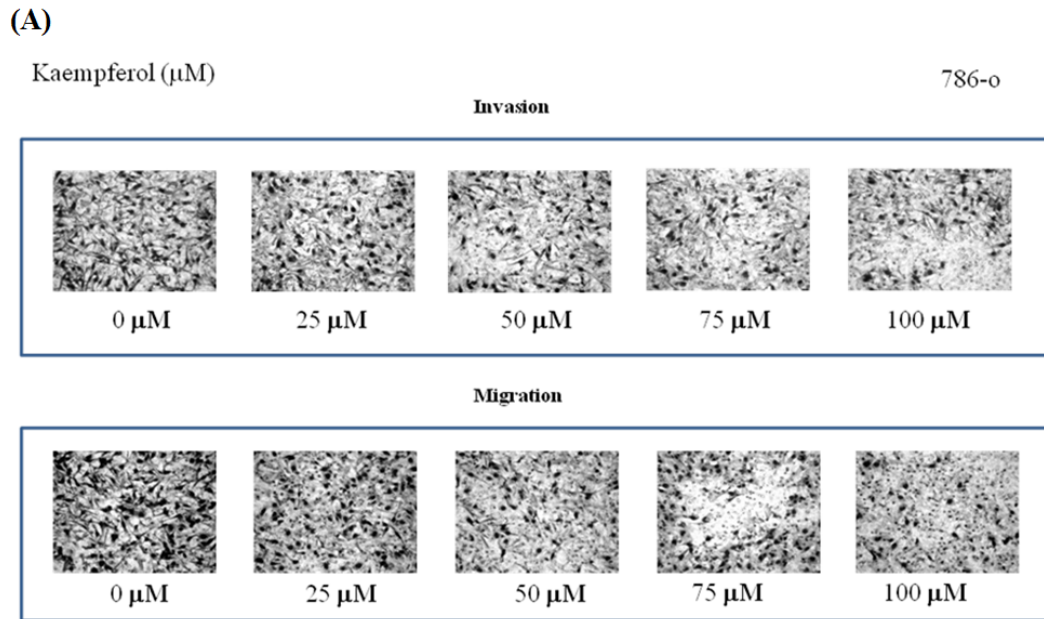


Figure 13. Effect of Daidzein on cell invasion and motility of 786-O cells. 786-O cells were treated with 0, 10, 20, or 50 $\mu\text{g/ml}$ of Daidzein for 24 hours and were then subjected to analyses for invasion and motility as described in Materials and Methods. Data represented the means \pm SD of at least 3 independent experiment. (*, $P < 0.05$)

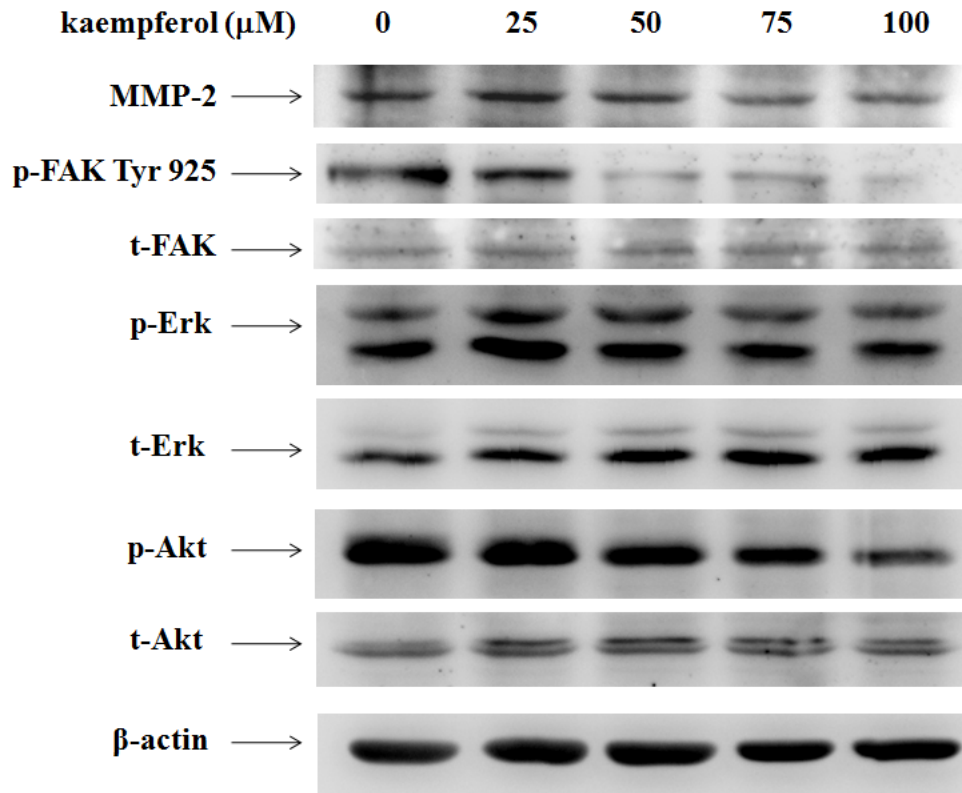


Figure 14. The effect of kaempferol on the phosphorylation of MMP-2, p-FAK Tyr925 and p-Akt protein. 786-O cells were treated with 0, 25, 50, 75 and 100 μM of kaempferol for 24 hours, and then cell lysates were subjected to SDS-PAGE followed by Western blotting with anti-MMP-2, phospho-FAK Tyr925 and anti-phospho-Akt antibodies. Signals of proteins were visualized with an ECL detection system.

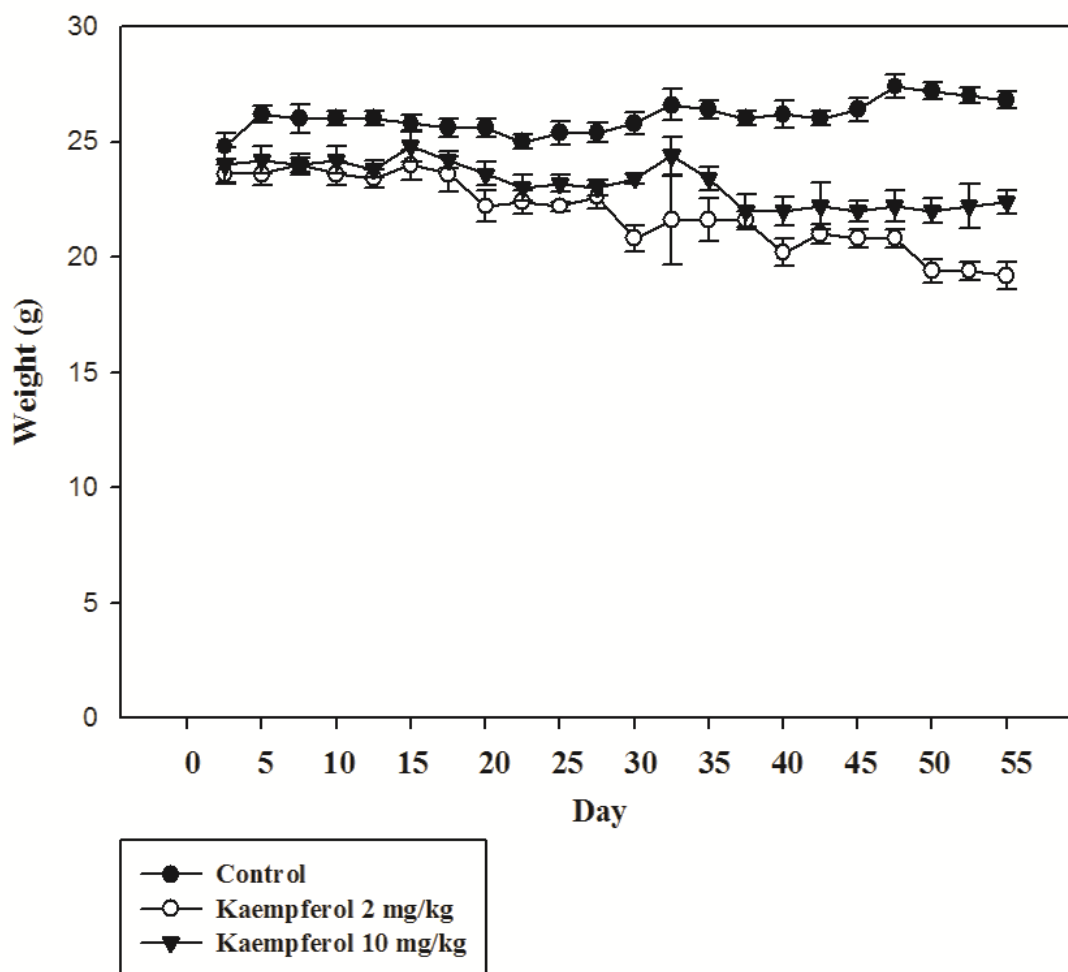


Figure 15. The *in vivo* anti-metastasis effect of after tail intravenous injection of 786-O cells, BALB/c mice were treated with kaempferol as described in Materials and Methods and then analysed for the body weight of mice. The values represented the means \pm SD (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; OAs compared with saline).

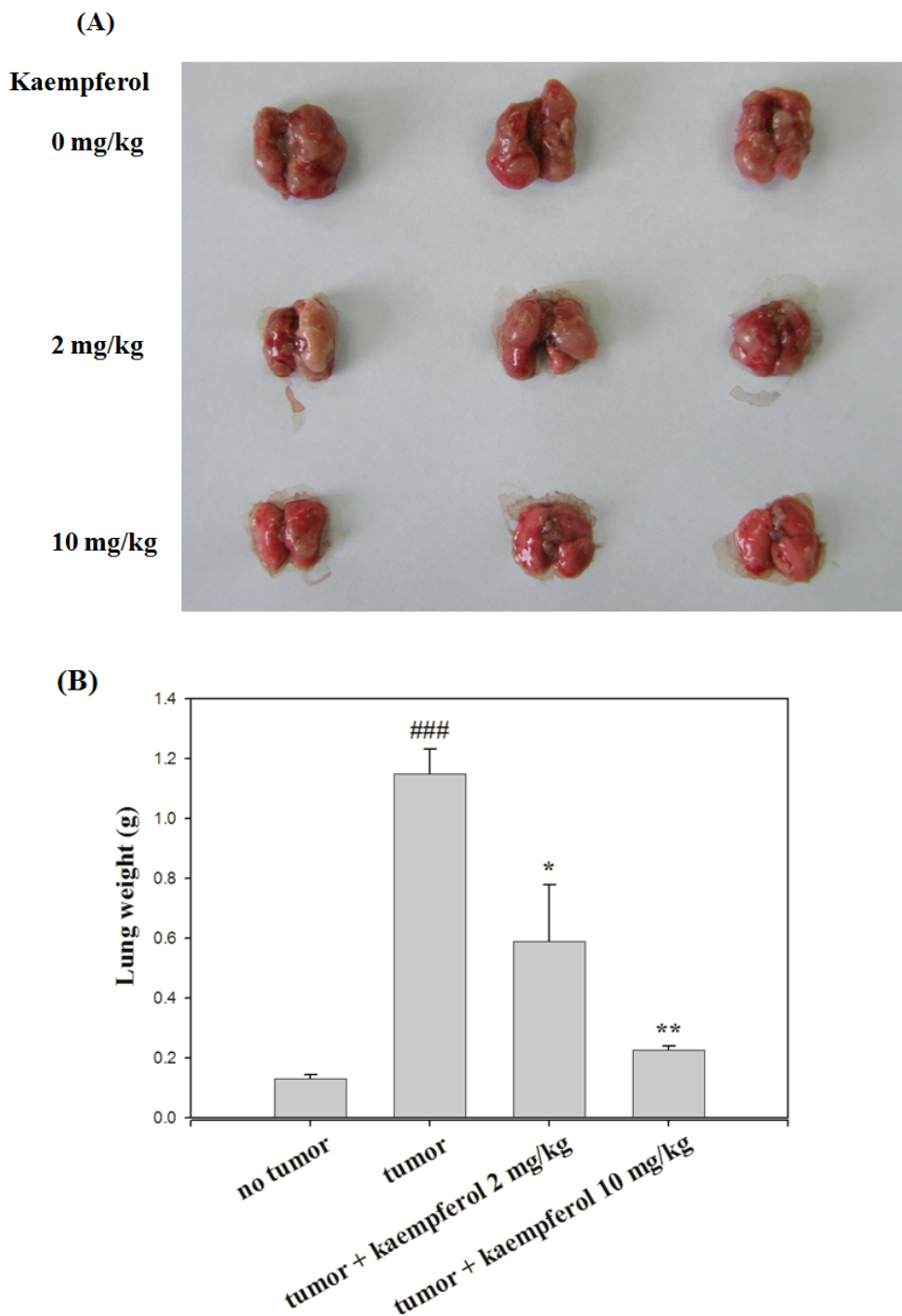


Figure 16 Suppression of lung metastasis of 786-O cells by kaempferol. (A) Photographs show lungs of 786-O cell-bearing mice. (B) 786-O cells were injected into the tail veins of 6-week-old female BALB/c nude mice. After injection of 786-O cells, kaempferol (0, 2 and 10 mg/kg/day) alone were administered oral gavage for 120 days to the berberine-treated groups and the control groups, respectively. Mice were sacrificed and the number of metastasis in the lung surface was counted on the

22 day after the cells were injected. *, $p < 0.05$; **, $p < 0.01$; ###, $p < 0.001$. Each value represents the mean \pm SE. * : Compare with kaempferol, # : Compare with no tumor.

國科會補助計畫衍生研發成果推廣資料表

日期:2012/10/30

國科會補助計畫	計畫名稱: 黃酮類化合物抑制不同腎臟癌細胞株(Caki-1, Caki-2, 786-0)轉移能力、誘導細胞凋亡及輔助抗癌藥之研究
	計畫主持人: 張滋榮
	計畫編號: 98-2314-B-040-004-MY3 學門領域: 腎臟科新陳代謝及內分泌
無研發成果推廣資料	

98 年度專題研究計畫研究成果彙整表

計畫主持人：張滋榮		計畫編號：98-2314-B-040-004-MY3					
計畫名稱：黃酮類化合物抑制不同腎臟癌細胞株(Caki-1, Caki-2, 786-0)轉移能力、誘導細胞凋亡及輔助抗癌藥之研究							
成果項目		量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）	
		實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比			
國內	論文著作	期刊論文	0	3	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	1	1	100%	人次	
		博士生	1	1	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%		章/本
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

<p style="text-align: center;">其他成果</p> <p>(無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)</p>	無
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	成果項目	量化	名稱或內容性質簡述
科 教 處 計 畫 加 填 項 目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與(閱聽)人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

達成目標

未達成目標（請說明，以 100 字為限）

實驗失敗

因故實驗中斷

其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文： 已發表 未發表之文稿 撰寫中 無

專利： 已獲得 申請中 無

技轉： 已技轉 洽談中 無

其他：（以 100 字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

實驗結果中證明，屬於黃酮類的 quercetin 和 baicalein 可以誘導腎臟癌細胞的凋亡甚至可能產生細胞自噬反應。而 luteolin 則可以與 抗癌藥物合併，增強 taxol 的癌細胞抑制效果。另一方面，kaempferol 則可以藉由 MMP-2、p-Akt 和 p-FAK Tyr925 來抑制腎臟癌細胞的轉移。在動物實驗方面也獲得證實。證實這些黃酮類化合物對於腎臟癌細胞的影響。未來 quercetin、baicalein、luteolin 和 kaempferol 將有可能被賦予腎臟癌治療上的應用價值。